The Cellular Production of Hydrogen Peroxide

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1. The enzyme–substrate complex of yeast cytochrome c peroxidase is used as a sensitive, specific and accurate spectrophotometric H_2O_2 indicator. 2. The cytochrome c peroxidase assay is suitable for use with subcellular fractions from tissue homogenates as well as with pure enzyme systems to measure H_2O_2 generation. 3. Mitochondrial substrates entering the respiratory chain on the substrate side of the antimycin A-sensitive site support the mitochondrial generation of H_2O_2. Succinate, the most effective substrate, yields H_2O_2 at a rate of 0.5nmol/min per mg of protein in state 4. H_2O_2 generation is decreased in the state 4 → state 3 transition. 4. In the combined mitochondrial–peroxisomal fraction of rat liver the changes in the mitochondrial generation of H_2O_2 modulated by substrate, ADP and antimycin A are followed by parallel changes in the saturation of the intraperoxisomal catalase intermediate. 5. Peroxisomes supplemented with uric acid generate extraperoxisomal H_2O_2 at a rate (8.6–16.4nmol/min per mg of protein) that corresponds to 42–61% of the rate of uric acid oxidation. Addition of azide increases these H_2O_2 rates by a factor of 1.4–1.7. 6. The concentration of cytosolic uric acid is shown to vary during the isolation of the cellular fractions. 7. Microsomal fractions produce H_2O_2 (up to 1.7nmol/min per mg of protein) at a ratio of 0.71–0.86mol of H_2O_2/mol of $NADP^+$ during the oxidation of NADPH. H_2O_2 is also generated (6–25%) during the microsomal oxidation of NADH (0.06–0.025mol of H_2O_2/mol of NAD^+). 8. Estimation of the rates of production of H_2O_2 under physiological conditions can be made on the basis of the rates with the isolated fractions. The tentative value of 90nmol of H_2O_2/min per g of liver at 22°C serves as a crude approximation to evaluate the biochemical impact of H_2O_2 on cellular metabolism.

The presence of a large quantity of catalase (cat.) (Sumner & Dounce, 1937; Agner, 1938) and its short half-life in the liver (Price *et al.*, 1962; Poole, 1969) suggest a significant physiological function for this haemoprotein (de Duve & Baudhuin, 1966; Deisseroth & Dounce, 1970). The discovery of the coupled oxidation described by Keilin & Hartree (1945):

$$Cat.-H_2O_2 + \underset{OH}{\overset{H}{>C<}} \longrightarrow Cat. + >C=O + H_2O$$

led to the distinction between the 'catalatic' and the 'peroxidatic' (Theorell, 1948; Chance, 1950) modes of action of catalase and established its capability of oxidizing a variety of substrates (Chance & Herbert, 1950). Subsequent investigations on the metabolisms of methanol (Chance, 1947; Aebi *et al.*, 1957*a*), ethanol (Jacobsein, 1952), formic acid (Aebi *et al.*, 1957*b*) and nitrite (Heppel & Porterfield, 1949)

indicate a probable role of liver catalase in the oxidation of these substances. de Duve & Baudhuin (1966) proposed an alternative possible role of catalase in carbohydrate metabolism by co-operation of the peroxisomal oxidases and the soluble glycolytic enzymes. Evidence for the significance of these postulat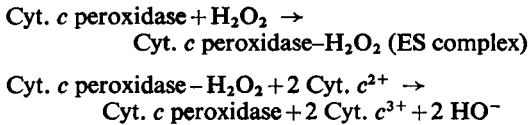ed functions is lacking, mainly owing to the lack of knowledge on the nature and effectiveness of the intracellular sources of H_2O_2.

A promising technique for the determination of H_2O_2-generation rates is afforded by the enzyme-substrate compounds of catalase and peroxidases, which have a characteristic high affinity for H_2O_2. The rates of formation of the catalase intermediate in subcellular suspensions (Chance & Oshino, 1971) and the steady-state concentration of the catalase intermediate in subcellular fractions (Chance & Oshino, 1971), in intact bacteria (Chance, 1950) or in

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perfused liver (Sies & Chance, 1970), have been used for this purpose.

A fluorescent hydrogen donor for the horseradish peroxidase reaction also affords an H_2O_2 indicator (Avi-Dor et al., 1954) and determinations by Loschen et al. (1971) established intact mitochondria as a novel source of H_2O_2 by the use of the scopoletin peroxidase method (Andreae, 1955; Perschke & Broda, 1961). Another fluorimetric method involving the use of the dye, diacetyldichlorofluorescein (Keston & Brandt, 1965), was previously utilized by Hinckle et al. (1967) to detect the evolution of H_2O_2 from submitochondrial particles. However, interferences in the above methods are to be expected owing to the broad specificity of horseradish peroxidase towards endogenous and exogenous hydrogen donors (Andreae, 1955).

Yeast cytochrome c peroxidase (Cyt. c peroxidase) has high specificity for reduced cytochrome c:

Cyt. c peroxidase $+ H_2O_2 \rightarrow$
    Cyt. c peroxidase–H_2O_2 (ES complex)

Cyt. c peroxidase $- H_2O_2 + 2$ Cyt. $c^{2+} \rightarrow$
    Cyt. c peroxidase $+ 2$ Cyt. $c^{3+} + 2$ HO$^-$

In the present paper we describe the use of the ES complex of cytochrome c peroxidase as a sensitive spectrophotometric H_2O_2 indicator. This method, when applied to subcellular fractions, reveals that in addition to the known peroxisomal oxidases, mitochondrial and microsomal electron-transport systems must be considered as intracellular sources of H_2O_2.

Materials and Methods

Yeast cytochrome c peroxidase, prepared by the method of Yonetani & Ray (1965), was kindly supplied by Professor T. Yonetani, Johnson Research Foundation, University of Pennsylvania. Glucose oxidase from Aspergillus niger (type II), pig kidney uricase (type V), ox liver catalase (type C-10), uric acid, D-alanine, NADH and NADPH were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Horseradish peroxidase (type II) was purchased from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A.

Rat liver fractions were prepared by the method of Schneider (1948) in MSE buffer (225 mM-mannitol, 75 mM-sucrose, 0.2 mM-EDTA, pH 7.2). The livers were perfused with MSE buffer before homogenization to eliminate haemoglobin. The pellet, after centrifugation of the homogenate at 700g for 10 min, was discarded. The supernatant (termed the 'homogenate') was centrifuged at 5000g for 10 min and the pellet, obtained after the 'fluffy layer' had been discarded, was washed. It was considered to consist of mainly heavy intact mitochondria and was termed the 'mitochondrial fraction'. The super-

natant fractions were centrifuged at 12000g for 10 min and then were washed, and the collected pellet including the fluffy layer, which consists of light mitochondria, peroxisomes, lysosomes and some microsomal membranes, was considered to be the 'peroxisomal fraction'. The 12000g supernatant was centrifuged at 104000g for 60 min to precipitate the 'microsomal fraction' and obtain the 'supernatant'.

The combined mitochondria–peroxisome fraction was used for the comparison of the rate of H_2O_2 generation and the steady state of the catalase intermediate as described by Chance & Oshino (1971). The trichloroacetic acid supernatant was prepared by treating a 1:5 (v/v) homogenate in water with 5% (v/v) trichloroacetic acid immediately after homogenization. After centrifugation at 4000g for 10 min the supernatant was removed, neutralized and assayed for uric acid. Sonication of peroxisomes was performed by using Sonifier Cell Disrupter, model W185, Ultrasonics Inc., Plainview, N.Y., U.S.A. Peroxisomes were suspended in MSE buffer at a protein concentration of 10 mg/ml and were sonicated three times for 30 s at 0°C.

Uric acid oxidation was determined spectrophotometrically at 293 minus 320 nm in 0.5 cm light-path cuvettes ($\Delta\epsilon_{mM} = 12.3$ litre·mmol^{-1}·cm^{-1}; Stimson & Reuter, 1943). Uric acid concentration in the supernatant was assayed by adding either 10μg of uricase/ ml and 0.1μM-haem of catalase, or peroxisomal fraction (0.05 mg/ml) to a supernatant diluted with 50 mM-potassium phosphate buffer, pH 7.6. Spectra were taken from 320–270 nm with a model 356 Perkin–Elmer double-beam spectrophotometer until no further decrease in E_{295} was observed.

Peroxisomal catalase was measured spectrophotometrically at 430 minus 407 nm ($\Delta\epsilon_{mM} = 100$ litre· mmol^{-1}·cm^{-1}) after conversion into its cyanide complex by addition of 0.4 mM-KCN (N. Oshino, unpublished work). Free catalase in the peroxisomal suspension, which was released from broken peroxisomes, was determined in the supernatant after dilution (1:6 to 1:150) and centrifugation (40000g, 15 min).
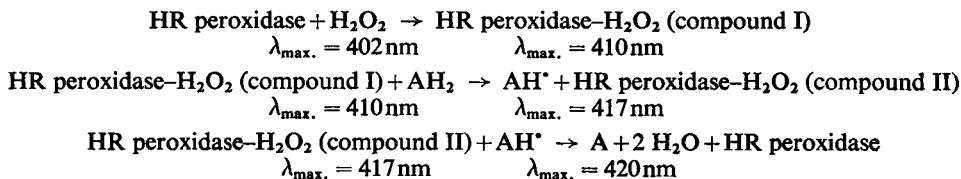
Nicotinamide nucleotides and cytochrome b_5 were determined spectrophotometrically at 350 minus 370 nm ($\Delta\epsilon_{mM} = 4.0$ litre·mmol^{-1}·cm^{-1}) and 424 minus 409 nm ($\Delta\epsilon_{mM} = 185$ litre·mmol^{-1}·cm^{-1}; Omura & Sato, 1964) respectively. The oxygen consumption of the homogenate was determined polarographically to be 350 nmol of O_2/min per g of liver.

Pigeon heart mitochondria were obtained as described by Chance & Hagihara (1963). Protein was measured by the biuret reaction (Gornall et al., 1949).

Procedure for the determination of the rate of generation of H_2O_2

This procedure measures the increase of absorbance at 419 nm (or 424 nm), with an active reference

wavelength at 407nm (or 400nm), of reaction mixtures containing $1-2\mu M$-cytochrome c peroxidase and about 0.1–0.3mg of protein/ml of the subcellular fraction to be studied. After calibration and equalization of the light-beams, the recording is started. On addition of appropriate substrates, the decrease in E_{407} (disappearance of free cytochrome c peroxidase) and the simultaneous increase in E_{419} (appearance of cytochrome c peroxidase–H_2O_2) are indicated as an increase in $\Delta E_{419-407}$, which follows a linear rate

solutions of both horseradish peroxidase (HR peroxidase) and cytochrome c peroxidase. The kinetics of formation of these ES complexes were compared under similar experimental conditions with pigeon heart mitochondria as the H_2O_2-generating system. Fig. 2 shows that the trace indicating the formation of the horseradish peroxidase intermediate is not linear. This is easily understandable, since the reaction involves two subsequent steps (Chance, 1952; George, 1953):

$$HR\ peroxidase + H_2O_2 \rightarrow HR\ peroxidase\text{–}H_2O_2\ (compound\ I)$$
$$\lambda_{max.} = 402\,nm \qquad\qquad \lambda_{max.} = 410\,nm$$

$$HR\ peroxidase\text{–}H_2O_2\ (compound\ I) + AH_2 \rightarrow AH^{\cdot} + HR\ peroxidase\text{–}H_2O_2\ (compound\ II)$$
$$\lambda_{max.} = 410\,nm \qquad\qquad \lambda_{max.} = 417\,nm$$

$$HR\ peroxidase\text{–}H_2O_2\ (compound\ II) + AH^{\cdot} \rightarrow A + 2\ H_2O + HR\ peroxidase$$
$$\lambda_{max.} = 417\,nm \qquad\qquad \lambda_{max.} = 420\,nm$$

until all the cytochrome c peroxidase is converted into its ES complex. Two wavelength settings have been used: 419 minus 407nm ($\Delta\epsilon_{mM} = 50$ litre·mmol^{-1}·cm^{-1}) and 424nm minus 400nm ($\Delta\epsilon_{mM} = 60$ litre·mmol^{-1}·cm^{-1}) (Yonetani, 1965). The second pair gives not only increased sensitivity for the ES complex, but also an undesirable increase of sensitivity to changes in light-scattering. The low protein concentration used in the assay diminishes the optical interference from cytochromes that occurs immediately on addition of the reagents, which thus does not affect the rate measurements and represents $<5\%$ of the total absorption change with mitochondria and $<20\%$ with microsomal fraction (reduction of cytochrome b_5, see Fig. 9). Both a model 356 Perkin-Elmer and an Aminco–Chance double-beam spectrophotometer were used for these determinations. All measurements were made at room temperature (21–23°C).

Results

Fig. 1 shows absolute spectra of horseradish peroxidase (a) and yeast cytochrome c peroxidase (b) in the presence of 0.3–1.0mg of pigeon heart mitochondria/ml. The absorption maxima of those peroxidases (Fig. 1) are at 402 and 407nm in the Soret region respectively (Chance, 1949; Yonetani & Ray, 1965). Addition of both succinate and antimycin A, which enhances the generation of H_2O_2 from mitochondria as described below, results in shifts of the absorption maximum to 417 and 419nm respectively, within a few minutes. The new derivatives were identified as horseradish peroxidase–H_2O_2 compound II and cytochrome c peroxidase ES complex respectively by comparison with the spectral characteristics reported by Yonetani (1965), as well as by control experiments in which H_2O_2 was added to

The H_2O_2-generation rate calculated from the steepest slope of the trace by using $\Delta\epsilon_{mM}$ (417–402nm) $= 50$ litre·mmol^{-1}·cm^{-1} is $0.4\mu M$/min per
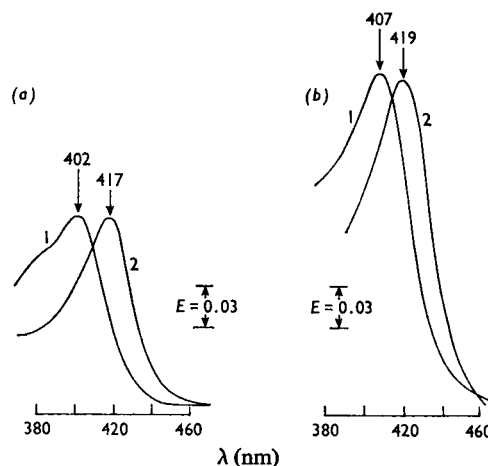

Fig. 1. *Spectra of horseradish peroxidase and cytochrome c peroxidase and their ES complexes with H_2O_2 generated by pigeon heart mitochondria*

(a) (1) Difference spectrum of $1.7\mu M$-horseradish peroxidase added to pigeon heart mitochondria (1.0mg of protein/ml) recorded against a mitochondrial suspension; (2) difference spectrum of the same mixture, after addition of 4mM-succinate and $0.5\mu M$-antimycin A. (b) (1) $2.2\mu M$-cytochrome c peroxidase added to a suspension of pigeon heart mitochondria (0.33mg of protein/ml) recorded against a mitochondrial suspension; (2) difference spectrum of the same mixture after addition of 4mM-succinate and $0.2\mu M$-antimycin A. The reaction medium consisted of 225mM-mannitol, 75mM-sucrose and 20mM-tris–HCl buffer, pH7.3.
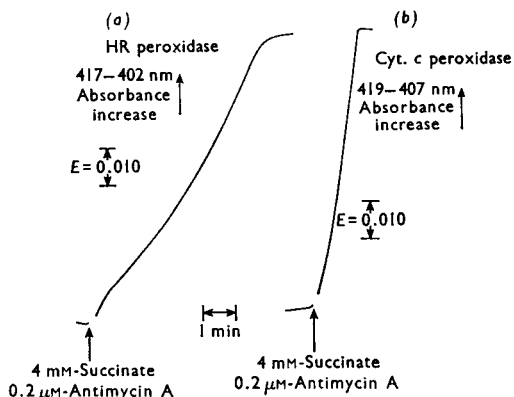
Fig. 2. *Kinetics of H_2O_2 production by pigeon heart mitochondria in the presence of succinate and antimycin A*

(*a*) Formation of horseradish peroxidase compound II; (*b*) formation of the ES complex of cytochrome *c* peroxidase. Experimental conditions are as in Fig. 1.

Fig. 3. *Relation between the amount of glucose oxidase and the rate of trapping of H_2O_2 by cytochrome c peroxidase*

For details see the text. The reaction medium was 225mM-mannitol, 75mM-sucrose, 30mM-tris–morpholinopropanesulphonic acid buffer, pH7.4, 5mM-glucose and 1.5 μM-cytochrome *c* peroxidase.

0.33mg of protein per ml. In contrast, the formation of the cytochrome *c* peroxidase–H_2O_2 intermediate is linear in time until all the cytochrome *c* peroxidase is converted into its ES complex, as shown by Fig. 2(*b*). Further, the rate of H_2O_2 generation calculated from this latter trace, by using $\Delta\epsilon_{mM}$ (419–417nm) = 50 litre·mmol^{-1}·cm^{-1}, is 1.2 μM/min per 0.33mg of protein per ml. This difference in rates might indicate an interference from an unidentified hydrogen donor, in the peroxidase or in the preparation, that precludes the use of horseradish peroxidase of this purity as a quantitative spectrophotometric H_2O_2 indicator.

Fig. 3 shows that the cytochrome *c* peroxidase assay can be applied to the detection and determination of H_2O_2-generation rates in the range 0.1–1.2 μM/min. When H_2O_2 is provided by the glucose–glucose oxidase system, the method gives a linear relationship between the rates of H_2O_2 production and the amount of added glucose oxidase.

The recovery of H_2O_2 from the glucose–glucose oxidase system by this method was further tested in the presence of subcellular fractions and is summarized in Table 1. Subcellular fractions contain their own H_2O_2-generating systems and their own endogenous substrates, and further these generation rates are enhanced by additions of specific substrates for those fractions (Table 1, column 1). The characteristics of H_2O_2 generation by the different subcellular fractions themselves are described in detail below.

Addition of glucose to the subcellular fractions does not stimulate H_2O_2 production by each fraction. The subsequent addition of glucose oxidase results in
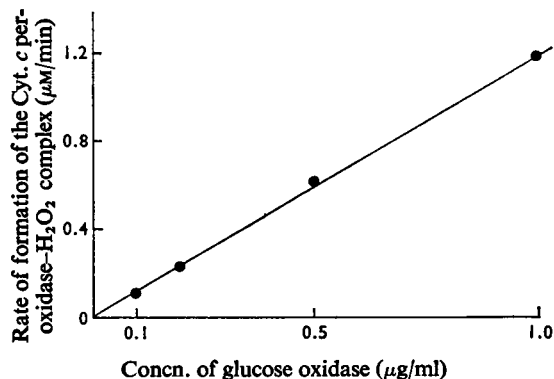
a rapid formation of the ES complex. The increase in the rate of H_2O_2 generation (Table 1, column 2 minus column 1) agrees with the H_2O_2-generation rate by the glucose–glucose oxidase system used with mitochondrial, microsomal and peroxisomal fractions, and indicates essentially a 95–103% recovery of the added H_2O_2-generating enzyme activity.

However, with the total homogenate and the supernatant fraction, the rate of formation of the cytochrome *c* peroxidase intermediate decreases with time and finally a steady state is reached. The concentration of the ES complex at the steady state depends on the rate of generation of H_2O_2. Further, the rates calculated from the initial slope of the traces indicate 80 and 75% recovery of activity in the supernatant and the whole homogenate respectively.

Possible factors interfering with the cytochrome *c* peroxidase assay under these experimental conditions are reduced cytochrome *c* and catalase. The establishment of a steady state of the cytochrome *c* peroxidase ES complex might be best explained by the existence of soluble cytochrome *c* and cytochrome *c* reductase.

To evaluate the interference by catalase with the cytochrome *c* peroxidase assay, the rate of formation of cytochrome *c* peroxidase ES complex was tested in the absence and in the presence of catalase. H_2O_2 was provided by the glucose–glucose oxidase system (Fig. 4). Substituting into eqn. (11) (see the Appendix) for $k_4 = 1.8 \times 10^7$M^{-1}s^{-1} (Chance *et al.*, 1952), $p/e \simeq 0.3$ and $k = 5 \times 10^7$M^{-1}s^{-1} (Chance *et al.*, 1967) and for 0.2 μM-cytochrome *c* peroxidase (e_1) and 1 μM-

Table 1. *Recoveries of the H_2O_2-generating activity of the glucose–glucose oxidase system in the presence of subcellular fractions from rat liver*

The reaction mixture contained, in a final volume of 3.0ml, 225mм-mannitol, 75mм-sucrose, 30mм-tris-morpholinopropanesulphonic acid buffer, pH7.4, and 2μм-cytochrome c peroxidase. The glucose–glucose oxidase system consisted of 5mм-glucose and 0.5μg of glucose oxidase/ml. H_2O_2 was determined as described in the Materials and Methods section.

	H_2O_2-generation rate (μм/min)		
Subcellular fraction and substrate	Control	+Glucose–glucose oxidase	Recovery (%)
None	—	0.64	—
Mitochondria (0.33mg of protein/ml)			
Endogenous substrate	0.03	0.65	97
+Succinate (4mм)	0.07	0.73	103
Peroxisomes (0.07mg of protein/ml)			
Endogenous substrate	0.01	0.66	101
+Uric acid (35μм)	0.66	1.32	103
Microsomes (0.36mg of protein/ml)			
Endogenous substrate	0.08	0.72	100
+NADH (30μм)	0.17	0.80	98
+NADPH (50μм)	0.21	0.82	95
Supernatant (0.7mg of protein/ml)			
Endogenous substrate	0.07* (8%)	0.51 (40%)	80
Homogenate (2.2mg of protein/ml)			
Endogenous substrate	0.45* (25%)	0.48* (50%)	75

* Initial rate. After approx. 1–2min, a steady state of the cytochrome c peroxidase–H_2O_2 compound is reached. The percentage of saturation of cytochrome c peroxidase as the cytochrome c peroxidase ES intermediate is indicated in parentheses beside the value for the rate of H_2O_2 generation.

haem of catalase (e) gives a ratio that graphically shows $dp_1/dt = -0.5\ dx/dt$,

$$\frac{dp_1}{dt} = -\frac{dx}{dt} \cdot 1 \bigg/ 1 + \frac{2 \times (1.8 \times 10^7) \times 0.3}{(5 \times 10^7) \times 0.2} = -0.5\frac{dx}{dt}$$

Thus a ratio of haem of catalase to cytochrome c peroxidase of 5:1 decreases the rate of dp_1/dt to one half. A similar calculation shows that $dp_1/dt = -0.95\,dx/dt$ at a ratio of haem of catalase to cytochrome c peroxidase of 0.25:1.

Application of the cytochrome c peroxidase to subcellular fractions

Mitochondrial generation of H_2O_2. Indirect (Chance & Oshino, 1971) and direct measurements (Loschen et al., 1971) pointed to intact mitochondria as a source of H_2O_2.

Fig. 5 illustrates the production of H_2O_2 by rat liver mitochondria. The rate of H_2O_2 generation is indicated as an upward slope owing to the formation of the cytochrome c peroxidase ES complex. Table 1 shows that the recovery of H_2O_2 by this assay is 97–103% and that, if intact mitochondria are used, cytochrome c located on the inner mitochondrial membrane cannot react with exogenous cytochrome c peroxidase, since the outer mitochondrial membrane is impermeable to cytochrome c peroxidase (Chance, 1971). At the beginning of the trace a slow endogenous rate of H_2O_2 production is recorded. Addition of succinate speeds up H_2O_2 production by a factor of six and, after that, the addition of ADP (state 4 → state 3 transition) (Chance & Williams, 1956) decreases it to a value similar to that in the absence of substrate. When all the added ADP is phosphorylated, there is an acceleration paralleling the changes in the mitochondrial metabolic state. A further addition of ADP produces the transition to the active state, with the corresponding slow rate of H_2O_2 production. The addition of antimycin A further stimulates the rate.

Table 2 shows the mitochondrial production of H_2O_2 with different substrates. All the tested mitochondrial substrates, i.e. malate+glutamate, succinate, palmitoylcarnitine and octanoate, stimulate H_2O_2 generation. Ascorbate, as a substrate that activates the cytochrome c to oxygen span of the respiratory chain, cannot be tested under these assay conditions, since it acts as a hydrogen donor for the cytochrome c peroxidase reaction (Yonetani & Ray,

1965). Among the active substrates, succinate is the most effective. The production of H_2O_2 in state 4 accounts for about 1–2% of the total O_2 consumption.
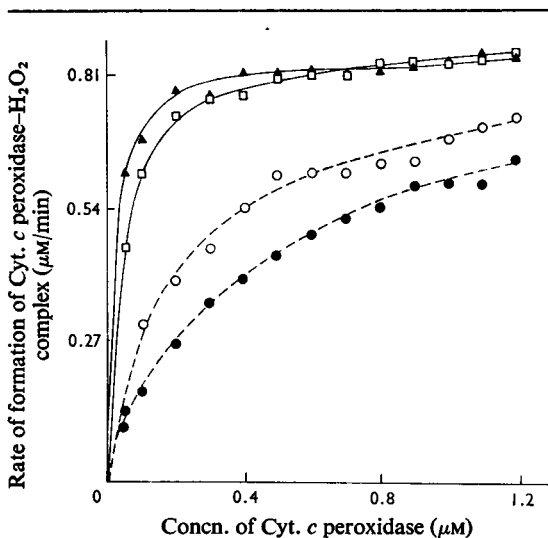

Fig. 4. *Rates of cytochrome c peroxidase ES complex formation in the presence and absence of catalase as a function of the cytochrome c peroxidase concentration*

For details see the text. The concentrations of haem of catalase were: ▲, 0; □, 0.2 μM; ○, 1 μM; ●, 2 μM. The H_2O_2-generating system consisted of 5 mM-glucose and 0.6 μg of glucose oxidase/ml. The reaction mixture contained 120 mM-KCl, 30 mM-tris–morpholinopropanesulphonic acid buffer, pH 7.4.

The other substrates were less effective and only increased the generation rate slightly over the basal rate supported by endogenous substrate. The rates were substantially decreased upon the transition from state 4 to state 3, by addition of either ADP or pentachlorophenol. It should be pointed out that the production of H_2O_2 supported by NAD-linked substrates, i.e. malate+glutamate (Table 2), is sensitive to rotenone.

Table 3 shows the effect of the mitochondrial metabolic state on the peroxisomal catalase. A correlation is observed between the rate of mitochondrial H_2O_2 generation (Table 3, column 1) and the steady-state concentration of the peroxisomal catalase intermediate (Table 3, column 2). The steady state of the intermediate follows the variations in the rate of mitochondrial H_2O_2 production. In the absence of mitochondrial H_2O_2 formation (i.e. in the presence of pentachlorophenol) the haem occupancy of the peroxisomal catalase with H_2O_2 (p/e) is 35% of its saturation value. This decrease in the steady-state concentration of the catalase intermediate (65 to 35%) corresponds to a decrease to less than one-third in the rate of H_2O_2 generation (B. Chance & N. Oshino, unpublished work).

Peroxisomal generation of H_2O_2. The peroxisome-rich fraction used in these experiments is not pure; it contains light mitochondria, rough microsomal membranes and lysosomes as well. The intracellular distribution of catalase activity in the subcellular fractions is: 60% of the total in the 'peroxisome-rich fraction', 8% in the heavy mitochondrial fraction and the remaining activity (25–30%) is recovered in the supernatant+microsomal fraction. Similarly when uricase activity was considered 65% was found in the
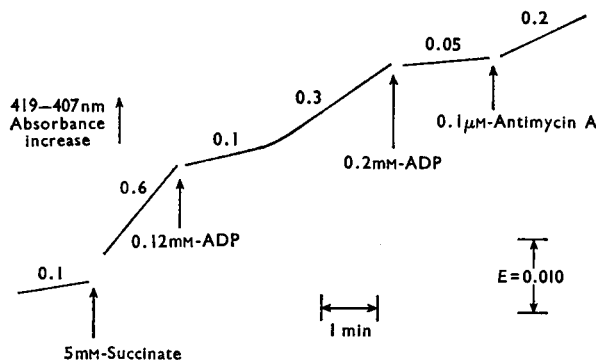

Fig. 5. *Generation of H_2O_2 by rat liver mitochondria (0.33 mg of protein/ml) detected by 1.4 μM-cytochrome c peroxidase*

For details see the text. The reaction medium was 225 mM-mannitol, 75 mM-sucrose, 4 mM-phosphate buffer and 20 mM-tris–morpholinopropanesulphonic acid buffer, pH 7.4. The numbers near the traces indicate nmol of H_2O_2/min per mg of protein.

Table 2. *Effect of different substrates on the generation of H_2O_2 by rat liver mitochondria*

Rat liver mitochondria (0.53 mg of protein/ml) were suspended in 225 mM-mannitol, 75 mM-sucrose, 4 mM-potassium phosphate, 25 mM-tris–morpholinopropanesulphonic acid, pH 7.4, and supplemented with 1.4 μM-cytochrome c peroxidase. The generation of H_2O_2 was recorded as described in the Materials and Methods section.

Substrate and additions	H_2O_2 generated (nmol/min per mg of protein)
Endogenous substrate	0.16
+Rotenone (0.6 μM)	0.08
+Pentachlorophenol (2 μM)	0.04
Malate (4 mM)+glutamate (4 mM)	0.19
+ADP (0.5 mM)	0.08
+Rotenone (0.6 μM)	0.10
Succinate (5 mM)	0.40
+ADP (0.5 mM)	0.06
Palmitoylcarnitine (10 μM)	0.23
+Pentachlorophenol (2 μM)	0.06
Octanoate (14 μM)	0.22
+Pentachlorophenol (2 μM)	0.04

Table 3. *Effect of the mitochondrial metabolic state on H_2O_2 generation and on the steady state of the catalase intermediate*

The reaction mixture was as described in Table 1 and contained 0.33 and 3.7 mg of protein/ml of rat liver mitochondrial–peroxisomal fraction for measurements of H_2O_2 generation and catalase intermediate respectively. Production of H_2O_2 was determined by using the cytochrome c peroxidase method as illustrated in Fig. 4. The percentage of saturation of the catalase intermediate was measured at 640 minus 660 nm in a double-beam spectrophotometer and was calculated as described by Chance & Oshino (1971). Pentachlorophenol at a final concentration of 2 μM (H_2O_2 production) or 8 μM (catalase intermediate) was added as a solution in dimethylformamide.

State	Addition	H_2O_2 production (μmol/min per mg of protein)	Saturation of the catalase intermediate (%)
1	None	0.15	62
4	Succinate (5 mM)	0.70	90
3	Pentachlorophenol	0.00	35
	+Antimycin A (0.4 nmol/mg of protein)	0.18	80
1	Uric acid (30 μM)	6.20	100
1	Ethanol (12 mM)	0.15	0

peroxisome-rich fraction, 10% in the mitochondrial fraction and 25% in the microsomal fraction.

Fig. 6 shows a typical example of peroxisomal H_2O_2 generation. Endogenous substrate affords only a slow rate at the beginning of the experiment. When D-alanine, the substrate of D-amino acid oxidase, is added, the rate of formation of the cytochrome c peroxidase ES complex is increased. This result indicates that in spite of the presence of intraperoxisomal catalase a fraction of the generated H_2O_2 diffuses across the membrane to the external reaction medium. Under similar experimental conditions, D-

alanine produced about 70% haem occupancy (p_m/e), in the steady-state concentration of the catalase intermediate. The addition of NaN_3, a catalase inhibitor, increases the rate of H_2O_2 diffusion outside the peroxisomes. The rates increase with increasing concentrations of azide and a saturation value seems to be reached at about 20 μM, an azide concentration that gives complete conversion of the catalase–H_2O_2 intermediate into catalase–nitric oxide complex (Lemberg & Foulkes, 1946).

Fig. 7 shows another typical case of peroxisomal H_2O_2 generation; in this case, the addition of uric
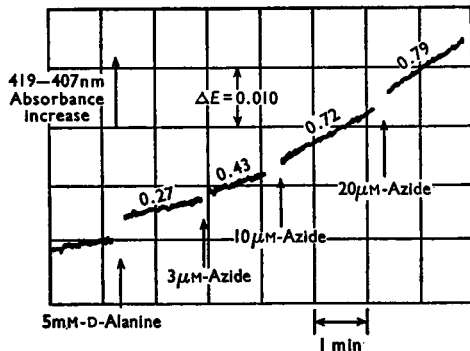
Fig. 6. D-*Alanine-induced* H_2O_2 *generation by the peroxisome-rich fraction of rat liver*

For details see the text. The reaction medium consisted of 225mM-mannitol, 75mM-sucrose, 30mM-tris–morpholinopropanesulphonic acid buffer, pH7.4, and 12μM-cytochrome c peroxidase. The peroxisomal protein concentration was 0.14mg/ml. The substrate was D-alanine (5mM).
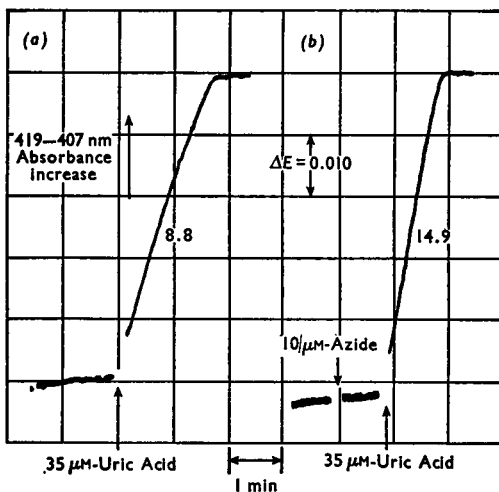

Fig. 7. *Uric acid-induced* H_2O_2 *generation by the peroxisome-rich fraction of rat liver*

Experimental conditions were as in Fig. 6, except that the peroxisomal protein concentration was 0.07mg/ml and the substrate was uric acid (35μM).

acid activates the most active of the peroxisomal H_2O_2-producing enzymes, uricase. In the left trace (*a*), the addition of uric acid results in formation of the cytochrome c peroxidase ES complex at a rate of 8.8nmol/min per mg of protein, which is accelerated

by a factor of 1.7 after addition of 10μM-NaN₃ (trace *b*).

Some characteristics of the peroxisomal fraction as a H_2O_2 generator are summarized in Table 4, Expt. A, in which the rate of H_2O_2 generation by intact peroxisomes in the presence of uric acid is compared with the rate of uric acid utilization under the same experimental conditions; it is found that about 42% of the H_2O_2 generated by the uricase reaction diffuses out of the peroxisomal membrane. Addition of azide up to a concentration of 20μM (no further increases are observed at higher concentrations) enhances the rate of H_2O_2 trapping by external cytochrome c peroxidase.

When the peroxisomal structure is disrupted either by treatment with deoxycholate or by sonication, about 90% of the generated H_2O_2 calculated from the rate of uric acid disappearance is recovered as cytochrome c peroxidase–H_2O_2 complexes. No effect of azide is observed with the disrupted peroxisomes.

When D-alanine is used as substrate, intact peroxisomes supplemented with azide give an H_2O_2-generation rate of 1.3nmol/min per mg of protein. This value accounts for about 85% of the value obtained with the deoxycholate-treated preparation. The measurement of H_2O_2 production in intact peroxisomes supplemented with azide or in deoxycholate-treated peroxisomes provides a sensitive assay for peroxisomal activities, which are otherwise difficult to estimate.

The generation of H_2O_2 by the peroxisomal fraction supported by endogenous substrate varied in the range 0.1–0.4nmol/min per mg of protein. In general, fresh preparations show a higher endogenous activity than the aged ones, and repeated washing of the peroxisomal pellet decreases the activity of a particular preparation. Freshly prepared supernatant, after separation of the microsomal fraction, was added to the peroxisome fractions (Table 4). Rates of 2.3 and 8.6nmol of H_2O_2/min per mg of protein are observed in intact and deoxycholate-treated peroxisomes respectively. The substance that is present in the supernatant fraction and stimulates H_2O_2 formation is identified as uric acid in Fig. 8. The amount of uric acid found in the supernatant corresponds to a cytosolic concentration of 3.2mM. When a similar experiment is carried out with a trichloroacetic acid supernatant a corresponding cytosolic concentration of 0.1mM-uric acid is found. Thus the accumulation of uric acid in the supernatant fraction is a consequence of the separation of the particulate peroxisomes (containing uricase) from the homogeneous phase containing the soluble enzymes of the purine-degradation pathway.

The lack of effect of azide in the deoxycholate-treated and in the sonicated peroxisomes (Table 4) seems to show that catalase in free solution at a concentration of 0.05–0.1μM (calculated from the

Table 4. *Production of H_2O_2 by peroxisomal preparations from rat liver*

Peroxisomal fractions were suspended in the buffer described in Table 1 and were assayed for H_2O_2 production spectrophotometrically at 419 minus 407nm in the presence of $1.2\mu M$-cytochrome c peroxidase at a protein concentration of 0.07–0.38mg/ml, and were assayed for oxygen uptake polarographically at a protein concentration of 0.7–2.8mg/ml, and were assayed for uric acid oxidation spectrophotometrically at 293 minus 320nm at a protein concentration of 0.76–1.54mg/ml. Uric acid was $40\mu M$ for the spectrophotometric determinations and $300\mu M$ for the polarographic assay. Oxygen uptake was measured in the presence of 34mM-ethanol.

Peroxisomal fraction and substrate+additions	Production of H_2O_2 (nmol/min per mg of protein)	Uptake of O_2 (nmol/min per mg of protein)	Uric acid oxidation (nmol/min per mg of protein)
Expt. A			
Intact peroxisomes			
Endogenous substrate	0.4	2.2	—
+Supernatant (0.5ml)*	2.3	—	—
+Uric acid	7.8	17.9	18.5
+Azide (7μM)	9.2	—	—
+Azide (20μM)	11.1	17.9	17.6
+Azide (40μM)	10.5	—	—
+D-Alanine (5mM)	0.56	4.9	—
+Azide (20μM)	1.30	—	—
Deoxycholate-treated peroxisomes			
Endogenous substrate	0.8	—	—
+Supernatant (0.5ml)*	8.6	—	—
+Uric acid	18.2	—	20.5
+Azide (20μM)	17.8	—	19.4
D-Alanine	1.53	—	—
Sonicated peroxisomes			
Endogenous substrate	2.0	—	—
+Supernatant (0.5ml)*	9.0	—	—
+Uric acid	19.2	—	21.0
+Azide (20μM)	18.6	—	21.0
Expt. B			
Intact peroxisomes			
Endogenous substrate	0.2	—	—
+Uric acid	16.4	—	27.0
+Azide (20μM)	24.5	—	25.5

* This was added to a final volume of 3.0ml, corresponding to approx. 50mg wet wt. of liver and carrying 2.07mg of protein.

catalase concentration in the peroxisomal fraction of 0.25nmol of haem of catalase/mg of protein) does not successfully compete with $1–2\mu M$ cytochrome c peroxidase for the common substrate. In Expt. B (Table 4) a more purified (three times washed) peroxisome-rich fraction was also used (the higher specific activity and the lower H_2O_2-generation rate supported by endogenous substrate should be noted). To estimate the intactness of the peroxisomal membrane, the amount of free catalase in this preparation was measured (see the Materials and Methods section) and was found to be 15% of the total catalase. The rate of formation of the cytochrome c peroxidase ES complex representing diffusion of H_2O_2 accounts for 61 and 95% of the rate of uric acid oxidation, which corresponds to the rate of intraperoxisomal H_2O_2 generation in the absence and in the presence of azide respectively.

Microsomal production of H_2O_2. When a microsomal suspension is assayed for H_2O_2 by the cytochrome c peroxidase test, a slow endogenous production that decays after 2 or 3min is detected. On addition of NADPH, the generation of H_2O_2 increases to values that account for 71–86% of the rate of nicotinamide nucleotide oxidation in the different buffers. The highest activity (1.7nmol of H_2O_2/min per mg of protein) is observed in the microsomal preparation suspended in the phosphate buffer. When
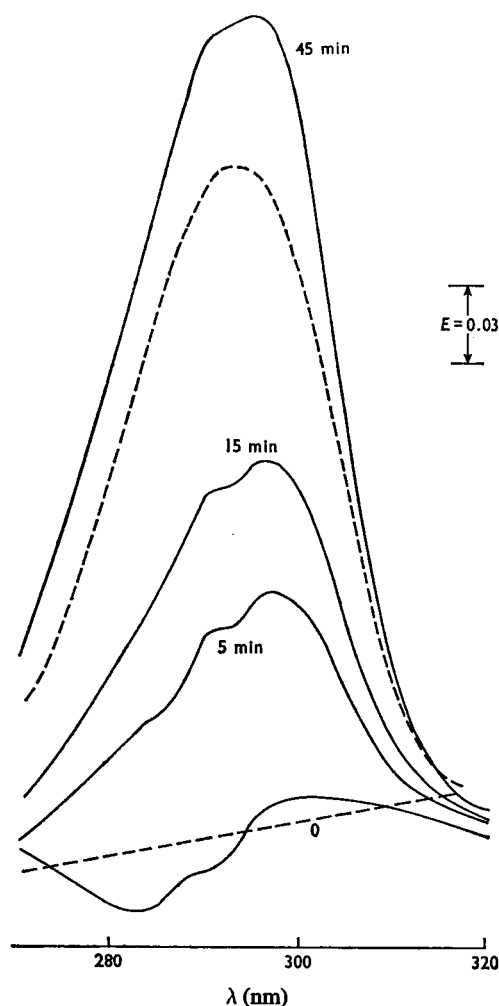
Fig. 8. *Difference spectra of the supernatant fraction after addition of 0.1 mg of uricase/ml and 0.03 μM-catalase to the reference cuvette*

For details see the text. The supernatant was used, diluted 1:6, in mannitol–sucrose–tris–morpholinopropanesulphonic acid buffer, pH 7.4. The broken line is the spectrum of a sample of uric acid. The light-path was 5 mm.

NADH is added as substrate the rates of H_2O_2 generation account for 6–25 % of the rate of nicotinamide nucleotide oxidation in the different buffers. The highest activity (0.47 nmol of H_2O_2/min per mg of protein) is observed in the microsomal preparation suspended in mannitol–sucrose–tris–morpholinopropanesulphonic acid buffer.

The effect of 20 μM-azide on the microsomal production of H_2O_2 was tested to evaluate any interference from contaminating catalase, but no increase in the rates of H_2O_2 generation was observed after addition of NaN_3.

The rate of auto-oxidation of cytochrome b_5 was tested with the microsomal preparation suspended in different buffers. Fig. 9 shows that cytochrome b_5 is oxidized in a first-order process after exhaustion of substrate (Chance & Pappenheimer, 1954; Oshino & Sato, 1971). The rate constants for the oxidation of cytochrome b_5 in the different buffers are listed in Table 5. The electron fluxes through this cytochrome are clearly smaller than the maximal microsomal rates of H_2O_2 generation, especially when compared with the NADPH-supported rates, and exclude the possibility of cytochrome b_5 being a significant microsomal generator of H_2O_2. If we assume, on the basis of the higher rates of H_2O_2 generation observed in the presence of NADPH, that the H_2O_2 generator is a member of the microsomal NADPH–oxidation pathway, the inverse relation between the rates of cytochrome b_5 oxidation and H_2O_2 generation found in the different buffers can be interpreted as modifications in the interaction between both microsomal systems of nicotinamide nucleotide oxidation.

Discussion

The aim of this paper is a double one. First, it introduces a new sensitive assay for the determination of H_2O_2 in biological samples. Secondly, by using the cytochrome *c* peroxidase assay, it gives the rates of production of H_2O_2 by the different subcellular fractions, which summed up provide an estimate of the cellular generation of H_2O_2.

The remarkable affinity of enzyme and substrate in the case of the peroxidases and the high extinction coefficients of the ES complexes afford spectrophotometric H_2O_2 indicators of high sensitivity. The use of cytochrome *c* peroxidase in preference to the several other peroxidases offers the further advantage of the unusual stability of its ES complex, presumably because of the high specificity of cytochrome *c* peroxidase towards hydrogen donors. These qualities afford a specific and sensitive indicator for H_2O_2 in the range 0.1–1.0 μM.

The production of H_2O_2 by submitochondrial particles was reported by Jensen (1966) and by Hinckle *et al.* (1967). The fact that intact mitochondria can generate H_2O_2 under physiological conditions was inferred from changes in the degree of saturation of the catalase intermediate after changes in mitochondrial metabolic states in the peroxisomal mitochondrial fraction of rat liver (Chance & Oshino, 1971). Direct measurement by Loschen *et al.* (1971) confirmed the alteration of H_2O_2 production on the state 4 → state 3 transition
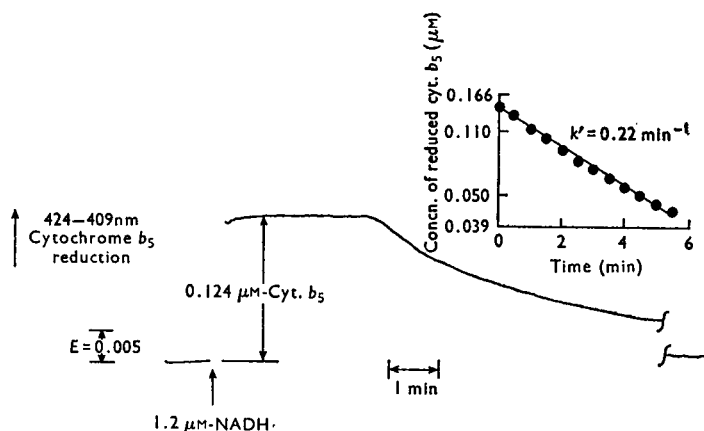
Fig. 9. *Rate of cytochrome b_5 oxidation in microsomal fractions*

The reaction mixture contained, in a final volume of 3.0ml, 1mg of protein of the microsomal fraction, 225mM-mannitol, 75mM-sucrose and 20mM-tris–morpholinopropanesulphonic acid buffer, pH7.4. After an addition of 1.2μM-NADH, the change in the redox state of cytochrome b_5 was measured at 424 minus 409nm. In the inserted small figure the concentration of reduced cytochrome b_5 at a given time in the reoxidation process is plotted against time. The apparent first-order rate constant (k') was estimated from the slope of the curve as described by Oshino & Sato (1971).

Table 5. *H_2O_2 production, nicotinamide nucleotide oxidation and cytochrome b_5 oxidation in rat liver microsomal fraction*

Microsomal fractions were suspended in the different buffers and were assayed for H_2O_2 production in the presence of 1.4μM-cytochrome c peroxidase at 0.27mg of protein/ml, were assayed for nicotinamide nucleotide oxidation at 0.36–0.72mg of protein/ml, and were assayed for cytochrome b_5 oxidation at 0.36mg of protein/ml. The composition of the buffers is as follows: A, 225mM-mannitol, 75mM-sucrose and 30mM-tris–morpholinopropanesulphonic acid buffer, pH7.4; B, 225mM-mannitol, 75mM-sucrose, 0.2mM-EDTA and 30mM-potassium phosphate buffer, pH7.4; C, 150mM-KCl and 30mM-tris–morpholinopropanesulphonic acid buffer, pH7.4; D, 100mM-potassium phosphate buffer, pH7.4. The electron rate (e^- rate) through cytochrome b_5 was calculated from e^- rate $= k' \cdot [b_5]$, and cytochrome b_5 concentration ($[b_5]$) was taken as our average value of 0.37nmol/mg of protein. k' was estimated as described in Fig. 9.

	Buffer A	Buffer B	Buffer C	Buffer D
Generation of H_2O_2				
(nmol/min per mg of protein)				
Endogenous substrate	0.11	0.11	0.13	0.14
+NADPH (50μM)	0.67	0.70	1.56	1.70
+NADH (33μM)	0.48	0.16	0.29	0.28
Nicotinamide nucleotide oxidation				
(nmol/min per mg of protein)				
+NADPH (50μM)	0.86	0.81	2.05	2.40
+NADH (33μM)	1.85	2.90	2.80	3.05
Cytochrome b_5 oxidation				
k' (min^{-1})	0.22	1.00	0.73	0.63
e^- rate (ne^-/min per mg of protein)	0.08	0.37	0.27	0.24

in pigeon heart mitochondria. Our present results report rates of H_2O_2 production associated with different substrates and metabolic conditions. It is apparent that all the mitochondrial substrates enter-

ing the respiratory chain before the antimycin A-sensitive site are able to support H_2O_2 generation. If we assume that there is only one H_2O_2 generator in mitochondria, it seems that this H_2O_2 generator is

a member of the respiratory chain or in equilibrium with it; the increased oxidation of the respiratory carriers in the active mitochondrial states prevents the formation of H_2O_2, whereas the reduced states enhance the rate of reaction with O_2.

The other system of electron transport in the cell, the membranes of the endoplasmic reticulum system, were claimed to be a source of H_2O_2 as early as 1957 by Gillette *et al.* (1957). Our results (Table 5) show that in the absence of hydroxylating substrates the microsomal oxidation of NADPH yields H_2O_2 almost quantitatively. As the ability of flavoproteins to generate H_2O_2 is well known (Dixon, 1971) and, further, the microsomal NADPH-specific flavoprotein exhibits unusual reactivity towards electron acceptors such as cytochrome c, it seems reasonable to assume that the NADPH–cytochrome c reductase might be the microsomal H_2O_2 generator.

NADPH is more effective than NADH in promoting the microsomal oxidation of methanol (Orme-Johnson & Ziegler, 1965) and ethanol (Lieber & DeCarli, 1970). This fact can be reinterpreted as the nicotinamide nucleotide specificity of the microsomal production of H_2O_2, which is able to oxidize alcohols through the peroxidatic reaction of catalase. In addition, it seems that H_2O_2 could be considered as the initiator of the process of microsomal-lipid peroxidation (Hoschein & Ernster, 1963; Ernster & Nordenbrand, 1967).

We have shown that some (40–80%) of the H_2O_2 generated in the peroxisomes is destroyed inside the organelle, and that the remaining 20–60% diffuses to the surrounding medium and is detected by the cytochrome c peroxidase assay. It is noteworthy that the rates measured with this method are rates of generation of free H_2O_2. In spite of being 'free' H_2O_2, no significant concentration of H_2O_2 is built up under the conditions of the experiment, as H_2O_2 is converted into the cytochrome c peroxidase–H_2O_2 intermediate.

The very low H_2O_2 concentration maintained in the medium surrounding the peroxisomes, because of the presence of cytochrome c peroxidase and the permeability of the peroxisomal membrane to H_2O_2, can be regarded as the cause of the apparent ineffectiveness of catalase in destroying the intraperoxisomally generated H_2O_2. A reaction sequence of the type:
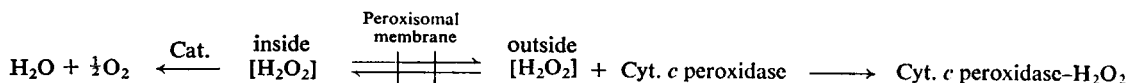
passage across the membrane of the internal H_2O_2. The high permeability of the peroxisomal membrane to H_2O_2 (de Duve, 1963; de Duve & Baudhuin, 1966) should be considered of primary physiological importance in allowing a rapid equilibration of the H_2O_2 concentrations at both sides of the peroxisomal membrane.

The observed increase in the haem occupancy of the peroxisomal catalase in parallel with the changes in mitochondrial H_2O_2 generation seems to be pertinent to this consideration. The possibility of H_2O_2 leaking out from catalase-loaded peroxisomes has been advanced by Poole (1968) on theoretical grounds.

Considering the whole rat liver homogenate, the rate of H_2O_2 production [38 nmol/min per g of liver at 22°C (Table 6), which accounts for about 10% of the total oxygen uptake] agrees with the reported rate of methanol oxidation (110 nmol/min per g of liver, at 37°C; Goodman & Tephly, 1968), a process catalysed by catalase that should be strictly limited by the supply of H_2O_2.

To consider the relative importance of the different subcellular fractions and the total cellular generation of free H_2O_2 in the whole liver, we have calculated the values shown in Table 6 from average values of specific activities and amount of protein isolated in the different fractions. Very approximate assumptions are made to estimate some approximate values that could approach the physiological conditions.

For instance: (*a*) the mitochondrial value is chosen by assuming that the succinate-supplemented state 4 approximates to the physiological liver condition (Scholz *et al.*, 1969); (*b*) the microsomal value is estimated by assuming a non-limiting concentration of NADPH and without consideration of the impact that hydroxylation of substrates could make on H_2O_2 production; (*c*) the peroxisomal fraction generates intraperoxisomal H_2O_2 at any rate between 44 and 172 nmol/min per g of liver, respectively the rates with endogenous substrate and after addition of a supernatant fraction in which accumulation of uric acid has been noticed. We estimate a mean value of 100 nmol/min per g wet wt. of liver, from which, assuming that the cytosolic concentration of H_2O_2 is negligible, approx. 30% would be able to diffuse out of the peroxisome. (The accumulation of uric acid, although regarded as an isolation artifact,

$$H_2O + \tfrac{1}{2}O_2 \; \underset{\text{Cat.}}{\longleftarrow} \; \underset{\text{inside}}{[H_2O_2]} \; \overset{\text{Peroxisomal membrane}}{\rightleftharpoons} \; \underset{\text{outside}}{[H_2O_2]} \; + \; \text{Cyt. } c \text{ peroxidase} \; \longrightarrow \; \text{Cyt. } c \text{ peroxidase–}H_2O_2$$

seems to be established. The extraperoxisomally generated H_2O_2 can be destroyed by catalase or can diffuse out to the external medium. The cytochrome c peroxidase assay reports on the unidirectional

calls our attention to the high activity of the purine-degradation pathway, a fact that should be further evaluated in connexion with intracellular H_2O_2 production.) Finally, (*d*) soluble enzymes and substrates

Table 6. *Estimation of the effectiveness of the intracellular sources of H_2O_2 in rat liver*

For details see the text. Experimental values are taken from Tables 1–5 and Figs. 5 and 6.

Fraction and substrate	Production of H_2O_2 (nmol/min per mg of protein)	Protein content (mg/g of liver)	Production of H_2O_2 (nmol/min per g of liver)
Mitochondria	—	25	—
Endogenous substrate	0.16		4
Succinate	0.50		12*
Microsomal fraction		25	
Endogenous substrate	0.13		3
NADPH	1.70		42*
Peroxisomes		20	
Intraperoxisomal endogenous substrate	2.2		44
+Supernatant	8.6		172
Estimated (see text)	5.0		100
Diffusing, estimated (see text)			30*
Supernatant		40	
Endogenous substrate	0.1		4*
Homogenate		140	
Endogenous substrate	0.27		38

* These values are considered as the physiological rates of H_2O_2 production.

generate H_2O_2 at the measured rate of about 4 nmol/min per g wet wt. of liver. The enzymes responsible for this generation have not been identified and could certainly include xanthine oxidase and peroxisomal oxidases that leaked out from broken peroxisomes.

In summary, the rate of free H_2O_2 production could be estimated at being of the order of 90 nmol/min per g wet wt. of liver. This free H_2O_2 is thought to build up a cytosolic steady-state concentration of H_2O_2, that (*a*) could force H_2O_2 across the peroxisomal membrane whenever the intraperoxisomal H_2O_2 concentration falls enough to create a gradient, or (*b*) could be utilized by soluble, cytosolic catalase acting, according to the supply of hydrogen donor, either in its catalatic or in its peroxidatic mode. The very existence of a cytosolic steady-state concentration of H_2O_2 and its eventual variations might be considered as a biochemical feature related to several biological phenomena, such as phagocytosis (Paul & Sbarra, 1968), oxygen poisoning (Gerschman, 1964) and radiosensitivity (Menzel, 1970).

The extrapolation of the rates estimated from isolated fractions to whole liver or even to liver slices appears to have some pitfalls, as the whole series of the biological regulatory devices may not be reconstructed by simple summation of subcellular fractions. However, by neglecting substrate regulation, it could be considered that H_2O_2 production might account for about 5% of the oxygen uptake in rat liver slices. On the one hand, the incomplete recovery of the subcellular fractions suggests that this value is an underestimate. On the other hand, the intracellular O_2 partial pressure (less than $50\mu\text{M-}O_2$; Kessler, 1968) could certainly be considered as rate limiting for the peroxisomal ($K_m = 100\mu\text{M-}O_2$; de Duve, 1963) and the microsomal ($K_m = 50\mu\text{M-}O_2$; Thurman *et al.*, 1972) H_2O_2-producing enzymes, thus establishing the mentioned percentage as an upper limit for whole liver.
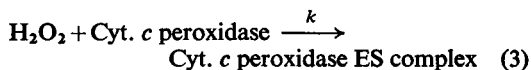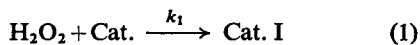
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APPENDIX

The interference by catalase in the cytochrome c peroxidase assay can be expressed as a set of equations in which catalase destroys the substrate for the cytochrome c peroxidase reaction. Taking into account only the forward reactions:

$$H_2O_2 + Cat. \xrightarrow{k_1} Cat. I \qquad (1)$$

$$Cat. I + H_2O_2 \xrightarrow{k_4} Cat. + H_2O + O_2 \qquad (2)$$

$$H_2O_2 + Cyt.\ c\ \text{peroxidase} \xrightarrow{k}$$
$$\text{Cyt.}\ c\ \text{peroxidase ES complex} \qquad (3)$$

where H_2O_2 is x; catalase (cat.) is e; catalase I is p; cytochrome c peroxidase is e_1; and cytochrome c peroxidase ES complex is p_1. These equations yield the following differential equations for the rate of H_2O_2 disappearance.

$$-\frac{dx}{dt} = k_1 x(e-p) + k_4 xp + kx(e_1 - p_1) \qquad (4)$$

$$\frac{dp_1}{dt} = kx(e_1 - p_1) \qquad (5)$$

$$\frac{dp}{dt} = k_1 x(e-p) - k_4 xp \qquad (6)$$

If we assume $\dfrac{dp}{dt} = 0$ then $k_1 x(e-p) = k_4 xp$ $\qquad (7)$

If we assume $p_1 \ll e_1$ then $\dfrac{dp_1}{dt} = kxe_1$ $\qquad (8)$

$$-\frac{dx}{dt} = 2k_4 xp + kxe_1 = x(2k_4 p + ke_1) \qquad (9)$$

$$\frac{dp_1}{dt} = ke_1 \cdot \left(-\frac{dx}{dt}\right) \cdot \frac{1}{2k_4 p + ke_1} \qquad (10)$$

$$\frac{dp_1}{dt} = -\frac{dx}{dt} \cdot \frac{1}{1 + 2k_4 p/ke_1} \qquad (11)$$