

## Properties of Glutathione Release Observed during Reduction of Organic Hydroperoxide, Demethylation of Aminopyrine and Oxidation of Some Substances in Perfused Rat Liver, and Their Implications for the Physiological Function of Catalase

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The enhanced reduction of t-butyl hydroperoxide by glutathione peroxidase is accompanied by a decrease in the cellular concentration of both glutathione and NADPH in isolated liver cells, resulting in the release of GSSG (oxidized glutathione) from the perfused rat liver. This phenomenon, first reported by H. Sies, C. Gerstenecker, H. Menzel & L. Flohé (1972) (*FEBS Lett.* 27, 171–175), can be observed under a variety of conditions, not only with the acceleration of the glutathione peroxidase reaction by organic peroxides, but also during the oxidation of glycollate and benzylamine, during demethylation of aminopyrine in the liver of the phenobarbital-pretreated rat and during oxidation of uric acid in the liver of the starved rat pretreated with 3-amino-1,2,4-triazole. The rate of release of GSSG is altered markedly by changes in the metabolic conditions which affect the rate of hepatic NADPH generation. Thus, regardless of whether achieved by enhanced oxidation of glutathione by glutathione peroxidase or by oxidation of NADPH through other metabolic pathways, an increase in the cellular concentration of GSSG appears to facilitate its release. It has been found that, in addition to the hexose monophosphate shunt, the mitochondrial NADH–NADP<sup>+</sup> transhydrogenase reaction plays an important role in supplying reducing equivalents to the glutathione peroxidase reaction and in maintaining the cellular oxidation–reduction state of nicotinamide nucleotides. Spectrophotometric analysis of the steady-state concentration of the catalase–H<sub>2</sub>O<sub>2</sub> intermediate with simultaneous measurement of the rate of release of GSSG leads to the conclusion that intracellular compartmentation of catalase in the peroxisomes and glutathione peroxidase in the cytosol and mitochondria distinguishes the reactivities of these enzymes one from the other, and facilitates their effective co-operation in hydroperoxide metabolism in the liver.

The erythrocyte is very susceptible to H<sub>2</sub>O<sub>2</sub> under certain conditions, even though it contains active catalase (Keilin & Hartree, 1945a; Mills & Randall, 1958). This observation, taken together with the discovery of the peroxidatic mode of reaction of catalase (Keilin & Hartree, 1945b, 1954), led those authors to deduce that the physiological function of this enzyme might be associated with its peroxidatic rather than its catalatic reaction. After the discovery of glutathione peroxidase (EC 1.11.1.9) by Mills (1957), Cohen & Hochstein (1963) reinvestigated the phenomenon of erythrocyte haemolysis caused by slowly diffusing H<sub>2</sub>O<sub>2</sub>, and concluded that under physiological conditions glutathione peroxidase linked to the pentose monophosphate-shunt activity

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represents the major pathway of H<sub>2</sub>O<sub>2</sub> metabolism in intact erythrocytes, leaving the physiological function of catalase in the erythrocyte obscure.

In our studies of the physiological function of catalase in rat liver, it was observed that the steady-state concentration of the catalase–H<sub>2</sub>O<sub>2</sub> intermediate Compound I (Chance, 1949) in the liver of the anaesthetized rat corresponded closely to the saturation concentration of the intermediate (Oshino *et al.*, 1975b). According to the calibration *in vitro* (Oshino *et al.*, 1974; Chance & Oshino, 1974), this observation is a direct indication that catalase *in situ* functions primarily in the catalatic rather than the peroxidatic mode. The rate of the catalase reaction with endogenously generated H<sub>2</sub>O<sub>2</sub> in the liver of the anaesthetized rat is 0.4 μmol of H<sub>2</sub>O<sub>2</sub>/min per g of liver (Oshino *et al.*, 1975b). In the perfused liver, increases in H<sub>2</sub>O<sub>2</sub> production obtained by infusion of uric acid were detected quantitatively as increased

turnover numbers of the catalase reaction (Oshino *et al.*, 1975a). Thus, in contrast with the case in the erythrocyte, these results seem to indicate an active role for the catalytic reaction of catalase in the liver.

However, it is also true that liver contains active glutathione peroxidase (Mills, 1960), which, with GSH\* as a specific hydrogen donor (Mills, 1957, 1959; Little & O'Brien, 1968), is capable of reducing many kinds of organic hydroperoxides (Little & O'Brien, 1968), including lipid peroxides (O'Brien & Little, 1969; Christophersen, 1969a) and peroxide derivatives of nucleic acids (Christophersen, 1969b) as well as  $H_2O_2$  (Mills, 1957; Flohé, 1969). Whereas catalase is concentrated in the peroxisome (de Duve & Baudhuin, 1966), 30% of the glutathione peroxidase is located in the matrix space of the mitochondrion and 70% in the cytosolic space (Green & O'Brien, 1970; Flohé & Schlegel, 1971). Thus the role of glutathione peroxidase in peroxide metabolism in the liver remains an open question.

Several clues to an approach to this problem were provided by observations that acceleration of the glutathione peroxidase reaction by either  $H_2O_2$  or alkyl hydroperoxide in the eye lens (Srivastava & Beutler, 1969a) and in the erythrocyte (Cohen & Hochstein, 1963; Jacob & Jandl, 1966; Srivastava & Beutler, 1969b; Srivastava *et al.*, 1974) resulted in the oxidation of cellular GSH with a subsequent release of GSSG from these preparations. Using t-butyl hydroperoxide and cumene hydroperoxides as substrates, Sies *et al.* (1972, 1973b, 1974) demonstrated that acceleration of the glutathione peroxidase reaction in perfused liver caused a release of GSSG into the effluent perfusion fluid. The rate of GSSG release appeared to be dependent on the rate of the glutathione peroxidase reaction.

A combination of the spectrophotometric measurement of the catalase- $H_2O_2$  intermediate Compound I, which is a specific indicator of the intracellular  $H_2O_2$  concentration (Sies *et al.*, 1973a; Oshino *et al.*, 1973a), with the monitoring of the glutathione peroxidase reaction via the rate of GSSG release provides a unique opportunity to analyse the interrelation of the reactions involved in hepatic peroxide metabolism. Since biological systems, such as the perfused liver and suspensions of liver cells, are not microscopically homogeneous, the experimental data have been evaluated in terms of (1) the way in which hydroperoxide is provided to the site of enzyme action, (2) the compartmentation of the two enzymes catalase and glutathione peroxidase, (3) the rate-limiting step of the glutathione peroxidase system, which consists of NADPH-generating systems, glutathione reductase and glutathione peroxidase, in liver, and (4) the characteristics of the enzyme reactions, i.e. the first-order

\* Abbreviations: GSH and GSSG, reduced and oxidized glutathione respectively.

kinetics of catalase with respect to  $H_2O_2$  concentration and the broad specificity of glutathione peroxidase towards organic hydroperoxides. In the present report, we describe the basic properties of the reactions that underly the phenomenon of t-butyl hydroperoxide-induced GSSG release in the perfused liver, and its implications for the possible roles of catalase and glutathione peroxidase in liver. These results and techniques have been applied to demonstrate the enhancement of lipid peroxidation in the perfused liver and lung under hyperbaric oxygenation, and the results of those applications are reported in Nishiki *et al.* (1976).

## Materials and Methods

### Materials

Enzymes and coenzymes were obtained from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or from Boehringer/Mannheim Co. (New York, NY, U.S.A.). 5,5'-Dithiobis-(2-nitrobenzoic acid) and t-butyl hydroperoxide were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and Matheson, Coleman and Bell Manufacturing Chemicals (East Rutherford, NJ, U.S.A.) respectively.

### Animal preparations

Male Sprague-Dawley rats (180–240 g), maintained on a commercial diet *ad libitum*, are designated fed rats, whereas starved rats are those deprived of food overnight. Phenobarbital (80 mg/kg body wt.) was injected intraperitoneally daily for 3 days at 10:00 h for phenobarbital-pretreated rats used on the fourth day. 3-Amino-1,2,4-triazole (1 g/kg body wt.) was injected intraperitoneally 1–2 h before the experiment for aminotriazole-pretreated rats.

### Perfusion and optical systems

The procedure and conditions of the haemoglobin-free non-circulating perfusion method for rat liver were as reported previously (Sies & Chance, 1970; Theorell *et al.*, 1972). In brief, the perfusion medium consisted of 115 mM-NaCl, 5.9 mM-KCl, 1.2 mM-MgCl<sub>2</sub>, 1.2 mM-NaH<sub>2</sub>PO<sub>4</sub>, 1.2 mM-Na<sub>2</sub>SO<sub>4</sub>, 2.5 mM-CaCl<sub>2</sub> and 25 mM-NaHCO<sub>3</sub>, equilibrated with O<sub>2</sub>/CO<sub>2</sub> (19:1). The flow rate of the perfusion was 28–34 ml/min and the temperature of the liver surface was 30 ± 2°C.

The time-sharing dual-wavelength spectrophotometer and fluorimeter (Theorell *et al.*, 1972; Chance *et al.*, 1975) was used for simultaneous spectrophotometric measurements of the oxidation-reduction state of cytochrome *c* at 550–540 nm and of the steady-state concentration of the catalase- $H_2O_2$  intermediate at 660–640 nm, and for fluorimetric

measurement of the redox state of nicotinamide nucleotides (excitation at 366 nm, emission at 460 nm). The fluorescence trace is described in terms of the intensity alteration relative to the initial intensity as a function of time. The concentration of  $O_2$  in the effluent was monitored by a Clark-type  $O_2$  electrode inserted into the tubing system just after the liver. Regardless of whether or not specific reference is made to such measurements, these four parameters were monitored in all the perfusion experiments to ensure well-perfused conditions.

#### *Determination of glutathione in the perfusate*

Since a specific release of GSSG rather than GSH has already been established by Sies *et al.* (1972, 1974) and was also confirmed in our preliminary experiments using the glutathione reductase (NADPH) reaction, total glutathione rather than GSSG was assayed as a routine in the effluent perfusate. Because the differences in glutathione concentrations were rather small, our assay procedures are described in detail, although the method itself is in principle the same as that reported by Owens & Belcher (1965) and others (Tietze, 1969; Berry & Friend, 1969).

The assay mixture, in a final volume of 2.5 ml, contained 0.05 M-potassium phosphate buffer, pH 7.4, 1 mM-EDTA, 0.1 mM-5,5'-dithiobis-(2-nitrobenzoic acid), 0.15 mM-NADPH and 0.1 ml or less of the effluent perfusate. After 2 min of preincubation, the reaction was initiated by the addition of 5.0  $\mu$ l of a solution of glutathione reductase. The  $A_{412}$  change was recorded for 3–5 min at 30°C with a full-scale deflexion of 0.5 absorbance unit and a chart speed of 1 cm/min. The reductase solution was prepared daily from a crystalline suspension of glutathione peroxidase (Boehringer/Mannheim; 5 mg/ml) by diluting it with 4 vol. of 0.1% bovine serum albumin/0.05 M-potassium phosphate buffer, pH 7.1. The linearity of the absorbance change with respect to time as well as glutathione concentration were examined carefully for each perfusate obtained under different conditions even in a single experiment, by using a standard of GSH (or GSSG) in a final concentration ranging from 0.1 to 2  $\mu$ M. The effluent perfusate (but not the fresh saline/bicarbonate perfusion solution) occasionally contained unidentified native inhibitor(s) of glutathione reductase; the degree of inhibition depended on both the glutathione concentration and the reductase concentration, and thus, in the presence of this inhibitor, the assay method becomes insensitive to concentrations of glutathione below a certain value. The existence of such inhibitor(s) in the effluent perfusate can be seen in the non-linear time-course of the absorbance change at low concentrations of glutathione, and/or by comparison of the absorbance changes

obtained with low concentrations of glutathione in the effluent perfusate with those in the presence of the fresh saline/bicarbonate solution. Under our assay conditions, the enzyme concentration was adjusted to produce  $\Delta A_{412}$  of 0.005–0.01 absorbance unit/min without glutathione and  $\Delta A_{412}$  of 0.02–0.03 absorbance unit with 0.2  $\mu$ M-GSH. The calculation of the glutathione concentration was based on the value of the internal standard determined for each set of experimental conditions, and the rate of glutathione release was expressed in terms of nmol of glutathione (equivalent to GSH)/min per g of liver in this report.

#### *Liver cell suspensions*

Liver cells were isolated by the procedure of Berry & Friend (1969) under the conditions described by Ontko (1972). About 85% of the cells were intact, as judged by Trypan Blue exclusion. The cells were incubated in the perfusion fluid described above, under a stream of  $O_2/CO_2$  (19:1) with magnetic stirring. The fluorimeter used in the perfusion experiment was rearranged for the measurement of nicotinamide nucleotide fluorescence from cell suspension at 30°C. *t*-Butyl hydroperoxide was either added in a pulse or infused continuously through 26-gauge stainless-steel tubing attached to an infusion pump, at rates between 1.5 and 30  $\mu$ l/min.

To measure the concentration of GSH and total glutathione in the suspension under different infusion rates of *t*-butyl hydroperoxide, 1.5 ml of an ice-cold solution of 7.5% metaphosphate was poured, at the desired moment, into 3.0 ml of the cell suspension in the measuring cuvette and, after centrifugation at 3000g for 10 min, the GSH concentration in the clear supernatant was determined by the method of Ellman (1959). By using the same sample, the total glutathione concentration was also measured as described for the assay of glutathione in the effluent perfusate. The ratio of GSH to total glutathione in normal cells was in the range 0.9–1.0 under our conditions, but the variation in the measured values seemed to be due to the inaccuracy of the 5,5'-dithiobis-(2-nitrobenzoic acid)-glutathione reductase method, since the liver homogenate, as described below, contained more inhibitor(s) of glutathione reductase than did the effluent perfusate.

#### *Suspensions of isolated mitochondria*

After perfusion of the rat liver with an ice-cold solution of 0.9% NaCl, a 10% (w/v) homogenate was prepared in 225 mM-mannitol, 75 mM-sucrose, 0.1 mM-EDTA and 10 mM-Tris/HCl buffer, pH 7.4. The mitochondrial fraction was obtained by serial centrifugations of the homogenate at 500g for 10 min and 5000g for 10 min. The supernatant and the

'fluffy' layer were discarded, and the pellet thus obtained was washed once with the homogenization medium and used as the mitochondrial fraction.

The kinetics of oxidation-reduction of nicotinamide nucleotide in mitochondrial suspensions were measured fluorimetrically as with liver cell suspensions, except that the reaction medium consisted of 20mM-Tris/HCl buffer, pH7.4, 4mM-potassium phosphate, pH7.4, 225mM-mannitol and 75mM-sucrose.

#### Other methods

t-Butyl hydroperoxide in the effluent perfusate was measured by using glutathione peroxidase in the presence of GSH, NADPH and a sufficient concentration of glutathione reductase. The glutathione peroxidase used was the partially purified preparation from rat liver described by Little & O'Brien (1968). The activity of lactate dehydrogenase and the concentrations of lactate, pyruvate,  $\beta$ -hydroxybutyrate and acetoacetate were assayed directly in the effluent perfusate or in the solution of deproteinized reaction mixture by standard enzymic spectrophotometric techniques (Bergmeyer, 1965).

#### Results

##### Oxidation of nicotinamide nucleotides and release of GSSG caused by infusion of $H_2O_2$ and t-butyl hydroperoxide

Infusion of t-butyl hydroperoxide into the perfused liver specifically enhances the glutathione peroxidase reaction, and the reduction of t-butyl hydroperoxide to t-butyl alcohol takes place with the concomitant oxidation of GSH to GSSG (Sies *et al.*, 1972). The regeneration of GSH from GSSG in the liver is achieved through the glutathione reductase (NADPH) system, which, in turn, results in stimulation of NADPH oxidation. Hence, as shown in Fig. 1(a), infusion of t-butyl hydroperoxide lowers the intensity of nicotinamide nucleotide fluorescence, indicating the oxidation of NADPH in the liver. Increasing the rate of t-butyl hydroperoxide infusion produces a further diminution of the steady state of nicotinamide nucleotide fluorescence. Thus the fluorescence of nicotinamide nucleotides in the perfused liver may be titrated by increasing the rate of t-butyl hydroperoxide infusion up to  $5 \mu\text{mol}/\text{min}$  per g of liver in the presence of 1mM-lactate and 0.3mM-pyruvate. After terminating the peroxide influence, the fluorescence intensity at first continues to decrease, but then recovers its initial intensity and occasionally a higher intensity is seen in a biphasic response; approximately one-half of the recovery takes place immediately and the other half follows slowly. The magnitude of the fluorescence change caused by an aerobic-

anaerobic transition, which mostly may be ascribed to the reduction of mitochondrial  $NAD^+$  under these conditions, is shown in the last portion of the trace of Fig. 1(a). The maximum extent of the fluorescence change produced by t-butyl hydroperoxide infusion is threefold greater than the change observed in the aerobic-anaerobic transition.

As demonstrated by Sies *et al.* (1972, 1974), the oxidation of nicotinamide nucleotides caused by infusion of t-butyl hydroperoxide is accompanied by an increase in the GSSG concentration in the effluent perfusate (Fig. 1b). On cessation of the peroxide infusion, the rate of GSSG release also decreases to the original basal rate. Throughout this experiment, t-butyl hydroperoxide was not detected in the effluent perfusate, indicating that all the infused peroxide had been decomposed by the liver. The activity of lactate dehydrogenase in the effluent perfusate was not increased by the peroxide infusion, in agreement with the results of Sies *et al.* (1972, 1974).

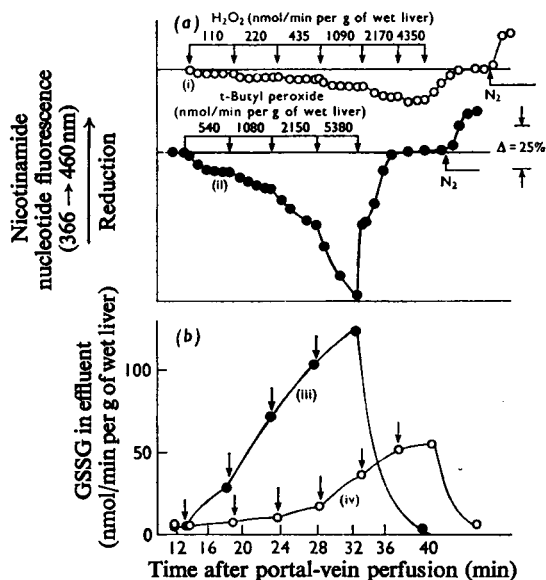


Fig. 1. Effect of  $H_2O_2$  and t-butyl hydroperoxide on the oxidation-reduction state of nicotinamide nucleotide (a) and on the rate of GSSG release from the perfused liver (b)

The liver of the fed rat was perfused with a saline/bicarbonate solution containing 1mM-lactate and 0.15mM-pyruvate at  $30^\circ\text{C}$ , as described in the text.  $H_2O_2$  (○) and t-butyl hydroperoxide (●) were infused at the various rates indicated on the Figure. The glutathione concentration in the effluent is expressed in terms of GSH equivalents in (b). Although traces (i) and (iv) and (ii) and (iii) were obtained from single perfused livers respectively, this result was confirmed in ten separate experiments performed under similar experimental conditions.

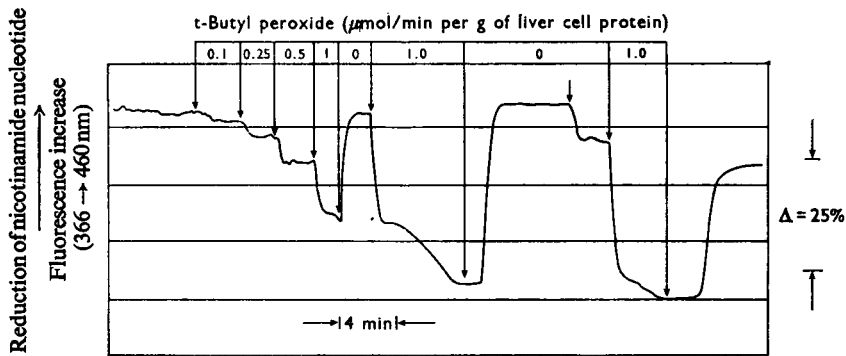


Fig. 2. Oxidation-reduction state of nicotinamide nucleotides in isolated liver cells during the reaction of glutathione peroxidase. Isolated liver cells at 5.1 mg of protein/ml were incubated with the medium used for the perfusion experiments. O<sub>2</sub>/CO<sub>2</sub> (19:1) was supplied constantly with magnetic stirring at 30°C. t-Butyl hydroperoxide was infused continuously into the suspension at the rates indicated on the Figure, and the fluorescence change of nicotinamide nucleotides was recorded at an emission wavelength of 460 nm, with excitation at 366 nm. Arrow on curve indicates the addition of pentachlorophenol (60 µM).

The infusion of H<sub>2</sub>O<sub>2</sub> causes similar results (Figs. 1a and 1b), but the extents of nicotinamide nucleotide oxidation and of GSSG release are much smaller than those observed with t-butyl hydroperoxide. This difference is due to the decomposition of H<sub>2</sub>O<sub>2</sub> by catalase.

*Relative redox states of nicotinamide nucleotides and of glutathione in the presence of t-butyl hydroperoxide*

To examine the relationship between the redox states of nicotinamide nucleotides and glutathione in liver, the intensity of nicotinamide nucleotide fluorescence was monitored in liver cell suspensions with continuous infusion of t-butyl hydroperoxide (Fig. 2). As has been observed in the perfused liver, stepwise increases in the rate of t-butyl hydroperoxide infusion up to 1 µmol/min per g of liver cell cause immediate decreases in fluorescence to lower steady-state values. Termination of the peroxide infusion at any of these steady states is followed by an immediate recovery of the original fluorescence intensity, indicating that there was no accumulation of t-butyl hydroperoxide in the system. The re-infusion of t-butyl hydroperoxide at 1 µmol/min per g of liver cell causes a decrease in the fluorescence intensity similar to that observed previously; however, under these conditions, the fluorescence could not be maintained constant but decreases progressively to the minimum value, where no further decrease was observed. After this minimum value is reached, the peroxide infusion is terminated and there follows a

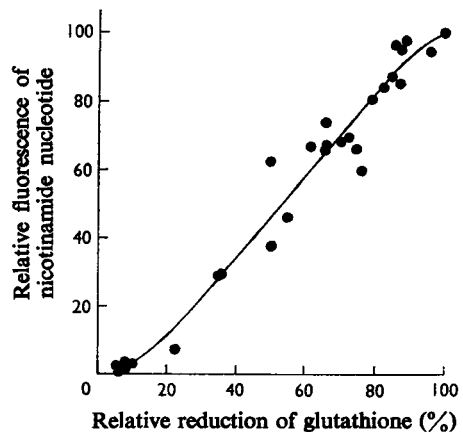


Fig. 3. Relative oxidation-reduction states of nicotinamide nucleotides and of glutathione in isolated liver cells during the reaction of glutathione peroxidase

The experimental conditions were as described for Fig. 2, except that the data points include the results of four separate experiments in which three different cell preparations and five different protein concentrations were examined. The relative reduction state of glutathione was determined with the aerobic steady-state concentration of GSH being taken as 100% (even though it corresponded to 95-100% of the total glutathione in the system). The relative fluorescence intensity of nicotinamide nucleotides was determined with the intensity in the aerobic steady state being taken as 100% and that with excess of t-butyl hydroperoxide as 0%.

recovery of the original intensity with a time delay of several minutes. The longer the duration of the minimum-fluorescence condition, the greater was the delay in the recovery, suggesting that the glutathione peroxidase system is saturated when *t*-butyl hydroperoxide is infused at a rate greater than  $1\ \mu\text{mol}/\text{min}$  per ml under these conditions. In separate experiments in which each infusion rate of *t*-butyl hydroperoxide was continued for 10 min, a steady state corresponding to that of Fig. 2 was maintained at rates below  $1\ \mu\text{mol}/\text{min}$  per ml. These results confirm the existence of two categories of changes in nicotinamide nucleotide fluorescence in response to *t*-butyl hydroperoxide infusion: the first corresponds to approx. 60% of the maximum signal and is sensitive to the low rate of hydroperoxide infusion, and the second corresponds to approx. 40% of the signal and is observable only at the higher infusion rate.

The latter portion of the trace of Fig. 2 indicates that uncoupling of mitochondrial function by pentachlorophenol abolishes the biphasicity of the nicotinamide nucleotide oxidation; under these conditions most of the signal responds sensitively to *t*-butyl hydroperoxide infusion.

The concentration of GSH in the liver cell suspension was assayed with varying rates of *t*-butyl hydroperoxide infusion, and the results are presented as a function of the relative intensity of nicotinamide nucleotide fluorescence (Fig. 3). When different cell concentrations are used, different infusion rates of *t*-butyl hydroperoxide are required to produce a given decrease of the relative fluorescence intensity; nevertheless, all the experimental points on this Figure indicate that decreases in the fluorescence intensity of nicotinamide nucleotides are accompanied by parallel oxidation of the cellular GSH. It should be noted that the lactate/pyruvate and  $\beta$ -hydroxybutyrate/acetoacetate ratios increase only slightly under these conditions, suggesting that the observed decreases in the nicotinamide nucleotide fluorescence may be attributable mostly to the oxidation of NADPH. In fact, direct chemical assays of NADPH/NADP<sup>+</sup> in the perfused liver by Sies *et al.* (1974) and in a suspension of liver cells by J. R. Williamson (personal communication) confirm that *t*-butyl hydroperoxide infusion causes marked oxidation of NADPH in these systems. It is therefore concluded that acceleration of the glutathione peroxidase reaction by infusion of *t*-butyl hydroperoxide produces decreases in the steady-state concentration of both GSH and NADPH in liver cells.

#### *Nature of NADPH-generating systems involved in peroxide metabolism in liver cells*

The parallel oxidation of GSH and NADPH raises questions as to which reaction is the rate-

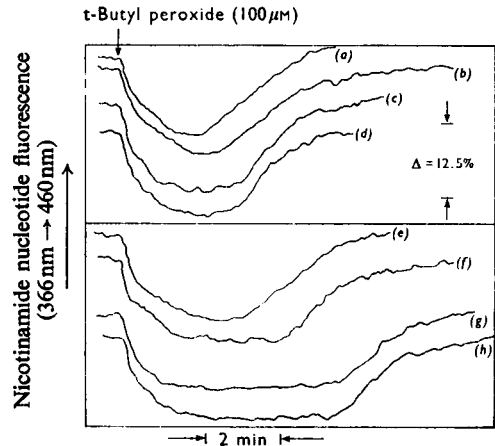


Fig. 4. Effects of various substances on the rate of *t*-butyl hydroperoxide reduction by isolated liver cells

Experimental conditions were as in Fig. 2, except that the liver cell concentration was 6.0 mg of protein/ml. *t*-Butyl hydroperoxide ( $100\ \mu\text{M}$ ) was added as a 'pulse' to the suspension at  $30^\circ\text{C}$ . These traces are representative of four separate experiments examined under different conditions. Curve (a) 0.5 mM-octanoate and 0.2 mM-amino-oxyacetate; curve (b) 0.5 mM-octanoate; curve (c) 10 mM-glucose and 0.2 mM-amino-oxyacetate; curve (d) 10 mM-glucose; curve (e) 0.5 mM-octanoate and 0.1 mM-ZnSO<sub>4</sub>; curve (f) 10 mM-glucose and 0.1 mM-ZnSO<sub>4</sub>; curve (g) 0.2 mM-amino-oxyacetate; curve (h) without added substrate.

limiting step in *t*-butyl hydroperoxide metabolism and what system is responsible for supplying reducing equivalents to the glutathione peroxidase reaction in the liver cell. The time-course of the change in nicotinamide nucleotide fluorescence after a single addition of different concentrations of *t*-butyl hydroperoxide to the liver cell suspension is examined in Fig. 4; it first decreases and then recovers its original intensity after the complete reduction of *t*-butyl hydroperoxide to *t*-butyl alcohol. The half-maximal decrease in the nicotinamide nucleotide fluorescence is found with an initial peroxide concentration of less than  $40\ \mu\text{M}$  in the presence of 10 mM-glucose. The sensitivity of the redox state to low concentrations of hydroperoxide strongly suggests that the regeneration of NADPH is the rate-limiting step in the reduction sequence of *t*-butyl hydroperoxide.

The mean rate of *t*-butyl hydroperoxide reduction may be approximated in terms of  $v = [\text{added } t\text{-butyl hydroperoxide}]/t_{\frac{1}{2}}$ , where  $t_{\frac{1}{2}}$  is the time required for half-maximal recovery of the nicotinamide nucleotide fluorescence. With a cell suspension of 4–6 mg of protein/ml, the maximum rate of *t*-butyl

hydroperoxide reduction is observed with initial peroxide concentrations of 150–200  $\mu\text{M}$ . In some cases, the maximum rate is maintained at higher initial peroxide concentrations, but more frequently such concentrations cause a progressive decrease in the overall rate of reduction as determined above.

Fig. 4 demonstrates the effects of some substrates and inhibitors on these reactions. The rate of t-butyl hydroperoxide reduction is almost twice as fast in the presence of octanoate (curve *b*) or glucose (curve *d*) than in the absence of these substrates (curve *h*).  $t_{\frac{1}{2}}$  decreases from 6.4  $\text{min}^{-1}$  (curve *h*) to 3.5  $\text{min}^{-1}$  (curve *b*) and 3.4  $\text{min}^{-1}$  (curve *d*).  $\text{ZnSO}_4$ , an inhibitor of the glutathione reductase (NADPH) system (Eggleston & Krebs, 1974), inhibits the reduction of t-butyl hydroperoxide; the addition of 0.1  $\text{mM}$ - $\text{ZnSO}_4$  increases the half-time in the presence of octanoate from 3.5  $\text{min}^{-1}$  (curve *b*) to 4.4  $\text{min}^{-1}$  (curve *e*), and from 3.4  $\text{min}^{-1}$  in the presence of glucose (curve *d*) to 5.1  $\text{min}^{-1}$  (curve *f*). Amino-oxyacetate, an inhibitor of glutamate-oxaloacetate transaminase, stimulates the peroxide decomposition slightly in the presence of octanoate ( $t_{\frac{1}{2}}$  decreases from 3.5  $\text{min}^{-1}$  to 3.1  $\text{min}^{-1}$  in curve *a*), but is without effect in the presence of glucose (curve *c*) or without exogenous substrate (curve *g*). Addition of oxanoate intensifies the biphasic response of nicotinamide nucleotide fluorescence to t-butyl hydroperoxide; this substance is a potent substrate for the mitochondrial respiratory system of isolated liver cells, being metabolized to acetate with concomitant generation of ATP and NADH exclusively in the mitochondrial compartment. Thus a possible interpretation of the stimulatory effect of octanoate and for the appearance of clear biphasicity in the oxidation of nicotinamide nucleotides is that the energy-dependent NADH-NADP<sup>+</sup> transhydrogenase of the mitochondria supplies reducing equivalents to the mitochondrial glutathione peroxidase reaction, and maintains a higher steady-state concentration of NADPH in the mitochondria.

#### *Glutathione peroxidase reaction in the isolated mitochondrial fraction*

Further support for this consideration is found in similar types of experiments using the mitochondrial fraction from rat liver. As shown in Fig. 5, the addition of 150  $\mu\text{M}$ -t-butyl hydroperoxide causes the oxidation of mitochondrial nicotinamide nucleotides, followed by a recovery to the original oxidation-reduction state. The time-course and relative degree of fluorescence change vary markedly depending on the kind of substrate present. In accord with the zero-order nature of the glutathione peroxidase reaction with respect to the peroxide concentration (Flohé, 1971), a continuous decrease in the fluorescence intensity is followed by an abrupt

increase after almost all the added t-butyl hydroperoxide has been reduced. Thus the time,  $t$ , shown in this Figure may be taken as inversely proportional to the rate of t-butyl hydroperoxide oxidation by mitochondria. Table 1 summarizes the values of  $t$  and the relative extents of nicotinamide nucleotide oxidation,  $p$ , obtained by the addition of 150  $\mu\text{M}$ -t-butyl hydroperoxide under various conditions. Since rat liver mitochondria contain endogenous substrates, a comparison of the effects of two consecutive additions of t-butyl hydroperoxide is given. Among the substrates used, succinate was the most effective in maintaining the highest steady-state concentration of reduced nicotinamide nucleotides, preventing their oxidation through the acceleration of the glutathione peroxidase reaction by t-butyl hydroperoxide (cf. also Fig. 5). The effect of succinate is decreased in the presence of antimycin A or pentachlorophenol, but not by the presence of rotenone. Thus the stimulatory effect of succinate, and probably that of octanoate as well, may be attributed to the effective operation of the energy-dependent NADH-NADP<sup>+</sup> transhydrogenase in providing reducing equivalents for the glutathione reaction under these conditions.

#### *Properties of the glycollate-dependent GSSG release from perfused liver*

The results obtained from isolated liver cells and isolated mitochondria indicate that the acceleration of the glutathione peroxidase reaction by t-butyl hydroperoxide is accompanied by decreases in the steady-state concentration of both NADPH and GSH, since the rate-limiting step in the overall reaction of t-butyl hydroperoxide reduction appears to be the regeneration of NADPH from NADP<sup>+</sup>. Before discussing the role of this phenomenon in the mechanism of GSSG release from the perfused liver, it is important to eliminate the possibility that this release is due to chemical modification of the cell membrane with consequent alteration in membrane permeability, or to modification of certain enzymes such as carrier proteins, by t-butyl hydroperoxide. For this reason,  $\text{H}_2\text{O}_2$  generation in the liver was stimulated to see whether or not it led to GSSG release. Infusion of substrates for  $\text{H}_2\text{O}_2$ -producing oxidases, such as urate and hypoxanthine, failed to cause the increase in GSSG release (see below), as reported by Sies *et al.* (1974). However, in contrast with their results, infusion of 2  $\text{mM}$ -glycollate stimulates GSSG release, as shown in Fig. 6. The increment in  $\text{O}_2$  consumption of 0.13  $\text{mM}$  caused by glycollate infusion corresponds to approx. 0.35  $\mu\text{mol}$  of  $\text{O}_2$ /min per g of liver. Since under these conditions  $\text{H}_2\text{O}_2$  is decomposed by the catalatic reaction of catalase (i.e.  $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ ), this rate of  $\text{O}_2$  consumption corresponds theoretically

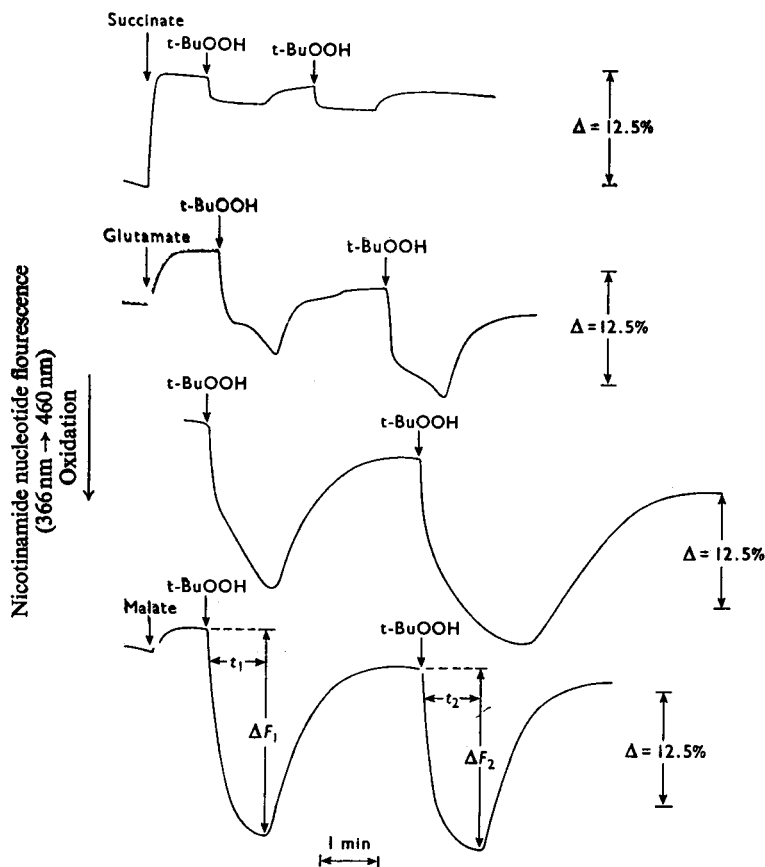


Fig. 5. Rate of hydroperoxide reduction by isolated mitochondria, and its effect on the redox state of nicotinamide nucleotides. The reaction mixture consisted of 225 mM-mannitol, 75 mM-sucrose, 0.1 mM-EDTA, 20 mM-Tris/HCl buffer, pH 7.4, 4 mM-potassium phosphate, pH 7.4, and rat liver mitochondria prepared as described in the text at a concentration of 7.1 mg of protein/ml. The oxidation and re-reduction of nicotinamide nucleotides caused by the addition of 0.15 mM-t-butyl hydroperoxide were measured fluorimetrically at 30°C; 5 mM-succinate, 5 mM-glutamate and 5 mM-malate (or -octanoate) were added as shown on the Figure.  $F$  represents the extent of fluorescence change in arbitrary units, and  $t$  the time required for consumption of the added t-butyl hydroperoxide.

to a maximum rate of  $\text{H}_2\text{O}_2$  production of  $0.7 \mu\text{mol}/\text{min}$  per g of liver, in agreement with our previous experimental finding of  $0.7 \mu\text{mol}/\text{min}$  per g of liver under these conditions, detected by spectrophotometric measurement of the catalase- $\text{H}_2\text{O}_2$  intermediate (Oshino *et al.*, 1975a). In other words, the  $\text{H}_2\text{O}_2$  produced by the glycollate oxidase reaction in the peroxisomes of perfused liver reacts predominantly with catalase, and thus the marked stimulation in the GSSG release observed with glycollate infusion is unlikely to be due to the acceleration of the glutathione peroxidase reaction by the additional  $\text{H}_2\text{O}_2$  produced by the glycollate oxidase reaction.

Fig. 6 also shows that glycollate infusion causes oxidation of nicotinamide nucleotides as well.

On termination of the glycollate infusion, the increased rate of  $\text{O}_2$  consumption decreases by two-thirds during the first minute and returns to the original basal rate during the next 10 min, following a time-course roughly parallel to that of the change in  $\text{H}_2\text{O}_2$  production measured by the catalase- $\text{H}_2\text{O}_2$  intermediate. However, the nicotinamide nucleotide fluorescence recovers more slowly, starting only after the appearance of the slow phase of decreasing  $\text{O}_2$  consumption, and the recovery of the GSSG release parallels that of the nicotinamide nucleotides. It is apparent that glycollate is converted into glyoxylate, with a concomitant production of  $\text{H}_2\text{O}_2$ . The accumulation of glyoxylate may elicit some as yet unidentified reactions such as transamination, which



Table 1. Activity in hydroperoxide reduction by isolated mitochondria and its effect on the oxidation-reduction state of nicotinamide nucleotides

The results obtained in the experiments shown in Fig. 5 are summarized. The extent of the decreases in the intensity of nicotinamide nucleotide fluorescence produced by the first and second additions of 150  $\mu$ M-t-butyl hydroperoxide are designated  $F_1$  and  $F_2$ , as illustrated in Fig. 5; the times required for the consumption of the added t-butyl hydroperoxide are similarly designated  $t_1$  and  $t_2$ .

Substrates	Inhibitors	$F_1$ (arbitrary units)	$t_1$		$F_2$ (arbitrary units)	$t_2$	
			(min)	(%)		(min)	(%)
None	None	7.3	1.24	100	8.4	2.08	100
Succinate (5mM)	None	1.5	0.92	135	1.3	0.96	217
	Rotenone (8 $\mu$ M)	6.3	1.04	119	8.2	1.16	179
	Antimycin A (2 $\mu$ g)	9.8	1.32	94	7.0	2.64	79
	Pentachlorophenol (10 $\mu$ M)	3.5	6.36	20	2.5	>8.8	<24
	Malonate (5mM)	6.6	1.52	82	6.3	>1.72	121
Malate (5mM)	None	9.2	1.16	107	8.2	1.12	186
	Antimycin A (4 $\mu$ g)	7.0	2.36	53	6.2	2.40	87
	Pentachlorophenol (10 $\mu$ M)	1.0	4.96	25	1.2	6.72	31
Glutamate (5mM)	None	4.6	1.04	120	4.7	1.16	179
	Pentachlorophenol (10 $\mu$ M)	3.9	>11.6	<11	*	—	—
Octanoate (0.2mM)	None	4.7	1.16	107	4.1	1.08	193
	Antimycin A (4 $\mu$ g)	9.5	>4.12	<30	*	—	—

\* These values could not be measured.

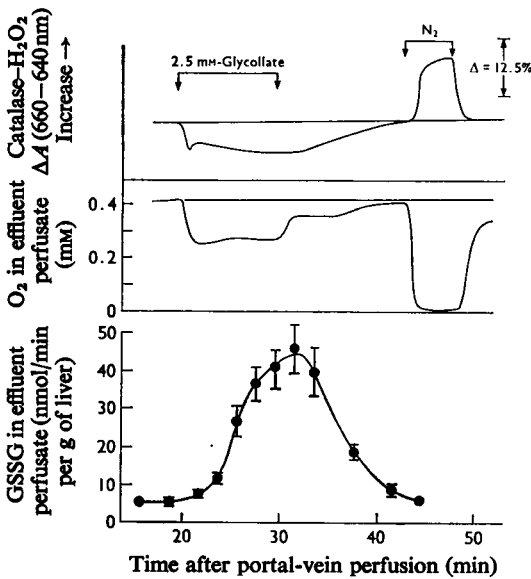


Fig. 6. GSSG release induced by glycollate oxidation in perfused liver

Perfusion conditions were as described for Fig. 1. Glycollate was infused at a concentration of 2.5 mM at a rate of 29–32 ml/min. The traces for nicotinamide nucleotide fluorescence and for O<sub>2</sub> consumption are representative of five separate experiments. The rate of GSSG release is given as the mean value  $\pm$  S.E.M. obtained in five separate experiments.

in turn may lead to the oxidation of NADPH. Although the reaction sequence of glycollate metabolism and the relationship of GSSG release to glycollate infusion have not yet been elucidated, the data of Fig. 6 provide evidence to indicate that the phenomenon of GSSG release can be observed with substances other than hydroperoxides. Thus it is not clear that GSSG release specifically accompanies the acceleration of the glutathione peroxidase reaction; it may be a common phenomenon observable when the steady-state concentrations of GSSG, and probably of NADP<sup>+</sup> as well, are increased.

GSSG release caused by the demethylation of aminopyrine in perfused liver

If, as suggested in Fig. 4, a decrease in the steady-state concentration of NADPH affects the oxidation-reduction state of GSH/GSSG through the glutathione reductase (NADPH) system, then the stimulation of the microsomal drug-metabolizing system, which requires NADPH as a specific electron donor, should also result in an increase in GSSG release. In preliminary experiments, infusion of 1 mM-aminopyrine into the perfused liver of the normal rat resulted in a very slight increase in the concentration of GSSG in the effluent (from 8 nmol/min per g of liver in the absence of aminopyrine to 10 nmol/min per g of liver when aminopyrine was present; mean value of three experiments). Similar experiments with the liver of the phenobarbital-pretreated

rat, in which higher activity of the microsomal drug-metabolizing enzymes was induced, show that aminopyrine activity increased more than twofold (Fig. 7). It is noteworthy that the enhanced

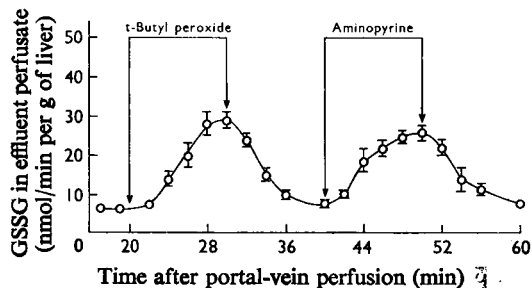


Fig. 7. GSSG release observed during demethylation of aminopyrine in the liver of phenobarbital-pretreated rats

Rats pretreated with 80 mg of phenobarbital/kg body wt. for 3 days were used on day 4. Perfusion conditions were as in Fig. 1. t-Butyl hydroperoxide (491 nmol/min per g of liver) and aminopyrine (2  $\mu$ mol/min per g of liver) were infused in that sequence for 10 min as indicated on the Figure. In separate experiments, the sequence of infusions was altered, but the results obtained were practically identical. The experimental points are mean values  $\pm$  S.E.M. of four separate experiments.

demethylation of aminopyrine under these conditions is accompanied by an increased rate of GSSG release, as shown in Fig. 7, comparable with that observed when NADPH oxidation via the reduction of t-butyl hydroperoxide proceeds at a rate of 491 nmol/min per g of liver.

Treatment of the phenobarbital-pretreated rat with 3-amino-1,2,4-triazole causes inactivation of more than 90% of the hepatic catalase, but does not alter the properties of the aminopyrine-dependent stimulation of GSSG release, indicating that  $H_2O_2$  is not involved in the aminopyrine effect (cf. Fig. 10). Since there is an increase in the GSSG release during aminopyrine demethylation in the liver of the phenobarbital-pretreated rat, but no increase when the aminopyrine is infused under anaerobic conditions where the drug-demethylation reaction does not proceed, it is concluded that, regardless of whether they are induced by enhanced oxidation of NADPH or by acceleration of GSH oxidation, increases in the intracellular GSSG concentration result in corresponding increases in the GSSG release from perfused liver.

#### Effect of metabolic states on the rate of GSSG release

Assuming the mechanism of GSSG release to be such that only the oxidized form of glutathione is

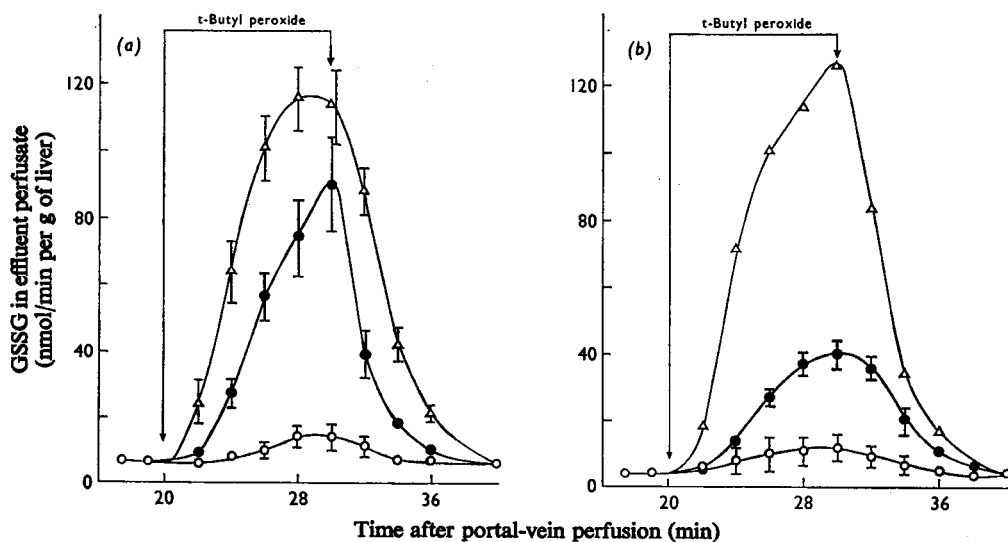


Fig. 8. Relationship between the rates of GSSG release and t-butyl hydroperoxide reduction by perfused liver under different metabolic conditions

The liver of the fed rat was perfused without exogenous substrates (a) or with 1 mM-lactate and 0.15 mM-pyruvate added (b). The rates of t-butyl hydroperoxide infusion were  $1335 \pm 133$  ( $\Delta$ ),  $775 \pm 0.04$  ( $\bullet$ ) and  $270 \pm 0.06$  ( $\circ$ ) nmol/min per g of liver in (a), and  $1021 \pm 100$  ( $\Delta$ ),  $716 \pm 0.05$  ( $\bullet$ ) and  $296 \pm 0.06$  ( $\circ$ ) nmol/min per g of liver in (b). Each point represents the mean  $\pm$  S.E.M. of five separate experiments, except that ( $\Delta$ ) in (b) is the mean value of three selected experiments; the results obtained with infusion of more than 1  $\mu$ mol of t-butyl hydroperoxide/min per g of liver in the presence of 1 mM-lactate and 0.15 mM-pyruvate varied greatly. The rate of GSSG released remained around 120 nmol (equiv. GSH)/min per g of liver under these conditions, but occasionally was more than 200 nmol/min per g of liver.

diffusible through the plasma membrane, the rate of GSSG release may be proportional to the steady-state concentration of GSSG in the cellular compartment adjacent to the plasma membrane. If this is the case, the rate of GSSG release elicited by a given rate of t-butyl hydroperoxide reduction should be affected by the capability of the NADPH regeneration in the liver under different metabolic conditions.

Fig. 8 compares the rates of GSSG release observed with three infusion rates of t-butyl hydroperoxide in the perfused liver of fed rats. Since no t-butyl hydroperoxide is detected in the effluent perfusate under these conditions, the rates of t-butyl hydroperoxide infusion can be taken to be the same as the rate of its reduction by the liver. The liver being perfused with or without added lactate and pyruvate seems to be able to maintain a constant cellular oxidation-reduction state of glutathione at rates of t-butyl hydroperoxide infusion of less than 300 nmol/min per g of liver, since only a slight increase in the rate of GSSG release is observed. As the infusion rate is increased to 775 or 716 nmol/min per g of liver, there is a significant difference in the rate of GSSG release; in the absence of exogenous substrate in the perfusate, the rate increases constantly up to  $90.7 \pm 13.0$  nmol/min per g of liver, but, in the presence of lactate and pyruvate, the increased GSSG release is clearly suppressed at  $40.4 \pm 3.8$  nmol/min per g of liver. When t-butyl hydroperoxide was infused for 20 min in a separate experiment, the GSSG release remained constant from 10 to 20 min under the latter conditions.

The system becomes more complex as the rate of GSSG release exceeds 100 nmol/min per g of liver. In the absence of substrates, the rate of GSSG release enhanced by 1335 nmol of t-butyl hydroperoxide/min per g of liver increases to 110 nmol/min per g of liver, but appears to decrease thereafter, as seen by the experimental points at 8 and 10 min of infusion. This tendency can be seen more clearly in the liver from starved rat, shown in Fig. 9. On the other hand, the rate of GSSG release enhanced by 1021 nmol of t-butyl hydroperoxide/g of substrate-supplemented liver increases continuously, reaching more than 200 nmol/min per g of liver on occasion.

The susceptibility of the GSSG release to t-butyl hydroperoxide infusion increases markedly in the perfused liver of the starved rat. As shown in Fig. 9, the infusion of t-butyl hydroperoxide at a rate as low as 362 nmol/min per g of liver results in the release of GSSG at 65 nmol/min per g of liver, and infusion of 10 mM-glucose (or 0.2 mM-octanoate) decreases this rate to less than 30 nmol/min per g of liver. The latter rate is lower than that observed with infusion of 296 nmol of t-butyl hydroperoxide/min per g of liver supplemented with lactate and pyruvate (cf. Fig. 8). Although Sies *et al.* (1974)

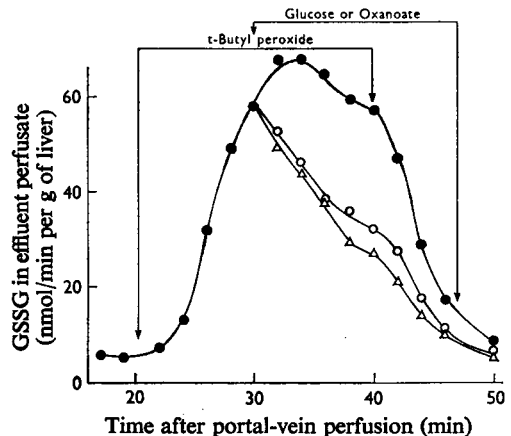


Fig. 9. Effects of glucose and octanoate on the rate of GSSG release in the perfused liver of starved rats

The liver of the starved rat was perfused with saline/bicarbonate solution without exogenous substrates (●). At 10 min after the initiation of t-butyl hydroperoxide infusion (362 nmol/min per g of liver) either 10 mM-glucose (○) or 0.25 mM-octanoate (△) was infused for 10 min, as indicated on the Figure. Although each experimental point was the mean value of three separate experiments, these results were confirmed in similar experiments with different concentrations of t-butyl hydroperoxide and octanoate.

(cf. their Fig. 3) reported no effect of starvation on the rate of GSSG release, they used very high rates of hydroperoxide infusion, which made such a comparison inadequate.

After the liver of starved rat had been perfused with 724 nmol of t-butyl hydroperoxide/min per g of liver for 20 min, the total glutathione concentrations in the liver homogenate and in the isolated mitochondrial fraction were determined. The total glutathione concentration of the homogenate was 21.0 nmol/mg of protein in the control liver and 12.7 nmol/mg of protein in the t-butyl hydroperoxide-treated liver, but the glutathione contents of mitochondria from control and treated livers were almost identical (5.7 and 5.6 nmol of glutathione/mg of protein respectively).

#### *Role of H<sub>2</sub>O<sub>2</sub> produced in the liver and the possible roles of catalase and glutathione peroxidase in peroxide metabolism*

In previous studies (Oshino *et al.*, 1975a) it was clearly demonstrated that H<sub>2</sub>O<sub>2</sub>, generated via the uricase reaction in the peroxisomes of perfused liver, is decomposed almost quantitatively by catalase under normal conditions. Accordingly, the rate of GSSG

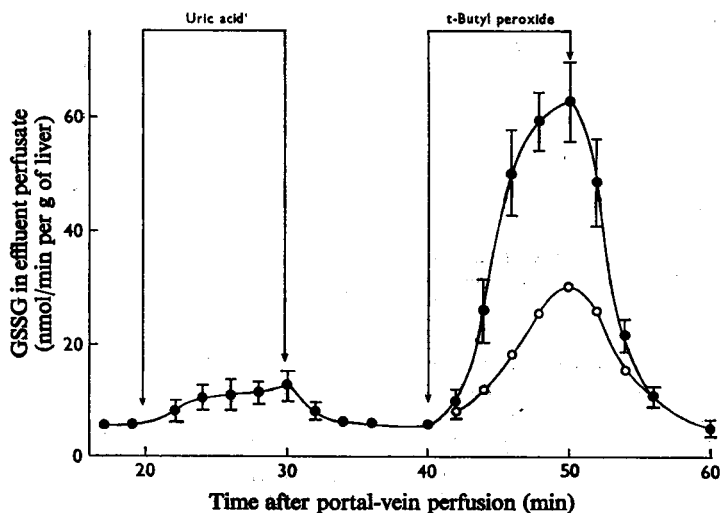


Fig. 10. GSSG release during oxidation of uric acid in the perfused liver of the aminotriazole-pretreated rat. The liver of the starved rat pretreated with 3-amino-1,2,4-triazole was perfused without exogenous substrates. Uric acid (1 mM) and t-butyl hydroperoxide at 362 nmol/min per g of liver (●) or 188 nmol/min per g of liver (○) ( $n = 2$ ) were infused for 10 min respectively, as indicated in the Figure. The rate of uric acid disappearance from the perfusate under these conditions was  $600 \pm 40$  nmol/min per g of liver and the rate of increased  $O_2$  consumption was  $418 \pm 27$  nmol/min per g of liver. Each point is the mean value  $\pm$  s.e.m. of five separate experiments. Although the experimental values are not shown, the infusion of uric acid into the control rat liver did not stimulate any detectable increase in the GSSG release.

release is not altered by infusion of urate up to 1 mM, even in the liver of the starved rat. In similar experiments using the liver of aminotriazole-pretreated starved rats, infusion of 1 mM-urate enhanced the  $O_2$  consumption over that of the control liver by  $418 \pm 27$  nmol of  $O_2$ /min per g of liver ( $n = 5$ ); the rate of urate oxidation was  $600 \pm 40$  nmol/min per g of liver. The ratio of urate oxidation to  $O_2$  consumed was 1.4, suggesting that, in spite of inactivation of more than 90% of the catalase by pretreatment with aminotriazole (Heim *et al.*, 1955; Oshino *et al.*, 1975b), the major portion of  $H_2O_2$  produced by the uricase reaction is decomposed by catalase operating in the catalatic mode in the liver. In accordance with this observation, the increment in GSSG release with infusion of uric acid is approx. 7 nmol/min per g of liver (from  $5.6 \pm 0.8$  to  $12.6 \pm 2.6$  nmol/min per g of liver), as shown in Fig. 10. For comparison, when t-butyl hydroperoxide is infused into the same liver at a rate of either  $362 \pm 12$  ( $n = 5$ ) or 188 ( $n = 2$ ) nmol/min per g of liver, the increments in GSSG release are  $56.9 \pm 7.6$  and 27 nmol/min per g of liver respectively (right-hand part of Fig. 10). Assuming that the increment in GSSG release is proportional to the rate of the glutathione peroxidase reaction under these conditions, the observed increment of 7 nmol/min per g of liver induced by urate oxidation at a rate of  $600 \pm 40$  nmol/min per g

of liver corresponds to the rate that would be observed on reduction of t-butyl hydroperoxide at a rate of 50 nmol/min per g of liver ( $= 188 \times 7/27$ , or  $362 \times 7/57$ ). In other words, less than 10% of the  $H_2O_2$  generated in the peroxisomes by the uricase reaction is decomposed by the glutathione peroxidase reaction in the liver in which more than 90% of the catalase has been inactivated.

The infusion of 0.29 mM-hypoxanthine into the liver of both starved and fed rats pretreated with aminotriazole causes an increase in  $O_2$  consumption of about 96 nmol/min per g of liver ( $n = 3$ ) and very little increase in the rate of GSSG release. However, infusion of 0.5 mM-benzylamine, a substrate for monoamine oxidase (Tabor *et al.*, 1954), stimulates GSSG release, as shown in Fig. 11. The increments in  $O_2$  consumption and GSSG release are 430 and 12 nmol/min per g of liver respectively for the fed rat, perfused in the presence of 1 mM-lactate and 0.3 mM-pyruvate. In a similar experiment carried out with the liver of starved rat in the absence of added substrates, the increments in  $O_2$  consumption and GSSG release were 622 and 119 nmol/min per g of liver respectively ( $n = 4$ ). Thus the rate of benzylamine-dependent GSSG release is also dependent on the metabolic state, as is the case with t-butyl hydroperoxide-induced GSSG release. The GSSG release induced by benzylamine in the perfused

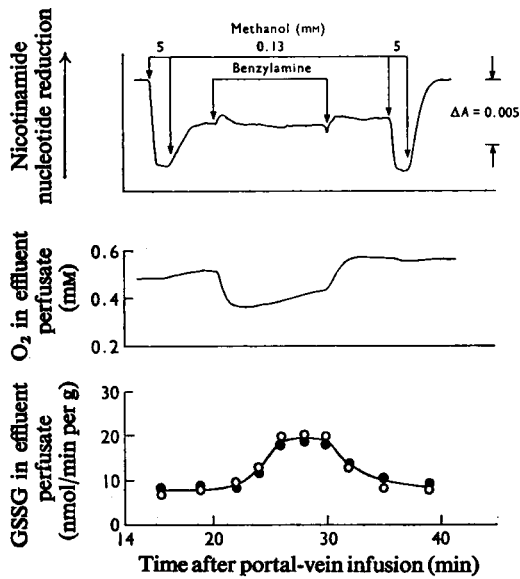


Fig. 11. Effects of benzylamine infusion on the steady state of the catalase  $H_2O_2$  intermediate, the rate of  $O_2$  consumption and the rate of GSSG release in perfused liver

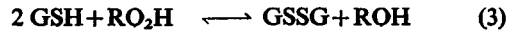
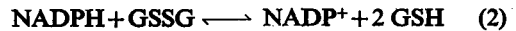
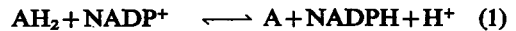
Perfusion conditions were as in Fig. 1. Methanol 0.13 or 5 mM and benzylamine (0.5 mM) were infused as indicated. The traces for the catalase- $H_2O_2$  intermediate and for  $O_2$  consumption are representative of results obtained in three separate experiments. The mean value of  $O_2$  consumption stimulated by benzylamine infusion was 430 nmol/min per g of liver under these conditions. The value for GSSG release was also the mean of three experiments. ○, Control rat; ●, 3-amino-1,2,4-triazole-pretreated rat.

liver of the fed rat with exogenous substrate, and of the starved rat without exogenous substrate, are equivalent to those induced by approx. 500 nmol of t-butyl hydroperoxide/min per g of liver under the same metabolic conditions.

In spite of the detectable increase in the rate of the GSH reaction, the steady-state concentration of the catalase- $H_2O_2$  intermediate increases only slightly during the oxidation of benzylamine (Fig. 11). The decrease in  $\Delta A_{660-640}$  is attributable to the absorbance change of a chromophore other than catalase, but, after correction for this contribution, the increment in the  $H_2O_2$  production detected by the catalase- $H_2O_2$  intermediate is so small, compared with that detected by  $O_2$  consumption and by the increase in GSSG release, that increased  $H_2O_2$  production does not seem to be the only cause of GSSG release.

## Discussion

The glutathione peroxidase system *in vivo* may be delineated by the following reactions:



where  $AH_2$  and A are the substrates for the NADPH-generating system in their reduced and oxidized forms respectively; R is H or an alkyl residue of corresponding hydroperoxides and alcohols. The studies carried out by Sies *et al.* (1972, 1974) on perfused liver and by Srivastava *et al.* (1974) on erythrocytes imply the complex nature of the metabolic changes associated with hydroperoxide metabolism; alterations in the concentration of cellular constituents such as ATP and  $\alpha$ -glycerophosphate as well as NADPH occur when the glutathione peroxidase reaction is enhanced by the addition of organic hydroperoxides. The present study has substantiated more clearly the possibilities for complex alterations of cellular metabolism which can be elicited by the acceleration of the peroxide reduction. In order, however, to evaluate the physiological implications of these possible metabolic changes, the events that occur under physiological conditions must be carefully distinguished from those that may occur only under conditions *in vitro*.

t-Butyl hydroperoxide and cumene hydroperoxide were assumed to be specific substrates for glutathione peroxidase in the work of Sies *et al.* (1972, 1974) and Srivastava *et al.* (1974). However, it has been reported by Kadlubar *et al.* (1973) that catalase is able to catalyse the cumene hydroperoxide-dependent oxidation of certain drugs, such as aminopyrine and benzphetamine. Kadlubar *et al.* (1973) also stated that 'it was possible to demonstrate by spectral means the formation of a catalase peroxyhaem (Compound I) upon the addition of cumene hydroperoxide'. As to the reactivity of catalase with t-butyl hydroperoxide, four negative results can be presented. (1) No decrease in the t-butyl hydroperoxide concentration, initially 5 and 1 mM, was found after incubation with 0.4  $\mu$ M ox liver catalase, with 50 mM-ethanol as hydrogen donor in 50 mM-potassium phosphate buffer, pH 7.5, for 10 min at 23°C. (2) Although the addition of ethyl hydroperoxide to a catalase solution causes a clear disappearance to the Soret band, indicating Compound I formation (Chance, 1949), the addition of t-butyl hydroperoxide up to 1 mM caused only a slight decrease in the absorbance at this wavelength. At acidic pH, this effect was intensified and led to a slow disappearance of the Soret band of catalase, but the time-course of the spectral change was not

that ascribable to the interconversion of free catalase into Compounds I or II but rather that which accompanies the destruction of the enzyme integrity. (3) *p*-Cresol is a hydrogen donor that stimulates the conversion of Compound I into Compound II (Keilin & Nicholls, 1958), and hence the addition of ethyl hydrogen peroxide or  $H_2O_2$  in the presence of *p*-cresol causes accumulation of Compound II in the reaction system (Oshino *et al.*, 1973*b*); no indication of the formation of Compound II was found in the presence of both *p*-cresol and *t*-butyl hydroperoxide. (4) Infusion of up to 1mM-*t*-butyl hydroperoxide into perfused liver did not produce any detectable change in  $A_{660-640}$  that could be ascribed to the formation of a catalase-hydroperoxide complex (Compound I or II). On this evidence, we consider that under normal conditions catalase is unable to react with *t*-butyl hydroperoxide at a rate that would be physiologically functional (Chance, 1951).

The peroxidase activity of cytochrome *P*-450 with various organic hydroperoxides has been demonstrated *in vitro* by Hrycay & O'Brien (1974). Cumene hydroperoxide and *t*-butyl hydroperoxide can replace NADPH and  $O_2$  in drug-metabolizing reactions dependent on cytochrome *P*-450, such as the hydroxylation of aromatic compounds (Rahimtula & O'Brien, 1974) and the C-oxidation of several amines (Kadlubar *et al.*, 1973). Even in the perfused liver of the phenobarbital-pretreated rat, we have failed to detect the spectral changes which may be ascribed to cytochrome *P*-450 on infusion of *t*-butyl hydroperoxide. Infusion of aminopyrine causes an increase in the rate of GSSG release (Fig. 7) and also the spectral change associated with the formation of a cytochrome *P*-450-substrate complex (N. Oshino, unpublished work). Further infusion of *t*-butyl hydroperoxide in the presence of aminopyrine results in a further increase in the GSSG release which is independent of the aminopyrine-induced GSSG release, but no effect on the steady-state concentration of the cytochrome *P*-450-substrate complex was observed under these conditions. The discrepancy between the reactivity of cytochrome *P*-450 with *t*-butyl hydroperoxide *in vivo* and *in vitro* may be mainly attributed to the low steady-state concentration of infused hydroperoxide due to its effective removal from the perfused liver by the glutathione peroxidase system under our experimental conditions.

Certain peroxides may be oxidants for the thiol groups of several enzymes and thus inhibit these enzymes. In fact, Green *et al.* (1971) reported a 50% inhibition of NADPH-isocitrate dehydrogenase activity by 1.2 $\mu$ M-linoleic acid hydroperoxide and by 0.25mM-cumene hydroperoxide; however, *t*-butyl hydroperoxide was without effect on this enzyme reaction. Srivastava & Beutler (1973) could not dis-

cover an inactivation greater than 25% of 19 enzymes in the erythrocyte after pretreatment with *t*-butyl hydroperoxide, but some cellular function had been impaired under their conditions, as indicated by a continual decrease in ATP concentration after this pretreatment. Under our experimental conditions for liver perfusion, the possibility that GSSG release might be due to chemical modification of the membrane, or to inhibition of certain enzymes by the peroxide, can be eliminated by the following considerations.

(1) Not only organic peroxides but also glycollate (Fig. 6), benzylamine (Fig. 11) and even uric acid (Fig. 10) stimulate the GSSG release. The stimulatory effects of these substances were observable only under the particular conditions specific for each; for example, the aminopyrine-dependent release of GSSG increased markedly in the liver of the phenobarbital-pretreated rat, in which the microsomal activity of aminopyrine demethylation was more than doubled. Similarly, uric acid caused a slight but distinct stimulation of GSSG release only when more than 90% of the catalase had been inactivated by aminotriazole pretreatment. These effects were not, of course, seen under anaerobic conditions, since aminopyrine demethylation and uric acid oxidation do not take place without oxygen.

(2) The extent of the GSSG release stimulated by any of the means described here is affected greatly by the metabolic conditions with respect to NADPH generation. Thus it is very likely that the cause of the GSSG release is an increase in the intracellular concentration of GSSG and its diffusion through the cell membrane. However, it must be noted that neither GSSG nor GSH could be incorporated from the perfusate into the liver cells (L. Flohé, personal communication). Thus the true mechanism for the anisotropic diffusion of GSSG through the plasma membrane remains unclear.

In contrast with the erythrocyte, where the major pathway for NADPH generation is the pentose monophosphate shunt (Cohen & Hochstein, 1963), several enzyme systems may operate in the liver. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, 'malic' enzyme, isocitrate dehydrogenase, glutamate dehydrogenase and the NADH-NADP<sup>+</sup> transhydrogenase function in different subcellular compartments and/or in different metabolic pathways. The stimulatory effects of succinate on the peroxide metabolism in isolated mitochondria (Table 1) and of octanoate in liver cells (Fig. 4) emphasize the importance of the contribution of the energy-linked NADH-NADP<sup>+</sup> transhydrogenase reaction (Klingenberg & Slenczka, 1959) in the maintenance of the cellular NADPH concentration. The capabilities of the other enzyme reactions in generating NADPH are confirmed by the stimulatory effects of glutamate and malate in

the mitochondrial fraction (Table 1) and of glucose in the liver cell suspension (Fig. 4), but further identification and quantification of the system by the pulse approach has not been possible.

Because of the zero-order nature of the glutathione peroxidase reaction with respect to the peroxide concentration (Flohé, 1971), the addition of a pulse of hydroperoxide, regardless of its concentration, causes near-maximal acceleration of the glutathione peroxidase reaction and results in marked oxidation of not only GSH and NADPH but also NADH. Such highly oxidized states will elicit various somewhat unphysiological effects on cell function. For example, liver cell respiration is apparently inhibited by addition of *t*-butyl hydroperoxide, as shown in Fig. 12, and part of the inhibitory effect disappears after the added hydroperoxide is consumed. Taking into account the extreme oxidation of nicotinamide nucleotides shown in Fig. 4, this reversible inhibition may be considered to result from an unusual decrease in the redox potential difference ( $\Delta E_h$ ) between the redox couples NADH/NAD<sup>+</sup> and O<sub>2</sub>/H<sub>2</sub>O, i.e. the free-energy span available for the phosphorylation of ADP, by which the rate of mitochondrial respiration is controlled (Wilson & Erecinska, 1972). In addition, such highly oxidized states may be accompanied by corresponding

decreases in the [ATP]/[ADP][P<sub>i</sub>] ratio, since the electron-transfer and phosphorylation systems of the mitochondria operate at near-equilibrium (Klingenberg & Schollmeyer, 1961; Wilson *et al.*, 1972). Stimulation of the pentose monophosphate pathway is also accompanied by a decrease in the efficiency of glycolytic ATP production. The simultaneous occurrence of all these events, which might be initiated by a pulse addition of organic hydroperoxide, increases further the possibility of deleterious side reactions, such as the chemical modification of the thiol-group-containing proteins, the unusual accumulation of certain metabolic intermediates, and the perturbation of the normal ionic environment due to the failure of energy production, which may finally lead to irreversible disordering of cellular function. For these reasons, the results obtained with pulse addition of *t*-butyl hydroperoxide may represent an intensification of the deleterious effects of hydroperoxides.

The steady-state-titration procedure of continuous infusion, on the other hand, allows exposure of the system to the lowest concentration of hydroperoxide, and the reduction of hydroperoxide by glutathione peroxidase takes place, with concomitant decreases in the steady-state concentrations of NADPH and GSH, which in turn stimulate NADPH generation in the liver. This procedure may be complicated by the fact that *t*-butyl hydroperoxide is diffused into the cell from the outside, in contrast with the physiological situation, where most peroxides are generated inside the cell. Thus, as noted in the Results section, the continuous infusion of a low concentration of *t*-butyl hydroperoxide causes a marked decrease in the glutathione concentration in the cytosolic compartment, but no loss of mitochondrial glutathione, although glutathione peroxidase exists in both compartments. The biphasic nature of the response of nicotinamide nucleotide fluorescence to *t*-butyl hydroperoxide infusion (Figs. 1 and 2) suggests that the mitochondrial glutathione peroxidase operated only at higher rates of hydroperoxide infusion under these experimental conditions. Of importance is the question of whether or not the mitochondrial and cytosolic NADPH-generating systems are capable of supporting the cytosolic and mitochondrial glutathione peroxidase reactions respectively. Although this would appear to be the case, as indicated by the profound effect of octanoate on GSSG release from the perfused liver of the starved rat, the fluorescence measurements do not provide conclusive evidence, since the fluorescence signal is derived from NADH and NADPH in both compartments. Further, the oxidation of NADPH appears to be accompanied by the reduction of NAD<sup>+</sup> (Fig. 6), making the analysis more difficult. Thus further quantification remains for further investigation.

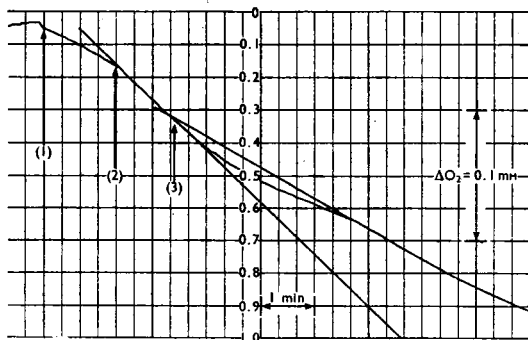


Fig. 12. Effect of *t*-butyl hydroperoxide on the respiration of isolated liver cells

Isolated liver cells [arrow (1), 0.1 ml; 4.1 mg of protein/ml] were suspended in Krebs-Ringer phosphate buffer (see Krebs & Henseleit, 1932), pH 7.4, and the O<sub>2</sub> consumption was measured at 30°C; 0.2 mM-octanoate [arrow (2)] and 0.75 mM-*t*-butyl hydroperoxide [arrow (3)] were added as indicated. Straight lines were drawn to clarify the transient inhibition of respiration by the hydroperoxide. This trace is representative of several experiments performed with different cell preparations, with different respiratory substrates, and with different concentrations of the hydroperoxide.

The rate of GSSG release induced by  $H_2O_2$  infusion was found to be about 40–50% of that induced by *t*-butyl hydroperoxide at a similar infusion rate; further,  $H_2O_2$ -induced release is saturated at approx. 50 nmol (GSH equiv.)/min per g of liver (Fig. 1). This result indicates that catalase decomposes approx. 50–60% of the infused  $H_2O_2$  when the infusion rate is less than  $2 \mu\text{mol}/\text{min}$  per g of liver. The saturation of the  $H_2O_2$ -dependent GSSG release emphasizes the capability of catalase to decompose unphysiological concentrations of  $H_2O_2$  effectively, but these same properties of catalase might lead to the incorrect conclusion that the catalytic activity of catalase is ineffective under physiological conditions, i.e. at low rates of  $H_2O_2$  generation, as has generally been thought to be the case (Keilin & Hartree, 1945*b*; Mills & Randall, 1958; Cohen & Hochstein, 1963). However, a number of factors, such as the compartmentation of enzymes and the site of  $H_2O_2$  production in the cell, must be taken into account when considering the physiological function of catalase and glutathione peroxidase.

Uricase is located exclusively in the peroxisome, and the major part of hepatic catalase is also concentrated in the peroxisome, surrounding the uricase core (de Duve & Baudhuin, 1966). Accordingly, the  $H_2O_2$  produced by the uricase reaction is decomposed exclusively by catalase (Oshino *et al.*, 1975*a*). Diffusion of  $H_2O_2$  from the peroxisome can be detected as a slight increase in GSSG release from perfused liver only when more than 90% of the hepatic catalase is inactivated by aminotriazole pretreatment (Fig. 10). The rate is equivalent to approx. 50 nmol of *t*-butyl hydroperoxide oxidized/min per g of liver, corresponding to less than 10% of the rate of uric acid oxidation under these conditions.

When stimulated by infusion of either octanoate or antimycin A, the enhanced  $H_2O_2$  production in the mitochondria is accompanied by an increase in the steady-state concentration of the catalase- $H_2O_2$  intermediate in the peroxisomes; the  $H_2O_2$  production of the liver increases from 50 to 170 nmol/min per g of liver with octanoate, and to 75 nmol/min per g of liver with antimycin A (Oshino *et al.*, 1973*a*). Although Sies *et al.* (1974) reported no increase in the GSSG release under these conditions, this should not be taken to indicate a preferential interaction of mitochondrial  $H_2O_2$  with catalase rather than glutathione peroxidase; as shown in the present study, the rate of GSSG release is altered by changes in metabolic conditions which affect the cellular oxidation-reduction state of NADPH (Figs. 6, 7, 8 and 9). Furthermore, it is not clear at present that the GSSG release is due solely to the acceleration of the glutathione peroxidase reaction, or to the increased concentration of  $NADP^+$ . Assuming that calibration by infusion of *t*-butyl

hydroperoxide infusion is applicable to this problem, the reduction of the hydroperoxide at the rate of 362 nmol/min per g of liver increases the GSSG release by only 25 nmol/min per g of liver in the presence of octanoate (Fig. 9), suggesting that under such conditions the glutathione peroxidase reaction might not be detectable at rates below approx. 100 nmol/min per g of liver. Similarly, when hypoxanthine is infused, the stimulation of the xanthine oxidase reaction causes an increase in the steady-state concentration of the catalase- $H_2O_2$  intermediate corresponding, at the maximum, to approx. 70 nmol of  $H_2O_2$  produced/min per g of liver, but the increase in the GSSG release is below the accuracy of the assay even in the liver of aminotriazole-pretreated starved rat. In the presence of these substances, the rate of  $H_2O_2$  decomposition through glutathione peroxidase is clearly less than that through catalase.

In contrast with the above-mentioned cases,  $H_2O_2$  produced in the monoamine oxidase reaction appears to be decomposed rather selectively by glutathione peroxidase (Fig. 11), although further investigation is needed, since one of the reaction products of benzylamine oxidation is  $NH_3$ , which may stimulate NADPH oxidation during the process of urea formation in the perfused liver (Sies *et al.*, 1973*a*).

The results of the present study suggest that compartmentation of enzymes in different subcellular organelles emphasizes the uniqueness of each enzyme's function, but allows them to co-operate effectively. The maximum activity of glutathione peroxidase determined *in vitro* is not to be expected *in vivo*, since the reaction is always accompanied by decreases in the steady-state concentrations of cellular NADPH and GSH, which may affect other metabolic processes. Taking into account this aspect of the glutathione peroxidase system, and taking the rate of  $H_2O_2$  production *in vivo* as  $0.4 \mu\text{mol}/\text{min}$  per g of liver (Oshino *et al.*, 1975*b*), it seems very likely that the unique capability of glutathione peroxidase in decomposing lipid hydroperoxides may operate effectively only when the system is exposed to a minimal input of  $H_2O_2$ . Such conditions are afforded and maintained by the first-order nature of the catalase reaction with respect to the  $H_2O_2$  concentration. Liver, leucocytes and erythrocytes possess co-operative catalase-glutathione peroxidase systems to deal with the hydroperoxides naturally produced in these biological materials. However, this hydroperoxide-detoxification system may not be as effective on exposure to extracellular hydroperoxides as it is for endogenously generated hydroperoxides. In this respect, it should be noted that many kinds of drugs exhibit their toxicity by inducing haemolysis and/or oedema; some of their toxic action may be ascribable to the production of



H<sub>2</sub>O<sub>2</sub> (or O<sub>2</sub><sup>-</sup>) by these autoxidizable drugs in the extracellular spaces, and/or to the over-oxidation of NADPH and GSH by drug metabolism.

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