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Biochemistry of the Peroxisome in the Liver Cell

By Helmut Sies[*]

The marker enzyme of the peroxisome—a phylogenetically old yet only recently discovered cell organelle—is catalase, a hemoprotein which decomposes hydrogen peroxide catalatically as well as peroxidatically. In the peroxisomes, catalase is associated with H_2O_2-producing oxidases and other enzymes. Also in parenchymal cells such as liver and kidney cells part of the reduction of oxygen occurs *via* formation of H_2O_2. A central role in peroxisomal H_2O_2-metabolism is played by the active intermediate, catalase-Fe^{3+}-H_2O_2 (Compound I), which is distinguished from free catalase by specific absorption bands. Organ photometry on intact hemoglobin-free perfused rat liver in order to measure Compound I selectively provides a direct insight into the dynamics of the H_2O_2 metabolism which takes place in the range of nanomolar concentrations. Endogenously, 1 g of liver forms approximately 50 nmol of H_2O_2 per min. The turnover number, which in the steady state is $<10\,min^{-1}$ in the cell as compared to $>10^8\,min^{-1}$ for the isolated enzyme with an excess of substrate, can be increased to approximately $10^2\,min^{-1}$ by intracellular stimulation of the H_2O_2 production (*e.g.* by glycolate or urate). The peroxidatic oxidation of hydrogen donors (*e.g.* methanol and ethanol), favored relative to the catalase pathway at low turnover numbers, is of importance in normal metabolism and in pathological conditions.

1. Introduction

As follows from Table 1, the subject of this report has a history going back approximately 150 years.

Research on the biological and chemical aspects of catalase and of hydrogen peroxide metabolism strongly revived in recent years. It received an essential impetus from cell biology. In 1954 *Rhodin* observed electron-optically cytoplasmic inclusions, "microbodies"[1], in the renal tubule cells of the mouse, which also occur in the liver cells[2] and in many other cell types of aerobic organisms. Little was known about the biochemical function of these cell organelles; in the beginning they were regarded as precursors of the mitochondria or as forms of lysosomes. As revealed in studies on homogenates and cell fractions, some oxidases as well as catalase are "particulate"[3–6]; however, they could not be related to the mitochondria, microsomes, or lysosomes. From the behavior of these enzyme activities during the isopyknic density-gradient centrifugation of cell fractions, and from morphological studies, *de Duve* and his co-workers concluded that these were associated with the "microbodies"[7–12]. The presence of H_2O_2-producing oxidases together with H_2O_2-decomposing catalase led to the concept of these cell organelles acting as a functional unit in H_2O_2 metabolism, "peroxisomes"[11,12].

While at the beginning of this century interest in research on the nature of cell respiration was focused on the hemoprotein catalase, it became almost forgotten after the discovery of the "Atmungsferment" cytochrome oxidase. Only in the last few years has it become clear that catalase plays an important part in its domain of biological oxidation, the key position being occupied by the active intermediate catalase-Fe^{3+}-H_2O_2 (Compound I), discovered by *Chance* in 1947[13] (Scheme 1a and 1b).

The function of the peroxisome in intracellular peroxidatic and catalatic reactions can only be partially understood in studies with isolated peroxisomes; rather, the system must be investigated in intact cells or organs. Spectrophotometry of intact organs, developed in recent years, affords a direct

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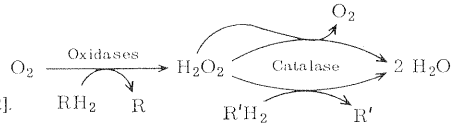
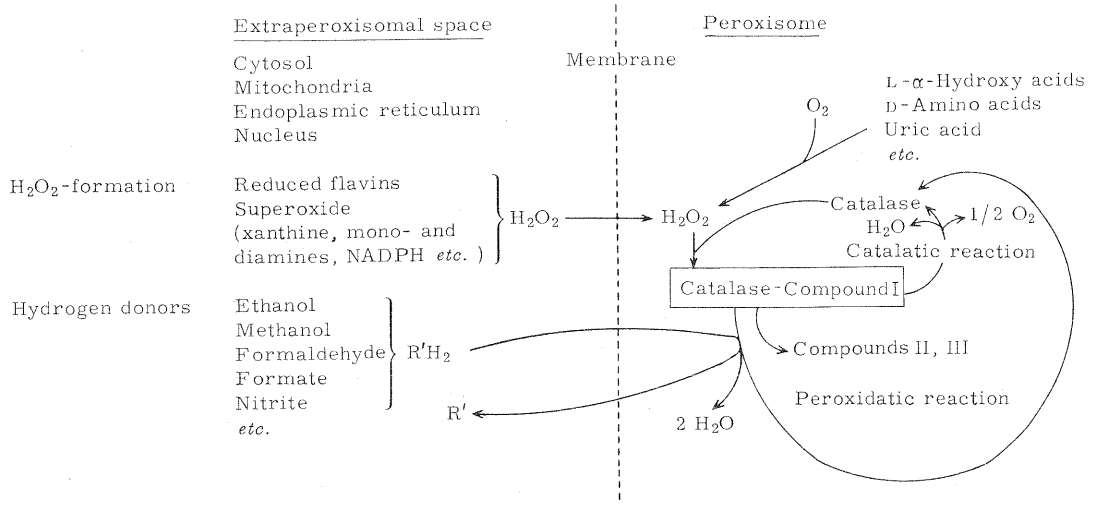
Table 1. Development of knowledge about H_2O_2, catalase, and peroxisomes.

Discovery	Year	Author	Ref.
H_2O_2 and its decomposition by tissue	1818	*Thénard*	[66]
Establishment of the name "catalase"	1901	*Loew*	[67]
Iron in the active group of catalase	1923	*Warburg*	[70]
Peroxidatic reaction of catalase	1936, 1945	*Keilin* and *Hartree*	[77, 78]
Crystallization of catalase	1937	*Sumner* and *Dounce*	[74a]
Crystallization of peroxidase	1942	*Theorell*	[74b]
Active intermediate (catalase-Fe^{3+}-H_2O_2 = Compound I)	1947	*Chance*	[13]
Compound I in bacteria	1952	*Chance*	[131]
"Microbodies"	1954	*Rhodin*	[1]
Glutathione peroxidase	1957	*Mills*	[143]
Peroxisome as a functional unit of H_2O_2 metabolism	1966	*deDuve* and *Baudhuin*	[12]
Inducibility of peroxisomes	1966	*Svoboda* and *Azarnoff*	[47]
Superoxide dismutase	1969	*McCord* and *Fridovich*	[124a]
Compound I in intact perfused rat liver	1970	*Sies* and *Chance*	[116]
Compound I in liver of anesthetized rat	1974	*Chance, Oshino, Sugano,* and *Jamieson*	[151]



Scheme 1a. *deDuve*'s scheme [12].



Scheme 1b. The role of oxidases and catalase and of catalase Compound I in peroxisomal H_2O_2 metabolism.

insight into cellular metabolism. The possibilities that organ photometry offers for research on the dynamics of intracellular compartmented processes can be demonstrated by the example of catalase Compound I in isolated perfused rat liver.

In spite of the efforts of many research groups, the manifold functions of the peroxisomes in animal cells are at present incompletely understood (cf. reviews on the cell biology of peroxisomes[12, 14–17], and on the enzymology of catalase[18–20]).

2. The Peroxisome

The peroxisome consists of a matrix surrounded by a unit membrane, and it contains a central core region called the crystalloid (Fig. 1).

Rat liver cells contain approximately 350—400 of these organelles. Table 2 gives a list of enzymes in rat liver peroxisomes, from which it follows that only about half of the peroxisomal protein is accounted for by the known enzyme activities. The nature of the remaining proteins is still unknown. Peroxisomes can be separated from other cell organelles by isopyknic density gradient centrifugation. In Figure 2 this is demonstrated by the localization of the activities of the marker enzymes of the peroxisomes, the mitochondria, microsomes, and lysosomes in the density gradient.

Table 2. Enzyme activities [a] found in rat liver peroxisomes (after [24—28]).

EC number	Enzyme (trivial name)	Fraction of peroxisomal protein [25] [%]
1.1.1.8	Glycerol-3-phosphate dehydrogenase	n. d. [b]
1.1.1.26	Glyoxylate reductase [NAD]	n. d.
1.1.1.29	Glycerate dehydrogenase [NAD]	n. d.
1.1.1.42	Isocitrate dehydrogenase [NADP]	≤ 25
1.1.1.—	Glyoxylate reductase [NADP]	n. d.
1.1.1.—	Glycerate dehydrogenase [NADP]	n. d.
1.1.3.1	Glycolate oxidase	n. d.
1.1.3.α	L-α-Hydroxy acid oxidase	≤ 3
1.4.3.3	D-Amino acid oxidase	≤ 2
1.7.3.3	Urate oxidase	≤ 10
1.11.1.6	Catalase	≤ 16
2.3.1.7	Carnitine acetyltransferase	n. d.

[a] Some of the homologous activities listed may be ascribed to the same enzyme.
[b] n. d. = not determined.

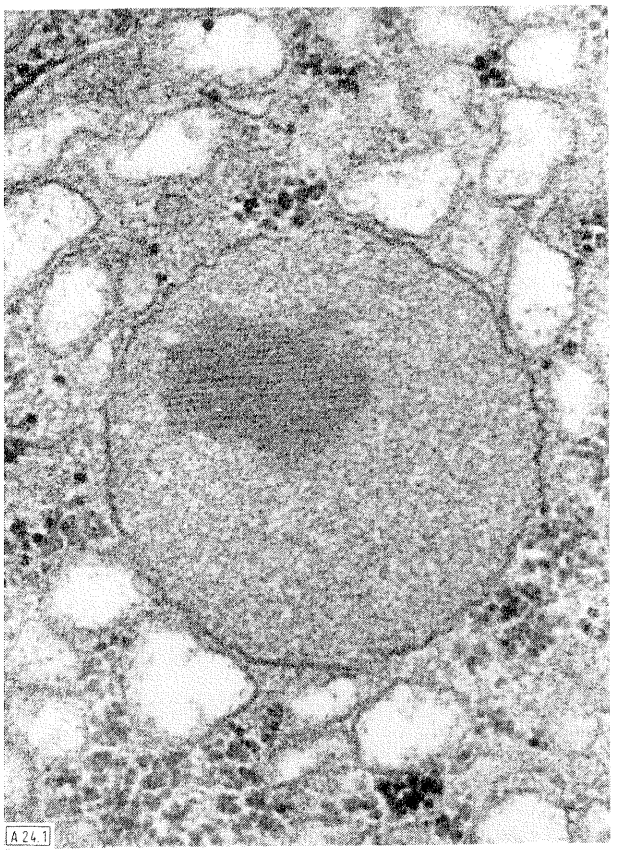


Fig. 1. Peroxisome from rat liver. The central region shows the urate oxidase present in the form of tubuli [21]. Magnification 82000 ×. The electron micrograph was obtained by courtesy of Prof. *F. Miller*.

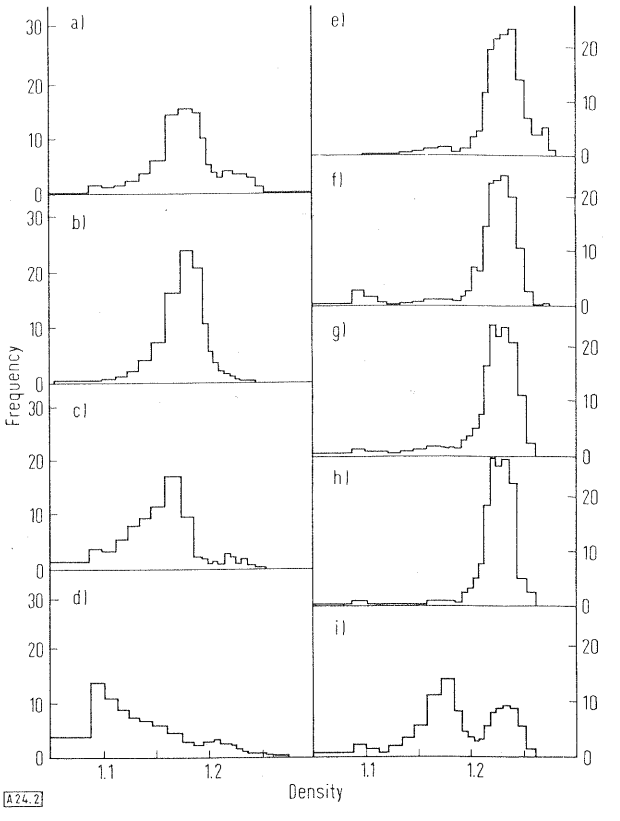


Fig. 2. Equilibrium density distribution of marker enzyme activities in a λ-fraction [10] from rat liver in a sucrose gradient. a) Total protein; below: marker enzymes for b) mitochondria (cytochrome oxidase), c) microsomes (glucose-6-phosphatase), and d) lysosomes (acid phosphatase); right: peroxisome enzymes: e) urate oxidase, f) catalase, g) D-amino acid oxidase, h) L-α-hydroxy acid oxidase, i) isocitrate dehydrogenase (at the density of 1.23 to 1.24). The $NADP^+$-dependent isocitrate dehydrogenase has a bimodal distribution. After treatment of the rat with Triton W-1339 [22] the lysosomes have a particularly low density (from [23]).

With respect to the functional morphology of the peroxisome, it may be mentioned that the organelle is freely permeable to low-molecular-weight solutes (zero osmotic space). Little

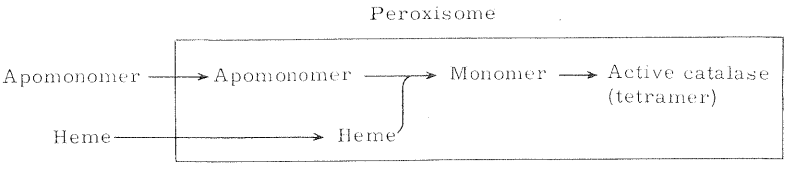
Table 3. Cell biology and cell chemistry of rat liver organelles with oxygen-activating systems.

Parameter	Peroxisomes	Mitochondria	Microsomes	Ref.
Volume fraction in the liver [%]	1.16	18.1	12.8	[30]
Number [ml^{-1}]	62×10^9	280×10^9	—	[30]
Equilibrium density in sucrose gradient [g/ml]	1.23	1.18		
Sucrose space [ml/g dry liver]	2.5	0.9	—	[31]
Osmotic space [ml/g dry liver]	0	0.6	—	[31]
Membrane	"Unit membrane"	Inner and outer membrane (one "unit membrane" each)	"Unit membrane", rough and smooth reticulum	
Phospholipid content [mg/mg protein]	0.09	0.20	0.32	[29]
Cardiolipin	−	+	−	
DNA	−	+	−	
Protein synthesis system	−	+	+	
"Terminal-oxidase"	Catalase	Cytochrome oxidase	Cytochrome P-450 "cytochrome b_5 oxidases"	
Inducibility of components	Hypolipidemic substances [a] salicylates	Thyroxine	Barbiturates, alicyclic hydrocarbons	
Marker enzyme	Catalase	Cytochrome oxidase, succinate dehydrogenase	Glucose 6-phosphatase, cytochrome P-450	
Half-life	1.5—2 d	Proteins of the inner membrane 12—13 d, those of the outer membrane 4 d	Proteins in rough and smooth reticulum 4 d	[32—36]

[a] Example: Clofibrate (ethyl 2-(*p*-chlorophenoxy)isobutyrate).

is known about specific permeability properties of the peroxisomal membrane, which resembles the microsomal membrane in its phospholipid composition[29]. Some properties of peroxisomes are compared in Table 3 with the properties of two other cell organelles with oxygen-activating systems, the mitochondria and the endoplasmic reticulum (microsomes).

mental evidence[56] (cf. also [57, 58]). The apomonomer, with a half-life of 14 min is brought into the peroxisome as such in a still unknown manner and there receives the heme group. The apomonomer and the monomer amount respectively to 1.6% and 0.5% of the total amount of catalase; their half-life is less than 1 h (Scheme 2).



Scheme 2. Biosynthesis of catalase (according to [56]).

2.1. Cytochemistry of Peroxisomal Catalase

Cytochemically, catalase is regarded as the marker enzyme of peroxisomes[37], on the basis of the 3,3′,4,4′-biphenyltetramine (diaminobenzidine) oxidase activity of catalase[38–41]. This peroxidatic activity of catalase is only observed after fixation of the enzyme, *e. g.* with glutaraldehyde. This applies both to catalase within the peroxisome in the cell[42] and to the isolated enzyme[43], and probably depends on a conformational change of the protein. This is also supported by the increase in reactivity for peroxidatic substrates if the enzyme has been subjected to trypsin degradation under mild conditions or to denaturation by alkali[44].

The question whether an appreciable amount of catalase exists extraperoxisomally *in vivo*[45] and whether this is demonstrable cytochemically[41], cannot yet be answered due to methodological reasons.

2.2. Turnover and Biogenesis of the Peroxisome

The organelle is subject to relatively rapid turnover *in toto*; its measured half-life is approximately 1.5—2 days[32, 33].

The stationary content of peroxisomes in the liver cell depends on numerous hormonal, nutritional, and pharmacological influences (cf.[15, 46]). For example, ethyl 2-(*p*-chlorophenoxy)-isobutyrate (Clofibrate) causes an increase in the number of peroxisomes[47], which increases the proportion of peroxisomes in the total volume of the liver cell from about 2% to about 17%[48]. This increase is related to an increase in the content of antigen reacting with anticatalase serum[49], *i. e.* the amount of catalase is also increased. The activity of the peroxisomal oxidases given in Table 2 remains unchanged[50], while the activity of the glycerol-3-phosphate dehydrogenase also increases[51]. Similar effects are observed *e. g.* with acetylsalicylic acid[52]. The increase in the number of peroxisomes by Clofibrate does not depend on catalase synthesis, since it also occurs when the formation of catalase is inhibited by allylisopropylacetamide[53].

The biogenesis of the organelle is still in dispute. Close morphological and biochemical relationships to the endoplasmic reticulum have been described repeatedly[54, 55]. Investigations on catalase biosynthesis are of special importance in this context. The hypothesis that catalase is first formed essentially within the peroxisomes from extraperoxisomally synthesized precursors has been supported by experi-

Histochemical attempts to establish the passage of active catalase through the free cytoplasm prior to incorporation into the peroxisome[41] led to equivocal results owing to methodological problems[59, 60]. In addition, the question whether catalase protein is synthesized *in vivo* on free and/or membrane-bound polysomes, has not been answered unambiguously[61–64]. *Redman et al.*[64] did not find catalase in the rough endoplasmic reticulum. Export proteins such as albumin and intracellular proteins such as catalase could therefore be synthesized at separate sites. *In vitro*, however, membrane-bound as well as free ribosomes can incorporate ^{14}C-leucine into enzymatically active catalase[62, 65].

3. Enzymology of Catalase

Catalase is a tetrameric enzyme with four probably identical subunits and four heme groups; the iron in the heme is trivalent. In contrast to hemoglobin, there are no indications of cooperativity between the subunits. Its molecular weight is about 240000 dalton. The methods of its isolation and crystallization, as well as its structural properties and the amino-acid composition, have been reviewed by *Deisseroth* and *Dounce*[20]. The catalatic action of catalase [equations (1a) and (1b), cf. Section 3.1] was one of the first enzymatic functions discovered. At the discovery of hydrogen peroxide in 1818 *Thénard* already observed that this substance is decomposed in the presence of animal tissues with evolution of oxygen[66], a reaction which also occurs on platinum surfaces. Catalase was at the center of intensive discussions concerning the nature of enzyme action[68, 69], in particular the heavy metal catalysis in enzymes[70–73], and after its crystallization[74] served as the object of numerous studies[18–20, 75, 76] on the biocatalysis by hemoproteins.

3.1. Catalase Complexes

Using rapid mixing methods, *Chance*[13] discovered spectrophotometrically an active intermediate, "Compound I", in the reaction of catalase with H_2O_2. Compound I is characterized by a decreased absorbance at 405 nm and an increased absorbance at 665 nm (Fig. 3). On the addition of alcohols to Compound I the absorbance again increases rapidly, *i. e.* free catalase is regenerated through the peroxidatic reaction discovered by *Keilin* and *Hartree*[77, 78] (eqs. (1a) and (1c)):

$$\underset{\text{(free catalase; } e)}{\text{Catalase-Fe}^{3+}} + \underset{(x)}{H_2O_2} \underset{k_3}{\overset{k_1}{\rightleftharpoons}} \underset{\text{(Compound I; } p)}{\text{Catalase-Fe}^{3+}\text{-}H_2O_2} \quad (1a)$$

$$\text{Catalase-Fe}^{3+}\text{-}H_2O_2 + H_2O_2 \xrightarrow{k'_4} \text{Catalase-Fe}^{3+} + 2H_2O + O_2 \quad (1b)$$

$$\underset{\text{(hydrogen donor; } a)}{\text{Catalase-Fe}^{3+}\text{-}H_2O_2 + C_2H_5OH} \xrightarrow{k_4} \text{Catalase-Fe}^{3+} + 2H_2O + CH_3CHO \quad (1c)$$

e, x, p, a, see Section 3.2.

The notation "catalase-Fe^{3+}-H_2O_2" for Compound I should indicate that the heme is occupied by H_2O_2 ("heme occupancy"). It is noteworthy that one mole of catalase does not simultaneously bind four moles of H_2O_2—*i. e.* one molecule per heme—but only approximately 1 to 1.6 moles of H_2O_2[79]. The other heme groups remain free. However, if an alkyl hydroperoxide, *e. g.* methyl or ethyl hydroperoxide, is used instead of H_2O_2, all four catalase subunits can be transformed into Compound I ([80], cf. [81]).

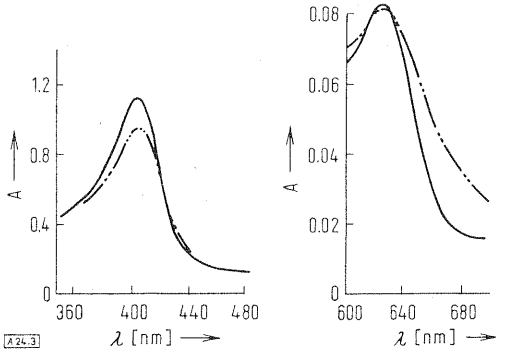


Fig. 3. Absorption spectra of purified catalase (——) from rat liver (catalase heme 9.3 μmol/l). Compound I (–···–) was obtained with H_2O_2 (23.5 μmol^{-1} min^{-1}) from the glucose oxidase reaction (after [82]).

The chemical structure of Compound I is still unknown[20]. Recent experiments with peracids indicate a reaction of the type:

$$\text{Enzyme} + H_2O_2 \rightarrow \text{Enzyme} = O + H_2O$$

which is probably followed by peroxidase[83–86].

The other catalase complexes (Compound II and Compound III) are catalatically inactive. Compound II has a low peroxidatic activity with respect to phenols. Indications of the occurrence of these compounds in normal intact liver are lacking[87], and therefore the compounds will not be discussed further here. Compound II is formed by the reduction of Compound I with H_2O_2, with phenols, or with ascorbate[88]. The relationships between Compounds I, II, and III and the donors have been reviewed by *Nicholls* and *Schonbaum*[18].

In the catalase-HCN complex[89, 90], which has a dissociation constant of 10^{-6} to 10^{-5} mol/l, each of the four heme groups carries one HCN molecule. The complex is characterized spectrophotometrically, *e. g.* by a minimum of 624 nm as compared to free catalase.

In the reaction between azide and Compound I the iron is reduced and catalase-Fe^{2+}-NO is formed[91–93].

A covalent complex is produced in an irreversible reaction between 3-amino-1,2,4-triazole[94, 95] and catalase in which a histidine group is involved[96]. The heme is then no longer[97] or almost not[98] capable of binding peroxides or ligands such as HCN.

3.2. Steady State Kinetics of Catalase

The kinetic constants k_1, k_4, and k'_4 of eqs. (1a)—(1c) were determined for the isolated enzyme (Table 4). For the kinetic analysis of the steady state of the catalase system it has been found, using *Chance*'s nomenclature[79, 103]:

Table 4. Kinetic constants of catalase [see eqs. (1a)—(1c)]. Some of the values were not measured directly but were calculated from indirect measurements. They indicate the order of magnitude of the values at 20—22 °C. More recent calculations [102] lead to slightly lower values of k_1 and k'_4.

Constant		Preparation	Ref.
k_1	1.7×10^7 l mol^{-1} s^{-1}	Rat liver	[79]
k'_4	2.6×10^7 l mol^{-1} s^{-1}	Rat liver	[79, 99] [a]
k_4	$(0.2—1.0) \times 10^3$ l mol^{-1} s^{-1}	Horse erythrocytes	[13, 79, 100] [b]
k_2	$(0—7.5) \times 10^{-3}$ s^{-1}	*M. lysodeikticus*	[101, 86]

[a] Calcd. by eq. (5a) with $p_M/e = 0.4$.
[b] For ethanol and methanol.

$$\frac{dp}{dt} = k_1 x(e-p) - k'_4 x p - (k_2 + k_4 a)p \quad (2)$$

$$\frac{dx}{dt} = \frac{dx_n}{dt} - k_1 x(e-p) - k'_4 x p + k_2 p \quad (3)$$

$$\frac{da}{dt} = -k_4 a p \quad (4)$$

e = concentration of the total catalase heme
p = concentration of Compound I
$x = H_2O_2$ concentration
dx_n/dt = steady state rate of H_2O_2 formation
a = hydrogen donor concentration.

In the case of a steady state of Compound I ($dp/dt = 0$) and the rate of H_2O_2 formation (dx_n/dt = const.), and for the condition $k_2 = 0$, the result for the fraction of the total catalase heme e existing as Compound I (p) (*i. e.* for the heme occupancy) is:

$$\frac{p_m}{e} = \left(1 + \frac{k'_4}{k_1} + \frac{k_4 a_0}{k_1 x_m}\right)^{-1} \quad (5)$$

where the subscript m denotes the steady state. As $x_m \rightarrow \infty$, or $a_0 = 0$ and $x_m \neq 0$, $p_m \rightarrow p_M$; thus

$$\frac{p_M}{e} = \left(1 + \frac{k'_4}{k_1}\right)^{-1} \quad (5a)$$

Equation (5a) demonstrates a simple relationship between the maximal heme occupancy p_M/e existing at the maximal rate of H_2O_2 formation and the second-order rate constants for the formation and catalatic degradation of Compound I.

Another useful relationship describes the catalase reaction for the condition that the hydrogen donor concentration a

is chosen so that $p_m = p_M/2$. In the steady state the donor concentration for half-maximal heme occupancy is directly proportional to the steady state rate of H_2O_2 formation dx_n/dt and inversely proportional to the total heme concentration e[103]:

$$a_{p_m = p_M/2} = \left(\frac{\left(3\frac{k'_4}{k_1} + 1\right)k_4}{2\left(\frac{k'_4}{k_1} + 1\right)^2} \right)^{\frac{1}{2}} \times \frac{1}{e}\frac{dx_n}{dt} \qquad (6)$$

$\frac{1}{e}\frac{dx_n}{dt}$ = steady state turnover number

From eq. (6) it follows that the catalase system does not have a "Michaelis constant" for H_2O_2 or for the hydrogen donor a. Rather, the ratio of the H_2O_2-formation rate and the concentration of the catalase heme (steady state turnover number) plays a role in the steady state concentration of compound I and hence in the donor concentration required for half-maximal decomposition of Complex I. As will be shown below, these relationships are important for the evaluation of the catalatic and peroxidatic reactions in biological systems.

In order to illustrate this point, two examples of experiments with isolated rat liver catalase are given. Figure 4 shows how the steady state heme occupancy p_m/e depends on the steady state turnover number. Both the H_2O_2 formation rate

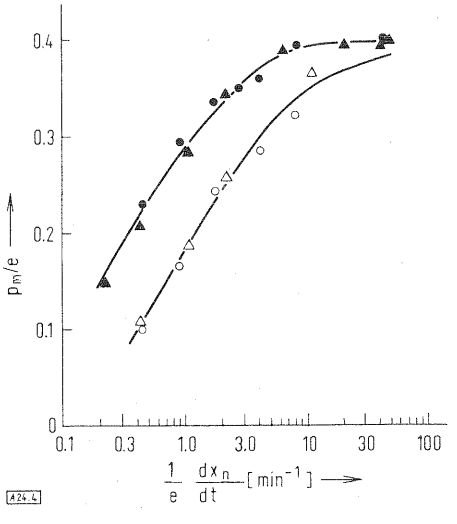


Fig. 4. Dependence of steady state heme occupancy of catalase (p_m/e) on the steady state turnover number (H_2O_2 formation rate/catalase heme concentration), measured with isolated rat liver catalase. The H_2O_2-formation rate (●, ○) was varied from 0.3 to 100 μmol l^{-1} min^{-1} and the catalase heme concentration (▲, △) from 0.1 to 12 μmol/l. Stimulation of the peroxidatic pathway by addition of ethanol (0.1 mmol/l) lowers the steady state heme occupancy (right-hand curve, open symbols). After [82].

(by varying the glucose plus glucose oxidase system) and the catalase heme concentration are varied. As expected, the curve shifts to the right when the peroxidatic pathway competes (by the addition of ethanol).

Figure 5 demonstrates how the partition into peroxidatic and catalatic reactions depends on the rate of H_2O_2 formation and on the concentration of hydrogen donor, in this case ethanol. An exclusively peroxidatic reaction can only be achieved at extremely high donor concentrations and at low turnover numbers[104, 105].

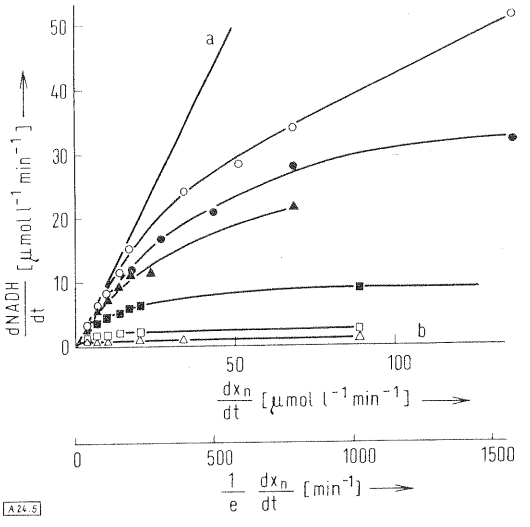


Fig. 5. Distribution of H_2O_2 decomposition between "peroxidatic" and "catalatic" pathways; a, 100% "peroxidatic"; b, 100% "catalatic" reaction. The oxidation rate of ethanol (given as the rate of formation of NADH) is illustrated graphically at several steady state H_2O_2 formation rates dx_n/dt for the following ethanol concentrations: ○, 100; ●, 50; ▲, 25; ■, 5; □, 1; △, 0.33 mmol/l (after [105]).

4. State of Catalase in Hemoglobin-Free Perfused Rat Liver

4.1. Organ Photometry of Catalase Compound I

Since it can be assumed that the H_2O_2 present in peroxisomes is indicated in the form of Compound I, the photometry of this complex allows a direct insight into the metabolism of H_2O_2 and its reaction partners. If the rat liver is perfused with a perfusion fluid free from hemoglobin, optical methods can be used without major difficulty[106–108]; the metabolism of the catalase-containing erythrocytes does not interfere. *Brauser*[109] used the light passing through the lobe of the perfused liver for absorbance measurements (photometry in transmitted light as opposed to reflection photometry), a method that was also used successfully on isolated tissues such as muscle preparations[110–112]. Changes in the steady state content of catalase Compound I in the liver can be detected in the blue (*e.g.* at 405 nm) and in the red spectral regions (*e.g.* at 660 nm).

The problem in the photometry of an organ, a multi-component system, of assigning the measured signals to transitions of specific components, is largely solved by the use of the dual wavelength technique[109–113] and of difference absorption spectra[114, 115]. Since other hemoproteins hardly interfere at the absorbance peak in the red region, the absorbance difference 660—640 nm proves to be best suited with the perfused liver for specific detection of the transition between catalase Compound I and catalase[116, 117].

As Figure 6 shows, the absorbance difference increases upon withdrawal of oxygen (causing an interruption of H_2O_2 formation) and upon the addition of an excess of methanol (causing peroxidatic degradation of Compound I). The two effects are not additive. They are two independent metabolic transitions in which catalase is regenerated from Compound I. The figure shows further that the absorbance difference decreases upon infusion of glycolate. This reflects the increase in intracellular

H_2O_2 production by the flavin enzyme, glycolate oxidase. When catalase is irreversibly inhibited by 3-amino-1,2,4-triazole, both the methanol effect and the effect of withdrawal of oxygen are almost completely lacking[116].

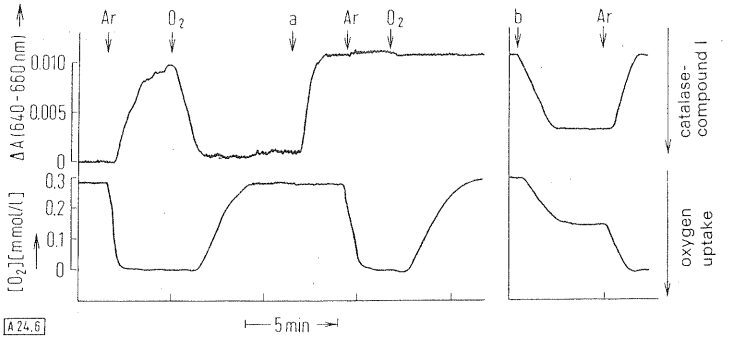


Fig. 6. Upper trace: Left: Recording of the absorbance difference ΔA (640 – 660 nm) of a perfused liver lobe. The decrease in the catalase Compound I by withdrawal of oxygen or by infusion of methanol (2.5 mmol/l at a) results in a rise of ΔA. Right: Re-formation of Compound I due to H_2O_2 formation from infused sodium glycolate (2mmol/l at b in the presence of 1mmol/l of methanol).
Lower trace: Oxygen concentration in effluent perfusate (after [116]).

4.2. Heme Occupancy of Catalase

Since a continuous endogenous H_2O_2 production in the liver (Figure 6) has been demonstrated, a system of measurement can be devised on the basis of the relationships obtained from the isolated enzyme, which provides information on the state of the enzyme in the intact organ. The heme occupancy $p_m/e = 0$ is obtained by an excess of methanol, the maximal heme occupancy $p_m/e = p_M/e$ by an excess of glycolate, and under the same conditions in the perfused organ the catalase-HCN complex is used as a measure of the total catalase heme.

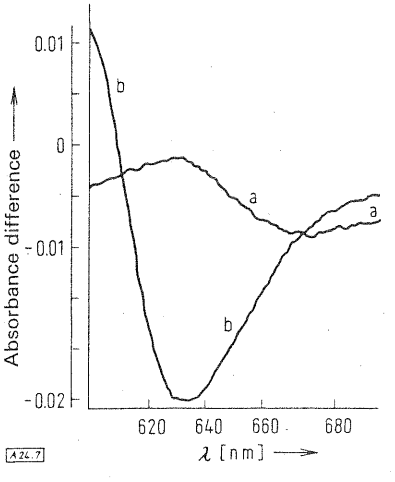


Fig. 7. Absorbance difference spectra of hemoglobin-free perfused rat liver. a) Catalase Compound I, b) HCN-catalase (as a measure of catalase heme). The reference spectrum is that of free catalase in the liver. The spectra correspond to metabolic steady states (see text); they were recorded with a Rapidspektroskop [118]. An instrument computer was used for signal averaging and subsequent digital subtraction according to the method of *Brauser et al.* [114] (after [82]).

Figure 7 shows two absorbance difference spectra. Sodium glycolate (2 mmol/l), methanol (4.8 mmol/l), and methanol (4.8 mmol/l) *plus* sodium cyanide (0.05 mmol/l) were successively infused into the perfusate entering the liver, for 2 to 3 min each. During each of the steady states corresponding to these three conditions 256 absorption spectra of a lobe of the liver were taken within 12 s in the range 600—700 nm and stored. Spectrum a corresponds to the absorbance difference of the liver, obtained by digital subtraction, between the state in the presence of methanol and that in the presence of glycolate, *i. e.* it shows the decrease of the maximal heme occupancy of catalase Compound I to zero heme occupancy. Spectrum b corresponds to the absorbance difference of the liver between the state in the presence of methanol plus cyanide and that in the presence of methanol, *i. e.* it shows the formation of HCN-catalase from free catalase (in the range around 610 nm there is a small contribution by cytochrome oxidase).

With the aid of the difference extinction coefficients[82] determined for catalase isolated from rat liver, a maximal heme occupancy $p_M/e = 0.38$ can be calculated from ΔA (640—660 nm) of spectra a and b. Hence the maximal heme occupancy of rat liver catalase *in vitro* (Fig. 4) and *in situ* (Fig. 7) are similar. According to eq. (5a), this means that the catalase molecule has a comparable rate constant ratio k'_4/k_1 at the dilution employed *in vitro* (approximately 10^{-6} mol/l with respect to heme) and with the concentration occurring within the peroxisome *in situ* (approximately 10^{-3} mol/l with respect to heme, see below).

The endogenous steady state of the heme occupancy can be measured by determining the extreme states with methanol and glycolate. In Figure 8 it is 3/4 of the maximal value.

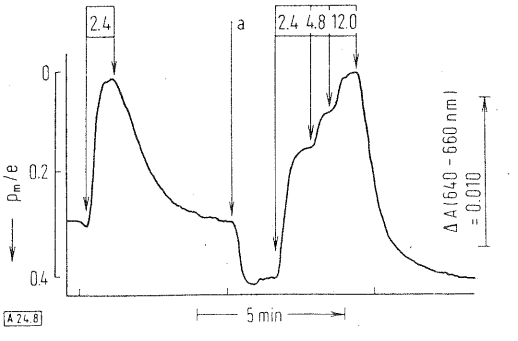


Fig. 8. Recording of the absorbance difference ΔA (640 — 660 nm) of a perfused liver lobe. Higher methanol concentrations are necessary (see eq. (6) and Fig. 4) for decomposition of Compound I during the stimulation of H_2O_2 formation by glycolate (addition of 2 mmol/l at a). The endogenous heme occupancy is 3/4 of the maximum value. The relevant methanol concentrations (in mmol/l) are given in the upper panel (after [82]).

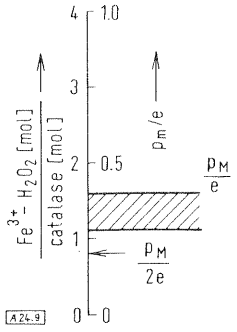


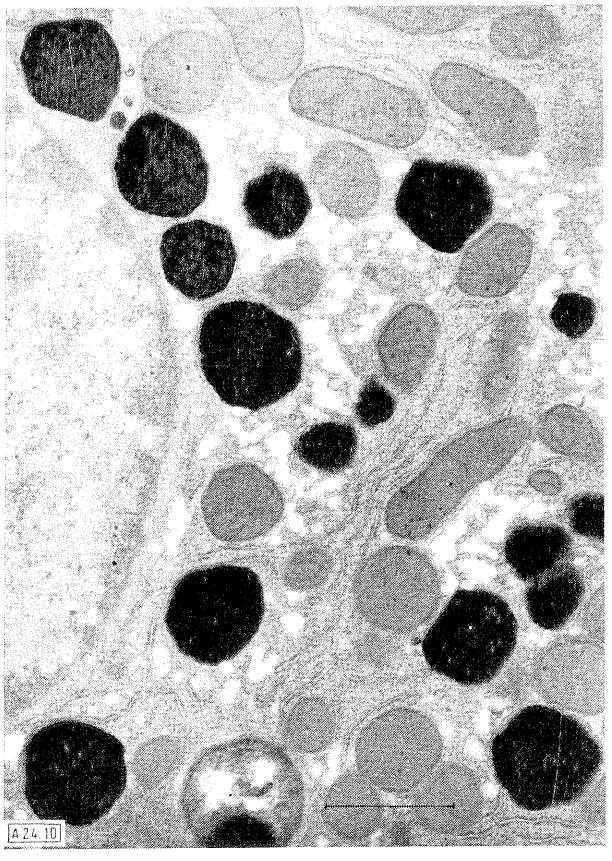
Fig. 9. Schematic representation of the heme occupancy p_m/e of catalase. The shaded region corresponds to the heme occupancy observed in the liver in the endogenous steady state. The maximum value p_M/e is produced by glycolate, urate, *etc.*, and the heme occupancy of zero by withdrawal of oxygen, or by methanol, ethanol, *etc.*

In addition, it can be seen that with increasing the rate of H_2O_2 formation the methanol concentration required for the decomposition of Compound I increases (cf. eq. (6)). The findings and definitions are summarized in Figure 9.

4.3. Catalase Content

Enzyme contents per gram of organ are usually determined from the activity measured in the homogenate and from the specific activity of the isolated enzyme, or by quantitative immunological methods. In another method, which does not require destruction of the tissue, the enzyme content is determined by photometric comparison with components of known content in the intact organ. A suitable reference component for catalase is cytochrome oxidase, the content of which in the liver is 18 nmol/g; the transition from normoxia to anoxia can be established by measuring the band at 607 nm[119]. The catalase content determined in this manner amounts to 1/3 of the content of cytochrome oxidase, *i.e.* 6 nmol/g or 24 nmol/g with respect to catalase heme (approximately 1.5 mg catalase protein per g of liver). From the data of *Price et al.*[33] and *Higashi* and *Peters*[34] results of 1 and 1.5 mg/g have been obtained. This corresponds to a catalase concentration in the peroxisome of approximately 0.5 mmol/l or 120 mg/ml.

For illustrative purposes a determination of the relative tissue content is shown in Figure 10. Here the catalase/cytochrome oxidase ratio increased from 0.33 to 1.1 because the number of peroxisomes was increased by prior treatment with ethyl 2-(*p*-chlorophenoxy)isobutyrate. This also follows from the electron micrograph of the perfused liver.



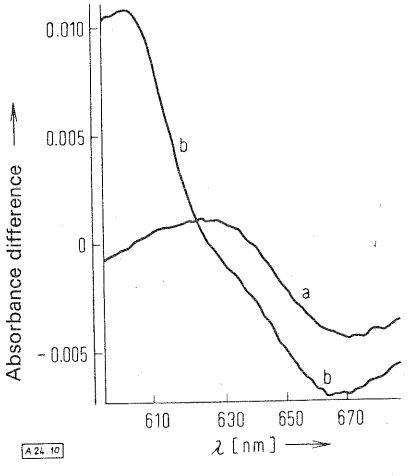


Fig. 10. Perfused liver of a rat pretreated with ethyl 2-(*p*-chlorophenoxy)isobutyrate. Bottom: Absorbance difference spectra, a) catalase minus catalase Compound I (cf. Fig. 7), b) cytochrome oxidase (through subsequent anoxia). The ratio of the catalase and cytochrome oxidase contents is 1.1 (as opposed to 0.33 for an untreated rat). Top: Electron micrograph of this liver after 1 h of perfusion. The number of peroxisomes (contrasted by the DAB reaction after [41]) has increased, while the number of mitochondria has remained unchanged (after [42]). The distance marker corresponds to 1 μm.

5. H_2O_2 Metabolism of the Liver Cell

5.1. H_2O_2 Formation

Identification of the substrates which form H_2O_2 in the liver in the endogenous steady state has not yet been performed. For example, uric acid is continuously formed from purine degradation, its concentration in rat liver amounting to 70 μmol/l[120]. In addition to urate oxidase, flavin-dependent enzyme systems come into consideration for H_2O_2 production[121], either directly or by means of the superoxide radical $O_2^{\cdot-}$[122, 123] through superoxide dismutase[124a–c]. However, the autoxidizability of flavin enzymes is quite variable, as is indicated by the distinction between flavin oxidases and flavin dehydrogenases (cf. reviews on acceptor specificities[125a, b]). In the case of xanthine oxidase a shift between oxidase and dehydrogenase activities was described[126].

Increments of H_2O_2 formation by the addition of H_2O_2-producing substrates can be determined directly by an increase in the methanol concentration required for the half-maximal decomposition of Compound I (cf. eq. (6)). The H_2O_2 formation of approximately 50 nmol min^{-1} g^{-1} determined in this

manner in the intact liver increases 10-fold in the presence of glycolate, and 15-fold in the presence of urate (Table 5). Hence it is in the order of magnitude of 1/100 to 1/10 of the total O_2 uptake. *Portwich* and *Aebi*[128] found comparable rates of H_2O_2 formation in liver slices and liver homogenates by measurement of the peroxidatic oxidation of ^{14}C-formate. The H_2O_2 concentration in the endogenous steady state is 10^{-9} mol/l and increases to 10^{-7} mol/l with maximal stimulation of the H_2O_2 formation by the substrates added[127]. Low steady state turnover numbers of $< 10\,min^{-1}$ to approximately $100\,min^{-1}$ at maximal stimulation are characteristic for the *in situ* condition. Isolated catalase is well-known for particularly high maximal turnover numbers of $> 10^8$ min^{-1}[129]. Hence catalase *in situ* operates in a region that favors the peroxidatic reaction relative to the catalatic reaction. However, this does not mean to say that at high turnover numbers the peroxidatic pathway would be inhibited. Some H_2O_2-producing enzymes of the liver are given in Table 6.

Table 5. Rate of H_2O_2 production in isolated hemoglobin-free perfused rat liver in several metabolic states. Determination by measurement of the heme occupancy [see eq. (6)] (after [127]). $\frac{1}{e}\cdot\frac{dx_n}{dt}$ = steady state turnover number, $dx_n/dt = H_2O_2$-formation rate.

Substrate or inhibitor	$[CH_3OH]$ at $p_M/2e$ [mmol/l]	$\frac{1}{e}\frac{dx_n}{dt}$ [min^{-1}]	$\frac{dx_n}{dt}$ [nmol/min][a]
L-Lactate 2 mmol/l, pyruvate 0.3 mmol/l	0.12 (0.09–0.16)	3.8	49
+ Antimycin A 8 µmol/l	0.18	5.8	75
+ Octanoate 0.3 mmol/l and Antimycin A 8 µmol/l	0.40	13.0	170
+ Oleate 0.1 mmol/l	0.16	5.1	66
+ Urate 1 mmol/l	—	54 [b]	750
+ Glycolate 3 mmol/l	—	34 [b]	490

[a] Per gram of liver.
[b] Titration in the presence of 0.6 mmol/l of methanol.

The question widely discussed at the beginning of this century, "Does hydrogen peroxide play any role in the living cell?" (cf. [130]), can be answered by stating that the production of H_2O_2 (as well as O_2^- and organic hydroperoxides) is a normal attribute of aerobic metabolism. A survey of the detection of H_2O_2 formation in living cells and cell fractions is given in Table 7.

The above-mentioned H_2O_2 formation rates may be regarded as the lower limits of the *in vivo* rates, because in the extraperoxisomal space other peroxidases, *e.g.* glutathione peroxidase[143,144], can compete with catalase for H_2O_2[145]. For example, oxidized glutathione appears in the effluent perfusate after infusion of H_2O_2[146], indicating the activity of glutathione peroxidase. About 1/3 of this enzyme is localized in the mitochondrial matrix and about 2/3 in the cytosol[147].

Table 6. H_2O_2-(or O_2^--)producing enzymes of the liver and their subcellular localization.

EC number	Enzyme (trivial name)	Localization
1.1.3.1	Glycolate oxidase	Peroxisome
1.1.3.α	L-α-Hydroxy acid oxidase	Peroxisome
1.1.3.8	L-Gulonolactone oxidase	
1.2.3.1	Aldehyde oxidase	
1.2.3.2	Xanthine oxidase	Cytosol
1.4.3.3	D-Amino acid oxidase	Peroxisome
1.4.3.4	Monoamine oxidase	Mitochondrial outer membrane
1.4.3.5	Pyridoxamine oxidase	
1.4.3.6	Diamine oxidase	Endoplasmic reticulum
1.6.99.1	NADPH cytochrome c reductase	Endoplasmic reticulum
1.6.99.3	NADH cytochrome c reductase	
1.7.3.3	Urate oxidase	Peroxisome, "core"
1.15.1.1	Superoxide dismutase	Cytosol, mitochondrial matrix

Under certain experimental conditions intraperoxisomally formed H_2O_2 can also leave the peroxisomes and be detected *e.g.* by the complex with cytochrome c peroxidase from yeast.

Table 7. Detection of H_2O_2 formation in living cells and subcellular fractions.

Source	Example	Detection by	Ref.
Bacteria	*Micrococcus lysodeikticus*	Catalase Compound I	[131]
Worms	*Ascaris lumbricoides*	Polarography	[132, 133]
Blood cells	Leukocytes	Diacetyldichlorofluorescein	[134]
Liver	Liver slices and homogenates	^{14}C-Formate → $^{14}CO_2$	[128]
	Isolated perfused rat liver	Catalase Compound I	[113]
Cell fractions			
Mitochondria	Liver (rat)	Catalase Compound I	[103]
		Cytochrome c peroxidase	[137]
	Heart (pigeon)	Scopoletin, horseradish peroxidase	[135]
		Cytochrome c peroxidase	[136]
	Yeast (*S. cerevisiae*)	Cytochrome c peroxidase	[138]
	Protozoa (*C. fasciculata*)	Cytochrome c peroxidase	[139]
Microsomes	Liver (rat)	Scopoletin, horseradish peroxidase	[140]
		Cytochrome c peroxidase	[137]
Peroxisomes	Liver (rat)	Cytochrome c peroxidase	[137]
Cytosol	Liver (rat)	Cytochrome c peroxidase	[137]
Submitochondrial particles	Liver (rat)	Diacetyldichlorofluorescein	[141]
		Polarography	[142]

The increase in heme occupancy of catalase upon infusion of octanoate and antimycin A (Table 5), which is attributed to increments of mitochondrial H_2O_2 production in the liver[127], is therefore particularly striking.

From experiments conducted so far on subcellular fractions[141, 99, 135–137] it follows that the mitochondrial H_2O_2 production depends on the control state[99] and is greatest[99, 135] in the state of respiratory control by the availability of phosphate acceptor ("State 4"[148]). The segment succinate-cytochrome b_{566} as well as the system of energy conservation are postulated to play a role in H_2O_2 formation[149, 136]. Since in this segment of the respiratory chain the transition from two-electron transfer to one-electron transfer takes place, the primary product could also be the superoxide radical O_2^-, which then dismutates into H_2O_2 and O_2. Experimental support for this possibility has been provided by *Loschen et al.*[150].

An increase in the oxygen pressure from atmospheric to 1.92 MPa (19 atm) increases the rate of H_2O_2 formation fourfold in pigeon heart mitochondria and 15- to 20-fold in rat liver mitochondria[136]. Thus, considerable differences should exist in the oxygen affinity of the H_2O_2-producing systems. Recently *Oshino et al.*[151a] were able to demonstrate *in situ* on anesthetized rats a fourfold increase of H_2O_2 production in the liver in the presence of glycolate when the animal was exposed to oxygen pressures of 1 atm.

A source of H_2O_2 that is normally negligible but which is worth mentioning is high-energy radiation. X-irradiation of neutral aqueous solutions produces H_2O_2 as a stable product in a concentration of approximately 3—4 $\mu mol\,l^{-1}\,kR^{-1}$[152, 153].

5.2. Hydrogen Donors for the Peroxidatic Reaction

The peroxidatic reaction, the "coupled oxidation" of *Keilin* and *Hartree*[77, 78], *i. e.* the combination of reactions (1a) and (1c), is important for the metabolism of a number of hydrogen donors in the liver, and thus for methanol[13, 154–157], ethanol[158], nitrite[159], and formate[160]. From the reaction sequence it follows that the rate of the oxidation of the hydrogen donors in the steady state cannot be greater than the rate of formation of hydrogen peroxide. With the exception of extremely high hydrogen donor concentrations, however, this value is not reached, as the catalase reaction competes with the peroxidatic reaction (cf. Figure 5)[105]. It should be mentioned that high donor concentrations can be obtained by exogenous supply (50 mmol of ethanol/l ≙ 2.3 %).

The titration curves of the steady heme occupancy of catalase in the perfused liver are different for the two primary alcohols methanol and ethanol (Figure 11). On the addition of 4-methylpyrazole the difference disappears; hence, this difference must be attributed to the activity of the alcohol dehydrogenase[127]. The titration curve for methanol remains unchanged on the addition of 4-methylpyrazole—an indication of the lack of reactivity of methanol with the dehydrogenase. These observations are in accord with the similarity of the rate constants k_4 in the reaction of catalase with methanol and ethanol[13].

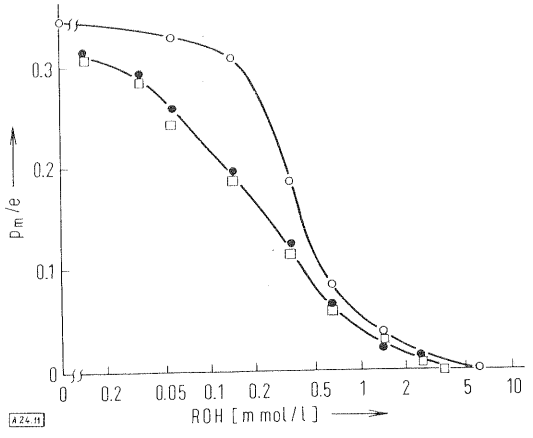


Fig. 11. Dependence of the heme occupancy of catalase (p_m/e) on methanol (□) and ethanol concentration in the perfusate (ROH). The ethanol titration was carried out in the presence (●) and in the absence (○) of 0.1 mmol/l of 4-methylpyrazole (after [127]).

The oxidation of ethanol *via* the catalase pathway is stimulated by increased H_2O_2 formation, as shown in Figure 12. By glycolate addition in the presence of methylpyrazole, the oxidation of ethanol per g of liver increases by approximately

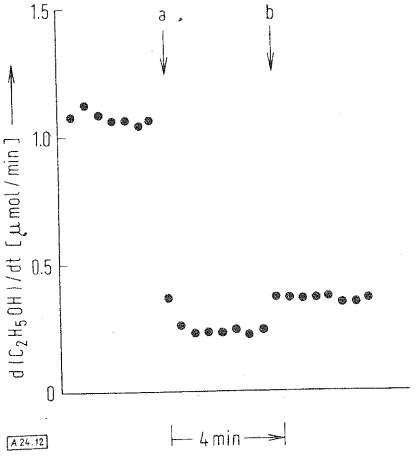


Fig. 12. Ethanol uptake by perfused rat liver, per g of liver [2 mmol of ethanol per l of influent perfusate]. Ethanol uptake in the presence of 90 µmol/l of 4-methylpyrazole (added at point a) is 22% of the control value and increases after the addition of 3.8 mmol/l of glycolate (at point b) to 34%. This increase corresponds, per g of liver, to about 150 nmol/min (after [161]).

150 nmol/min. The stimulation of H_2O_2 production per g liver under these conditions is approximately 400 nmol/min, so that about 35—40% of the H_2O_2 additionally produced is used for the peroxidatic reaction. As expected, this proportion increases at higher ethanol concentrations[162]. The rate of the oxidation of methanol also increases on addition of glycolate[163]. Since the peroxidatic oxidation of ethanol is not connected with NAD^+ reduction, this pathway can be of use[116, 164] for avoiding the negative shift caused by the alcohol dehydrogenase in the redox potential of the cytosolic free NAD^+ system[165–169].

Currently, the possibility is being considered whether this could be utilized to influence liver metabolism in alcoholism. *Blomstrand* and *Kager*[170] demonstrated on human subjects that the inhibition of fatty acid oxidation and the accumulation of triglycerides by ethanol can be largely suppressed by the addition of 4-methylpyrazole. Therefore, the use of pyrazole

derivatives concomitant with a stimulation of the peroxidatic pathway of ethanol oxidation opens interesting perspectives. According to recent studies, the peroxidatic oxidation of ethanol by catalase should be responsible[105, 137, 140, 174] for the activity of a special "microsomal ethanol-oxidizing system" (MEOS)[171–173]. The NADPH-specificity of the microsomal activity is attributed in this case to the coenzyme specificity of an autoxidizable microsomal flavoprotein[137, 175]. Should this explanation be correct, the oxidation of ethanol in intact endoplasmic reticulum without catalase should not play a physiologically important role (for discussion, see [176]).

Of the aldehydes, catalase only acts on formaldehyde; acetaldehyde is not oxidized[177]. Hence, by measuring catalase Compound I one can follow the formation of ethanol from acetaldehyde[178] in the liver[171].

The question of the nature of the endogenous hydrogen donors is still largely unsettled. Only for ethanol has a continuous supply from the intestinal tract of rats been verified by determinations in portal and in caval blood[179]. Methanol can be formed continuously with the help of pectase by hydrolytic cleavage from pectins; in this manner in human subjects approximately 10—20 mmol of methanol are set free per day[180].

deDuve and *Baudhuin*[12] postulated that the peroxidatic oxidation of α-hydroxy acids plays a role in gluconeogenesis since it might be linked to an oxidation of extramitochondrial reducing equivalents (NADH). Experimental proof of this hypothesis is still lacking.

The possible function of catalase in lipid metabolism of the liver, as already postulated by *Novikoff* and *Shin*[54] and also suggested by the effects of the hypolipidemically active drugs, also remains obscure. Acatalasemic strains of mice as compared to wild strains contain a diminished serum concentration of triglycerides and cholesterol[181]. A possible reason for this observation could reside in a higher content of catalase degradation products within the peroxisomes. It is known that acatalasemia in mice can be attributed to a thermolabile variant of catalase[182], possibly resulting in an increased level of degradation products (*e.g.* subunits). A fall in the blood lipids was produced experimentally by injection of catalase subunits with peroxidatic activity[183–185]. Recently, the lack of peroxisomes in the liver, which contained considerable lipid deposits, was described in a human patient with a fatal cerebro-hepato-renal syndrome (Zellweger's disease)[186].

6. Outlook

The methods of acquiring biochemical information on intracellular processes in intact surviving organs allow insight into the metabolism of hydrogen peroxide taking place in the range of nanomolar concentrations. Constant production of H_2O_2 is a normal feature of liver metabolism. Exogenous influences, *e.g.* the effects of radiation[187, 188] or oxygen and ozone poisoning[189, 190], could not be discussed here in detail.

Corresponding to our still limited knowledge of the metabolism of endogenous H_2O_2-producing substrates and the endogenous hydrogen donors, we are still at the beginning of a full comprehension of the function of liver catalase (cf.[12, 18, 20]). Also, phenomena such as the decreased activity of catalase in tumor cells[191] and the inhibition of catalase activity by tumor extracts[192], which is reported to be based upon formation of Compound II[193, 194], still require clarification. In contrast, for the plant variants of peroxisomes, *e.g.* the glyoxysomes, clear concepts already exist regarding their function in fat and carbohydrate metabolism (glyoxylate cycle), as well as in photorespiration and photosynthesis (reviews in [17, 195]). In particular, the implication attached to the term peroxisome requires further elucidation for animal tissue cells. With the steadily increasing number of known enzyme activities in peroxisomes and peroxisome-containing cell types with biphenyltetramine oxidase activity (cf. the peroxisomes in the small intestine[196] and the myocardium[197, 198], in steroid-secreting cells such as the interstitial cells (Leydig cells) of the testes[199] and the adrenal cortex[200]) and with the rapid development of biochemical and cytological methods, further aspects are expected in the near future on the cell biology of catalase, which was once described as a "fossil enzyme"[18].

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