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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) generation. 3. Mitochondrial substrates entering the respiratory chain on the substrate side of the antimycin A-sensitive site support the mitochondrial generation of \( \text{H}_2\text{O}_2 \). Succinate, the most effective substrate, yields \( \text{H}_2\text{O}_2 \) at a rate of 0.5nmol/min per mg of protein in state 4. \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) (up to 1.7nmol/min per mg of protein) at a ratio of 0.71–0.86mol of \( \text{H}_2\text{O}_2 \)/mol of NADPH. \( \text{H}_2\text{O}_2 \) is also generated (6–25%) during the microsomal oxidation of NADH (0.06–0.025mol of \( \text{H}_2\text{O}_2 \)/mol of NADH). 8. Estimation of the rates of production of \( \text{H}_2\text{O}_2 \) under physiological conditions can be made on the basis of the rates with the isolated fractions. The tentative value of 90nmol of \( \text{H}_2\text{O}_2 \)/min per g of liver at 22°C serves as a crude approximation to evaluate the biochemical impact of \( \text{H}_2\text{O}_2 \) 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):

\[
\text{Cat.} - \text{H}_2\text{O}_2 + \overset{\text{O}}{\text{C}} = \overset{\text{H}}{\text{C}} + \text{H}_2\text{O}
\]

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., 1957a), ethanol (Jaccobsein, 1952), formic acid (Aebi et al., 1957b) 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 postulated functions is lacking, mainly owing to the lack of knowledge on the nature and effectiveness of the intracellular sources of \( \text{H}_2\text{O}_2 \).

A promising technique for the determination of \( \text{H}_2\text{O}_2 \)-generation rates is afforded by the enzyme–substrate compounds of catalase and peroxidases, which have a characteristic high affinity for \( \text{H}_2\text{O}_2 \). 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...
perfused liver (Sies & Chance, 1970), have been used for this purpose.

A fluorescent hydrogen donor for the horseradish peroxidase reaction also affords an H$_2$O$_2$ indicator (Avidor et al., 1954) and determinations by Loschen et al. (1971) established intact mitochondria as a novel source of H$_2$O$_2$ by the use of the scopoletin peroxidase method (Andreae, 1955; Perschke & Broda, 1961). Another fluorometric method involving the use of the dye, diacetyl dichlorofluorescein (Keston & Brandt, 1965), was previously utilized by Hinckle et al. (1967) to detect the evolution of H$_2$O$_2$ 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).

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

Cyt. c peroxidase + H$_2$O$_2$ →

Cyt. c peroxidase–H$_2$O$_2$ (ES complex)

Cyt. c peroxidase – H$_2$O$_2$ + 2 Cyt. c$_{2+}$ →

Cyt. c peroxidase + 2 Cyt. c$_{3+}$ + 2 H$^+$

In the present paper we describe the use of the ES complex of cytochrome c peroxidase as a sensitive spectrophotometric H$_2$O$_2$ 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$_2$O$_2$.

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 MIE buffer (225 mm-mannitol, 75 mm-sucrose, 0.2 mm-EDTA, pH 7.2). The livers were perfused with MIE buffer before homogenization to eliminate haemoglobin. The pellet, after centrifugation of the homogenate at 700 g for 10 min, was discarded. The supernatant (termed the 'homogenate') was centrifuged at 5000 g 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 12000 g 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 12000 g supernatant was centrifuged at 104000 g for 60 min to precipitate the 'microsomal fraction' and obtain the 'supernatant'.

The combined mitochondria–peroxisomal fraction was used for the comparison of the rate of H$_2$O$_2$ generation and the steady state of the catalase intermediate as described by Chace & 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 4000 g for 10 min the supernatant was removed, neutralized and assayed for uric acid. Sonication of peroxisomes was performed by using Sonifer Cell Disruptor, 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 ($\Delta\varepsilon_{\text{nm}} = 12.3 \text{litr} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$) (Stimson & Reutter, 1943). Uric acid concentration in the supernatant was assayed by adding either 10 }&mu;g of uricase/ml and 0.1 }&mu;M-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 $E_{325}$ was observed.

Peroxisomal catalase was measured spectrophotometrically at 430 minus 407 nm ($\Delta\varepsilon_{\text{nm}} = 100 \text{litr} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$) 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 (40000 g, 15 min).

Nicotinamide nucleotides and cytochrome $b_5$ were determined spectrophotometrically at 350 minus 370 nm ($\Delta\varepsilon_{\text{nm}} = 4.0 \text{litr} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$) and 424 minus 409 nm ($\Delta\varepsilon_{\text{nm}} = 185 \text{litr} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$; Omura & Sato, 1964) respectively. The oxygen consumption of the homogenate was determined polarographically to be 350 mmol of O$_2$/min per g of liver.

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

Procedure for the determination of the rate of generation of H$_2$O$_2$

This procedure measures the increase of absorbance at 419 nm (or 424 nm), with an active reference
wavelength at 407 nm (or 400 nm), of reaction mixtures containing 1–2 µM-cytochrome c peroxidase and about 0.1–0.3 mg 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 until all the cytochrome c peroxidase is converted into its ES complex. Two wavelength settings have been used: 419 minus 407 nm ($\Delta E_{407} = 50$ litre·mmol$^{-1}$·cm$^{-1}$) and 424 nm minus 400 nm ($\Delta E_{400} = 60$ 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, 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 0.3–1.0 mg of pigeon heart mitochondria/ml. The absorption maxima of those peroxidases (Fig. 1) are at 402 and 407 nm 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 419 nm 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 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):

$$\text{HR peroxidase} + H_2O_2 \rightarrow \text{HR peroxidase} - H_2O_2 \text{ (compound I)}$$

$$\lambda_{\text{max}} = 402 \text{ nm}$$

$$\text{HR peroxidase} - H_2O_2 \text{ (compound I)} + AH_2 \rightarrow AH^+ + \text{HR peroxidase} - H_2O_2 \text{ (compound II)}$$

$$\lambda_{\text{max}} = 417 \text{ nm}$$

$$\text{HR peroxidase} - H_2O_2 \text{ (compound II)} + AH^+ \rightarrow A + 2 H_2O + \text{HR peroxidase}$$

$$\lambda_{\text{max}} = 420 \text{ nm}$$

The $H_2O_2$-generation rate calculated from the steepest slope of the trace by using $\Delta E_{407}$ (417–402 nm) = 50 litre·mmol$^{-1}$·cm$^{-1}$ is 0.4 µM/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](image)
0.33 mg of protein per ml. In contrast, the formation of the cytochrome c peroxidase–H₂O₂ 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 H₂O₂ generation calculated from this latter trace, by using Δε_{459-417} (419 - 417 nm) = 50 litre·mmol⁻¹·cm⁻¹, is 1.2 μM/min per 0.33 mg 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 H₂O₂ indicator.

Fig. 3 shows that the cytochrome c peroxidase assay can be applied to the detection and determination of H₂O₂-generation rates in the range 0.1–1.2 μM/min. When H₂O₂ is provided by the glucose–glucose oxidase system, the method gives a linear relationship between the rates of H₂O₂ production and the amount of added glucose oxidase.

The recovery of H₂O₂ 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 H₂O₂-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 H₂O₂ generation by the different subcellular fractions themselves are described in detail below.

Addition of glucose to the subcellular fractions does not stimulate H₂O₂ 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 H₂O₂ generation (Table 1, column 2 minus column 1) agrees with the H₂O₂-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 H₂O₂-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 H₂O₂. 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. H₂O₂ was provided by the glucose–glucose oxidase system (Fig. 4). Substituting into eqn. (11) (see the Appendix) for k₄ = 1.8 x 10⁷ M⁻¹ s⁻¹ (Chance et al., 1952), p/c = 0.3 and k = 5 x 10⁴ M⁻¹ s⁻¹ (Chance et al., 1967) and for 0.2 μM-cytochrome c peroxidase (e₇) and 1 μM-

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**Fig. 2. Kinetics of H₂O₂ 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 H₂O₂ by cytochrome c peroxidase**

For details see the text. The reaction medium was 225 mm mannitol, 75 mm sucrose, 30 mm tris–morpholinopropanesulphonic acid buffer, pH 7.4, 5 mm glucose and 1.5 μM-cytochrome c peroxidase.
Table 1. Recoveries of the H$_2$O$_2$-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, 225mm-mannitol, 75mm-sucrose, 30mm-tris-morpholinopropanesulphonic acid buffer, pH 7.4, and 2µM-cytochrome c peroxidase. The glucose--glucose oxidase system consisted of 5mm-glucose and 0.5µg of glucose oxidase/ml. H$_2$O$_2$ was determined as described in the Materials and Methods section.

<table>
<thead>
<tr>
<th>Subcellular fraction and substrate</th>
<th>H$_2$O$_2$-generation rate (µM/min)</th>
<th>+Glucose--glucose oxidase</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Control 0.64</td>
<td>0.73</td>
<td>103%</td>
</tr>
<tr>
<td>Mitochondria (0.33mg of protein/ml)</td>
<td>Endogenous substrate 0.07</td>
<td>0.73</td>
<td>103%</td>
</tr>
<tr>
<td></td>
<td>+ Succinate (4mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peroxisomes (0.07mg of protein/ml)</td>
<td>Endogenous substrate 0.01</td>
<td>0.66</td>
<td>101%</td>
</tr>
<tr>
<td></td>
<td>+ Uric acid (35µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomes (0.36mg of protein/ml)</td>
<td>Endogenous substrate 0.08</td>
<td>0.72</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>+ NADH (30µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ NADPH (50µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant (0.7mg of protein/ml)</td>
<td>Endogenous substrate 0.07* (8%)</td>
<td>0.51 (40%)</td>
<td>80%</td>
</tr>
<tr>
<td>Homogenate (2.2mg of protein/ml)</td>
<td>Endogenous substrate 0.45* (25%)</td>
<td>0.48* (50%)</td>
<td>75%</td>
</tr>
</tbody>
</table>

* Initial rate. After approx. 1–2min, a steady state of the cytochrome c peroxidase--H$_2$O$_2$ 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$_2$O$_2$ generation.

The reaction rate of catalase (e) gives a ratio that graphically shows dp$_1$/dt = −0.5 dx/dt,

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

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$_2$O$_2$. Indirect (Chance & Oshino, 1971) and direct measurements (Loschen et al., 1971) pointed to intact mitochondria as a source of H$_2$O$_2$.

Fig. 5 illustrates the production of H$_2$O$_2$ by rat liver mitochondria. The rate of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ production is recorded. Addition of succinate speeds up H$_2$O$_2$ 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$_2$O$_2$ production. The addition of antimycin A further stimulates the rate.

Table 2 shows the mitochondrial production of H$_2$O$_2$ with different substrates. All the tested mitochondrial substrates, i.e. malate+glutamate, succinate, palmitoylcarnitine and octanoate, stimulate H$_2$O$_2$ 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, 1971).
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.

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).

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

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**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$\mu$M; ○, 1$\mu$M; ●, 2$\mu$M. The $H_2O_2$-generating system consisted of 5$\mu$M-glucose and 0.6$\mu$g of glucose oxidase/ml. The reaction mixture contained 120$\mu$M-KCl, 30$\mu$M-tris-morpholinopropanesulphonic acid buffer, pH 7.4.

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

For details see the text. The reaction medium was 225$\mu$M-mannitol, 75$\mu$M-sucrose, 4$\mu$M-phosphate buffer and 20$\mu$M-tris–morpholinopropanesulphonic acid buffer, pH 7.4. The numbers near the traces indicate nmol of $H_2O_2$/min per mg of protein.
peroxisome-rich fraction, 10% in the mitochondrial fraction and 25% in the microsomal fraction.

Fig. 6 shows a typical example of peroxisomal H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ intermediate into catalase–nitric oxide complex (Lemberg & Foulkes, 1946).

Fig. 7 shows another typical case of peroxisomal H$_2$O$_2$ generation; in this case, the addition of uric acid produced a steady-state concentration of the catalase–H$_2$O$_2$ intermediate.

Table 2. Effect of different substrates on the generation of H$_2$O$_2$ 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$_2$O$_2$ was recorded as described in the Materials and Methods section.

<table>
<thead>
<tr>
<th>Substrate and additions</th>
<th>H$_2$O$_2$ generated (nmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous substrate</td>
<td>0.16</td>
</tr>
<tr>
<td>+ Rotenone (0.6 μM)</td>
<td>0.08</td>
</tr>
<tr>
<td>+ Pentachlorophenol (2 μM)</td>
<td>0.04</td>
</tr>
<tr>
<td>Malate (4 mM) + glutamate (4 mM)</td>
<td>0.19</td>
</tr>
<tr>
<td>+ ADP (0.5 mM)</td>
<td>0.08</td>
</tr>
<tr>
<td>+ Rotenone (0.6 μM)</td>
<td>0.10</td>
</tr>
<tr>
<td>Succinate (5 mM)</td>
<td>0.40</td>
</tr>
<tr>
<td>+ ADP (0.5 mM)</td>
<td>0.06</td>
</tr>
<tr>
<td>Palmitoylcarnitine (10 μM)</td>
<td>0.23</td>
</tr>
<tr>
<td>+ Pentachlorophenol (2 μM)</td>
<td>0.06</td>
</tr>
<tr>
<td>Octanoate (14 μM)</td>
<td>0.22</td>
</tr>
<tr>
<td>+ Pentachlorophenol (2 μM)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 3. Effect of the mitochondrial metabolic state on H$_2$O$_2$ 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$_2$O$_2$ generation and catalase intermediate respectively. Production of H$_2$O$_2$ 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$_2$O$_2$ production) or 8 μM (catalase intermediate) was added as a solution in dimethylformamide.

<table>
<thead>
<tr>
<th>State</th>
<th>Addition</th>
<th>H$_2$O$_2$ production (μmol/min per mg of protein)</th>
<th>Saturation of the catalase intermediate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0.15</td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td>Succinate (5 mM)</td>
<td>0.70</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>Pentachlorophenol</td>
<td>0.00</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>+ Antimycin A</td>
<td>0.18</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>(0.4 nmol/mg of protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Uric acid (30 μM)</td>
<td>6.20</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>Ethanol (12 mM)</td>
<td>0.15</td>
<td>0</td>
</tr>
</tbody>
</table>
The acid activates the most active of the peroxisomal H$_2$O$_2$-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 8.8 nmol/min per mg of protein, which is accelerated by a factor of 1.7 after addition of 10$\mu$M-Na$_3$N$_2$(trace b).

Some characteristics of the peroxisomal fraction as a H$_2$O$_2$ generator are summarized in Table 4, Expt. A, in which the rate of H$_2$O$_2$ 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$_2$O$_2$ generated by the uricase reaction diffuses out of the peroxisomal membrane. Addition of azide up to a concentration of 20$\mu$M (no further increases are observed at higher concentrations) enhances the rate of H$_2$O$_2$ 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$_2$O$_2$ calculated from the rate of uric acid disappearance is recovered as cytochrome c peroxidase–H$_2$O$_2$ 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$_2$O$_2$ generation rate of 1.3 nmol/min per mg of protein. This value accounts for about 85% of the value obtained with the deoxycholate-treated preparation. The measurement of H$_2$O$_2$ 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$_2$O$_2$ by the peroxisomal fraction supported by endogenous substrate varied in the range 0.1–0.4 nmol/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 peroxisomal fractions (Table 4). Rates of 2.3 and 8.6 nmol of H$_2$O$_2$/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$_2$O$_2$ formation is identified as uric acid in Fig. 8. The amount of uric acid found in the supernatant corresponds to a cytosolic concentration of 3.2 mM. When a similar experiment is carried out with a trichloroacetic acid supernatant a corresponding cytosolic concentration of 0.1 mM-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 0.05–0.1 $\mu$M (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 407 nm in the presence of 1.2 $\mu M$-cytochrome c peroxidase at a protein concentration of 0.07–0.38 mg/ml, and were assayed for oxygen uptake polarographically at a protein concentration of 0.7–2.8 mg/ml, and were assayed for uric acid oxidation spectrophotometrically at 293 minus 320 nm at a protein concentration of 0.76–1.54 mg/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 34 mM-ethanol.

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

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

catalase concentration in the peroxisomal fraction of 0.25 nmol 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 3 min 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 (1.7 nmol of $H_2O_2$/min per mg of protein) is observed in the microsomal preparation suspended in the phosphate buffer. When
The effect of 20μM-azide on the microsomal production of H₂O₂ was tested to evaluate any interference from contaminating catalase, but no increase in the rates of H₂O₂ generation was observed after addition of NaN₃.

The rate of auto-oxidation of cytochrome b₅ was tested with the microsomal preparation suspended in different buffers. Fig. 9 shows that cytochrome b₅ 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 b₅ in the different buffers are listed in Table 5. The electron fluxes through this cytochrome are clearly smaller than the maximal microsomal rates of H₂O₂ generation, especially when compared with the NADPH-supported rates, and exclude the possibility of cytochrome b₅ being a significant microsomal generator of H₂O₂. If we assume, on the basis of the higher rates of H₂O₂ generation observed in the presence of NADPH, that the H₂O₂ generator is a member of the microsomal NADPH-oxidation pathway, the inverse relation between the rates of cytochrome b₅ oxidation and H₂O₂ 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 H₂O₂ in biological samples. Secondly, by using the cytochrome c peroxidase assay, it gives the rates of production of H₂O₂ by the different subcellular fractions, which summed up provide an estimate of the cellular generation of H₂O₂.

The remarkable affinity of enzyme and substrate in the case of the peroxidases and the high extinction coefficients of the ES complexes afford spectrophotometric H₂O₂ 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 H₂O₂ in the range 0.1–1.0μM.

The production of H₂O₂ by submitochondrial particles was reported by Jensen (1966) and by Hinckle et al. (1967). The fact that intact mitochondria can generate H₂O₂ 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 H₂O₂ production on the state 4 → state 3 transition.

---

**Fig. 8.** *Difference spectra of the supernatant fraction after addition of 0.1mg of uricase/ml and 0.03μM-catalase to the reference cuvette*

For details see the text. The supernatant was used, diluted 1:6, in mannitol–sucrose–tris–morpholinopropanesulphonic acid buffer, pH7.4. The broken line is the spectrum of a sample of uric acid. The light-path was 5mm.

NADH is added as substrate the rates of H₂O₂ generation account for 6–25% of the rate of nicotinamide nucleotide oxidation in the different buffers. The highest activity (0.47nmol of H₂O₂/min per mg of protein) is observed in the microsomal preparation suspended in mannitol–sucrose–tris–morpholinopropanesulphonic acid buffer.
**CELLULAR PRODUCTION OF HYDROGEN PEROXIDE**

The reaction mixture contained, in a final volume of 3.0 ml, 1 mg of protein of the microsomal fraction, 225 mM-mannitol, 75 mM-sucrose and 20 mM-tris-morpholinopropanesulphonic acid buffer, pH 7.4. After an addition of 1.2 μM-NADH, the change in the redox state of cytochrome b₅ was measured at 424 minus 409 nm. In the inserted small figure the concentration of reduced cytochrome b₅ 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. H₂O₂ production, nicotinamide nucleotide oxidation and cytochrome b₅ oxidation in rat liver microsomal fraction**

Microsomal fractions were suspended in the different buffers and were assayed for H₂O₂ production in the presence of 1.4 μM-cytochrome c peroxidase at 0.27 mg of protein/ml, were assayed for nicotinamide nucleotide oxidation at 0.36–0.72 mg of protein/ml, and were assayed for cytochrome b₅ oxidation at 0.36 mg of protein/ml. The composition of the buffers is as follows: A, 225 mM-mannitol, 75 mM-sucrose and 30 mM-tris-morpholinopropanesulphonic acid buffer, pH 7.4; B, 225 mM-mannitol, 75 mM-sucrose, 0.2 mM-EDTA and 30 mM-potassium phosphate buffer, pH 7.4; C, 150 mM-KCl and 30 mM-tris-morpholinopropanesulphonic acid buffer, pH 7.4; D, 100 mM-potassium phosphate buffer, pH 7.4. The electron rate (e⁻ rate) through cytochrome b₅ was calculated from e⁻ rate = k' [b₅], and cytochrome b₅ concentration ([b₅]) was taken as our average value of 0.37 nmol/mg of protein. k' was estimated as described in Fig. 9.

<table>
<thead>
<tr>
<th>Generation of H₂O₂ (nmol/min per mg of protein)</th>
<th>Buffer A</th>
<th>Buffer B</th>
<th>Buffer C</th>
<th>Buffer D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous substrate</td>
<td>0.11</td>
<td>0.11</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>+NADPH (50 μM)</td>
<td>0.67</td>
<td>0.70</td>
<td>1.56</td>
<td>1.70</td>
</tr>
<tr>
<td>+NADH (33 μM)</td>
<td>0.48</td>
<td>0.16</td>
<td>0.29</td>
<td>0.28</td>
</tr>
<tr>
<td>Nicotinamide nucleotide oxidation (nmol/min per mg of protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+NADPH (50 μM)</td>
<td>0.86</td>
<td>0.81</td>
<td>2.05</td>
<td>2.40</td>
</tr>
<tr>
<td>+NADH (33 μM)</td>
<td>1.85</td>
<td>2.90</td>
<td>2.80</td>
<td>3.05</td>
</tr>
<tr>
<td>Cytochrome b₅ oxidation k' (min⁻¹)</td>
<td>0.22</td>
<td>1.00</td>
<td>0.73</td>
<td>0.63</td>
</tr>
<tr>
<td>e⁻ rate (ne⁻/min per mg of protein)</td>
<td>0.08</td>
<td>0.37</td>
<td>0.27</td>
<td>0.24</td>
</tr>
</tbody>
</table>

in pigeon heart mitochondria. Our present results report rates of H₂O₂ production associated with different substrates and metabolic conditions. It is apparent that all the mitochondrial substrates entering the respiratory chain before the antimycin A-sensitive site are able to support H₂O₂ generation. If we assume that there is only one H₂O₂ generator in mitochondria, it seems that this H₂O₂ 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 \( \text{H}_2\text{O}_2 \), whereas the reduced states enhance the rate of reaction with \( \text{O}_2 \).

The other system of electron transport in the cell, the membranes of the endoplasmic reticulum system, were claimed to be a source of \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) almost quantitatively. As the ability of flavoproteins to generate \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \), which is able to oxidize alcohols through the peroxidatic reaction of catalase. In addition, it seems that \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \). In spite of being 'free' \( \text{H}_2\text{O}_2 \), no significant concentration of \( \text{H}_2\text{O}_2 \) is built up under the conditions of the experiment, as \( \text{H}_2\text{O}_2 \) is converted into the cytochrome \( c \) peroxidase-\( \text{H}_2\text{O}_2 \) intermediate.

The very low \( \text{H}_2\text{O}_2 \) concentration maintained in the medium surrounding the peroxisomes, because of the presence of cytochrome \( c \) peroxidase and the permeability of the peroxisomal membrane to \( \text{H}_2\text{O}_2 \), can be regarded as the cause of the apparent ineffectiveness of catalase in destroying the intraperoxisomal generated \( \text{H}_2\text{O}_2 \). A reaction sequence of the type:

\[
\text{H}_2\text{O} + \frac{1}{2}\text{O}_2 \xrightarrow{\text{Cat.} \text{ inside [H}_2\text{O}_2\text{]}} \xrightarrow{\text{Peroxisomal membrane}} \xrightarrow{\text{outside [H}_2\text{O}_2\text{]}} \xrightarrow{\text{Cyt. c peroxidase}} \text{Cyt. c peroxidase-\text{H}_2\text{O}_2}
\]

seems to be established. The extraperoxisomal generated \( \text{H}_2\text{O}_2 \) can be destroyed by catalase or can diffuse out to the external medium. The cytochrome \( c \) peroxidase assay reports on the unidirectional passage across the membrane of the internal \( \text{H}_2\text{O}_2 \).

The high permeability of the peroxisomal membrane to \( \text{H}_2\text{O}_2 \) (de Duve, 1963; de Duve & Baudhuin, 1966) should be considered of primary physiological importance in allowing a rapid equilibration of the \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) generation seems to be pertinent to this consideration. The possibility of \( \text{H}_2\text{O}_2 \) leaking out from catalase-loaded peroxisomes has been advanced by Poole (1968) on theoretical grounds.

Considering the whole rat liver homogenate, the rate of \( \text{H}_2\text{O}_2 \) 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 & Tephy, 1968), a process catalysed by catalase that should be strictly limited by the supply of \( \text{H}_2\text{O}_2 \).

To consider the relative importance of the different subcellular fractions and the total cellular generation of free \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) production; (c) the peroxisomal fraction generates intraperoxisomal \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) is negligible, approx. 30\% would be able to diffuse out of the peroxisome. (The accumulation of uric acid, although regarded as an isolation artifact,
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 re-constructed 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 μM-O$_2$; Kessler, 1968) could certainly be considered as rate limiting for the peroxisomal ($K_m = 100$ μM-O$_2$; de Duve, 1963) and the microsomal ($K_m = 50$ μM-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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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:

\[
\begin{align*}
\text{H}_2\text{O}_2 + \text{Cat.} & \xrightarrow{k_1} \text{Cat. I} \\
\text{Cat. I} + \text{H}_2\text{O}_2 & \xrightarrow{k_4} \text{Cat.} + \text{H}_2\text{O} + \text{O}_2 \\
\text{H}_2\text{O}_2 + \text{Cyt. c peroxidase} & \xrightarrow{k} \text{Cyt. c peroxidase ES complex}
\end{align*}
\]

(1) (2) (3)

where \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) disappearance.

\[
\frac{dx}{dt} = k_1 x (e-p) + k_4 xp + k x (e_1 - p_1)
\]

(4)

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

(5)

(6)

If we assume \( p_1 = e_1 \) then \( \frac{dp_1}{dt} = kxe_1 \)

(7)

(8)

\[
\frac{dx}{dt} = 2k_4 xp + kxe_1 = x(2k_4 p + ke_1)
\]

(9)

\[
\frac{dp_1}{dt} = \frac{1}{2k_4 p + ke_1}
\]

(10)

\[
\frac{dp_1}{dt} = \frac{1}{1 + 2k_4 p/ke_1}
\]

(11)

1972