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Hydroperoxide Metabolism in Mammalian Organs

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I. Introduction and Historical Background 527
II. Enzymes Metabolizing Oxygen-Reduction Products 531
A. Enzymes utilizing free hydroperoxides 531
B. Enzymes utilizing bound hydroperoxides 542
C. Enzymes utilizing superoxide anion 545
III. Methods for Determining Oxygen-Reduction Products and Related Chemical Species in Biological Systems 547
A. Determination of hydrogen peroxide 547
B. Determination of superoxide anion 553
C. Detection of hydroxyl radical 554
D. Determination of lipid and organic peroxides 555
E. Detection of singlet molecular oxygen 555
IV. Cellular Sources of Hydrogen Peroxide 556
A. Subcellular fractions 557
B. Hydrogen peroxide production at different oxygen concentrations 561
V. Intracellular Regulation of Oxygen-Reduction Products 564
A. Steady-state intracellular concentrations of hydrogen peroxide and superoxide anion 564
B. Levels of enzyme activity 565
C. Cellular redox state 570
VI. Oxygen Toxicity and Defense Against Oxygen-Reduction Products 574
A. Oxygen toxicity 574
B. Defense against toxic products of oxygen reduction 578
VII. Lipid Peroxidation 579
A. Lipid and organic peroxide formation 579
B. Biochemical consequences of lipid peroxidation 582
C. Physiological consequences of lipid peroxidation 583
VIII. Hydrogen Peroxide as a Useful Metabolite 585
A. Phagocytosis 586
B. Alcohol oxidation 587
IX. Conclusion 589

I. INTRODUCTION AND HISTORICAL BACKGROUND

The existence of significant levels of potentially dangerous oxidants in cells and tissues under physiological conditions has frequently been proposed but had previously received little quantitative support. Current approaches to the direct measurement of the intracellular concentration of hydrogen peroxide, together with a better understanding of the experimental and theoretical basis for hydrogen peroxide generation and utilization in cells and tissues, now afford an appropriate background for a review of the physiological and biochemical aspects of intracellular hydroperoxide metabolism in mammalian systems.

Although carbon monoxide- and cyanide-insensitive respiration, which bypasses the sequential reduction of oxygen via the respiratory carriers with the concomitant phosphorylation of ADP to ATP, has long been established, the physiological significance of a number of intermediates of oxygen reduction, produced in this or other intracellular reactions, has been recognized only in the last decade. This review focuses on hydrogen peroxide generation and utilization, emphasizing the function of catalase, the enzyme chiefly responsible for the intracellular regulation of H_2O_2. The reactions of glutathione peroxidase with H_2O_2 and organic hydroperoxides are evaluated, as is the role of superoxide dismutase in converting the superoxide anion O_2^- to H_2O_2. The overall scheme of Figure 1, showing the interaction of these pathways, serves as the background for this study.

The metabolic implications of the continuous production of hydroperoxides, including the physiological role of catalase in the oxidation of methanol, ethanol,

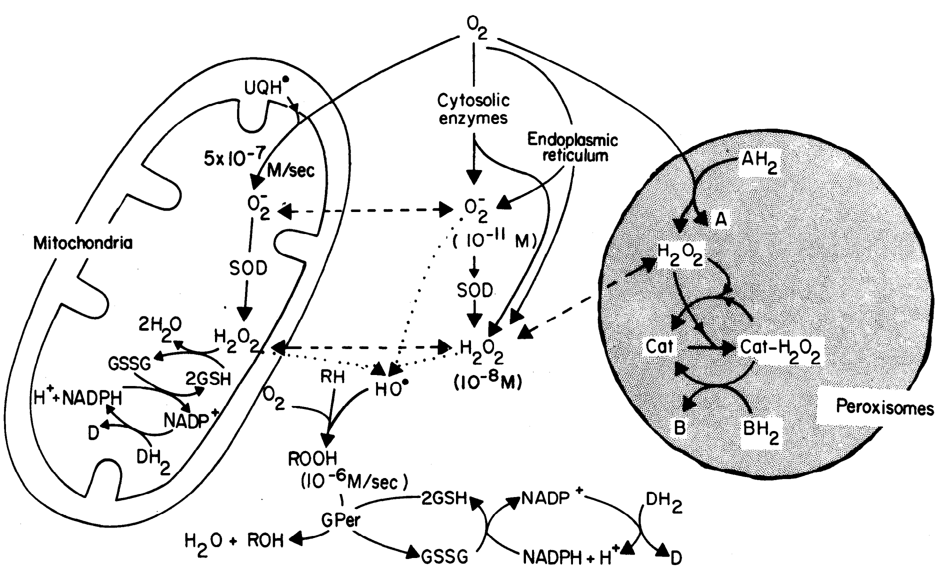

FIG. 1. General scheme of roles of catalase, glutathione peroxidase, and superoxide dismutase in different subcellular locations. Concentrations and formation rates of oxygen metabolites are estimated. UQH·, ubiquinone radical; GSSG, oxidized glutathione; GSH, reduced glutathione; DH_2 and D, a nonspecified NADP reducing system; SOD, superoxide dismutase; NADPH and NADP, nicotinamide adenine dinucleotide phosphate; O_2^- superoxide anion; HO·, hydroxyl radical; ROOH, an alkyl hydroperoxide; GPer, glutathione peroxidase; Cat, catalase; B and BH_2, hydrogen donors of a specificity appropriate to catalase, such as ethanol.

etc., in its peroxidatic mode, and the relation of the glutathione peroxidase reaction to the thiol and NADPH systems of the cell are discussed. The contribution of oxygen metabolites to a number of biological phenomena such as oxygen toxicity, radiation sensitivity, phagocytosis, and senescence is considered.

Much of the new information on H_2O_2 metabolism has been acquired by an approach based on optical techniques with catalase used as an indicator of intracellular H_2O_2. This enzyme forms an intermediate, compound I, discovered by Chance in 1947 (84) that has distinctive optical properties (see sect. II, *A1*). Compound I was soon observed in living bacterial cells (*Micrococcus lysodeikticus*) where the intracellular H_2O_2 concentration was estimated to be 10^{-8} M under the usual growth conditions and "about four times as much peroxide was decomposed catalatically as peroxidatically in the respiring cell" (93). These studies were extended to mammalian tissues by Sies and Chance (476) using absorption spectrophotometry of isolated perfused rat liver, and compound I is now employed as an intracellular indicator of H_2O_2 concentration even in the intact organ in situ (395). At present, there is ample evidence that univalent and divalent reduction of oxygen is a universal attribute of aerobic life and that powerful metabolic activities have been developed to deal with the potentially harmful intermediates, in some cases utilizing them for cellular function such as alcohol oxidation or phagocytosis.

The study of enzyme-substrate compounds has been of the greatest importance in biochemical studies of the mechanism of enzyme reactions and now has an essential role in organ physiology. This is appropriate since, from the historical point of view, the exploration of enzyme reactions began in physiology. The beginnings of enzymology were accompanied in the early years of the twentieth century by formulations of mechanisms of enzyme action by Henri (220) in 1903 and by Michaelis and Menten (349) in 1913 that were based on the concept of the combination of enzyme with substrate to form intermediate compounds.

More sophisticated reaction mechanisms, proposed by Briggs and Haldane in 1925 (70), were considered particularly appropriate for enzymes utilizing H_2O_2, termed "hydroperoxidases" by Theorell (517). Keilin and Mann (269), Stern (499), and Theorell (516) all identified intermediates in the reactions of hydroperoxidases with peroxides that they believed to be related to the "enzyme-substrate compounds" proposed by Henri and by Michaelis and Menten.

Kinetic studies revealed the true nature and function of these intermediates. Two types, primary and secondary, are involved in peroxidase reactions, whereas only the primary type has been found to be functional in the reactions of catalase (86, 87, 93, 113). Early in vivo experiments with *Micrococcus lysodeikticus* (93) demonstrated the primary intermediate of catalase and hydrogen peroxide in living cells and its reactions with ethanol, nitrite, and formate. These observations of the catalase compound lay fallow for nearly 20 years, until improved techniques for the study of perfused organs, a greater interest in intracellular H_2O_2, and the development of more sensitive instrumentation led to similar observations of the primary catalase-H_2O_2 intermediate in the perfused liver (392, 393, 475, 476). These studies have been followed by investigations of H_2O_2 generation in isolated organelles (66), which identified the microsomes and mitochondria, as well as the peroxisomes studied by de Duve (143–145), as significant contributors to this phenomenon. The discovery of the role of the mitochondria in peroxide generation (63, 66, 108, 317) was the most surprising and affords the conceptual basis for a revolution in our consideration of the problem of hydrogen-transfer reactions in mitochondria and the mechanism of intracellular H_2O_2 generation.

The concept of direct hydrogen transfer to oxygen with the concomitant generation of hydrogen peroxide had its proponents and opponents among the giants of biochemistry and physiology 50 years ago: Thunberg, Wieland, Willstätter, Warburg, Keilin, and others. The controversy centered about the mechanisms and relative significance of hydrogen transfer and electron transfer in biological oxidations. Wieland and Mitchell (550, 551) favored a theory of substrate activation in which hydrogen atoms from the substrate molecule combined with oxygen to form hydrogen peroxide. Warburg's concept of oxygen activation (537, 538), together with Keilin's proposal of electron transfer through a cytochrome chain (266), suggested that electrons were transferred to oxygen, which is then completely reduced and combines with hydrogen ions to form water only at the last step.

Finally, in 1929 Warburg's "*atmungsferment*" (540) could be identified with Keilin's cytochrome *c* oxidase, and this component was recognized as the generalized, carbon monoxide- and cyanide-sensitive, oxygen-consuming catalyst of the respiratory chain. This landmark discovery eclipsed for several decades the investigation of univalent oxygen reduction leading to the superoxide anion O_2^- or divalent reduction leading to H_2O_2. The eclipse was due in part to the lack of adequate methods for the direct demonstration of hydrogen peroxide generation in the cell and in part to Warburg's opposition to the idea. He is believed to have stated that Wieland has processed whole dogs and has not found one drop of H_2O_2!

A test of Wieland's hypothesis depended on the direct demonstration of H_2O_2 formation in substrate oxidation in vivo and in model systems. Despite numerous attempts, Wieland (550) was never able to identify H_2O_2 in animal tissues. This probably was not so much a fault of the analytical technique as a failure to comprehend how rapidly H_2O_2 disappears in tissues after an animal is killed. Tissue ischemia causes liver H_2O_2 to fall essentially to zero in less than 30 s, as determined by direct observations of the decrease of the concentration of the catalase-H_2O_2 intermediate (476).

A number of hints that the Warburg-Keilin scheme did not account fully for all the cellular oxygen reduction were provided by the cyanide- and antimycin A-insensitive respiration observed to a limited extent in mitochondria isolated from eukaryotic cells (488) and as a major pathway in bacterial respiratory systems and in mitochondria from higher plants (223, 312). In some plants the magnitude of the cyanide-insensitive respiration is so great, accounting for as much as 60–95% of the total respiration (50, 102), that it can scarcely be attributed to incomplete inhibition of the cytochrome oxidase system by cyanide, strongly suggesting the involvement of alternative pathways for oxygen reduction. The possibility that the antimycin-insensitive respiration of mitochondria may be generally related to the formation of hydrogen peroxide has recently been verified for animal as well as for plant mitochondria, with the use of a sensitive method for the detection of H_2O_2 by its binding to yeast peroxidase (60, 63, 442).

As early as 1946, Michaelis (348) proposed that the oxidation of bivalent organic molecules proceeds in two compulsory univalent steps, the intermediate being a free radical; according to this theory, intermediates of oxygen reduction would be a universal attribute of aerobic life, which must have developed effective mechanisms to deal with the highly reactive toxic products O_2^- and H_2O_2. It thus has been a matter of great interest to discover why cytochrome oxidase, which controls the main pathway of cellular oxygen reduction, has not been shown to generate significant amounts of such intermediates, although there have been intense searches for its interaction with O_2^- and H_2O_2 and its potential for generating radical intermediates is known (184). On the basis of optical studies of oxy- and peroxycytochrome oxidase (110, 111), it is now clear that the intermediates of oxygen reduction remain within the active site of cytochrome oxidase until the final reaction stage of water is achieved (110). It is possible of course that cytochrome oxidase is distinguished from most other oxidases by its unique coupling of oxygen reduction to the formation of ATP (374) and that for this reason, as well as for protection against cellular intoxication, the intermediates remain within the active site.

II. ENZYMES METABOLIZING OXYGEN-REDUCTION PRODUCTS

A. *Enzymes Utilizing Free Hydroperoxides*

1. *Catalase*

Catalase is present in virtually all mammalian cell types. A wide range of catalase concentrations is observed, and it is difficult to identify a mammalian cell in which no catalase is present (517). In many cases, the enzyme is localized in subcellular organelles such as the peroxisomes (microbodies) of liver and kidney or in much smaller aggregates such as the microperoxisomes found in a variety of other cells (144, 145, 382).

The chemical properties of catalases from different sources and of their functional enzyme-substrate compounds have been reviewed by Chance (87), Nicholls and Schonbaum (373), Brill (72), Deisseroth and Dounce (146), and, most recently, Schonbaum and Chance (459). The basic features of the catalase molecule are set forth in Table 1.

TABLE 1. *Selected enzymological features of catalase, glutathione peroxidase, and superoxide dismutase of rat liver*

	Catalase	Glutathione Peroxidase: Se dependent	Glutathione Peroxidase: Non Se dependent; glutathione S-transferase B	Superoxide Dismutase
Enzyme structure				
Molecular weight	240,000	76,000	46,000	33,000
Subunits	4	4	2	2
Active center group	Fe^{3+}-protoporphyrin	Selenium		Copper-zinc (cytosol) Manganese (mitochondria)
Enzyme function				
Reaction	$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$ (catalatic) $H_2O_2 + AH_2 \rightarrow 2H_2O + A$ (peroxidatic)	$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$		$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$
Subcellular distribution	Peroxisome, cytosol?	Cytosol, mitochondrial matrix		Cytosol, mitochondrial matrix
Rate constants	$k_1 = 1.7 \times 10^7 M^{-1} \cdot s^{-1}$ $k_4' = 2.6 \times 10^7 M^{-1} \cdot s^{-1}$ $k_4 = .2 - 1 \times 10^3 M^{-1} \cdot s^{-1}$	$5 \times 10^7 M^{-1} \cdot s^{-1}$		$2.4 \times 10^9 M^{-1} \cdot s^{-1}$

The need for a thorough understanding of the catalase reaction mechanism is underlined by the confusion that has arisen from inadequate comprehension of its enzymatic action. Catalase appears to be the only enzyme that exhibits dual activities with completely different kinetic characteristics. The principal reactions relevant to the biological function of catalase are based on the spectroscopic and kinetic evidence for the active intermediate, compound I. The simple sequence of consecutive reactions for the catalase mechanism proposed some time ago (84) still seems to represent the most accurate description of catalase action (see 459). The three key steps below provide at present a complete description of the kinetics of catalase in the catalatic (*Eqs. 1* and *2*) and peroxidatic (*Eqs. 1* and *3*) function:

$$\text{catalase-Fe}^{3+} + H_2O_2 \xrightarrow{k_1} \text{compound I} \quad (1)$$

$$\text{compound I} + H_2O_2 \xrightarrow{k_4'} \text{catalase-Fe}^{3+} + 2H_2O + O_2 \quad (2)$$

$$\text{compound I} + \underset{\text{(donor)}}{AH_2} \xrightarrow{k_4} \text{catalase-Fe}^{3+} + 2H_2O + A \quad (3)$$

In these equations, the intermediate compound I is not given a formal chemical structure. Although it is undoubtedly an oxidation product of the reaction of iron and peroxide, the tendency of the electrons of the ferric iron to delocalize to the periphery of the porphyrin, and probably toward the peroxide to varying extents, by analogy with oxyhemoglobin (34, 81), makes an exact structure meaningless. Thus a ferric peroxide (Fe^{3+}—HOOH) and a covalently bonded Fe^{5+}=O afford examples of no electron transfer between the donor, Fe^{3+}, and the acceptor, H_2O_2, on the one hand and complete charge separation on the other. The green compound I observed in cells and tissues involves the transfer of two electrons from the iron to the peroxide, and thus peroxide is reduced and can no longer be dissociated. Compound I may have as yet undetected precursor such as Fe^{3+}—HOOH, containing ferric iron and the unaltered peroxide molecule. Such an intermediate must have a very short lifetime since it has eluded all kinetic methods so far applied to the study of this interesting enzyme-substrate interaction.

The optical spectra of the free enzyme and its enzyme-substrate compound are illustrated in Figure 2. The decrease in absorbance at 405 nm in the region of the γ-band or Soret band (84) and the red shift of the α-band to 660 nm (92) are characteristic of the "primary" green enzyme-substrate compounds of the hydroperoxidases. These types of spectral shifts are characteristic of heme compounds in which the porphyrin ring is affected. The distinct spectroscopic properties of compound I that aided in its discovery have also been pivotal in the detection of H_2O_2 in intact tissues.

A more generalized scheme for the chemistry of the catalase reaction is shown in Figure 3, which indicates two cycles of catalase: the upper portion indicates the catalatic cycle, and the lower portion indicates the peroxidatic

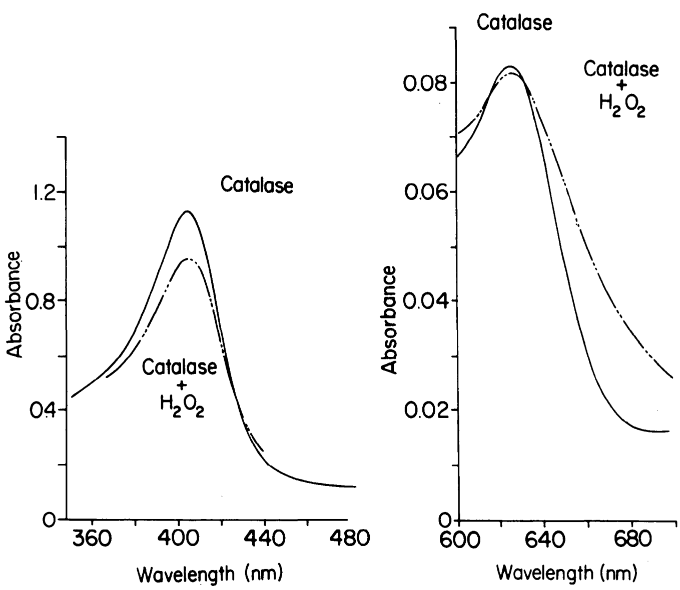

FIG. 2. Absolute spectra of purified catalase from rat liver. Catalase-H_2O_2 (compound I) as generated from glucose *plus* glucose oxidase systems exhibits characteristic absorbance changes in Soret band (*left*) and near infrared (*right*) spectral regions. [From Sies et al. (476).]

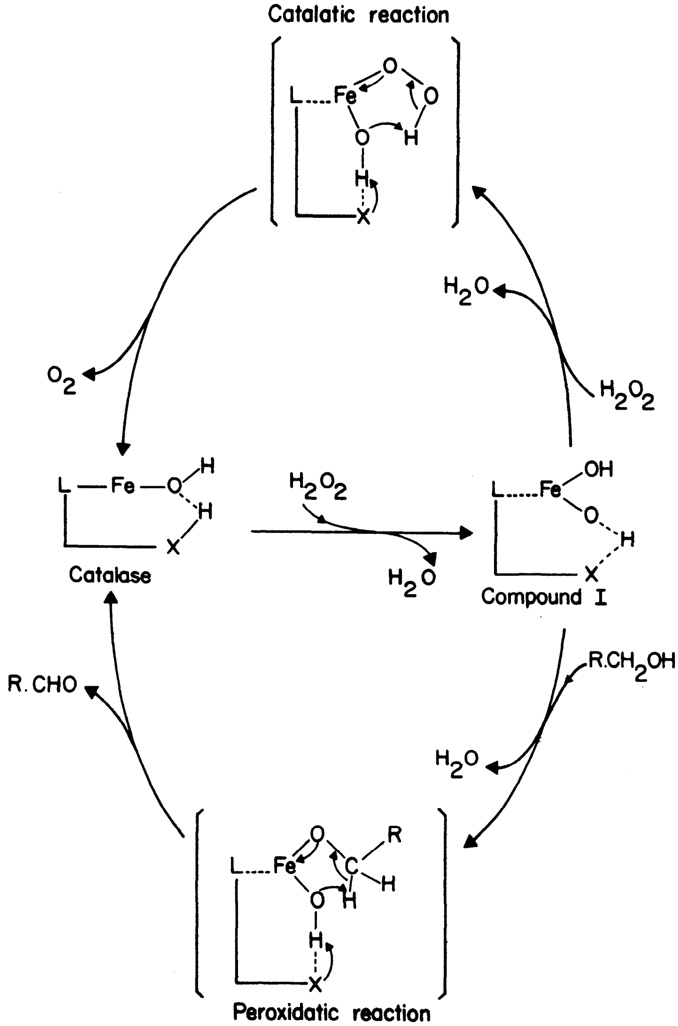

FIG. 3. Proposed mechanism of the reactions of catalase compound I. [Modified from Schonbaum and Chance (459).]

cycle. The formation of compound I by reaction with peroxide is indicated across the middle of the diagram; it is structured here as a peroxide compound involving a substrate binding site on the protein molecule, X. The catalatic reaction with the second molecule of H_2O_2 forms an as yet unidentified ternary intermediate from the remnants of the two molecules of peroxide. Free catalase is regenerated and molecular oxygen is released. Evolved oxygen is in the ground triplet state; less than 0.5% of the total is released as singlet molecular oxygen (419).

The peroxidatic reaction involves the formation of a similar unidentified intermediate of compound I and the alcohol RCH_2OH. In this case, the product is the aldehyde. Similar mechanisms apply to the oxidation of other substrates. Catalase is remarkable in that a common intermediate serves the two pathways.

The specificity of catalase for peroxides is high; only hydrogen, methyl, and ethyl hydroperoxides give appreciable activity. It is especially noteworthy that *t*-butyl peroxide does not react with catalase, but is a substrate of glutathione peroxidase. Hydrogen donors for catalase include a similar sequence of aliphatic alcohols, with high activities for methyl and ethyl and low activities for butyl and higher homologues. However, some unusual alcohols are also oxidized (see Table 2).

All four catalase hemes can combine with H_2O_2 to form compound I. However, the unique feature of the consecutive reactions of *Equations 1* and *2* is that compound I is in a steady state with H_2O_2 acting as both oxidizing and reducing substrate. Thus, at any moment only a fraction of the catalase heme is bound to H_2O_2 in the form of compound I, and that fraction reaches a maximal level determined by the ratio of the rate constants k_1 and k_4'. This ratio can be calculated from *Equations 1*, *2*, and *3* as shown below. The development of these equations can be found in previous work (101, 109); they are included here in simplified form since they form the basis for the discussion that follows.

$$\mathrm{d[compound\ I]}/\mathrm{d}t = \mathrm{k_1[H_2O_2][free\ catalase]} - k_4'[\mathrm{H_2O_2}][\mathrm{compound\ I}] - k_4[\mathrm{donor}][\mathrm{compound\ I}] \quad (4)$$

$$\mathrm{d[H_2O_2]}/\mathrm{d}t = \frac{\mathrm{d[H_2O_2]generated}}{\mathrm{d}t} - k_1[\mathrm{H_2O_2}][\mathrm{free\ catalase}] - k_4'[\mathrm{H_2O_2}][\mathrm{compound\ I}] \quad (5)$$

$$\frac{\mathrm{d[donor]}}{\mathrm{d}t} = -k_4[\mathrm{donor}][\mathrm{compound\ I}] \quad (6)$$

The following relations were developed on the assumption that the rate constant for the dissociation of compound I was negligible, as required by the

TABLE 2. *Reduction of compound I by two-, three-, and four-carbon alcohols*

	Alcohol				
	Ethyl	Propyl	Butyl	Allyl	Propargyl
k_4 app, $M^{-1} \cdot s^{-1}$	1020	6.5	0.4	330	2500

Compound I: horse erythrocyte catalase, 5 mM phosphate, pH 7, 25°C. [From Schonbaum and Chance (459).]

nature of the compound in which electrons are transferred from the iron to the peroxide. For the steady state of compound I and of the rate of H_2O_2 generation, the fraction of total catalase heme existing as compound I is given by:

$$[\text{compound I}]/[\text{total catalase heme}]_{\text{steady state}} = (1 + k_4'/k_1 + k_4[\text{donor}]/k_1[H_2O_2])^{-1} \quad (7)$$

Equation 7 indicates that the catalatic reaction does not have a Michaelis constant (K_m) since the term k_4'/k_1 is independent of the H_2O_2 concentration. The next term inside the parentheses, identified with the peroxidatic reaction, does not give a unique K_m for the donor since $[H_2O_2]$ is a variable. Where $k_1[H_2O_2] \gg k_4[\text{donor}]$, the reaction is largely catalatic and the occupancy of catalase heme in the form of compound I is given by:

$$[\text{compound I}]_{\text{maximal}}/[\text{total catalase heme}] = (1 + k_4'/k_1)^{-1} \quad (8)$$

Thus a simple relationship exists between the maximal heme occupancy at the maximal rate of H_2O_2 utilization and the second-order rate constants for the formation and catalatic degradation of compound I. Values for maximal heme occupancy range from 0.3 to 0.5 (101).

Another useful relationship describes the peroxidatic reaction of catalase when the donor concentration is chosen to give half-maximal heme occupancy (109). In the steady state, this donor concentration, $[\text{donor}]_{1/2}$ or $[A]_{1/2}$, is directly proportional to the steady-state turnover number of catalase:

$$[\text{donor}]_{1/2} = \text{constant} \cdot \frac{\text{steady-state rate of } H_2O_2 \text{ generation}}{[\text{total catalase heme}]} \quad (9)$$

where the constant is:

$$\left(\frac{3(k_4'/k_1) + 1}{2(k_4'/k_1 + 1)^2} \cdot k_4\right)^{-1}$$

Equation 9 is perhaps the most useful one for the studies of catalase function in situ, since the donor concentration that decreases the concentration of compound I from maximal to half-maximal is readily determined by a titration of the system with donor, as described in detail below (sect. III*A*). The steady-state rate of H_2O_2 production in the system is the sum of the rates from all the sources of H_2O_2 generation that are available to catalase.

A further equation can be derived to specify the steady-state H_2O_2 concentration for the condition that the catalase is "saturated" with H_2O_2. *Equation 10* is of special significance in physiological systems since it shows that a given rate of H_2O_2 utilization will be consistent with a wide range of catalase and H_2O_2 concentrations; the lower the catalase concentration, the higher the H_2O_2 concentration and vice versa:

$$-d[H_2O_2]/dt = 2k_4'[H_2O_2][\text{compound I}] = 2k_1[H_2O_2][\text{free catalase}] \quad (10)$$

Thus, the physiological or pathological variation of catalase concentration in different organs and tissues will lead to different steady-state levels of H_2O_2 concentration for the same rate of H_2O_2 generation. For example, one expects a low H_2O_2 concentration in those organs having a high catalase content, such as liver and kidney, and a much higher H_2O_2 concentration in other organs, such as heart and brain, possessing a low catalase content (393, 515). Apparently catalase is uniquely fitted to provide a homeostasis of H_2O_2 concentration according to *Equation 10*, as a consequence of the catalatic mode of action in which, as the rate of H_2O_2 generation for a given catalase concentration increases, the amount of free H_2O_2 also increases. Whereas in Michaelis-Menten enzyme systems this rise of substrate concentration could exceed the Michaelis constant for the system so that saturation of the enzyme activity would occur, we can see from *Equations 7* and *8* that no Michaelis constant exists for the catalatic reaction. Thus, the enzyme activity will increase linearly with the available H_2O_2 concentration over wide ranges, thereby maintaining a controlled intracellular H_2O_2 concentration. This safety feature of catalase regulation is a unique property of the consecutive reaction of two molecules of H_2O_2 with the enzyme.

The primary effect of inhibiting the enzyme by azide or cyanide is to diminish the effective enzyme concentration available for catalatic function, causing a correlated increase of the steady-state H_2O_2 concentration (*Eq. 10*). This in turn increases the turnover number of residual catalase and diminishes somewhat the fraction of H_2O_2 that is expended in hydrogen donor oxidation, but leaves the overall situation largely unchanged.

A general quantitation of the partitioning of catalase activity between the catalatic and peroxidatic modes is explained by *Equations 11* and *12*, which embrace the empirical observations of Keilin and Hartree (267, 268) on coupled oxidations. Similar equations have been derived both by ourselves (104, 109) and by Clayton (123).

$$-d[H_2O_2]/dt = 2k_4'[H_2O_2][\text{compound I}] + k_4[\text{donor}][\text{compound I}] \quad (11)$$

This equation contains both a catalatic term, $k_4'[H_2O_2][\text{cmpd I}]$, and a peroxidatic term, $k_4[\text{donor}][\text{cmpd I}]$. When $[H_2O_2]$ is low, the peroxidatic pathway will predominate; when $[H_2O_2]$ is high, the catalatic pathway will predominate.

These two functions are illustrated in Figure 4, where the rate of ethanol oxidation is plotted against the rate of H_2O_2 generation at different initial concentrations of ethanol. The peroxidatic pathway predominates at low rates of H_2O_2 formation and therefore at low steady-state turnover numbers of catalase, as well as at high concentrations of ethanol.

An explicit formulation of the partitioning of activities between the catalatic and peroxidatic pathways is provided by the simplified formulation of *Equation 12*. The efficiency, F, is defined as the ratio of the rate of hydrogen donor oxidation, $d[\text{donor}]/dt$, to the rate of total H_2O_2 utilization, $d[H_2O_2]/dt$. Thus,

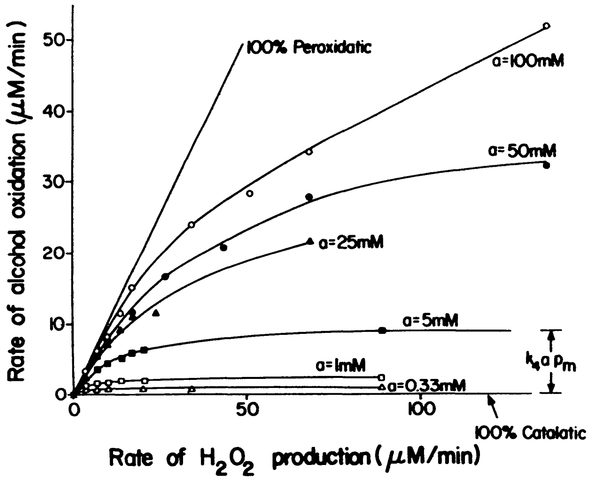

FIG. 4. Partitioning of H_2O_2 decomposition by catalase between peroxidatic and catalatic reactions. Production of NADH from ethanol (plotted on *ordinate*) is shown as a function of H_2O_2 production (plotted on *abscissa*) at different initial concentrations of ethanol as indicated. [From Oshino et al. (396).]

as F approaches 1, the peroxidatic pathway predominates; as F approaches 0, the catalatic pathway predominates. The formula that correlates heme occupancy with efficiency is given by

$$[\text{compound I}]/\text{total catalase heme} = (1 - F)/(2.5 + 0.5F) \qquad (12)$$

with $k_4'/k_1 = 1.5$ for rat liver catalase (109, *Eqs. 4i,j*).

A second type of useful equation is expressed in terms of the percentage of maximal saturation of catalase with H_2O_2

$$\%\ \text{maximal saturation} = (1 - F)/(1 + 0.2F) \qquad (13)$$

Thus, the value of F at $[A]_{1/2}$, the hydrogen donor concentration giving 50% saturation, corresponds to a peroxidatic efficiency of $5/9$ or 45%. An efficiency of 90% requires a saturation value of about 8%.

These relationships are plotted in Figure 5*A*, together with the experimental data obtained in vitro. The most efficient utilization of H_2O_2 for the peroxidatic oxidation of hydrogen donors is at low heme occupancy or, as above, at low steady-state turnover numbers. A 50% efficiency is obtained when the heme occupancy is 45% of maximal (396). The graph in Figure 5*A* emphasizes low ethanol concentrations, whereas that in Figure 5*B* plots the equation in semilogarithmic coordinates and thus covers a wider range of alcohol concentrations. At the higher concentrations of alcohol, the amount of compound I present is very small, and a decrease of available catalase heme due to added inhibitor or to a physiological change will cause a relatively small decrease in the efficiency, as pointed out above for *Equation 10*. The relationship depends on the value of k_4 for the particular alcohol used (see also sect. VIII*B*).

a) Assay of catalase activity. The rate of H_2O_2 decomposition in catalatic activity is directly proportional to the amount of enzyme at a fixed H_2O_2 con-

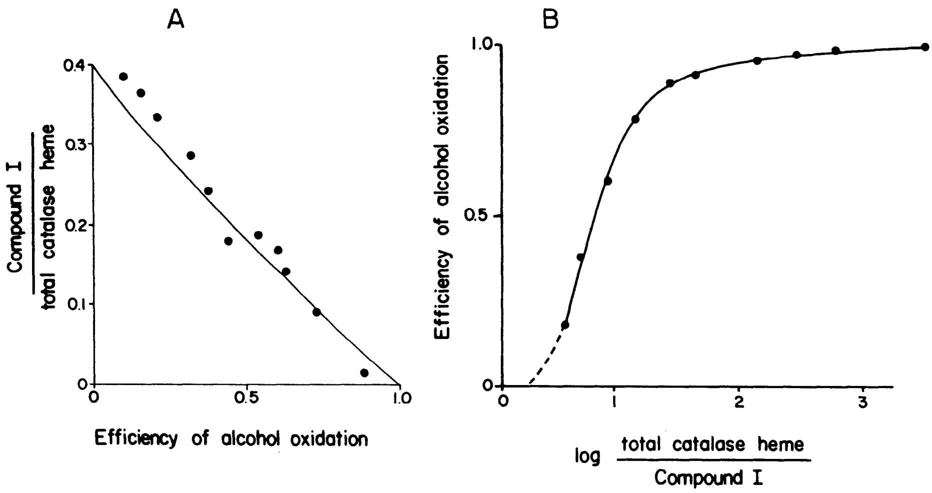

FIG. 5. Efficiency of alcohol oxidation and its relationship to the level of catalase compound I. [Modified from Chance and Oshino (109) and Oshino et al. (396).]

centration and obeys first-order kinetics with respect to H_2O_2 concentration. For this reason, both the assay conditions and the expression of the results must be stated precisely in order to permit comparisons. The rate constant (k) for the overall H_2O_2 utilization is given by $k = (2.3/t) \log [H_2O_2]_0/[H_2O_2]_t$, where $[H_2O_2]_0$ and $[H_2O_2]_t$ are the H_2O_2 concentrations at $t = 0$ and after a time t, respectively; k is related to the catalase content of the sample as $k = k_1'[e]$, where $k_1' = 4.6 \times 10^7 M^{-1} \cdot s^{-1}$ (475) and [e] is the catalase heme concentration. The breakdown of H_2O_2 may be determined by following spectrophotometrically the decrease of absorbance at 240 nm or polarographically in terms of oxygen evolution (3, 91, 95, 103). An apparent k_1' is calculated by dividing k by a suitable reference unit, i.e., milligrams of protein or milliliters of blood. Detailed descriptions of catalase assays have been published elsewhere (3, 95).

2. Glutathione peroxidase

a) Biochemical function of glutathione peroxidase. Glutathione peroxidase, discovered in 1957 by Mills (354), catalyzes the reaction of hydroperoxides with reduced glutathione (GSH) to form oxidized glutathione disulfide (GSSG) and the reduction product of the hydroperoxide

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O \quad (14)$$

This enzyme is found in high activity in liver and erythrocytes, where it was first discovered; in moderate activity in heart and lung; and in low activity in muscle (118, 355). Glutathione peroxidase is specific for its hydrogen donor, GSH, and nonspecific for the hydroperoxide. This notable lack of substrate specificity extends the range of substrates from H_2O_2 to organic hydroperoxides, among which fatty acid hydroperoxides of various structures and nucleotide- or steroid-derived hydroperoxides are of particular interest. Thus, although glutathione peroxidase shares the substrate H_2O_2 with catalase, it alone can react effectively with organic hydroperoxides as well.

The protein chemistry and kinetic mechanism of glutathione peroxidase have been described in detail in recent years, and surveys have been presented by Flohé (167, 169) and by Ganther et al. (187). Some features are presented in Table 1. The enzyme contains selenium (170), most probably as part of the active center, but no other prosthetic groups such as heme, flavin, or other metal constituents. The native enzyme is made up of four subunits and contains four atoms of selenium per molecule. Little is known at present about the state and nature of the selenium compound in the active center, although Wendel et al. (545) demonstrated that the selenium moiety can undergo substrate-linked redox changes. The X-ray photoelectron spectroscopic signal of the 3d electrons of enzyme-bound selenium shifts from 54.4 to 58.0 eV on addition of H_2O_2 and shifts back to 54.4 eV on addition of reduced glutathione. These observations are in accord with a functional role for selenium in the active center, but require further chemical support. No selenium-containing peptides have yet been isolated from purified enzyme. Nevertheless, a 2.5-Å X-ray crystallographic study revealed subunit symmetry (295) and the precise coordinates of the four selenium atoms in the tetrameric enzyme molecule (294).

The kinetic mechanism of glutathione peroxidase formally resembles that of heme peroxidases. The initial velocity pattern of glutathione peroxidase from bovine blood and rat liver is in agreement with a sequential reaction mechanism (171)

$$E_{red} \xrightarrow{ROOH \searrow \quad \nearrow ROH,\, H_2O} E_{ox} \xrightarrow{2GSH \searrow \quad \nearrow GSSG} E_{red} \qquad (15)$$

Thus, the maximum velocity of the reaction at a given concentration of GSH is independent of the nature of the hydroperoxide, as has been experimentally verified with H_2O_2 and ethyl, *t*-butyl, and cumene hydroperoxides (205). The rate constant, k_1, for the reaction of reduced glutathione peroxidase with H_2O_2 has been found to be approximately $5 \times 10^7\ M^{-1} \cdot s^{-1}$ (glutathione peroxidase subunit concentration), similar to that found with catalase.

The subcellular distribution of glutathione peroxidase in rat liver is complementary to that of catalase; two-thirds of the enzyme is in the cytosol and one-third in the mitochondria, and there is no glutathione peroxidase in the peroxisomes (172). Thus, the characteristic sharing of H_2O_2 metabolism between glutathione peroxidase and catalase has a basis in the distribution of the two enzymes. Diffusion of glutathione peroxidase within the cell is required in order for it to reach the hydrophobic lipid hydroperoxide substrates associated with membrane structures. However, recent observations (334) have failed to detect hydroxy fatty acids, the expected product, in the phospholipids of mitochondria and microsomes.

In the steady state, regeneration of GSH by reduction of GSSG is required. The NADPH-dependent GSSG reductase has a subcellular distribution similar to that of glutathione peroxidase (172). Oxidation of NADPH (see Fig. 1 and sect. V, *C2*) links the operation of glutathione peroxidase with the NADPH-linked substrates.

b) Assay of glutathione peroxidase activity. Glutathione peroxidase is usually determined by following spectrophotometrically or fluorometrically the rate of NADPH oxidation coupled to glutathione peroxidase activity in a reaction mixture containing NADPH, excess glutathione reductase, GSH, *t*-butyl or cumene hydroperoxide, and the sample in the presence of sodium azide (204, 301, 412). Activity is expressed in units of micromoles per minute referred to milligrams of protein, nanograms of hemoglobin, or grams of tissue. This assay includes measurement of the activity of glutathione peroxidase and of the non-selenium-dependent activity of glutathione transferases (204, 301).

3. Heme peroxidases

Heme peroxidases are among the most extensively studied enzymes because of their wide distribution (especially in plant tissues), their chemical stability, and the colored enzyme-substrate intermediates of their catalytic action. Horseradish peroxidase has been the prototype heme peroxidase for the investigation of reaction rates and mechanisms (87, 88, 91). A detailed review of peroxidases has been done by Saunders et al. (457) and, more recently, by Yamazaki (555); in the present review the properties of the major heme peroxidases of mammalian origin are briefly summarized.

Mammalian peroxidases seem to have prosthetic groups different from protoheme and the heme is tightly bound to the apoprotein (555). This binding is considered to be covalent, except in thyroid peroxidases, where heme can be extracted and then reconstituted with full restoration of enzyme activity (288).

Lactoperoxidase, which is present in the mammary and salivary glands (366), utilizes common hydrogen donors such as pyrogallol, guaiacol, ascorbic acid, and benzidine (457) and can iodinate tyrosine (43). Since lactoperoxidase, peroxide, and halides can inhibit the aerobic growth of some microorganisms (251), it has been suggested that this peroxidase may be a growth inhibitor for oral bacteria (366).

Peroxidases from eosinophils (17) and intestinal mucosa (498) have spectral properties similar to those of lactoperoxidase. Although it has been claimed that the various peroxidases isolated from animal tissue, with the exception of thyroid peroxidase and myeloperoxidase, are simply a single enzyme carried to the tissues by the eosinophils (453), more recent reports (121, 319) indicate an organ origin, at least for uterine peroxidase. Uterine peroxidase activity is known to be markedly increased by estrogen or luteinizing hormone administration (11, 318, 319).

Thyroid peroxidase is firmly bound to the particulate fraction of thyroid homogenates and catalyzes the oxidation of iodide to iodine in the gland, playing an important role in the synthesis of thyroid hormones (457).

Myeloperoxidase, which is found in neutrophils, has unique spectral properties (462). Unlike other peroxidases, it contains two iron atoms per molecule (10, 387), with heme groups that are similar to heme *a* and exhibit different reactivities toward H_2O_2 (10) and cyanide (388). Myeloperoxidase utilizes phenols, quinols, ascorbic acid, etc. as hydrogen donors and has a slight catalase activity (9). Although the biochemical details of phagocytosis are still being debated, there is no doubt that myeloperoxidase has a physiological function in the phagocytic cells (see sect. VIII*A*).

B. Enzymes Utilizing Bound Hydroperoxides

1. Cytochrome oxidase

Cytochrome a_3 is the terminal oxidase of the main pathway of cellular respiration. The nature and mechanism of the oxygen reaction of this hemoprotein, first revealed as such by Warburg's immortal photochemical action spectrum of its CO compound, until recently have remained an enigma. Although the Warburg photochemical action spectrum (540) and subsequent photodissociation difference spectra (83, 94) clearly identified Keilin's "cytochrome oxidase" (265) as a heme that forms photodissociable compounds with CO, no evidence for an "oxycytochrome oxidase" with a dissociable oxygen was obtained until low-temperature studies of the photolysis reactions of cytochrome oxidase in the frozen state in the presence of oxygen revealed a highly dissociated oxy compound, termed compound A, which is quite stable below −100°C (110). The kinetics of formation of this compound and of a series of subsequent intermediates in which oxygen is partially reduced and cytochrome oxidase partially oxidized (compounds B and C) illustrate possible steps in the reaction sequence. Compound B oxidizes cytochrome *c* and appears to be an intermediate in the much more rapid oxidation reaction observed at room temperature (96).

The role of peroxides in the oxidase reactions is relevant here. It is known that catalase does not inhibit the cytochrome oxidase reactions and further that peroxides are not a substrate for cytochrome oxidase action (90). Apparently oxygen reduction occurs within the active site of cytochrome oxidase, but the evidence for intermediates of oxygen reduction demonstrates that, at least at low temperatures, oxygen reduction is not a concerted reaction but rather a stepwise electron transfer from the iron and copper components of cytochrome oxidase to reduce oxygen ultimately to water. One intermediate, compound B, is of special interest: it appears to contain both oxidized iron and oxidized copper, as judged from optical and electron paramagnetic resonance signals, and is presumably a peroxy compound (110). The peroxide is tightly bound to the heme of the oxidase until its eventual reduction to water, suggesting that the tight binding of cytochrome oxidase to peroxide prevents the release of the dangerous oxygen-reduction products that, if released into the external medium at the rates characteristic of cytochrome oxidase action, would surely lead to a sufficiently high level of radical intermediates leading to lipid peroxidation to be deleterious to cell function. However, some minor portion of the radical intermediates escapes from the active site of cytochrome oxidase and initiates the radical-dependent sulfite oxidation (184).

Figure 6 attempts to rationalize the tight binding of peroxide to the active site of cytochrome oxidase by postulating a "pocket" or "crypt" in the active site in which radical intermediates are retained until water is formed. Indeed, the fact that oxycytochrome oxidase is formed in an oxygen-dependent bimolecular reaction even at −100°C suggests a pocket in the molecule that is isolated from the surrounding frozen environment. The reaction sequence is depicted to be

$$\underset{\text{(reduced oxidase)}}{a_3^{2+} + O_2} \longrightarrow \underset{\text{(compound A)}}{a_3^{2+} \cdot O_2} \longrightarrow \underset{\text{(compound A')}}{a_3^{3+} \cdot O_2^-} \qquad (16)$$

However, even at these low temperatures, one of the two copper atoms of cytochrome oxidase is oxidized:

$$\underset{\text{(reduced oxidase)}}{a_3^{2+}Cu^{1+} + O_2} \longrightarrow \underset{\text{(compound A)}}{a_3^{2+} \cdot O_2 \cdot Cu^{1+}} \longrightarrow \underset{\text{(compound B)}}{a_3^{3+} \cdot O_2^{2-} \cdot Cu^{2+}} \qquad (17)$$

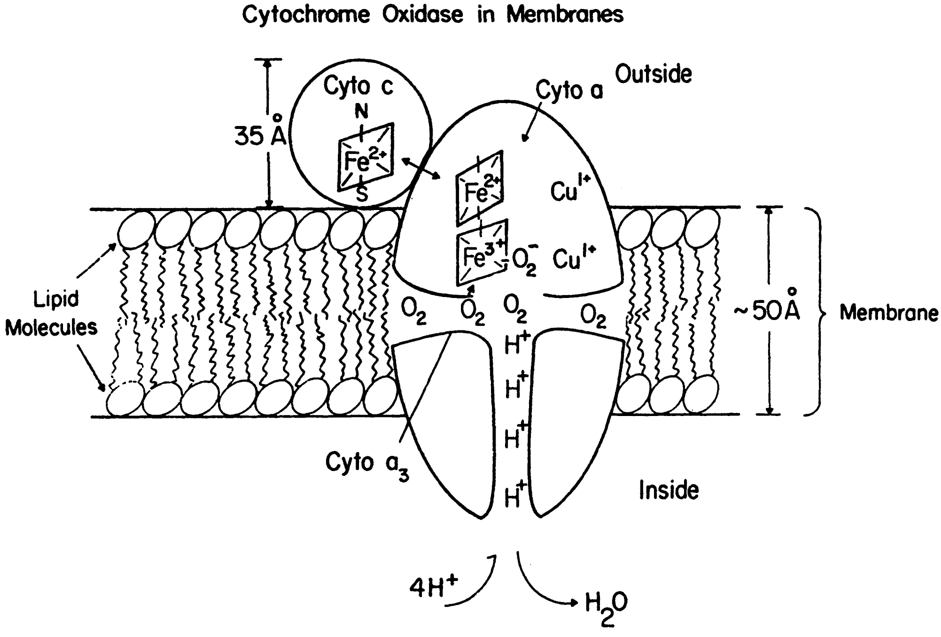

FIG. 6. Generation of bound oxygen intermediates at active site of cytochrome oxidase.

Thus, the radical intermediates are further reduced immediately and bound as in *Equation 17*. The further reduction of peroxide to water is made possible by the two additional heme iron and copper components of cytochrome oxidase

$$4H^+ + \underset{\text{(compound B)}}{(a^{2+}Cu_a^{1+})\cdot(a_3^{3+}\cdot O_2^{2-}\cdot Cu_{a_3}^{2+})} \rightarrow \underset{\text{(oxidized oxidase)}}{(a^{3+}Cu_a^{2+})\cdot(a_3^{3+}Cu_{a_3}^{2+})} + 2H_2O \quad (18)$$

Thus, the four reducing equivalents of cytochrome oxidase are available to complete the reduction of oxygen to water without the release of O_2^- or H_2O_2. The overall process requires only very low oxygen concentrations (10^{-7} M) for maximal activity.

The functional role of the partially reduced oxidase in cytochrome *c* oxidation has been demonstrated (112). The kinetics of the oxidation of reduced cytochrome *c* by compound B at $-90°C$ suggest that a peroxidase-type reaction ensues and that cytochrome *a* and Cu_a are nonfunctional at low temperatures and at higher temperatures may serve as an electron reserve or ballast to ensure the rapid reduction of cytochrome oxidase intermediates (112).

The valency states of iron and copper in these intermediates are not conclusively established, and they are further complicated by the equilibrium of cytochrome *c* with cytochrome *a* and its associated copper atom. Nevertheless, cytochromes *c* and c_1 and an associated iron-sulfur protein add to the reducing capability of cytochrome oxidase and the associated respiratory carriers. Many other properties of these electron carriers are relevant to ATP formation, a topic beyond the scope of this review. It suffices to say that the oxidase is a large molecule (~240,000 daltons for the dimeric unit), located in the membrane as indicated in Figure 6, with hemes perpendicular to the plane of the membrane and presumably near enough to cytochrome *c* and to each other to permit electron transfer by thermally assisted electron tunneling (107).

2. Cytochrome P_{450}

The electron transfer chain from NADPH to cytochrome P_{450}, localized in the endoplasmic reticulum membrane of liver and also in the mitochondria of the adrenal cortex and other organs, catalyzes monooxygenation reactions of endogenous substrates or foreign substances such as drugs. This field has been recently reviewed by Orrenius and Ernster (390) and Gunsalus et al. (203). There is now evidence for a peroxide intermediate, $R^+\cdot Fe^{3+}\cdot O_2^{2-}$ (159), as indicated in the proposed mechanism of Figure 7; the remaining intermediates are hypothetical. The activated oxygen is bound to the terminal oxidase, cytochrome P_{450}. Hamilton (211) and Ullrich (525) propose an oxene or oxenoid mechanism. In contrast to cytochrome oxidase, P_{450} releases O_2^- or H_2O_2 under certain conditions (227, 379, 489).

Organic hydroperoxides such as cumene hydroperoxide (259, 430) and the inorganic compounds NaIO and $NaClO_2$ (241) support monooxidation catalyzed

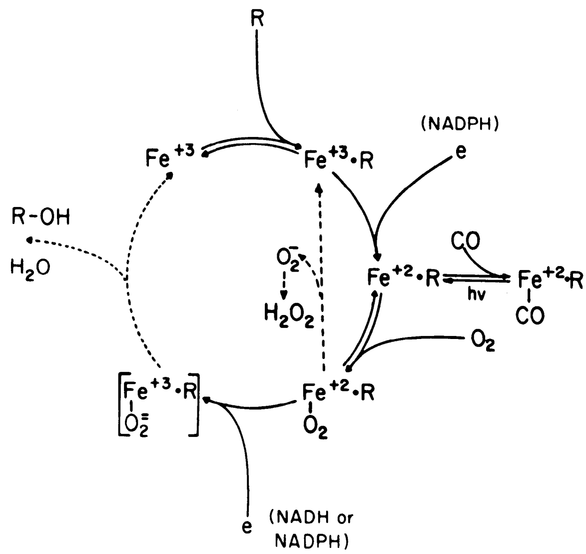

FIG. 7. Reaction mechanism of cytochrome P_{450}. [From Estabrook and Werringloer (160).]

by cytochrome P_{450} in the absence of oxygen, lending further support for the role of peroxidic intermediates in the catalytic mechanism. Hydroperoxide can be utilized as oxidant for alcohol in a reaction catalyzed by rat liver microsomes (431). Cumene hydroperoxide forms the peroxide intermediate of Figure 7 more effectively than does H_2O_2 (431).

C. Enzymes Utilizing Superoxide Anion

1. Superoxide dismutase

Superoxide anion dismutase [superoxide dismutase (SOD)] is a widely distributed enzyme that, unlike catalase, exists in a variety of forms (183, 186). This copper- and zinc-containing enzyme found in the cytosol of eukaryotic cells is identical to the long-recognized proteins termed erythrocupreins, hepatocupreins, cerebrocupreins, etc. Its enzymatic activity was discovered by McCord and Fridovich in 1969 (338). This form of the enzyme, also found in erythrocytes as well as in the mitochondrial intermembrane space, is sensitive to high concentrations of cyanide. Mitochondria also contain, in the matrix space, a distinctive cyanide-insensitive manganese enzyme similar to that found in prokaryotes (182). In addition, a ferrienzyme has been identified in bacteria (426, 559) that appears to be located at the periplasmic space (202).

The dismutation of superoxide anion leads to the formation of H_2O_2 and O_2 by a reaction that occurs spontaneously and is also catalyzed by superoxide dismutase:

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (19)$$

Thus, SOD and catalase share the property of a sequential pathway involving consecutive reactions in which two identical substrate molecules dismutate to higher and lower oxidation states. Superoxide anions dismutate nonenzymatically in a second-order reaction that is pH dependent due to the charged and uncharged forms of these radicals; the reaction has an observed half time of about 7 s at pH 9.5, as measured after pulse radiolysis at an O_2^- concentration of 1.5–2.0 $\times$ 10^{-7} M (280). These decay times decrease to approximately 0.5 ms in the presence of 3.5 $\times$ 10^{-7} M SOD (280) due to the high second-order velocity constant for the pH-independent SOD-catalyzed reaction (1.8–2.4 $\times$ 10^9 $M^{-1} \cdot s^{-1}$) (165, 280, 450), which is apparently diffusion limited (450).

Some data for the copper-zinc enzyme are given in Table 1. The mechanism of action of this enzyme involves successive reduction and oxidation of copper (165)

$$\text{E-}Cu^{2+} + O_2^- \rightarrow \text{E-}Cu^{1+} + O_2 \quad (20)$$

$$\text{E-}Cu^{1+} + O_2^- + 2H^+ \rightarrow \text{E-}Cu^{2+} + H_2O_2 \quad (21)$$

As shown above, the copper moiety is alternately reduced by one superoxide anion and oxidized by the next. These reactions may be compared with those of catalase shown in *Equations 1* and *2*, which are in the opposite sequence, i.e., the metal is alternately oxidized and reduced. Presumably, as an intermediate has been identified in the SOD reaction, it should follow that this reaction mechanism shares other characteristics with the catalase reaction mechanism: partial occupancy of the enzyme by the substrate, the lack of a Michaelis constant, and a linear increase of enzyme activity with increasing substrate concentration (450). The reaction mechanism may also resemble those of copper ions and Cu^{2+}-amino acid complexes (256, 427) in solution, which also can catalyze the dismutation reaction; however, the SOD reaction, with the protein-bound copper ions, is more effective by orders of magnitude. The back reaction (the reverse of that shown in *Eq. 21*) is observed at high concentrations (in the mM range) of H_2O_2 that inactivate SOD (69).

Superoxide dismutase activity appears to be present in those subcellular compartments where O_2^- formation may occur. Several groups of investigators (400, 407, 524, 543, 544) have shown that the greater part of the SOD activity in rat and chicken liver is in the cytosol, with between 15% and 20% in the particulate fraction. The particulate activity is associated with the mitochondria, with half of it confined to the matrix space and the other half between the inner and outer membranes. At variance with an earlier report (523), peroxisomes showed no SOD activity (407, 524).

a) Assay of superoxide dismutase activity. Assays for SOD activity are usually based on the inhibition of a reaction in which superoxide anion is a reactant. Xanthine oxidase is the most commonly used source of O_2^-

and the most widely used detection systems are adrenochrome formation and cytochrome *c* reduction (see sect. III*B*). Epinephrine oxidation at alkaline pH provides a system in which no supplemental source of O_2^- is needed. Superoxide dismutase may also be assayed by its ability to increase the rate of the riboflavin-sensitized photooxidation of *o*-dianisidine (362). In all cases, the expression of enzyme activity may be made in terms of "units" (one unit is the amount of enzyme that causes 50% inhibition of the detection reaction) or, more adequately, as equivalent to an absolute enzyme concentration by referring the determined inhibition to concentration-effect curves (176, 338). For further information on SOD assay methods, see Fridovich (183, 186).

III. METHODS FOR DETERMINING OXYGEN-REDUCTION PRODUCTS AND RELATED CHEMICAL SPECIES IN BIOLOGICAL SYSTEMS

The low physiological levels of H_2O_2 and O_2^- maintained by the enzyme systems that actively metabolize them, together with their rapid disappearance in anoxic tissue, render virtually impossible the direct measurement of the steady-state concentrations of these metabolites in biological systems, as indeed Wieland's negative results (550) testify. Although the presence of catalase, glutathione peroxidase, or superoxide dismutase prevents direct chemical analysis, these enzymes afford the direct and indirect chemical approaches described below.

A. Determination of Hydrogen Peroxide

The methods that follow may be considered quantitative, and some may be used intracellularly. Table 3 lists examples for applications in biological systems. They may be divided into those that employ direct spectroscopic measurement of the enzyme-substrate compounds of catalase and peroxidases and those that measure the appearance of the oxidation product or the consumption of the hydrogen donor for these enzyme systems. The almost irreversible reaction of enzyme and substrate, combined with the intense absorption bands of hemoproteins, makes the enzyme-substrate compound a uniquely sensitive detector of H_2O_2. However, methods that use the reaction product can be arranged so that the product accumulates over a period of time, thus amplifying the response and increasing the sensitivity.

1. Indication of intracellular hydrogen peroxide

Optical detection of redox states of electron carriers and of enzyme-substrate compounds is a direct consequence of the greatly increased sensitivity of optical measurements made possible by the double-beam or dual-wavelength spectrophotometric technique in which light-scattering changes are largely canceled (89).

TABLE 3. *Detection of formation of hydrogen peroxide in living cells and subcellular fractions*

Cell or Subcellular Fraction	Source	Detection by	Ref.
Bacteria	*Micrococcus lysodeikticus*	Catalase compound I	93
Worms	*Ascaris lumbricoides*	Manometry, polarography	73, 115
Blood cells	Leukocytes	Diacetyldichlorofluorescein, horseradish peroxidase	402
		Scopoletin, horseradish peroxidase	445
Liver	Liver slices and homogenates	[^{14}C]formate-$^{14}CO_2$	421
	Isolated perfused rat liver	Catalase compound I	392, 475, 476
	In situ organ, anesthetized rat	Catalase compound I	395
Mitochondria	Liver (rat)	Catalase compound I	66, 108
		Cytochrome *c* peroxidase	66
	Heart (pigeon)	Scopoletin, horseradish peroxidase	317
		Cytochrome *c* peroxidase	63
	Yeast (*S. cerevisiae*)	Scopoletin, horseradish peroxidase	158
		Cytochrome *c* peroxidase	59
	Protozoa		
	C. fasciculata	Cytochrome *c* peroxidase	293
	T. cruzi	Horseradish peroxidase	67
	Plant (*P. aureus*)	Cytochrome *c* peroxidase	442
Microsomes	Liver (rat)	Scopoletin, horseradish peroxidase	519
		Cytochrome *c* peroxidase	66, 519
Peroxisomes	Liver (rat)	Cytochrome *c* peroxidase	66
Cytosol	Liver (rat)	Cytochrome *c* peroxidase	66
Submitochondrial particles	Heart (beef)	Diacetyldichlorofluorescein, horseradish peroxidase	228
		Scopoletin, horseradish peroxidase	314, 315, 377
		Polarography	253
		Cytochrome *c* peroxidase	61, 62

[Modified from Sies (470), with additional references.]

a) Direct monitoring of catalase intermediate. The direct optical measurement of the steady-state concentration of catalase compound I in any given system, be it isolated catalase, catalase-containing organelles, perfused organs, or organs in situ, has both quantitative and qualitative advantages for indicating H_2O_2 concentrations and production rates. This method has been used extensively with perfused organs, in which the catalase intermediate is monitored, for example, through a liver lobe or through the cortical region of the kidney.

The obvious advantage of the method is its specificity, i.e., the compound being measured is in direct steady-state equilibrium with cellular H_2O_2, so that rapid changes in H_2O_2 generation rates, or indeed in hydrogen donor concentration, may be measured continuously. Furthermore, by plotting the profile of the intermediate concentration versus the concentration of added donor, a great deal of information on the total system can be obtained.

The data obtained in this way are at present our best estimates of the intracellular H_2O_2 concentration reacting directly with catalase. This is a highly relevant parameter, particularly when H_2O_2 is generated in the peroxisome. The question of whether the cellular H_2O_2 concentration is uniform or whether severe concentration gradients exist requires two H_2O_2-detecting systems in different parts of the cell, as provided by catalase and glutathione peroxidase (376).

The rationale of the method is based on the fact that heme occupancy of catalase, i.e., the total catalase heme present in the form of compound I, depends in a characteristic way on the rate of H_2O_2 formation ($d[H_2O_2]/dt$), the total catalase heme concentration ([cat]) and the hydrogen donor concentration ([A]). The hydrogen donor concentration required for half-maximal steady-state occupancy ($[A]_{1/2}$) is related simply to $d[H_2O_2]/dt$ and [cat] (see sect. II, *Eq. 9*). For the particular condition of A being methanol or ethanol and for the perfused rat liver

$$d[H_2O_2]/dt = [A]_{1/2}[cat] \cdot (32 \times 10^3 \ M^{-1} \cdot min^{-1}) \qquad (22)$$

$[A]_{1/2}$ is detected experimentally by titration of the catalase intermediate, with double-beam (dual-wavelength) spectrophotometry of suspensions of subcellular organelles, isolated cells, or intact organs. The total catalase heme concentration [cat] is readily determined by the conversion of catalase to the cyanide compound (85, 393). Figure 8 illustrates the method for the quantitative determination of the intracellular H_2O_2 generation rate. The methanol titration of the catalase intermediate gives the critical parameter, $[A]_{1/2}$. The addition of a series of methanol concentrations to the perfused liver, starting with a concentration high enough to diminish the level of the catalase intermediate nearly to zero and following with decreasing methanol concentrations (~2 mM gives 90% efficiency), causes successive increases of the absorbancy at 660 nm relative to 640 nm that represent increasing concentrations of the catalase intermediate. It can be readily determined from the chart or from the graph derived from it that 0.16 mM methanol, or a 45% peroxidatic reaction (see sect. II, *A1*), corresponds to the $[A]_{1/2}$ value. Complete conversion to catalase-H_2O_2 is assumed to be obtained on infusion of glycolate, giving a completely catalatic reaction with $F' = 0$. This value of $[A]_{1/2}$ corresponds to a catalase turnover of 5.1 min^{-1} and, with a normal catalase heme content of 20 nmol/g tissue, to a rate of 102 nmol H_2O_2/min per g of tissue, as shown in Figure 8*B* or calculated from *Equation 9* (109). An in situ control of the method is afforded by the balance between the oxidation rate of added urate and the extra H_2O_2 formation rate calculated with this method (Fig. 9).

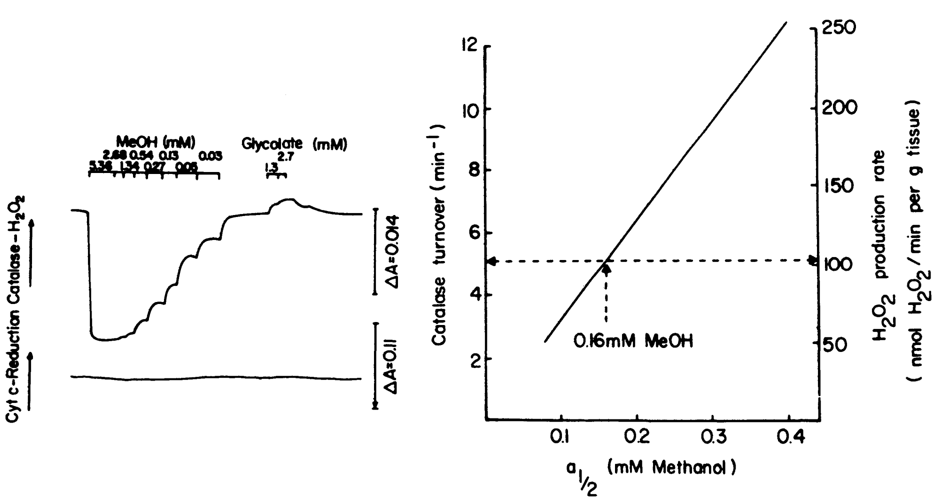

FIG. 8. Recording of catalase-H_2O_2 (compound I) from a lobe of perfused liver by dual-wavelength absorbance spectrophotometry at 640–660 nm. Trace represents methanol titration for determination of $A_{1/2}$ from intact organ (compare *Eq. 22*). [From Oshino et al. (393).]

b) Hydrogen donor oxidation. Measurement of substrate oxidation by the catalase-H_2O_2 intermediate provides a useful approach, particularly when catalase is the only enzyme dealing with the substrate and when endogenous substrates for catalase are at levels sufficiently low that they do not compete with the added substrate. Methanol and formate have been used, and as the concentration of these donors is increased the efficiency of the peroxidatic reaction approaches 100%. Thus, Mannering et al. (323, 513, 528) and Aebi et al. (4, 421) have measured the oxidation of [^{14}C]methanol and [^{14}C]formate, respectively, to form $^{14}CO_2$ in various tissues ranging from liver slices to intact animals. Extrapolation to 100% efficiency may be necessary but difficult to achieve in intact organs. An error is possible, however, since the 10-formyltetrahydrofolate dehydrogenase pathway, for example, also participates in the conversion of formate to CO_2 in intact cells (287) and organs (533a). Other donors of higher activity, such as propargyl alcohol (Table 2), may also be useful.

2. Measurement of hydrogen peroxide release

The methods described below measure the H_2O_2 released from the enzymes or subcellular organelles into the reaction medium, or from the tissue into the perfusate, and may underestimate the rate of H_2O_2 formation for two reasons: *a*) the presence of competing hydrogen donors in the sample or in the perfusion medium; *b*) the intervention of intracellular or intraorganelle enzymes such as catalase or glutathione peroxidase, which actively metabolize H_2O_2 and permit only a fraction to diffuse from the sample to the indicator. The methods de-

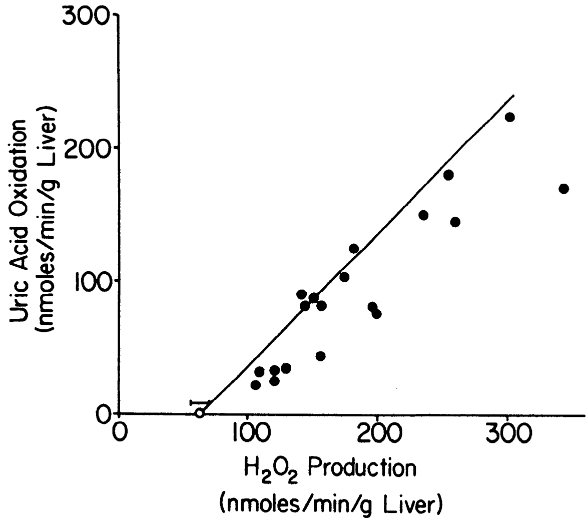

FIG. 9. Balance between rate of uric acid oxidation and extra H_2O_2 production rate calculated according to *Equation 22* in perfused rat liver. [From Oshino et al. (394).]

scribed here have been utilized mostly with subcellular fractions and are not appropriate to intact systems.

a) Cytochrome c peroxidase. Cytochrome *c* peroxidase reacts with H_2O_2 to form a stable enzyme-substrate compound (557, 558) that has an absorption maximum at 419 nm, whereas that of the free enzyme is at 407 nm ($E_{419-407}$ = 50 $mM^{-1} \cdot cm^{-1}$). The formation of the intermediate is studied by dual-wavelength spectrophotometry (66) at 419–407 nm or, for more sensitivity, at 424–400 nm (Fig. 10). Thus, H_2O_2 formation rates as low as 0.1 μM/min can be determined with accuracy because of the essentially irreversible reaction of enzyme and substrate ($K_D \sim 10^{-8}$ M). Cytochrome *c* peroxidase offers the advantages of stability of the peroxidase-H_2O_2 compound and high specificity for its hydrogen donor, reduced cytochrome *c*.

This method has been applied to the determination of H_2O_2 production in subcellular fractions of rat liver (66) and in various mitochondrial preparations (63, 65, 66, 78, 293, 442). Catalase present in the sample can be inhibited with azide without interference with the assay (66); alternatively, if enough catalase is present, its interference with the determination of H_2O_2 generation can be evaluated by measurement of the amount of catalase present (cf. 66, Appendix). In cases where the wavelengths 419–407 nm are inconvenient due to interfering pigments, the cytochrome *c* peroxidase reaction can also be used to measure H_2O_2 production in the presence of catalytic amounts of cytochrome *c* peroxidase and substrate amounts of reduced cytochrome *c*. Such samples must be free of cytochrome oxidase or cytochrome *c* reductase and indeed of O_2^-, which also reduces cytochrome *c*.

b) Horseradish peroxidase and coupled oxidation of hydrogen donors. Horseradish peroxidase oxidizes various hydrogen donors in the presence of H_2O_2. Photometric determination of the concentration of such hydrogen

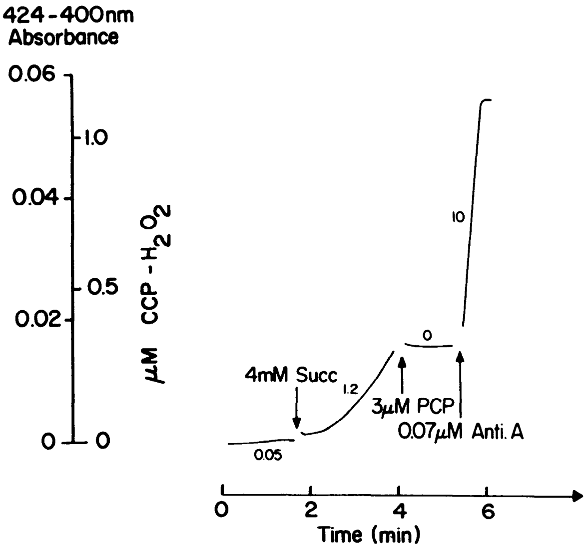

FIG. 10. Formation of H_2O_2 in pigeon heart mitochondria as detected by cytochrome *c* peroxidase method. [From Chance et al. (100).]

donors is widely used, e.g., in the clinical assay of glucose where the addition of glucose oxidase leads to the formation of a molar amount of H_2O_2 equivalent to the molar amount of glucose present (52). Many hydrogen donors have been used for measuring H_2O_2 formation in biological systems, e.g., benzidine, guaiacol, *o*-dianisidine, etc. (5, 20).

Fluorescent hydrogen donors or oxidation products are particularly suitable for such measurements in samples with low H_2O_2 formation rates. Scopoletin, which was introduced as a fluorescent hydrogen donor by Andreae in 1955 (16) and further studied by Perschke and Broda (408), is a coumarin derivative with excitation and emission maxima at about 360 and 450 nm, respectively; it loses its fluorescence when oxidized in the peroxidase reaction. The fluorometric method can be calibrated with appropriate systems such as glucose and glucose oxidase or urate and uricase, and rates of H_2O_2 formation in the range of 1 μM/min can be detected easily. This method has been applied to the measurement of H_2O_2 production in isolated mitochondrial and microsomal fractions (65, 314, 317, 519) and in leukocytes during phagocytosis (445). Diacetyldichlorofluorescin is oxidized by the peroxidase reaction to a fluorescent product (272); this assay has been applied to submitochondrial particles (228).

Formation of horseradish peroxidase compound II can also be studied as an indication of H_2O_2 generation (65, 66) with peroxidase preparations that are free of endogenous hydrogen donor. However, the broader specificity of the horseradish enzyme for various hydrogen donors, compared with cytochrome *c* peroxidase, may cause underestimation, and controls with cytochrome *c* peroxidase are advisable (65). Catalase interference may be evaluated by determining the peroxidase-to-catalase heme ratio (65).

c) Glutathione peroxidase and glutathione disulfide reductase. Release of H_2O_2 from biological samples can be coupled to NADPH oxidation through glutathione peroxidase and glutathione disulfide reductase. The assay can be used in the presence of cyanide (313), but in many preparations cyanide-insensitive NADPH oxidase activity may present experimental obstacles.

d) Oxygen formation by catalatic reaction. In catalase-free biological samples generating H_2O_2, the addition of catalase leads to the formation of oxygen by the catalatic reaction (see sect. II, *Eqs. 1* and *2*). This approach may be used with catalase-free subcellular fractions or tissues; in the latter case, it requires perfusion of the organ with catalase and the oxygen evolution may be measured polarographically in the effluent. The method is useful if sufficient H_2O_2 is being generated and in the presence of low concentrations of hydrogen donors; with high concentrations of hydrogen donors the technique is only semiquantitative because peroxidatic activity (see sect. II, *Eqs. 1* and *3*) leads to an underestimation of the H_2O_2 present. Because of these difficulties, methods employing peroxidases are preferable.

3. Other methods

The assays below do not provide adequately quantitative information, but in some cases they may be useful.

a) Myoglobin conversion. Myoglobin can be used as an indicator of cellular H_2O_2 generation by studying the conversion to the H_2O_2 compound "ferryl" myoglobin. This indication is rather slow because of the slow reaction of myoglobin with H_2O_2, and the steady-state accumulation of the ferryl compound may be slight due to the very active reductase present. However, Kaplan-Bresler (262) has used this approach to detect H_2O_2 in muscle tissue. The ferryl compound is slow to react with hydrogen donors, and therefore it is difficult to quantitate the H_2O_2 generation by titration.

b) Aminotriazole inhibition of catalase activity. Since compound I, rather than free catalase, reacts with 1,2,4-aminotriazole to be converted to an inactive alkylated derivative (217, 325), this inhibition depends on the presence of intracellular H_2O_2 and therefore may be used for assaying H_2O_2 generation (217, 325).

B. Determination of Superoxide Anion

The steady-state concentration of O_2^- can be measured directly by ultraviolet absorption (165, 280, 450) or by integration of the spectrum obtained by electron paramagnetic resonance (EPR) spectroscopy of frozen samples (31, 165). However, these assays can scarcely be employed with biological systems because of the low steady-state concentrations of O_2^- in the presence of even minimal amounts of SOD. The spontaneous decay of O_2^- also contributes to the failure of direct physical methods for its detection. Unfortunately, there is no available assay for O_2^- based on a spectroscopically detectable intermediate of O_2^- with any of the dismutases, similar to the assay for H_2O_2 with the catalase intermediate in cells and tissues; it is necessary to fall back on assays of the rate of O_2^- generation either by detection of O_2^- itself or by detection of H_2O_2 as the product of the dismutation reaction.

The chemical methods integratively measure the rates of O_2^- formation by trapping it with suitable spectrophotometric indicators in reactions that are sensitive to SOD addition. The concentration of the trapping agent is adjusted to compete effectively with the dismutation reaction so that nearly all the O_2^- can be detected.

The reduction of cytochrome *c*, which was used in the discovery of SOD activity (338), is most often employed. Superoxide anions actively reduce cytochrome *c*; some details of the process are known (77, 283, 465), as well as the second-order velocity constant of the reaction (1.1×10^5 $M^{-1} \cdot s^{-1}$) at pH 8.5 (297). The use of acetylated cytochrome *c* (23) has the advantage of being applicable to samples having cytochrome *c* reductase and cytochrome oxidase activities. The sensitivity of the assay is reasonably good, allowing detection of O_2^- at concentrations of 10^{-7}–10^{-8} M.

The irreversible oxidation of epinephrine to adrenochrome, which is conveniently monitored spectrophotometrically at 480 nm, or better at 480–575 nm by double-beam spectrophotometry, provides a sensitive assay for O_2^- at concentrations in the range of 10^{-7} M (78, 360). However, the specificity of the reaction appears to be rather poor. Formation of the rather stable semiquinone of another catechol compound, Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid), by O_2^- allowed kinetic studies by EPR (353a, 353b). The reduction of nitroblue tetrazolium allows the determination of O_2^- levels of 10^{-8} M due to the large absorption change (E_{490} = 100 $mM^{-1} \cdot cm^{-1}$), but the assay appears to be somewhat unspecific, since only partial inhibition by superoxide dismutase has been noted (13, 47, 357, 552). Other methods monitor nitroformate accumulation (140, 428), dichlorophenol reduction (177), nitrite formation (156), etc.

The chemiluminescence from the reactions of oxidized luminol (197, 233, 316) or luciferin (221, 222) with superoxide anions, which can be measured in a scintillation counter, provides highly sensitive assays. It has been claimed that the luminol assay is reliable at O_2^- concentrations below 10^{-10} M (426) and that luciferin is even more sensitive than luminol (221). Concerning luminol at least, the specificity of the reaction is rather poor, and the involvement of $HO\cdot$ radicals is likely (44, 233).

C. Detection of Hydroxyl Radical

Gas chromatographic analysis of the generation of ethylene from methionine as an assay for hydroxyl radicals (48, 57) is doubtful due to the unspecific fragmentation of the thioether (424a). Alternatively, the use of methionine in the presence of pyridoxal phosphate has been proposed (280a). The sensitive bleaching of *p*-nitrosodimethylaniline (*p*-NDA), with ϵ_{440} = 34 $mM^{-1} \cdot cm^{-1}$ (45, 286), can be used only in aqueous systems since the nitroso compound binds covalently to lipids (173a). Optimal systems may well be the spin trapping of hydroxyl radicals by nitroso compounds in combination with EPR detection (252a, 296a) or identification of hydroxylation products (78a, 207a).

D. Determination of Lipid and Organic Peroxides

The total steady-state concentrations of lipid and other organic peroxides can be evaluated after extraction with chloroform-methanol or *t*-butanol and subsequent analysis of the iodine formation from a KI solution in acidic medium (353). The oxidation of leucodyes (236) or of Fe^{2+} (350) may also provide a suitable assay for extracted peroxides. Malonaldehyde is formed in extensive lipid peroxidation after rupture of the carbon chain of unsaturated fatty acids, especially linolenic acid; it usually is determined colorimetrically by the thiobarbiturate reaction (53, 398). Malonaldehyde is not the major degradation product, accounting for only about 10% of the total oxygen uptake by the lipid (232); however, this relatively simple and sensitive method for determining a minor by-product has provided a major analytical tool for the study of lipid peroxidation in biological systems through the last three decades. A comprehensive review of the method, its applications, and its limitations has been given by Barber and Bernheim (32). Lipid peroxides can also be measured by the formation of conjugated diene hydroperoxides that show an increased absorption at 230–235 nm (331). In the presence of proteins having free amino groups, lipid peroxides form fluorescent products of the Schiff-base type after prolonged incubation (510).

The glutathione peroxidase turnover, which can be estimated from the efflux of glutathione disulfide from the cell (477, 478, 484), might be developed into a sensitive assay for lipid peroxide production in biological systems such as intact cells and perfused organs (74, 391). The specificity of glutathione peroxidase allows this assay to extend from lipid peroxides (119) to other hydroperoxides such as those of nucleic acids (120) and steroids (311); on the other hand, since H_2O_2 is also a substrate for glutathione peroxidase, H_2O_2 generation must also be determined in this assay. In order to establish the relative contributions of lipid and hydrogen peroxides in the turnover of glutathione peroxidase, an independent measurement of H_2O_2 generation with the catalase intermediate must be made (376).

The rate of generation of the saturated hydrocarbons ethane, propane, and pentane evolved on lipid peroxidation (148, 281, 310, 443) that can be measured in the expired air may provide adequately quantitative data on the rates of lipid peroxidation occurring in the intact animal under physiological conditions.

E. Detection of Singlet Molecular Oxygen

The difficulty in identifying singlet oxygen ($^1O_2$), the electronic excited state of oxygen, in biological systems is due chiefly to the quenching effect of water; $^1O_2$ in aqueous solution decays to the triplet ground state with a half-life of 2 μs (15, 175, 192, 290, 375). The observation that D_2O increases the lifetime of singlet oxygen by a factor of 10 (375) suggests better assay conditions. Singlet oxygen is also quenched by such specific substances as carotenoids (14) and by diazabicyclooctane (175). Various furan derivatives scavenge singlet oxygen (270, 276, 419). The chromatographic determination of the reaction product (270, 399, 419, 541) is a sensitive assay procedure. When singlet oxygen decays to the ground state in aqueous solution, photoemission occurs at 634, 703, 1,070, and 1,269 nm (273, 274, 466) with a very weak band at 578 nm (466). Light emission at the 578- to 580-nm band is favored in the presence of lipids, such as in microsomal preparations and liposomes (368, 501). The very weak light emission observed from organs in situ may be due to the production of singlet oxygen in vivo (511, 512), but further studies are needed (162a).

IV. CELLULAR SOURCES OF HYDROGEN PEROXIDE

A number of enzymes of rat liver catalyze univalent or divalent reduction of oxygen; they are listed in Table 4 and their subcellular location is indicated. Similar lists have been compiled by others (58, 150, 219), but a complete and detailed identification of enzymes and substrates that form H_2O_2 is not yet possible. However, the maximal relative contribution from the various sources can be estimated from the rates of H_2O_2 formation by isolated subcellular fractions. Mitochondria, microsomes, peroxisomes, and cytosolic enzymes have all been recognized as effective H_2O_2 generators, contributing in rat liver, respectively, 15%, 45%, 35%, and 5% to the cytosolic H_2O_2 level at a Po_2 of 158 mmHg when fully supplemented by their substrates (66).

TABLE 4. *Liver enzymes producing hydrogen peroxide or superoxide anion and their subcellular localization*

EC Number	Enzyme (Trivial Name)	Localization
1.1.3.1	Glycolate oxidase	Peroxisome
1.1.3α	L-α-Hydroxyacid 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

[From Sies (470).]

A. Subcellular Fractions

1. Mitochondria

Mitochondrial membranes were recognized as a possible physiological source of H_2O_2 when submitochondrial particles obtained by sonication or alkaline treatment showed the production of minute amounts of H_2O_2 (228, 253). Further studies with the peroxisomal-mitochondrial fraction of rat liver showed that mitochondrial substrates and inhibitors were effective modulators of the level of the peroxisomal catalase intermediate (108). However, the cytochrome *c* peroxidase and horseradish peroxidase-scopoletin assays for H_2O_2 were required in order to elucidate the properties of mitochondrial H_2O_2 generation.

Isolated mitochondria produce H_2O_2 at rates that depend primarily on their metabolic state. Under physiological conditions, H_2O_2 generation is greatest in the controlled state 4 (63, 317), characterized by a high degree of reduction of the respiratory carriers; here, the respiratory rate is determined by the availability of the phosphate acceptor, ADP (114). In the presence of either NAD-linked substrates or succinate, state 4 mitochondria from rat liver or from rat or pigeon heart generate about 0.3–0.6 nmol H_2O_2/min per mg of protein (63, 66, 377). This H_2O_2 generation represents approximately 2% of the total oxygen utilization under these conditions (100). Formation of H_2O_2 in isolated mitochondria is increased severalfold in the presence of antimycin A, in which case it accounts for most of the oxygen consumption (63, 100, 317); it is inhibited by rotenone when supported by NAD-linked substrates (63, 66) and is increased under alkaline conditions (63, 317). These findings apply to protozoan and to plant mitochondria as well (67, 293, 442).

Surprisingly, no H_2O_2 (or O_2^-) production or catalase activity was reported for brain mitochondria (492). However, a subsequent report identified both O_2^- production and SOD activity in brain mitochondria (179). The flavoprotein dihydroorotate dehydrogenase is also a source of O_2^- in rat liver mitochondria (177, 178).

Submitochondrial particles from rat and beef heart are effective sources of H_2O_2 (61, 228, 314–316, 377). These particles are devoid of auxiliary dehydrogenases, such as the flavoproteins of fatty acid oxidation, so that H_2O_2 formation can be directly related to the components of the respiratory chain. Since the transition from two-electron to one-electron transfer takes place in the succinate dehydrogenase-cytochrome *b* segment of the respiratory chain, the formation of O_2^- as a primary product of oxygen reduction at this site has been investigated. In the presence of succinate and antimycin A, submitochondrial particles washed extensively to remove SOD generate from 4 to 7 nmol O_2^-/min per mg of protein, giving O_2^-/H_2O_2 ratios of 1.5–2.1 (60–62, 149, 316) and indicating that O_2^- is indeed an almost stoichiometric precursor of mitochondrial H_2O_2. Accordingly, mitochondrial membranes must produce about 24 nmol O_2^-/min per g of tissue in the liver to account for the measured H_2O_2 production (66) and are apparently one of the main physiological sources of superoxide anions. Most of these superoxide anions are readily converted to H_2O_2 by SOD, maintaining an estimated intramitochondrial steady-state O_2^- concentration of 8×10^{-12} M or greater (524).

In order to identify the source of reducing equivalents for the generation of superoxide anions, succinate dehydrogenase has been detached by alkaline treatment; the depleted membranes generated H_2O_2, thus ruling out a major role for the succinate dehydrogenase flavoprotein in H_2O_2 production (62, 315). On the other hand, acetone extraction and supplementation with exogenous ubiquinones gave preparations in which the rate of H_2O_2 production was linearly related to the amount of reducible quinone in the membranes (62, 63). Both NADH-ubiquinone reductase and ubiquinol-cytochrome *c* reductase, which have ubiquinone as the main common component, generate O_2^- and H_2O_2; this activity is rotenone-sensitive in the NADH-ubiquinone reductase (78). It has been proposed that ubisemiquinone and ubiquinol are chiefly responsible for mitochondrial peroxide generation in a nonenzymatic reaction (60, 62, 78). The energy-conserving reactions involving cytochrome b_{566} have been postulated to be involved in H_2O_2 formation either directly or indirectly (314, 315), but seem less important than the reduced forms of ubiquinone.

2. *Peroxisomes*

Peroxisomes of liver and other organs contain a number of H_2O_2-generating enzymes, including some flavoproteins, D-amino-acid oxidase, L-α-hydroxyacid oxidase, fatty acyl-CoA oxidase, and, in nonprimates, the copper enzyme urate oxidase. Table 5 gives some properties of these enzymes and a more comprehensive list is given in the recent review by Masters and Holmes (327).

The recent observation by Lazarow and de Duve (305) of a palmitoyl-CoA–dependent formation of H_2O_2 in isolated peroxisomal fractions of rat liver

TABLE 5. *Properties of peroxisomal oxidases of rat liver*

Enzyme	Activity Content at 37°C, μmol/min per g liver	Peroxisomal Protein, %	K_m	Rate in O_2/ Rate in Air
Urate oxidase	3.1	10 (core)	Urate, 0.02 mM	1.8
L-α-hydroxyacid oxidase	1.1	3	Glycolate, 0.25 − 5 mM $K_m(O_2)$ = 0.4 mM L-α-OH-isocaproate, 3.4 mM	
D-Amino-acid oxidase	1.4	2	D-norvaline, 0.06 mM D-alanine, 1.8 mM (kidney enzyme)	2.4

[Modified from Sies (471); original data from additional references (151, 292, 305–307, 321, 340, 394).]

adds fatty acids to the list of important potential substrates in peroxisomes. The rate of H_2O_2 formation was similar to that of urate oxidation, 3.5 μmol/min per g of liver at 37°C. However, clofibrate and other hypolipodemic drugs that increase the number of hepatic peroxisomes cause an 11- to 18-fold increase in the rate of palmitoyl-CoA oxidation by liver homogenates (302).

Earlier reports of H_2O_2 formation on addition of octanoate to the perfusate (393) as shown in Table 6 may suggest that H_2O_2 generation also occurs with fatty acids of medium chain length in the intact organ. The principal peroxisomal substrate in the rat liver, uric acid, is continuously formed in the purine degradation pathway; its concentration in rat liver cytosol is 70–100 μM (66, 154). In isolated peroxisomal preparations, catalase destroys most of the H_2O_2 formed by urate oxidase within the organelle, but between 11% and 42% of the H_2O_2 diffuses to the external medium (66). A theoretical consideration of the properties of intact peroxisomes sets the diffused H_2O_2 at 2% of that generated within the organelle (417). The discrepancy between the observed and calculated rates of H_2O_2 release may reflect the degree of damage suffered by the peroxisome during isolation. There has been no report of O_2^- formation in peroxisomal H_2O_2 generation.

3. Endoplasmic reticulum

The membranes of the endoplasmic reticulum were early associated with H_2O_2 generation by Gillette et al. (191). Microsomes produce H_2O_2 when supplemented with NADH or, more actively, with NADPH, where rates of 6–15 nmol H_2O_2/min per mg of protein in control and phenobarbital-treated rats, respectively, are reported (66, 227, 519). This is a factor of considerable im-

TABLE 6. *Rates of hydrogen peroxide production in isolated hemoglobin-free perfused rat liver from measurement of catalase heme occupancy*

Substrates or Inhibitors	$A_{1/2}$ for Methanol, mM	$(dx_n/dt)\cdot(1/e)$, turnover number/min	dx_n/dt, nmol H_2O_2/min per g liver wet wt
Lactate, 2 mM; pyruvate, 0.3 mM	0.12 (0.09–0.16)	3.8	49
+ Antimycin A, 8 μM	0.18	5.8	75
+ Octanoate, 0.3 mM	0.40	13.0	170
+ Octanoate, 0.03 mM; antimycin A, 8 μM	0.24	7.4	96
+ Oleate, 0.1 mM	0.16	5.1	66
+ Xylitol, 5.2 mM	0.15	4.8	62
+ Urate, 1 mM		54*	750
+ Glycolate, 3 mM		34*	490

* Titration performed with 0.6 mM methanol initially present. [From Oshino et al. (393).]

portance in evaluating the ethanol-oxidizing capabilities of isolated catalase-containing microsomal fractions (see sect. VIII*B*). However, so far there is little conclusive evidence of H_2O_2 formation by the endoplasmic reticulum in intact cells (394, 474).

The autoxidation of cytochrome P_{450} provides O_2^-, and recent reports indicate that isolated microsomes produce considerable amounts of O_2^-, with rates of the order of 2–10 nmol/min per mg of protein (38, 160, 356). The flavoprotein-NADPH-cytochrome *c* reductase system and cytochrome P_{450} are the most likely sources of H_2O_2 and O_2^- in these membranes (227, 379, 489).

4. *Cytosolic enzymes*

Cytosolic enzymes such as xanthine oxidase and aldehyde oxidase may contribute to the cellular production of H_2O_2. Although quantitation of their activity in the supernatant after cell fractionation sets their relative contribution at about 5% of the cellular H_2O_2 production (66), it is difficult to evaluate their activity under physiological conditions. The contribution of xanthine oxidase is verified by the observed accumulation of its product, uric acid, in homogenates after separation of the uricase-containing peroxisomes (66). The H_2O_2 production supported by endogenous substrate in perfused rat liver, compared with liver in situ (Table 7), suggests that extrahepatic substrates contribute to H_2O_2 production in the liver.

Xanthine oxidase and aldehyde oxidase produce a minor fraction of cellular O_2^-; moreover, xanthine oxidase activity is only partially expressed as O_2^- formation (185).

5. *Nucleus*

The observation of respiratory activity in isolated nuclei is at variance with the recognized biological function of the nucleus. However, consistent reports seem to indicate that, at least in calf and rat thymus, nuclei can catalyze a slow

TABLE 7. *Rates of hydrogen peroxide production in the rat in vivo*

Conditions	Assay Method	H_2O_2 Production, μmol/min per 100 g body wt	Ref.
Anesthetized, air	Catalase compound I	1.45*	395
Anesthetized, glycolate, 100% O_2	Catalase compound I	5.32*	395
Germfree rat	[^{14}C]formate-$^{14}CO_2$	1.88	30
After contamination with intestinal flora	[^{14}C]formate-$^{14}CO_2$	2.27	30
Intact rat	[^{14}C]methanol-$^{14}CO_2$	1.24†	323

* H_2O_2 production in liver only. † Calculated from V_{max} of methanol oxidation; however, recovery of H_2O_2 in methanol oxidation seems not to be 100%.

energy-yielding respiratory activity (134). The nuclear fraction isolated from rat liver (133) and ascites tumor cells (36) seems to contain flavins and cytochromes. In ascites tumor cell nuclei, where a microsomal type of electron transfer takes place (36), respiratory activity is associated with the production of O_2^-, at a rate of 3.2 nmol O_2^-/min per mg of protein (37), and H_2O_2. Superoxide dismutase activity has also been detected (37).

6. Nonenzymatic sources of hydrogen peroxide

Autoxidation of thiols (358) or other soluble reduced cell constituents are of unknown importance under physiological conditions.

B. Hydrogen Peroxide Production at Different Oxygen Concentrations

1. Hydrogen peroxide in normoxia

The rates of H_2O_2 production observed in normoxia in isolated organelles, perfused liver, and liver in situ in anesthetized rats are in agreement. The measured rate of 380 nmol H_2O_2/min per g of liver in the normal anesthetized rat (395) corresponds approximately with the rate of 160 nmol/min per g of liver at 22°C calculated from the isolated subcellular fractions fully supplemented with their substrates (66). Production of H_2O_2 in perfused liver is only a fraction of that in the liver in situ, about 50–80 nmol/min per g of liver (Tables 6 and 8), possibly indicating the depletion of the perfused organ due to lack of both hepatic substrates and hormonal stimuli. In the perfused liver, H_2O_2 production

TABLE 8. *Production of hydrogen peroxide in rat liver under normal and hyperoxic conditions*

Substrate and Oxygen	H_2O_2 Production, nmol/min per g of liver wet wt		
	Subcellular fractions	Perfused liver	Anesthetized rat
Endogenous			
+ 0.2 atm O_2	160	82[a]	380[b]
+ 1 atm O_2	200	102[a]	
+ 6 atm O_2	260	102[a,c]	340
2 mM glycolate			
+ 0.2 atm O_2	600		760
+ 1 atm O_2	860	740–1,070[d]	1,330
+ 6 atm O_2	1280	820[c]–1,420[d]	1,180

Assay methods were: direct H_2O_2 assay for subcellular fractions and methanol oxidation via catalase-H_2O_2 for perfused liver and anesthetized rat. [a] Perfused with 1 mM lactate, 0.15 mM pyruvate. [b] Tissue Po_2 = 50 mmHg. [c] 5 atm O_2. [d] Perfused with a mixture of urate, glycolate, octanoate, lactate, and pyruvate. [From Boveris and Chance (64), Boveris et al. (66), and Oshino et al. (394, 395).]

can be stimulated up to 15-fold depending on the substrate added; e.g., urate addition (Table 6) causes generation of 750 nmol H_2O_2/min per g of liver at 30°C. Under such conditions, as much as half the oxygen utilized in the liver may be converted to H_2O_2 and used in alcohol oxidation, compared with the small fraction in the absence of added substrates.

Production of H_2O_2 in the intact animal, measured by the rate of [^{14}C]formate oxidation (323, 421), is of the same order of magnitude as that in the liver in situ. Table 7 summarizes the results obtained with labeled hydrogen donors and direct spectroscopy of the catalase-H_2O_2 intermediate. Although absorption spectrophotometry is more difficult in the blood-perfused liver, the value of 1,450 nmol H_2O_2/min per 100-g rat would indicate that about 75% of the total H_2O_2 generated by the animal may be attributed to the liver (395).

In summary, a considerable fraction of the oxygen consumed in the liver is converted to H_2O_2. In the perfused liver, the value of this fraction ranges between 5 and 50% depending on the supply of peroxisomal substrates (393, 477) and in the liver in situ it accounts for about 10–15% of the total oxygen uptake (395).

2. *Hydrogen peroxide production in hypoxia*

Since the oxygen affinity of cytochrome oxidase [$k_{0.5}(O_2) = 0.01-1\ \mu M$] is much greater than those of the isolated microsomal and peroxisomal oxidases, the flux through the latter might be expected to be more sensitive to hypoxia than the flux through cytochrome oxidase. However, the activities of all three systems in the perfused liver are diminished to roughly the same extent in hypoxia (394, 471, 472). With glycolate as substrate, H_2O_2 generation in the perfused liver is halved at an intracellular Po_2 that caused a 10% increase in the level of reduction of cytochrome *c*; the $k_{0.5}$ value of glycolate oxidase for oxygen has been reported to be 0.4 mM (394), whereas that of cytochrome oxidase is less than 0.1 μM. With lactate-pyruvate as substrate, the half-maximal rate of H_2O_2 formation was observed at an intracellular Po_2 that caused nearly the same (40%) increase in cytochrome *c* reduction (394, 472). Similarly, removal of urate from the perfusate, indicating uricase activity, exhibits an oxygen dependency similar to that of cytochrome oxidase during stationary states of hypoxia (Fig. 11).

The dichotomy of the in vitro and in vivo results can be explained by the steepness of the oxygen gradient that may cause enzymes of vastly different oxygen affinities to appear to have the same oxygen affinity. The oxygen gradient in the intercapillary space of the Krogh tissue cylinder is so steep that there is either sufficient oxygen for the operation of most of the enzymes or insufficient oxygen for the operation of any of them. The existence of intra- or intercellular oxygen gradients may similarly explain the experimental observations. In isolated hepatocytes, where intercellular oxygen gradients are diminished, the rate of urate removal is substantially diminished during a slight

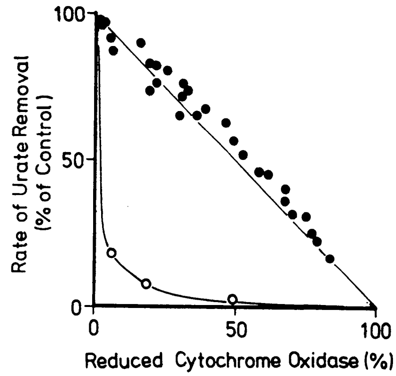

FIG. 11. Relationship between rate of urate removal and degree of reduction of cytochrome oxidase in isolated hepatocytes (○) and in perfused liver (●). Data are interpreted to indicate steep O_2 gradients during hypoxia in perfused liver. [Sies (471, 472).]

hypoxia that leads to only a small reduction of cytochrome oxidase (Fig. 11), a result that supports the possibility of marked oxygen gradients within the liver sinusoid along the lobule from the periportal to the pericentral region (471, 472). Similar results have been obtained in rat heart, with oxymyoglobin and cytochrome *c* used as indicators (97, 509). Thus the steepness of tissue oxygen gradients causes oxidases of dissimilar K_m values to behave as if their K_m values were identical.

3. Hydrogen peroxide production in hyperoxia

Table 8 shows rates of H_2O_2 formation in various rat liver preparations under normal and hyperoxic conditions. Isolated pigeon heart and rat liver mitochondria show a marked increase in H_2O_2 generation when exposed to oxygen under high pressure (63, 64), in agreement with the almost linear dependence of mitochondrial O_2^- production on oxygen tension (60). Moderate increases of H_2O_2 production in hyperoxia are also measured in the peroxisomal fraction of rat liver in the presence of endogenous substrate or when supplemented with glycolate (64). On the other hand, microsomal H_2O_2 formation is not affected by hyperoxia. Pure oxygen and hyperbaric oxygen enhance H_2O_2 generation at the subcellular level and in isolated liver cells from 60% to 200% (98). The effect is less marked, about 15% to 40%, in the perfused liver of normal and tocopherol-deficient rats (376).

The hyperbaric response appears to be greatly diminished at the organ level in vivo (395). The tissue oxygen level may be limited by the microvascular response or the endogenous rate of H_2O_2 generation may be substrate limited. The rate of H_2O_2 production in the liver in situ was not increased by hyperbaric oxygenation (395). When the peroxisomal fraction, the perfused liver, or the liver in situ is supplemented with glycolate, there is a large increase in H_2O_2 formation under hyperbaric oxygenation (Table 8) and the majority of liver respiration leads to H_2O_2 formation.

V. INTRACELLULAR REGULATION OF OXYGEN-REDUCTION PRODUCTS

A. Steady-State Intracellular Concentrations of Hydrogen Peroxide and Superoxide Anion

Although present in low concentrations, hydrogen peroxide and superoxide anion are normal metabolites in the aerobic cell. A remarkable multiplicity of cellular sources is found (see Fig. 1). The level of superoxide anion, the more reactive species, is maintained at 10^{-12}–10^{-11} M by SOD (524), whereas the level of hydrogen peroxide, which is less reactive, is regulated at concentrations up to 3 orders of magnitude greater, 10^{-9}–10^{-7} M, depending on the H_2O_2 production (393). Since these values have been calculated for H_2O_2 in equilibrium with peroxisomal catalase, they may slightly overestimate the actual concentration in the presence of glutathione peroxidase. The panoply of cellular sources for H_2O_2 is modulated by a series of controls, both physiological and biochemical, such as the transition from the resting state 4 to the active state 3 and the supply of oxygen or substrate.

There has been much discussion as to whether catalase or glutathione peroxidase is the predominant enzyme in regulating intracellular H_2O_2 levels. Both fulfill important metabolic functions in controlling H_2O_2 concentrations at different levels and in different parts of the cell. Catalase is especially effective as a "safety valve" for dealing with the large amounts of H_2O_2 that may be generated in the peroxisomes. Glutathione peroxidase is capable not only of utilizing hydroperoxides but also of metabolizing H_2O_2 in both the cytosolic and mitochondrial compartments. In fact, no enzymatic pathway is used completely to the exclusion of the other in metabolizing H_2O_2. In rat liver, with a GSH concentration of about 10 mM the relative reaction rates for catalase and glutathione peroxidase depend on the localized concentrations of both enzymes and of H_2O_2 within the cell. The compartmentation of catalase in the peroxisomes and of glutathione peroxidase in the cytosol and the mitochondria facilitates their effective collaboration in H_2O_2 metabolism, each enzyme being chiefly responsible for the decomposition of H_2O_2 generated at the intracellular site at which the enzyme is located. However, it has been shown that addition of the peroxisomal substrate glycolate to perfused liver activates glutathione peroxidase (391, 479). This response could indicate the presence of cytosolic glycolate oxidase or the existence of at least minimal intracellular H_2O_2 gradients from the peroxisome to the cytosol.

The steady-state levels of H_2O_2 in the cytosol may allow a portion to diffuse out of the cell into the interstitial fluid and subsequently to blood catalase. Such diffusion provides an efficient defense mechanism for those organs low in catalase, such as brain, lung, and heart (384, 515), or those lacking effective concentrations of glutathione peroxidase, such as muscle (355). Biological membranes are highly permeable to H_2O_2; the permeability constants of 0.2 cm/min for peroxisomal membranes (143) and 0.04 cm/min for erythrocyte plasma membranes (371) may be compared with those for water in a wide variety of cell membranes, which range from 0.02 to 0.42 cm/min (147). The H_2O_2 permeability of the erythrocyte membrane is higher than that for most nonelectrolytes; it is close to that for methanol and lower than the oxygen permeability (166, 371).

Superoxide anion may be generated by flavin enzymes and iron-sulfur proteins (Table 3; 186, 219, 326, 359). The rates of O_2^- generation in subcellular fractions suggest that cytosolic enzymes such as xanthine oxidase and aldehyde oxidase are minor contributors to the total cellular O_2^- production. Conversely, the rates of O_2^- generation in the mitochondrial and microsomal fractions indicate that the membrane-bound multienzyme redox systems are the chief sources of O_2^- in the cell.

B. Levels of Enzyme Activity

For all three enzymes—catalase, glutathione peroxidase, and superoxide dismutase—different factors influencing the level of enzyme activity have been recognized. Although the mechanisms underlying the control of enzyme synthesis and the regulation of steady-state levels are not completely understood at present, the presence of the appropriate substrate usually is regarded to be the inducer of increased enzyme synthesis. However, complex metabolic pathways make the causes of enzyme induction difficult to assign. Thus the following discussion may be considered a report of a variety of observations on these three enzymes.

1. Catalase

a) Bacteria. Anaerobic bacteria show very low or no catalase activity, whereas aerobic bacteria have significant catalase levels (80, 339). The ability of the aerobic microorganisms to survive when exposed to air or H_2O_2 is related to their catalase activity (342, 343). Some facultative anaerobes, such as the eukaryote *Saccharomyces cerevisiae* (201) and the *Escherichia coli* K-12 his$^-$ (200), show higher catalase levels when grown under aerobic conditions. On the other hand, *E. coli* B and *Streptococcus faecalis* show similar catalase concentrations whether grown under aerobic or anaerobic conditions, but they do show different SOD levels (199). Catalase synthesis is induced in anaerobically grown *Rhodopseudomonas spheroides* on contact with air and also after addition of H_2O_2 (124, 125), a substrate that appears to induce its enzyme (125). Adaptation studies in bacteria should cover the three related enzymes, catalase, glutathione peroxidase, and SOD, in order to achieve full physiological meaning.

b) Peroxisomes. The content of catalase and other enzymes in the peroxisomes of the liver cell depends on numerous hormonal, nutritional, and pharmacological influences (239, 502, 503); nevertheless, the coordination of the enzyme assembly may occur in the peroxisome itself (145, 303, 304). A number of hypolipidemic agents, e.g., clofibrate (4-ethyl-2-*p*-chlorophenoxyisobutyrate), increase the percentage of peroxisome volume in the liver cell from about 2% to about 17% (302, 434, 502). The number of peroxisomes (502) as well as the amount of antigen reacting with anticatalase serum (434) both increase; i.e., the total amount of catalase protein is increased. An increase in the number of peroxisomes is also obtained when catalase synthesis is inhibited by allylisopropylacetamide (433). However, the activity of some H_2O_2-forming oxidases is unchanged after clofibrate treatment (21). Thus, a complex and as yet unresolved array of factors appears to participate in the regulation of the steady-state enzyme content of the peroxisomes. Recently, proliferation of a peroxisome-associated polypeptide in rat liver has been reported (435).

Biogenesis of the peroxisome has been studied in considerable detail by de Duve's group (144, 303, 304, 383, 418), but the mechanism is not yet fully elucidated. The peroxisome in toto is subject to a relatively rapid turnover; its measured half-life is approximately 1.5–2 days (418, 422). Labeling studies support the hypothesis that catalase is formed within the peroxisome from extraperoxisomally synthesized precursors (144, 303, 304). The apomonomer for catalase, with a half-life of 14 min, is transferred into the peroxisomes in an unknown manner and receives its heme group there. The apomonomer and the monomer account for 1.6% and 0.5% of the total catalase, respectively (303, 304). There have been conflicting reports on the existence of intact extraperoxisomal catalase (226, 235, 264, 438, 454), due to the fragility of the peroxisomes. In species other than the rat, considerable catalase activity has been reported in the soluble cytoplasmic space of the liver (235).

The "peroxisome concept" of de Duve and Baudhuin (145), implying the close association of product-specific oxidases with catalase in these organelles, has been treated in detail in a number of reviews (42, 145, 239, 327, 470). In recent years, peroxisomelike structures have been demonstrated in almost every type of mammalian cell in which they have been sought. The analytical tool for such histochemical determinations is the oxidation of diaminobenzidine (DAB). The peroxisome concept is based on studies of rat liver and kidney, where the microbodies are about 0.5–1.0 μm in diameter. Rat liver contains about 400 peroxisomes (Fig. 12*A*) per hepatocyte, with a slight preponderance in the centrilobular as contrasted with the periportal regions of the lobule. In 1972 Novikoff and Novikoff (384) described another category of DAB-oxidizing particles, terming them "microperoxisomes" (Fig. 12*B*) because of their smaller diameter (0.2–0.3 μm). Although the identity of catalase with the DAB-oxidizing activity of these particles has not yet been completely established, several lines of evidence support a close relationship to the peroxisomes of liver and kidney: *a*) the occurrence of D-amino acid oxidase activity in microperoxisome-containing fractions of mouse heart muscle (225) and in epithelial cells of guinea pig small intestine (132), *b*) the limitation that only the glutaraldehyde-fixed samples exhibit DAB activity, and *c*) the sensitivity of the reaction to aminotriazole.

Table 9 lists tissues that contain peroxisomes and microperoxisomes and also provides a summary of the metabolic function on which the assignments

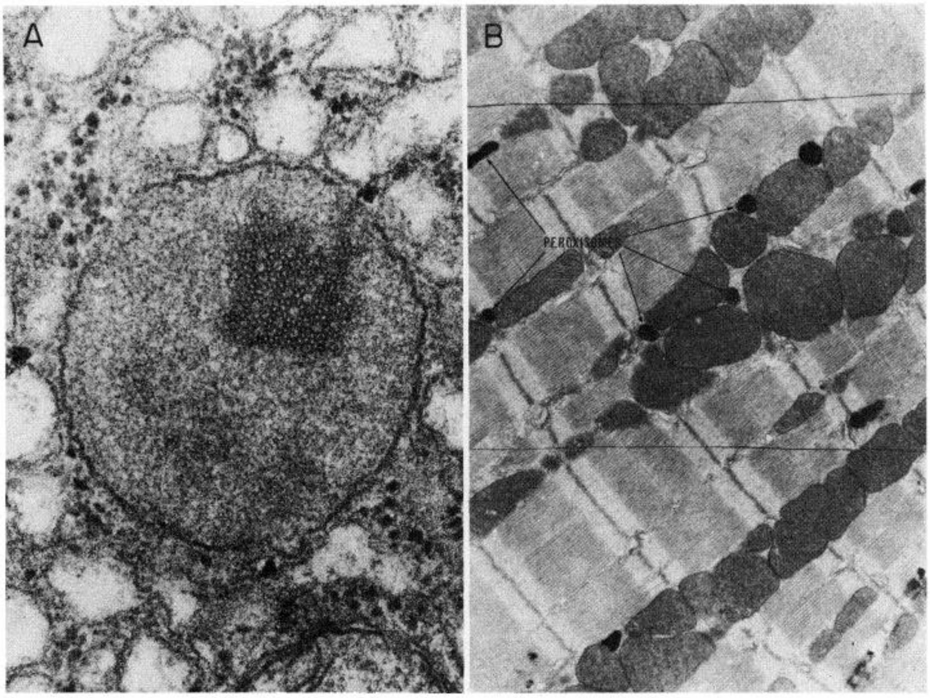

FIG. 12. Peroxisome from rat liver (A), and microperoxisomes from mouse myocardium (B). Microperoxisomes are stained with DAB procedure. [Courtesy of Prof. F. Miller and Priv. Doz. V. Herzog.]

of these structures to cellular processes are based. As noted initially by Novikoff and Shin (383), there seems to be a close relationship between lipid metabolism and the prevalence of peroxisomes, as in Zellweger's disease (194, 532), or an increase in microbody number, as in Reye's syndrome (461, 514; see, however, 504).

c) Assay of peroxisomal activity. The peroxidatic activity of biological samples can be assayed conveniently by the formation of histochemically demonstrable oxidation products of the hydrogen donor 3,3′-diaminobenzidine(biphenyltetramine) (DAB). Since localized peroxidatic activities can be altered by simple changes in incubation conditions, it is possible to distinguish, for example, between the DAB oxidase activities of mitochondrial inner membranes and those of peroxisomes. The latter activity occurs under alkaline conditions and has been used extensively to indicate the peroxidatic activity of catalase (161, 381). The hydrogen donor activity of DAB with catalase is not a property of the native enzyme but is conferred on the hemoprotein by fixation with glutaraldehyde. The cross-linked enzyme both in situ in the peroxisome and in the isolated state (224) can react with the bulky hydrogen donor (480). The DAB oxidase activity of the mitochondrial membrane has been attributed to cytochrome oxidase (467).

TABLE 9. *Tissues containing peroxisomes or microperoxisomes and selected metabolic functions*

Tissue	Cell	Function	Ref.
Liver	Hepatocyte	Fatty acid oxidation	451
	Sinus-lining cells		161
Kidney			
Glands			
Adrenal	Cortical	Steroid production	46, 55
Pancreas	Acinar	Exocrine	208
Lacrimal			
Parotid			
Thyroid	Epithelial		208
Harder's	Sebaceous	Lipid production	
Meibomian			
Preputial			
Muscle			
Heart		Lipid oxidation?	209, 225
Skeletal			
Smooth (aorta)			469
Nerve			
Cerebrum	Cortical		
Cerebellum	Cortical		
Retina	Pigment, epithelial	Esterification of vit. A	308
Dorsal root ganglion	Neuron, Schwann		122
Testis	Leydig	Steroid production	436
Ovary	Stromal		56
Corpus luteum	Luteal		
Placenta	Decidua		
Lung	Alveolar	Phagocytosis	410
Intestine	Epithelial		132, 384
Interscapular fat body	Brown fat cells	Thermogenesis	12

[Modified from Hruban et al. (240), with additional references.]

d) Acatalasemia. Disorders of the catalase level, particularly in the erythrocyte, are known as acatalasia or acatalasemia, and were first observed by Takahara in 1952 (507, 508). Acatalasemia, a genetic defect leading to a decrease of catalase activity early in the life of the erythrocyte to about 1% of that in the control, measured by the catalatic reaction in erythrocytes, has been of considerable interest in hematology and in catalase enzymology (8, 508). The acatalasemic erythrocytes show increased methemoglobin formation on oxidative stress (6), indicating the role of catalase in H_2O_2 removal. The mutation has been shown by Aebi et al. (8) to consist of a thermolabile variant of catalase with a half-life of 18 min at 37°C, compared with 68 min for active catalase. The defect occurs only in the erythrocyte; however, it is compensated for by enhanced rates of catalase synthesis in the liver, causing a 40% increase in the liver catalase level compared with that in the control. So far, no catalase-free mouse has been obtained; presumably, such a mutation would be lethal. Thus, the presence of catalase in liver and presumably in kidney as well, together with residual catalase and other enzymes of the erythrocyte, protects acatalasemic patients, in accord with clinical observations.

2. *Glutathione peroxidase*

The level of glutathione peroxidase in tissues apparently undergoes changes on alterations in the substrate supply, as suggested by the increased activity in intestinal mucosa and liver after oral administration of peroxidized lipids (437) and in lung after lipid peroxidation due to exposure to ozone (118) or to 85–90% oxygen for 14 days (275). Moreover, the relationship of the glutathione peroxidase level in rat liver to the estrogen cycle, or to the estrogen supply in ovariectomized rats (413), may be a result of a change in the profile of polyunsaturated lipids. A clinical genetic defect in which the glutathione peroxidase level decreases to one-fourth of the control value in the erythrocytes of patients homozygous for the trait has been reported (369). As in acatalasemia, there were no overt pathological symptoms, but the tendency to hemolysis was increased in the presence of oxidizing agents (369). The 50% increase in the glutathione peroxidase activity in erythrocytes from trisomy 21 patients suggests a localization of the gene for glutathione peroxidase on chromosome 21 (487).

Disorders of the enzyme level are also observed in selenium deficiency, since glutathione peroxidase is a selenoenzyme. Schwarz and Foltz (464) in 1957 discovered the essential role of selenium as a trace element in mammals because of its prophylactic action on dietary liver necrosis in rats. Since then, numerous symptoms, ranging from degenerative lesions in pancreas, heart, liver, muscle, and skin to kwashiorkor in man, have been attributed to selenium deficiency (75, 169). Thus, although the experimental models of glutathione peroxidase deficiency induced by a selenium-deficient diet can provide useful information on the physiological role of this enzyme, additional functions of selenium, such as its postulated relationship to vitamin E, unsaturated lipids, and sulfur-containing amino acids (234), render this approach more complex. Two glutathione peroxidase activities have been described in rat liver. The recent observation of hydroperoxide removal concomitant with selenium deficiency in the rat (74, 301) indicates that this reaction is catalyzed by a selenium-independent glutathione peroxidase. This enzyme has been identified as glutathione transferase B (423). Although the GSSG level in selenium-deficient rats does not respond to H_2O_2 infusion into the perfused liver, GSSG is released with infusion of *t*-butyl hydroperoxide as substrate for the transferase (74). Furthermore, the distribution of selenium-dependent and selenium-independent enzymes varies widely in different organs and animal species (300).

3. Superoxide dismutase

Correlations of the SOD level with biological phenomena that would be expected to increase the O_2^- level within the cell provide the current rationale for an understanding of the physiological role of this enzyme. McCord et al. (339) noted that the aerobic bacteria contain both SOD and catalase, whereas aerotolerant anaerobes have ample SOD and low catalase activity. Obligate anaerobes appear to lack both enzymes, suggesting that in the aerobic or aerotolerant bacteria the stress of toxic reaction products causes an increased concentration of the two enzymes that deal with the oxygen intermediates. However, exceptions have been noted (339), and the determination of enzyme activity must be supplemented with direct measurements of H_2O_2 and O_2^- production.

An increase in the oxygen tension from anaerobic to hyperbaric levels has been correlated with an increased SOD activity suggesting increased intracellular levels of O_2^- in *S. faecalis, E. coli, Pseudomonas leiognathi,* and *S. cerevisiae* (199–202, 425). Paralleling the increase in dismutase activity, ranging from 6- to 20-fold in the various microorganisms, there is an increase in the resistance to hyperbaric oxygen (199–201). Paraquat-supplemented *E. coli* exposed to normal oxygen tension also shows an enhanced synthesis of SOD and an increased resistance to hyperbaric oxygen (216). Since paraquat is an O_2^- generator and since oxygen tension was not increased, biosynthesis of SOD in *E. coli* appears to result from increased intracellular concentrations of O_2^- or of a closely related substance (216).

Mammalian tissues apparently lack this marked response. After a 7-day exposure to 85% oxygen, a 45% increase in dismutase activity was found in rat lung and a 12% increase in rat brain (138); no change in dismutase activity was observed in heart, kidney, liver, or blood, presumably because the tissue Po_2 was not increased. The phenomenon was not seen in hamsters and was less marked in guinea pigs and mice (138). The proliferation of alveolar type II cells (1) may explain the increase of SOD activity in the lung. Conflicting reports indicate specific increases in either the mitochondrial (275, 500) or the cytosolic (137) enzyme. Exposed rats show enhanced resistance to further hyperoxic exposure (138). Neonatal rat lung is particularly responsive to hyperoxia, showing a 40–70% increase in SOD activity after both in vivo and in vitro exposures to 95% oxygen (500). Trysomy 21 causes increased SOD levels in human erythrocytes and in the cytosol of blood platelets (486).

C. Cellular Redox State

1. Intracellular glutathione

The oxidation-reduction state of the reduced glutathione–oxidized glutathione couple is of major importance in cellular metabolism since, at about 5 μmol/g of rat liver, it is the largest mobile thiol redox system of the cell.

For a full discussion of the metabolic role of glutathione, the reader is referred to the proceedings of five symposia (18, 131, 139, 168, 485), two recent reviews (51, 344), and a monograph (255).

Several groups have reported that up to one-third of the total cellular glutathione is present as mixed disulfides (215, 365), among which those of coenzyme A (496), cysteine, proteins such as hemoglobin or albumin, and proteins from the crystalline lens have been specifically identified (51, 344). The enzymatic reduction of such mixed disulfides is catalyzed by several routes, either directly, at the expense of NADPH, by glutathione reductase or CoA-SSG reductase, or indirectly by thiol transferases with GSH, which is regenerated from GSSG by NADPH-dependent glutathione reductase. The first of the thiol transferases was described by Racker in 1955 (429), and a short review on this field has been provided by Mannervik and Eriksson (324).

The oxidation of reduced glutathione is related to the reduction of hydroperoxides by glutathione peroxidase activity (see sect. II, *A2*), and oxidized glutathione is reduced by the NADPH-dependent glutathione reductase. Hydroperoxide utilization is thus coupled to NADPH oxidation (Fig. 13; 479) and, by the transhydrogenase pathway, to the mitochondrial redox state. The NADPH-generating reactions include the pentose phosphate pathway, the isocitrate dehydrogenase, and the malic enzyme. In turn, these may affect such NADPH-consuming processes as lipogenesis, monooxygenations, and ureogenesis. The major metabolic impact of perturbing the redox state of free thiols is just beginning to be elucidated, but it is clearly mediated by enzymatic and nonenzymatic reactions linked to mixed disulfides and involving thiol transferases and transhydrogenases. Several complex biological phenomena have been

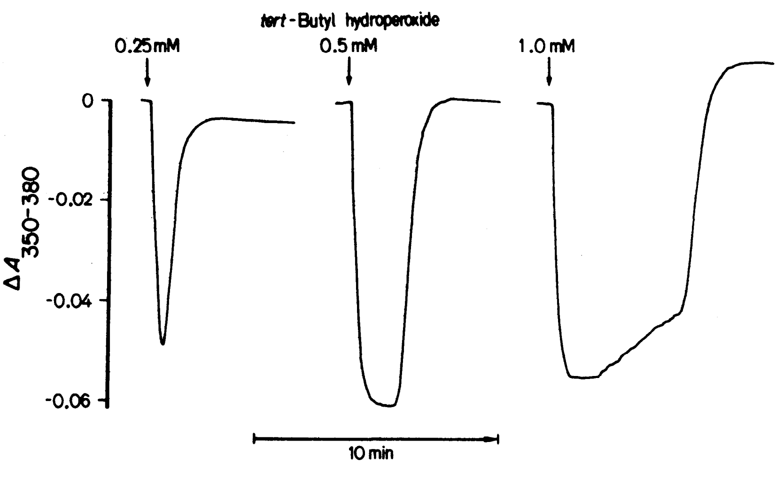

FIG. 13. Oxidation of NADPH in isolated hepatocytes on addition of *t*-butyl hydroperoxide. Time for recovery is increased with higher hydroperoxide concentrations, which also are associated with higher rates of lipid peroxidation detectable as thiobarbiturate-reactive material. [From Sies and Summer (482).]

correlated with the thiol redox state, including cell division (35), protein synthesis (284), hormone release (218), and neurotransmitter release and memory (284). Further, it was proposed that the binding of insulin to its receptors triggers the oxidation of specific sulfhydryl groups in a membrane component involved in the regulation of fat cell hexose transport to the active disulfide form (141). Guanylate cyclase activation can be promoted by peroxides, hydroperoxides, free radicals, and dehydroascorbic acid and can be prevented by glutathione and other thiols (193).

At present, no unequivocal statement can be made about the redox state of glutathione. The equilibrium constant for the reaction of glutathione reductase (531) is $K = ([GSSG][NADPH])/([GSH^2][NADP^+]) = 0.02\ M^{-1}$ (pH 7.0, 38°C, ionic strength = 0.25 M), corresponding to a midpoint potential of −291 mV. Due to this exceptionally high midpoint potential for an NADP-linked system, and to the fact that [GSH] is present to the second power in the equilibrium expression, the cellular ratio of [GSH]/[GSSG] would be roughly 10^6 at the usual NADPH redox potential of approximately −400 mV (473), if thermodynamic equilibrium prevails in the reaction. Reports of GSSG levels ranging from 1% to 3% of the GSH level (520, 546) indicate large deviations from equilibrium, perhaps as a result of rate limitations by glutathione reductase (533) or by the NADPH supply. Possibly glutathione reductase does not operate at sufficiently high activity in situ to maintain equilibrium concentrations; when assayed at 0.3 mM NADPH and 2.5 mM GSSG, the glutathione reductase activity is 7–10 μmol GSSG reduced/min per g of liver at 30°C. The K_m of isolated glutathione reductase from rat liver is 50 μM for GSSG and 3 μM for NADPH (364).

2. Glutathione disulfide release

Addition of hydroperoxides leads to an increased release of GSSG from isolated cells and perfused organs (477, 493, 495); *t*-butyl and cumene hydroperoxides are especially effective and have been used with isolated perfused rat liver (477, 482, 487), intact isolated hepatocytes (391, 478), and erythrocytes (493). Release of GSSG also occurs when glutathione is oxidized by nonenzymatic reactions (285, 495). Under normal conditions, it is assumed that the oxidized glutathione release reflects the intracellular oxidation of GSH by glutathione peroxidase. Reduced glutathione (347) and cytosolic enzymes such as lactic dehydrogenase (477, 482) are not released from the cell in excess of a basal rate (39) on hydroperoxide addition, indicating that the cell membrane is not damaged. Whereas there is an approximately linear relationship between GSSG release and hydroperoxide reduction (Fig. 14), a further correlation with intracellular concentrations of GSH and GSSG would be very useful. In liver, GSSG release occurs into bile (484). Thus GSSG appears to be acted on by the biliary excretory system as the "glutathione-S-conjugate of glutathione." On the other hand, GSH release occurs into the caval perfusate. Release of GSSG has been correlated with glutathione turnover in erythrocytes (490). Perfused rat liver

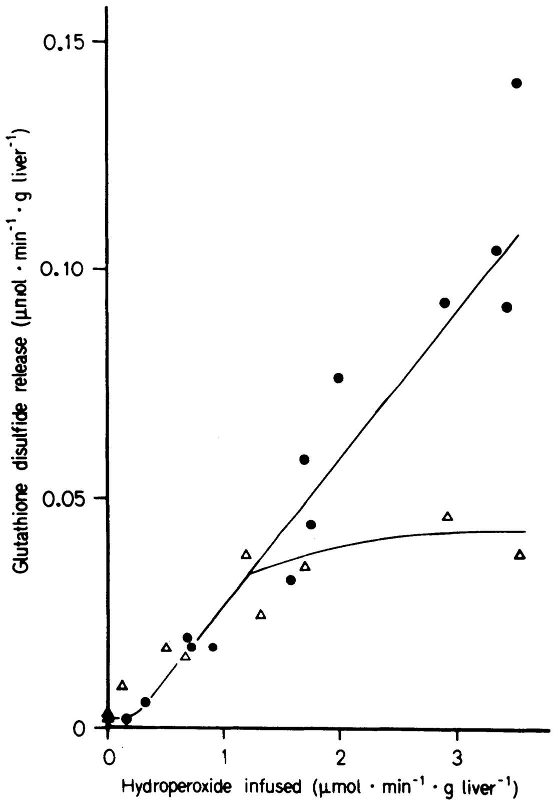

FIG. 14. Rate of glutathione disulfide (GSSG) release from perfused liver as a function of rate of hydroperoxide infusion. Hydroperoxide not reactive with catalase [*t*-butyl hydroperoxide (●)] shows approximately linear dependence, whereas H_2O_2 (△) exhibits saturation of effect due to action of catalase. [Modified from Sies and Summer (482).]

releases from 1 to 2 nmol GSSG/min per g of liver (391, 478, 482) and about 10 nmol GSH/min per g of liver (39), possibly reflecting the normal aerobic turnover of glutathione.

3. Reduced nicotinamide adenine dinucleotide phosphate

As shown in Figure 13, the addition of a small amount of an organic hydroperoxide, such as *t*-butyl hydroperoxide, to perfused liver or to isolated hepatocytes leads to a rapid and substantial temporary decrease of pyridine nucleotide absorption, which is mainly due to oxidation of NADPH (391, 477, 478, 483), with a subsequent recovery. This sequence would be expected from the scheme of Figure 1. With higher concentrations of added hydroperoxide, NADPH is largely oxidized and its concentration falls to the minimal steady-state level observed in intact cells; at this level secondary effects, such as lipid peroxidation (shown by the accumulation of malondialdehyde), set in (482). Furthermore, the time required for pyridine nucleotide to regain the original steady-state level of reduction depends in part on the presence of glucose, indicating the role of the pentose phosphate pathway in providing reducing equivalents (482). These and other observations suggest that the addition of organic hydroperoxides, applied with due caution (391, 482), may afford a useful approach to a number of experimental problems in intact biological systems. Recent studies with *t*-butyl hydroperoxide-supplemented rat liver mitochondria (481) have shown effects of the glutathione peroxidase of the matrix space on the pattern of substrate oxidations; ketoacid oxidases, dependent on coenzyme A and lipoamide, were the main target sites. In view of the known steady-state formation of mitochondrial O_2^- and H_2O_2, a connection between the resulting oxidation of mitochondrial GSH and NADPH and the regulation of mitochondrial substrate oxidations was proposed.

4. *Pentose phosphate pathway*

Oxidation of glutathione increases the flux through the pentose phosphate pathway (154, 250); increased turnover of this pathway also occurs under oxidative stress in erythrocytes (354), liver (482), ascites tumor cells (238), leukocytes (439), lens (414, 494), and lung (40). This pathway is of particular interest in the erythrocyte, where it is the only NADPH-regenerating system; other reactions contribute to NADPH production in liver cells. The role of glutathione in regulating the pentose phosphate pathway seems to be exerted at the level of the enzyme glucose-6-phosphate dehydrogenase. Eggleston and Krebs (154) have suggested that GSSG counteracts the inhibition of glucose-6-phosphate dehydrogenase by NADPH, thus controlling the first enzyme in this pathway. The glutathione peroxidase reaction stimulates the pentose phosphate pathway both by relieving the inhibition of the dehydrogenase and by contributing the cofactor, $NADP^+$.

VI. OXYGEN TOXICITY AND DEFENSE AGAINST OXYGEN-REDUCTION PRODUCTS

A. *Oxygen Toxicity*

The phenomenon of oxygen toxicity was first noted a century ago by Bert (54). Oxygen toxicity develops in animals exposed to oxygen tensions above 2 or 3 atm; the first overt symptoms are the appearance of generalized convulsions (468, 497, 527), followed by severe pulmonary damage (252, 526, 554). Some years ago, Gerschman et al. (189, 190) proposed that an increase of oxidizing free radicals to toxic levels in cells and tissues was the cause of the toxicity of both X irradiation and oxygen. Recent data on the rates of generation and decomposition of the intermediates of oxygen reduction and on their specific effects on cellular components fill some of the gaps in the earlier work. A review of the effects of hyperoxia on hydroperoxide metabolism has been recently provided by Chance and Boveris (98). In spite of microvascular control of the tissue oxygen levels, oxygen pressurization increases the generation of O_2^-

[implicated by Fridovich (186)] and H_2O_2, leading to an increased steady-state concentration of these intermediates (Table 8). The response, which may be limited by the substrate supply, is intrinsically immediate (63, 64). The effect of hyperbaric oxygen on O_2^- and H_2O_2 production has been considered in section IV, *B3*.

Nishiki et al. (376) have given some quantitative data on the enhanced turnover of glutathione peroxidase caused by hyperbaric oxygen (Fig. 15*A*). On pressurization to 4.1 atm oxygen over a 15-min interval, the release of GSSG increases by about 4 nmol/min per g of liver, corresponding (if one assumes that titration curves made by infusing *t*-butyl hydroperoxide may apply) to 100 nmol ROOH/min per g in control (air) and 190 nmol ROOH/min per g under hyperbaric oxygenation; the situation is similar in perfused lung (Fig. 15*B*). The organs of tocopherol-deficient animals are much more sensitive, the increase in GSSG release being equivalent to 320 nmol ROOH/min and 540–610 nmol/min per g of weight in the perfused liver and lung, respectively.

These results agree with previous reports of increased lipid peroxidation correlated with increasing oxygen pressure in vivo in brain (254) and erythrocytes (257), where the process was also enhanced in starved and vitamin E-deficient animals (345, 346, 522).

The immediate release of GSSG on oxygen pressurization may indicate that increased lipid peroxidation is a primary metabolic change, leading as well to NADPH oxidation (Fig. 1). Another primary biochemical change, observed in a variety of intact organs after oxygen pressurization, is the oxidation of pyridine

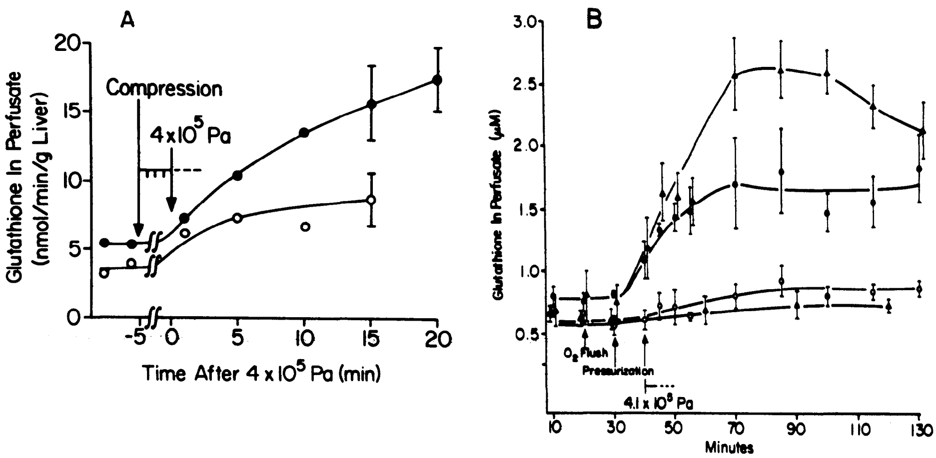

FIG. 15. The effect of diet on GSSG release from 2 perfused organs exposed to hyperbaric oxygenation. *Ordinate*, pressure in pascal units; *abscissa*, GSSG release per unit time. Glutathione release from control and tocopherol-deficient rat liver (*A*) and lung (*B*). *A*: ○ normal starved, pressurized; ● tocopherol-deficient, pressurized livers. *B*: ▲ normal fed, not pressurized; ○ normal fed, pressurized (same results obtained with starved); ● tocopherol-deficient fed; △ tocopherol-deficient starved. [From Nishiki et al. (376).]

nucleotide (105, 106). Studies with a time-sharing fluorometer-reflectometer for measuring the redox state of pyridine nucleotides on the cortex of the brain of the intact unanesthetized rat, combined with electroencephalographic recordings of electrical activity, establish that NAD(P)H oxidation precedes modified electrical activity by about 7 min and the onset of convulsions by about 15 min (333). Intracellular metabolite changes secondary to the increased concentration of oxygen intermediates and lipid peroxides apparently lead to enhanced neuronal membrane permeability, especially to K^+ ions, giving first an alert response on the electrocorticogram and later a typical grand mal activity with generalized convulsions (332).

1. Toxicity of hydrogen peroxide and superoxide anion

The toxic action of H_2O_2 and O_2^- apparently is due to the capacity of these intermediates of oxygen reduction to generate other reactive oxygen species such as the hydroxyl radical and singlet oxygen, which then initiate a radical chain reaction leading to extensive lipid and organic peroxide formation. Besides being effective enzyme inhibitors, lipoperoxides affect membrane-bound multienzyme systems and membrane permeability (see sect. VII*A*).

Hydrogen peroxide itself seems to be quite unreactive; it does not induce lipid peroxidation (561), high-amplitude swelling (370), or inhibition of respiratory control (22) in isolated mitochondrial membranes. On the other hand, H_2O_2 reportedly inactivates transforming DNA, the effect being apparent after 10 min with 2 mM H_2O_2 (180, 181); it induces chromosomal aberrations after a 16- to 36-h incubation of ascites tumor cells in 100 mM H_2O_2 (460) and produces base liberation and backbone breakage of DNA (441). These points may be relevant to theories of aging (see below). However, the last reaction (441) is extremely slow, with a half-time of 40 h at 50 mM H_2O_2; the effects have been attributed to oxygen-radical formation (441), since Fe^{3+} ions were present (181, 337, 534).

Superoxide radicals produced by autoxidation of photoreduced FMN or by the xanthine oxidase reaction inactivate a protein (ribonuclease), a lipoprotein (lysine tRNA ligase), and a ribonucleoprotein (bacteriophage R-17) (299, 351) and kill bacteria (299) and myoblasts (351, 352).

2. Radiation sensitivity

The biological action of ionizing radiation seems to consist of the primary formation of a series of unstable chemical species and radicals derived from water radiolysis, such as hydrated electrons, hydrogen atoms, and hydroxyl radicals (26). The oxygen-enhancing effect may be partially due to O_2^- formation by the reaction of hydrated electrons or hydrogen atoms with oxygen (136, 361), and it may also be due to the addition of oxygen to OH-induced radicals with the formation of peroxy radicals (456a). Aebi et al. (2, 5) have shown that X irradiation of neutral aqueous solutions produces H_2O_2 as a stable product at about 3–4 μM/kR; these rates increase slightly in the presence of certain amino acids. Apparently, radiation toxicity involves essentially the same oxygen intermediates that are responsible for oxygen toxicity (189, 190, 346, 361), but the details of the effects may differ according to the experimental systems studied.

3. Artificial hydrogen peroxide and superoxide anion generators

It has been known since the early experiments of Battelli and Stern (41) and of Wieland and Mitchell (551) that supplementing respiratory systems with autoxidizable electron acceptors such as methylene blue affords an efficient cyanide-insensitive source of H_2O_2. The general reactions may be formulated as follows, where Q represents an unidentified and autoxidizable quinoid redox catalyst, with a midpotential of approximately −40 to +40 mV

$$NAD(P)H_2 + Q \rightarrow NAD(P) + QH_2 \quad (23)$$

$$QH_2 + O_2 \rightarrow Q + H_2O_2 \quad (24)$$

$$QH_2 + O_2 \rightarrow QH^{\cdot} + H^+ + O_2^- \quad (25)$$

The quinone reductase reaction is readily catalyzed by mitochondrial and microsomal membranes, in which NADH and NADPH are the specific reductants (231, 452). If the redox potential of the quinone is high enough, succinate may also act as reducing agent. Quinol oxidation occurs, yielding H_2O_2, or O_2^-, or both (359). Structural requirements for monovalent or bivalent electron transfer in this reaction have been shown (78).

A long series of substances may fit in this chemical scheme, but only some of those that are biologically active are considered here. Some antibiotics are typical: streptonigrin (182, 186, 231, 549), toxoflavin (298), and pyocyanin, active only in aerobic cultures, exert their action by generating O_2^- and H_2O_2; mitomycin is an H_2O_2 generator (521); and β-lapachone, an antimicrobial agent, generates both O_2^- and H_2O_2 (152). Some toxic substances appear to function according to this mechanism as well: the antitumor drug adriamycin, an O_2^- generator, causes lipid peroxidation and cardiotoxicity (25, 195, 367, 515); paraquat, another O_2^- generator, produces lipid peroxidation and lung fibrosis (19, 76, 216). Menadione, a generator of O_2^- and H_2O_2 (78, 359), increases the rate of alcohol oxidation when added to microsomes (397). Menadione and Sinkavit, the phosphate derivative of the quinol form, have been reported as active radiosensitizers in isolated cell cultures and human tumors (363). A rational chemotherapy may be approached by designing drugs with O_2^-- or H_2O_2-generating capabilities, depending on the content of catalase or SOD of the target cell type.

4. Senescence

A widely discussed theory of aging, first proposed by Szilard (505) and extended by Orgel (389), considers the process in terms of the accumulation of molecular damage to informational molecules. This damage may well be produced by free radicals, especially oxygen intermediates such as O_2^- and HO· (157, 186, 214), which randomly alter DNA molecules or other components of the protein-synthesizing system. Although most of the damage induced by the oxygen intermediates would be dealt with by specialized systems for genetic repair, the glutathione peroxidase reaction reducing thymine hydroperoxide (120) might constitute an additional defense. Some support for the theory of aging as a result of informational damage produced by continuous oxidative stress and cumulative errors of the antioxidant defense could be claimed from the fact that administration of antioxidants, such as 2-mercaptoethylamine, butylated hydroxytoluene (213, 214), and 2-ethyl-6-methylhydroxypyridine (157), increases the life-span in mice.

Lipofucsin, which accumulates in heart, brain, testes, etc., from unsaturated lipids, was suggested to be an "age pigment" many years ago (212). The fluorescence properties of lipofucsin are similar to those of the reaction product of malonaldehyde with proteins or nucleic acids. Speculation that deposited lipofucsin represents the sites where in vivo lipid peroxidation has taken place (510) has led to a number of correlations between Schiff-base fluorescence properties, lipofucsin content, and lipid peroxidation. Especially in rat testes, which accumulate fairly large amounts of lipofucsin (440), fluorescent products of lipid peroxidation are deposited even on a nutritionally complete and "antioxidant-sufficient" diet (510). Since there is apparently no mechanism for degrading lipofucsin into harmless metabolic debris, its accumulation could be integratively proportional to the occurrence of lipid peroxidation. However, there are several theories of aging, and it is not clear whether lipid peroxidation is a cause or an effect or whether vitamin E is oxidized before or after lipid peroxidation in "antioxidant-sufficient" cells and tissues.

5. Tumors

The cells from experimental tumors show a low level of activity of the enzymes dealing with oxygen intermediates and hydroperoxides, probably because of loss of cellular differentiation. Catalase (68, 198, 536, 539), superoxide dismutase (68, 149, 409), and glutathione peroxidase (68, 409) are diminished in neoplastic tissue. Catalase activity has been reported lowered also in the normal tissues of tumor-bearing animals (198).

B. Defense Against Toxic Products of Oxygen Reduction

The principal defense against toxic products of oxygen reduction lies at the level of the tissue oxygen tension, which is maintained at rather low levels, first by the microvascular system and then by the steep tissue oxygen gradient (97). The arterioles are sensitive to high oxygen pressure and cause a decrease in the blood circulation to the organ in question, e.g., to the brain, so that the effects of hyperbaric oxygen are greatly decreased in organs other than the lung.

Second, the high affinity of cytochrome oxidase for oxygen, together with the relatively large intercapillary distances for active organs such as brain and heart, leads to oxygen tension gradients from the capillary to the "lethal corner" of the Krogh tissue cylinder (291) that may diminish the oxygen concentration 100-fold or even 1,000-fold from that of the blood vessel.

A further and as yet unrecognized protection against toxic oxygen-reduction products is shown by new evidence for the mechanism of cellular oxygen reduction by the main chain of electron transfer in the mitochondria. This evidence suggests that there is a site in cytochrome oxidase that retains the intermediates of oxygen reduction until water is formed (110, 111).

Furthermore, the evidence suggests not only that the intermediates are retained by the enzyme, but also that there is a "pocket" capable of retaining a number of oxygen molecules, and presumably their intermediate reaction products as well, without communication to the external medium. These data are supplemented by spectroscopic studies at low temperature that identify cytochrome oxidase and two other intermediates that may well involve bound oxygen-reduction products. Thus, in mitochondria it is not the oxidase but rather the ubiquinone-cytochrome *b* region of the respiratory chain that is considered, especially in state 4, to generate O_2^- (63).

The specific enzymes that deal with oxygen-reduction products released from mitochondria and other intracellular sources are superoxide dismutase, catalase, and glutathione peroxidase (Fig. 1). A key feature of this set of defenses is that they seem to be localized at the sites where their appropriate intermediates accumulate. For example, in the peroxisomes, catalase deals so efficiently with H_2O_2 generated by uricase that no H_2O_2 is detected by cytosolic and mitochondrial glutathione peroxidase during urate oxidation (391, 478). Similarly, H_2O_2 infused into the liver is largely decomposed by glutathione peroxidase and is unable to reach the peroxisomes. Mitochondrial SOD readily converts the bulk of mitochondrial O_2^- to H_2O_2. The recent report of catalase in the matrix space of rat heart mitochondria (378) suggests that this catalase may add a special "safety valve" for regulating the intramitochondrial H_2O_2 concentration in a tissue that is relatively deficient in catalase (225, 515).

VII. LIPID PEROXIDATION

A. Lipid and Organic Peroxide Formation

Although direct evidence for the occurrence of lipid peroxidation in intact biological systems is scarce, extensive peroxidation is readily observed after tissue disruption. The phenomenon is easily demonstrated on aerobic incubation of homogenates or subcellular fractions, and in recent years the peroxidation of membrane structures has been associated with a number of pathological phenomena (415). The aerobic environment involves a potential threat for polyunsaturated lipids. Since the major polyunsaturated fatty acids implicated in lipid peroxide formation are linolenic and arachidonic acids, the degree of possible peroxidation damage depends on the fatty acid profile of the phospholipids and other membrane components. Direct comparisons between different types of membranes therefore must take into account the amount of total polyunsaturated lipids. Other cell components, such as nucleic acids, are also prone to peroxidation through similar reactions.

1. Radical Chain Reaction

Although the chemical details of the chain reaction that leads to lipoperoxide formation and later to extensive lipoperoxidation are not satisfactorily understood at present, the initial steps apparently involve the formation of an organic free radical by hydrogen abstraction at the allylic position, subsequent diene conjugation, and formation of the corresponding peroxide radical by incorporation of molecular oxygen (424). The stable products of this process are the hydroperoxides (32, 142, 424), as shown in Figure 16. Molecular oxygen, singlet oxygen, superoxide anion, hydroxyl radical, and hydrogen peroxide concentrations have been proposed by various workers as rate limiting in the process of lipid peroxidation. It seems likely that all these molecular species participate in the free-radical chain reaction that produces lipid peroxidation and that the chemical and kinetic details of the chain reaction differ in the variety of biological systems in which lipoperoxidation occurs. Hence, lipoperoxide formation will not depend on the appearance of a single oxidizing species, but rather will include all species in variable proportions in different systems.

A direct effect of molecular oxygen, probably mediated through nonspecific catalysts initiating and propagating the free-radical reaction, has been proposed

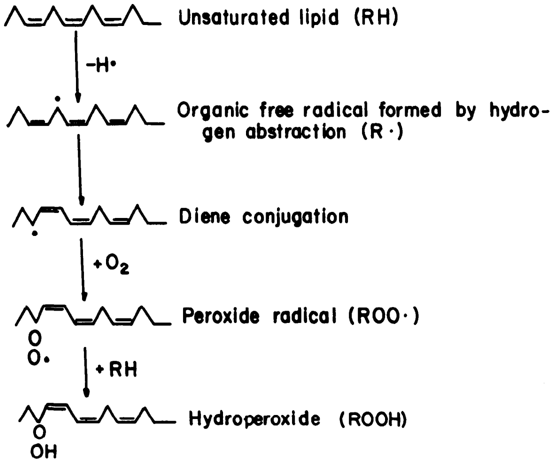

FIG. 16. Scheme of initial steps of process of lipid peroxidation. [From Barber and Bernheim (32).]

to explain the effect of high-pressure oxygen on glutathione disulfide release from perfused organs (376). Hydrogen peroxide is capable of reacting with unsaturated alkyl chains introducing peroxide groups (535), but this property apparently is not expressed under conditions that lead to lipoperoxidation in biomembranes (561).

The protective effect of SOD in a variety of experimental situations strongly indicates a major role for the superoxide anion in the lipoperoxidation process (76, 127, 270, 403, 404, 561). Different interpretations have been offered for this effect, with superoxide anions (403), hydroxyl radicals (174, 174a, 270, 271, 276, 561), or singlet oxygen (76, 270, 271, 561) being proposed as the actual initiator of the radical chain.

The hypothesis that the formation of hydroxyl radical and singlet oxygen may be the rate-limiting steps in the chain reaction that, in its various modalities, leads to lipoperoxidation in biological systems could fit most of the data. Hydroxyl radicals chiefly generated by a catalyzed Haber-Weiss reaction (206, 207b, 337) would account for the protective effect of catalase (270, 271, 403, 561), SOD (270, 271, 403, 404, 561), and hydroxyl radical scavengers such as mannitol (174) or benzoate (174). Singlet oxygen as an intermediate rather than inducing lipid peroxidation (368, 501, 501a) may be quenched by β-carotene (15) or trapped by furan derivatives (270, 404, 296b).

Singlet oxygen produced by photosensitization or radiofrequency discharge does cause peroxidation of liposomes by a process that can be prevented by β-carotene (15); the β-carotene does not, however, seem very effective in inhibiting lipoperoxidation in microsomal preparations and linolenic acid suspension (270, 368, 501). The thermodynamic (281a) and kinetic (282) feasibilities of $^1O_2$ formation have been evaluated.

Hydroxyl radical formation is almost exclusively considered in terms of its generation through the Haber-Weiss reaction:

$$H_2O_2 + O_2^- \rightarrow O_2 + HO^- + HO\cdot \qquad (26)$$

Although attempts to demonstrate a reaction between O_2^- and H_2O_2 have failed (207, 335, 444), metal catalysis, especially porphyrin iron, might make $HO\cdot$ radical generation in biological systems a reality. The formation of complexed superoxide anion ($Fe^{3+}-O_2^-$), similar to cytochrome oxidase intermediate compound A′ (see sect. II, *B1*), and complexed H_2O_2 ($Fe^{3+}-H_2O_2$) may be kinetically efficient in consuming H_2O_2 and O_2^-, respectively, yielding effective rates of $HO\cdot$ generation (246, 282). Recently, McCord and Day (337) and Halliwell (207b) have shown that ethylenediaminetetraacetate (EDTA)-chelated iron indeed catalyzes the Haber-Weiss reaction with hydroxyl radical formation. The efficiency of the process, in terms of $HO\cdot$ formed per O_2^- consumed, was at least 8% in the presence of 5 μM iron and 30 μM H_2O_2. Since O_2^- and H_2O_2 compete for EDTA-Fe^{2+}, the effective electron donor for the homolytic breakdown of H_2O_2, a somewhat lower efficiency may be expected at lower H_2O_2 concentrations. After considering the rate of O_2^- production and the H_2O_2 concentration, HO· generation in rat liver cytosol may be estimated to be on the order of $10^{-12}-10^{-9}$ M/s. This rate is at least 3 orders of magnitude smaller than the best estimates of the rate of lipid peroxide formation (see below), implying that the radical chain reaction (Fig. 16) acts as an amplification factor.

2. Rate of lipid and organic hydroperoxide formation

Since the very existence of lipid peroxidation is still under debate, at present only very approximate estimations can be made of the rate at which this process occurs. Nevertheless, ethane formation and glutathione release provide promising approaches to the problem. Ethane formation can be monitored in the expired air (see below); this may be an optimal approach for studying lipid peroxidation in intact systems. Perfused rat liver releases 1–2 nmol GSSG/min per g of liver (39, 391, 478), a value corresponding to approximately 30–60 nmoles ROOH/min per g of liver on the basis that GSSG release reflects about 3% of the turnover of glutathione peroxidase (391, 482).

B. Biochemical Consequences of Lipid Peroxidation

The breakdown pathways for peroxidized lipids are even less clearly established than the mechanisms of their formation. It suffices here to note that the stable end products include ethane, propane, and pentane (148, 281, 310, 443) and malonaldehyde (32).

The sequels of polyunsaturated fatty acid peroxidation include *a*) perturbation of membrane microarchitecture due to the introduction of hydrophilic functions, even after reduction to the corresponding hydroxylipid; *b*) inhibition of enzyme activity by hydroperoxides; and *c*) subsequent reactions of the breakdown products, such as aldehydes. Oxygenated derivatives of fatty acids assembled as membrane constituents will produce an alteration of membrane structure. In addition, the lipoperoxide-inducing oxidizing free-radical reaction will rupture the carbon chain of membrane constituents. The cumulative effect may be the pathological alteration of membrane permeability.

Isocitrate dehydrogenase activity, which is sensitive to inhibition by linoleic acid hydroperoxide (196, 385), is completely abolished during the lipid peroxidation of liver mitochondria induced by ferrous ions (341). Modification of sulfhydryl groups and/or a methionine residue have been shown to be the cause of this loss of activity (129, 130). Some other enzyme activities have also been shown to decrease during lipid peroxidation (33, 117, 230, 341). Note that lipoperoxide-induced enzyme inactivation is apparently a rather slow process (196).

Breakdown products may have a bearing on what are considered to be the toxic effects of lipid peroxidation. There are two possible pathways for malondialdehyde degradation: oxidation by aldehyde dehydrogenase with further metabolic conversion (237, 416, 432) and the formation of Schiff bases, e.g., with α-amino groups of lysine. The latter has been studied intensively by Tappel and his colleagues (116, 117, 510), who have demonstrated cross-linking in a number of enzymes; for example, ribonuclease A becomes cross-linked in the presence of either peroxidizing lipid or added malonaldehyde. Albumin generally develops such cross-linking on storage, since a large portion of lipids bound to it are polyunsaturated. Experimentally, such cross-linking of polypeptide chains is indicated by specific fluorescence properties with excitation maxima around 350 nm and emission maxima around 430 nm. The fluorescent chromophore is generally considered to be an *N,N'*-disubstituted 1-amino-3-iminopropene, R—N=CH—CH=CH—NH—R (510), resulting from the reaction of malondialdehyde with amino compounds; thus, other types of amino compounds such as amines, RNA, DNA, and phospholipids may also form such products. It is of particular interest that the fluorescence properties of such cross-linked products resemble those of lipofucsin, the "age pigment." Lipofucsin granulae have been observed cytochemically to be in close proximity to microperoxisomes in human hepatocytes (382).

Lipid hydroperoxide formation is much more likely in membranes of animals that have been on diets deficient of vitamin E (tocopherol) or selenium. The antioxidant capability of selenium is due to its role in glutathione peroxidase, but the recognized effectiveness of tocopherol as an antioxidant is not so well understood; thus far, no regenerating reaction that reduces the product, tocoquinone, has been found. In addition to breaking or preventing radical chains (510), tocopherol may participate in ubiquinone metabolism (463). Under physiological conditions, the presence of catalase, glutathione peroxidase, and SOD may diminish the flux of radicals sufficiently that the small amounts of vitamin E [~1 per 1,000 molecules of polyunsaturated fatty acid (510)] operate as a second line of defense, dealing with unusual "overflows" of radicals.

C. Physiological Consequences of Lipid Peroxidation

1. Membrane damage

a) Hemolysis. Lipid peroxides, H_2O_2, and perhaps O_2^- and other oxidants contribute to the instability of the erythrocyte cell membrane. Catalase and glutathione peroxidase probably play complementary roles in protecting the erythrocyte from hemolysis due to H_2O_2, since their rate constants are roughly equal (Table 1) and since a calculation based on the content of the two enzymes in the erythrocyte shows that the rates of H_2O_2 decomposition by the two systems are comparable (7, 372). However, human erythrocytes deficient in glutathione peroxidase are more fragile than those from acatalasemic subjects when exposed to oxidative stress, suggesting that lipid hydroperoxides are more important than H_2O_2 in causing hemolysis. Thus, reduction of unsaturated membrane lipid hydroperoxides by glutathione peroxidase is important in counteracting hemolytic damage. Fee and Teitelbaum (164), working with erythrocytes from vitamin E-deficient rats, showed that SOD plays a protective role against hemolysis induced by dialuric acid and that catalase plus SOD provided the highest degree of protection, suggesting that the hydroxyl radical may be a factor in membrane lipid peroxidation.[1] The Haber-Weiss reaction (206) has often been regarded as a causative agent in hemolysis, most recently by Cohen and Heikkila (127) and by Kellogg and Fridovich (271), but the participation of hydroxyl radical and/or singlet oxygen in the hemolytic process has not been unanimously accepted. In fact, Fee et al. (163) specifically exclude the Haber-Weiss reaction from the dialurate-induced hemolysis in vitamin E-deficient red cells, suggesting instead that reactive dialurate-derived compounds, such as a peroxy derivative (491) or the dialurate radical (296), might react with the membrane or serve as precursors of the active substance. On this basis, the protective effect of superoxide dismutase could be to protect catalase from reacting with O_2^- to form compound III (386); interestingly enough, Fee et al. (163) propose that catalase reacts directly with the unknown deleterious intermediate, implying a catalytic function for catalase in addition to the known catalatic and peroxidatic modes of action.

The enhanced hemolytic sensitivity of erythrocytes deficient either in glucose-6-phosphate dehydrogenase (126, 128) or in tocopherol (126) indicates the protective roles of the NADPH-linked pentose phosphate shunt and glutathione peroxidase activity and vitamin E in the erythrocyte membrane (126).

b) Mitochondria, lysosomes, and microsomes. The functions of catalase, glutathione peroxidase, and superoxide dismutase as protective agents have been further studied in model systems. The role of lipid peroxidation in the "high-amplitude" swelling of isolated rat liver mitochondria has been elucidated (173, 245), and it has been shown that the phenomenon can be prevented by these three enzymes. For example, GSH-induced swelling has been shown to be prevented by the addition of glutathione peroxidase (370).

Catalase and glutathione peroxidase have a partially protective effect on isolated mitochondrial inner membranes, and SOD substantially delays oxidative damage (561). The effect is greater than linear with added enzymes, indicating that reactive intermediates such as the HO· radical may participate in lipid peroxidation. Similar results have been obtained with isolated lysosomes (174) protected by HO· scavengers such as ethanol, benzoate, or mannitol.

The mechanism of microsomal lipid peroxidation has been extensively investigated, notably by McCay and co-workers (174, 276, 328–330, 411) and Pederson and Aust (403–406), following the early work of Hochstein and Ernster (229, 232), who showed that isolated microsomal fractions from rat liver undergo lipid peroxidation concomitant with an enhanced oxygen uptake

[1] Lynch and Fridovich (318a) have shown that membranes of vesicles formed from washed erythrocyte stroma were markedly sensitized to the lytic attack of enzymatically generated superoxide anion by preloading the membranes with lipid hydroperoxide by exposure to a photochemical flux of singlet oxygen, thus pointing out that superoxide anion must have functioned as a precursor of more reactive species.

and an oxidation of NADPH on addition of ADP complexed with Fe^{3+}. Superoxide anions have been considered to initiate microsomal lipoperoxidation by producing either singlet oxygen (403, 404) or hydroxyl radicals (174, 276). Alkoxy radicals have also been proposed as the main chain propagator (405).

Isolated rat liver microsomes metabolize hydroperoxides via an NADPH-dependent peroxidaselike reaction (242–244) involving cytochrome P_{450}, which accounts for about half the hydroperoxides decomposed; the other half is probably removed nonenzymatically in the form of lipid peroxidation (482). Cytochrome b_5 becomes fully oxidized on addition of hydroperoxides, probably by interaction with cytochrome P_{450}; this effect is also observed in isolated hepatocytes, even though lipid peroxidation is largely suppressed by glutathione peroxidase and other defense systems (478, 479). Cytochrome P_{450} can produce O_2^- by autoxidation (227, 379, 489), and added hydroperoxides have been shown to support cytochrome P_{450}-dependent hydroxylations (155, 259, 430). However, hydroperoxides lead to the destruction of the heme groups of cytochrome P_{450}, so that such experiments are feasible for only a few minutes.

2. Toxicity of administered lipid peroxides

Although the beneficial effects of polyunsaturated fatty acids in counteracting high cholesterol levels and atheromatous disease have been highly publicized, little note has been taken of the fact that ingestion of such substances increases the capacity for forming lipid peroxides. Organic peroxides, hydroperoxides, and autoxidized unsaturated fatty acids are quite toxic, with LD_{50} for mice of 4–20 μmol/mouse (236). Feeding autoxidized fats to laboratory animals produces toxic and possibly carcinogenic effects; decreasing the amount and degree of unsaturated fats in the diet decreased mortality in mice (214). The dietary use of fatty acids prone to oxidation has therefore been questioned (548).

VIII. HYDROGEN PEROXIDE AS A USEFUL METABOLITE

The hypothesis that hydrogen peroxide may be a useful metabolite involves a dichotomy of viewpoints. On the one hand, H_2O_2 can be considered a dangerous substance: injections of glucose oxidase into the bloodstream and the consequent H_2O_2 generation can be lethal, due to the continuous conversion of hemoglobin to methemoglobin. Much of the cell machinery is geared to the control of the H_2O_2 level; for example, catalase, one of the most potent enzymes, is present in high localized concentrations and exhibits kinetics appropriate to the regulation of H_2O_2 levels: i.e., the rate of H_2O_2 decomposition proceeds linearly through the higher physiological and pathological range of H_2O_2 concentrations. A further back-up regulation is provided by glutathione peroxidase. With this multiplicity of controls, it is scarcely conceivable that H_2O_2 performs a beneficial metabolic function. On the other hand, it was suggested early in the study of catalase reactions that the peroxidatic reaction in particular (268) might be useful to cell function, and indeed this reaction appears in phagocytosis and in several syntheses, particularly those of thyroid hormones (457) and probably those of prostaglandins (210, 420, 529, 553). A fuller understanding of catalase function required the development of better instrumentation, and recent results shed new light on its role in phagocytosis and alcohol oxidation.

A. *Phagocytosis*

Biochemically and clinically oriented research groups have clearly demonstrated that polymorphonuclear leukocytes (24, 24a, 142a, 248, 402, 444a, 445) and alveolar macrophages (188) produce H_2O_2, $^1O_2$, and $HO\cdot$ in a process that is stimulated dramatically during phagocytosis. The importance of this biological mechanism is emphasized by the enhanced susceptibility to infection of patients with dysfunction of leukocyte H_2O_2 generation, as in chronic granulomatous disease (27), myeloperoxidase deficiency (279), and leukocyte glucose-6-phosphate dehydrogenase deficiency (29, 135).

The longstanding observation (248, 402, 445) that H_2O_2 is formed during the large increase of cyanide-insensitive oxygen uptake and increased pentose phosphate shunt activity (49, 458) accompanying phagocytosis is frequently associated with the suggestion that NAD(P)H oxidation is a source of H_2O_2 (28, 79, 249, 263, 449, 458, 552). More recently, it has been shown that O_2^- is also produced during phagocytosis (24b, 153, 277–279, 455, 456, 560) and that glutathione reductase activity is also increased (380, 439). The localization of NADPH oxidase and its activation in phagocytosis strongly suggest that H_2O_2 is involved in killing the bacteria, but the exact chemical species involved are not yet clarified; the participation of O_2^-, $^1O_2$, and $HO\cdot$ radicals has also been suggested (277, 279, 289, 447, 560). It has been proposed that O_2^- production by polymorphonucleates may account for synovial fluid deterioration in inflammatory arthritis (336).

Increased NADPH activity is also shown by cytosolic (28) and granular (401, 448) fractions. The granular fraction isolated from phagocytizing leukocytes shows a 10-fold increase in H_2O_2 production compared with granules isolated from resting cells (260). The fact that after isolation the granules retain the stimulated condition suggests an imprinted message. The isolated granules also produce O_2^- in an amount that corresponds to 24% of the H_2O_2 generated (260), and they contain a cyanide-insensitive component spectroscopically identical to myeloperoxidase (261). The peroxide generator has not yet been identified, although a peroxidase-oxidase mechanism involving the granule myeloperoxidase-NADPH oxidase complex has been proposed (261, 555, 556). In addition, myeloperoxidase and halide ions provide a bactericidal system (278), but halogenation is not bactericidal per se (446), and the exact role of myeloperoxidase in phagocytosis is not yet understood.

Scavengers for singlet oxygen and hydroxyl radical (258, 278) partially impair the destruction of bacteria, which takes place within a phagocytic vesicle. This phagosome is formed by fusion of the myeloperoxidase-containing granules with invaginations of the plasma membrane (71, 351, 506) that contains the NADPH oxidase that is apparently the major source of H_2O_2 and O_2^- (Fig. 17; 24, 24a). Phagocytosis in the presence of catalase or superoxide dismutase exhibits reduced bactericidal action (258, 322) but at the same time allows prolonged life of the leukocytes (456). Catalase activity was strongly correlated with mouse lethality on exposure to 15 strains of *Staphylococcus aureus*, whereas there was no apparent relation between virulence and SOD activity in the same strains (322).

It is clear that oxygen metabolites, highly toxic in mammalian cells, do indeed fulfill a useful function in phagocytosis. Compartments for phagocytosis may exist within the leukocyte, since catalase and SOD are located in the cytosol of the phagocyte (455) and presumably protect the cell from O_2^- and H_2O_2 damage; nevertheless, bactericidal action leads the leukocyte to an early death.

B. Alcohol Oxidation

Keys to the useful peroxidations of catalase may be found in its high localized concentration, so that the turnover number of the enzyme, the H_2O_2 concentration, and the fractional saturation of catalase are low and the efficiency of H_2O_2 in peroxidatic oxidations approaches 100%. Such conditions might be obtained with H_2O_2 generated in the mitochondria and diffusing throughout the cell at low and uniform concentrations to the peroxisomes, where it could be efficiently used in ethanol oxidation (99). If, however, H_2O_2 were generated directly in the peroxisomes at a high rate (as, e.g., by uric acid oxidation), then the H_2O_2 level would be controlled locally by the catalatic reactions and the

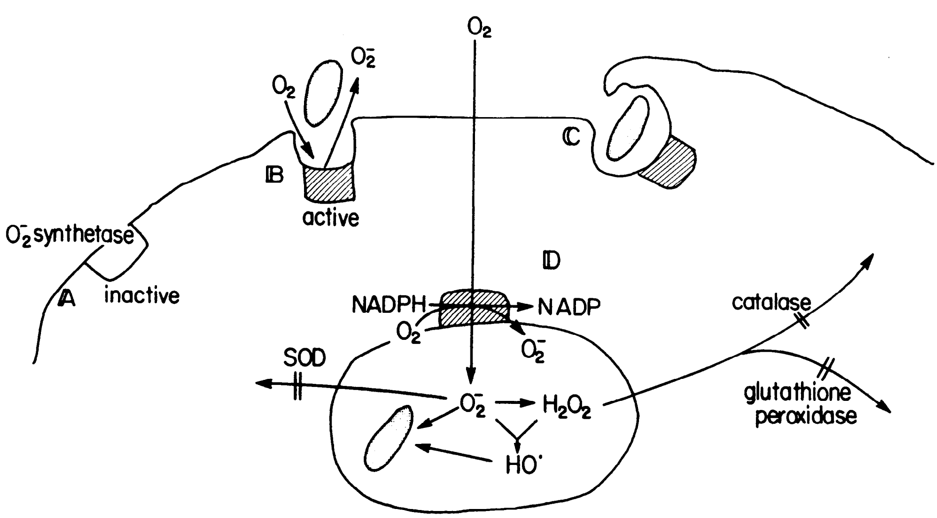

FIG. 17. Proposed role of "superoxide anion synthetase" and oxygen intermediates in phagocytosis. [From Michelson (351).]

increment of cytosolic H_2O_2 would be small. Under these conditions, the efficiency of ethanol oxidation by peroxisomal catalase might be low.

One of the puzzling inconsistencies of nature's design is that catalase is restricted to react only with H_2O_2. If, indeed, teleology and autonomy were first principles, catalase should be capable of decomposing lipid peroxides as well as hydrogen peroxide and short-chain peroxides. Instead, another enzyme—glutathione peroxidase—decomposes long-chain peroxides, and glutathione peroxidase and catalase together cover the gamut of peroxide reactions of the cell. This obligatory collaboration is all the more puzzling when it is remembered that both the mitochondria and the peroxisomes generate H_2O_2. However, exclusive subcellular localization may be the guiding principle, providing for catalase in the peroxisomes and glutathione peroxidase in the cytosol and the mitochondria.

One use for catalase is the oxidation of ethanol without the formation of large amounts of NADH in the liver cytosol (518). The redox imbalance caused by excessive alcohol intake is thought to be at least a starting point for a wide variety of pathological consequences of alcoholism. In the liver, catalase seems to provide an innocuous metabolic pathway for alcohol oxidation. The dependence on this and other pathways can be determined in vivo by use of appropriate levels of substituted pyrazoles that inhibit the alcohol dehydrogenase pathway and leave the alternate pathways of alcohol oxidation unchanged (518).

Although pyrazole studies do not delineate the alternate pathways, the directly observed reaction of the catalase-H_2O_2 compound with alcohol in the liver in vivo affords firm evidence of its function in tissues and is strongly

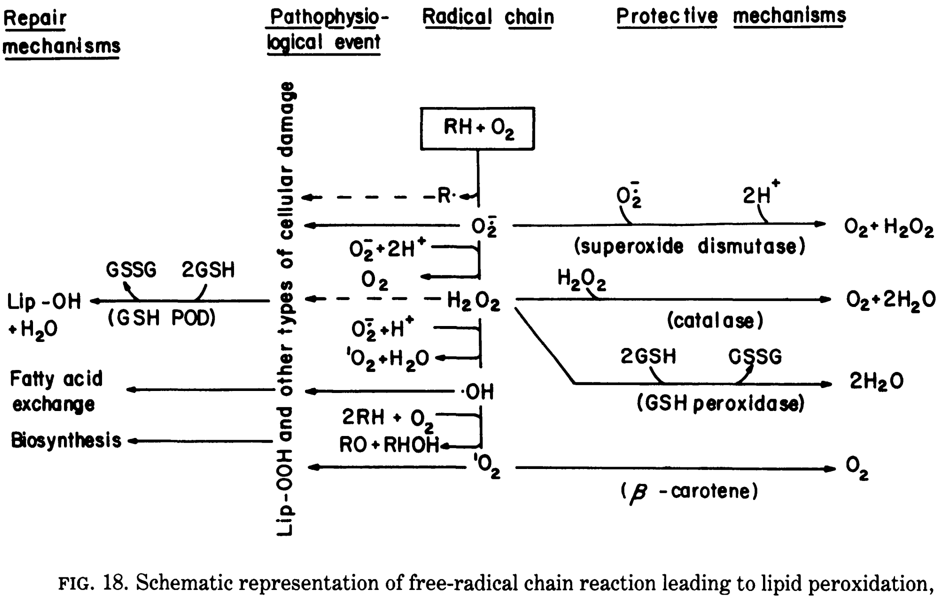

FIG. 18. Schematic representation of free-radical chain reaction leading to lipid peroxidation, possible pathophysiological implications, and protective mechanisms. [Modified from Flohé et al. (169).]

supported by classic experiments with formate and methanol. Lieber and his colleagues (309) have been the nearly exclusive supporters of the view that a microsomal ethanol-oxidizing system is functional in the intact liver (247, 397, 519, 530, 547). However, the evidence in favor of this pathway lacks both quantitative in vitro studies, with a highly purified system under controlled conditions, and a clear demonstration of increased NADPH oxidation in the liver in situ metabolizing alcohol. This need for further study, together with considerations of the complicated mechanism for controlling the NADPH/NADP redox state, suggests that whatever the upshot of the controversy over a microsomal ethanol-oxidizing system may be, the system does not offer a pathway of alcohol oxidation that prevents overreduction in the cytosol.

The question of whether a significant amount of unknown hydrogen donor exists in the metabolizing liver is largely resolved by the extrapolation of methanol and ethanol titrations in the perfused and intact organ. Generally, these titrations indicate that the endogenous donor is present at concentrations of less than 30 μM (393) in ethanol equivalents. However, the nature of such endogenous donors is not known, and indeed many important pathways could be affected by catalase reacting with such pathways in vivo.

IX. CONCLUSION

The cell employs several lines of defense against the toxic products of oxygen reduction (Fig. 18). The first is systemic protection against high oxygen tensions at the cellular level. The second is the intracellular localization of the enzymes appropriate to the decomposition of the toxic intermediates at or near the site where they are generated, together with steep gradients of the reactive species themselves. A third line of defense is provided by radical scavengers such as α-tocopherol and β-carotene, which also have the advantage of being appropriately distributed in the membranes where lipid peroxidation might occur. A fourth level of protection is provided by glutathione peroxidase, which reacts directly with lipid peroxides.

Finally, recent understanding of the beneficial action of H_2O_2 in phagocytosis and in ethanol oxidation suggests caution in condemning any metabolite as useless until its functions in toto are thoroughly understood.

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