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EVIDENCE THAT PEROXIDATION OF LYSOSOMAL MEMBRANES IS INITIATED BY HYDROXYL FREE RADICALS PRODUCED DURING THE ACTIVITY OF CERTAIN FLAVIN ENZYMES

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KUO-LAN FONG

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EVIDENCE THAT PEROXIDATION OF LYSOSOMAL MEMBRANES IS INITIATED BY HYDROXYL FREE RADICALS PRODUCED DURING THE ACTIVITY OF CERTAIN FLAVIN ENZYMES

APPROVED BY

DISSERTATION COMMITTEE

EVIDENCE THAT PEROXIDATION OF LYSOSOMAL MEMBRANES IS INITIATED BY HYDROXYL FREE RADICALS PRODUCED DURING THE ACTIVITY OF CERTAIN FLAVIN ENZYMES

By Kuo Lan Fong

Major Professor: Paul B. McCay, Ph.D.

The nature of the free radicals produced during the activity of several oxidative enzymes, using lysosomes as a sensitive indicator, has been studied. These radicals appear to play important roles in biological oxidations involving one electron transfer. The enzymes investigated in this work are flavoproteins: microsomal NADPH oxidase, partially purified cytochrome P450 reductase, and a highly purified xanthine oxidase. These enzymes, when operating upon their own substrate in vitro, produce a factor which causes the release of acid hydrolases from lysosomes. The factor appears to be a free radical involved in the activity of these enzymes, the generation of which is dependent on Fe^{3+} bound to the membrane in which the enzyme is located, or in the case of the purified enzymes, Fe^{3+} which is added in chelated form to maintain it in solution. The free radical component is apparently responsible for the lipid peroxidation in the microsomal membranes which occurs during NADPH-dependent electron transport. It was found that preparation of either lysosomes or microsomes from rats supplemented with α-tocopherol has a protecting effect on lysosomal integrity in the microsomal NADPH oxidase systems. These interactions suggest possible mechanisms which might promote necrosis through the release of lysosomal enzymes in vivo, especially in animals deficient in dietary scavenging components.

The lysosomal lysis caused by these 3 enzyme systems is prevented a) by inhibitors of lipid peroxidation, b) by prior heat denaturation of the enzymes, and c) by including free radical-trapping agents in the incubation system. Preincubation of these enzyme systems in the presence of its substrate and chelated iron for 30 minutes, followed by the addition of 1.0 mM Mn^{2+} and then followed by the addition of lysosomes, results in no disruption of these particles. The role of Mn^{2+} appears to be to displace Fe^{3+} from an essential position on the enzyme required to generate the radicals. The factor causing lysosomal disruption is formed only during the activity of the respective enzymes.

The addition of superoxide dismutase enhances the lysosomal membrane breakdown during the microsomal NADPH oxidase system and xanthine oxidase system, and prevents the lysosomal membrane breakdown during the cytochrome P450 reductase system.

The studies described in this report indicate that the hydroxyl free radicals are derived from the activity of certain flavin enzymes (probably metalloflavin enzymes), and that superoxide anion must play a role in the formation of the $HO\cdot$ radicals. Since adequate chelated iron is present in the cytosol of cells to promote these reactions, these studies also suggest that the activity of such flavin enzymes may contribute to the turnover of membrane polyunsaturated fatty acids, or possibly initiate membrane lesions under some conditions, and carries certain implications concerning the process of aging.

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CHAPTER I

INTRODUCTION

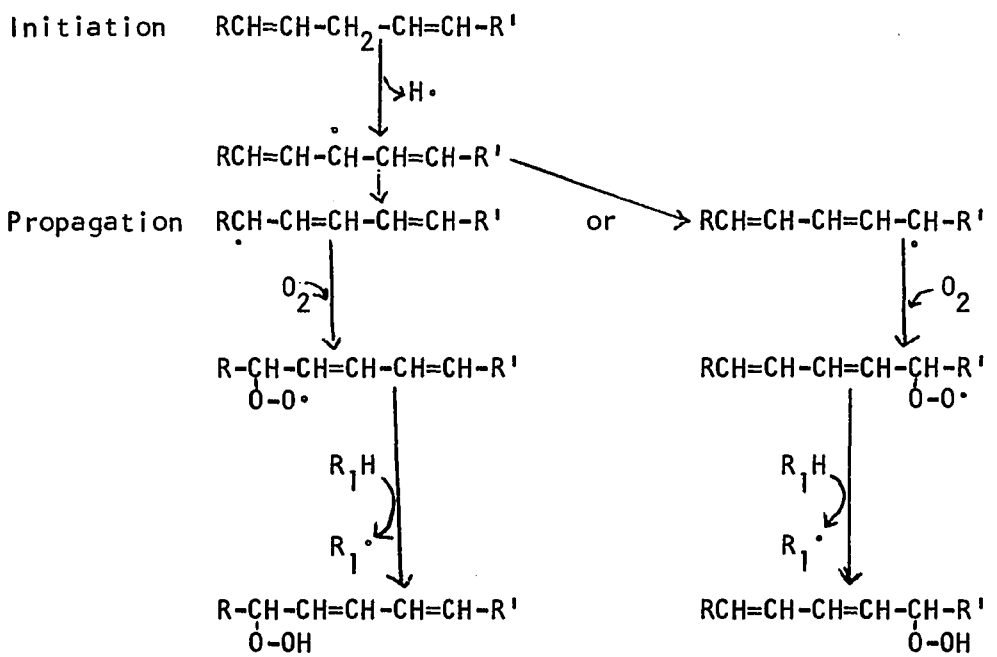
Animal tissues are rich in polyunsaturated fatty acids which are found primarily in ester linkage to the various phospholipids of cellular membranes. Lipid peroxidation in membranes results in oxidative deterioration of polyunsaturated lipids, and is a consequence of a reaction between oxygen and unsaturated lipid involving free radical intermediates which produces semistable peroxides. Lipid peroxidation and other free radical reactions probably constitute an on-going mechanism of cellular membrane damage in all metabolizing animal cells, judging by the accumulation of lipofuscin and ceroid pigments in animal tissues during their life-time which are believed to be end-products of membrane lipid peroxidation (1). In vitro lipid peroxidation has been studied in model systems for several years and many, although not all, of the basic mechanisms and properties of such systems are known. Peroxidation of pure lipids is thought to be an autocatalytic series of reactions involving a free radical mechanism (2,3). The process can be divided into four phases.

1) Initiation---formation of a free radical on a polyunsaturated lipid by hydrogen abstraction at a methylene group of a methylene-interruped (1, 4) pentadiene portion of the lipid structure; this methene radical (H-C.), which is in the unconjugated diene configuration, shifts to the more stable conjugated 1,3 diene radical configuration.

2) Propagation---formation of a peroxy radical by reaction of the diene free radical with molecular oxygen. Subsequent abstraction of H atoms from other lipids to form hydroperoxides and more free radicals occurs.

3) Hydroperoxide breakdown---homolytic chain cleavage with formation of more free radicals, carbonyl compounds and other substances.

4) Chain termination---radical products react with each other or other substances to form stable products.



Peroxide breakdown: aldehydes, ketones, alcohols, carboxylic acids, polymers, etc.

Chain termination:

$$R\text{-}CH{=}CH\text{-}CH{=}CH\text{-}\underset{\underset{O\text{-}O\cdot}{|}}{CH}\text{-}R' + X \longrightarrow \text{Inactive Products}$$

$$R\cdot + X \longrightarrow \text{Inactive Products}$$

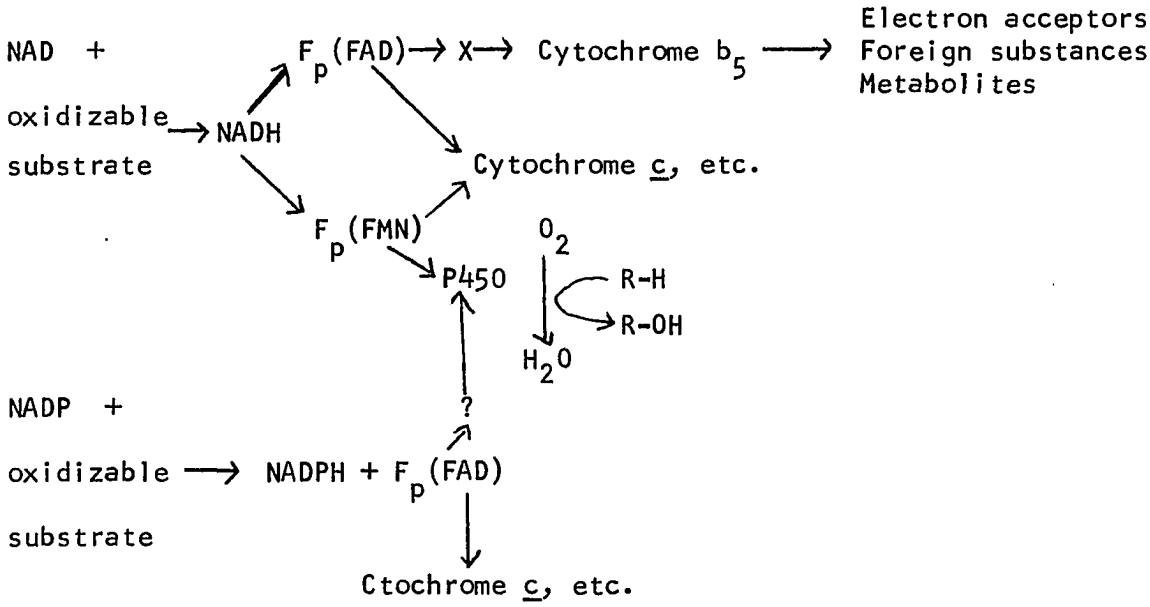
R_1H may be an unsaturated fatty acid with a methylene group of a methylene interupped (1, 4) pentadiene portion present and X is either another free radical or compound which may react with a free radical to form other substances which may then be stable and inactive.

Reports of lipid peroxidation <u>in vivo</u> have been reviewed by Tappel (4). The first observation of lipid peroxidation related to the function of an enzyme has been reported to occur with the rat liver microsomal enzyme L-gulonolactone oxidase in α-tocopherol-deficient animals (2, 5). However, it has been difficult to demonstrate directly that lipid changes occurred during the function of this enzyme (3). Microsomes from several tissues contain a variety of mixed function oxidases, which are concerned with the metabolism of substances foreign, and often toxic (termed "xenobiotics") to the metabolic network. The mixed function oxidases are involved in the metabolism of normal components such as steroids. These oxidases appear to constitute an electron transport system situated in the membrane of the endoplasmic reticulum (6). The activity of the mixed-function oxidases conform to the general reaction:

$$NADPH + H^+ + R\text{-}H + O_2 \longrightarrow NADP^+ + R\text{-}OH + H_2O$$

The relationships between the components of the microsomal electron transport system have been established by: a) studies on isolated components; b) studies of the effects of various electron acceptors and inhibitors; c) studies on the induction of certain components of the system by drugs;

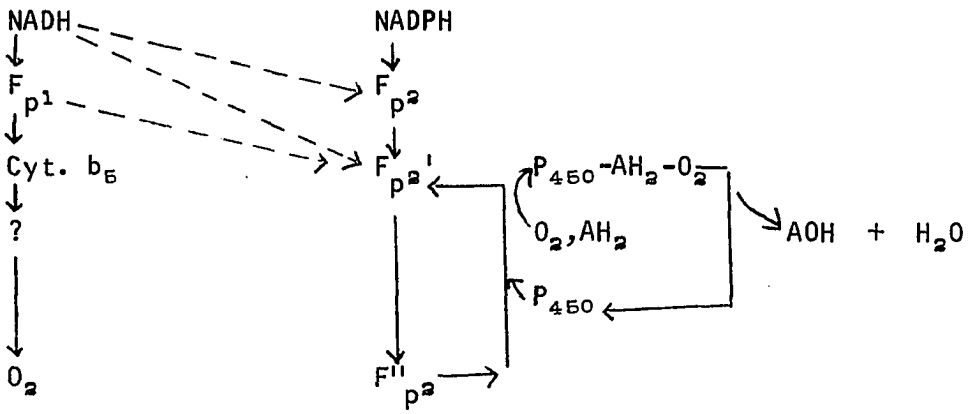
and d) studies concerning the appearance of various enzymic activities in the liver of fetal and developing animals. These studies have led to the following scheme of action which is a composite of those given by Siekevitz, Mason, Ernster and Sato (7, 8, 9, 10, 11).



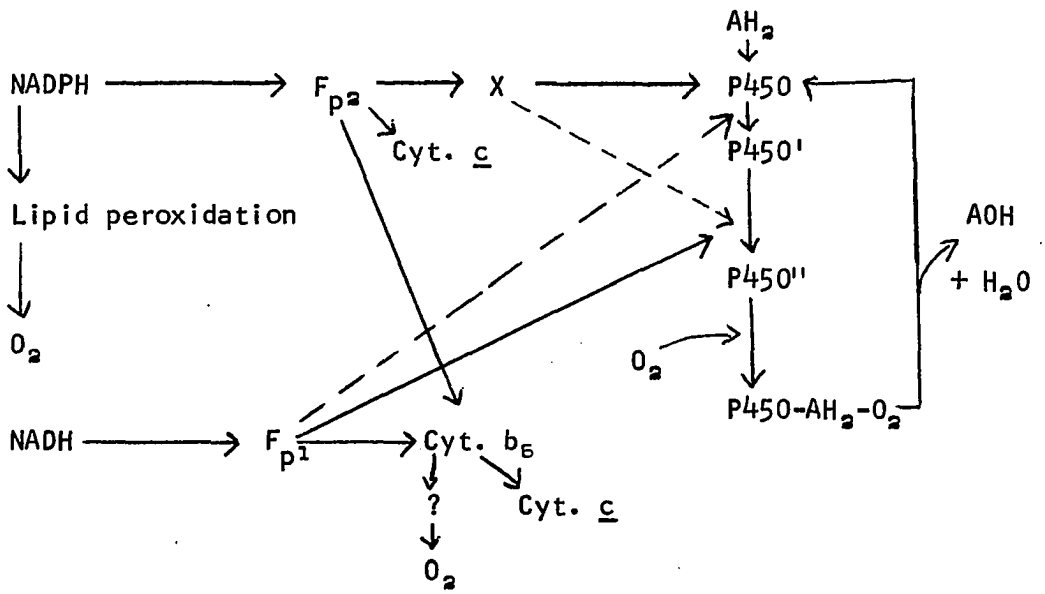
Where NADPH and NADH are tri- and diphosphopyridine nucleotides, respectively, F_p(FAD) and F_p(FMN) are flavoproteins containing flavin adenine dinucleotide and flavine mononucleotide, respectively.

Recently, Cohen and Estabrook investigated a cooperative interaction between NADH and NADPH-linked electron transport pathways in microsomes by studying codeine, ethylmorphine, and aminopyrine demethylation (12). Two hypotheses, both including an NADPH-sensitive "valve" which controls the flow of electrons from NADH to cytochrome P450, were proposed to explain the synergism between NADH and NADPH. The site of interaction is the essential difference between the two hypotheses. One hypothesis proposes that the NADPH-cytochrome c reductase flavoprotein is the site of NADH-NADPH interaction. In this scheme, F_{p1} and F_{p2} represent NADH-cytochrome b_5 reductase and NADPH-cytochrome c reductase respectively.

The single and double primes of F_{p_2} indicate the half- and fully reduced forms of the flavoprotein, respectively.

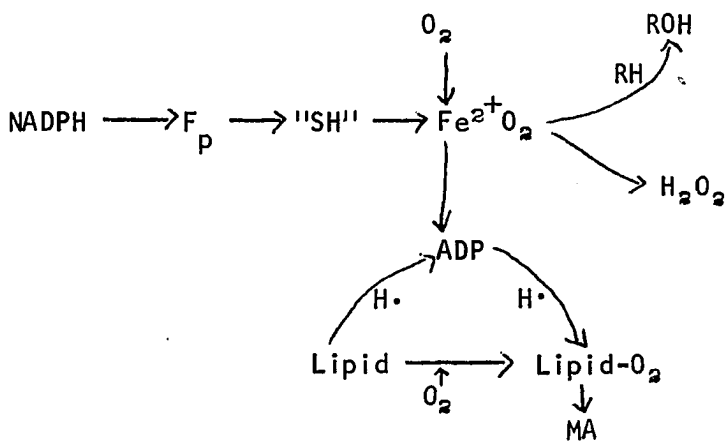


The second mechanism (shown below) suggests that the NADPH-NADH effect is due to an interaction at the level of cytochrome P450. In this scheme, F_{p_1} and F_{p_2} represent NADH-cytochrome b_5 reductase and NADPH-cytochrome c reductase, respectively, P450' indicates cytochrome P450 was "half reduced" by a single electron and P450'' means the cytochrome P450 in the "fully reduced form". The reduced and oxidized substrates are indicated by AH_2 and AOH, respectively.



Hochstein and Ernster first described the related reaction of lipid peroxidation in the pathway of microsomal electron transport for cytochrome

P450 reduction and the activation of oxygen in "mixed function oxidases". It was found that liver microsomes, in the presence of reduced triphosphopyridine nucleotide, ferric ion, and adenine dinucleotide phosphate, catalyze the rapid formation of a 2-thio-barbituric acid (TBA)-reacting compound which was believed to be malondialdehyde, a product formed in the peroxidation of unsaturated lipids. They proposed the first mechanism of NADPH lipid peroxidation (13).



The formation of this thiobarbituric acid-reacting substance during the reaction could be prevented by an inhibitor of microsomal electron transport such as para-chloromercuribenzoate (PCMB), the metal chelating agent ethylenediaminetetraacetate (EDTA), and several anti-oxidants such as ethoxyquin, diphenylphenylenediamine and α-tocopherol. The reaction could be inactivated by heat treatment of microsomes prior to incubation with NADPH. On the basis of these results they believed that formation of an ($ADP\text{-}Fe^{2+}O_2$) intermediate catalyzed lipid peroxidation in the NADPH oxidase system.

The presence and necessity of NADPH oxidase activity and its involvement in the catalysis of microsomal lipid peroxidation as well as the identification of malondialdehyde as the chromogenic substance reacting with thiobarbituric acid was demonstrated by McCay and colleagues

(20, 21, 22, 53) and confirmed by Wills (70) and others. Poyer, by studying the dependence for Fe^{3+} on oxygen consumption and malondialdehyde formation during the enzymic oxidation of NADPH, proposed a mechanism for free radical formation in lipid peroxidation (14). He showed that the addition of free radical trapping compounds will inhibit the O_2 utilization and malondialdehyde formation for the NADPH oxidase system both in vivo and in vitro. This inhibition indicates that a free radical species is formed in the process of microsomal lipid peroxidation. A hypothesis was proposed that there might be an enzymic complex such as that depicted in Figure 1, which contained a reducible sulphydryl group bound to protein (represented by the rectangular bar) and which might also have some other ligand (X), such as a carboxylate group, capable of binding Fe^{3+}. The change in redox potential of the reduced enzyme after Fe^{3+} binding to sulphydryl groups of the enzyme could lead to the formation of a reduced oxygen free radical species, as appears to be the case for xanthine oxidase. The radical species may then cause lipid peroxidation of the polyunsaturated fatty acids of the microsomal membrane by promoting hydrogen abstraction from methylene carbons. The reduction of the Fe^{3+} to Fe^{2+} in association with the formation of the $O_2^{\cdot-}$ radical must also be considered in this system. In the presence of H^+ ions, the $O_2^{\cdot-}$ radical would be converted to the HOO· radical. However, at pH 7.4, peroxyl free radicals would exist in the $O_2^{\cdot-}$ form.

Since 1959, it has been postulated that free radicals are necessary intermediates in biological oxidation-reduction systems (15) and a number of electron resonance studies of enzyme reactions have been performed to investigate the details of the reaction mechanisms. Many laboratories have investigated the formation and the importance of super-

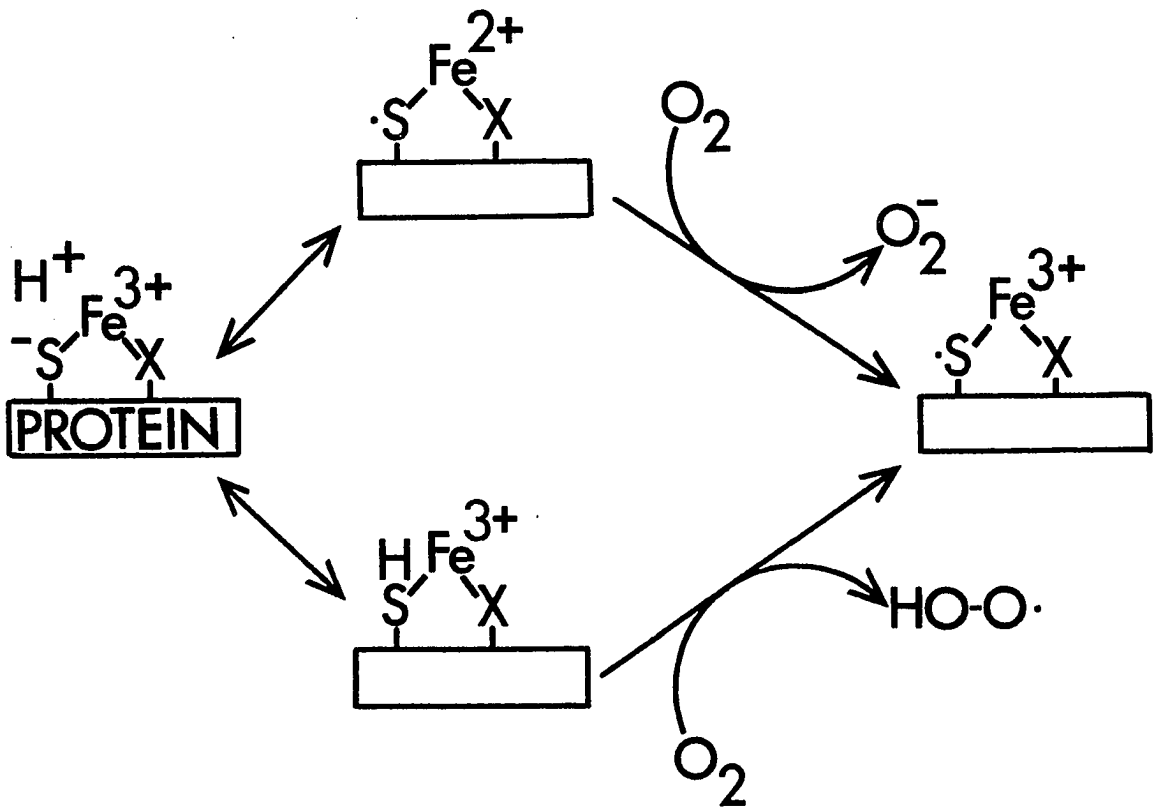


Figure 1. Proposed Mechanism for Free Radical Formation.

oxide anion radical in a wide range of biological oxidation-reduction systems (16, 18, 77), and the discovery of the superoxide anion dismutase activity of erythrocuprein (16) has provided an important tool for detecting the participation of the superoxide anion in systems in which the radical is generated in the reaction under observation.

The liver microsomal drug-metabolizing enzyme system has been solubilized and separated into 3 fractions containing cytochrome P450, cytochrome P450 reductase, and lipids, all of which are needed to reconstitute the catalytic activity of hydroxylation of a variety of steroids, drugs, and other substances, when the fractions are recombined (17). Strobel and Coon (18) proposed a mechanism of enzymic hydroxylation involving superoxide anion bound to cytochrome P450 by studying the effect of superoxide generation and dismutation on liver microsomal hydroxylation reactions. They showed that superoxide dismutase inhibits hydroxylation of benzphetamine by a reconstituted, purified rat liver drug metabolizing system in the presence of NADPH and molecular oxygen as well as in a superoxide generating system (xanthine and xanthine oxidase) containing cytochrome P450 which is capable of promoting hydroxylating reactions in the absence of NADPH (Fig. 2).

Aust and Pederson (19) provided evidence that superoxide was generated by a purified NADPH-cytochrome c reductase from rat liver microsomes and suggested that the radical may be responsible for lipid peroxidation. However, the presence of EDTA, a potent inhibitor of lipid peroxidation in biological materials, is required in their system (20), and therefore the reaction they are studying may involve entirely different mechanisms than that under consideration in this dissertation.

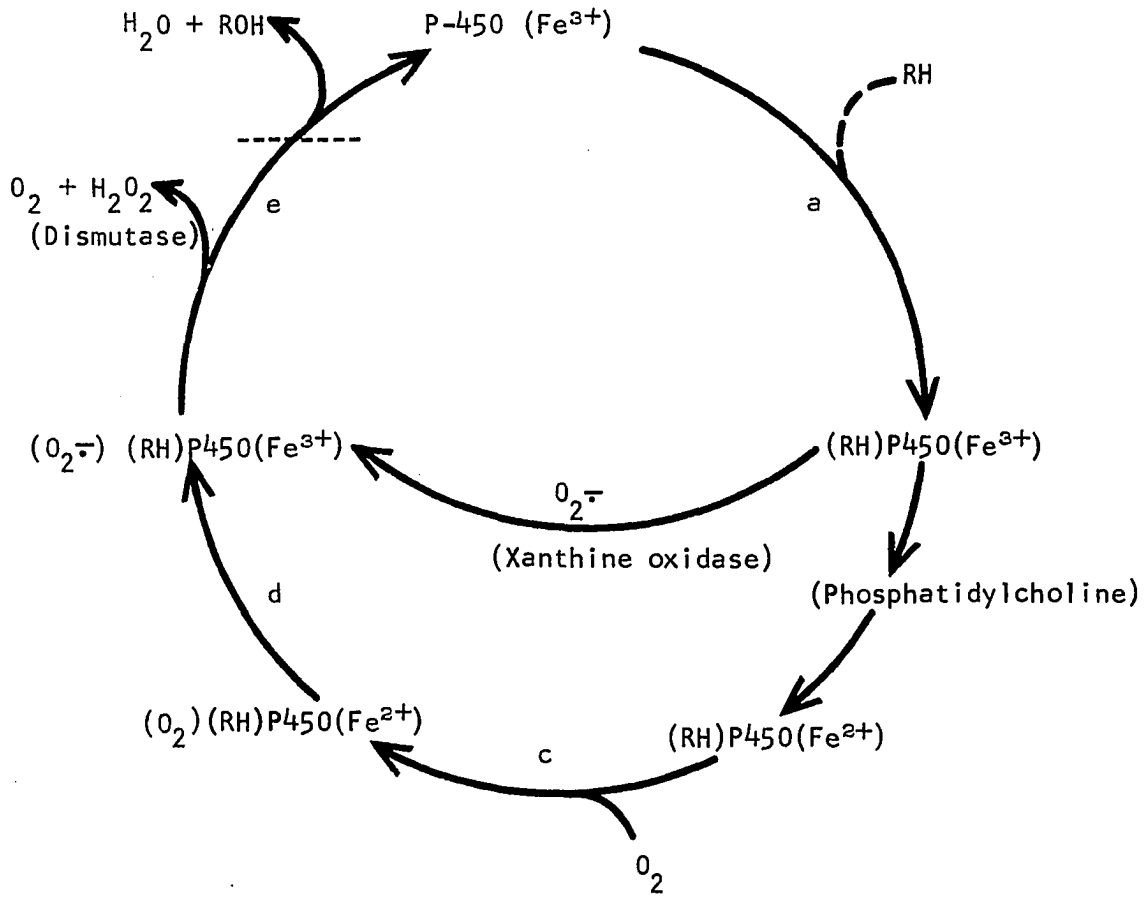


Figure 2. Mechanism of hydroxylation reaction catalyzed by cytochrome P450.

In our laboratory, Pfeifer and McCay (21) have reported that liver microsomes oxidizing NADPH in the presence of physiological levels of iron and ADP, produced a factor with properties of a free radical capable of hemolyzing normal erythrocytes. The evidence for presuming the factor must be a free radical has been given. The erythrocytes were protected from hemolysis under any one of three different conditions: 1) when a free radical trapping agent was added to the incubation system; 2) when the animals which were donors of the erythrocytes were supplemented with α-tocopherol, and 3) when the inhibitors of NADPH oxidation were added to the reaction systems. This factor is also apparently responsible for the lipid alterations which occur in the microsomal membrane itself during NADPH-dependent electron transport (22). The production of the factor occurred only when NADPH was being actively oxidized by microsomes. The conditions of the enzymic reaction producing the radicals appeared compatible with in vivo conditions in the liver cell and suggested that other membraneous organelles in the cell might be altered in certain situations. The formation of free radicals by other mechanisms during the normal course of metabolism (23) associated with the activity of certain types of flavin enzymes may represent a steady source of membrane alterations, one to which lysosomes are particularly susceptible (24).

Rat liver lysosomes are characterized by a property which has always been considered of fundamental importance: The structure-linked latency of their enzymes. This latency has been attributed to the existence around the lysosomes of a membrane-like barrier of a lipoprotein nature restricting the accessibility of their internal hydrolases to

external substrates. The activation and release of lysosomal enzymes depend on the limiting membrane surrounding the particles. If the membrane becomes labilized or ruptured, lysosomal enzymes are free and active. Lysosomal enzymes are bound and essentially inactive in the intact lysosomal membrane. A comprehensive survey and classification of lysosomal enzymes has been given (25). These lysosomal hydrolytic enzymes are normally concerned with the digestion of cell nutrients, turnover of cell protein and organelles, tissue remodeling, lysis of invaders and autolysis of dead cells. The leakage of lysosomal enzymes into the cytoplasm could lead to damage or the digestion of essential cellular components.

It has recently been reported that lysosomes from neurons of older animals are more permeable than those in neurons of younger animals (73). Drugs which stabilize lysosomal membranes have been shown to lengthen the life of Drosophila melanogaster (74). Other studies have shown that such lysosome stabilizers lengthen the life of several types of cells in culture (75, 76). A recent development has been the demonstration by Sullivan and Debusk that there is a correlation between the occurrence of unstable lysosomal membranes and the appearance of altered proteins in Neurospora (77).

There is a proportional release of hydrolytic enzymes with time when free lysosomes are suspended in rapidly peroxidizing linoleate emulsions (24). Similar results were obtained by exposing lysosomes to free radicals generated by the decomposition of hydrogen peroxide. It was observed that it takes several hundred times fewer peroxide molecules per unit surface area to cause maximum release of enzymes from a lysosome than it does to swell and lyse mitochondria. The explanation is that

lysosomes possess a single membrane while mitochondria have a double membrane. They conclude that lysosomes are extremely sensitive to peroxides and free radicals, and that lysosomal membrane damage can readily release hydrolytic enzymes leading to varying degrees of non-specific lysis of cell components.

The purpose of this study was:

1. To determine whether or not lysosomal disruption could occur as a result of exposure of these particles to free radicals generated by the function of the microsomal NADPH oxidase system,

2. To study the effect of α-tocopherol and other radical scavengers on the reaction,

3. To study the effect of free radicals generated by a partially purified NADPH-cytochrome P450 system and by various flavin enzymes which are known to produce superoxide anion when acting on their substrates and how their activity affects lysosomal membranes,

4. To determine the nature and mechanism of action of the actual free radical factor responsible for lysosomal lysis.

CHAPTER II

MATERIALS AND METHODS

Reagents

All chemicals and solvents were reagent grade and were used as obtained except where specified otherwise.

Adenosine 5'-diphosphate, sodium (ADP) was obtained from P-L biochemicals, Inc., Milwaukee, Wisconsin.

The following chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri: Horseheart cytochrome c, Type III; Triton X-100, o-nitrophenyl-d-galactoside, 2-hydroxy-5-nitrophenyl sulfate, 4-nitrocatechol, p-nitrophenyl-β-d-galactoside, and NADPH (nicotine adenine dinucleotide phosphate, reduced form).

Xanthine was purchased from Calbiochem, Sandiego, Calif.

Tris (hydroxymethyl) amino methane, chloroform, methanol, sucrose, mannitol, and trichloroacetic acid were obtained from Fisher Scientific Company, Philadelphia, Pennsylvania.

Triton WR-1339 (oxyethylated tertiary octylphenol polymethylene polymer) was purchased from Ruger Chemical Co., Irvington, New Jersey.

Benzoic acid was a product of Curtin Scientific Co., Tulsa, Oklahoma.

The following chemicals were obtained from Eastman Organic

Chemicals, Rochester, New York: sodium ethylenediaminetetraacetate, 2-thiobarbituric acid, aniline, Santoquin, N-methylaniline, diphenylamine, and o-nitrophenol.

Hydrogen peroxide (30%) was obtained from Merck & Co., Inc., Rahway, New Jersey.

P-nitrophenol and p-nitrophenylphosphate, disodium salt, were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin.

Partially purified NADPH-cytochrome P450 reductase was a gift from Dr. Anthony Y. H. Lu (36), Dept. of Biochemistry, Hoffmann-LaRoche, Inc., Nutley, New Jersey.

Highly purified preparations of xanthine oxidase and bovine superoxide anion dismutase were prepared in the laboratory of Dr. Bernard Keele, University of Alabama College of Dentistry, Birmingham, Alabama.

Instruments and Equipment

Centrifugations were done using either a Spinco Model L Ultracentrifuge or a Beckman Model L2-65 Ultracentrifuge, both made by Beckman Instruments, Palo Alto, California. Enzyme incubations were carried out in a Dubnoff shaking waterbath equipped with a constant termperature regulator from Precision Scientific Company, Chicago, Illinois.

Spectrophotometric measurements were made using a Beckman DU2, from Beckman Instruments Company, South Pasadena, California.

Spectrophotometric assays were performed with a Gilford model 2400 recording spectrophotometer.

Animals

The white albino rats used in this study were derived from the Holtzman-Sprague-Dawley strain bred in our laboratory. The rats were fed

either a stock rat pellet diet or a synthetic diet, the compositions of which are shown below. Animals fed the stock pellet ration until they were 11 to 12 weeks of age (300 to 350 grams in weight) were selected for use in these studies. In some cases, experimental animals were maintained on a Torula yeast diet (26) for a period of 18 to 21 days (80 to 100 grams in weight) were used.

Materials for Diets

Vitamins (except α-tocopheryl acetate), cod liver oil, casein and Alphacel (a pure powdered cellulose added for bulk) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Stripped lard (α-tocopherol and other volatile materials removed by molecular distillation), α-tocopheryl acetate and α-tocopherol were obtained from Distillation Products Industries, Rochester, New York.

Experimental Diets

α-tocopherol-deficient diets

The experimental diet used was following the method of Schwarz (26).

Composition of Vitamin Mixture

Calcium pantothenate	0.044%
Choline chloride	1.000%
Menadione	0.010%
Niacin	1.000%
Pyridoxin HCl	0.020%
Riboflavin	0.025%
Thiamin HCl	0.040%

Composition of Salt Mixture

$CaHPO_4$	10.930%
Ca Lactate	26.310%
$CuSO_4 \cdot 5H_2O$	0.022%
Fe Citrate.$3H_2O$	2.450%
KH_2PO_4	19.450%
$MgSO_4$	5.280%
$MnSO_4 \cdot 7H_2O$	24.500%
NaCl	4.070%
$Na_2HPO_4 \cdot 7H_2O$	6.900%
$ZnSO_4 \cdot 7H_2O$	0.038%

Composition of Basal Diet

Cod liver oil	2.000%
Lard, stripped	5.000%
Salt mixture	5.000%
Sucrose	57.750%
Vitamin mixture	0.250%
Yeast	30.000%

The basal diet was mixed with Alphacel in a ratio of 10 parts diet to 1 part Alphacel.

α-Tocopherol-supplemented diets

In several experiments, α-tocopherol was added to the stock pellet ration which had been finely ground. The amount added was 30 mg of α-tocopherol acetate per 100 grams of ground pellet ration. In some of the experiments α-tocopherol was administered by supplementing the

α-tocopherol-deficient diet described above with 50 mg of α-tocopherol acetate per 100 grams of diet. In other experiments, α-tocopherol was administered *in vivo* by intraperitoneal injection of 75 mg of the vitamin/kg body weight 12 hours prior to sacrificing. The injection solution was prepared by dissolving the α-tocopherol in 1 volume of ethanol and 9 volumes of 16 Tween 80 in 0.85% (w/v) NaCl solution and stirring vigorously.

Stock Ration (Pellet Form)

Rats which were not maintained on an experimental diet were fed a commercial pellet ration from Rockland Laboratories, Teckland Incorporated, Monmouth, Illinois. This diet was composed of the following ingredients: soybean meal, ground wheat, ground yellow corn, fish meal, pulverized barley, wheat midlings, dehydrated alfalfa meal, pulverized oats, feeding oat meal, dried skim milk, 1% animal fat, vitamin A palmitate, irradiated dried yeast, niacin, pantothenate calcium, riboflavin supplement, 0.5% dicalcium phosphate, copper oxide, traces of manganese oxide, menadione, vitamin B_{12}, 1% calcium carbonate, 1% sodium carbonate, cobalt carbonate, iron carbonate, calcium iodate and zinc oxide. The manufacturer guaranteed the following composition: crude protein, 24%; crude fat, 4%; and crude fiber, 6%.

Experimental Procedures

Preparation and Handling of Lysosomes

Lysosomes were isolated from liver tissue by the procedure of Leighton *et al.* (27) as modified by Tulsiani and Carubelli (28). This procedure takes advantage of the fact that the hepatocytes accumulate

the Triton WR-1339 within the lysosomal particles, causing their density to be less than that of other cellular particles. Therefore, by using appropriate centrifugation gradients, essentially pure preparations of lysosomes can be obtained. Experiments described in this dissertation demonstrate that lysosomes obtained in this manner behave in the various reaction systems in the same way as lysosomes isolated by the differential centrifugation procedure described below not involving injection of Triton WR-1339.

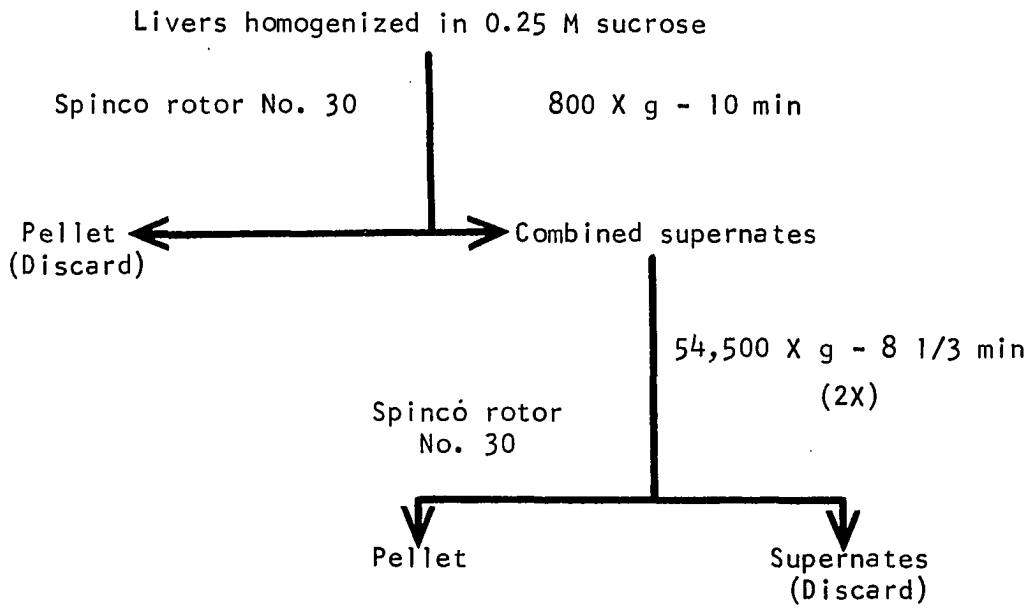
Male rats (300-350 grams) were injected intraperitoneally with an aqueous solution (26% w/v) of Triton WR-1339 at a dosage of 85 mg/100g of body weight 3 1/2 days before sacrificing. They were fasted for the last 12 hr of that period and then killed by a blow on the head. The livers were rapidly dissected out, rinsed in cold 0.25 M sucrose (all sucrose solutions contained 0.001 M tetrasodium EDTA) and the adhering fat, connective tissue and blood clots were removed. The liver tissue was cut into small pieces and homogenized in 5 volumes of the same sucrose medium. All steps were performed at 0-4°. Homogenization was performed in a smooth-walled glass homogenizer fitted with a mechanically driven Teflon pestle (Arthur H. Thomas Company, Philadelphia). The pestle was driven at about 1,000 rpm by a fixed hand drill. The glass homogenizer, maintained in a beaker containing a mixture of crushed ice, was pushed upward until all the tissue had been forced above the pestle, and then lowered. In each operation, which was performed with only a single up and down pass on the liver, moderate pressure was applied to the homogenizer for the purpose of avoiding the excessive shearing forces generated by high vertical velocity gradients. The homogenate was centrifuged for

10 min at about 2,900 rpm. The cytoplasmic extract (supernatant fraction) was diluted with 0.25 M sucrose to obtain a 1:10 dilution (w/v) and was centrifuged at 25,000 rpm (54,500 x g) for 8 1/2 min. The supernatant and the loose pink layer on the surface of the sediment were removed as thoroughly as possible. The pellet was suspended in homogenizing medium and centrifuged again as above. After the sediment was washed for a second time, it was fractionated on a discontinuous sucrose gradient by the procedure of Trouet (29). The washed pellet was resuspended in 45% (w/v) sucrose (density 1.21) in a volume equivalent to 1 ml per gram of original liver. A 10 ml sample of this suspension, containing mitochondria and lysosomes, was placed in the bottom of a plastic tube fitting the SW 25.1 rotor and then 10 ml of 34.5% (w/v) sucrose (density 1.155) and 5 ml of 14.3% (w/v) sucrose (density 1.06) were carefully layered in that order on top of the particulate suspension. Three such tubes were then centrifuged for 2 hr at 25,000 rpm (63,581 x g) in the Beckman Ultracentrifuge Model L or L2-65. The Triton-filled lysosomes were collected at the interface between the two upper layers while most of the mitochondria and peroxisomes remained in the bottom layer. The lysosomal suspension was diluted with 0.25 M sucrose and centrifuged for 30 min at 25,000 rpm (54,500 x g) to obtain the lysosomes in the form of a pellet.

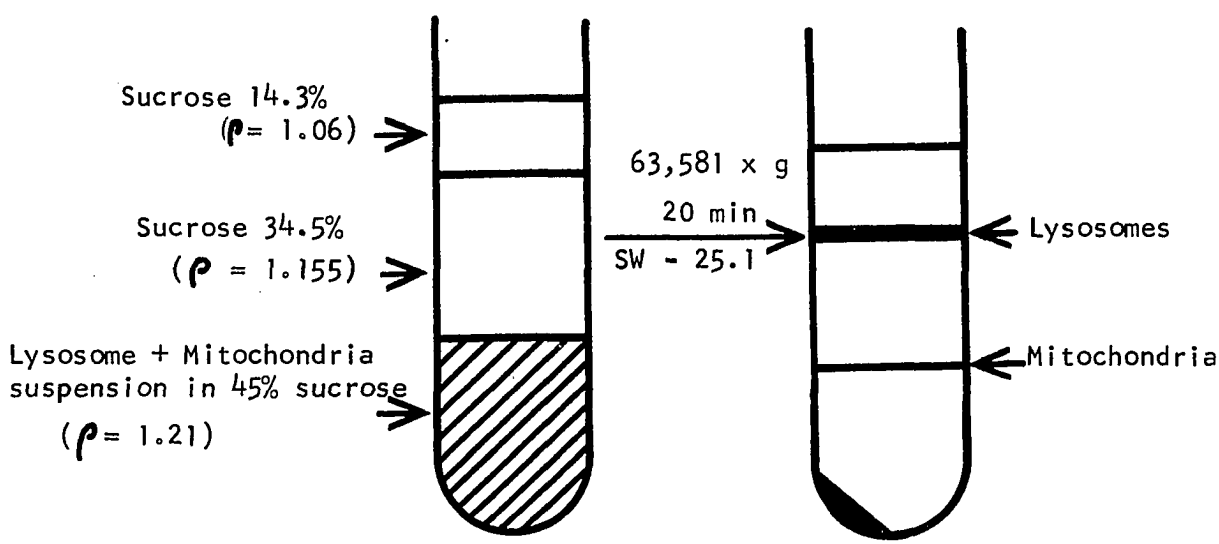
Isolation of Lysosomes by Differential Centrifugation and Density Gradient Centrifugation

The lysosomes were isolated by the method of Ragab, et al (40). Rats were sacrificed as previously described after fasting them for 24 hrs. The livers were rapidly dissected and washed with 0.25 M sucrose (all sucrose solutions contained 0.001 M tetrasodium salt of EDTA). The livers

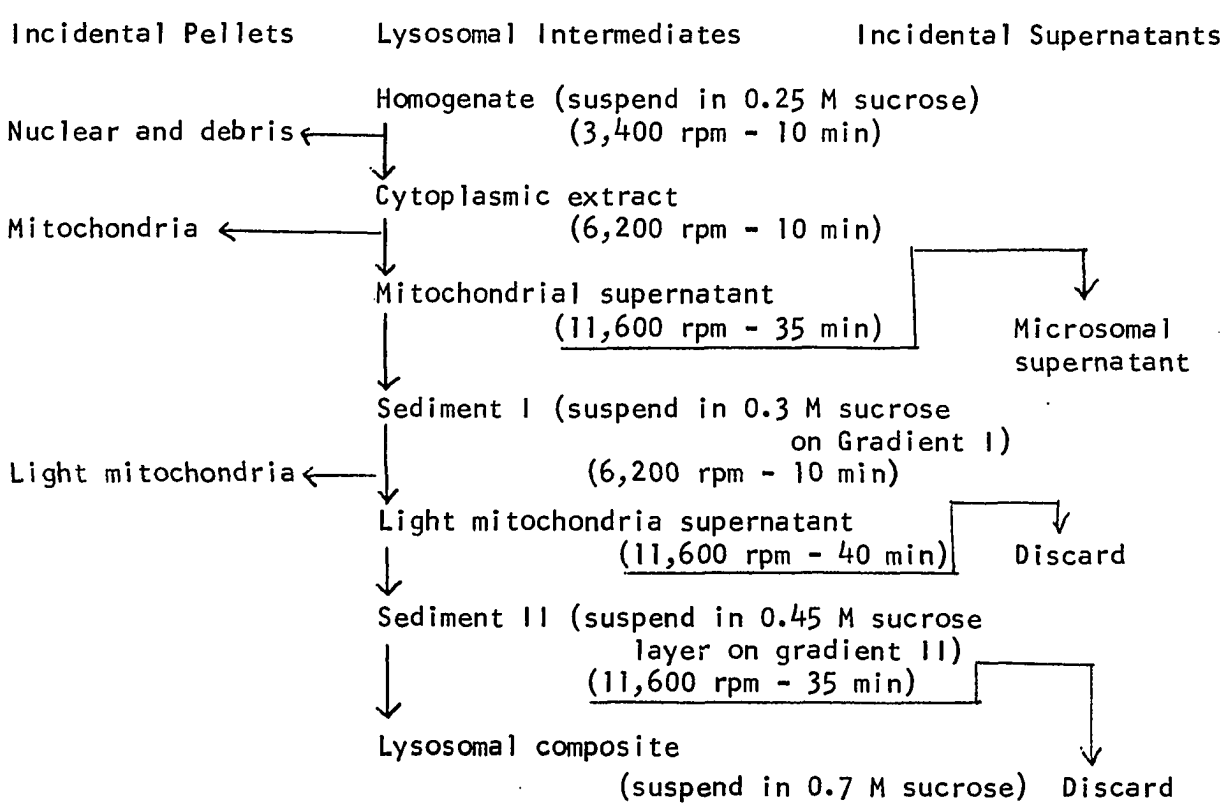
Rats injected intraperitoneally with 85 mg of Triton WR-1339 per 100 g body weight 3 1/2 days before sacrifice



Resuspended in 45% sucrose
layered beneath a sucrose gradient



were minced and homogenized in 0.25 M sucrose (1:8 w/v). The rat liver homogenate was successively centrifuged as shown below. All centrifugations were done in a Spinco Model L or L 2-65 preparative ultracentrifuge with Rotor 30. The discontinuous sucrose gradients were prepared as follows from bottom to top: Gradient I, 14 ml 0.60 M sucrose, 12 ml 0.45 M sucrose, and 10 ml 0.30 M sucrose in which a portion of sediment I was suspended; Gradient II, 14 ml 0.70 M sucrose, 12 ml 0.60 M sucrose, and 10 ml 0.45 M sucrose in which a portion of sediment II was suspended. Sediment I, sediment II, mitrochondrial, and lysosomal composite fractions were resuspended by minimum homogenization with the Potter-Elvehjem glass homogenizer.



Microsomes

Male rats were killed by a blow on the head and exsanguinated by severing the neck vessels. The liver was removed, washed with 0.15 M

buffer, pH 7.4, so that 0.1 ml of the suspension was equivalent to 1 mg microsomal protein.

Enzyme Assay Procedures

A. Enzyme systems tested for free radical generation.

Liver lysosomes prepared as described above. Lysosomal pellets were used immediately after each preparation and suspended in 0.7 M sucrose. The latency of lysosomal enzymes was always determined on lysosomal particles suspended in 0.7 M sucrose. Total activities of the enzymes were determined on lysosomal suspensions pretreated for 30 minutes at 0^{o} with the non-ionic detergent Triton X-100 at a final concentration of 0.1% (31). Various appropriate controls were included in all experiments. The total volume of every system, including the different controls, was 1 ml. The controls were constituted by incubating the lysosomes (0.3-0.5 mg protein/ml of reaction system) at 37^{o} under any of the following conditions.

1. with buffer only
2. with substrate only
3. with cofactor only
4. with enzyme only
5. with substrate and enzyme
6. with substrate and cofactor
7. with cofactor and enzyme.

The incubation time was varied depending on the nature of the experiments. In this study, the effect of free radicals generated by the catalytic activity of various flavin enzymes on the stability of lysosomal membranes was tested. (Two ml cuvettes with a 1 mm light pathway were used in all spectrophotometric assays.)

1. Microsomal NADPH oxidase system. One ml of the complete experimental incubation system contained lysosomes (0.3-0.5 mg protein/ml of reaction system), microsomes (1 mg protein/ml of reaction system), 0.4 mM ADP, 0.012 mM $FeCl_3$, 0.7 M sucrose, 0.015 M tris, pH 7.5, and 0.3 mM NADPH. The incubations were performed in a Dubnoff apparatus at 37°. After the incubations were carried out, Mn^{2+} was added to the reaction systems at a final concentration of 1.0 mM to stop further action on the lysosomal particles. The reaction systems were then assayed immediately for release of acid hydrolases from the lysosomes.

2. Purified NADPH-cytochrome P450 reductase system. A typical 1 ml reaction mixture had the following composition: lysosomes (0.3-0.5 mg protein/ml of reaction system), NADPH-cytochrome P450 reductase (0.066 mg protein/ml of reaction system), 0.3 mM NADPH, 2 mM ADP, 0.12 mM $FeCl_3$, 0.015 M tris, pH 7.5, and 0.7 M sucrose. The reaction mixture was incubated at 37° for 30 minutes and followed by addition of 1.0 mM Mn^{2+} to stop further reaction. Assays for the release of acid hydrolases from the lysosomes followed immediately.

3. Xanthine oxidase system. The incubation system contained lysosomes (0.3-0.5 mg protein/ml of reaction system), xanthine oxidase (0.219 mg protein/ml of reaction system), 0.1 mM xanthine, 2 mM ADP, 0.12 mM $FeCl_3$, and 0.7 M sucrose. The total volume of reaction mixture was 1 ml. The incubation time was 30 minutes. The incubations were performed in a Dubnoff apparatus at 37°. The reactions were stopped by the addition of 1.0 mM Mn^{2+}. The activity of lysosomal acid hydrolases released from lysosomes was determined immediately.

B. Lysosomal hydrolase enzyme assays.

The activity of lysosomal enzymes available to react with substrates was determined under conditions which would insure the integrity of particles remaining at the end of the prior incubation, as much as possible, namely, by means of an assay at 37°, maintaining the same sucrose concentrations in the assay system as in the suspension of isolated lysosomes (0.7 M sucrose). Any increase in enzyme available brought about by the specific exposure of the lysosomes to the enzymic reactions described above gives a quantitative measurement of the release of the enzymes resulting from the effect of that treatment on the lysosomes. All experiments were done with suitable controls and number of repetitions to assure validity of results. All the reagents for acid hydrolase assays described below were made up in 0.7 M sucrose.

1. Acid phosphatase. The composition of the reaction systems, the controls, and conditions of incubation are as describedin part A above except for the necessary additions described below, since the assays were performed on the same systems in which the lysosomes were initially exposed to a particular enzyme. At the end of the initial exposure period, 1.0 mM Mn^{2+} was added to the reaction system to stop further attacks on the lysosomes (20). Acid phosphatase activity was determined essentially according to the method of Fukuzawa et al. (32) by adding 0.1 ml of 250 mM sodium p-nitrophenyl phosphate and 0.1 ml of 50 mM of sodium acetate buffer, pH 5.0 to each ml of lysosomal incubation systems to be analyzed. The reaction was stopped after 10 minutes incubation at 37° by the addition of 0.25 ml of 10% (w/v) sodium hydroxide solution. After adequate dilution with distilled water (about 1:20), the optical density of yellow

supernatant was measured at 420 nm. The amount of sodium-p-nitrophenyl phosphate hydrolyzed was estimated from a standard curve using p-nitrophenol as standard.

2. Aryl sulfatase. Aryl sulfatase activity was measured by using the dipotassium salt of 2-hydroxy-5-nitrophenyl sulfate as the substrate under conditions which measure the combined activities of aryl sulfatases A and B (33). After exposure of the lysosomes to one of the particular enzymic reactions described above, the action on the lysosomes was stopped by the addition of 1.0 mM Mn^{2+} (final concentration). Then, 0.2 ml of 0.17 M 2-hydroxy-nitrophenyl sulfate, and 0.2 ml of 1.5 M of sodium acetate buffer, pH 5.0 were added to each tube. Total volume for these assays was 1.4 ml. After incubation for 15 min at 37°, the reaction was stopped by the addition of 3 ml of 2% (w/v) phosphotungstic acid in 0.1 N HCl. The precipitated proteins were removed by centrifugation. To 1.5 ml of the supernatant fraction was added 3.5 ml of alkaline quinol reagent for the final color development. (The alkaline quinol reagent was made by combining 1 part of 4% (w/v) quinol in 0.1 N HCl and 20 parts of 5% Na_2SO_3 in 2.5 N NaOH). The optical density of the color was determined at 520 nm. A standard curve was constructed using 2-hydroxy-5-nitrophenol as standard.

3. β-galactosidase. The activity of β-galactosidase was assayed by the use of the substrate 0-nitrophenyl-β-d-galactopyranoside. After prior exposure of the lysosomes to one of the radical generating systems described above and terminating the attack on the lysosomes by addition of 1.0 mM Mn^{2+}, the β-galactosidase activity released was assayed according to the method of Sellinger, et al. (34). This was done by adding 0.1 ml of

0.025 M substrate and 0.1 ml of pyridine-HCl buffer, pH 5.0 to each ml of lysosomal prior incubation systems. The total volume was 1.2 ml after these additions. After incubation for 15 minutes at 37°, the reaction was stopped by addition of 1.5 ml of 2.75% (w/v) trichloroacetic acid. A clear supernatant was obtained after centrifugation. To 2.0 ml of the clear supernatant were added 0.5 ml of 0.5 M NaOH and 1.0 ml of 0.25 M Na_2CO_3-glycine buffer, pH 10.0. The enzyme activity was determined by measuring the amount of o-nitrophenol formed by its absorbance at 420 nm. A standard curve was constructed using o-nitrophenol as standard.

C. Assay of superoxide anion generation during xanthine oxidase activity.

This assay was performed by the method of McCord et al. (37) with slight modification. The reduction of cytochrome c by superoxide anion was performed in 0.05 M potassium phosphate, pH 7.8, containing 0.1 mM xanthine, xanthine oxidase (0.015 mg protein/ml of assay system), and cytochrome c (0.129 mg protein/ml of assay system). The amount of cytochrome c reduced was determined by measuring the increase in absorbancy at 550 nm, using 27,700 as the molar absorbancy of cytochrome c.

D. Assay of uric acid production by xanthine oxidase activity.

Reactions were performed in a total volume of 1.5 ml. The assay system consisted of 0.05 M potassium phosphate buffer, pH 7.8, 0.1 mM xanthine, and xanthine oxidase (0.015 mg protein/ml of reaction system). Uric acid formation was determined by measuring the increase in absorbancy at 280 nm.

E. Assay for formation of malondialdehyde.

The enzymic systems described under part A of "Enzyme assay procedures" were terminated by the addition of 0.5 ml of 5% (w/v) trichloroacetic acid (TCA). One ml of 5% (w/v) thiobarbituric acid (TBA) was then added and the mixtures were heated at 60° for 90 min. After cooling, 1.0 ml of 70% (w/v) TCA was added to each tube and swirled gently. The samples were then centrifuged and optical density of clear pink supernatant fraction was determined at a wavelength of 532 nm. Samples that were turbid were extracted with chloroform (to remove dispersed lipid) and the optical density of the clear aqueous was then determined. If the sample color was too intense to be read directly, suitable dilutions were prepared by addition of a mixture with the same reagent composition as that of the sample.

Determination of Protein

The amount of protein was estimated by the method of Lowry et al. (35). Samples of lysosomal suspensions, microsomal suspensions and of the purified enzymes containing 20 to 100 μg of protein were diluted to 1.0 ml with distilled water and added to 1.0 ml of the alkaline copper sulfate solution described for this method. After standing for 15 min at room temperature, 0.1 ml of 1 N phenol reagent was added to the sample mixture and the tube was shaken with a Vortex mixer immediately. The sample was then allowed to stand at room temperature for 30 min, after which the absorbance at 500 nm was determined. Bovine serum albumin was used as a standard.

CHAPTER III

RESULTS

Lysosomal Disruption by a Free Radical-like Component Generated during Microsomal NADPH oxidase Activity

Evidence has been demonstrated for the production of a highly transient factor having the properties of a free radical during the oxidation of NADPH by liver microsomes (21). This factor is apparently responsible for the peroxidative chain scission of phospholipid polyunsaturated fatty acyl groups which occur in the microsomal membrane itself during NADPH-dependent electron transport. The production of this component by the enzyme system is enhanced in microsomes from tocopherol-deficient rats and depressed in animals supplemented with an additional increment of α-tocopherol.

This being the case we opted to determine if the factor was capable of causing a decrease in the stability of lysosomes, since these organelles are often in close proximity to elements of the endoplasmic reticulum, and because lack of a suitable radical-trapping agent in animal diet results in intracellular structural deterioration in certain tissues characterized by increased lysosomal hydrolase activity (38). The ensuing studies demonstrated that incubation of lysosomes with microsomes which are in the process of oxidizing NADPH enzymically results in a progressive increase of free lysosomal hydrolase activity. Figure 3 illustrates the

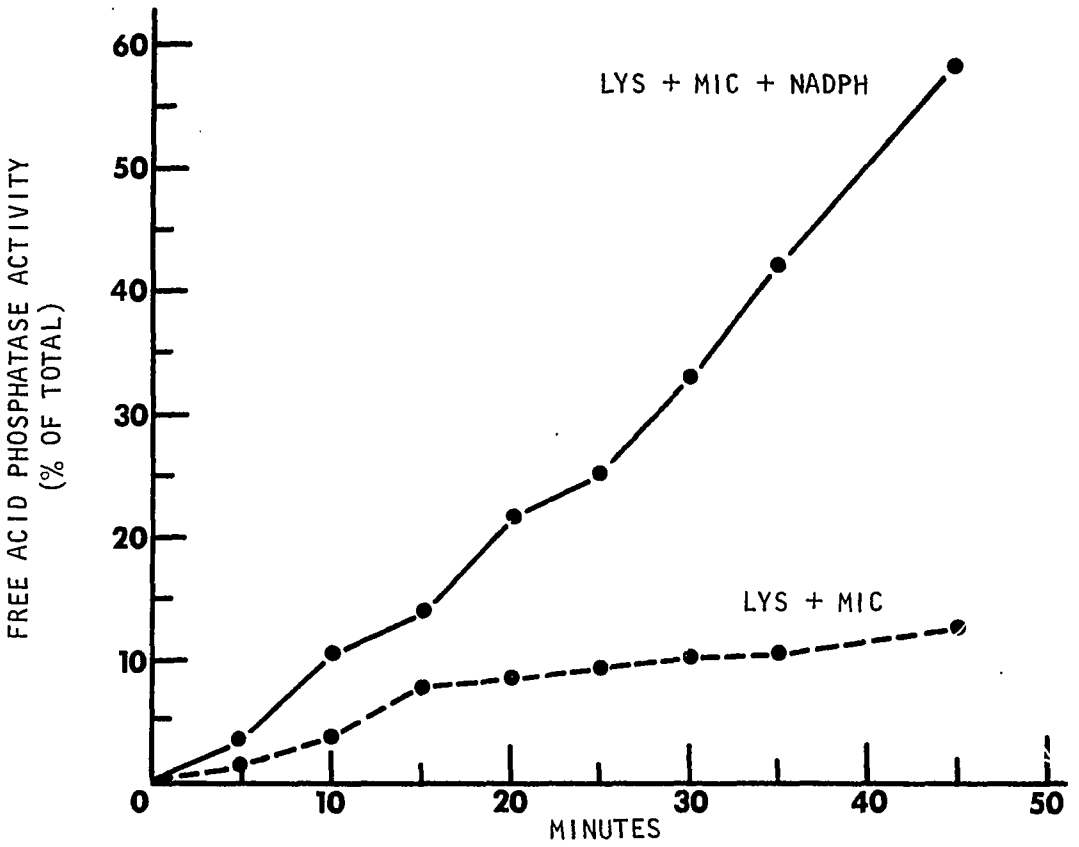


Figure 3. Progress curve of the release of acid phosphatase from lysosomes exposed to microsomal NADPH oxidase activity. The reaction systems contained lysosomes (Lys), (0.3 mg protein/ml reaction system); microsomes (Mic), (1.0 mg protein/ml reaction system); NADPH (where indicated), 0.3 mM; Fe^{3+}, 0.012 mM; 0.015 M Tris-HCl buffer, pH 7.4; sucrose 0.7 M. Incubation was carried out at 37°. The reaction was stopped at the different time intervals by the addition of 1.0 mM Mn^{2+} (final concentration), and the activity of free acid phosphatase was determined immediately as described under Experimental Procedures.

course of release of free acid phosphatase from lysosomes exposed to microsomes which are in the process of oxidizing NADPH enzymically. In this case, about 60% of the total phosphatase activity was released in 45 min. A small percentage of this lysosomal enzyme was released in the control system, presumably due to shaking during incubation and to thermal activation. This lytic action caused by the microsomal NADPH oxidase system is accompanied by the formation of significant amounts of malondialdehyde (Table 1). Table 1 also demonstrates the requirement for Fe^{3+} to effect deterioration of the lysosomal membranes. Without the substrate for the enzymic reaction (NADPH), however, the addition of the iron had very little effect on the lysosomes. Evidence will be presented with other data to demonstrate the necessity for enzymic oxidation of NADPH to occur before attack on the lysosomes occurs. The presence of the ADP is required to promote the solubility of Fe^{3+} in the form of a chelate, otherwise very low concentrations of iron would exist in solution because of its low solubility. Although significant amounts of acid phosphatase and malondialdehyde were formed in the system: Lysosomes + Microsomes + NADPH, more than three times as much acid phosphatase was released and twice as much malondialdehyde was formed when chelated iron was included in the system. The results indicate that a peroxidative attack on lysosomal membrane lipids was associated with release of the acid hydrolase. The amounts of ADP and Fe^{3+} required for this activity are compatible with physiological levels of these substances found in the liver cytosol.

Table 2 shows analyses of three different hydrolase enzymes which were activated during NADPH oxidation by microsomes, all of which indicate lysosomal membrane deterioration. Incubation of lysosomes + microsomes

TABLE 1

A CORRELATION BETWEEN MALONDIALDEHYDE FORMATION AND ACID PHOSPHATASE RELEASED FROM LYSOSOMES EXPOSED TO MICROSOMAL NADPH OXIDASE SYSTEMS

Additions to incubation	Free acid phosphatase released	Malondialdehyde formation
	% of Total	mμ mole/ml incubation system
Mic	4.99	4.59
Lys	8.99	2.24
Lys + Mic	12.99	5.71
Lys + Mic + ADP-Fe^{3+}	19.99	8.85
Lys + Mic + ADP-Fe^{3+} + NADPH	94.99	30.24
Lys + ADP-Fe^{3+} + NADPH	19.49	8.74
Lys + Mic + NADPH	29.39	13.44
Lys + ADP-Fe^{3+}	12.99	5.60

The incubation medium was 0.7 M sucrose in 0.015 M Tris-HCl buffer, pH 7.4. Additions were made as indicated. Concentration of lysosomes, microsomes, ADP-Fe^{3+}, and NADPH were as described under Experimental Procedures. Total activity of acid phosphatase of lysosomes was measured after treatment of the particles with 0.1% Triton X-100. The percent of total activity of acid phosphatase released was determined by comparison of each value to the maximum released by treatment with Triton X-100. The latter treatment is described under Experimental Procedures. The incubation time was 45 min.

TABLE 2

RELEASE OF 3 DIFFERENT ACID HYDROLASE ENZYMES FROM THE LYSOSOMES DURING NADPH OXIDATION BY MICROSOMES

		Additions to incubation medium		
		Lys	Lys + Mic + ADP-Fe^{3+}	Lys + Mic + ADP-Fe^{3+} + NADPH
Acid phosphatase μmoles p-Nitrophenol/10 min/ mg protein (avg. 5 exp.)		1.92	4.22	16.91
β-galactosidase μmoles O-Nitrophenol/ 15 min/mg protein	#1	0.19	0.25	1.10
	#2	0.08	0.10	0.92
Aryl Sulfatase μmoles O-Nitrocatechol 15 min/mg protein	#1	0.64	0.87	2.39
	#2	0.73	1.26	2.89

The incubation medium was 0.7 M sucrose in 0.015 M Tris-HCl buffer, pH 7.4. Additions were made as indicated. The concentration of lysosomes, microsomes, ADP-Fe^{3+}, and NADPH were as described under Figure 1. The total volumes of the reaction systems were 1.0 ml. The reaction was carried out at 37^{o} for 45 min. Assays for the activities of acid phosphatase, aryl sulfatase, and β-galactosidase were as described under Experimental Procedures. Activity of enzymes was expressed by μmoles product/10 min/mg protein.

+ NADPH + ADP-Fe^{3+} causes the values for free hydrolase activity of acid phosphatase, β-galactosidase and aryl sulfatase to rise significantly. The controls (lysosomes + microsomes + ADP-Fe^{3+}) showed very little activity of these lysosomal enzymes.

Enzymic oxidation of NADPH by liver microsomes is markedly heat labile. Mild warming of microsomes (65^{o} for one minute) results in complete loss of this enzyme activity and their ability to produce the factor which promotes lysosomal lysis (Table 3). This table demonstrates that the release of the hydrolases must be a consequence of enzymic oxidation of NADPH by microsomes. Only in the system in which NADPH oxidation was occurring does significant hydrolase release occur. Omitting NADPH resulted in essentially no activation of hydrolase activity. The exposure of the lysosomes to NADPH alone did not cause release of hydrolases. The most significant data here shows that when the microsomes had been subjected to a brief heat treatment (a treatment which totally inactivates the enzymic oxidation of NADPH (20)) prior to addition to the reaction system, release of the hydrolases was abolished. In subsequent studies, only measurements of acid phosphatase is shown since it was established that the other hydrolases exhibited identical patterns of release.

A study was done to demonstrate that there is no accumulation of any product which is responsible for lysosomal lysis. The microsomes are subjected to prior incubation in the presence of NADPH and ADP-Fe^{3+} for 30 min, followed by the addition of 1.0 mM Mn^{2+} (which totally inhibits further peroxidation of lipids in biological systems (20)). At this point addition of lysosomes was made. The results show that no lysosomal disruption occurred during a subsequent 30 min incubation (Table 4). Thus

TABLE 3

RELEASE OF LYSOSOMAL HYDROLASES BY MICROSOMAL NADPH OXIDASE ACTIVITY

Components added to incubation medium[a]	Acid phosphatase activity	Aryl sulfatase activity	β-galactosidase activity
	μmoles product/10 min/mg protein		
Lys	1.91	.49	.05
Mic	1.18	.11	.00
Lys + Mic	3.82	.84	.07
Lys + NADPH (0.3 μmole)	2.06	.53	.11
Lys + Mic + NADPH	25.74	1.93	.61
Lys + Mic (Δ)[b] + NADPH	1.62	1.08	.08
Lys + Mic + NADPH + Mn^{2+}	6.32	.87	.29
Lys + Triton X-100	29.41	3.40	1.33

[a]The incubation medium, quantity of components added and conditions of incubation were as described in Table 2. Incubation time was 45 min.

[b]Mic (Δ) = Microsomes heated at 65° for 1 min before addition to the reaction system.

TABLE 4

DEMONSTRATION THAT LYSOSOMAL LYSIS IS NOT DUE TO ANY PRODUCT FORMED DURING THE MICROSOMAL NADPH OXIDASE SYSTEMS

Systems analyzed	Free acid phosphatase released
	% of Total
Control enzyme system	15.19
Experimental enzyme system	83.99
Preincubated experimental[a] enzyme system	18.39

[a]Experimental enzyme system incubated for 30 min without lysosomes followed by the addition of 1.0 mM Mn^{2+}. Lysosomes were then added and the system was incubated for another 30 min. The release of acid phosphatase was measured as described under Table 1.

whatever factor causes the lysosomal lysis exists only during the enzymic activity. Evidence that a factor having free radical properties with a brief half-life was produced by the microsomal system and was responsible for the release of the lysosomal hydrolases is shown on Table 5. If free radical-trapping agents or compounds capable of reacting with free radicals are added to the reaction system, the release of the hydrolases from the lysosomes is largely, and in some cases completely, abolished.

Table 5 shows that Santoquin (ethoxyquin), diphenylamine and N-methylaniline at a final concentration of 1.0 mM were all effective in preventing the release of the lysosomal hydrolases during the oxidation of NADPH by liver microsomes. The protection was not due to the inactivation of the lysosomal hydrolases by these substances nor due to the interference with the activities of these three enzymes per se. The addition of Triton X-100 to duplicate systems at the end of the incubation resulted in release of fully active hydrolases. It appears that the oxidation of NADPH by microsomes initiated formation of radicals which, in the presence of Fe^{3+}, attack the lysosomal membrane.

Further Evidence for Free Radicals as the Lysosomal Lytic Agent

The lipid peroxidation reaction in the system is eliminated if the microsomes are obtained from animals supplemented with somewhat higher than normal levels of α-tocopherol (39). Several groups of rats were given the same laboratory ration as those used in all other experiments except that the ration was supplemented with 30 mg percent of α-tocopherol. The animals were fed the diets for at least 10 days before sacrifice. Other groups of rats were injected with 75 mg/Kg of body weight of tocopherol (as described under Experimental Procedures) instead of dietary supplement,

TABLE 5

EFFECT OF FREE RADICAL-SCAVENGING AGENTS ON THE RELEASE OF LYSOSOMAL HYDROLASES BY MICROSOMAL NADPH OXIDASE ACTIVITY

Components added to incubation medium[a]	Acid phosphatase activity	Aryl sulfatase activity	β-galactosidase activity
	μmoles product/10 min/mg protein		
Lys + Mic	3.82	.58	.13
Lys + Mic + NADPH	24.41	1.60	.73
Lys + Mic + NADPH + Santoquin (1 μmole)	6.18	.66	.13
Lys + Mic + NADPH + N-methylaniline (1 μmole)	4.41	.62	.27
Lys + Mic + NADPH + Diphenylamine (1 μmole)	10.29	.85	.13
Lys + Triton X-100	29.41	3.15	1.07

[a]The incubation medium, quantity of components added, and conditions of incubation were as described in Table 2 except as indicated. Incubation time was 45 min.

administered 12 hours before use. Microsomes were prepared from animals from the various groups. These various microsomal preparations were incubated with NADPH and with aliquots of lysosomes from the same lysosomal preparation. The release of hydrolytic enzymes by incubating lysosomes with microsomes from animals fed the standard laboratory ration in the presence of NADPH and ADP-Fe^{3+} is shown in Table 6. The results showed in all cases a significant reduction in lysosomal lysis after tocopherol administration by either method. In addition, Figure 4 also shows the progressive release of acid phosphatase from lysosomes incubated in systems containing microsomes from livers injected with α-tocopherol 12 hrs before use, in which case one observes less hydrolase release. This inhibition of lysosomal lysis during the enzyme reaction would appear to be due to a decreased attack on lysosomal membrane lipids judging by the decrease in malondialdehyde by microsomes from α-tocopherol-supplemented animals. The control system (containing no NADPH) showed essentially no difference in acid phosphatase activity during the time period of the experiment. It was of interest to determine whether or not an inhibition of acid phosphatase release would occur in lysosomes derived from animals supplemented with α-tocopherol during incubation with the NADPH oxidase system of normal microsomes and, in addition, to determine the effect of normal microsomes oxidizing NADPH systems on lysosomes derived from α-tocopherol deficient animals. Three groups of rats were analysed as illustrated in Figure 5. Some of the rats were fed a standard laboratory ration, while others were fed a Torula yeast diet deficient in α-tocopherol (which was as described under Experimental Procedures) for a period of 2 weeks. The latter group were in an early stage of liver necrosis. Still others were fed the Torula yeast diet supplemented with 50 mg of α-tocopherol.

TABLE 6

EFFECT OF SUPPLEMENTING α-TOCOPHEROL TO RATS DONATING THE LIVER MICROSOMES ON THE RELEASE OF ACID PHOSPHATASE FROM LYSOSOMES BY MICROSOMAL NADPH OXIDASE ACTIVITY

Method of tocopherol supplementation[a]	Level of tocopherol supplement given to microsome donors	Acid phosphatase released
		% of Total
Diet	None	100.00
Diet	30 mg/100 g diet	43.75
Intraperitoneal injection	None	100.00
Intraperitoneal injection	75 mg/Kg body weight	52.75

Incubation systems were assembled as described under Experimental Procedures with the addition of 0.1 ml of microsomes from one of the supplemented animals per ml of reaction system. The incubation period was 45 min at 37°. Values are expressed as the percentage of acid phosphatase release obtained with microsomes from animals fed the standard laboratory ration.

[a]All animals in these experiments were fed the standard laboratory ration. Tocopherol supplementation by diet was given by mixing with the standard laboratory ration (see Experimental Procedures).

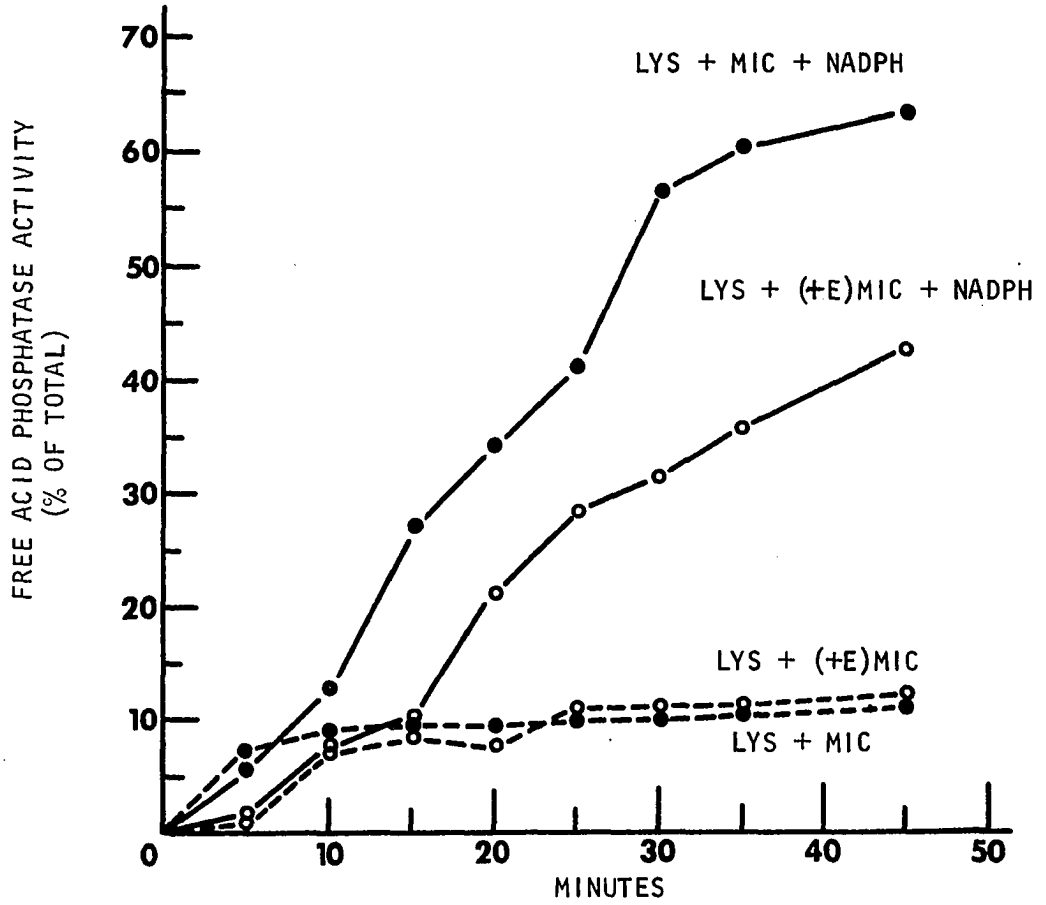


Figure 4. Effect of supplementing α-tocopherol on the release of acid phosphatase from liver lysosomes by microsomal NADPH oxidase activity. Normal rats fed a stock ration were injected with α-tocopherol (75 mg/kg body weight) 12 hrs prior to preparing microsomes. The conditions were as in Fig. 3. (+E)mic = microsomes isolated from α-tocopherol-injected animals; other particles were from normal animals. Equivalent amounts of lysosomes from the same lysosomal preparation were used in all systems.

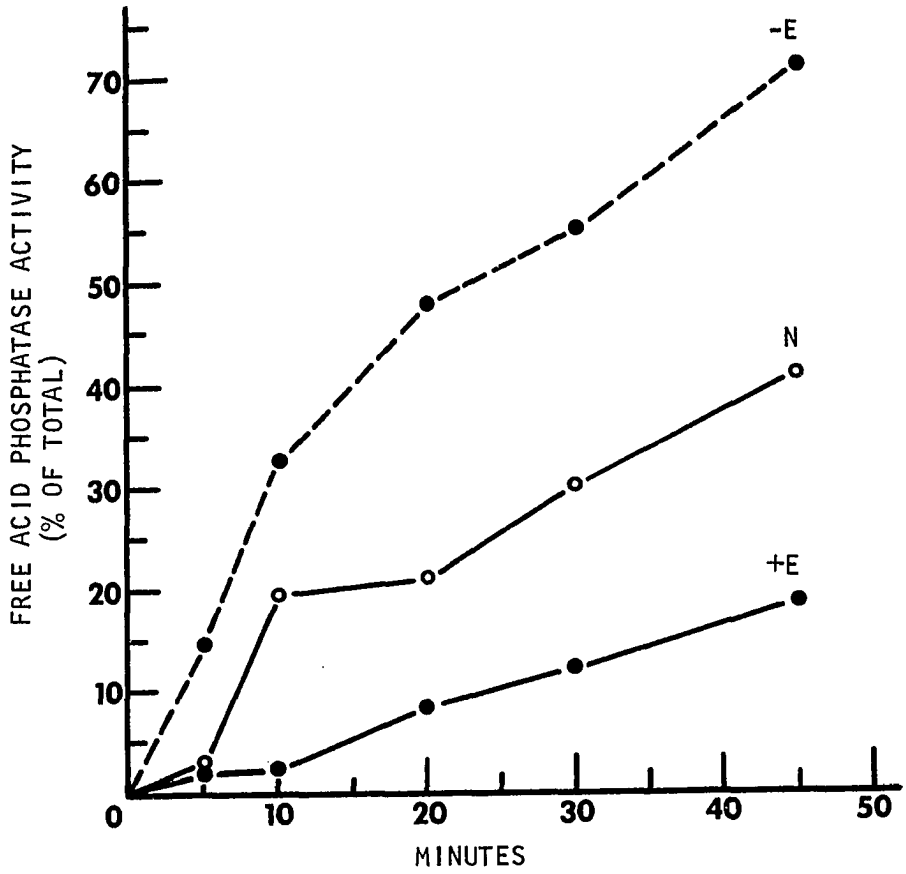


Figure 5. Effect of dietary levels of α-tocopherol on the release of acid phosphatase from liver lysosomes by microsomal NADPH oxidase activity. N = lysosomes from normal rats; -E = lysosomes from rats fed α-tocopherol - deficient diet (15); +E = lysosomes from rats fed -E diet supplemented with 50 mg% α-tocopherol. The conditions of the experiment are as in Fig. 3. -EC, NC and +EC are 45 min values for control systems in which no NADPH is added.

Lysosomes were isolated from all three of these types of animals and exposed to microsomal NADPH oxidizing systems. Lysosomes from tocopherol-supplemented animals showed least acid phosphatase release by the microsomes oxidizing NADPH systems. Lysosomes from normal animals showed an intermediate susceptibility. The significant fact is that lysosomes from animals deficient in α-tocopherol showed the greatest susceptibility and are considerably more susceptible to lysis by the radical-like factor than are lysosomes from α-tocopherol-supplemented animals. These results were not due to the difference in lysosomal thermal stability since lysosomes from the different levels of α-tocopherol rats incubated with microsomes from the same preparation, respectively, (containing no NADPH) had essentially the same small extent of lysis as those isolated from rats fed the standard ration. Thus the prevention of lysosomal acid phosphatase release during the microsomal NADPH oxidase reaction would appear due to radical scavenging by the vitamin E in the lysosomal membrane. Because the effect of this factor on lysosomes used in these studies may have been dependent on the lysosomes having been isolated by the Triton injection procedure, similar experiments were conducted with lysosomes which were isolated from rats using a discontinuous sucrose gradient (40) without previous injection of the rats with Triton. The results were identical with those reported above.

Lysosomal Lysis Caused by Purified NADPH Cytochrome P450 Reductase

The liver microsomal hydroxylation system which metabolizes drugs and steroids was separated into fractions containing cytochrome P450, cytochrome P450 reductase, and phospholipid, all of which are needed for catalytic activity (17). Experiments were designed to study

the origin of the apparent radical which attacks the lysosomal membrane. A purified NADPH-cytochrome P450 reductase was incubated in a system containing NADPH and lysosomes. The basic medium contained 0.7 M sucrose in 0.015 M Tris-HCl buffer, pH 7.4. Incubation was performed in a Dubnoff shaking waterbath apparatus for 30 min at 37°. The data (Table 7) shows that this system is effective in causing lysis of a large portion of the lysosomes in the presence of ADP and Fe^{3+} at a final concentration of 0.1 mM. An appreciable amount of malondialdehyde is formed simultaneously in the system. Table 8 shows the effect of NADPH oxidation by purified NADPH cytochrome P450 reductase on the release of lysosomal acid phosphatase. Only in the system in which NADPH oxidation was occurring in the presence of Fe^{3+} (chelated by ADP) does significant hydrolase release occur. No acid phosphatase was released from lysosomes in the systems omitting NADPH. Systems containing boiled NADPH-cytochrome P450 reductase resulted in essentially no activation of hydrolase activity. A small percentage of acid phosphatase released from lysosomes incubated in the presence of NADPH and ADP-Fe^{3+} was presumably due to slight contamination of lysosomes by microsomes. Potent inhibitors of lipid peroxidation such as Mn^{2+}, Co^{2+} and Ce^{3+} prevented the lysosomal lysis and lipid peroxidation caused by the purified cytochrome P450 reductase systems. Furthermore, if the enzymic reaction is subjected to prior incubation for 30 min and then inactivated by the addition of 1.0 mM Mn^{2+} (which has been shown to inhibit lipid peroxidation associated with the oxidation of NADPH by cytochrome P450 reductase), the addition of lysosomes at that point produces no membrane breakdown during a subsequent incubation (Table 9). This study apparently eliminates the possibility that any stable product accumulates

EFFECT OF PURIFIED CYTOCHROME P450 REDUCTASE ACTIVITY ON LYSOSOMAL MEMBRANES REQUIREMENT FOR CHELATED IRON TO EFFECT PEROXIDATIVE MEMBRANE DAMAGE

Additions to incubation medium	Free acid phosphatase released	Malondialdehyde formed
	% of Total	mμ moles/ml reaction system
Lys	8.99	2.24
Lys + cyt P450 red[a]	11.49	3.58
Lys + ADP-Fe^{3+}	12.99	5.82
Lys + cyt P450 red + ADP-Fe^{3+}	18.99	9.41
Lys + cyt P450 red + ADP-Fe^{3+} + NADPH	42.99	18.14
Lys + ADP-Fe^{3+} + NADPH	19.41	8.51
Lys + cyt P450 red + ADP-Fe^{3+}[b] + NADPH	16.99	7.50
Lys + cyt P450 red + NADPH	12.99	4.93

The basic medium contained 0.7 M sucrose in 0.015 M Tris, pH 7.4. Additions were made as indicated in the table. Lysosomes (Lys), (0.3-0.5 mg protein/ml reaction system); NADPH, 0.3 mM; ADP, 20 mM; Fe^{3+}, 0.12 mM; purified NADPH dependent-cytochrome P450 reductase (cyt P450 red), 0.066 mg protein/ml reaction system. Incubations were carried out at 37° for 30 min and followed by addition of 1.0 mM Mn^{2+} to stop further action on lysosomes. Assay of acid phosphatase released was described under Table 1. Malondialdehyde formation was described under Experimental Procedures.

[a] purified NADPH dependent cytochrome P450 reductase (cyt P450 red)

[b] In this system, the iron concentration was 0.012 mM instead of 0.12 mM, and the ADP was 0.4 mM instead of 2.0 mM.

TABLE 8

RELEASE OF LYSOSOMAL ACID PHOSPHATASE BY PURIFIED NADPH-DEPENDENT CYTOCHROME P450 REDUCTASE ACTIVITY

Additions to the incubation medium	Free acid phosphatase released	Malondialdehyde formation
	% of Total	mμ moles/ml reaction system
Lys	3.30	2.69
Lys + ADP-Fe^{3+}	6.61	2.57
Lys + ADP-Fe^{3+} + cyt P450 red	7.66	2.80
Lys + ADP-Fe^{3+} + cyt P450 red + NADPH	69.42	9.07
Lys + ADP-Fe^{3+} + cyt P450 red (Δ) + NADPH	16.52	3.58
Lys + ADP-Fe^{3+} + NADPH	17.35	3.36
Lys + ADP-Fe^{3+} + cyt P450 red + NADPH + Mn^{2+} a	9.91	2.46
Lys + ADP-Fe^{3+} + cyt P450 red + NADPH + Co^{2+} a	14.05	2.69
Lys + ADP-Fe^{3+} + cyt P450 red + NADPH + Ce^{3+} a	12.39	2.64

The basic incubation medium, quantity of components added, and conditions of incubation were as described in Table 7 except where indicated.

[a]The final concentration of Mn^{2+}, Co^{2+}, and Ce^{3+} was 1.0 mM.

(Δ) Cyt P450 red heated at 100° for 5 min.

TABLE 9

DEMONSTRATION THAT LYSOSOMAL LYSIS IS NOT DUE TO ANY ACCUMULATIVE, STABLE PRODUCT FORMED DURING THE PURIFIED NADPH-DEPENDENT CYTOCHROME P450 REDUCTASE ACTIVITY

System analyzed	Free acid phosphatase released
	% of Total
Lys + cyt P450 red + ADP-Fe^{3+}	5.00
Lys + cyt P450 red + ADP-Fe^{3+} + NADPH	44.17
Lys[a] + cyt P450 red + ADP-Fe^{3+} + NADPH	10.50

The composition of the systems and conditions of incubation were the same as in Table 8, except as indicated. Measurements of free acid phosphatase activity was then assayed according to Experimental Procedures. The basic medium, incubation conditions and quantity of components added were the same as described under Table 7.

[a]Lys + cyt P450 red + ADP-Fe^{3+} + NADPH = System containing cyt P450 red + ADP-Fe^{3+} + NADPH incubated for 30 min <u>without lysosomes</u>. This was followed then by the addition of 1.0 mM Mn^{2+} to stop further potential peroxidative reactions. Lysosomes were then added and the system was incubated for another 30 min.

in the system which is responsible for the lysosomal lysis during the incubation period.

The addition to the system of structurally unrelated free radical-trapping agents (such as Santoquin and diphenylamine) at the same concentration as that used to inhibit NADPH-dependent microsomal phospho-lipid peroxidation, greatly inhibited lysis of lysosomes promoted by cytochrome P450 reductase activity (Table 10). These studies indicate that a free radical species is involved in the lysosomal degradation process.

Lysosomal Lysis Caused by Xanthine Oxidase Activity

It is well known that superoxide anion is generated by milk xanthine oxidase when acting on its substrate in the presence of oxygen (41). In order to determine the effect of the superoxide radical on the stability of the lysosomal membrane, the behavior of lysosomes in the presence of a highly purified xanthine oxidase and xanthine was studied. The basic incubation medium contained 0.7 M sucrose only. The incubation time was 30 min. Although it was determined that this degree of xanthine oxidase activity produced a sufficient flux of $O_2^{\cdot-}$ (superoxide anion radical) to reduce cytochrome c at a rate of 4 μmoles/min, little or no degradation of the lysosomal membrane occurred during the incubation period since significant release of acid phosphatase and formation of malondialdehyde did not occur (Table 11). Even the addition of 0.01 mM Fe^{3+} (chelated with ADP) did not facilitate an attack on the membrane. However, addition of 0.1 mM Fe^{3+} resulted in a vigorous disruption of the lysosomes and production of malondialdehyde. Omission of the substrate (xanthine) from this system resulted in no lysosomal membrane breakdown,

TABLE 10

EFFECT OF FREE RADICAL-SCAVENGING AGENTS ON THE RELEASE OF LYSOSOMAL ACID PHOSPHATASE BY PURIFIED NADPH-CYTOCHROME P450 REDUCTASE

Components added to incubation medium	Free acid phosphatase released	Malondialdehyde formation
	% of Total	mμ moles/ml reaction system
Lys + ADP-Fe^{3+} + cyt P450 red	17.46	6.72
Lys + ADP-Fe^{3+} + cyt P450 red + NADPH	63.25	15.46
Lys + ADP-Fe^{3+} + cyt P450 red + NADPH + Santoquin (1 μmole)	18.69	6.94
Lys + ADP-Fe^{3+} + cyt P450 red + NADPH + Diphenylamine (1 μmole)	10.24	6.27

The incubation medium, quantity of components added, and conditions of incubation were as described in Table 7 except where specifically indicated.

TABLE 11

EFFECT OF XANTHINE OXIDASE ACTIVITY ON LYSOSOMAL MEMBRANES--- REQUIREMENT FOR CHELATED IRON TO EFFECT MEMBRANE DAMAGE

Additions to incubation medium	Free acid phosphatase released	Malondialdehyde formation
	% of Total	mμ moles/ml reaction system
Lys	13.99	1.57
Lys + Xanthine	8.99	1.57
Lys + Xanthine + Xanthine oxidase	17.99	2.46
Lys + Xanthine + Xanthine oxidase + ADP-Fe^{3+}	64.00	6.50
Lys + Xanthine oxidase + ADP-Fe^{3+}	10.99	2.91
Lys + ADP-Fe^{3+}	5.19	2.46
Lys + Xanthine + Xanthine oxidase + ADP-Fe^{3+a}	17.01	2.69

The incubation medium consisted of 0.7 M sucrose only. Additions were made as indicated. Lysosomes (0.3 mg protein/ml incubation system). Xanthine, 0.1 mM; Xanthine oxidase (.219 mg protein/ml incubation system). ADP, 2.0 mM; Fe, 0.12 mM except as indicated. Acid phosphatase release was measured as described under Experimental Procedures. Incubation time was 30 min.

[a]The Fe^{3+} concentration in this experiment was 0.012 mM.

indicating that the activity of xanthine oxidase was necessary to promote the disruption, but that a concentration of iron between 0.01 mM and 0.1 mM was required in order to effect an attack of $O_2^{\cdot-}$ on the lysosomal membrane. The addition of lipid peroxidation inhibitor (Mn^{2+}, Co^{2+} or Ce^{3+}) to the xanthine oxidase system which attacks lysosomes, prevented the lytic action just as it did in the microsomal system and the purified cytochrome P450 reductase system (Table 12). Again, xanthine oxidase previously incubated in the presence of xanthine and chelated iron for 30 min, followed by the addition of Mn^{2+}, and then followed by the addition of lysosomes, results in no disruption of these particles during a subsequent 30 min incubation (Table 13). This fact demonstrated that there is no significant accumulation of any product which is responsible for the lytic action. The following experiment was designed to determine if a radical species is involved in the lysosomal lysis caused by xanthine oxidase activity in the presence of chelated iron. An inhibitory effect of lysosomal lysis was observed on the addition of free radical trapping agents to the system (Table 14). These agents have been shown neither to interfer with xanthine oxidase activity per se nor to interfer with the subsequent assay for acid phosphatase activity.

Effect of Superoxide Dismutase

The discovery of superoxide dismutase, which catalyzes the dismutation of $O_2^{\cdot-}$ to oxygen and hydrogen peroxide (37) has provided a useful tool in probing the reactions involving oxygen radicals (42). It has previously reported that superoxide anion did not appear to be the peroxidizing agent produced by microsomal electron transport, since superoxide dismutase had no effect on microsomal lipids in that system under

TABLE 12

RELEASE OF LYSOSOMAL ACID PHOSPHATASE BY XANTHINE OXIDASE ACTIVITY

Components added to incubation medium	Free acid phosphatase released	Malondialdehyde formation
	% of Total	mμ moles/ml reaction system
Lys + ADP-Fe^{3+} + Xanthine oxidase	18.51	2.69
Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine	54.62	7.17
Lys + ADP-Fe^{3+} + Xanthine oxidase (Δ) + Xanthine	24.99	2.24
Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine + Mn^{2+} (1 μmole)	21.29	2.24
Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine + Co^{2+} (1 μmole)	21.85	2.58
Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine + Ce^{3+} (1 μmole)	23.14	2.87

The incubation medium, quantity of components added, and conditions of incubation were as described in Table 11.

Xanthine oxidase (Δ) = Xanthine oxidase heated in boiling water for 2 min before addition to the reaction system.

TABLE 13

DEMONSTRATION THAT LYSOSOMAL LYSIS IS NOT DUE TO ANY SIGNIFICANT AMOUNT OF PRODUCT ACCUMULATED DURING THE ACTIVITY OF THE XANTHINE OXIDASE SYSTEM

System analyzed	Free acid phosphatase released
	% of Total
Lys + ADP-Fe^{3+} + Xanthine oxidase	9.59
Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine	48.98
[a]Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine	15.99

The incubation medium, quantity of components added and conditions of incubation were as described in Table 11.

[a]Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine = System containing ADP-Fe^{3+} + Xanthine oxidase + Xanthine incubated for 30 min followed by the addition of 1.0 mM Mn^{2+} to stop further reaction. Lysosomes were then added and the system was incubated for another 30 min. The activity of acid phosphatase release was measured as described in Table 1.

TABLE 14

EFFECT OF FREE RADICAL-SCAVENGING AGENTS ON THE RELEASE OF LYSOSOMAL ACID PHOSPHATASE BY XANTHINE OXIDASE ACTIVITY IN THE PRESENCE OF ADP-Fe^{3+}

Components added to incubation medium	Free acid phosphatase released	Malondialdehyde formation
	% of Total	mμ moles/ml reaction system
Lys + ADP-Fe^{3+} + Xanthine oxidase	20.49	4.94
Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine	56.55	10.98
Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine + Santoquin (1 μmole)	19.67	5.04
Lys + ADP-Fe^{3+} + Xanthine oxidase + Xanthine + Diphenylamine (1 μmole)	20.49	4.48

The incubation medium, quantity of components added, and conditions of incubation were as described in Table 11 except as indicated.

the conditions studied (43). It was of interest to determine the effect of dismutase on the lysosomal membrane breakdown caused by the free radical-like component. In the experiments which were conducted, it was observed that the addition of superoxide dismutase usually enhanced the peroxidative breakdown of the lysosomes during the activities of the microsomal NADPH oxidase and xanthine oxidase systems (Table 15). However, the addition of superoxide dismtase caused a substantial reduction in lysosomal breakdown and in malondialdehyde production during incubation of the purified cytochrome P450 reductase system (Table 16). The protection afforded by the dismutase was only slightly reduced by boiling this enzyme for several minutes. Forman and Fridovich have recently reported that the holoenzyme is rather stable to temperature (44).

Effect of Fe^{3+} on Lysosomal Disruption and Lipid Peroxidation

In order to effect an attack on lysosomal membrane, a certain concentration of iron was required to be present either during the activity of the xanthine oxidase system or during the activity of the purified cytochrome P450 reductase system (refer to Tables 7 and 11). The addition of iron to the microsomal NADPH oxidase system is not always required unless the microsomes had been thoroughly washed during their preparation (Table 1). Fe^{3+} has been shown to be reversibly bound to the microsomal enzyme system and is lost in variable amounts during the isolation of microsomes. It has been proven previously that Mn^{2+} inhibits phospholipid peroxidation by competing with Fe^{3+} for binding sites on the microsomes (and presumably on the enzyme) (43). The fact that Mn^{2+} inhibits lysosomal lysis indicates that iron may participate in the process of generating the radical effective

TABLE 15

ENHANCEMENT OF MICROSOMAL NADPH OXIDASE-CATALYZED AND XANTHINE OXIDASE-CATALYZED DISRUPTION OF LYSOSOMAL MEMBRANES BY SUPEROXIDE DISMUTASE

Additions to incubations buffer[a]	Free acid phosphatase released	Malondialdehyde formation
	% of Total	mμ moles/ml reaction system
Lys + Mic + NADPH + $ADP\text{-}Fe^{3+}$	90.00	33.56
Lys + Mic + NADPH + $ADP\text{-}Fe^{3+}$ + S.O.D.[b]	130.00	34.29
Lys + Xanthine + Xanthine oxidase + $ADP\text{-}Fe^{3+}$	38.00	5.82
Lys + Xanthine + Xanthine oxidase + $ADP\text{-}Fe^{3+}$ + S.O.D.[b]	99.00	9.63

[a]The incubation medium for the microsomal NADPH oxidase system contained 0.7 M sucrose in 0.015 M Tris, pH 7.4, plus additions as indicated in the Table. The basic medium for xanthine oxidase systems contained 0.7 M sucrose only, as well as the indicated additions. The quantity of components added is described under Experimental Procedures except where indicated otherwise. Incubation time was 30 min.

[b]S.O.D. = Superoxide dismutase, 0.066 mg protein/ml incubation system.

TABLE 16

INHIBITION OF PURIFIED NADPH-CYTOCHROME P450 REDUCTASE-CATALYZED LYSOSOMAL MEMBRANE DAMAGE BY SUPEROXIDE DISMUTASE

Additions to incubation buffer	Free acid phosphatase released	Malondialdehyde formation
	% of Total	mμ moles/ml reaction system
Lys + cyt P450 red + ADP-Fe^{3+}	12.50	4.48
Lys + cyt P450 red + ADP-Fe^{3+} + NADPH	58.23	8.06
Lys + cyt P450 red + ADP-Fe^{3+} + NADPH + S.O.D.[a]	26.25	5.60
Lys + cyt P450 red + ADP-Fe^{3+} + NADPH + S.O.D. $(\Delta)^{b}$	35.00	4.93

The incubation medium and conditions of incubation were the same as described in Table 7 except where indicated.

S.O.D.[a] = Superoxide dismutase, 0.066 mg protein/ml incubation system.

S.O.D. $(\Delta)^{b}$ = Dismutase heated in boiling water for half an hour.

in damaging the lysosomal membrane. Furthermore, the addition of EDTA, a metal chelating agent, at a concentration of 1.0 mM totally prevents lysosomal membrane breakdown (Table 17).

Evidence that Release of the Acid Hydrolases is Caused by Peroxidative Damage of Unsaturated Lysosomal Membrane Lipids

Damage to subcellular particles is usually associated with the membranous portion of the particles. Lipid peroxidation has led to membrane breakdown in hemolysis of erythrocytes of vitamin E deficient animals (45). It has been shown previously in these studies that there was a correlation between lipid peroxidation and acid phosphatase release. Further evidence was given that acid phosphatase release from lysosomes is caused by peroxidative damage to unsaturated lipids. Table 18 shows the addition of phospholipids extracted from microsomes to systems in which lysosomes are disrupted by flavin enzyme activity resulted in inhibition of acid phosphatase release and enhancement of malondialdehyde production, presumably by competition of the phospholipids, for the radicals produced. The results indicate that the phospholipids competed for the radicals generated by the enzyme system and prevented an attack on lysosomal membrane lipids.

Evidence that Superoxide Anion is not a Reactive Radical Toward Biological Membranes

McCord and Fridovich have shown that superoxide anion ($O_2^{\cdot-}$) is an effective reductant of cytochrome c and superoxide dismutase can reduce the level of the $O_2^{\cdot-}$ flux and thereby inhibit the reduction of cytochrome c (16, 37, 41). In the studies reported in this dissertation, we used the reduction of cytochrome c by xanthine oxidase activity as an indicator of

TABLE 17

EFFECT OF EDTA ON FLAVIN ENZYME CATALYZED LYSOSOMAL LYSIS

Enzyme system to which lysosomes were exposed	Free acid phosphatase released	
	Additions to system	
	% of Total	
	None	EDTA
Microsomal NADPH oxidase	87.50	3.35
Purified cyt P450 red	58.23	8.33
Xanthine oxidase	66.39	17.59

The incubations were described under Experimental Procedures. EDTA was added at a final concentration of 1.0 mM. Incubations were carried out for 30 min at 37°. Acid phosphatase released was determined as described under Experimental Procedures.

TABLE 18

EFFECT OF ADDED PHOSPHOLIPID ON LYSOSOMAL MEMBRANE INTEGRITY AND MALONDIALDEHYDE FORMATION DURING LYSOSOMAL MEMBRANE BREAKDOWN CAUSED BY THE ACTIVITY OF CERTAIN FLAVIN ENZYMES

Enzyme system to which lysosomes were exposed	Effect of added phospholipids on the: Release of lysosomal acid hydrolase		Formation of Malondialdehyde	
	% Total activity		mμ moles/ml system	
	None	Phospholipids	None	Phospholipids
Microsomal NADPH oxidase	74.67	18.99	15.68	31.36
Purified cyt P450 red	56.32	11.49	8.18	26.65
Xanthine oxidase	22.00	0.00	5.8	22.40

Phospholipids extracted from microsomes was described under Experimental Procedures. The incubations were described under Methods. Phospholipid added was equivalent to that extracted from 1 mg of microsomal protein/ml reaction system. Incubation was carried out for 30 min at 37°. Acid phosphatase release was measured as described under Experimental Procedures.

dismutase to verify the generation of superoxide anion and the activity of the dismutase. We have shown that the addition of free radical scavenging agents such as diphenyl-p-phenylenediamine and Santoquin to this system do not interfer with the reduction of cytochrome c. The reduction of cytochrome c in the presence of xanthine and xanthine oxidase is not inhibited by the addition of 1.0 mM (final concentration) of any one of the following compounds: ethanol, benzoate and mannitol. The importance of this finding will be explained below. However, the reduction of cytochrome c by the xanthine-xanthine oxidase system is markedly inhibited by addition of Mn^{2+} (1.0 mM final concentration), ADP-Fe^{3+} (0.1 mM and 0.01 mM final concentrations), and, of course, by superoxide dismutase (0.011 mg protein/ml reaction system, Fig. 6). This inhibition by Mn^{2+} and ADP-Fe^{3+} was shown not to interfere with the activity of the xanthine oxidase activity judging by the measurement of uric acid production. These results, plus the fact that xanthine oxidase activity itself produces no lysosomal membrane disruption or malondialdehyde production, indicate that the superoxide anion radical is not the radical species which attacks the lysosomal membrane lipids. Only when chelated inorganic iron is present in the system are the systems effective in exerting membrane damage. The exception is the microsomal NADPH oxidase system in which the microsomes have not been washed too thoroughly as to reduce the Fe^{3+} bound to the membrane below the effective level. Under the latter conditions, the radical-scavenging agents mentioned above are effective protectors of lysosomal membrane lipids (data has been shown previously). It is worth noting that both the ADP-Fe^{3+} complex and Mn^{2+} inhibit cytochrome c reduction by xanthine oxidase activity. Mn^{2+} is a

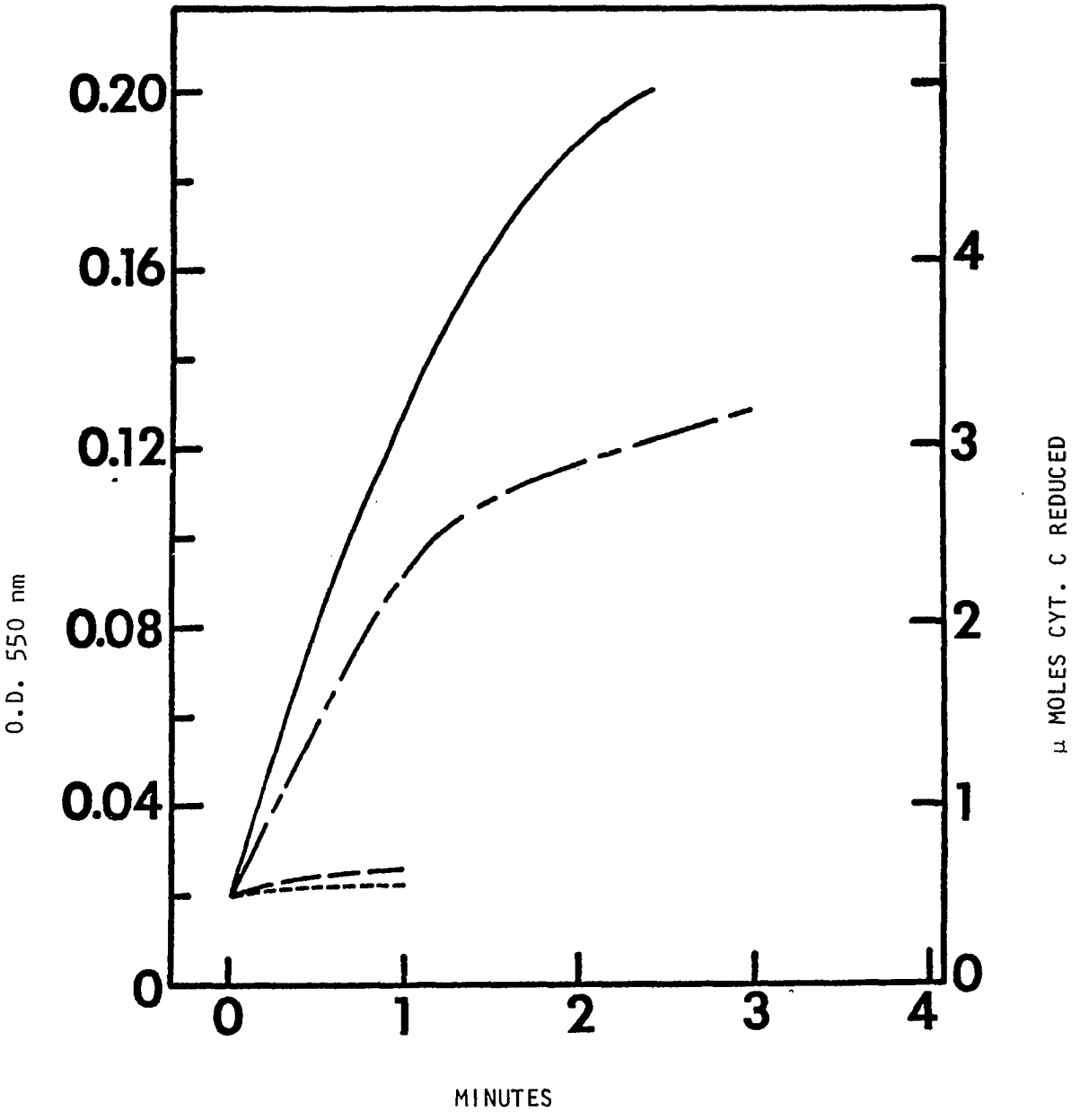


Figure 6. The reduction of cytochrome c by superoxide anion generated by the xanthine oxidase system. The composition and conditions of the assay are explained under Experimental Procedures.

———— no inhibitors added

— - — 2 mM of ADP and 0.1 mM Fe^{3+} added

— — — superoxide dismutase (0.011 mg protein/ml reaction system)

------- 1.0 mM $MnCl_2$

potent inhibitor of lipid peroxidation while the ADP-Fe^{3+} complex promotes peroxidative damage to biological membranes.

Evidence that the Radical which Promotes Peroxidative Damage to Lysosomal Membrane is HO·

As mentioned above superoxide radicals produced by the purified NADPH-cytochrome P450 reductase and the xanthine oxidase systems clearly do not produce peroxidative membrane damage except in the presence of chelated Fe^{3+}. It was reasonable to assume that the superoxide anion ($O_2^{\cdot-}$), in the presence of this form of iron, is diverted to a reaction which forms a radical that attacks membrane unsaturated lipids to a measurable degree. Therefore, studies were carried out to determine whether or not HO· is a reactive radical toward lysosomal membranes. The following experiments support the conclusion that the effective radical produced is HO·. The addition of scavengers with specificity for HO· radicals causes a marked reduction in membrane damage. Table 19 shows that addition of ethanol, benzoate, and mannitol, all known to be scavengers of the HO· radical (46), provided a significant degree of protection against lysosomal membrane lysis. It was also mentioned previously that the radical scavenging agents apparently do not interact with $O_2^{\cdot-}$ since addition of the compounds at the same concentration in the xanthine-xanthine oxidase systems catalyzing the reduction of cytochrome c do not interfer with the reduction of the latter. Furthermore, the protection afforded by the addition of these substances was not the result of the scavengers causing inactivation of the lysosomal hydrolases. Thiw was shown by the addition of Triton X-100 to duplicate systems at the end of the incubation, causing the liberation of the full complement of lysosomal hydrolases. All exhibited maximum

TABLE 19

EVIDENCE THAT THE FACTOR WHICH EFFECTS LYSOSOMAL LYSIS IS THE HYDROXYL FREE RADICAL

Enzyme system	Additions to the system			
	Free acid phosphatase released			
	% of Total			
	None	Ethanol	Benzoate	Mannitol
Xanthine oxidase	39.99	5.00	3.00	14.00
Microsomal NADPH oxidase	84.34	23.00	17.47	18.07
Purified cyt P450 reductase	39.17	5.50	9.58	10.83

The incubations were as described under Experimental Procedures. Final concentrations of ethanol, benzoate and mannitol were 1.0 mM. Incubation was carried out for 30 min at 37°. Acid phosphatase was measured as described under Experimental Procedures.

activity even in the presence of the radical scavengers. It was also shown that the addition of catalase to the xanthine-xanthine oxidase system and to the microsomal NADPH-cytochrome c reductase system resulted in a marked inhibition of membrane damage (Table 20).

TABLE 20

EFFECT OF CATALASE ON FLAVIN ENZYME CATALYZED LYSOSOMAL LYSIS

Enzyme system	Additions to the system	
	Free acid phosphatase released	
	% of Total	
	None	Catalase
Microsomal NADPH oxidase	84.34	8.43
Xanthine oxidase	39.99	3.00

The incubations were described under Methods. Catalase 0.029 mg protein/ml incubation system. Incubation was carried out for 30 min at 37°. Acid phosphatase release was measured as described under Experimental Procedures.

CHAPTER IV

DISCUSSION

Lysosomal Disruption by a Free Radical-like Component Generated by Microsomal NADPH oxidase Activity

Previous studies suggested that the nature of the reactions catalyzed by the microsomal mixed function oxidase system is mediated by free radicals (14, 47). However, negative results were obtained when attempts to demonstrate radicals attributable to enzyme activity in the system were made by electron spin resonance techniques (43). This was not unexpected since the effective concentration of such radicals must be very low due to their apparently extreme reactivity and consequent short half-life (48). We recently demonstrated generation of a factor having free radical properties during the oxidation of NADPH by normal liver microsomes under conditions resembling those existing *in vivo*. Erythrocytes were used as indicators to demonstrate the presence of the radicals. This fact suggested that other membraneous organelles in the cell might be altered under certain intracellular conditions. Since lysosomal membranes are particularly susceptible to structural damage, we studied the effect of the enzyme-generated factor on lysosomes. The results indicate that liver lysosomes release a considerable quantity of their hydrolase contents as a result of exposure to these enzyme-generated radicals. Previous studies of Zalkin *et al.* showed that muscle

tissue from tocopherol-deficient rabbits contains an excess of free lysosomal hydrolases correlated with the development of muscle degeneration. They concluded that the increase in hydrolases must have been a consequence of macrophage invasion initiated by tissue damage resulting from lipid peroxidation (38). In our studies, however, we showed that preparation of either lysosomes or microsomes from rats supplemented with α-tocopherol has a protecting effect on lysosomal integrity in this system. This, plus the effect of radical scavengers *in vitro*, together with the fact that the microsomal NADPH oxidase system generates a radical-like factor, suggests that the activity of this enzyme system could cause hydrolase release *in vivo* especially in the deficiency of a radical scavenging agent such as α-tocopherol.

It is possible that radical-like factors such as those associated with microsomal NADPH oxidase activity could be responsible for the initial lesion in tocopherol absorption or deficiency diseases by triggering the intracellular release of lysosomal hydrolases *in vivo*. These enzymes would then have the potential to cause cell injury and/or death and possibly focal necrosis, thereby promoting invasion of phagocytes which could then account for the very large increase in total content of hydrolases in the tissue as necrosis progresses. If lysosomal particles were caused to release hydrolases into the cell by some such mechanisms, it is possible that disruption of intracellular structures and processes might occur, especially if acidic lipoproteins (71) and other strongly acidic substances (49) contained in the lysosomes are released as well. Evidence that hydrolases released *in vivo* cells can cause severe damage to cell structure was provided by Allison and Patton (50) whose studies indicated that intracellular lysosomal

DNase release could cause multiple chromosome breaks. Other structural abnormalities were noted also. Pearce reported that primary release of lysosomal enzymes may occur in some myopathies and followed by macrophage invasion (51). Exposure of lysosomes to non-enzymic systems involving free radicals with resulting release of hydrolase enzymes was reported by Desai et al. (52) who subjected lysosomes to γ-irradiation and to auto-oxidizing substances and obtained release of hydrolases. A mechanism through which such lysosomal disintegration could occur in vivo is suggested by the studies presented in this report, in that constitutive enzyme systems can produce apparently highly reactive free radicals which are capable of destabilizing the lysosomal membrane. A mechanism such as this for promoting hydrolase release would undoubtedly contribute to cell damage seen in certain physiological states such as a deficiency of a free radical scavenging agent (as α-tocopherol). The latter appears to function as a stabilizer of biological membranes against alterations caused by radicals produced by the endogenous enzymes (53). Indeed, lysosomes obtained from normal rats which had been given an additional supplement of α-tocopherol were demonstrated in this study to be resistant to the lytic process. It appears that at least one function of α-tocopherol is to serve as a terminator for undesirable chain-reactions initiated by radicals produced in the normal function of certain endogenous enzymes.

The Nature of the Free Radical which Attacks Lysosomal Membranes

Generally, electron paramagnetic resonance is now the method for detecting and identifying free radical species. However, the usefulness of electron paramagnetic resonance is limited by instrument

sensitivity (the minimum useful radical concentration being in the region of 10 μM) and by lack of time resolution, since it usually takes several minutes to obtain an electron paramagnetic resonance spectrum. It is not surprising that thus far only the most stable oxygen radical, $O_2^{\cdot-}$, on the alkaline side of neutrality, has been conclusively identified by electron paramagnetic resonance in a biochemical system (54). However, the superoxide anion radical would not be expected to be detected by electron paramagnetic resonance in biological systems containing Fe^{3+} (55). The sensitivity of the method in which the lysosome is used as a free radical detector is apparent and this may be a method for detecting other oxidoreductase systems which may produce highly reactive radicals or radical-like components, the course of whose production cannot be observed by other means. The mechanisms of lipid peroxidation in biological systems are not well defined, but we view such attacks on membrane lipids as a consequence of certain types of radical-producing oxidoreductase reactions (56) and believe that the attacks are facilitated by the ordered arrangement of the lipids. The results of these studies suggest that the peroxidation of membrane lipids associated with the activity of some metalloflavin oxidoreductase system is the result of the interaction of the membrane lipids with HO· radicals which are secondary to the production of superoxide anions. The enzymes which we have studied have all been reported to produce superoxide anion through interaction of the reduced enzyme with molecular oxygen (18, 47, 57, 58). We have been uniformly unable to show that systems known to produce superoxide anion result in a direct attack either on biological membranes or on lipids extracted from membranes. However, in the presence of chelated iron, all

such systems producing superoxide anion radicals caused a peroxidative attack on lysosomal membranes. These effects are clearly not due to the chelated iron itself. The data indicate that the membrane disruption occurs only during the ongoing enzymic reaction and is not due to any of the components of the system required to produce the reaction. For instance, allowing any of these enzyme systems to incubate in the presence of its substrate and chelated iron for 30 minutes at 37^{o}, followed by the addition of 1.0 mM Mn^{2+} (which totally inhibits further peroxidation of lipids in biological systems) and then followed by the addition of lysosomes, results in no disruption of these particles. Thus no lytic agent formed during the reaction accumulates in the system. The factor causing lysosomal disruption is formed only during the activity of the respective enzymes. Furthermore, systems in which the enzymes had been heat-activated caused no lysosomal breakdown. In addition, the action of structurally unrelated free radical-scavenging agents in preventing lysosomal breakdown during the various enzymic reactions further adds to the possibility that the lytic agent is a free radical resulting from the activity of these oxido-reductase systems, but the superoxide anion itself is not the damaging factor. Since it is known that these systems produce superoxide anion, it would appear that the addition of iron either reacts with the superoxide anion to produce a different type of radical or that the iron interacts with the enzyme itself resulting in the generation of a different type of radical. The inhibitory effect of EDTA (refer to Table 17), provides further evidence that the superoxide anion is not a reactive radical toward biological membranes, and shows that the presence of iron is necessary for lysosomal disruption in these studies. EDTA is known to

be a chelating agent and has been shown to enhance the rate of cytochrome c reduction during sulfite oxidation, and that superoxide dismutase eliminated this effect of EDTA (42). The action of superoxide dismutase in these systems studied in this dissertation is not always consistent. In some systems it may cause enhancement of lysosome breakdown. In others it may cause inhibition. This may occur for the following reasons. One of the enzyme reactions studied was the xanthine oxidase system which produces both $O_2^{\cdot-}$ and H_2O_2 (59). The secondary reaction of $O_2^{\cdot-}$ with H_2O_2 would be expected to form OH· to a certain extent (59). The addition of chelated iron to the system could, however, accelerate the formation of OH· by reacting with the hydrogen peroxide as reported by Haber and Weiss (60), especially if the iron is reduced by the superoxide being formed in the system. The redox potential of $O_2^{\cdot-}$ (65) would favor the reduction of the chelated iron complex. Thus the rate of HO· formation (and, therefore, the flux) could then become sufficient to produce degradation of the lysosomal membrane. Therefore, the enhancement of lysosomal disruption caused by the addition of superoxide dismutase can occur under the following condition: if the flux of $O_2^{\cdot-}$ is sufficient, the addition of superoxide dismutase may serve to enhance the formation of hydrogen peroxide and facilitate an even greater formation of OH· radical when iron is present in the system. On the other hand, the prevention of lysosomal breakdown caused by the addition of superoxide dismutase may occur under the following condition: if the formation of $O_2^{\cdot-}$ and H_2O_2 is occurring at a slower rate in the reaction, addition of the dismutase may inhibit the attack on the lysosomes by lowering the $O_2^{\cdot-}$ level to the point that the chelated iron is not reduced at a significant rate.

Thus, the formation of HO• through interaction of the reduced iron chelate with H_2O_2 would be inhibited and damage to the membrane prevented. This concept is strengthened by the fact that the addition of catalase to either the xanthine oxidase system or the microsomal NADPH-cytochrome c reductase system results in the inhibition of lysosomal membrane damage (Table 19). The role of iron in the function of the active site of xanthine oxidase has been investigated. Evidence is provided to suggest that the iron atoms on the xanthine oxidase are necessary for the formation of an oxygen-free radical during normal operation of the enzyme (61). It is interesting that the effect of either Mn^{2+} or ADP-Fe^{3+} on the superoxide anion-mediated reduction of cytochrome c by xanthine oxidase system are both inhibitory since Mn^{2+} is a very effective inhibitor of lipid peroxidation while the chelated iron complex promotes peroxidative attack on membranes by these enzymes. Our interpretation of these observations is that Mn^{2+} may displace iron atoms on the xanthine oxidase enzyme essential for the production of superoxide anion just as it displaces iron from microsomes which is essential for peroxidative attacks (Poyer, J.L. and McCay, P.B., unpublished results). The ADP-Fe^{3+}, however, may exert its effect on the reduction of cytochrome c by the reaction:

$$O_2\cdot^- + \text{ADP-}Fe^{3+} \longrightarrow O_2 + \text{ADP-}Fe^{2+}$$

as mentioned above. The overall scheme for membrane lipid degradation catalyzed by these flavin enzymes may be represented as follows:

$$\text{flavin enzyme} + \text{substrate} \longrightarrow \text{reduced flavin enzyme} + \text{product}$$

$$\text{reduced flavin enzyme} + O_2 \longrightarrow \text{flavin enzyme} + O_2\cdot^-$$

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$H_2O_2 + O_2^{\cdot-} \longrightarrow O_2 + HO\cdot + OH^-$$

$$O_2^{\cdot-} + ADP\text{-}Fe^{3+} \longrightarrow O_2 + ADP\text{-}Fe^{2+}$$

$$ADP\text{-}Fe^{2+} + H_2O_2 \longrightarrow ADP\text{-}Fe^{3+} + HO\cdot + OH^-$$

This scheme demonstrates the possible role of membrane bound iron or added iron (chelated to maintain it in solution) in enhancing HO· production, and suggests that intracellular enzymic generation of $O_2^{\cdot-}$ may couple to certain non-enzymatic reactions (62) on or in subcellular organelles to perturb membrane structure unless there is adequate radical scavenging. The fact that three different specific scavengers for HO· (ethanol, benzoate and mannitol) each protect the lysosomal membrane from degradation, when added to any of the systems which have been described, is strong evidence that the destructive entity, regardless of how it originates, must be the hydroxyl radical. This would also explain the negative results produced by electron spin resonance studies since the HO· at the reaction temperature has a half-life in the region of a few tenths of a microsecond only (63). In addition, the superoxide would not be expected to be detected in biological systems containing Fe^{3+} (55). This concept agrees with the fact that the peroxidation of biological membrane lipids is very rapid during the activity of these enzymes. The hydroxyl radical is extremely reactive while $O_2^{\cdot-}$ is a rather unreactive radical (64) and, furthermore, superoxide is reducing in nature rather than oxidative, having a E_o' of -0.45V (65). In conclusion it should be pointed out that while this destructuve reaction of oxido-reductase systems on lysosomes (and also on erythrocytes, microsomes and mitochondria) has only been observed <u>in vitro</u>, it is possible that phenomena similar to this <u>in vivo</u> may occur when certain flavoprotein enzymes (probably metalloflavoproteins) function at or near maximal <u>in vivo</u>

rates. It would seem that the results of this study and those of others are sufficiently suggestive to stimulate a search for similar reactions *in vivo*. For example, when orotic acid is administered to animals, there is resulting liver damage (66) which is associated with the degeneration of subcellular organelles (67). Dihydroorotic acid dehydrogenase appears to be similar to xanthine oxidase in producing superoxide anion (68). Whether or not the activity of such enzymes cause alteration of cellular lipids may depend upon the localization of the enzyme in the cell with respect to constituent polyunsaturated lipids and the amount of free radical scavengers (such as α-tocopherol) present in the lipid phase of the membranes. Scholan and Boyd have shown, for example, that aberrant products are formed by the drug metabolizing system in animals deficient in free radical scavengers (69). The amount of ADP and inorganic iron in cells is more than adequate to facilitate the processes described.

CHAPTER V

SUMMARY

The nature of free radicals produced during the activity of several oxidative enzymes, using lysosomes as a sensitive indicator, has been studied. These radicals appear to play important roles in biological oxidations involving one electron transfer. The enzymes investigated in this work are flavoproteins: microsomal NADPH oxidase, partially purified cytochrome P450 reductase, and a highly purified xanthine oxidase. These enzymes, when operating upon their own substrate *in vitro*, produce a factor which causes the release of acid hydrolases from lysosomes. The factor appears to be a free radical involved in the activity of these enzymes, the generation of which is dependent on Fe^{3+} bound to the membrane in which the enzyme is located, or, in the case of the purified enzymes, Fe^{3+} which is added in chelated form to maintain it in solution. The free radical component is apparently responsible for the lipid peroxidation in the microsomal membranes which occurs during NADPH-dependent electron transport. It was found that preparation of either lysosomes or microsomes from rats supplemented with α-tocopherol has a protecting effect on lysosomal integrity in the microsomal NADPH oxidase systems. These interactions suggest possible mechanisms which might promote necrosis through the release of lysosomal enzymes *in vivo*, especially in animals deficient in dietary scavenging components.

The lysosomal lysis caused by these 3 enzyme systems is prevented a) by inhibitors of lipid peroxidation, b) by prior heat denaturation of the enzymes, and c) by including free radical-trapping agents in the incubation system. Preincubation of these enzyme systems in the presence of its substrate and chelated iron for 30 minutes, followed by the addition of 1.0 mM Mn^{2+} and then followed by the addition of lysosomes, results in no disruption of these particles. The role of Mn^{2+} appears to be to displace Fe^{3+} from an essential position on the enzyme required to generate the radicals. The factor causing lysosomal disruption is formed only during the activity of the respective enzymes.

The addition of superoxide dismutase enhances the lysosomal membrane breakdown during the microsomal NADPH oxidase system and xanthine oxidase system, and prevents the lysosomal membrane breakdown during the cytochrome P450 reductase system.

The studies described in this report indicate that the hydroxyl free radicals are derived from the activity of certain flavin enzymes (probably metalloflavin enzymes), and that superoxide anion must play a role in the formation of the HO· radicals. Since adequate chelated iron is present in the cytosol of cells to promote these reactions, these studies also suggest that the activity of such flavin enzymes may contribute to the turnover of membrane polyunsaturated fatty acids, or possibly initiate membrane lesions under some conditions, and carries certain implications concerning the process of aging.

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