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The Properties of Hydrogen Peroxide Production under Hyperoxic and Hypoxic Conditions of Perfused Rat Liver

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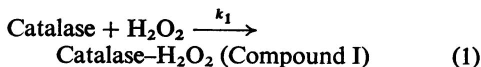
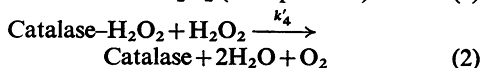
The properties of H_2O_2 production in the 'haemoglobin-free', 'non-circulatory' perfused liver of rats were examined. The H_2O_2 production with 1 mM-lactate and 0.15 mM-pyruvate was 82 nmol/min per g of liver or 333 nmol/min per 100 g body wt. in the liver of fed rats at 30°C. This rate decreased to almost half in the livers of starved and phenobarbital-pretreated rats. When H_2O_2 production was stimulated by urate infusion, almost all of the H_2O_2 produced by the uricase reaction was decomposed by the catalase reaction. During the demethylation reaction of aminopyrine, no change in H_2O_2 production was detected by the present method; thus microsomal H_2O_2 production observed in isolated subcellular fractions appeared not to contribute significantly to the H_2O_2 production in the whole organ. Whereas the rate of the glycollate-dependent H_2O_2 production was halved at an intracellular O_2 concentration that caused a 10% increase in the reduction state of cytochrome *c*, the half-maximal rate of H_2O_2 production with lactate and pyruvate was observed at an O_2 concentration that caused a 40% increase in the reduction state of cytochrome *c* in the liver. No further increase in the rates of H_2O_2 production was obtained by increasing O_2 pressure up to 5×10^5 Pa. The rate of ethanol oxidation through the catalase 'peroxidatic' reaction varied, depending on the substrate availability. The maximal capability of this pathway in ethanol oxidation reached approx. 1.5 μmol/min per g of liver, when a mixture of urate, glycollate and octanoate was infused to enhance H_2O_2 production.

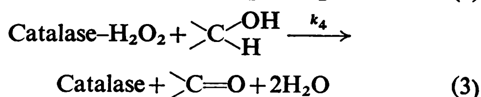
The existence of a catalase–H_2O_2 intermediate (Compound I) has been previously demonstrated in respiring micro-organisms (Chance, 1952, 1954) and perfused rat liver (Sies & Chance, 1970; Sies *et al.*, 1973). Catalase decomposes H_2O_2 in two different reaction modes, 'catalatic' (eqns. 1 and 2) and 'peroxidatic' (eqns. 1 and 3) (Keilin & Hartree, 1945; Chance, 1949):



$$\text{Catalase} + H_2O_2 \xrightarrow{k_1} \text{Catalase–}H_2O_2\ (\text{Compound I}) \quad (1)$$



$$\text{Catalase–}H_2O_2 + H_2O_2 \xrightarrow{k'_4} \text{Catalase} + 2H_2O + O_2 \quad (2)$$



$$\text{Catalase–}H_2O_2 + {>}C{<}^{OH}_{H} \xrightarrow{k_4} \text{Catalase} + {>}C{=}O + 2H_2O \quad (3)$$

Regardless of the concentration of H_2O_2 being supplied in the system, the steady-state concentration of the catalase–H_2O_2 intermediate is determined by rate constants k_1 and k_4 and corresponds to 40% of the total haem concentration of rat liver catalase if the catalase reaction is strictly in its 'catalatic' mode (Chance & Oshino, 1971, 1973; Oshino *et al.*, 1973*b*). The contribution of the 'peroxidatic' mode in the overall reaction increases with increasing concentration of hydrogen donor, and the steady-state concentration of the catalase–H_2O_2 intermediate decreases in such a way that the hydrogen donor concentration ($a_{1/2}$) required for a half-maximal saturation of catalase–H_2O_2 formation is proportional to the turnover number of the catalase reaction ($1/e\cdot dx_n/dt$):

$$1/e\cdot dx_n/dt = Ka_{1/2} \quad (4)$$

where e is the catalase haem concentration and dx_n/dt is the rate of H_2O_2 generation. K is a constant specified for each hydrogen donor and is $31.5\,min^{-1}\cdot mM^{-1}$ for methanol and ethanol (Chance & Oshino, 1973; Oshino *et al.*, 1973*a*). Thus by infusing a known concentration of methanol and measuring the steady-state concentration of the catalase–H_2O_2 through a lobe of the perfused liver, the rate of H_2O_2 interaction with catalase in liver may be measured (Oshino *et al.*, 1973*a*). This method is non-destructive, sensitive and also is applicable to a variety of purposes and thus was used not only in the measurement of H_2O_2 production but also in the measurement of alcohol dehydrogenase-dependent ethanol oxidation in the perfused liver (Lindros *et al.*, 1974).

H_2O_2, as an oxidant and also as a reductant, has

long been known to be toxic to certain bacteria (McLeod & Gordon, 1923; Clayton, 1961) and to modify cellular components. However, more recently it has been recognized that more reactive and potentially hazardous species such as O_2^- and $OH^•$ are produced from various enzyme systems *in vitro* (Beauchamp & Fridovich, 1970; Fong *et al.*, 1973). These molecules, in turn, may either attack biological constituents (Lavelle *et al.*, 1973) or eventually be converted into H_2O_2 by non-enzymic dismutation or by superoxide dismutase, and then into H_2O by catalase, glutathione peroxidase and other peroxidases. Fong *et al.* (1973) demonstrated that formation of the most hazardous species, $OH^•$, appears to depend not only on a steady-state concentration of O_2^- but also on the free H_2O_2 concentration in the system. Various co-operative systems seem to have evolved as a protective mechanism against possible hazardous side effects of O_2 metabolism. These include superoxide dismutase, catalase and peroxidases. One approach to the understanding of this overall protective mechanism is to examine the properties of cellular H_2O_2 production. Oxidases such as uricase, glycollate oxidase and xanthine oxidase produce H_2O_2 or O_2^- as the natural reaction products and are obviously one of the sources of H_2O_2 production. Formation of O_2^- or H_2O_2 from a variety of redox components besides the enzymes mentioned above has been detected *in vitro* (Dixon, 1971; Boveris *et al.*, 1972; Nakamura & Kimura, 1972; Loschen *et al.*, 1974). Since these reactions belong to the category of 'auto-oxidation', apparent K_m values of oxygen may be rather high, as was observed in the case of the microsomal H_2O_2 production ($K_m = 50\,\mu$M) (Thurman *et al.*, 1972), and indeed hyperbaric oxygen greatly augmented H_2O_2 generation in isolated mitochondrial fractions under certain conditions (Boveris & Chance, 1973).

Therefore in the present study we have attempted to identify the probable sources of H_2O_2 in the perfused livers of normal rats and in those from rats subjected to various pretreatments. The effect of hyperoxic and hypoxic conditions on the formation of H_2O_2 was also tested. Although the perfusion system employed was the 'haemoglobin-free', 'non-circulatory' system, in which artificial washout phenomena of metabolic intermediates and anomalously low metabolic rates may occur, our attempts were expanded to compare the results in this study with those obtained in the liver *in situ*, as reported in the accompanying paper (Oshino *et al.*, 1975).

Materials and Methods

Pretreatment of animals

Male Holzman rats (200–260g in weight) maintained on a commercial diet *ad libitum* were designated 'fed' rats, whereas 'starved' rats were those deprived of food overnight. Phenobarbital (8mg/100g body wt.) was injected intraperitoneally daily for 3 days at 10:00a.m., to minimize the effect of the drug on food uptake. Such pretreated rats were used on day 4 as 'phenobarbital-pretreated fed' rats.

Perfusion and optical systems

The 'haemoglobin-free', 'non-circulatory' perfusion of rat liver has been reported previously (Sies & Chance, 1970; Sies *et al.*, 1973). Dual-wavelength spectrophotometry was used as described previously (Theorell *et al.*, 1972) for the simultaneous measurement of the catalase–H_2O_2 intermediate (Compound I) at 660–640nm and cytochrome *c* at 550–540nm. The latter provides a constant monitor of the occurrence of possible intracellular hypoxia. Oxygen concentration in the influent or effluent fluid was determined by a Clark-type oxygen electrode inserted into the perfusion system before, or after, the liver. Substrates were usually added to the perfusion-fluid reservoir, whereas other substances were infused into the perfusion medium immediately before entering the liver, at constant rates calculated to provide the desired arterial concentrations.

Liver perfusion under hyperoxic conditions

The perfusion and detecting systems were as described for normal perfusion procedure, except that Krebs–Ringer phosphate buffer (Krebs & Henseleit, 1932) rather than saline–bicarbonate solution (Sies & Chance, 1970) was used to avoid problems in gassing with CO_2 mixture in hyperbaric conditions. Reservoirs for the perfusion fluid were placed inside the Bethlehem Steel pressure chamber (model 1836-HP), which was maintained at 30±0.5°C. However, for safety reasons the peristaltic pump was placed outside the chamber. The perfusion fluid, equilibrated with the desired pO_2, passed through an inlet in the pressure vessel wall, to the exterior, through the pump and then through a further insert in the chamber wall to the liver inside the chamber. The flow rate of perfusion was monitored and maintained constant by a flow regulator in the tubing system between the peristaltic pump and the chamber. Flow regulation was necessary, as control experiments showed that large changes in flow rate occurred with this system on pressurization. The optical measurements were performed with the aid of optical light-pipes sealed into the chamber wall.

Catalase content in liver

The method reported previously (Oshino *et al.*, 1973*a*) was used with some modifications. After perfusion of the liver with the saline–bicarbonate

mixture, water was removed from the liver as thoroughly as possible by blotting with sheets of filter paper. After weighing and cooling the liver, a 20% (w/v) homogenate was prepared by using a mixture of 1% Triton X-100, 1% sodium deoxycholate, 20mM-methanol and 0.1M-potassium phosphate buffer, pH7.5. The homogenate was then centrifuged at 10000*g* for 10min and 104000*g* for 60min. The catalase haem concentration in the clear supernatant obtained was measured at 660–630nm as described by Oshino *et al.* (1973*a*). Measurements were taken of four different dilution factors for each sample.

Rate of H_2O_2 production in the perfused liver

Measurements of the rate of H_2O_2 production were made by the steady-state titration of the catalase–H_2O_2 intermediate at 660–640nm by using methanol as a hydrogen donor. The method and the properties of the reactions included were reported in detail previously (Sies *et al.*, 1973; Oshino *et al.*, 1973*a,b*). In short, methanol was infused at concentrations sufficient to decompose the catalase–H_2O_2 completely in the liver in which a steady-state condition with a particular substrate or at a desired oxygen concentration had been established. The methanol concentration was then decreased in a stepwise fashion to zero, usually by six to eight steps. As the concentration of methanol was decreased, an increased amount of the catalase–H_2O_2 intermediate was established. For each step, 3–5min was allowed to establish a new steady-state. Such a titration can be seen in Fig. 2. Infusion rates of methanol were between 1.5 and 300μl/min added to perfusion fluid flowing at 28–36ml/min in various experiments. The saturation of the catalase–H_2O_2 formation was measured by infusing urate (at a final concentration of 0.5mM) or glycollate (at a final concentration of 1mM) after methanol titration. We could then plot the fractional saturation of the catalase–H_2O_2 intermediate as a function of the infused methanol concentrations. In this way the methanol concentration ($a_{1/2}$) required for a half-maximal saturation of the catalase–H_2O_2 formation was obtained. The catalytic-centre activity of the catalase reaction was estimated from the $a_{1/2}$ value, by using eqn. (4). The rate of H_2O_2 production was calculated from the catalytic-centre activity by using the catalase haem content in liver shown in Table 1.

Rate of ethanol oxidation in the perfused liver

The rate of ethanol oxidation independent of the catalase pathway was determined by comparing the titration curve of the catalase–H_2O_2 and ethanol with the titration curve with methanol. The principle of the determination was based on the fact that the efficiency of methanol and ethanol as hydrogen donors for the catalase 'peroxidatic' reaction is almost identical (Chance, 1949; Chance & Oshino, 1973) and thus the difference between the curves is due to disappearance of ethanol, mostly via alcohol dehydrogenase. The rationale is amplified in the Results section and the validity of the method has been presented in detail elsewhere (Lindros *et al.*, 1974).

K_m value of glycollate oxidase for O_2

Glycollate oxidase of rat liver was partially purified from isolated peroxisomal–mitochondrial fraction (5000*g* for 10min to 8000*g* for 10min) by sonication, $(NH_4)_2SO_4$ fractionation and subsequent DEAE-cellulose column chromatography as described by Nakano *et al.* (1968). The rate of H_2O_2 production from the glycollate oxidase reaction was measured fluorimetrically by coupling the reaction with the horseradish peroxidase–scopoletine reaction (Loschen *et al.*, 1971). Fluorescence intensity was calibrated with a known concentration of H_2O_2, which was determined by the cytochrome *c*–cytochrome *c* peroxidase method (Oshino *et al.*, 1973*b*). The reaction mixture contained, in a final volume of 30ml, Krebs–Ringer bicarbonate buffer (or 0.1M-potassium phosphate buffer, pH7.4), 1mM (or 0.3mM)-sodium glycollate, 2μM-scopoletine, 0.05μM-horseradish peroxidase and appropriate amounts of glycollate oxidase preparation. The observation cuvette was a cylindrical shape with a total volume of 35ml and was closed with a rubber stopper having a gas inlet and outlet. A Clark-type oxygen electrode was inserted into the reaction mixture through the stopper. The reaction mixture was bubbled with a stream of desired gas mixture (N_2, O_2 and 5% CO_2), and when the O_2 concentration reached the desired point the tube for gassing was removed and the cuvette was closed while stirring continuously under a stream of the gas mixture. When the O_2 concentration reached a steady state, scopoletine, horseradish peroxidase and glycollate oxidase were added and the oxidation of scopoletine by the peroxidatic reaction, which is accompanied by a decrease in fluorescence intensity, was measured at 460nm with excitation at 366nm. Although the preparation showed contamination with catalase, the concentrations of glycollate oxidase used were so small (H_2O_2 production of 1–0.5μM/min at 23°C) that almost all of the H_2O_2 produced was detected by horseradish peroxidase under this condition.

Other procedures

Urate oxidation by perfused liver was determined by measuring and comparing the influent and effluent urate concentrations spectrophotometrically at 293–320nm. Glutathione, which leaked out from the liver

to the perfusate, was determined by the method of Owens & Belcher (1965).

Materials

Purified horseradish peroxidase was kindly supplied by Dr. M. Tamura of Johnson Research Foundation. Scopoletine was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and aminopyrine was from Aldrich Chemical Company, Milwaukee, Wis., U.S.A. The other materials used were reagent grade.

Results

Catalase content in rat liver

An accurate determination of the catalase haem content in the liver is required for the measurement of the rate of H_2O_2 production by the perfused liver. Our previous measurement of the catalase content gave a rather lower value (13 nmol of haem/g of liver) (Oshino *et al.*, 1973*a*) than those reported by others (13–22 nmol/g of liver) (Price *et al.*, 1962; Higashi & Peters, 1963; Sies, 1971). This discrepancy may be due to variations in water content of the liver after perfusion, as well as seasonal variation of the catalase content reported by Price *et al.* (1962). The determination was repeated and the results obtained are presented (Table 1) as the content per g of liver and also as the content per 100 g body wt. The liver wet weight as measured after perfusion is 4.0 g/100 g body wt. in the fed rats and 3.0 g/100 g body wt. in the starved rats. Corresponding to these changes in the liver weight, the specific content of catalase alters from 19.2 in the fed rats to 24.8 nmol of haem/g of liver in the starved rats. However, no significant difference was found between 78 nmol for the fed rats and 74 nmol for the starved rats when compared on a 100 g body wt. basis.

In fed rats pretreated with phenobarbital for 3 days, the catalase haem content was 19.6 nmol/g of liver. However, the ratio of liver weight to body weight increased and thus liver catalase content per 100 g body wt. rose to 102 nmol. These values (Table 1) were used for calculation of the H_2O_2 production in the present study.

Correlation between the rates of urate oxidation and of H_2O_2 production

The validity of the direct spectrophotometric readout method was checked against an alternative procedure for determining H_2O_2 production. The second method chosen was the experimental measurement of the rate of urate oxidation by the perfused liver. In Fig. 1 the rates of H_2O_2 production determined by the catalase method are compared

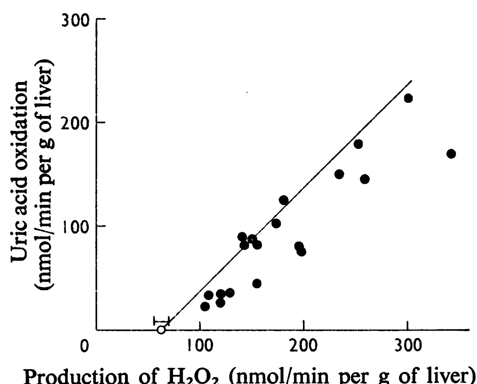


Fig. 1. *Rates of uric acid oxidation and of H_2O_2 production measured in the perfused liver*

The livers of fed rats were perfused with various concentrations of urate (0–100 μM) added to the saline–bicarbonate buffer. The temperature of the perfused liver was 30°C. The flow rate of the perfusate was 28–32 ml/min. The rates of urate oxidation and H_2O_2 production were measured as described in the Materials and Methods section. The theoretical values are given as a solid line on 1:1 stoicheiometry of urate oxidation to H_2O_2 production. The results obtained in seven separate experiments are given in this Figure. Each point represents one independent measurement. The rate of H_2O_2 production without urate (○) was 66±6 nmol/min per g of liver in seven experiments.

Table 1. *Catalase content in rat liver*

After perfusion of liver with saline–bicarbonate buffer for 5 min, catalase content was determined spectrophotometrically as described in the Materials and Methods section. The results are given as means ±S.E.M. with the numbers of experiments in parentheses.

Condition	Liver wt. (g wet wt./100 g body wt.)	Catalase haem content (nmol/g of liver)	Catalase haem content (nmol/100 g body wt.)
Fed	4.05±0.05 (11)	19.2±0.60 (5)	76.8±2.4 (5)
Starved	3.0 ±0.19 (9)	24.8±0.69 (6)	74.4±2.1 (6)
Phenobarbital (3 days)	5.2 ±0.37 (6)	19.6±0.33 (6)	101.7±1.7 (6)

Table 2. *H_2O_2 production in the perfused livers of fed, starved and phenobarbital-pretreated rats*

The rates of H_2O_2 production were measured based on the equation: $1/e \cdot dx_n/dt = 31.5a_{1/2}$. $a_{1/2}$, methanol concentration required for half-maximal saturation of the catalase–H_2O_2 intermediate; e, catalase content in liver (see Table 1); dx_n/dt, rate of H_2O_2 production. Livers were perfused with saline–bicarbonate solution containing 1.0mM-lactate and 0.15mM-pyruvate at 30°C. The results are given as means±S.E.M. with the numbers of experiments given in parentheses.

Conditions		$a_{1/2}$ (mM)	Catalytic-centre activity ($1/e \cdot dx_n/d_t$) (min^{-1})	Rate of H_2O_2 production (nmol/min per g of liver)	(nmol/min per 100g body wt.)
Fed	(17)	0.135±0.009	4.28±0.27	82.2±5.2	332.9±21.0
Starved	(6)	0.065±0.008	2.05±0.25	50.8±6.2	154.4±18.7
Phenobarbital (3 days)	(6)	0.085±0.010	2.68±0.31	52.4±6.2	272.5±32.0

with the rates of urate oxidation, as measured by the difference in urate concentrations between influent and effluent perfusate at various influent urate concentrations. The rate of endogenous H_2O_2 production without lactate or pyruvate was 66nmol/min per g of liver. Experimental data obtained in the presence of various concentrations of urate were distributed near and slightly below the theoretical values (solid line) in this plot, suggesting that some underestimation in the rate of uric acid oxidation or overestimation in the catalase content may be involved. It should be noted that the rate of urate oxidation depends on whether or not whole liver lobes are thoroughly perfused, and will be lower when perfusion is less than complete. The steady state of the catalase–H_2O_2 intermediate, however, was measured through a portion of the largest liver lobe with simultaneous measurement of the redox state of cytochrome *c*, by which normoxic condition of the perfused liver lobe could be confirmed. Therefore we consider that the deviation of the experimental points from the theoretical values are most likely due to a slight underestimation in the rate of urate oxidation.

H_2O_2 production in the perfused livers of the fed and starved rats

The catalytic-centre activity of the catalase reaction was found to be significantly lower in the perfused liver from starved rats compared with fed rats, under the conditions described in Table 2. A portion of the decrease in turnover number resulted from the increase in the specific content of liver catalase in the starved rats, but when calculated in terms of 100g body wt. the rate of H_2O_2 production still corresponded to only 46% the rate observed in fed rats. In the fed rats the rate of H_2O_2 production was 82nmol/min per g of liver or 333nmol/min per 100g body wt. with 1mM-lactate and 0.15mM-pyruvate.

Microsomal H_2O_2 production in the perfused liver: effect of phenobarbital pretreatment

It is known that pretreatment of rats with phenobarbital causes a profound increase in the endoplasmic reticulum (Remmer & Merker, 1963; Conney & Burns, 1959) and thereby increases the liver content of NADPH–cytochrome *c* reductase and cytochrome *P*-450 (Orrenius *et al.*, 1965), which are components of the microsomal drug-hydroxylation system (Estabrook *et al.*, 1963). In measurements with isolated microsomal fractions *in vitro*, this pretreatment resulted in a significant increase in the rate of NADPH-dependent H_2O_2 production (Hildebrandt *et al.*, 1973). As shown in Table 2, however, we could not detect any increased H_2O_2 production in the perfused liver of fed rats which were pretreated with phenobarbital for 3 days. Indeed H_2O_2 production was decreased after pretreatment with phenobarbital. The specific rate of H_2O_2 production observed with 1mM-lactate and 0.15mM-pyruvate was 50.8nmol/min per g of liver or 272nmol/min per 100g body wt. Similar experiments where the titration of the catalase–H_2O_2 intermediate was carried out with formate, which is another hydrogen donor for catalase but may not be a substrate for 'microsomal ethanol-oxidizing system' (Lieber & DeCarli, 1970), confirmed this result; the formate concentration required for half-maximal formation of the catalase–H_2O_2 intermediate was 1.8mM for the liver of fed rats and 1.3mM for the phenobarbital-pretreated rats, which indicated that H_2O_2 production was less rapid in the latter condition. This latter result appears to eliminate the possibility that an enhanced oxidation of methanol by 'microsomal ethanol-oxidizing system' caused an underestimation in our measurement.

Microsomal H_2O_2 production: effect of aminopyrine on the rate of H_2O_2 production

As described above, an increase in the content of endoplasmic reticulum was not accompanied by an

increase in the rate of H_2O_2 production, indicating that H_2O_2 production from the microsomal electron-transfer system is negligible in the perfused liver. To confirm this observation, the effect of aminopyrine was tested on the rate of H_2O_2 production in the perfused liver from control fed rats. The $E_{660-640}$ decreased on infusion of aminopyrine; however, this absorbance change was also observed in the presence of 10mM-ethanol, where the steady-state concentration of the catalase–H_2O_2 intermediate was virtually zero. The absorbance change observed with aminopyrine infusion corresponded to 10–20% of the maximal change associated with the catalase–H_2O_2 intermediate, and a half-maximal change in the absorbance was observed with aminopyrine concentration of approx. 0.15mM. The change in O_2 consumption accompanying the absorbance change induced by 0.2mM-aminopyrine was 0.36μmol of O_2/min per g of liver (a mean value of three separate experiments). Therefore aminopyrine-induced change in $E_{660-640}$ may be attributed to the substrate-induced spectral change of cytochrome *P*-450 in this wavelength pair (Waterman *et al.*, 1973). In spite of such an interaction of the drug with cytochrome *P*-450 in the perfused liver, the rate of H_2O_2 production was unchanged in the presence and absence of aminopyrine; the $a_{1/2}$ values were 0.13mM in both conditions.

As reported by Sies *et al.* (1972) interaction of peroxides with glutathione peroxidase in the perfused liver is accompanied by a release of oxidized glutathione into the effluent perfusate. There was a possibility that H_2O_2 produced from the microsomal electron-transfer system could be specifically detoxified through the glutathione peroxidase system. However, only a very slight change in the rate of glutathione release was observed in the presence of 0.5mM-aminopyrine; the rates were 9.9 and 8.3nmol/min per g of liver with and without aminopyrine, respectively (mean values of three separate experiments).

Effect of hyperbaric oxygen on the rate of H_2O_2 production

In view of the observation made previously that the rates of H_2O_2 generation in isolated mitochondrial fractions were increased by hyperbaric oxygen (Boveris & Chance, 1973), the effect of hyperoxic conditions on the intact perfused liver was determined by a special apparatus, which permits perfusion under pressure up to 5×10^5Pa. An experiment performed at ambient pressures and then at high-pressure O_2 is shown in Fig. 2. An initial titration with the desired substrates (a mixture of octanoate, urate, glycollate, lactate and pyruvate in this particular experiment) was carried out at ambient pressure, whereas subsequent titrations were performed under increasing oxygen pressures at 2.3×10^5, 3.7×10^5 and 5×10^5Pa. As seen in these traces, compression itself does not interfere with measurement if the flow rate of perfusate is maintained constant, as discussed in the Materials and Methods section.

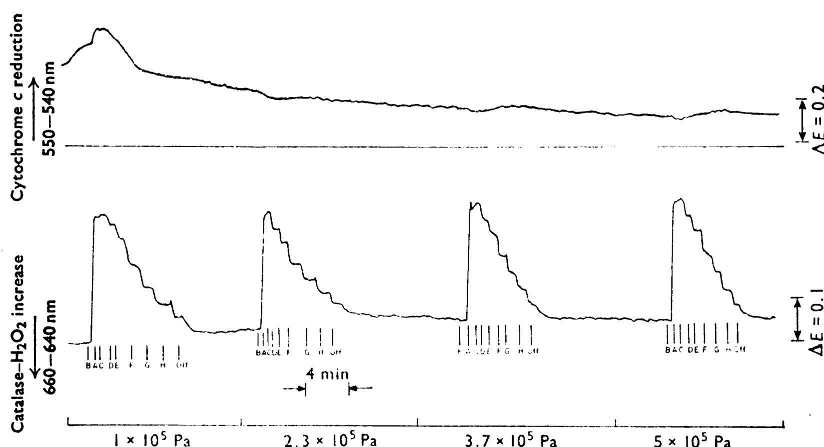


Fig. 2. *Methanol titration of the steady state of catalase–H_2O_2 intermediate under hyperoxic conditions*

The livers of fed rats were perfused with a mixture of urate (0.75mM), glycollate (1mM), octanoate (1mM), lactate (1mM) and pyruvate (0.15mM) added to the Krebs–Ringer phosphate buffer under O_2 pressures of 1×10^5, 2.3×10^5, 3.7×10^5 and 5×10^5Pa, as described in the Figure. The flow rate of the perfusate was maintained at 34ml/min. The methanol concentrations infused were changed at the indicated points: A, 132; B, 66; C, 28; D, 13; E, 6.6; F, 2.8; G, 1.3; H, 0.66mM. This trace was representative of three separate perfusions under the same conditions.

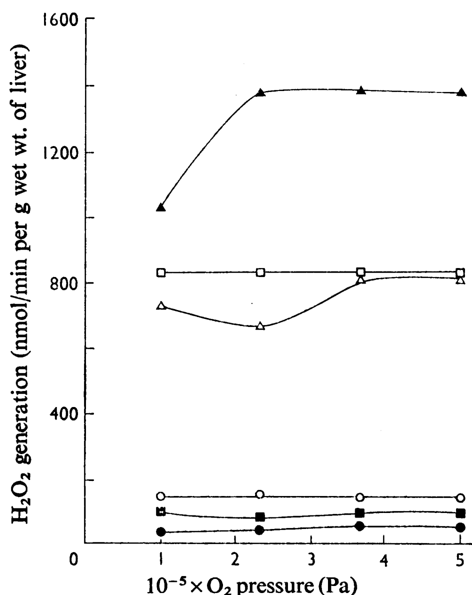


Fig. 3. *Effect of O_2 pressures on the rate of H_2O_2 production with various substrates*

The rate of H_2O_2 production was measured, as described in the Materials and Methods section, from the results of the methanol-titration experiments as shown in Fig. 2. Each point was a mean value of two to four separate experiments. □, 0.75mM-urate; △, 1mM-glycollate; ○, 1mM-octanoate; ■, 1mM-lactate plus 0.15mM-pyruvate; ●, 5mM-glucose or 2mM-propan-1-ol or without substrate; ▲, a mixture of urate, glycollate, octanoate, lactate and pyruvate.

Fig. 3 summarizes the results which were obtained with various substrates, alone or combined, under the hyperoxic conditions mentioned above. It may be seen that the rate of endogenous H_2O_2 production was not affected by O_2 concentrations above 1×10^5 Pa. Glucose addition had no effect. Propan-1-ol at 2mM is known to cause a reduction of NAD^+ to NADH due to the liver alcohol dehydrogenase reaction and the extent of the nicotinamide nucleotide reduction is comparable with that observed by an infusion of 2mM-ethanol, which is near the maximal reduction value achieved with ethanol infusion (Lindros *et al.*, 1974). Such a high reduction state of the $NADH/NAD^+$ couple, however, did not produce any enhancement of H_2O_2 production, either at 1×10^5 Pa or under hyperbaric O_2. Octanoate (1mM), as reported previously (Oshino *et al.*, 1973*a*), caused an increase in the H_2O_2 production, presumably from mitochondria, whereas substrates for peroxisomal oxidases such as urate and glycollate produced large increases in the rate of H_2O_2 production at ambient pressure. However, hyperoxia up to 5×10^5Pa did not increase the H_2O_2 production supported by any of these diverse substrates. Only when a mixture of the various substrates was used did hyperoxia exert an apparently stimulant effect on the H_2O_2 generation. Under these conditions increasing O_2 to 2.3×10^5Pa caused some increase of the H_2O_2 production with no additional effect at higher pressure. However, we believe this is due to slight hypoxia occurring initially due to the greatly increased O_2 consumption caused by the summation of the activities of various oxidases, and due to the low affinity of glycollate oxidase for O_2. This effect is illustrated in the experiment shown in Fig. 2. The reduction state of cytochrome *c* appeared to be initially somewhat increased and unstable. Infusion of a high concentration of methanol under these conditions was accompanied by a further reduction of cytochrome *c*, due to decreased O_2 production in the conversion of the catalase reaction from its 'catalatic' into its 'peroxidatic' mode, suggesting that slight hypoxia was induced under these conditions. Such a reduction of cytochrome *c* on addition of hydrogen donor was not seen in other titrations when the perfusate was equilibrated with 100% O_2. With O_2 pressure at and above 2.3×10^5Pa, cytochrome *c* became and remained oxidized, with no reduction during titration, and the rate of H_2O_2 production remained constant at 1.5μmol/min per g of liver.

H_2O_2 production under hypoxic conditions

The existence of tissue gradients and oxygen consumption by tissue invalidates the use of the O_2 concentration in the influent fluid to provide quantitative data on O_2 concentration at the cellular level. This is especially true where low O_2 concentrations are used to equilibrate the perfusing fluid. To measure the effect of low O_2 concentrations on the rate of H_2O_2 production rate in perfused liver therefore the glycollate oxidase activity and the redox state of cytochrome *c* were used as intracellular references for O_2. The effect of O_2 concentrations on the activity of rat liver glycollate oxidase is shown in Fig. 4. As suggested by de Duve & Baudhuin (1966), this enzyme showed a low affinity for O_2 and the half-maximal activities were observed at an O_2 concentration of approx. 0.3mM in Krebs–Ringer bicarbonate buffer and also in 0.1M-potassium phosphate buffer, pH 7.4 (K_m value obtained in a double-reciprocal plot was approx. 0.4mM).

Fig. 5 shows the representative titration curves in the perfused liver when the influent fluid was equilibrated with 95, 74, 55 or 42% O_2. With the former three O_2 concentrations, the titration curves are monotonic and would be linear on a double-logarithmic co-ordinate as obtained in a homogeneous system such as in a purified catalase system (Oshino

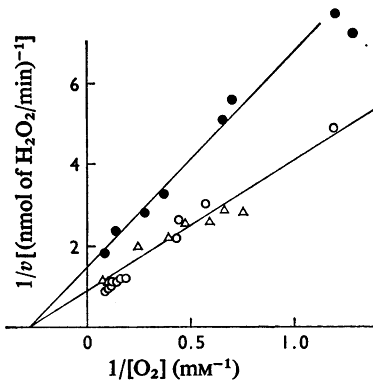


Fig. 4. *Effect of O_2 concentrations on the activity of rat liver glycollate oxidase*

The activity was measured fluorimetrically at 23°C under various O_2 concentrations as described in the Materials and Methods section. ●, Glycollate (1mM) in Krebs–Ringer bicarbonate buffer, pH 7.4; ○, glycollate (1mM) in 0.1M-potassium phosphate buffer, pH 7.4; △, glycollate (0.3mM) in 0.1M-potassium phosphate buffer, pH 7.4.

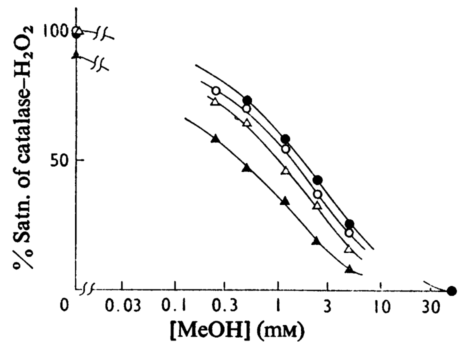


Fig. 5. *Methanol-titration curves of the catalase–H_2O_2 intermediate under hypoxic conditions*

The liver was perfused with glycollate (1mM), lactate (1mM) and pyruvate (0.15mM) added to the saline–bicarbonate solution. The oxygen concentrations in the influent perfusate were changed by equilibrating with gas mixtures of different ratio of O_2 and N_2 (CO_2 was maintained at 5%). ●, 95% O_2; ○, 74% O_2; △, 55% O_2; ▲, 42% O_2.

et al., 1973*a*). This suggests that the turnover number of the reaction of individual catalase molecules is similar throughout the tissue and thus suggests that the cellular O_2 is neither high enough to operate the glycollate oxidase at the maximal rate nor low enough to ensure that no glycollate oxidase is operating.

The rates of H_2O_2 production measured from a series of such titration experiments are shown in Fig. 6. Glycollate-dependent H_2O_2 production decreased as O_2 concentration in the influent fluid was decreased, and reached its half-maximal rate at an O_2 concentration corresponding to 47% saturation (Fig. 6*a*). This latter condition produced approx. 10% increase in the reduction state of cytochrome *c* as compared with the extent of reduction observed by aerobic to anaerobic transition. According to Sugano *et al.* (1974), who determined the relation between O_2 concentration and the redox state of mitochondrial cytochrome *c* in the presence of various substrates, a half-maximal reduction of mitochondrial cytochrome *c* in the presence of succinate, malate and glutamate was observed at an O_2 concentration of 0.4μM. When measured with respiring yeast cells, a half-maximal reduction of cytochrome *c* was observed at an O_2 concentration of approx. 1μM (Oshino *et al.*, 1973*c*). Thus 10% reduction of cytochrome *c* observed in the perfused liver may suggest that the oxygen concentration near the mitochondrial compartment is at least below 20μM. This latter O_2 concentration is approx. 15 times lower than the O_2 concentration indicated by the glycollate oxidase activity. However, as reported by Owen & Wilson (1974), the redox state of mitochondrial cytochromes is affected not only by O_2 concentration but also by the ratios of (NADH)/(NAD^+) and (ATP)/(ADP)(P_i). Whether this increase in the reduction state of cytochrome *c* observed in the perfused liver was solely due to a decrease in the O_2 concentration or was affected significantly by changes in (NADH)/(NAD^+) and (ATP)/(ADP)(P_i) is at present not clear.

If mitochondrial O_2 consumption is inhibited, as by antimycin A, the diffusion gradient of O_2 may be broadened. Continual infusion of antimycin A and glycollate shifted the apparent half-maximal O_2 concentration for H_2O_2 production from 47% to 30% in the influent perfusate. The O_2 concentration in the peroxisomal spaces as indicated by the glycollate oxidase activity under this condition appears to be close to that in the influent perfusate (Fig. 6*b*). A significant increase in the reduction state of cytochrome *c* was observed only when the O_2 concentration of the influent fluid fell to below 10% saturation. In the presence of antimycin A therefore the curves of glycollate oxidase activity and of the redox state of cytochrome *c* are clearly separated from each other. With isolated mitochondrial fractions, 50% reduction of cytochrome *c* in the presence of antimycin A was observed at an O_2 concentration of 0.04μM (Sugano *et al.*, 1974).

In contrast with the glycollate oxidase reaction, the H_2O_2 generation supported by lactate and pyruvate did not decrease until a considerable cytochrome *c* reduction occurred (Fig. 6*c*). At half-maximal rate of H_2O_2 production, the O_2 concentration in liver corresponded to that required to produce

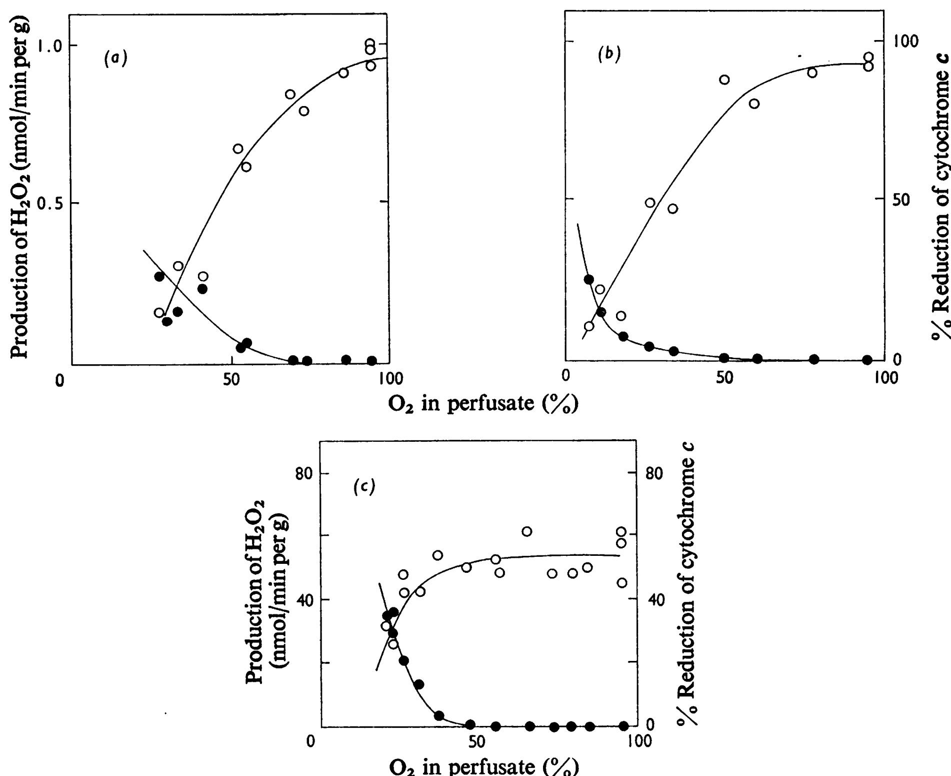


Fig. 6. *Effects of low oxygen concentrations on the H_2O_2 production*

The rates of H_2O_2 production under various O_2 concentrations (○) were measured from the results of the methanol-titration experiments represented in Fig. 5. For the % reduction of cytochrome *c* (●), the extents of reduction observed under aerobic and anaerobic conditions were tentatively taken as 0 and 100% respectively. Antimycin A in 70% dimethylformamide solution was infused at a concentration of 8 μM during the titration experiments. (*a*) 1 mM-Glycollate; (*b*) 1 mM-glycollate plus antimycin A; (*c*) 1 mM-lactate plus 0.15 mM-pyruvate. The results obtained in three (*a*), three (*b*) and seven (*c*) separate perfusions are given in this Figure.

approx. 40% increase in the reduction state of cytochrome *c*. When urate was used as a substrate, the half-maximal rate of H_2O_2 production in the perfused liver was observed at an O_2 concentration in the influent fluid of 40% saturation, in which approx. 25% increase in the reduction state of cytochrome *c* was observed. The situation was between those observed with glycollate and with lactate and pyruvate in Fig. 6.

Contribution of the catalase 'peroxidatic' reaction in ethanol metabolism

As seen in Fig. 2 and in a previous publication (Oshino *et al.*, 1973*a*), an infusion of ethanol or methanol causes a decrease in the steady-state concentration of the catalase–H_2O_2 intermediate, which provides direct evidence for the transition of the catalase reaction from its 'catalatic' to its 'peroxidatic' mode (Oshino *et al.*, 1973*b*). It is also obvious that in the presence of excess of ethanol the rate of the catalase-dependent ethanol oxidation must approach the rate of H_2O_2 production. As described under 'Effect of hyperbaric O_2 on the rate of H_2O_2 production', the rate of H_2O_2 production and thus the maximal rate of the catalase-dependent ethanol oxidation varied, depending on the kinds of substrate available for H_2O_2 production, from 0.06 to 1.5 μmol/min per g of liver. Methanol and ethanol are equally effective as hydrogen donors for the peroxidatic reaction (Chance, 1949; Chance & Oshino, 1973). But as ethanol is metabolized relatively rapidly by alcohol dehydrogenase, the concentration of ethanol which must be continually infused to produce a certain steady-state concentration of the catalase–H_2O_2 intermediate will be higher than that

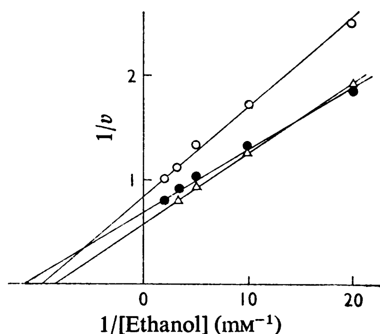


Fig. 7. *Rate of the catalase-independent ethanol oxidation in the perfused liver*

The determination method was as described in the Materials and Methods section. Each experimental point represents a mean value of four separate experiments. The livers of the fed (●), starved (△) and phenobarbital-pretreated fed (○) rats were used. The $V_{max.}$ calculated from these plots were 1.2 for phenobarbital-pretreated, 1.5 for fed and 1.8 μmol/min per g of liver for starved rats respectively.

of methanol necessary to maintain the same steady state of the catalase–H_2O_2 intermediate. The difference between the concentrations of ethanol and of methanol to produce a certain steady state of the catalase–H_2O_2 will be a reflection of the rate of ethanol consumption by pathways other than catalase, notably alcohol dehydrogenase. The ethanol oxidation rate at each steady state was obtained by multiplying this concentration difference by the flow rate of perfusate and then dividing by the wet weight of liver. The maximal rate ($V_{max.}$) was measured by plotting the result in a double-reciprocal plot as described previously (Lindros *et al.*, 1974). As shown in Fig. 7, $V_{max.}$ of the ethanol oxidation by alcohol dehydrogenase is 1.45 and 1.8 μmol/min per g of liver for fed and starved rats respectively. The phenobarbital pretreatment caused almost no change in the alcohol dehydrogenase-dependent ethanol oxidation (1.2 μmol/min per g of liver or 6.1 μmol/min per 100g body wt.). When comparing the rate of H_2O_2 production shown in Table 2 with the rate of the catalase-independent ethanol oxidation (Fig. 7), it is concluded that the contribution of the catalase 'peroxidatic' reaction in ethanol metabolism is approx. 3–6% of the alcohol dehydrogenase reaction when livers from starved or fed rats respectively metabolized lactate and pyruvate. However, it must be borne in mind that the rate of endogenous H_2O_2 production may be minimal in the 'non-circulatory' and 'haemoglobin-free' perfused system compared with those in more physiological perfusion systems. In fact, as will be seen in the accompanying paper (Oshino *et al.*, 1975), the contribution of the liver catalase in ethanol oxidation of anaesthetized rats increased to 10% of the total ethanol consumption in the whole body simply because the rate of endogenous H_2O_2 production in the liver *in situ* is approx. 0.38 μmol/min per g of liver, due presumably to a supply of circulatory substrates.

Discussion

Value of the method

Determination of the rate of H_2O_2 production by the usual chemical and enzymic methods (Chance & Maehly, 1955; Thurman *et al.*, 1972; Boveris *et al.*, 1972) are not applicable to the perfused liver system, where active H_2O_2-decomposing enzymes such as catalase and glutathione peroxidase function and decrease free H_2O_2 concentration to below 0.01 μM (Oshino *et al.*, 1973*a*). Spectrophotometric measurement of the catalase–H_2O_2 intermediate, which is directly involved in the H_2O_2 decomposition process of the liver, is the only method sensitive enough to detect free H_2O_2 in this very low concentration range. The validity of this direct-read-out technique as a quantitative method was confirmed in the present study by satisfactory agreement between the rate of H_2O_2 production calculated by the catalase method and the rate calculated from urate oxidation (Fig. 1). As Sies *et al.* (1972) pointed out, hepatic glutathione peroxidase also operates as a H_2O_2-decomposing system, and thus the amount of H_2O_2 detected by the present method has to be considered as the amount of H_2O_2 which is free to interact with catalase. However, H_2O_2 produced in the peroxisomes by urate oxidase was almost totally decomposed by the catalase pathway, as is evident from Fig. 1. Sies *et al.* (1974) reported that the rate of GSSG release associated with glutathione peroxidase action was not increased on an infusion of urate. In preliminary experiments recently carried out, the contribution of the glutathione peroxidase pathway in H_2O_2 metabolism was found to be not large in the perfused liver under normal conditions (N. Oshino & B. Chance, unpublished work).

The 'non-recirculatory' perfusion of the liver was used in these studies to facilitate the wash-out of one reagent in order that it be followed by another. Although this appears to be an inappropriate procedure in studying some metabolic systems based on the assumption of equilibrium in the whole system, it appears that a high degree of reproductibility was obtained in the repeated 'infusion' of various substrates. However, as mentioned previously (Oshino *et al.*, 1973*a*), some substrates for H_2O_2 production were washed out during the first 10min of perfusion. It may well be that depletion of unknown and unrecognized cofactors has occurred in these

experiments, and thus the rates of H_2O_2 production, especially without substrates, may be less than those which may be observable under physiological conditions.

Another problem in our perfusion system is that the perfusate does not contain blood or haemoglobin. These additions are believed to ensure an adequate oxygenation of the liver. However, the O_2 affinity of haemoglobin (P_{50}) is approx. 30mm of Hg (approx. 50μM), and hence a significant deoxygenation of haemoglobin does not occur until O_2 concentration decreases below 0.2mM. In other words the presence of haemoglobin may not increase free O_2 concentration significantly under normal perfusion conditions. Fig. 6 shows that the O_2 supply in our perfusion system is enough to maintain the glycollate oxidase activity at near maximal. Thus the O_2 concentration in the peroxisomal space in our perfused liver is, at least, higher than that expected in the liver of the air-breathing rat *in vivo*.

Origin of H_2O_2 in the liver cell

The origin of H_2O_2 in the perfused liver is of particular interest in relation to the progress in the study of lipid peroxidation and of the formation of radical species in O_2 metabolism. As an approximation from the results observed in subcellular fractions of rat liver, Boveris *et al.* (1972) estimated the rate of H_2O_2 production to be 12 for mitochondria, 42 for microsomal systems, 172 for peroxisomes and 4nmol/min per g of liver for supernatant fractions. Obviously the rate of H_2O_2 production from the peroxisome was not significant in the present system of liver perfusion unless urate or glycollate was infused. As reported in the accompanying paper (Oshino *et al.*, 1975), however, the rate of H_2O_2 production in the liver of anaesthetized rats is approx. 0.38μmol/min per g of liver, and more than half of this rate may be accounted for as being of peroxisomal origin.

In contrast with the results reported by Boveris *et al.* (1972), the contribution of the microsomal electron-transport system in H_2O_2 production seems not to be significant in the perfused liver. This conclusion is derived from several lines of evidence. An increase in the hepatic content of the drug-hydroxylation system by phenobarbital treatment caused no increase, and in fact caused a decrease in the rate of H_2O_2 production, which can be detected by the catalase–H_2O_2 intermediate (Table 2). Thurman & Scholz (1969) detected expected rates of drug hydroxylation in the perfused liver of the phenobarbital-pretreated fed rats, and Sies & Brauser (1970) could detect reduction of cytochrome *P*-450 under similar conditions. The aminopyrine-dependent oxygen uptake was measured in the present study and was as high as 0.36μmol/min per g of liver. In these conditions aminopyrine infusion did not produce an oxidation of nicotinamide nucleotide (a decrease in nicotinamide nucleotide fluorescence) as did t-butyl peroxide infusion. This latter substance is known to react with the glutathione peroxidase and thereby cause a remarkable oxidation of NADPH (Sies *et al.*, 1974). Therefore the supply of NADPH appears not to limit the microsomal H_2O_2 production in the perfused liver. The H_2O_2 production from isolated microsomal fraction has been reported to be approx. 2nmol/min per mg of protein (Boveris *et al.*, 1972) and to be increased up to 7–10nmol/min per mg of protein after phenobarbital pretreatment (Hildebrandt *et al.*, 1973). Assuming a value of 20mg of microsomal protein/g of liver, the expected rate of microsomal H_2O_2 production would be expected to exceed 140nmol/min per g of liver in the liver of phenobarbital-pretreated rats. This value cannot be explained by the values found in the present study. A hypothesis was recently reported that microsomal cytochrome *P*-450, during the drug-hydroxylation reaction, functions either in a 'peroxidase' or in an 'oxygenase' mode, depending on the spin state of cytochrome *P*-450 haem iron (Hildebrandt *et al.*, 1973). The effect of aminopyrine on the rate of H_2O_2 production was therefore examined and no difference was observed in the rate of H_2O_2 production with and without aminopyrine. The rate of GSSG release from the liver, which was ascribed to the glutathione peroxidase reaction (Sies *et al.*, 1972, 1974) was also unchanged under these conditions. The apparent K_m value of oxygen for the microsomal H_2O_2 production was reported to be 50μM (Thurman *et al.*, 1972), yet the rate of H_2O_2 production in perfused liver was not affected by decreasing O_2 concentration until a significant reduction of cytochrome *c* occurred, nor was it increased under hyperoxic conditions (Figs. 3 and 5). Taking all these lines of evidence together, it is concluded that the microsomal H_2O_2 production observed *in vitro* is not significant in the perfused liver.

In the perfused liver of the starved rats, the rate of H_2O_2 production is below half the value observed with fed rats (Table 2). Since the perfusion system is a 'non-circulatory' system, very low concentration of endogenous substrates for the peroxisomal oxidases may be expected after the first 10min perfusion (Oshino *et al.*, 1973*a*). This situation is also confirmed by the difference in O_2 requirements for H_2O_2 production between endogenous and glycollate-supplemented livers (Fig. 5). Thus the difference observed in fed and starved rats may have resulted from differences in the metabolic states of mitochondria of livers from fed and starved rats. Brauser *et al.* (1972) reported that the state of mitochondrial respiration is near State 3 (or State 2) in the perfused liver of starved rats and is closer to State 4 in fed rats (for definition of States, see Chance & Williams, 1956). In other words the redox states of

the mitochondrial electron carriers are lower in the latter than in the former. Clear stimulation of H_2O_2 production accompanied by transition of mitochondrial respiration from State 3 to 4 was observed *in vitro* (Chance & Oshino, 1971; Boveris *et al.*, 1972). Infusion of antimycin A or octanoate causes stimulation of H_2O_2 production in the perfused livers, associated with increases in the reduction states of several mitochondrial components (Oshino *et al.*, 1973*a*), whereas reduction of cytosolic NAD^+ by infusion of propan-1-ol did not affect the H_2O_2 production significantly (Fig. 3). Therefore endogenous H_2O_2 production detected in the perfused liver is most likely of mitochondrial origin.

H_2O_2 production and O_2 toxicity

One of the recent hypotheses concerning the primary mechanism of O_2 toxicity is that production of superoxide anion (O_2^-) is stimulated under hyperoxic conditions, which, in turn, creates modifications of various cellular constituents (Fridovich, 1972). In the presence of abundant superoxide dismutase (McCord & Fridovich, 1969), as in the perfused liver, the major fraction of O_2^- formed would be expected to be converted into H_2O_2 and O_2. In the system *in vitro*, a number of redox components show autooxidizability with O_2 and some of these produce O_2^- (McCord & Fridovich, 1970; Nakamura & Kimura, 1972; Misra & Fridovich, 1972). Fig. 3 shows, however, that increases in the oxygen pressure up to 5×10^5 Pa did not cause any clear change in the H_2O_2 production in the perfused liver. The intracellular O_2 concentrations under these conditions are not known; however, we found the K_m value of O_2 for glycollate oxidase reaction to be of the order of 0.4mM (Fig. 4), whereas the apparent half-maximal rate of glycollate-dependent H_2O_2 production was observed under the condition where liver was perfused with the medium saturated with 47% O_2 (Fig. 5). Therefore the perfusion at 5×10^5 Pa must certainly maintain high intracellular O_2 concentrations. The method used for detection of H_2O_2 is quite sensitive, as discussed above, and, hence, the negative results observed under hyperoxic conditions suggest that the primary events associated in the O_2-toxicity phenomenon are not produced via the reactions resulting in a formation of detectable quantities of H_2O_2 in the perfused liver. This observation agrees well with the results of experiments *in vivo* which are reported in an accompanying paper (Oshino *et al.*, 1975). However, production of O_2^- or H_2O_2 as an aetiological factor in hyperbaric oxygen toxicity cannot be completely ruled out. Fong *et al.* (1973) have shown that incubation of lysosomes with microsomal fractions in the presence of NADPH, EDTA and Fe^{3+} produce complete lysis of lysosomal membranes accompanied by peroxidation of lipid within 30min. When the rate of NADPH oxidation in the system and the presence of superoxide dismutase and catalase contaminating the fractions are considered, such a drastic effect observed *in vitro* suggests that only a small quantity of the hazardous oxygen metabolites could be sufficient to initiate oxygen toxicity.

Catalase and ethanol metabolism

Because of recent controversial arguments on 'microsomal ethanol-oxidizing system' (Lieber & & DeCarli, 1970; Lieber *et al.*, 1974; Thurman *et al.*, 1972, 1974), it is worth while discussing briefly the contribution of catalase in ethanol metabolism. With lactate and pyruvate as substrates the rate of H_2O_2 production was 51 and 82nmol/min per g of liver in the perfused livers of starved and fed rats respectively (Table 2). These values represent the maximal rate of the catalase-dependent ethanol oxidation under these conditions. The maximal rate of the catalase-independent ethanol oxidation measured in the present study was 1.5 and 1.8 μmol/min per g of liver or 5.9 and 5.4 μmol/min per 100g body wt. in the perfused liver of fed and starved rats at 30°C, respectively (Fig. 7). The sum of the rates in these two metabolic pathways is in agreement with the overall rate of ethanol oxidation measured in the perfused liver by another method (2.0 μmol/min per g of liver at 37°C; Lindros *et al.*, 1972). It should be noted that the catalase-independent ethanol oxidation measured in the present study was totally sensitive to 4-methylpyrazole (Oshino *et al.*, 1973*a*). Also, the determinations were performed with ethanol and methanol concentrations below 1mM, which is far lower than the apparent K_m value of ethanol for 'microsomal ethanol-oxidizing system' (8–12mM; Lieber & DeCarli, 1970; Thurman *et al.*, 1972), and hence the rate estimated by the present method should not include any significant activity due to this 'microsomal ethanol-oxidizing system'. These results therefore suggest that, in spite of recent arguments on the microsomal ethanol-oxidizing capacity based on the data obtained in isolated microsomal fraction or in partially purified system (Lieber *et al.*, 1974; Thurman *et al.*, 1974), neither the specific enzyme system (microsomal ethanol-oxidizing system) nor the activity of the catalase 'peroxidatic' reaction supported specifically by the microsomal H_2O_2 production appears to have significant physiological meaning in the haemoglobin-free perfused liver.

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