

A MICROSOMAL PEROXIDASE UTILIZING A LIPID
PEROXIDE SUBSTRATE

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A MICROSOMAL PEROXIDASE UTILIZING A LIPID
PEROXIDE SUBSTRATE

A thesis

by

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the degree of Master of Science

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ABSTRACT

Linoleic acid hydroperoxide (LAHPO) when incubated with heme compounds or liver microsomes is rapidly decomposed, presumably by a free radical mechanism, to yield a complex range of products. In this study, a spectrophotometric method has been developed for investigating the peroxidase reaction using *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as hydrogen donor and LAHPO as substrate. The reaction was found to be first-order with respect to catalyst concentration and first-order with respect to LAHPO concentration.

The mitochondrial and microsomal fractions from rat liver exhibited the highest peroxidase activity per mg protein. The microsomal peroxidase activity had a pH optimum of 4.7, was inhibited 50% by 1 mM cyanide and was unaffected by EDTA. The peroxidase activity of microsomes was enhanced 2- to 8-fold by reagents that converted cytochrome P-450 to P-420 (e.g., lysolecithin, *p*-hydroxymercuribenzoate, *N*-bromosuccinimide, iodine, trypsin, deoxycholate). Microsomes from phenobarbital-injected rats exhibited a 2.5-fold higher specific P-450 content and showed a similarly enhanced peroxidase activity.

LAHPO destroyed cytochrome P-450 rapidly and inhibited demethylation activity in liver microsomes. Cytochrome b₅ had low peroxidase activity whereas microsomal "P-450 particles"

containing P-450 as the sole protoheme constituent were very active. It was concluded that cytochrome P-450 is responsible for most of the peroxidase activity of liver microsomes. A mechanism for the microsomal peroxidase activity is proposed in which LAHPO oxidizes the P-450 thiol ligand to form "high spin" P-420 which then acts as a peroxidase.

The peroxidase properties of cytochrome P-420, in its "high spin" and "low spin" forms, were next investigated using a preparation of P-420 from hepatic microsomal "P-450 particles". The peroxidase activity of "high spin" P-420 was inhibited 50% by 1 mM cyanide, was completely abolished by boiling and was unaffected by EDTA. The cyanide difference spectrum of oxidized "high spin" P-420 revealed a peak at 426 nm, a trough at 403 nm and a binding constant for cyanide of about 1 mM. "High spin" and "low spin" cytochromes P-420 were rapidly destroyed when incubated with LAHPO.

A comparison of the effectiveness of various heme compounds in catalyzing the peroxidase reaction showed that "high spin" P-420 was the most effective catalyst. Other high spin heme compounds such as hematin and methemoglobin were also very effective catalysts whereas low spin hemoproteins such as "low spin" P-420, "low spin" P-450, cytochrome b_5 , and oxyhemoglobin exhibited much lower catalytic activities. "High spin" P-420 and thyroid peroxidase showed remarkably similar spectral properties and it is suggested that the two peroxidases may be identical hemoproteins.

ABBREVIATIONS

The following abbreviations are used: LAHPO, linoleic acid hydroperoxide; TMPD, N,N,N'N'-tetramethyl-*p*-phenylenediamine; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; ESR, electron spin resonance; DEAE-cellulose, diethylamino-ethyl-cellulose.

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INTRODUCTION

1. Peroxide formation in non-biological systems

Hydroperoxides comprise the majority of the products formed during the early stages of autoxidation of unsaturated lipids (1,2). Peroxide formation occurs by the initial formation of a radical species (1). O_2 , because of its diradical nature, readily reacts with the initial radical species to form a peroxy radical which can then propagate by a chain reaction. The chain reaction is terminated by radical-radical interaction or by radical scavengers such as antioxidants or hydrogen donors (1,2).

Heavy metals, especially those having two valency states (iron, copper, cobalt), catalyze the peroxidation of unsaturated lipids. Metal catalysis of lipid peroxidation is believed to be involved with the chain initiation reaction (3). Heavy metals also catalyze the autoxidation of various hydrogen donors such as ascorbic acid, hydroquinone, dihydric phenols, etc. with the resultant formation of hydrogen peroxide.

Tappel (2) has shown that hemoglobin and other hematin compounds catalyze the peroxidation of unsaturated fatty acids. The mechanism proposed for the pro-oxidant activity of hematin compounds is based on their known ability to decompose lipid peroxides (4). Once the fatty acid peroxide free radical has been formed, it can initiate the peroxidation of an adjacent unsaturated fatty acid and thus the process can continue as a

chain reaction as long as adjacent unsaturated fatty acids are available.

2. Peroxide formation in biological systems

Peroxides in biological systems may be dietary in origin or generated intracellularly. Thus, hydrogen peroxide is probably formed as a result of the action of intracellular flavoprotein oxidases. Unsaturated fatty acids of intracellular membrane phospholipids readily undergo peroxidation in the presence of oxygen (1). Other types of peroxides produced in animal or plant cells include hydroperoxides of nucleic acids (5), squalene (6), certain amino acids, flavins and pteridines (7), and cholesterol and some steroids (8).

a. Metal-catalyzed peroxidation

Tissues and tissue extracts contain metal ions known to catalyze the peroxidation of pure unsaturated lipids (9). The most active catalysts are Co^{2+} and Mn^{2+} in the concentration range of 10^{-4} to 10^{-3}M , whilst Cu^{2+} , Fe^{2+} , and Fe^{3+} are weakly active (10). The catalytic activity of Fe^{3+} can be markedly enhanced by addition of hydrogen donors such as ascorbic acid or cysteine which reduce Fe^{3+} to the more active Fe^{2+} state. Addition of EDTA inhibits the reaction.

In vivo, most metal ions are complexed with amino acids or proteins. In tissue homogenates lipid peroxide formation is probably catalyzed by the most abundant of the

catalytic metals, namely iron, either in the free state or bound in a porphyrin complex. Sufficient iron is also present in mitochondria and microsomes to catalyze peroxidation of the lipids of these subcellular particles when ascorbic acid or cysteine are added to maintain iron in the ferrous state (11, 12).

b. Hematin-catalyzed peroxidation

All of the hematin compounds occurring in nature catalyze the peroxidation of unsaturated lipids (2, 4) and indeed hemoproteins are very effective catalysts. Tappel (4) suggests that the critical reaction in hematin-catalyzed unsaturated lipid peroxidation is the catalytic decomposition of peroxide into free radicals. Evidence is presented indicating no valence change of the hematin compound during the reaction. The formation of an activated coordination complex between the hematin catalyst and the lipid peroxide is postulated. Once formed the hematin-peroxide complex decomposes into free radicals by homolytic scission of the -O-OH bond of the hydroperoxide. The free radicals thus formed can initiate the peroxidation of adjacent unsaturated fatty acids.

Tarladgis (13) claims that the spin state of Fe in heme compounds rather than the oxidation state of the metal governs the catalytic effectiveness. The high spin state is the active form and any treatment (e.g., suitable ligands) which converts the high spin state into a low spin form delays

the onset of lipid peroxidation.

c. Enzymically produced peroxides

Lipoxidase is an enzyme which catalyzes the oxidation of methylene-interrupted polyunsaturated fatty acids by oxygen in which the double bonds have the cis-configuration. However, lipoxidases have been found only in legumes, some cereal grains, and oily seeds (4).

Hochstein and Ernster (14) found an enzymic system in liver microsomes which catalyzes the peroxidation of microsomal lipids in the presence of oxygen, NADPH, Fe^{2+} , and a nucleoside di- or triphosphate.

An NADPH oxidase requiring Mn^{2+} may be responsible for most of the H_2O_2 present in polymorphonuclear leukocytes (15) or thyroid gland (16). In these tissues, NADPH would probably be supplied by the hexose monophosphate shunt. The H_2O_2 formed during phagocytosis may be involved in the destruction of bacteria by leukocytes.

Free reduced flavins are reoxidized very rapidly by molecular oxygen to form H_2O_2 . This reactivity is retained with several flavoprotein oxidases such as glucose oxidase, D-amino acid oxidase, xanthine oxidase, urate oxidase, glycollate oxidase, monoamine oxidase, and others. Peroxisomes, a microbody subcellular fraction, are believed to be the site of hydrogen peroxide metabolism in the cell since many flavoprotein oxidases and catalase are located in these microbodies.

d. Drug-catalyzed peroxide formation

The high concentration of oxyhemoglobin in erythrocytes can be a powerful source of oxygen for the oxidation of various hydrogen donors, e.g., drug metabolites. Hydrogen peroxide readily forms in erythrocytes by the autoxidation of administered drug metabolites. In individuals with glucose-6-phosphate dehydrogenase deficiency, this can lead to hemolysis. The drugs that are readily autoxidized in erythrocytes include antimalarials, primaquine and other aminoquinolines, phenylhydrazine, menadione hydroxylamine, and a drug found in fava beans (17). Aniline, acetanilide, phenacetin, and sulfonamide are also converted in vivo into autoxidizable derivatives (18).

Most drugs and foreign compounds are metabolized in the microsomal fraction of liver. The reaction requires NADPH and O_2 and is catalyzed by the oxygen-activating enzyme known as cytochrome P-450. Lipid peroxidation is known to occur in liver microsomes during the enzymic oxidation of NADPH (14).

Administration of carbon tetrachloride to rats produces considerable accumulation of fat in liver. The necrogenic effect of CCl_4 is dependent on a limited metabolism of the solvent to a more toxic product. It has been suggested that this activation occurs in the endoplasmic reticulum by an interaction of carbon tetrachloride with an endogenous radical. A sequence of reactions is postulated whereby carbon tetrachloride is homolytically split and the free radicals produced initiate peroxidation of the unsaturated fatty acid side

chains of structural lipids in the endoplasmic reticulum. It has been suggested that the NADPH-ADP-Fe²⁺ system of liver microsomes is a possible route for such an activation stage resulting in subsequent propagation of lipid peroxidation (19).

3. Lipid peroxidation in subcellular fractions

Lipid peroxidation occurs very rapidly in many tissues as soon as the cells are damaged, e.g., during homogenization. Subcellular particles or cell suspensions readily undergo lipid peroxidation when incubated with metal ions (11), ascorbate (20), pyridine nucleotides (14), hydrogen peroxide (21), GSH (21), CCl₄ (19), or by the effects of ionizing irradiation (1). Peroxidation has been demonstrated in mammalian liver preparations including homogenates, mitochondria, microsomes, and microsomes + cytosol fractions (22).

Rat liver mitochondria and brain mitochondria readily undergo peroxidation in the presence of Fe²⁺ (23, 24). In liver, lipid peroxidation is associated with ascorbate or GSH + GSSG-induced swelling of mitochondria. Tappel (25) has reported lipid peroxidation in the lysosomal fraction of mammalian liver. Hochstein and Ernster (14) found an enzymic system in liver microsomes which catalyzes the peroxidation of microsomal lipids in the presence of O₂, NADPH, ADP, and Fe²⁺.

4. Consequences of Peroxidation

Hydrogen peroxide formation in biological systems has been associated with very few pathological changes since the toxic effects of H_2O_2 are somewhat limited due to the enzyme catalase which decomposes the peroxide rapidly to molecular oxygen. In contrast, lipid peroxides are not destroyed by a catalase mechanism (26).

Lipid peroxidation in biological systems is associated with many pathological changes such as hyperoxia, hemolysis, ageing, atherosclerosis, formation of fatty liver, thrombosis, irradiation damage, skin inflammation, and muscular dystrophy associated with vitamin E or selenium deficiency (see review by Barber (1)). The administration of antioxidants could prevent some of these effects and is indirect evidence that peroxidation may be responsible for these pathological changes.

Hydroperoxides are a source of damaging free radicals that can destroy many biological compounds (4, 25). The damaging effects of lipid peroxides on biochemical cellular constituents and subcellular fractions have been reported (23-27). Lipid peroxidation in subcellular fractions leads to increased membrane permeability, lysis, and formation of membrane ghosts (23, 25). Tappel (25) has demonstrated inactivation of mitochondrial enzymes, damage to cytochromes, and release of hydrolases from lysosomes as a result of in vitro lipid peroxidation. Hochstein and Ernster (27) have reported inactivation

by peroxides of enzymes associated with the membranes of the endoplasmic reticulum. Many enzymes believed to contain essential thiol groups were inactivated as a result of lipid peroxidation in subcellular fractions (28, 29). The inactivation of enzymes by lipid peroxides has been shown to be usually the result of the oxidation of essential thiol groups by the peroxide or by oxidizing radicals formed during peroxide decomposition (28-30).

5. Peroxide decomposition

Hydrogen peroxide in the erythrocyte is decomposed rapidly by the enzymes catalase and glutathione peroxidase (31). Many hemoprotein peroxidases that rapidly decompose hydrogen peroxide have been found in other tissues (32, 33).

The lipid peroxides formed in tissues are rapidly decomposed (1, 25, 34-37). Recently, O'Brien (34) has studied the decomposition of linoleic acid hydroperoxide by metal ions, heme compounds, and nucleophiles in an attempt to understand further the intracellular mechanisms for peroxide decomposition. Two types of mechanisms were associated with the decomposition of the hydroperoxide by subcellular fractions: 1) a non-enzymic and cytochrome-catalyzed decomposition by the mitochondrial and microsomal fractions resulting in a complex range of products presumably formed as a result of interaction of free radical intermediates; 2) a reduction of the hydroperoxide by glutathione

of the cytosol fraction catalyzed by glutathione peroxidase and resulting in the formation of monohydroxy linoleic acid (34, 35).

The relative importance of each of these pathways in the cell cannot be assessed. The cytosol-located glutathione peroxidase with its wide hydroperoxide specificity (36) could probably catalyze the interaction in the cell between glutathione and hydroperoxides of intracellular membrane phospholipids. The resulting formation of hydrophilic hydroxy fatty acids could affect membrane function and the oxidized glutathione formed would be expected to decrease intracellular NADPH and activate the hexose monophosphate shunt (37). The absence of glutathione in the mitochondrial matrix (P.C. Jocelyn, personal communication) suggests that the glutathione peroxidase of the matrix (38) has no effect on hydroperoxides formed by the mitochondrial inner membrane and that the cytochrome-catalyzed mechanism of peroxide decomposition predominates.

Tam and McKay (39) have shown that the phospholipid peroxides formed enzymically when microsomes are incubated with NADPH and ADP rapidly undergo cleavage producing a variety of carbonyl-containing residues. The resulting membrane disruption could be important in vivo in organelle ageing. The complex range of products formed suggests that the decomposition of hydroperoxides involves

free radical intermediates which in the cell would be expected to oxidize intracellular hydrogen donors as well as inactivate enzymes. The membrane-bound peroxidases responsible for lipid peroxide decomposition could therefore be important intracellular sites of free radical production and consequently sites of hydrogen donor oxidation.

In the following work, the peroxidase responsible for the rapid lipid peroxide decomposition by the endoplasmic reticulum of the liver cell has been isolated, characterized, and identified. Linoleic acid hydroperoxide was used as a substrate and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) as the hydrogen donor.

MATERIALS AND METHODS

Materials

Linoleic acid, nucleotides, trypsin, trypsin inhibitor, p-hydroxymercuribenzoate, heme compounds, glucose-6-phosphate dehydrogenase, lysolecithin, sodium dodecyl sulfate, sodium cholate, sodium deoxycholate, and DEAE-cellulose were purchased from Sigma. TMPD was obtained from British Drug Houses, Toronto. Cumene hydroperoxide was supplied by Matheson, Coleman and Bell, Cincinnati. Lubrol WX was kindly donated by Imperial Chemical Industries, Providence, Rhode Island. CO was prepared from sodium formate and concentrated sulfuric acid and purified by passing through an NaOH solution. All other reagents were of the highest grade commercially available.

Preparation of LAHPO

The method of O'Brien (34) was followed for the preparation and purification of LAHPO. The concentration was determined by measuring the extinction at 233 nm in ethanol ($E_{233\text{nm}}^{\text{mM}} = 25.25/\text{cm}$) (40).

Assay of peroxidase activity

Peroxidase activity was assayed at 23° with TMPD as hydrogen donor, using a system containing 0.05 M Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM TMPD, 50 μM LAHPO and a suitable amount of catalyst in a final volume of 3 ml. The rate of TMPD

oxidation was followed spectrophotometrically at 610 nm in the first minute of reaction by measuring the formation of Wurster's blue free radical (41). Calculation of the rate of TMPD oxidation was made using an extinction coefficient of $11.6 \text{ cm}^{-1} \text{ mM}^{-1}$ (41). In all cases, the rate of the reaction in the absence of LAHPO was subtracted.

Preparation of microsomes

Male albino rats (200-250 g) of the Wistar strain were fasted for 10 hours prior to sacrifice and the livers thoroughly perfused in situ with 0.9% NaCl solution. A 10% (w/v) homogenate was prepared in 0.3 M sucrose-2 mM EDTA (pH 7.4) and centrifuged at $11,700 \times g$ for 20 minutes in a refrigerated centrifuge. The pellet was discarded and the microsomes sedimented by centrifugation at $105,000 \times g$ for 60 minutes. The microsomes were resuspended in 0.15 M KCl - 2 mM EDTA (pH 7.4) and again centrifuged as above. The washed microsomes were finally suspended in 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 25% (v/v) glycerol unless otherwise stated, and were stored anaerobically at -20° until further use. Such preparations were free from hemoglobin contamination and could be stored for several weeks without any appreciable loss of peroxidase activity.

Preparation of "P-450 particles"

"P-450 particles" free from cytochrome b_5 and hemoglobin

contamination were prepared from perfused liver microsomes by anaerobic digestion with trypsin according to the method of Ichikawa and Yamano (42). The "P-450 particles" were suspended in 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 25% (v/v) glycerol and stored at -20° until further use. Such preparations could be stored for several weeks without any appreciable loss of peroxidase activity.

Preparation of cytochrome P-420

High spin P-420 was prepared by anaerobic incubation of "P-450 particles" (5 mg protein/ml) with 1.5% (w/v) lubrol WX and 2 mM p-hydroxymercuribenzoate in 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 3% (v/v) glycerol. The mixture was stirred in ice for 60 minutes and then used immediately. The preparation was designated "high spin" P-420 (43, 44).

Low spin P-420 was prepared by anaerobic incubation of "P-450 particles" (5 mg protein/ml) with 0.5% (w/v) sodium deoxycholate in 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 3% (v/v) glycerol. The mixture was stirred in ice for 30 minutes and then used immediately. The preparation was designated "low spin" P-420 (42,45).

Preparation of oxyhemoglobin

Oxyhemoglobin was prepared from pig blood by application of the hemolysate, obtained by the method of Little et al

(46), onto a DEAE-cellulose column (5 x 45 cm) previously equilibrated with 0.1 M Tris-HCl (pH 7.5). The oxyhemoglobin was eluted with Tris buffer containing 0.1 M KCl. The concentration was determined by measuring the extinction at 415 nm ($E_{415nm}^{mM} = 128/cm$) (47). The preparation was found to be devoid of GSH peroxidase activity, assayed as described by Little et al (46).

Phenobarbital administration

Male albino rats (150-200 g) were injected intraperitoneally with phenobarbital (50 mg/kg body wt) twice daily for 5 days as described by Ernster and Orrenius (48). Microsomes were prepared from perfused liver and peroxidase activity, cytochrome b_5 and P-450 contents measured. Control rats were injected with 0.9% NaCl. Determinations were made on the pooled livers of 4 rats.

Measurement of P-450 destruction by LAHPO

A mixture of microsomes (15 mg protein/ml) was treated anaerobically for 30 minutes at 23° with 1.4% (w/v) lubrol WX in 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 25% (v/v) glycerol. The lubrol-treated microsomes (2.5 mg protein/ml) containing 0.6nmoles P-450 per mg protein were incubated for 10 minutes at 23° with 0.1 mM LAHPO in 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 12% glycerol in a final volume of 3 ml. After incubation, the CO-difference

spectra of dithionite-treated samples were measured and the destruction of P-450 determined.

Measurement of inhibition of enzymic demethylation by LAHPO

A suspension containing 0.4 ml microsomes (16.5 mg protein/ml), 0.1 ml of 30 mM EGTA, 0.1 ml of 1.5 M Tris-HCl (pH 7.4), varying concentrations of LAHPO, and deionized water in a final volume of 1.3 ml was incubated at 37° for 5 minutes. Control incubations with linoleic acid substituted for LAHPO were performed. The amount of enzymic demethylation of p-chloro-N-methylaniline was determined after incubation for 20 minutes essentially by the method of Kupfer and Bruggeman (49).

Analytical methods

Cytochromes P-450 and P-420 were determined from the CO-difference spectrum of dithionite-treated samples as described by Imai and Sato (50). Cytochrome b_5 in microsomes was determined from the difference spectrum between the oxidized and NADH-reduced form. For the determination of solubilized cytochrome b_5 , the difference spectrum between the oxidized and dithionite-reduced sample was measured. Calculation of the content was made using an extinction coefficient increment ($\Delta E_{424-409 \text{ nm}}$) of $185 \text{ cm}^{-1} \text{ mM}^{-1}$ (51).

NADPH-cytochrome c reductase was assayed by the method of Williams and Kamin (52). NADH-cytochrome c reductase was assayed at 23° by measuring the reduction of cytochrome c

(50 μ M) at 550 nm in a 3 ml system containing 0.05 M Tris-HCl (pH 7.4), 0.1 mM NADH and enzyme. Succinate dehydrogenase was assayed by the method of Pennington (53). Protein was determined by the method of Lowry et al (54) using bovine serum albumin as standard.

Solubilization of peroxidase activity, cytochrome P-420, cytochrome b_5 , and microsomal enzymes by deoxycholate and elution from a DEAE-cellulose column

Microsomes were prepared from perfused rat liver and suspended in 0.3 M sucrose - 2 mM EDTA (pH 7.5). Microsomal constituents were solubilized by deoxycholate treatment by a modification of the method of Lu et al (55). A microsomal suspension (15 mg protein/ml) was incubated anaerobically at 0° for 60 minutes with 1% (w/v) sodium deoxycholate in 0.1 M sodium citrate buffer (pH 7.5) containing 15% (v/v) glycerol, 2 mM EDTA and 0.15 M sucrose in a final volume of 50 ml. The suspension was centrifuged for 2 hours at 105,000 x g and the pellet was discarded. The deoxycholate solubilization converted over 90% of microsomal cytochrome P-450 into the modified P-420 form which was recovered exclusively in the supernatant fraction. The supernatant layer contained over 90% of the total protein and 90% of the peroxidase activity originally present in the incubation medium.

The microsomal extract was filtered through cheese-cloth, diluted with 3 volumes of cold deionized water, and

placed onto a DEAE-cellulose column (2.5 x 80 cm) previously equilibrated with 0.1 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 0.05% (w/v) deoxycholate. A fraction containing no peroxidase activity was eluted from the column with 1 liter of starting buffer containing 0.1 M KCl. A fraction which contained cytochrome P-420 and peroxidase activity was then eluted with 1 liter of buffer solution containing 0.2 M KCl. Cytochrome P-420 and peroxidase activity was also eluted with 1 liter of buffer mixture containing 0.3 M KCl. A fraction containing cytochrome b₅, P-420, peroxidase activity, and NADH-cytochrome c reductase was eluted with 1 liter of a similar solution containing 0.5 M KCl. All operations were performed at 4°.

RESULTS

The peroxidase assay

The peroxidase reaction was investigated using TMPD as hydrogen donor, linoleic acid hydroperoxide (LAHPO) as substrate, and several heme compounds as catalysts. The oxidation of TMPD was followed at 610 nm by measuring the rate of formation of the Wurster's blue free radical (41). The initial reaction rate was found to be fairly constant in the first minute of reaction but the rate gradually fell off with time because of catalyst destruction by LAHPO.

There are several advantages in using TMPD as hydrogen donor in the peroxidase assay: 1) the oxidation of TMPD results in the formation of mainly one oxidation product, the Wurster's blue free radical (41), which can be conveniently measured in aqueous solution at 610 nm; 2) the Wurster's blue free radical is very stable (41) even at low pH values; 3) the reaction rate in the absence of peroxide is almost negligible; 4) the reaction rate in the absence of catalyst is almost negligible.

The peroxidase properties of several heme compounds such as cytochrome c, methemoglobin, myoglobin, and hematin were examined. Maintaining the TMPD concentration at 0.2 mM, the peroxidase reaction was found to be first-order with respect to catalyst concentration and first-order with respect to LAHPO concentration. The range of LAHPO concentrations investigated was 5 μ M to 75 μ M.

The effect of pH on the peroxidase activity of

several heme compounds was also examined. Hemin, methemoglobin, and myoglobin were found to have an acid pH optimum between pH 4.5 and 4.7 whereas cytochrome c exhibited maximal catalytic activity at pH 6.2.

The efficiency of TMPD oxidation by LAHPO in the presence of an excess amount of heme catalyst was investigated using a 10-fold excess of TMPD to LAHPO. The stoichiometry was determined under anaerobic conditions to prevent autoxidation of TMPD. It was found that approximately 1.4 moles of TMPD were oxidized to Wurster's blue per mole of LAHPO in the presence of cytochrome c or methemoglobin. As LAHPO has two oxidizing equivalents, this represents a 70% efficiency. Since peroxides are known to attack readily hematin rings of hemoproteins (26, 34), the hematin group of cytochrome c or methemoglobin probably competes with TMPD for the oxidizing species formed during peroxide decomposition. TMPD has two reducing equivalents (56) and it was found that Wurster's blue itself could be oxidized by relatively high concentrations of LAHPO to a colorless oxidation product. This further oxidation of Wurster's blue was found to be a very slow reaction compared to the rate of oxidation of TMPD. In view of the relative rates of oxidation of TMPD and Wurster's blue and the high ratio of TMPD/LAHPO used, it is unlikely that any significant oxidation of Wurster's blue occurred during the measurements of the stoichiometry or during the peroxidase assay. Many hydrogen donors other than TMPD were oxidized by LAHPO in the presence of various hemoproteins (30, 37).

TABLE I

SUBCELLULAR DISTRIBUTION OF PEROXIDASE ACTIVITY IN RAT LIVER^a

<u>Fraction</u>	<u>Peroxidase Activity</u>		<u>Succinate Dehydrogenase</u>		<u>NADPH-cyt. c Reductase</u>	
	<u>% of total</u>	<u>Units^b/mg protein</u>	<u>% of total</u>	<u>Units^c/mg protein</u>	<u>% of total</u>	<u>Units^b/mg protein</u>
Mitochondrial	58	16	77	81	11	4
Lysosomal	9	5	5	10	20	15
Microsomal	22	8	3	4	69	33
Soluble	11	1	15	5	0	0

^a Subcellular fractions of perfused rat liver were prepared by the method of Sedgwick and Hubscher (66). The fractions were assayed for protein, peroxidase activity, succinate dehydrogenase, and NADPH-cyt. c reductase as described in "Materials and Methods". All fractions were sonicated prior to assay.

^b One unit is equivalent to an absorbance change of 0.01 per minute.

^c One unit is equivalent to an absorbance change of 1.0 per 15 minutes.

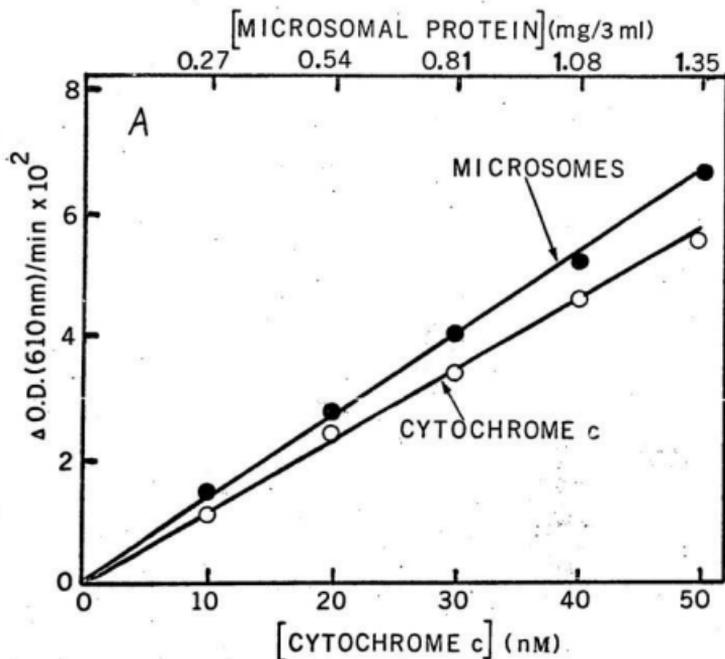


FIGURE 1A.

The effect of catalyst concentration on the rate of TMPD oxidation by LAHPO.

Peroxidase activity was measured with TMPD as hydrogen donor at 610 nm as described under "Assay of peroxidase activity".

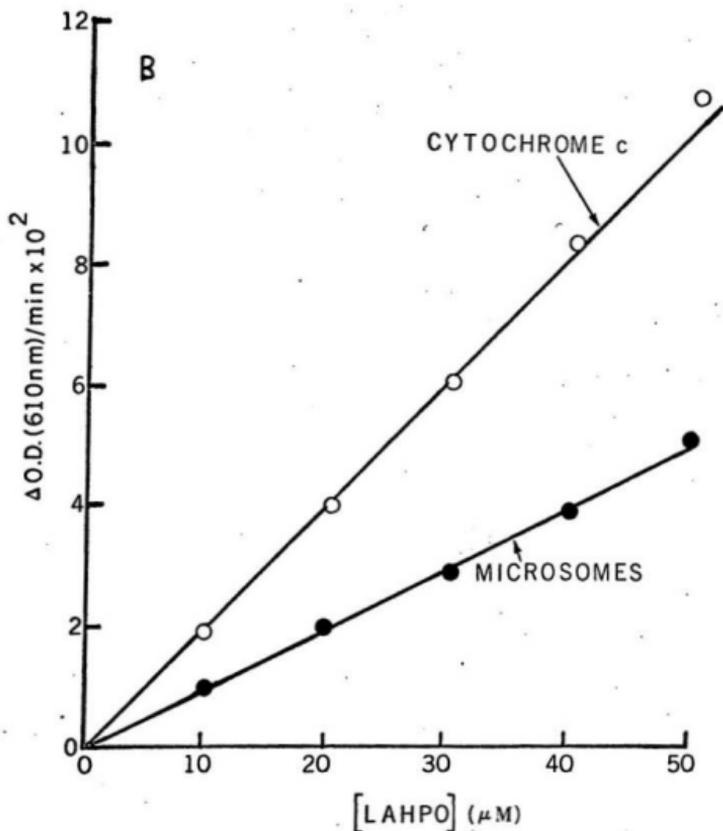


FIGURE 1B.

The effect of LAHPO concentration on the rate of catalyzed TMPD oxidation.

The peroxidase reaction was measured at 23° in a 3 ml solution containing 0.05 M Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM TMPD, 0.1 μM cyt. c or 1.1 mg microsomal protein, and indicated amounts of LAHPO. Reaction rates were corrected for TMPD oxidation in the absence of LAHPO.

Subcellular distribution of peroxidase activity in rat liver

The subcellular distribution of peroxidase activity, succinate dehydrogenase (a mitochondrial marker), and microsomal NADPH-cytochrome c reductase is presented in Table I. It is seen that 58% of the peroxidase content of the nuclei-free homogenate was recovered in the mitochondrial fraction and 22% in the microsomal fraction. The mitochondrial fraction exhibited twice the specific activity of the microsomal fraction. Cytochrome c may be partly responsible for the mitochondrial decomposition of the hydroperoxide since cytochrome c-deficient mitochondria were found to be 70% less effective at decomposing LAHPO (35). Hemoglobin appears to be a constituent of lysosomes (57) and may be partly responsible for the peroxidase activity of the lysosomal fraction. However, the intracellular distribution of microsomal NADPH-cytochrome c reductase showed that 20% of the activity was recovered in the lysosomal fraction and hence some of the peroxidase activity of lysosomes may be due to microsomal contamination. The properties of the microsomal peroxidase were next investigated in detail.

Properties of microsomal peroxidase

The kinetic properties of microsomal peroxidase were investigated using TMPD as hydrogen donor and LAHPO as substrate. Maintaining the TMPD concentration at 0.2 mM, the peroxidase reaction was found to be first-order with respect to catalyst concentration and first-order with respect to LAHPO concentration. The range of LAHPO concentrations investigated was 5 μ M to 75 μ M. Figure 1 presents the kinetics of the peroxidase reaction using liver microsomes and cytochrome c as catalysts.

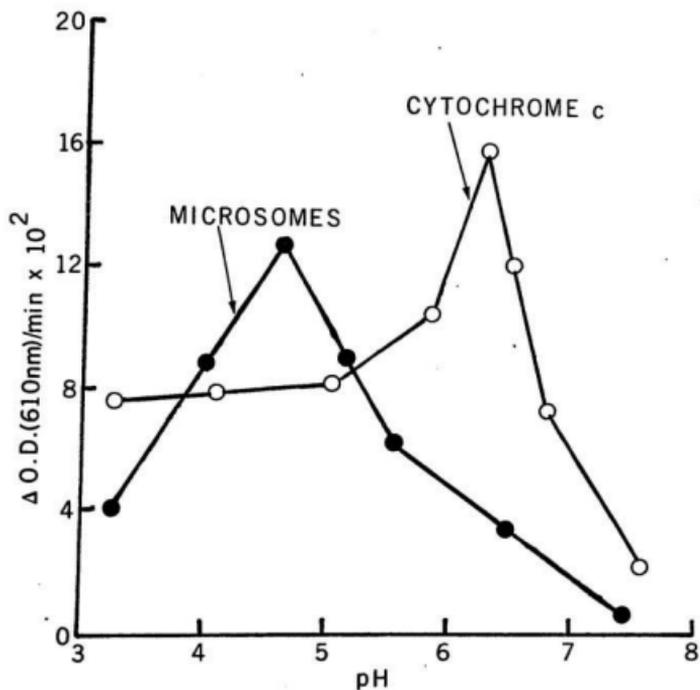


FIGURE 2.

The effect of pH on the peroxidase activity of liver microsomes and cyt. c.

The peroxidase reaction was measured at 23° in a 3 ml system containing 0.2 mM TMPD, 1 mM EDTA, 10 μM LAHPO, and 0.2 μM cyt. c or 0.53 mg microsomal protein. The pH was adjusted with HCl or NaOH.

TABLE II

PEROXIDE SPECIFICITY OF MICROSOMAL PEROXIDASE AND CYTOCHROME c^a

<u>Hydroperoxide</u>	<u>Peroxidase Activity</u>	
	Δ O.D. <u>610nm</u> /min	
	<u>Microsomes</u>	<u>Cytochrome <u>c</u></u>
LAHPO	0.110	0.115
Cumene hydroperoxide	0.044	0.002
H ₂ O ₂	0.006	0.004

^a Peroxidase activity was assayed at 23^o in a 3ml system containing 0.05 M Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM TMPD, 50 μ M hydroperoxide, and 2.3 mg microsomal protein or 0.1 μ M cyt. c. Reaction rates were corrected for TMPD oxidation in the absence of peroxide.

The effect of pH on the peroxidase activity of microsomes and cytochrome c is illustrated in Figure 2. Microsomes were shown to have an acid pH optimum of about 4.7 whereas cytochrome c exhibited maximal catalytic activity at pH 6.2.

A comparison of the peroxide specificity of microsomal peroxidase with that of cytochrome c is presented in Table II. It is shown that LAHPO is approximately 30 times more effective than H_2O_2 and nearly 60 times more effective than cumene hydroperoxide in oxidizing TMPD in the presence of cytochrome c. With microsomes as catalyst, however, LAHPO was found to be 20 times more effective than H_2O_2 and almost 3 times as effective as cumene hydroperoxide. Part of the effectiveness of LAHPO may be due to its surface-active properties which would enable it to expose the heme groups of microsomal cytochromes and enhance peroxidase activity (26).

Several hydrogen donors such as guaiacol, pyrogallol, o-dianisidine, hydroquinone, catechol, resorcinol, and others were tested in an attempt to obtain a suitable measurement of microsomal peroxidase activity but no satisfactory reaction rate at pH 7.4 could be obtained at the concentrations of LAHPO normally employed in the standard TMPD assay. Hence, the use of TMPD as hydrogen donor was the most satisfactory method available for measuring the peroxidase reaction under the conditions described.

TABLE III

EFFECT OF VARIOUS MODIFIERS ON PEROXIDASE ACTIVITY^a

<u>Modifier</u>	<u>Peroxidase Activity (%)</u>			
	<u>Microsomes</u>	<u>Methemoglobin</u>	<u>Hematin</u>	<u>Cytochrome c</u>
None	100	100	100	100
<u>p</u> -Hydroxymercuribenzoate (0.1 mM)	260	74	83	87
EDTA (10 mM)	101	100	100	100
Lubrol WX (0.004%)	130	150	100	0
Triton X-100 (0.002%)	99	140	100	0
Cyanide (1 mM)	50	44	40	83
Cyanide (10 mM)	20	13	7	40
Azide (10 mM)	90	64	71	73
Boiling ^b	55	20	84	65
Imidazole (1 mM)	77	110	120	111
Aniline (100 mM)	170			
Pyridine (100 mM)	168			
Aminopyrine (10 mM)	101			

- a Peroxidase activity was determined using different catalysts in the presence of specified conc. of various modifiers. Assays were carried out as described under "Assay of Peroxidase Activity". The conc. of catalysts used were: microsomes (1.1 mg protein/3 ml reaction vol.); methemoglobin (0.03 μ M); hematin (0.1 μ M); and cyt. c (0.1 μ M).
- b Catalysts were heated for 10 minutes at 90° and sonicated prior to assay.

Table III compares the effects of various modifiers on the peroxidase activity of liver microsomes and several heme compounds. It is seen that EDTA had no effect on the peroxidase activity of microsomes and heme compounds and, therefore, inorganic metals are unlikely to be involved. The microsomal peroxidase was inhibited only 45% by boiling which rules out an enzymic microsomal peroxidase such as lactoperoxidase or myeloperoxidase. The sulfhydryl reagent, *p*-hydroxymercuribenzoate, produced a 2.6-fold stimulation of microsomal peroxidase activity but exerted an inhibitory effect on the catalytic activities of methemoglobin, hematin, and cytochrome c. The nonionic detergents, lubrol WX and Triton X-100, completely abolished the peroxidase activity of cytochrome c, had no effect on the hematin-catalyzed reaction and stimulated the catalytic activity of methemoglobin. The catalytic activity of microsomes was unaffected by Triton X-100 but was enhanced by lubrol WX. The differences in catalytic properties of the various catalysts indicate that the peroxidase activity of microsomes is not due to hematin or contaminating cytochrome c or hemoglobin.

Cyanide (10 mM) exerted a strong inhibitory effect on the catalytic activities of the heme compounds and inhibited the microsomal peroxidase 80%, which suggests that a hemoprotein or heme compound may be responsible for the peroxidase activity of microsomes. Azide produced a somewhat less pronounced

inhibition. Since the two hemoproteins, cytochrome b₅ and hemoprotein P-450, account for virtually all of the protoheme in the microsomal fraction of liver (51) and only cytochrome P-450 combines with cyanide (44, 58), this indicates a possible involvement of P-450 in the peroxidase mechanism of liver microsomes.

The effects of hemichrome formation were investigated (Table III). Imidazole produced a slight reduction in microsomal activity but stimulated the activities of the other catalysts. Imidazole is known to form hemichromes with various heme compounds including cytochrome P-450 (42). Aniline and pyridine, which are known to interact with cytochrome P-450 in liver microsomes to produce Type II spectral changes (58), stimulated the activity of microsomes about 1.7-fold. In contrast, aminopyrine, which produces Type I spectral changes in liver microsomes (58), had no effect.

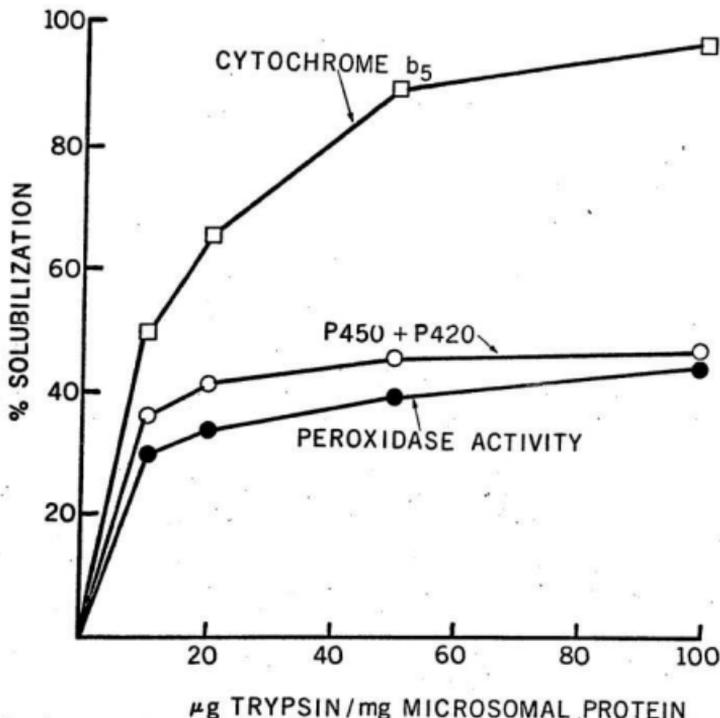
These results seem to indicate that a heme compound other than methemoglobin, cytochrome c, or hematin is responsible for the peroxidase activity of liver microsomes. As will be seen later, reagents that cause an increase in the activity of liver microsomes (e.g., p-hydroxymercuribenzoate, lubrol WX, aniline) are also able to convert hemoprotein P-450 into the modified P-420 state (43, 44, 50, 59).

Purification of microsomal peroxidase activity

Anaerobic digestion of liver microsomes with trypsin has been shown to solubilize cytochrome b₅ completely and to

FIGURE 3.

Solubilization of peroxidase activity from liver microsomes by trypsin treatment.



A mixture of microsomes (10 mg protein/ml) containing 0.6 nmoles P-450 per mg protein, 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 25% (v/v) glycerol, and indicated amounts of trypsin was incubated anaerobically at 4° for 12 hr. The reaction was stopped by addition of trypsin inhibitor (1 mg/mg trypsin). Cyt. b₅, P-450 and P-420, and peroxidase activity were determined from a portion of the digest whereas the rest was centrifuged at 105,000 x g for 2 hr. The % solubilization of cyt. b₅, P-450 and P-420, and peroxidase activity was determined from the distribution of these components in the supernatant and pellet fractions.

convert cytochrome P-450 into the modified P-420 form (60). Fig. 3 illustrates the effectiveness of trypsin in solubilizing cytochrome \underline{b}_5 , P-450 and P-420, and peroxidase activity from liver microsomes. A striking resemblance in the pattern of solubilization of peroxidase activity and P-450 plus P-420 is observed. The trypsin digestion solubilized about 40% of the total peroxidase activity and this corresponded to the amount of P-450 plus P-420 released. Over 95% of the cytochrome \underline{b}_5 was released, about 60% of the protein was solubilized, and 35% of the P-450 was converted into the modified P-420 state. The trypsin treatment also enhanced peroxidase activity about 2-fold, probably as a result of P-450 conversion to P-420.

The pellets obtained after centrifugation of the digest retained most of the peroxidase activity and contained most of the P-450 plus P-420 still attached to the microsomal membrane. The results indicate that cytochrome P-450 plus P-420, rather than cytochrome \underline{b}_5 , is responsible for the observed peroxidase activity of liver microsomes during trypsin digestion.

The two hemoproteins, cytochrome \underline{b}_5 , and P-450, are present in approximately equal amounts in liver microsomes and account for virtually all of the iron protoporphyrin in the fraction (51, 61). P-450 is very labile to a variety of treatments by which it is converted to a spectrally modified form called P-420 (51, 59, 60). However, satisfactory preparations

of hepatic microsomal P-450 practically free from cytochrome b_5 and P-420 have been obtained by several investigators using different procedures (42, 44, 60). Such preparations were termed "P-450 particles" because of their high specific content of P-450 relative to cytochrome b_5 .

In this report, "P-450 particles" were prepared by trypsin digestion of liver microsomes according to the procedure of Ichikawa and Yamano (42). The preparation was found to contain about 0.7 nmoles P-450 per mg protein and a negligible amount of cytochrome b_5 . The content of P-420 in the particles represented not more than 5% of the total heme. The peroxidase activity of microsomal "P-450 particles" corresponded closely to that originally present in liver microsomes when activities were expressed per mole of P-450. Under the standard conditions of the peroxidase assay (see "Assay of peroxidase activity"), approximately 30 moles of TMPD were oxidized per minute per mole of P-450. On the other hand, when a purified preparation¹ of cytochrome b_5 was used in the peroxidase assay, about 6 moles of TMPD were oxidized per minute per mole of cytochrome b_5 . Hence, cytochrome P-450 appears to be responsible for most of the peroxidase activity of "P-450 particles" and liver microsomes.

The effect of phenobarbital administration to rats on microsomal peroxidase activity

Ernster and Orrenius (48) have demonstrated the induction

of cytochrome P-450 and other constituents of liver microsomes by phenobarbital administration to rats. On the other hand, cytochrome b_5 showed no appreciable increase in specific content. In this study, administration of 50 mg phenobarbital per kg body weight twice daily for 5 days increased the specific activity of peroxidase in liver microsomes about 2.5-fold. This activation was accompanied by a parallel increase in specific content of P-450 whereas cytochrome b_5 showed no appreciable increase in amount.

Destruction of P-450 by LAHPO

It was reported previously (26, 34, 35) that LAHPO is decomposed by heme compounds which are themselves damaged in the reaction and that the microsomal decomposition of LAHPO also results in catalyst inactivation. In our study, the effect of LAHPO on cytochrome P-450 in liver microsomes was examined and further evidence indicating that P-450 is responsible for LAHPO decomposition was found. Microsomes suspended in Tris-EDTA-lubrol mixture (pH 7.4) were incubated with LAHPO in the presence of glycerol (see "Materials and Methods") and the P-450 content was determined. Lubrol WX was used in the incubation medium because of its clarifying and stabilizing action on microsomes whereas glycerol was included to prevent conversion of P-450 to P-420 by lubrol WX (43, 44). It was noticed that at 23^o, incubation of 40 nmoles LAHPO per mg protein resulted in 58% destruction of microsomal hemoprotein P-450 at pH 7.4.

Since cytochrome P-450 is involved in many drug hydroxylation and demethylation reactions (62), it was decided to determine the effect of LAHPO on the demethylation activity of liver microsomes using linoleic acid as a control. Liver microsomes were incubated with LAHPO for 5 minutes at 37° as described in "Materials and Methods". After incubation, the amount of enzymic demethylation of p-chloro-N-methylaniline was determined. It was observed that 0.1 mM LAHPO inhibited enzymic demethylation 43%, probably as a result of P-450 destruction, whereas the same concentration of linoleic acid reduced demethylation activity only 19%. The linoleic acid-induced inhibition of demethylation may have occurred as a result of linoleic acid hydroperoxide formation from linoleic acid during the incubation period which would result in the subsequent destruction of P-450, or the inhibition may be due to the binding of linoleic acid to P-450 which would consequently lead to inhibition of the demethylation reaction (63).

Changes in microsomal peroxidase activity associated with the interconversion of P-450 and P-420

The conversion of P-450 to P-420 in liver microsomes has been shown to be caused by a wide variety of reagents such as proteases, sulfhydryl reagents, surface-active agents, lipophilic substances, chelating agents, strong oxidizing agents, protein denaturants, and acidic or alkaline pH (42-45, 50, 59-61, 64, 65). Since the peroxidase activity of liver microsomes was previously found to be stimulated by several of

TABLE IV

EFFECT OF REAGENTS WHICH CONVERT CYTOCHROME P-450 TO P-420 ON MICROSOMAL PEROXIDASE ACTIVITY^a

<u>P-450 Modifier</u>	<u>Peroxidase Activity(%)</u>	<u>Conversion to P-420(%)</u>
None	100	0
Lysolecithin (0.4%)	640	80
N-Bromosuccinimide (0.4 mM)	360	48
p-Hydroxymercuribenzoate (0.4 mM)	260	64
Iodine (0.2 mM)	220	45
Trypsin ^b (0.1%)	240	70
Sodium Dodecyl Sulfate (0.1%)	370	100
Urea (4 M)	142	55
Triton X-100 (0.1%)	104	3
Sodium Deoxycholate (1%)	194	100
Sodium Cholate (1%)	164	60
Sodium Cholate (1%) + 25% Glycerol ^c	107	12
Acid Treatment ^d (pH 3)	340	
Alkali Treatment ^d (pH 11)	280	

-
- a Microsomes at a conc. of 5.3 mg protein per ml were incubated at 23° for 15 minutes with specified conc. of various reagents in 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 0.06 M sucrose. After incubation the suspensions were diluted with an equal vol of 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA. The peroxidase activity was measured and the extent of conversion of P-450 to P-420 determined.
- b The incubation time was 30 minutes.
- c Microsomes were incubated with sodium cholate under the conditions described above. After incubation the test suspension was diluted with an equal vol of 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 50% (v/v) glycerol. The peroxidase activity and the extent of conversion of P-450 to P-420 were determined.
- d A solution of microsomes containing 10.6 mg protein per ml and 1 mM EDTA was incubated with acid or alkali at the specified pH for 2 minutes at 23°. After incubation the solution was neutralized with alkali or acid and peroxidase activity determined on a portion of the mixture.

these reagents (see Table III), it was decided to determine the extent of conversion of P-450 to P-420 and the effect of these reagents on microsomal peroxidase activity. The results (Table IV) illustrate that reagents which converted P-450 to P-420 also enhanced peroxidase activity. However, reagents which are known to convert cytochrome P-450 to a high spin form of P-420 (e.g., lysolecithin, N-bromosuccinimide, iodine, p-hydroxymercuribenzoate) (42-45, 50, 65), produced a 4- to 8-fold² increase in peroxidase activity whereas substances that convert cytochrome P-450 to low spin P-420 (e.g., deoxycholate, cholate, trypsin) (42, 43, 45) caused only a 2- to 3.5-fold stimulation². Lysolecithin, N-bromosuccinimide, and iodine exerted the most pronounced stimulation whereas the nonionic detergent, Triton X-100, produced no appreciable effect.

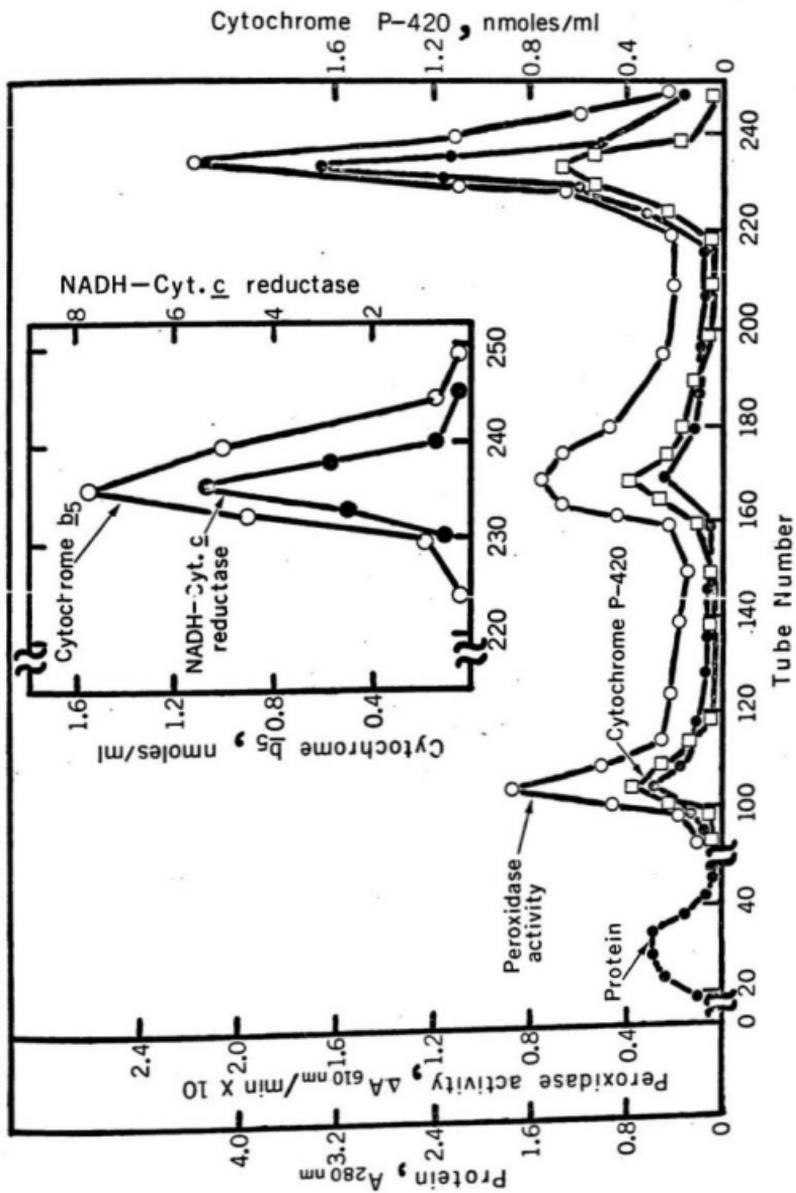
Ichikawa and Yamano (45) have reported that polyols such as glycerol can reconvert detergent-produced P-420 to P-450 under appropriate conditions. Therefore, the effect of glycerol on the cholate-induced conversion was examined (Table IV). It was found that the cholate-induced conversion could be nearly completely reversed by glycerol. Furthermore, the enhanced peroxidase activity of the cholate-treated microsomes was decreased to normal levels as a result of the reversion of P-420 to P-450.

Several investigators (42-45, 50) have demonstrated that the conversion of P-450 to P-420 is affected profoundly by pH of the medium. At pH values lower than 6 and higher than 8, some conversion to P-420 was observed which became irreversible at extreme pH values. When liver microsomes were incubated at acid or alkali pH and the suspensions neutralized after incubation, approximately a 3-fold stimulation of peroxidase activity accompanied the conversion of P-450 to P-420 (Table IV).

The above results seem to indicate that the conversion of P-450 to P-420 by various treatments is responsible for the observed increase in peroxidase activity of liver microsomes.

Elution of peroxidase activity, cytochrome P-420, and microsomal components from a column of DEAE-cellulose

Lu et al (55) have reported resolution of hepatic microsomal cytochromes P-450, P-420 and \underline{b}_5 by DEAE-cellulose chromatography of microsomes treated with sodium deoxycholate. In this study, chromatography of deoxycholate-treated rat liver microsomes on DEAE-cellulose was repeated using an excess of deoxycholate in order to convert hemoprotein P-450 into the modified P-420 state. Assays for cytochromes P-420 and \underline{b}_5 , microsomal enzymes, peroxidase activity, and protein were carried out on the various fractions (Fig. 4).



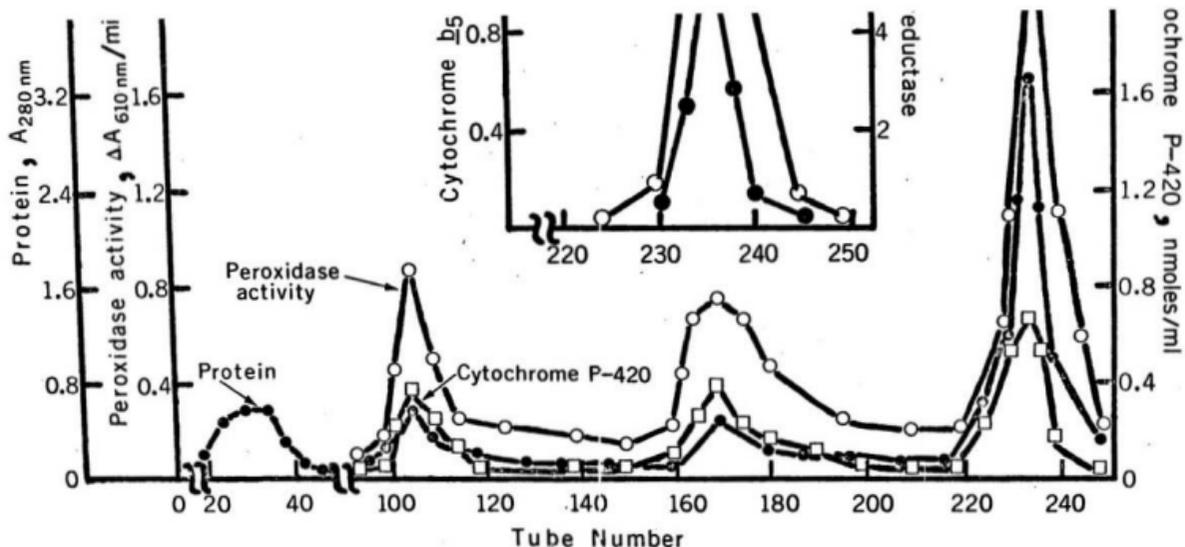


FIGURE 4.

Elution of peroxidase activity, cytochrome P-420, and several microsomal components from a column of DEAE-cellulose.

Perfused liver microsomes were treated with deoxycholate and the supernatant containing P-420, peroxidase activity, and microsomal components was eluted from a DEAE-cellulose column as described in "Materials and Methods"; 15 ml fractions were collected. Protein was measured at 280 nm whereas other components were assayed as described in "Materials and Methods". The NADH-cyt. c reductase activity ($\Delta A_{550\text{ nm}}/\text{min}$) and other values are expressed per ml of column effluent.

It is seen that four major protein bands emerged from the DEAE-cellulose column after deoxycholate treatment of microsomes (Fig. 4). Cytochrome P-420 was eluted from the column in the second protein peak (tube number 105) which also contained a considerable amount of peroxidase activity. In addition, P-420 was eluted in the third (tube number 170) and fourth (tube number 235) protein peaks which also contained peroxidase activity.

Although chromatography of microsomes treated with deoxycholate resolves several microsomal enzyme activities, it does not efficiently separate hemoprotein P-420 from cytochrome b₅ which has been reported to possess low peroxidase activity (see Purification of microsomal peroxidase activity in "Results" Section). Furthermore, cytochrome P-420 was somewhat degraded during the column run probably as a result of lipid peroxidation (51,64). Inclusion of EDTA in the buffer eluant did not suppress the destruction of the hemoprotein to any appreciable extent.

Peroxidase properties of cytochrome P-420

The peroxidase properties of cytochrome P-420, in its "high spin" and "low spin" forms, were next investigated using linoleic acid hydroperoxide (LAHPO) as substrate and TMPD as the hydrogen donor. Cytochrome P-420 was prepared from microsomal "P-450 particles" as described in "Materials and Methods". Maintaining the TMPD concentration at 0.2 mM, the peroxidase reaction was found to be first-order with respect to P-420

concentration and first-order with respect to LAHPO concentration, as previously reported for other heme catalysts (see The peroxidase assay in "Results" section). The range of LAHPO concentrations investigated was 5 μM to 60 μM . The pH optimum of the reaction could not be determined due to rapid appearance of turbidity at low pH, probably the result of aggregation of P-420 molecules (64). However, the catalytic effectiveness of P-420 was over 10 times greater at pH 6 than at pH 10. The low activity of P-420 at pH 10 is probably partly due to destruction of the hemoprotein in alkaline media (64).

The efficiency of TMPD oxidation by LAHPO in the presence of an excess amount of P-420 was investigated using a 10-fold excess of TMPD to LAHPO. The stoichiometry was determined under anaerobic conditions to prevent autoxidation of TMPD. It was found that approximately 1.5 moles of TMPD were oxidized to Wurster's blue per mole of LAHPO in the presence of "high spin" or "low spin" P-420. As LAHPO has two oxidizing equivalents, this represents a 75% efficiency. Since the heme rings of hemoproteins are very sensitive to attack by lipid peroxides (26, 34), the heme group of P-420 probably competes with TMPD for the oxidizing species formed during peroxide decomposition. TMPD has two reducing equivalents (56) and Wurster's blue was oxidized by relatively high concentrations of LAHPO to a colorless oxidation product. This further oxidation of Wurster's blue was found to be a very slow reaction compared to the rate of oxidation of TMPD. In view of the relative rates of oxidation of TMPD and Wurster's blue and the

TABLE V

PEROXIDE SPECIFICITY OF CYTOCHROME P-420^a

<u>Hydroperoxide</u>	<u>Peroxidase Activity</u>	
	<u>ΔO.D._{610nm}/min</u>	
	<u>"high spin" P-420</u>	<u>"low spin" P-420</u>
LAHPO	0.272	0.151
Cumene hydroperoxide	0.005	0.003
H ₂ O ₂	0.004	0.006

^a P-420 was prepared as described in "Materials and Methods". Peroxidase activity was assayed at 23° in a 3 ml solution containing 0.05 M Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM TMPD, 50 μM hydroperoxide and 0.12 μM "high spin" P-420 or 0.23 μM "low spin" P-420. Reaction rates were corrected for TMPD oxidation in the absence of peroxide.

TABLE VI

EFFECT OF MODIFIERS ON THE PEROXIDASE ACTIVITY OF P-420^a

<u>Modifier</u>	<u>Peroxidase Activity (%)</u>	
	<u>"high spin" P-420</u>	<u>"low spin" P-420</u>
None	100	100
EDTA (10 mM)	100	100
Pyridine (100 mM)	145	114
Cyanide (1 mM)	50	73
Cyanide (10 mM)	14	37
Azide (10 mM)	101	98
Fluoride (10 mM)	106	90
Imidazole (5 mM)	106	90
Boiling ^b	0	30

^a Peroxidase activity was determined as described in "Materials and Methods" using 0.12 μM "high spin" P-420 or 0.23 μM "low spin" P-420 and specified concentrations of various modifiers.

^b Catalysts were heated for 10 minutes at 90° and sonicated prior to assay.

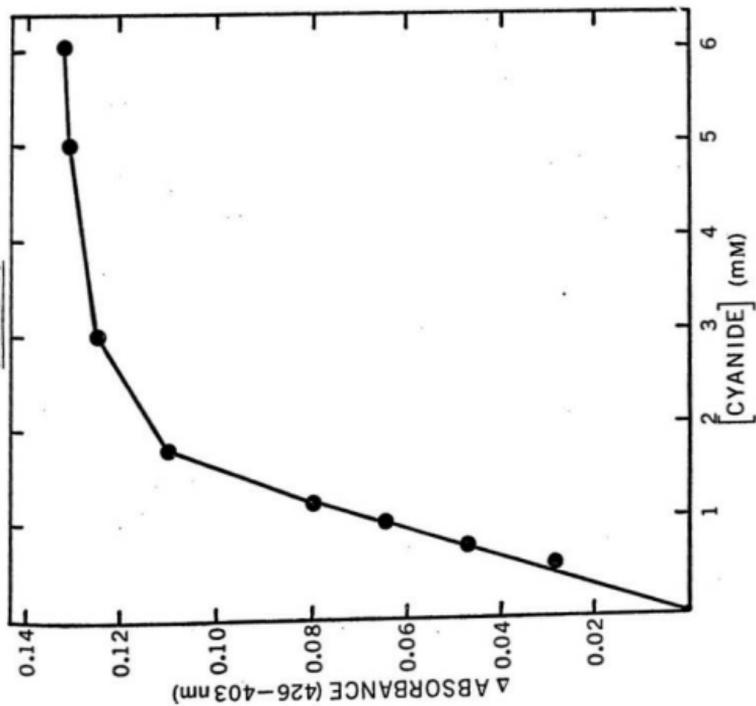
high ratio of TMPD/LAHPO used, it is unlikely that any significant oxidation of Wurster's blue occurred during the measurements of the stoichiometry or during the peroxidase assay. Many hydrogen donors other than TMPD are oxidized by LAHPO in the presence of various hemoproteins (30, 37).

The peroxide specificity of cytochrome P-420, in its "high spin" and "low spin" forms, is presented in Table V. It is shown that LAHPO is about 25 to 50 times more effective than cumene hydroperoxide and H_2O_2 in oxidizing TMPD in the presence of cytochrome P-420. Previous results (See Table II) also showed that LAHPO was much more effective than either cumene hydroperoxide or H_2O_2 in oxidizing TMPD in the presence of other hemoproteins. Part of the effectiveness of LAHPO may be attributed to its surface-active properties which might enable it to expose the heme groups of hemoproteins and enhance peroxidase activity (26).

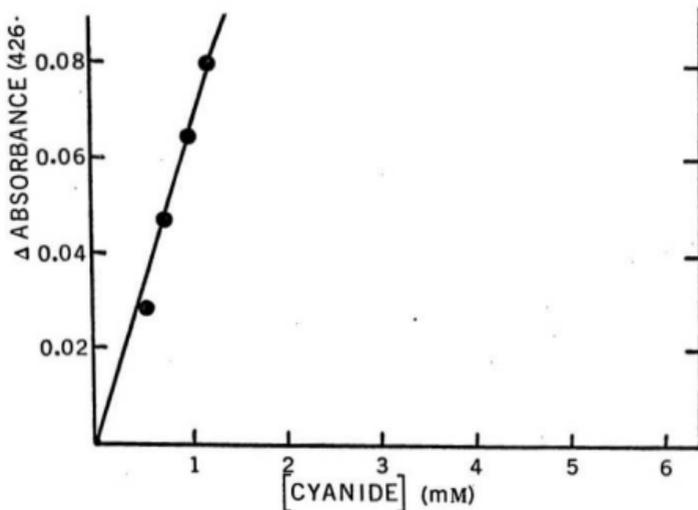
Table VI compares the effects of various modifiers on the peroxidase activities of "high spin" and "low spin" P-420. EDTA had little effect on the peroxidase activity of P-420 which suggests that inorganic metals are unlikely to be involved. The catalytic activity of "high spin" P-420 was completely abolished by boiling whereas the "low spin" hemoprotein-catalyzed reaction was inhibited to a lesser extent.

Cyanide (10 mM) exerted a strong inhibitory effect on both peroxidase activities, inhibiting the "high spin" hemoprotein-catalyzed reaction 86% and the "low spin" hemoprotein-catalyzed reaction 63%. Cyanide (1 mM) inhibited

FIGURE 5.



Cyanide saturation curve of oxidized "high spin" P-420.



Cyanide saturation curve of oxidized "high spin" P-420.

A mixture of "high spin" P-420 (2.5 mg protein/ml) suspended in 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA, 1 mM p-hydroxymercuribenzoate, 0.7% lubrol WX and 1.9 μ M P-420 was treated with cyanide and the difference spectrum measured at 23 $^{\circ}$ in a final vol of 1 ml.

the peroxidase activity of "high spin" P-420 by 50% and also inhibited the activity of microsomal hemoprotein P-450 to the same extent (see Table III). Pyridine stimulated the activities of both hemoproteins whereas fluoride, azide and imidazole produced no significant effect.

Since the "high spin" and "low spin" preparations of P-420 are free from cytochrome b_5 , contain no detectable hemoprotein other than P-420, and catalyze a cyanide-sensitive and an EDTA-insensitive peroxidase reaction, the peroxidase activity observed is probably mainly due to the catalytic effectiveness of P-420.

Cyanide difference spectrum of "high spin" P-420

Microsomal hemoprotein P-450 has a low binding affinity for cyanide (44) and yet the peroxidase activity of the hemoprotein is markedly inhibited by relatively low concentrations of cyanide (see Table III). Since cyanide exerted a strong inhibitory effect on the peroxidase activities of "high spin" and "low spin" P-420, the spectral properties and cyanide binding affinity of the two forms of P-420 were therefore examined. The difference spectrum of oxidized + cyanide versus oxidized "high spin" P-420 gave a peak at 426 nm and a trough at 403 nm. Titration of "high spin" P-420 with cyanide and measurements of the cyanide difference spectrum between λ_{\max} of 426 nm and λ_{\min} of 403 nm revealed a binding constant for cyanide of about 1 mM (Fig. 5). In addition, the peroxidase

TABLE VII

DESTRUCTION OF CYTOCHROME P-420 BY LAHPO^a

<u>Hemoprotein</u>	<u>Destruction by LAHPO (%)</u>
"high spin" P-420	59
+ 10 mM cyanide	36
"low spin" P-420	45
+ 10 mM cyanide	38

^a A mixture containing 2.5 mg protein/ml and 1.8 μ M P-420 in a final volume of 3 ml was incubated for 5 minutes at 23^o with 25 μ M LAHPO in 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 3% (v/v) glycerol. After incubation the CO-difference spectrum of dithionite-treated samples was measured and the destruction of P-420 determined. The effect of cyanide on P-420 destruction by LAHPO was determined by preincubation of the P-420 suspension with 10 mM cyanide prior to addition of LAHPO.

activity of "high spin" P-420 was inhibited 50% by 1 mM cyanide (See Table VI). On the other hand, "low spin" P-420 did not combine with 1 mM cyanide.

Other hemoproteins such as methemoglobin, metmyoglobin, and thyroid peroxidase exhibit similar spectral properties with cyanide (67,68) to our "high spin" P-420 preparation whereas low spin P-450 has different spectral properties when treated with cyanide (69).

Destruction of P-420 by LAHPO

Omura and Sato (51,64) have reported that cytochromes P-450 and P-420 in "CO-binding particles" are very labile to oxygen and have attributed the instability of the hemoproteins to lipid peroxidation. P-420 was found to be much more labile to oxygen than P-450. In contrast, cytochrome b_5 even in the presence of "CO-binding particles" was very stable to oxygen. The destruction of microsomal cytochrome P-450 by LAHPO has already been reported in this work. Thus, the effect of LAHPO on cytochrome P-420 was investigated (Table VII). It is seen that at 23^o, incubation of 10 μ moles LAHPO per mg protein resulted in 59% destruction of "high spin" P-420 and 45% destruction of "low spin" P-420 at pH 7.4. Inclusion of 10 mM cyanide in the incubation medium offered some protection to the hemoprotein. Thus, cytochrome P-420 is very susceptible to destruction by lipid peroxides.

Comparative efficiencies of various heme catalysts for peroxidase activity and LAHPO decomposition

Table VIII compares the rates of LAHPO decomposition

TABLE VIII

COMPARATIVE EFFICIENCIES OF HEME CATALYSTS FOR PEROXIDASE ACTIVITY AND LAHPO DECOMPOSITION^a

<u>Heme catalyst</u>	<u>Peroxidase Activity</u> Moles TMPD oxidized/min per mole of heme	<u>Rate constants for LAHPO decomposition</u> k(1/mole per min) x 10 ⁻⁴
"high spin"cytochrome P-420 ^b	200	
Hematin	156	20
Methemoglobin	122	25
Cytochrome <u>c</u>	96	13
"low spin"cytochrome P-420 ^b	60	
"low spin"cytochrome <u>P-450</u> ^c	30	
Cytochrome <u>b₅</u> ^d	6	
Oxyhemoglobin ^b	5.2	6
Myoglobin	4.3	5.6
Horseradish Peroxidase	1.9	0.2
Catalase	0.01	0.001
Lactoperoxidase ^e	< 0.01	< 0.001

Methemoglobin	122	20
Cytochrome <u>c</u>	96	25
"low spin"cytochrome P-420 ^b	60	13
"low spin"cytochrome P-450 ^c	30	
Cytochrome <u>b</u> ₅ ^d	6	
Oxyhemoglobin ^b	5.2	6
Myoglobin	4.3	5.6
Horseradish Peroxidase	1.9	0.2
Catalase	0.01	0.001
Lactoperoxidase ^e	< 0.01	< 0.001

^a The rates of LAHPO decomposition by heme compounds were measured at 233 nm by the method of O'Brien (34) whereas peroxidase activities were determined as described previously in "Materials and Methods". Values are expressed per mole of heme.

^b Prepared as described in "Materials and Methods".

^c Microsomal "P-450 particles" free of cytochrome b₅ and containing "low spin" P-450 (42) were used in the determination.

^d Cytochrome b₅ was prepared by deoxycholate treatment of microsomes and DEAE-cellulose chromatography as described in "Materials and Methods".

^e Lactoperoxidase was obtained from Calbiochem.

by heme compounds with their respective peroxidase activities. The results clearly show that "high spin" P-420 is the most effective catalyst. "High spin" P-420 was over 3 times more effective than "low spin" P-420 and almost 7 times more effective than "low spin" P-450 in catalyzing the peroxidase reaction. Hematin was found to be more effective than most hemoproteins presumably because the protein portion of hemoproteins sterically hinders the interaction between the iron residue and the lipid peroxide. Oxyhemoglobin was much less effective than methemoglobin and this was probably the result of steric hindrance by the oxygen molecule preventing the interaction of the hydroperoxide with the iron residue. Cytochrome b_5 and myoglobin had low peroxidase activity whereas catalase, horseradish peroxidase, and lactoperoxidase did not exhibit catalytic activity to any appreciable extent.

The results show that the high spin (70) heme compounds such as "high spin" P-420, hematin and methemoglobin were the most effective catalysts whereas the low spin (42,70) hemoproteins such as "low spin" P-420, "low spin" P-450, cytochrome b_5 and oxyhemoglobin exhibited much lower catalytic activities.

DISCUSSION

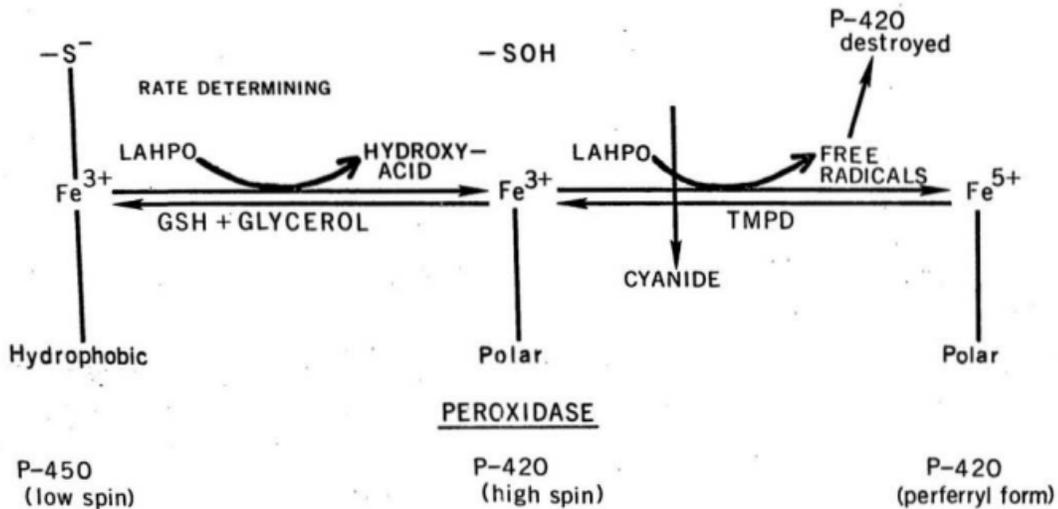
This study has investigated the mechanism of lipid peroxide decomposition by liver microsomes using TMPD as the hydrogen donor and linoleic acid hydroperoxide (LAHPO) as substrate. Several lines of evidence have been produced implicating cytochrome P-450 as the microsomal peroxidase responsible for rapid lipid peroxide decomposition: 1) Microsomal "P-450 particles" containing cytochrome P-450 as the sole protoheme constituent were very active in catalyzing the peroxidase reaction; 2) trypsin digestion of microsomes produced a striking resemblance in the pattern of solubilization of peroxidase activity and cytochromes P-450 plus P-420; 3) microsomes from phenobarbital-treated rats exhibited a 2.5-fold higher specific P-450 content and showed a similarly enhanced peroxidase activity; 4) the microsomal decomposition of LAHPO results in catalyst inactivation (35) and cytochrome P-450 was rapidly destroyed when incubated with LAHPO; 5) the peroxidase activity of microsomes was enhanced 2- to 8-fold by reagents that converted cytochrome P-450 to P-420; 6) the peroxidase activity of microsomes was inhibited by cyanide which is known to combine with microsomal cytochrome P-450 (44, 69). It therefore seems reasonable to conclude that cytochrome P-450 is responsible for the rapid lipid peroxide decomposition by liver microsomes.

Microsomal cytochrome P-450 acts as a site of both oxygen and substrate activation for hydroxylations of lipid soluble drugs (48, 58, 62). Thus, the reactive area of P-450, i.e., the vicinity of the heme, seems to be imbedded in a highly

hydrophobic portion of P-450 protein or phospholipid of the microsomal membrane (43, 50, 59, 71) and may account for the anomalous spectral properties of the hemoprotein. P-450 can be converted by a wide variety of treatments to a spectrally modified form called P-420 which shows normal spectra for a hemoprotein (42-45, 50, 51, 59, 60, 64, 65). It was found that this conversion results in a marked increase in peroxidase activity (see Table IV). The conversion to P-420 is likely accompanied by marked alterations of the hydrophobic environment around the heme or by changes in P-450 conformation (43, 50, 59, 71, 72) which probably results in increased exposure of the heme group to the environment and renders the protein-bound iron of P-450 readily available for catalysis of lipid peroxide decomposition.

Cytochrome P-450 in its oxidized form is detectable by ESR spectroscopy in a low spin state (42) and can be converted by various treatments to a high spin form of P-420 (42-45, 59, 61, 65). Several lines of evidence suggest that the thiol group is a ligand in low spin hemoprotein P-450: 1) The thiol reagents, *p*-hydroxymercuribenzoate and *p*-chloromercuriphenylsulfonate, convert low spin P-450 to high spin P-420 (43, 59); 2) the thiol oxidants, iodine and N-bromosuccinimide (73), convert low spin P-450 to high spin P-420 (65); 3) GSH and other thiols convert high spin P-420 to low spin P-450 (42-45, 59); 4) Bayer et al (74) found a close similarity in the physical properties of low spin P-450 and various model iron-heme thiol complexes;

SCHEME I. MECHANISM FOR MICROSOMAL PEROXIDASE



5) Ullrich et al (75) examined the hydroxylation of cyclohexane by model systems consisting of Fe^{2+} ions, thiol compounds, and oxygen and found that these systems carry out hydroxylation reactions by the same mechanism as microsomal P-450; 6) Jefcoate and Gaylor (76) investigated the binding of n-propylmercaptide with metmyoglobin and found a close similarity in the physical properties of the metmyoglobin-thiol complex and low spin P-450.

A mechanism for microsomal peroxidase which involves the thiol ligand of P-450 is proposed in Scheme I. As peroxides can oxidize sulfhydryl groups of proteins to higher oxidation products (30), it is suggested that LAHPO oxidizes the thiol ligand of low spin hemoprotein P-450 to a sulfenic acid derivative and modifies the coordination thereby producing a high spin form of P-420 which is a very powerful peroxidase. In the process, the hydrophobic environment of the heme is probably altered. LAHPO converts ferric hemoprotein P-420 to a higher oxidation state, the perferryl form, which oxidizes TMPD to Wurster's blue. Cyanide complexes with the ferric form of P-420 and inhibits the formation of the perferryl form thus inhibiting peroxidase activity. Perferryl forms of hemoproteins have been implicated in the peroxidase activities of various hemoproteins (37, 77).

Murakami and Mason (43) examined the binding of cyanide to high spin P-420 and observed the disappearance of a high spin ESR-detectable signal upon treatment of the hemoprotein with

cyanide. Our results show that the peroxidase activity of microsomal hemoprotein P-450 was inhibited 50% by 1 mM cyanide (see Table III) and yet cytochrome P-450 has a binding constant for cyanide of 6-8 mM (69). The peroxidase activity of "high spin" P-420 was also inhibited 50% by 1 mM cyanide (see Table VI) and a binding constant for cyanide of about 1 mM was obtained (see Fig. 5). The similar inhibitions by cyanide of the peroxidase activities of "high spin" P-420 and microsomal P-450 again suggest a role for "high spin" P-420 in the peroxidase mechanism of microsomal P-450 (see Scheme I).

Jefcoate and Gaylor (76) suggested the coordination of histidine and cysteine in low spin hemoprotein P-450 and found that the histidine could be displaced by various lipophilic ligands. Low spin P-420 is assumed to contain the same coordinating ligands as low spin P-450 (43, 59, 76, 78). The low binding affinities of "low spin" P-420 and cytochrome P-450 (69) for cyanide could be explained if the heme iron is still coordinating with a strong donor group such as a thiol (43, 76, 78). Then, relatively high concentrations of cyanide would be required to displace the coordinating ligands in P-450 and "low spin" P-420. On the other hand, the high sensitivity of "high spin" P-420 to cyanide can be explained if the heme iron is not coordinated to a thiol ligand.

LAHPO has been shown to cause extensive damage to microsomal hemoprotein P-450. In Scheme I, the lipid free radicals produced during LAHPO decomposition could account for P-450 damage by destroying the cytochrome P-420 catalyst. Other hemoproteins

that decompose LAHPO are also destroyed in the process (26, 34, 37). Cytochrome P-420, in its "high spin" and "low spin" forms, was found to be very susceptible to destruction by LAHPO (see Table VII). This sensitivity of cytochrome P-420 to destruction by lipid peroxides may be attributed to its molecular conformation whereby the heme moiety is probably readily exposed to the environment (42-45, 50, 71, 72) and is extremely susceptible to attack by the lipid peroxide or by oxidizing free radicals formed during peroxide decomposition (26, 37). Cyanide partially inhibits destruction of cytochrome P-420 presumably by forming a complex with the central iron atom rendering the modified hemoprotein less effectual for catalyzing peroxide breakdown, thereby inhibiting the formation of powerful oxidizing free radicals.

A comparison of the effectiveness of various heme compounds in catalyzing the peroxidase reaction showed that "high spin" P-420 was the most effective catalyst (see Table VIII). Other high spin (70) heme compounds such as hematin and methemoglobin were also very effective catalysts whereas low spin (42, 70) hemoproteins such as "low spin" P-420, "low spin" P-450, cytochrome b_5 , and oxyhemoglobin exhibited lower catalytic activities. Thus, the spin state of iron in heme compounds may be an important factor in determining their catalytic effectiveness. Since "high spin" P-420 is presumed to contain an unoccupied coordination site to the iron of the heme as a result of disruption of the thiol ligand (43, 44, 65, 78) the catalytic effectiveness of the "high spin" hemoprotein is probably due to the

increased accessibility of the iron residue to the lipid peroxide which would inevitably result in rapid catalysis of hydroperoxide decomposition.

The complex range of products formed when lipid peroxides are decomposed by the microsomal fraction of liver (35) suggests that free radical intermediates are involved, which in the cell would be expected to oxidize intracellular hydrogen donors. Thus, the microsomal peroxidase, i.e., cytochrome P-450, can be an important intracellular site for free radical production and consequently a site for hydrogen donor oxidation. The various intracellular hydrogen donors that have been found to be oxidized during lipid peroxide decomposition include ascorbate, urate, cysteine, ubiquinol, α -tocopherol, NAD(P)H, GSH, tyrosine and tryptophan (?).

The consequences of intracellular P-450 destruction by lipid peroxides could be serious. Hemoprotein P-450 is the oxygen-activating pigment involved in a number of mixed function oxidation reactions, e.g., hydroxylation reactions important in steroid synthesis and metabolism, cholesterol side-chain cleavage; ω -oxidation of fatty acids; drug hydroxylation; detoxification reactions; and oxidative demethylation of carcinogens (48, 58, 62, 79-82). It is therefore not surprising that LAHPO was found to inhibit the demethylation of *p*-chloro-N-methylaniline. Kitabchi (79) has shown that in vitro microsomal lipid peroxidation results in the inhibition of C-21 hydroxylation of progesterone and Wills (83) has demonstrated inhibition of various hydroxylation and demethylation reactions. Lipid

peroxidation may also explain the high in vivo turnover of cytochrome P-450 in the endoplasmic reticular membrane (84) compared with other cytochromes in the cell. On the other hand, lipid peroxidation which results in the formation of radical species by peroxidase action (1, 4, 25, 26) may actually enhance certain types of steroid hydroxylation and oxidation reactions (85-87).

Cytochrome P-450 is present in a large variety of tissues (61) and may function in the decomposition of hydroperoxides other than LAHPO (e.g., H_2O_2 , steroid hydroperoxides, etc.). Thus, if steroid hydroxylation involves the initial formation of steroid hydroperoxides (88), cytochrome P-450 could play a role in steroid metabolism by converting these hydroperoxide intermediates to hydroxysteroids. The microsomal peroxidase could be involved in the oxidation and subsequent glutathione conjugation of those steroids able to act as hydrogen donors for the peroxidase (89). The hemoprotein may also play a part in the peroxidase-catalyzed inactivation of estrogens in the uterus (89).

It is of interest to note that adrenalectomy and castration of rats diminish the specific content of hepatic microsomal P-450 about 5-fold and this is accompanied by a similar decrease in steroid hydroxylation activity (90). Injection of steroids restores the P-450 level back to normal and also restores the hydroxylation activity. Thus, cytochrome P-450 and consequently microsomal peroxidase may be under steroid control.

Cytochrome P-420 seems to be identical with hemoprotein 559, a peroxidase which has been localized in the microsomal fractions of several bacterial and plant tissues and in

TABLE IX

COMPARISON OF ABSORPTION SPECTRA OF "HIGH SPIN" P-420 AND THYROID PEROXIDASE^c

<u>Spectra</u>	<u>"High spin" P-420^b</u>	<u>Thyroid peroxidase</u>
	<u>nm</u>	<u>nm</u>
Oxidized (Soret)	406 (43)	406 (100)
Oxidized	535 (64)	533 (68)
Reduced (Soret)	426 (43)	424 (68)
Reduced (β)	530 (64)	528 (68)
Reduced (α)	559 (64)	558 (68)
Reduced + CO	421 (64)	420 (68)
Oxidized + CN^-	417 (this work)	416 (68)
Difference spectra (oxidized + CN^-)		
<u>versus</u> oxidized		
Peak	426 (this work)	427 (68)
Trough	403 (this work)	404 (68)
Difference spectra (reduced + CO)		
<u>versus</u> reduced		
Soret	420 (this work)	418 (68)

^a The reference numbers are shown in parentheses.

Oxidized	535 (64)	533 (68)
Reduced (Soret)	426 (43)	424 (68)
Reduced (β)	530 (64)	528 (68)
Reduced (α)	559 (64)	558 (68)
Reduced + CO	421 (64)	420 (68)
Oxidized + CN^-	417 (this work)	416 (68)
Difference spectra (oxidized + CN^-)		
<u>versus oxidized</u>		
Peak	426 (this work)	427 (68)
Trough	403 (this work)	404 (68)
Difference spectra (reduced + CO)		
<u>versus reduced</u>		
Soret	420 (this work)	418 (68)

^a The reference numbers are shown in parentheses.

^b The spin state of P-420 in reference (64) was not determined but it has been reported that phospholipase treatment of microsomes, as was used to prepare P-420, converts P-450 irreversibly to high spin P-420 (45).

mammalian microsomes of heart, brain, adrenal medulla, retinal pigment epithelium and leukocytes (91-95). Cytochrome P-420 may play a role in the in vivo peroxidase-mediated catecholamine synthesis and melanin formation in retinal pigment epithelium, melanocytes, mast cells, leukocytes, brain cells, and adrenal medulla (94-96). An enzymically active P-420 has been implicated in the mixed function oxidase system of Pseudomonas aminovorans (97) and possibly, P-420 may be involved in certain mammalian hydroxylation and demethylation reactions (51,64,55, 81, 98). It will be interesting to see if peroxide intermediates are involved in these reactions.

The microsomes of thyroid gland contain a hemoprotein called thyroid peroxidase (67, 68, 99, 100) which possesses very high peroxidase activity. Thyroid peroxidase appears to be spectrally similar to "high spin" P-420 (Table IX) which is also a very active peroxidase (See Table VIII). The two hemoproteins contain ferriprotoporphyryn IX as a heme prosthetic group (64, 68), and the peroxidase activities of both hemoproteins are inhibited by cyanide and boiling (see Table VI) (68). Thyroid microsomes contain no cytochrome P-450 and no microsomal Fe_x signal (67). The ESR signal indicates the presence of P-450 or low spin P-420 (43, 44). However, the possibility of high spin P-420 (which has no Fe_x signal) being present in thyroid microsomes has not been investigated. Possibly, thyroid peroxidase and high spin P-420 are identical hemoproteins and the thyroid tissue may have converted its P-450 to high spin P-420 for the specialized synthesis of thyroxine (100).

FOOTNOTES

¹ A purified preparation of cytochrome b₅ from rat liver microsomes was obtained by deoxycholate solubilization and DEAE-cellulose chromatography according to the method of Lu et al (55). The concentration of cytochrome b₅ was determined from the difference spectrum between the oxidized and dithionite-reduced form using an extinction coefficient increment (ΔE 424-409 nm) of $185 \text{ cm}^{-1} \text{ mM}^{-1}$ (51).

² Based on 100% conversion of P-450 to P-420.

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