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CHAIN-SCISSION OF MICROSOMAL PHOSPHOLIPIDS OCCURRING DURING THE ENZYMIC OXIDATION OF NADPH; REACTION MECHANISM AND PRODUCTS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

JOE LEE POYER

Oklahoma City, Oklahoma

1969

CHAIN-SCISSION OF MICROSOMAL PHOSPHOLIPIDS OCCURRING

DURING THE ENZYMIC OXIDATION OF NADPH;

REACTION MECHANISM AND PRODUCTS

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CHAPTER I

INTRODUCTION

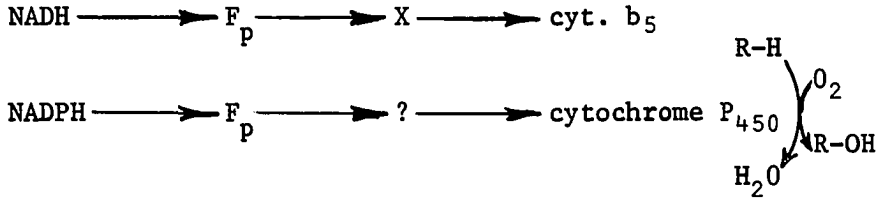
Vesicular particles (microsomes) formed from the endoplasmic reticulum of liver upon homogenization contain a variety of "mixed function" oxidases, which are concerned with the metabolism of normal metabolites such as steroid hydroxylation; and also with substances foreign, and often toxic, named "xenobiotics," to the metabolic network, such as aromatic hydrocarbons, methylated alkaloids, and other drugs. These "mixed function oxidases" conform to the general reaction:

$$NADPH + H^+ + R\text{-}H + O_2 \rightarrow NAD^+ + R\text{-}OH + H_2O.$$

These oxidases involve an electron transport chain in the endoplasmic reticulum, only some of the components of which have been isolated and partially characterized. Two cytochromes unique to the cytoplasmic reticulum, b_5 and P_{450}, have been isolated and their properties have been demonstrated to be distinct from those found in mitochondria. Also, flavoproteins that oxidize reduced pyridine nucleotides have been isolated and extensively studied, and another

cytochrome studied by Mason (19), called "Fe-X" has been isolated and is believed to function in a manner analogous to that of cytochrome a or a_3 of the mitochondrion. The rate of electron flow, as measured by various electron acceptors, appears to be approximately of equal magnitude in mitochondria and microsomes.

The relationships between the components of the microsomal electron transport system have been established by (a) studies of the effects of various electron acceptors and inhibitors, (b) studies of the appearance of various enzymic activities in the developing liver, and (c) studies of the rates of induction of the components by drugs. These studies indicate the following electron transport scheme, which is a composite of those given by Siekewitz, Ernster, and Mason and Sata (35, 25, 19, 24):



At the present time there appear to be two distinct electron transport chains, one utilizing NADH and the other utilizing NADPH.

In the pathway of microsomal electron transport for cytochrome P_{450} reduction and the activation of oxygen in "mixed function oxidases" the related reaction of lipid peroxidation has been described. This reaction was first described by Hochstein and Ernster and has been more extensively investigated by others although in no case was any study of lipids performed in order to verify the nature of extent of oxidation. It was found that when reduced nicotinamide adenine dinucleotide

phosphate (NADPH) was added to rat liver microsomes, the consumption of oxygen was greatly enhanced by the presence of ferric iron and pyrophosphates, with the concomitant occurrence of lipid peroxidation, as evidenced by the formation of a thiobarbituric acid-reacting substance, which was believed to be malonaldehyde. As the result of studies of the effects of various electron acceptors, electron chain blocking agents, chelating agents, drugs, and anti-oxidants (free-radical trapping agents) several proposals were advanced for the mechanism of the interaction of the observed lipid peroxidation with the microsomal electron transport system.

The first mechanism to be proposed was that of Hochstein and Ernster (13) (Fig. 1) and was based on the observation that a thiobarbituric acid (TBA) reacting chromogen, believed to be the result of

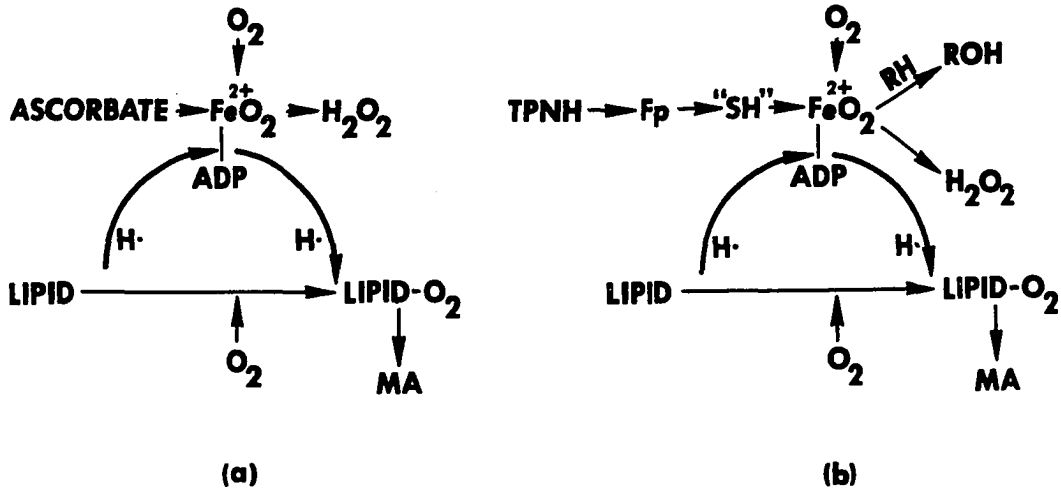


Figure 1

lipid peroxidation, could be prevented by the metal chelating agent EDTA, several anti-oxidants such as diphenylphenylenecliamine and tocopherol, and para-chloromercuribenzoate which is an inhibitor of microsomal electron transport; and inactivation by heat treatment. They also observed the nonenzymic formation of a TBA-reacting substance in the presence of ascorbic acid, which was found to be inhibited only by EDTA and the anti-oxidants, but not by the electron transport inhibitors. This activity was not heat sensitive. On the basis of these results they proposed for both the iron-catalized oxidation of ascorbate (a) and the NADPH oxidase system (b) the formation of an ($ADP-Fe^{2+}O_2$) intermediate which catalized the lipid peroxidation.

Subsequently, Orrenius, Dallner, and Ernster (25), demonstrated that drugs undergoing oxidative demethylation, such as aminopyrene and codeine, inhibited the formation of the TBA-reacting chromogen. They interpreted this result as a competition between the process of lipid peroxidation and that of drug demethylation for a common NADPH-oxidizing enzyme (Fig. 2). They proposed that a pyrophosphate-iron complex (ADP-Fe) interacted with the electron transport system of the NADPH-oxidizing microsomal system and catalyzed lipid peroxidation. Later, Beloff-Chain et al. (2), by studying the interdependence of the effects of Fe^{2+} and Fe^{3+}, ADP, and antimycin A on the microsomal lipid peroxidation, and by measuring the redox state of the microsomal cytochrome b_5, reduced by NADPH in the presence of the above reagents, proposed (Fig. 3) that the microsomal electron transport system activated by ADP reduced Fe^{3+} to Fe^{2+}, which is then responsible for the observed lipid peroxidation. Marks and Hecker (18) observed that Fe^{2+} at low concentration (10^{-5} M)

catalyzed the NADPH-oxidase lipid peroxidation, but that the lipid peroxidation became nonenzymic and independent of NADPH at higher Fe^{2+} concentrations (10^{-3} M). They proposed that the Fe^{2+} (10^{-5} M) effect was identical to that proposed by Hochstein and Ernster (above), while the nonenzymic Fe^{2+} (10^{-3} M) effect resembled that proposed by Beloff-Chain et al. (above).

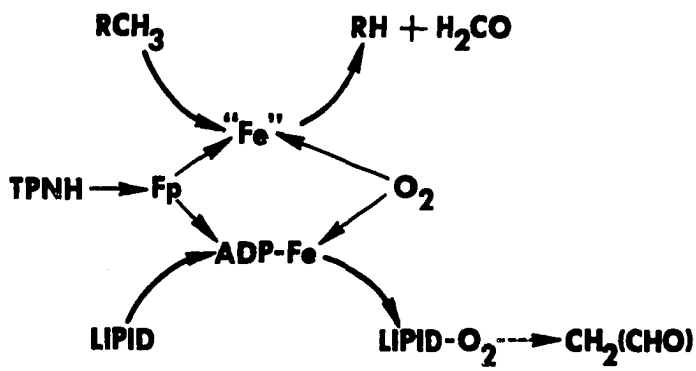


Figure 2

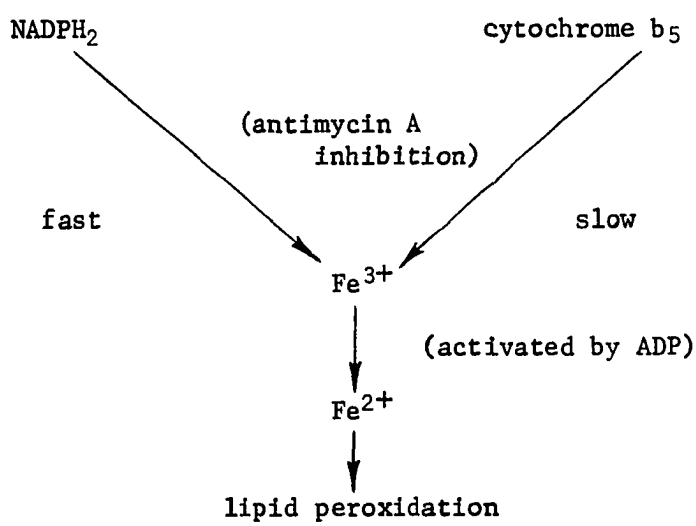
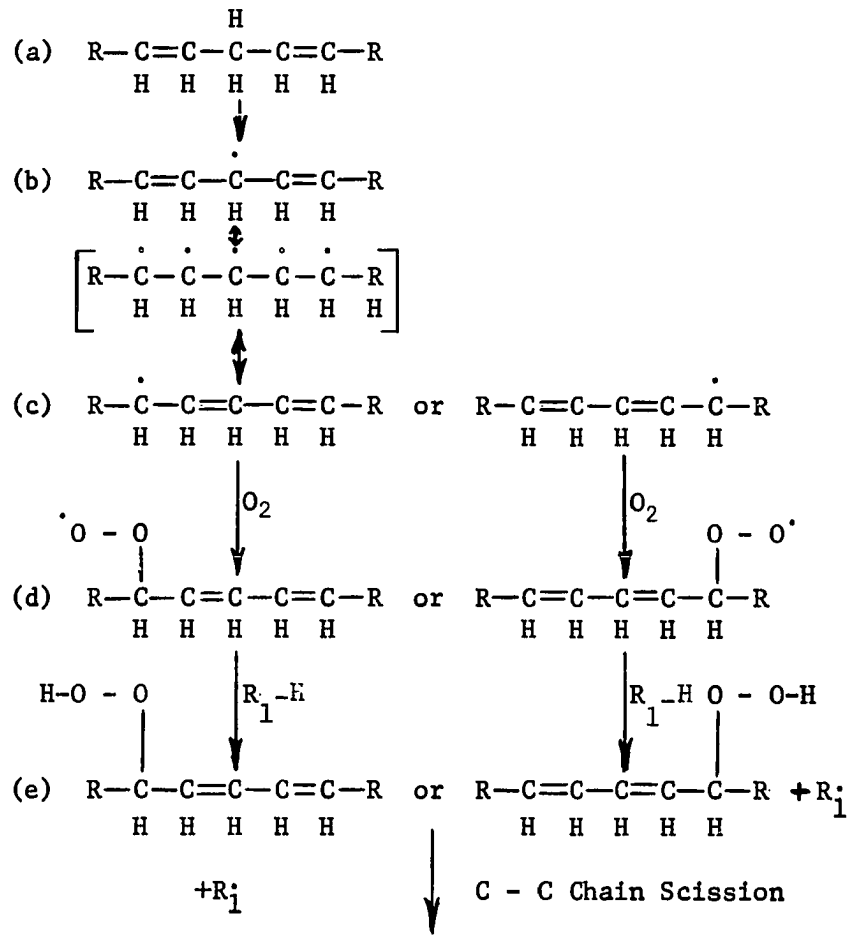


Figure 3

"Lipid peroxidation," in vitro, has been studied in model systems for several years and some, although not all, of the basic properties and mechanisms involved in such systems are known. "Lipid peroxidation" is thought to be an autocatalytic series of reactions (8) involving a free radical mechanism:



the initial event being the attack of a methylene group of a methylene-interrupted (1,4-) pentadiene structure (a). This forms a methine radical H—C· (b) which in the diene environment shifts to the more stable (lower energy state) conjugated 1,3 diene radical configuration (c). This diene free-radical is capable of interacting with molecular oxygen to form a peroxy radical (d), which can then attack another 1,4-

pentadiene structured molecule (R-H) to form a conjugated diene hydroperoxide (e) and another conjugated diene radical (c) giving rise to a chain reaction process. In addition radicals may interact to form chain-scission products. The conjugated diene hydroperoxides have been characterized as intermediates in this process by Privett and others (7, 8, 29). However, nothing is known concerning the mechanism of the formation of the carbonyl products from the conjugated diene hydroperoxides or the cleavage of the carbon-carbon bonds necessary for the formation of the carbonyl products.

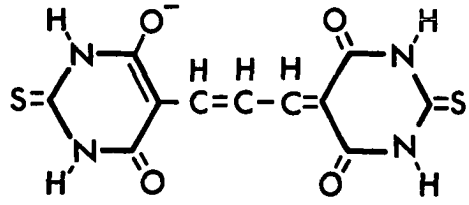
It is significant that for the 1,4-pentadiene system with only one CH_2 group between two ethylene groups, no carbon-carbon bond chain-scission occurs which produces the 3 carbon dialdehyde, malonaldehyde $H-\overset{O}{\overset{\|}{C}}-CH_2-\overset{O}{\overset{\|}{C}}-H$. For the case of a more unsaturated methylene-interrupted compound, which may be regarded as an example of the next higher homologue, such as linoleic acid:

$$\underset{9}{C}=\underset{10}{C}-\underset{11}{CH_2}-\underset{12}{C}=\underset{13}{C}-\underset{14}{CH_2}-\underset{15}{C}=\underset{16}{C}$$

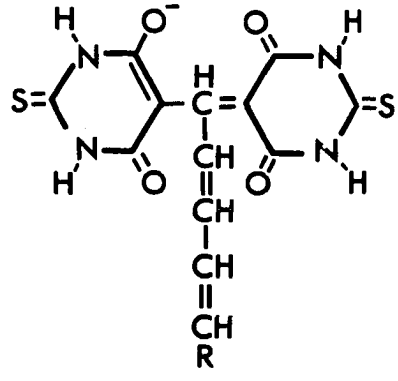
it would be predicted from the above mechanism that mono-hydroperoxides would be formed at C-numbers 9, 13, 14, and 16. These four mono-hydroperoxides have indeed been found. Also, multiple carbon-carbon chain-scission produced carbonyl compounds are formed. It is especially noteworthy that peroxidation of this homolog forms malonaldehyde as a product of the multiple carbon-carbon chain-scission decomposition reactions, as well as a number of other carbonyl compounds, including conjugated dienealdehydes. Higher homologs, such as arachidonic or docosapentenoic acid exhibit even greater susceptibility to free radical

attack, especially in the presence of molecular oxygen, and also form malonaldehyde and complex mixtures of carbonyl products as a result of carbon-carbon chain-scission, including conjugated dienealdehydes.

Malonaldehyde reacts with 2-thiobarbituric acid (TBA) to form the following chromogen (34, 36, 37):



This chromogen has a maximum absorbance at 532 mμ and is used as an index of lipid peroxidation. However, conjugated dienealdehydes also react with TBA to form a chromogen (14):



having a maximum absorbance at 532 mμ but which has a much lower molar absorbance.

The purpose of this study was: (1) to determine what products

formed during the process of lipid peroxidation react with 2-thiobarbituric acid to form a chromogen absorbing at 532 mμ, (2) to determine whether or not Fe^{3+} is a cofactor in the processes of lipid peroxidation, (3) to determine the effects of inhibitory substances, such as metallic ions and free radical trapping compounds, on lipid peroxidation, and (4) to compare the properties of nonenzymic lipid peroxidizing systems with that of the enzymic NADPH-requiring lipid peroxidation.

CHAPTER II

MATERIALS AND METHODS

Materials

Animals

Adult male albino rats (180-250 g), bred and maintained in this laboratory, were used in these experiments. These rats were originally derived from the Holzman-Sprague Dawley strain and are now highly inbred. Animals were watered with distilled water and fed either a commerical pellet diet or a synthetic diet described below.

Materials for Diets

Casein, vitamins (except α-tocopheryl acetate), cod liver oil 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-tocopherol and α-tocopheryl acetate were obtained from Distillation Products Industries, Rochester, New York.

Experimental Diets

α-Tocopherol-deficient diets. The experimental diet used was that of Young and Dinning as modified by Caputto et al. (4). The salt mixture and vitamin mixture were prepared according to the method of Hubbell et al. (16).

Composition of Vitamin Mixture

Inositol	22.5 g
Choline Chloride	22.5 g
Nicotinamide	4.5 g
Pyridoxine HCl	112.5 mg
Thiamine	112.5 mg
Riboflavin	112.5 mg
Calcium Pantothenate	225.0 mg
Folic Acid	112.5 mg
2-Methylnapthoquinone	5.6 mg
Vitamin B_{12}	1.0 mg
Biotin	1.1 mg
Dextrose	100.0 g

Composition of Salt Mixture

$CaCO_3$	54.300 %	KH_2PO_4	21.200 %
KCl	11.200 %	NaCl	6.900 %
$MgCO_3$	2.500 %	$MgSO_4$	1.600 %
$FeSO_4.7H_2O$	0.090 %	$MnSO_4.H_2O$	0.035 %
$AlK(SO_4)_2.12H_2O$	0.017 %	KI	0.008 %

Composition of Basal Diet

	Percent Composition
Casein, vitamin free	17.0
Sucrose	37.3
Corn Starch	36.0
Lard	3.0

	Percent Composition
Cod Liver Oil	3.0
Salt Mixture	3.0
Vitamin Mixture	0.7

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

Stock Diet (Pellet Diet)

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

Reagent Chemicals

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

Nicotinamide adenine diphosphonucleotide phosphate, reduced (NADPH), sodium D-glucose-6-phosphate, and purified glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP-oxidoreductase) were obtained

from Sigma Chemical Company, St. Louis, Missouri.

The following chemicals were obtained from Fisher Scientific Company, Philadelphia, Pennsylvania: tris (hydroxymethyl) amino methane, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, cerous sulfate, trichloroacetic acid, and sodium chloride.

The following chemicals were obtained from Eastman Organic Chemicals, Rochester, New York: sodium ethylenediaminetetraacetate, 2-thiobarbituric acid, cysteine hydrochloride, mercaptoacetic acid, aniline, dimethyl aniline, p-nitroaniline, ethyl p-aminobenzoic acid, diphenyohenylenediamine, and mercaptoethanol.

Pyridine aldehydeoxime 2-mercaptoethylamine and 4,4'-sulfonyldianiline were obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin.

Sephadex G-10 was obtained from Pharmacia, Uppaala, Sweden.

Ascorbic acid was obtained from Nutritional Biochemicals Corp.

Adenosine diphosphate (ADP) was obtained from P-L Biochemicals, Milwaukee, Wisconsin.

Methyl aniline hydrochloride was obtained from K and K Laboratories, Plainview, New York.

D-cysteine, cysteine methoxyester, and N-acetyl cysteine were obtained from Cyclo Chemical Corp., Los Angeles, California.

Instruments and Equipment

Spectrophotometric measurements were made either using a Beckman DU-2 from Beckman Instruments Company, South Pasadena, California, or a Cary Model 14 Recording Spectrophotometer, Applied Physics Corporation, Pasadena, California. Centrifugations were done using an

International Refrigerated Centrifuge, International Equipment Company, Boston, Massachusetts and a Spinco Model L Ultracentrifuge, Beckman Instruments Company, Spinco Division, Palo Alto, California. Enzyme incubations were performed in a Dubnoff Shaker equipped with a constant temperature water bath, Precision Scientific Company, Chicago, Illinois. Fluorometric analyses were done using an Aminco-Bowman Spectrophotofluorometer, American Instrument Company, Inc., Silver Spring, Maryland. Oxygen consumption studies were done using the Gilson Differential Respirometer, Gilson Medical Electronics, Middleton, Wisconsin.

Methods

Preparation of Rat Liver Microsomes

Male rats (180-250 g) were stunned by a blow on the head and exsanguinated by severing the neck vessels. The liver was removed, chilled with cold potassium phosphate buffer (0.15 M, pH 7.5), blotted dry and weighed. Five milliliters of cold phosphate buffer (0^{o} C) was added to each gram of liver and then homogenized using a monogenizer of the Potter-Elvehjem type. The homogenate was centrifuged in a refrigerated centrifuge at 8,000 x g for 15 minutes to sediment cellular debris, nuclei, and mitochondria. The supernatant fraction so obtained was then centrifuged for 90 minutes in a 30 rotor at 30,000 rpm (105,000 x g) in a Spinco Model L Ultracentrifuge in order to sediment the microsomes. The microsomal pellet was resuspended in the same volume of cold phosphate buffer and recentrifuged for 60 minutes at 30,000 rpm. This washing process was repeated once. The buffer was drained from the final microsomal pellet and the pellet was stored at

-20°C. The frozen pellet of microsomes was thawed immediately before use and suspended by homogenization in tris-HCl buffer (0.1 M, pH 7.5) so that 1 ml of the suspension was equivalent to the microsomes from 1 gram of liver wet weight. Once thawed, microsomes were not refrozen for later use since it was found that repeated freezing and thawing caused an increase in the thiobarbituric acid (TBA) chromogen in the control incubation systems.

Incubation Systems. All incubation systems contained microsomal particle suspensions equivalent to 0.1 gram of rat liver (wet weight)/ ml, which is equivalent to 1 mg ± 10% microsomal protein/ml, 4 m M ADP, 1.2 X 10^{-5} M Fe^{3+}, and 0.1 M tris buffer at pH 7.5. NADPH was used as substrate at 0.3 m M, ascorbic acid at 10^{-3} M, and all thiol compounds used as substrates at 4 X 10^{-3} M unless otherwise specified. Incubation systems having a NADPH regenerating system contained in addition to NADPH as substrate 6 m M glucose-6-phosphate and 0.5 units of glucose-6-phosphate dehydrogenase/ml.

O_2 utilization measurements were done on 2 ml incubation volumes using the Gilson Respirometer. The study of Figure 25 utilized a 5 ml incubation system, from which 0.5 ml reaction aliquots were withdrawn at the appropriate time intervals.

Assay of formation of TBA chromogen. A typical assay mixture contained per ml: 0.1 ml of microsomes, 4.0 μmoles ADP, 1.2 X 10^{-5} M Fe^{3+}, and 0.9 ml of tris-HCl buffer (0.1 M, pH 7.5) were incubated with (Experimental) and without (Control) 0.3 μmoles of NADPH at 37° C in air. One milliliter incubations were carried out in test tubes in a Dubnoff incubator. The enzyme reaction was terminated by the addition of 0.5 ml

of 35% (weight/volume; W/V) trichloroacetic acid (TCA). One milliliter of 0.75% TBA (W/V) was added and the mixtures were heated in a boiling water bath for 15 minutes according to the method of Ottolenghi (24). After cooling, 1 ml of 70% TCA (W/V) was added to each tube and swirled gently. The samples were then centrifuged and the optical density of the clear pink supernatant was determined at 532 mμ. In many instances sample colors were too intense to be read directly and were first diluted with an acid dilution mixture with the same composition as that of the sample. Samples that were turbid were extracted with chloroform (to remove the lipid turbidity) and the optical density of the clear aqueous layer was determined.

Sephadex G-10 Column Chromatography. A column of Sephadex G-10 (Pharmacia) 1.35 x 100 cm was prepared in potassium phosphate buffer (0.1 M, pH 7.2 containing 0.1 M NaCl). The column was allowed to equilibrate for one week before use. A 2 ml sample containing either authentic malonaldehyde (28) or the chromogen reacting with TBA which is produced in the NADPH-oxidase system was applied to the column and eluted with 0.1 M phosphate - 0.1 M NaCl buffer at a rate of 0.5 ml/minute. Two different pH conditions were used: one at pH 7.2 and another at pH 2.8. Fractions of 1.0 ml volume were collected in tubes containing the reagents used for the TBA assay for malonaldehyde.

Spectrofluorometric Methods for Malonaldehyde determination. The microsomal incubation system was diluted 1:1 with distilled water and to a 0.1 ml aliquot of this was added 0.9 ml of dimethyl formamide and 2.0 ml of a dimethylformamide solution containing 1% ethyl p-aminobenzoate (or 4,4'-sulfonyldianiline) and 1% concentrated (38%)

hydrochloric acid. The mixture was heated in a boiling water bath for 5 minutes, cooled, and 0.5 ml of 10% NaOH solution added. The resulting solution was read in the spectrofluorometer with an excitation wavelength of 475 mμ and an emission (detection) wavelength of 545 mμ. The meter deflection reading was compared to that of a standard graph (Figure 4) to determine the malonaldehyde content of the sample. This method was adapted from that of Sawicki, Stanley, and Johnson (33).

p-Nitroaniline Spectrophotometric Assay for Malonaldehyde. This malonaldehyde determination method was performed identically to that reported by Sawicki, Stanley, and Johnson (33).

Fe estimation. To 30 ml of 1.5 M phosphate buffer was added 8 mg of sodium dithionite ($Na_2S_2O_4$), to reduce all Fe to the Fe^{2+} state, and one ml of a 0.1 M solution of pyridine-2-aldehyde oxime. The optical density was read in a 10 cm path length cell at 520 mμ in a Cary spectrophotometer. The Fe^{2+} content was then calculated using a molar absorbance value of 11,200.

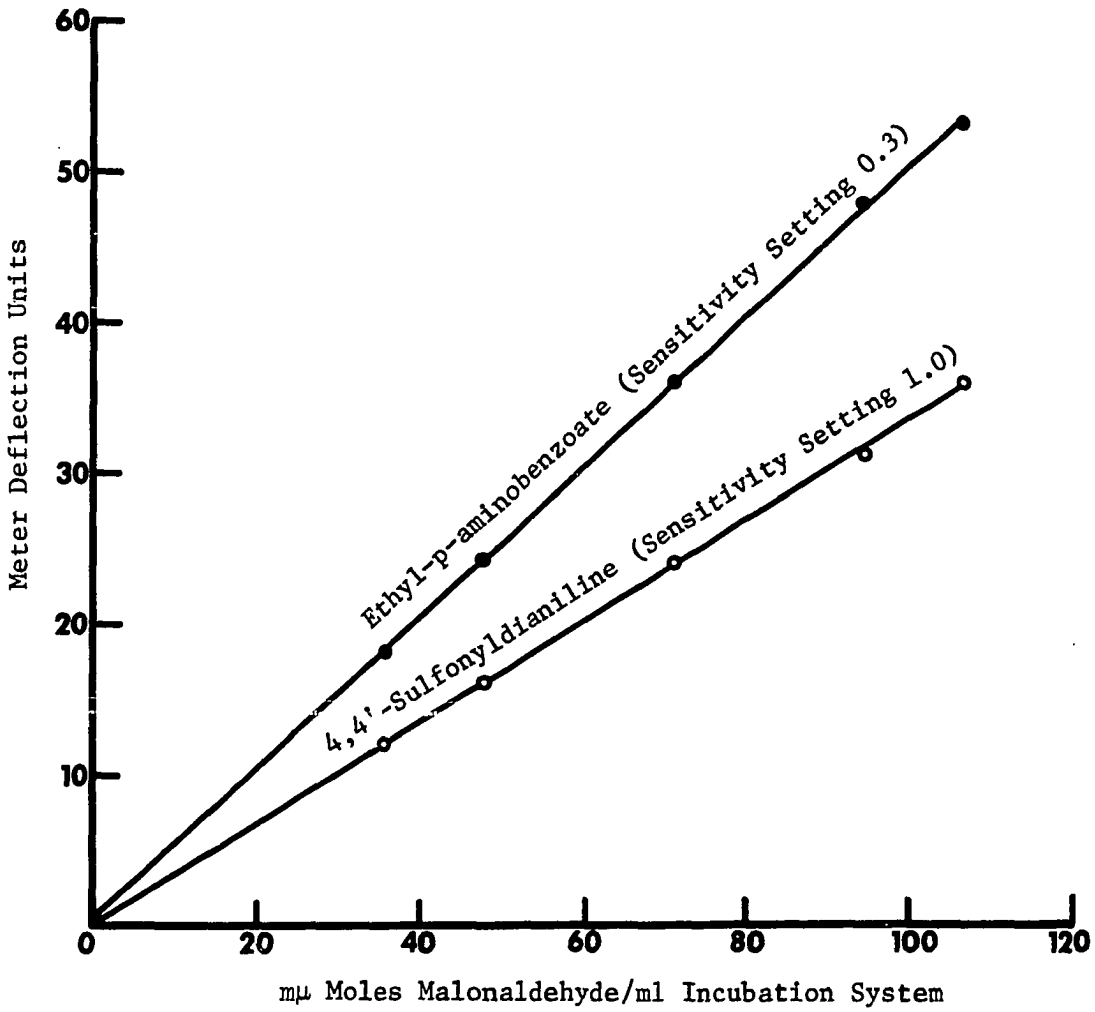


Figure 4. Spectrofluorophotometric Determination of Malonaldehyde

CHAPTER III

RESULTS

Studies by Saslow and Waravdekar (31, 32), and others (14), indicated that malonaldehyde might not be the product of autocatalytic lipid peroxidation which reacts with TBA to form the 532 mμ-absorbing chromogen, and that it might not be formed at all during the peroxidation process. Also, it has been demonstrated that conjugated dienealdehydes were formed during autocatalytic lipid peroxidative processes, and Schmidt (34) has shown that conjugated dienealdehydes react with TBA to form a chromogen having maximum absorption in the 530 - 532 mμ region. As a result of the above studies it was necessary to demonstrate that malonaldehyde is indeed a product of microsomal NADPH-oxidase-catalyzed phospholipid peroxidation. Furthermore, it was necessary to demonstrate that it is the substance which reacts with TBA to form the chromogen used as an index of the extent of peroxidative cleavage of the β-fatty acids of membrane-bound phospholipids and that it accounts for all of the color formation.

A simple kinetic study was first done to determine any differences or similarities between the rates of reaction of (1) TBA and malonaldehyde, (2) TBA and a conjugated dienealdehyde, hexadienealdehyde, and (3) TBA and the products of the microsomal NADPH-oxidase-catalyzed

lipid peroxidation. The results are shown in Figure 5. The product(s) of the enzymic peroxidation of microsomal phospholipids had a reaction rate with TBA identical with that of an authentic sample of malonaldehyde, while the rate for the reaction of the hexadienealdehyde with TBA was distinctly different.

A logarithmic plot also showed a difference between the rates of reaction of hexadieneal and malonaldehyde with TBA. A further effort was made to distinguish by chemical means if malonaldehyde is the TBA-reacting product of the NADPH-oxidase catalyzed lipid peroxidation system. A spectrophotometric assay for malonaldehyde described by Sawicki, Stanley, and Johnson, and an adaption of two spectrofluorometric methods also described by them were used, and the results obtained were compared to those utilizing the TBA assay method for malonaldehyde. Table 1 shows a comparison between calculated values using the two spectrofluorometric methods (ethyl p-aminobenzoate and 4,4'-sulfonyldianiline), the p-nitroaniline spectrophotometric method, and the TBA assay method. The quantitative results are in good agreement for the four methods of malonaldehyde estimation.

Finally, a comparison of the behavior of malonaldehyde and the TBA-reacting product of NADPH-oxidase-catalyzed phospholipid peroxidation on Sephadex G-10 chromatography was made similarly to that described by Kwon (10, 11). When the pH of a Sephadex G-10 column is shifted from 7.2 to 2.8 there is a shift in the elution volume for malonaldehyde (10, 11), presumably because of the shift from a β-keto enolate structure (a) to an hydrogen bonded β-keto enol structure (b)

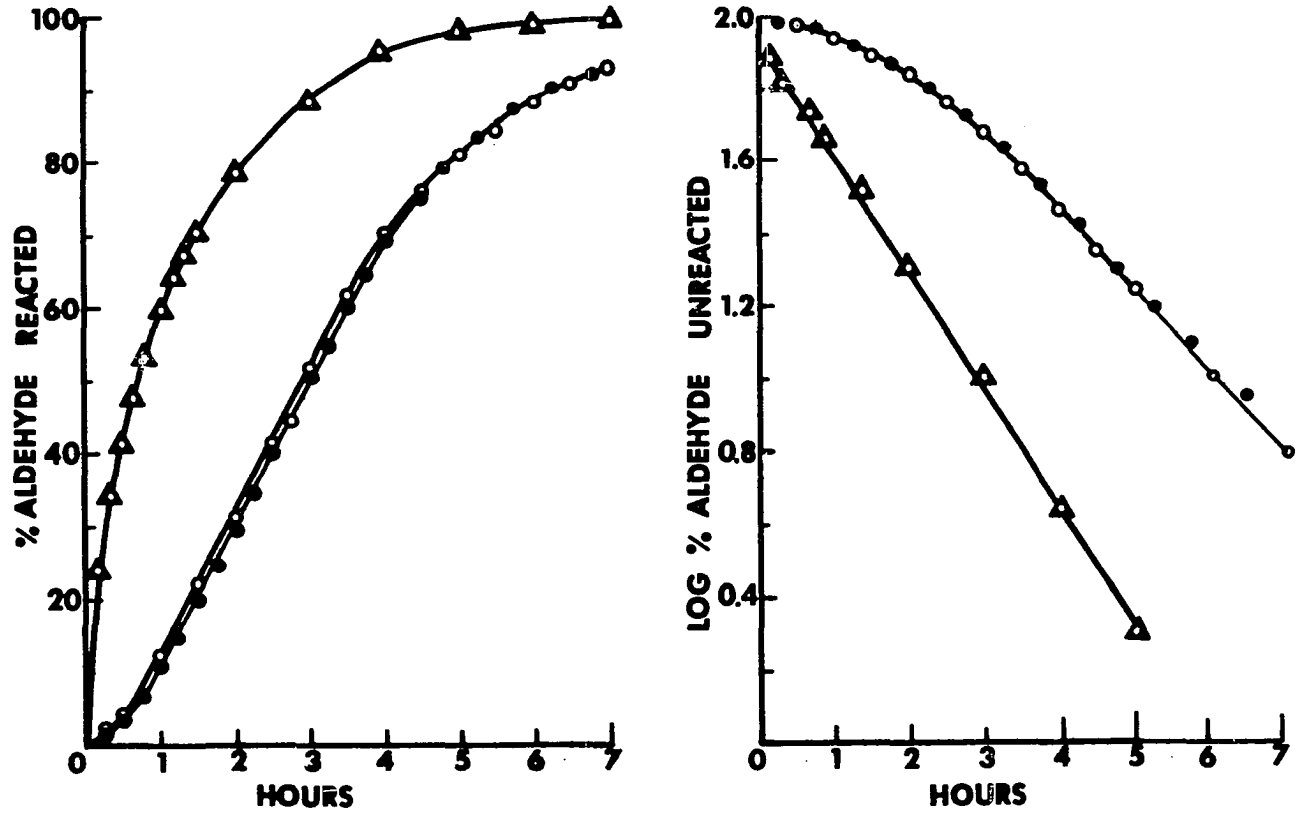


Figure 5. Kinetics of 532 mμ Absorbing Chromogen Formation

TABLE 1

COMPARISON OF MALONALDEHYDE DETECTION METHODS

	mμ Moles Malonaldehyde per ml Incubation System			
	EPAB[a]	SDA[b]	TBA[c]	PNA[d]
Microsomes only	0.2	0.2	0.2	0.4
Complete Incubation System Number				
1	69.6	68.2	71.8	72.7
2	53.2	51.4	50.9	50.1
3	78.0	76.6	80.6	78.9
4	80.2	81.5	84.6	83.7

a) EPAB - ethyl p-aminobenzoate
b) SDA - sulfonyldianiline
c) TBA - 2-thiobarbituric acid
d) PNA - p-nitroaniline

(a) (b)

Figure 6 shows that the TBA-reacting product of the microsomal NADPH-oxidase-catalyzed lipid peroxidation has the same elution characteristics as malonaldehyde at both pH 7.2 and 2.8 using Sephadex G-10. The column used had a void volume of 59 ml as determined using Dextran Blue 2,000. The V_e/V_o ratio (where V_e equals the elution volume of the applied sample and V_o equals the void volume) for a column at pH 2.8 was 2.42, which is in good agreement with the value of 2.42 obtained by Kwon (10, 11). However, the V_e/V_o ratio at pH 7.2 was 2.05 compared to a value of 1.93 obtained by Kwon. Table 2 shows the quantitative elution characteristics for both malonaldehyde and the product of the microsomal NADPH-oxidase lipid peroxidation. The recovery of material applied to the column in both cases was about 97%. This indicates that there is only one molecular species reacting with 2-thiobarbituric acid produced by the microsomal NADPH-oxidase lipid peroxidation system, and the data presented are regarded as sufficient evidence that the product is indeed malonaldehyde.

Kinetic Studies

An understanding of the kinetics of an enzymic action is usually useful for determining the mode of action or excluding certain modes of action of inhibitors or activators of enzyme catalyzed reactions. Since it has been demonstrated that Mn^{2+}, Co^{2+}, and Ce^{3+} ions, as well as chelating agents such as EDTA have a drastic inhibitory

Figure 6. Elution Characteristics of Malonaldehyde and the NADPH-oxidase TBA Chromogen Forming Lipid Peroxidation Product on a Sephadex G-10 Column.

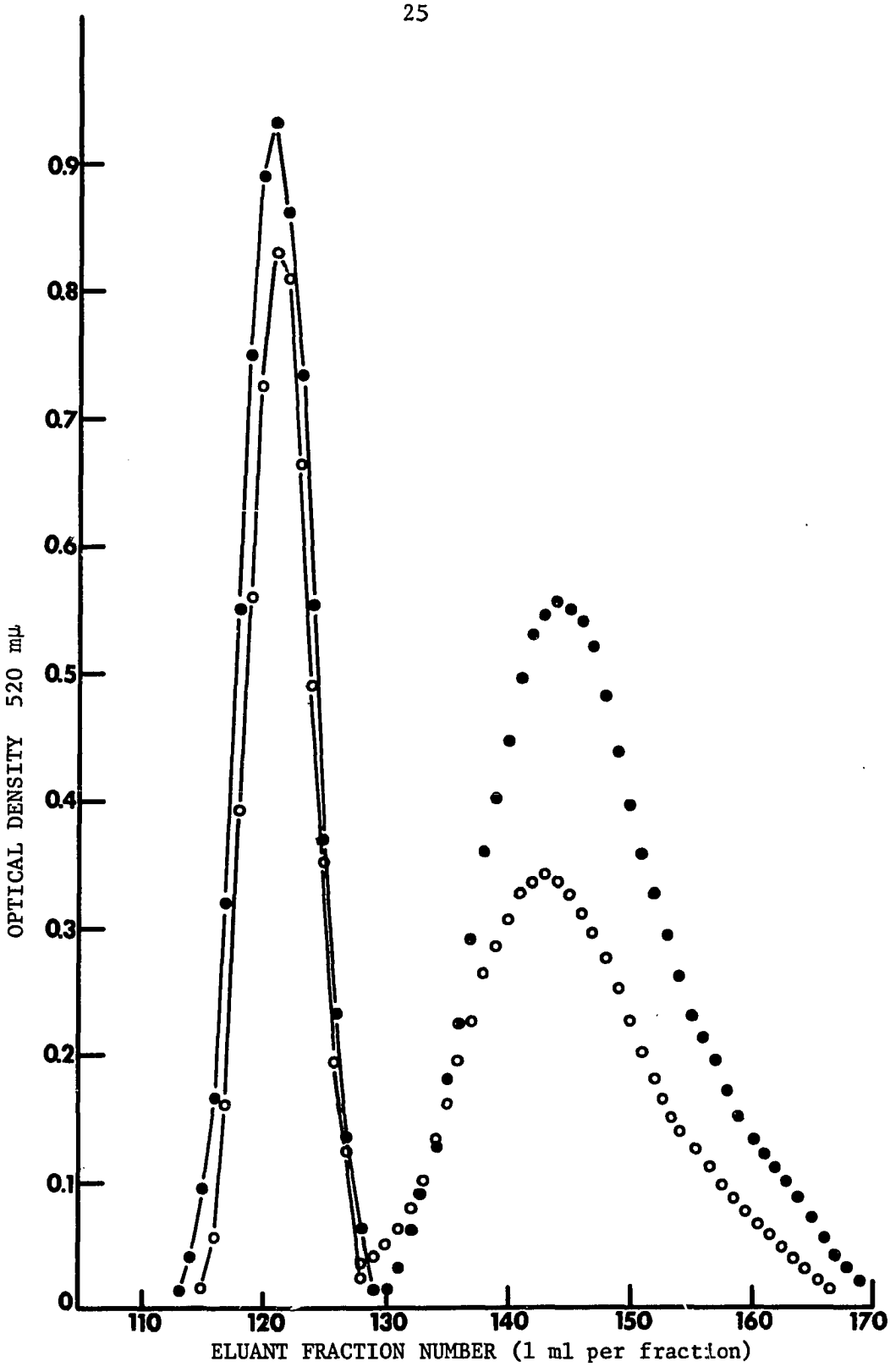

OPTICAL DENSITY 520 mμ
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
110
120
130
140
150
160
170
ELUANT FRACTION NUMBER (1 ml per fraction)
●—● Malonaldehyde pH 7.2; ● Malonaldehyde pH 2.8
o—o NADPH-oxidase lipid peroxidation product pH 7.2
o NADPH-oxidase lipid peroxidation product pH 2.8

TABLE 2

THE ELUTION OF MALONALDEHYDE FROM
SEPHADEX G-10 COLUMN

	mμ moles malonaldehyde		pH
	charged	eluted	
Authentic Malonaldehyde	109	106	7.2[a]
NADPH-oxidase Product	86	85	7.2
Authentic Malonaldehyde	163	158	2.8[b]
NADPH-oxidase Product	104	101	2.8

a) 0.1 M phosphate buffer + 0.1 M NaCl
b) 0.1 M phosphate buffer + 0.1 M NaCl + HCl to achieve pH 2.8

effect (19, 26) on the microsomal NADPH-oxidase catalized lipid peroxidation system (Table 3), a series of kinetic experiments were done to determine if Mn^{2+}, Co^{2+}, and Ce^{3+} ions were of the competitive or non-competitive inhibition type. Experiments were also designed to determine, if possible, the inhibition dissociation constants, K_I, of the inhibitor complex for each of the respective metal ions Mn^{2+}, Co^{2+}, and Ce^{3+}.

TABLE 3

INHIBITION OF MALONALDEHYDE FORMATION

Additions[a]	mμMoles Malonaldehyde
None	86
Mn^{2+}	2
Co^{2+}	2
Ce^{3+}	3
EDTA	1

a) All additions are 10^{-3} M.

To determine the inhibitory nature of the metal ions, incubations were carried out at varying concentrations of Fe^{3+} and at two different inhibitor concentrations (1.5×10^{-5} M and 3×10^{-5} M Mn^{2+} and Co^{2+}), as well as incubations with no inhibitors added. Both the NADPH-oxidase and ascorbic acid systems were done for comparison. When Lineweaver-Burk (14) (double reciprocal) plots were made from the resulting data (Figures 7, 8, 9, and 10) they indicate a competitive type of

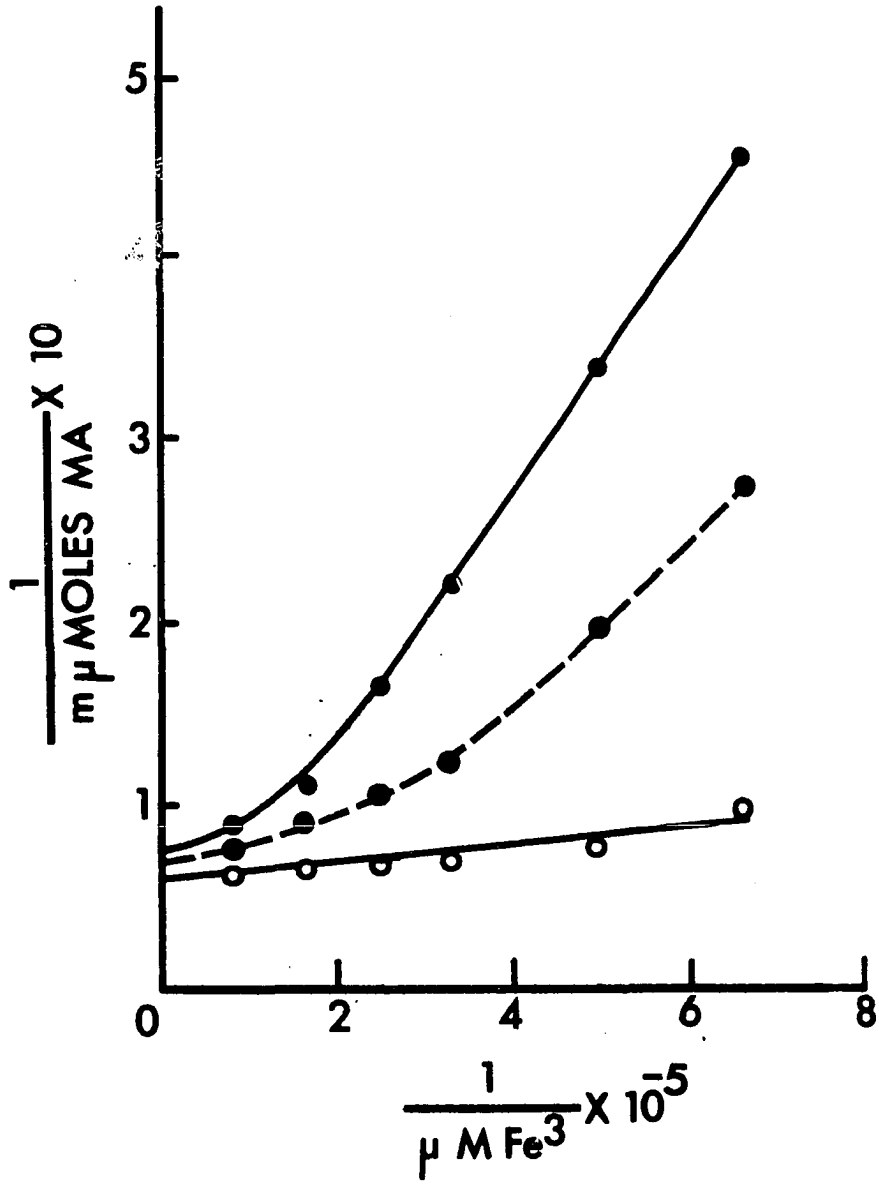


Figure 7. Lineweaver-Burk(Double Reciprocal) Plot of the Effect of the Inhibitory Mn^{2+} Ion on the Formation of Malonaldehyde at Varying Fe^{3+} Concentrations. 0.3 mM NADPH used as substrate. Incubation time 2.5 minutes.

o——o No Mn^{2+} added to incubation system
●– –● 1.5 X 10^{-5} M Mn^{2+}
●——● 3 X 10^{-5} M Mn^{2+}

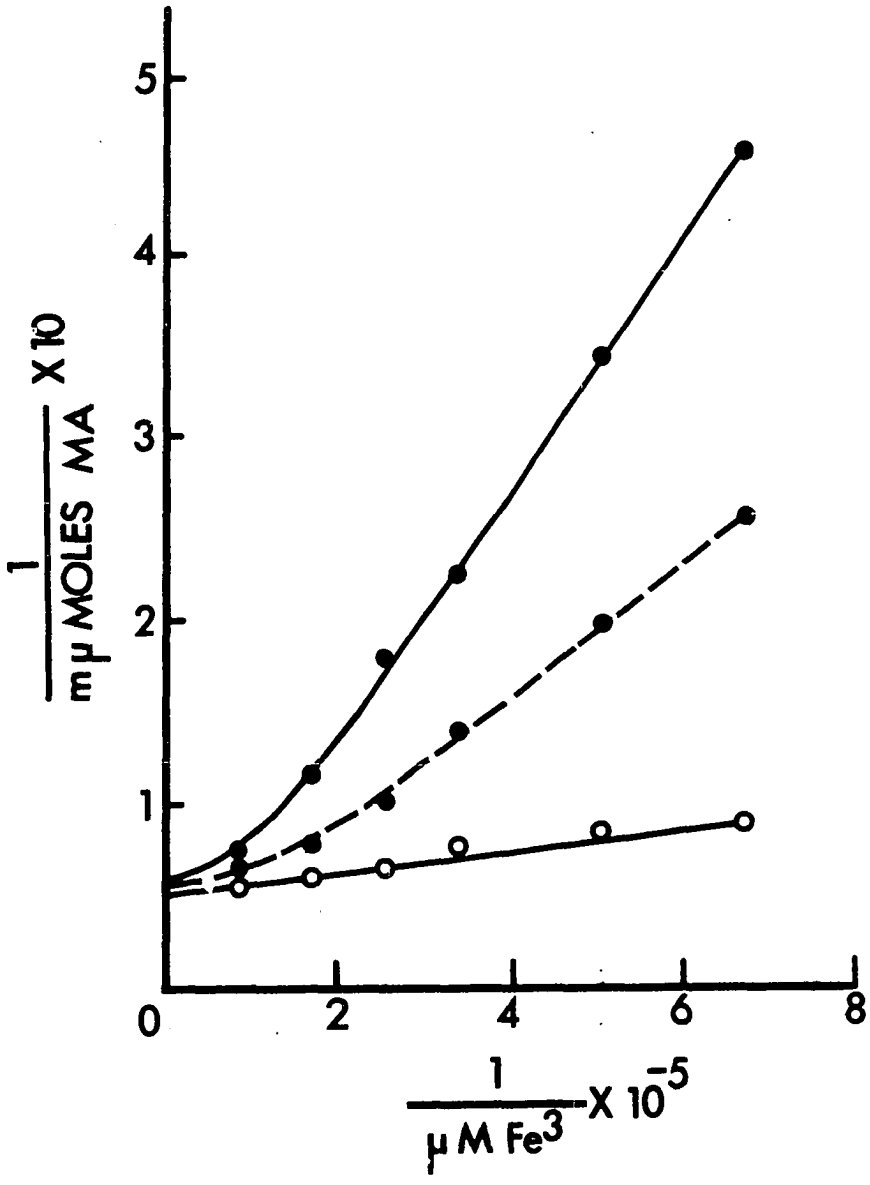


Figure 8. Lineweaver-Burk(Double Reciprocal) Plot of the Effect of the Inhibitory Co^{2+} Ion on the Formation of Malonaldehyde at Varying Fe^{3+} Concentrations. 0.3 mM NADPH used as substrate. Incubation time 2.5 minutes.

o——o No Co^{2+} added to incubation system
•– –• 1.5×10^{-5} M Co^{2+}
•——• 3×10^{-5} M Co^{2+}

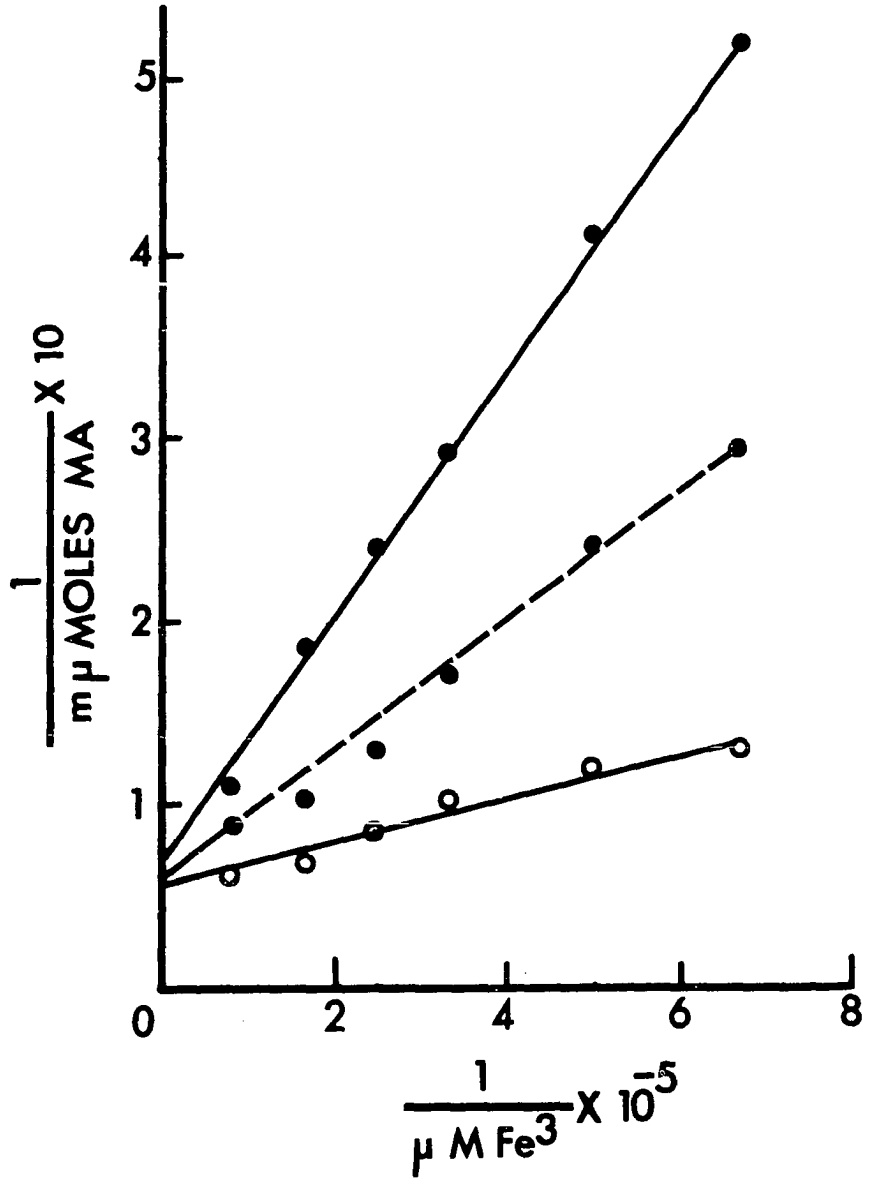


Figure 9. Lineweaver-Burk(Double Reciprocal) Plot of the Effect of the Inhibitory Mn^{2+} Ion on the Formation of Malonaldehyde at Varying Fe^{3+} Concentrations. Ascorbic acid used as substrate at 10^{-3} M. Incubation time 2.5 minutes.

o——o No Mn^{2+} added to incubation system
•– –• 1.5 X 10^{-5} M Mn^{2+}
•——• 3 X 10^{-5} M Mn^{2+}

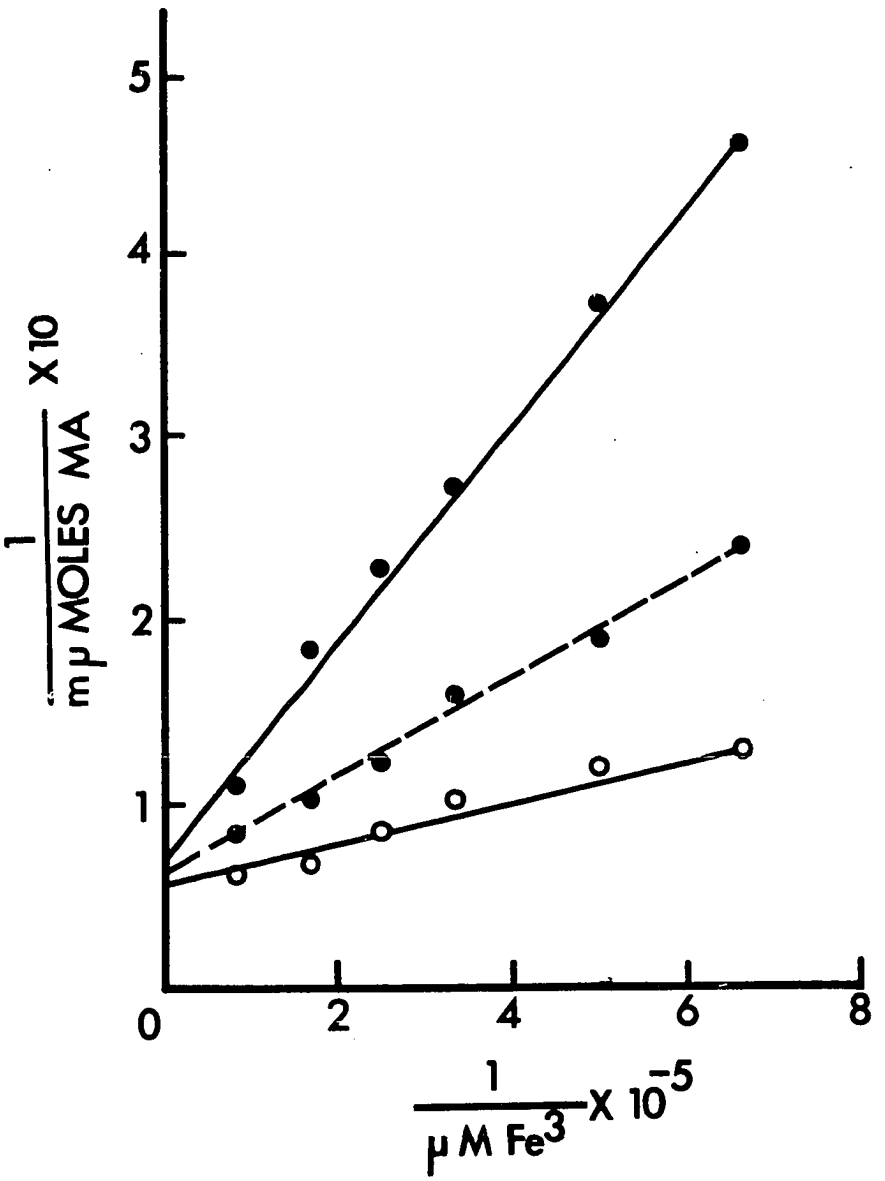


Figure 10. Lineweaver-Burk(Double Reciprocal) Plot of the Effect of the Inhibitory Co^{2+} Ion on the Formation of Malonaldehyde at Varying Fe^{3+} Concentrations. Ascorbic acid used as substrate at 10^{-3} M. Incubation time 2.5 minutes.

o——o No Co^{2+} added to incubation system
●– –● 1.5 X 10^{-5} M Co^{2+}
●——● 3 X 10^{-5} M Co^{2+}

inhibition for the case of ascorbic acid used as substrate, but the results for the NADPH-oxidase system were somewhat ambiguous, due to the curvature of the lines as they approach the ordinate. A competitive inhibition type of situation was suspected because of the convergence of the lines toward the ordinate, but additional data were necessary to clarify the true situation.

When a graph of malonaldehyde formed versus the amount of Fe^{3+} added was made (Figure 11) it was observed that when no Fe^{3+} was added an appreciable amount of malonaldehyde formation was still observed. A deviation between the expected and observed malonaldehyde formation is represented by the difference between the solid and dashed curves in Figure 11. This deviation was thought to be caused by Fe^{3+} bound to the microsomes from the 0.15 M phosphate buffer used during the course of their preparation.

When the phosphate buffer used for the preparation of the microsomes was tested for Fe^{3+} content it was found to contain 2.2×10^{-6} M Fe^{3+}. An "Fe^{3+}-free" potassium phosphate buffer was then prepared by passing a 1.5 M solution through a column of Chelex-100 resin to remove as much of the contaminating Fe^{3+} as possible. This treatment removed all but 1/40 or 2.5% of the Fe^{3+}, which reduced the Fe^{3+} concentration of the Chelex treated phosphate buffer to 5.5×10^{-8} M. This is about the lowest concentration of Fe^{3+} which can be obtained using Chelex-100 resin (22). When 2.5 minute (initial velocity) incubations were performed with varying Fe^{3+} concentrations, using microsomes prepared with the "Fe^{3+}-free" phosphate buffer, rate curves were obtained (Figure 12) which were now in agreement with what would be theoretically expected

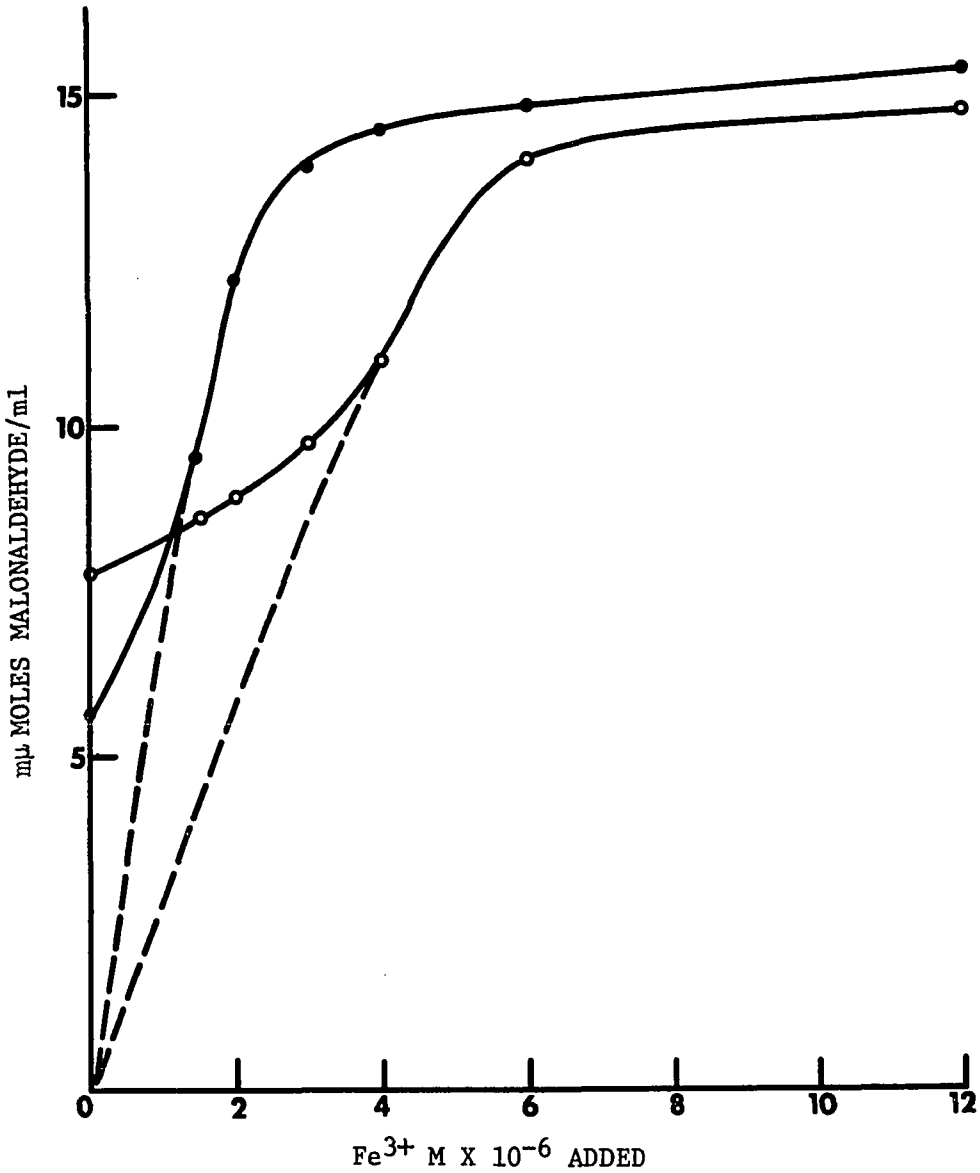


Figure 11. The Rate of Malonaldehyde Formation at Different Fe^{3+} Concentrations Incubated for 2.5 minutes.

●——● 0.3 mM NADPH as substrate
o——o 10^{-3} M Ascorbic acid as substrate

(5), and with the dashed-line portion of the curves of Figure 11.

When Lineweaver-Burk (double reciprocal) plots of the data from Figure 12 were made the K_m value for the Fe^{3+} ion could be calculated from the intercept on the abcissa ($1/\mu MFe^{3+}$ axis). From these calculations using NADPH, ascorbic acid, and 2-mercaptoacetic acid as substrates, the K_m values for Fe^{3+} of Table 4 were obtained.

TABLE 4

Fe^{3+} K_m VALUES[a]

Substrate	Special Conditions	K_m
Ascorbic acid	None	1.6×10^{-6} M
2-mercaptoacetic acid	None	1.5×10^{-6} M
NADPH	None	6.7×10^{-7} M
Ascorbic acid	Heated Microsomes[b]	6.3×10^{-7} M
Mercaptoacetic acid	Heated Microsomes	6.6×10^{-7} M

a) Determined graphically from Lineweaver-Burk double reciprocal plot of Figure 13.

b) 65° C for one minute.

It is interesting to note that the K_m values for ascorbic acid and 2-mercaptoacetic acid were almost identical, and that for the case of microsomes heated at 65° C for 1 minute, the K_m values shifted to a lower value, but the values for the two substrates were very close, though shifted.

An attempt was next made to determine the inhibition dissociation constants, if possible, for the inhibitory ions Mn^{2+}, Co^{2+}, and

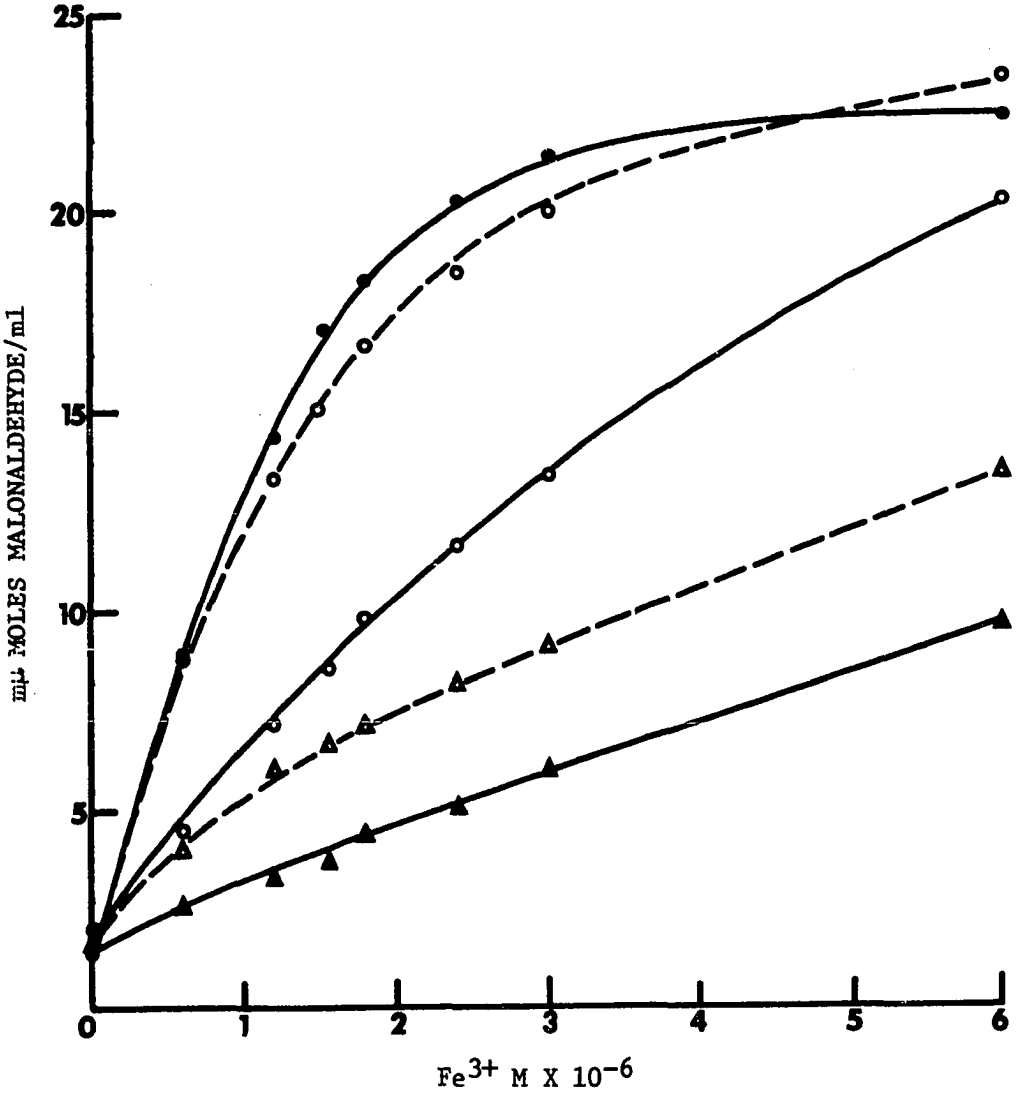


Figure 12. The Dependence of Malonaldehyde Formation on Fe^{3+} Concentration. All incubations done for 2.5 minutes.

- ●——● 0.3 mM NADPH as substrate
- o——o 10^{-3} M Ascorbic acid as substrate
- ▲——▲ 2 X 10^{-3} M 2-mercaptoacetic acid as substrate
- o--o Microsomes heated 1 minute at 65°C + 10^{-3} M ascorbic acid
- ▲--▲ Microsomes heated 1 minute at 65°C + 2 X 10^{-3} M 2-mercaptoacetic acid

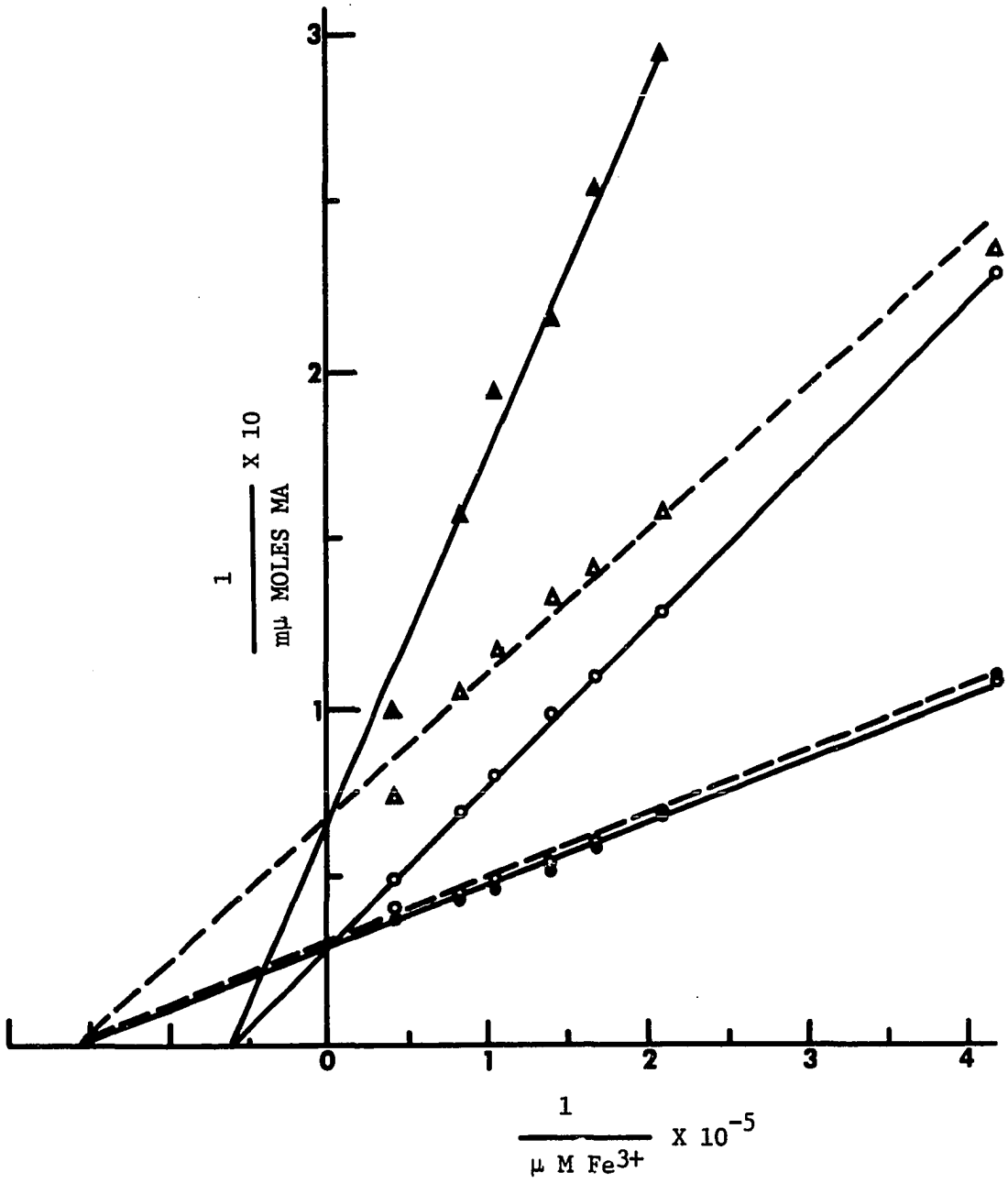


Figure 13. Lineweaver-Burk(Double Reciprocal) Plot of Malonaldehyde Formed and Amount of Fe^{3+} Added to Microsomes Prepared in an Iron-free Buffer. Incubation time 2.5 minutes.

●——● 0.3 mM NADPH as substrate
o——o 10^{-3} M Ascorbic acid as substrate
▲——▲ 2 X 10^{-3} M 2-mercaptoacetic acid as substrate
o- -o Microsomes heated 1 minute at 65°C + 10^{-3} M Ascorbic acid
▲- -▲ Microsomes heated 1 minute at 65°C + 2 X 10^{-3} M 2-mercaptoacetic acid

Ce^{3+}, and to determine if competitive inhibition is the case for NADPH used as substrate, since the Lineweaver-Burk plots were ambiguous.

Microsomes prepared with the "Fe^{3+}-free" phosphate buffer were used for these measurements, although this is not really relevant, since the only important criterion is that two different concentrations of Fe^{3+} be used (5). When plots of the data obtained were made of the reciprocal of malonaldehyde formed versus the concentration of the inhibitor used (using two different Fe^{3+} concentrations) as described by Dixon (6), the results of Figures 14 - 20 were obtained. These results indicated a competitive type of inhibition. The inhibition dissociation constants, K_I, obtained are shown in Table 5.

TABLE 5

COMPETITIVE INHIBITOR DISSOCIATION CONSTANTS, K_I

Substrate	Inhibitor	Dissociation Constant, K_I
NADPH	Mn^{2+}	3.6×10^{-6} M
Ascorbic acid	Mn^{2+}	3.6×10^{-6} M
2-mercaptoacetic acid	Mn^{2+}	3.6×10^{-6} M
NADPH	Co^{2+}	1.2×10^{-5} M
Ascorbic acid	Co^{2+}	1.2×10^{-5} M
NADPH	Ce^{3+}	5×10^{-5} M
Ascorbic acid	Ce^{3+}	5×10^{-5} M

Free Radical Nature of Lipid Peroxidation

It has been demonstrated that lipid peroxidation is inhibited by certain compounds, such as dietary α-tocopherol (4), which are

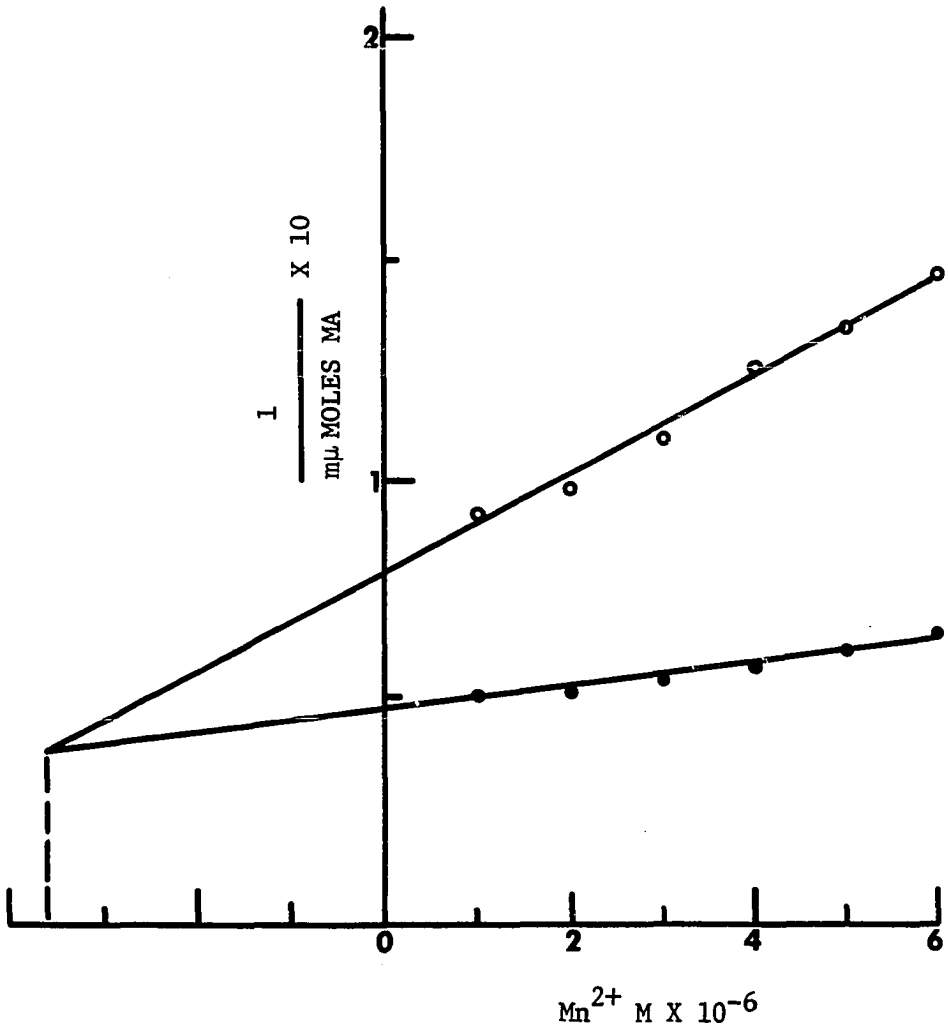


Figure 14. Determination of the Inhibition Dissociation Constant, K_I, for Mn^{2+} Using 0.3 mM NADPH as Substrate. Incubation time 2.5 minutes.

●——● 6 X 10^{-6} M Fe^{3+}
o——o 3 X 10^{-6} M Fe^{3+}

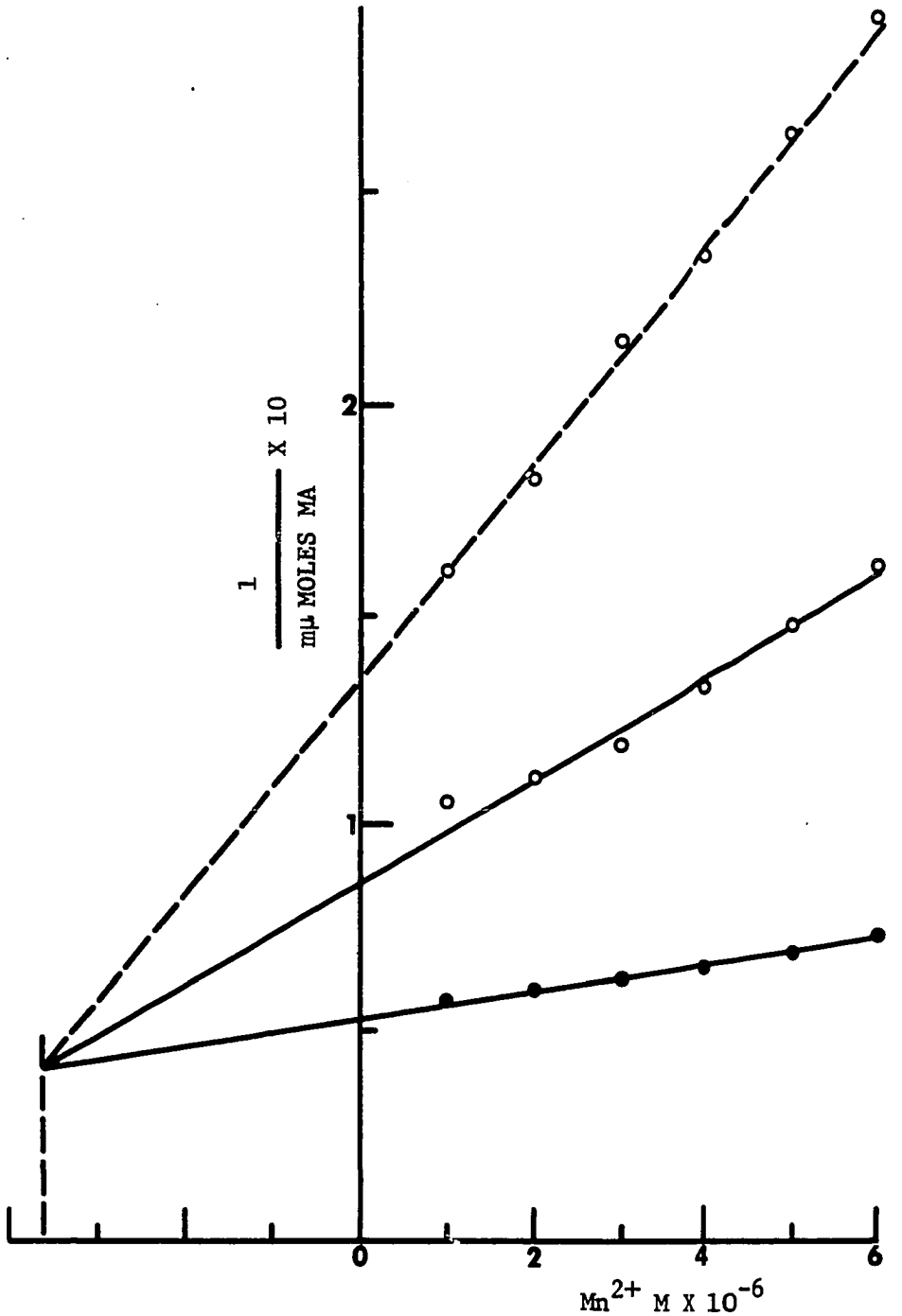


Figure 15. Determination of the Inhibition Dissociation Constant, K_I, for Mn^{2+} Using 10^{-3} M Ascorbic Acid as Substrate. Incubation time 2.5 minutes.

●——● 6 X 10^{-6} M Fe^{3+}
o——o 3 X 10^{-6} M Fe^{3+}
o--o 1.5 X 10^{-6} M Fe^{3+}

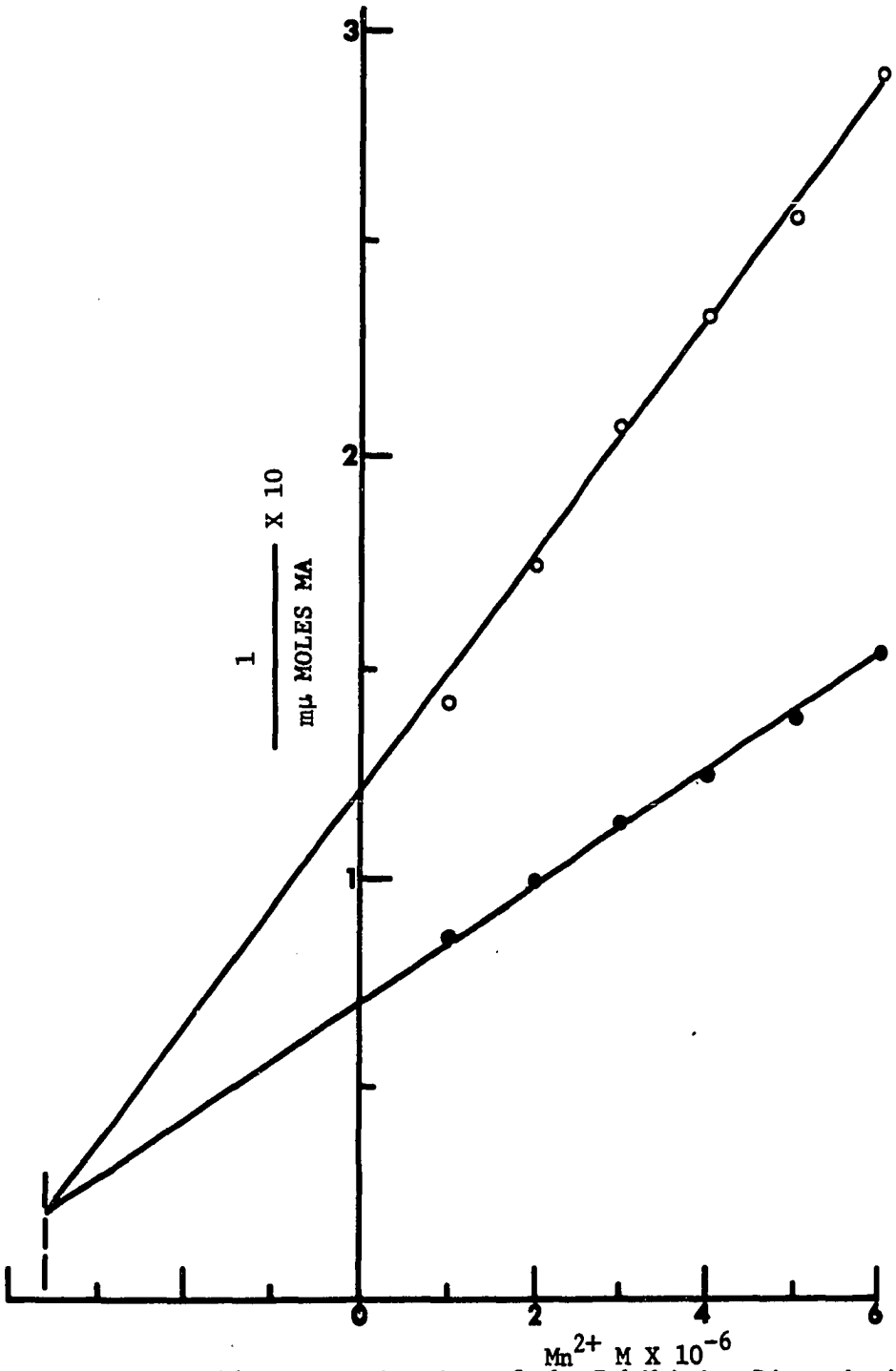


Figure 16. Determination of the Inhibition Dissociation Constant, K_I, for Mn^{2+} Using 2 X 10^{-3} M Mercaptoacetic Acid as Substrate. Incubation time 2.5 minutes.

●——● 6 X 10^{-6} M Fe^{3+}
o——o 3 X 10^{-6} M Fe^{3+}

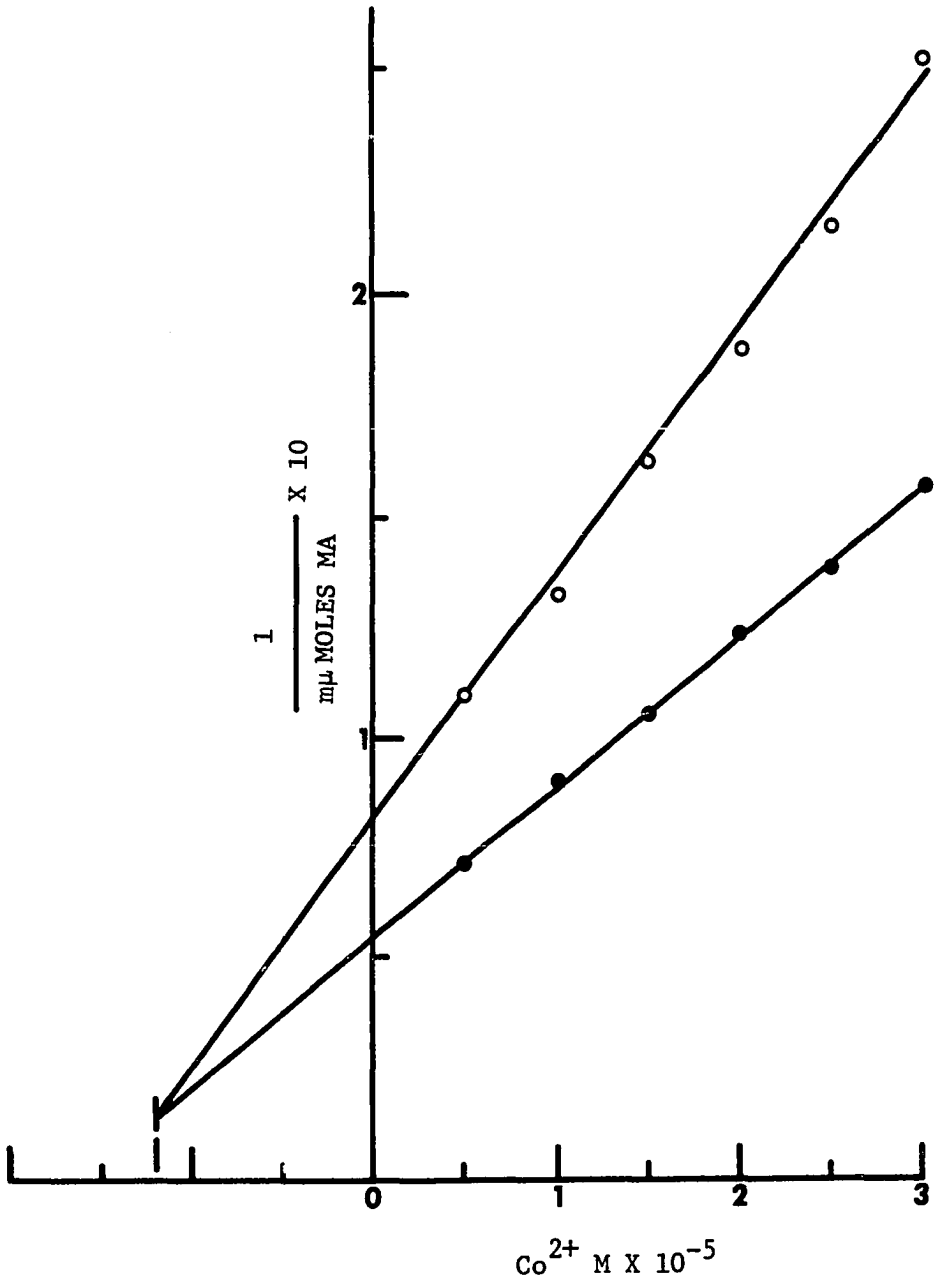


Figure 17. Determination of the Inhibition Dissociation Constant, K_I, for Co^{2+} Using 0.3 mM NADPH as Substrate. Incubation time 2.5 minutes.

●——● 6 X 10^{-6} M Fe^{3+}
o——o 3 X 10^{-6} M Fe^{3+}

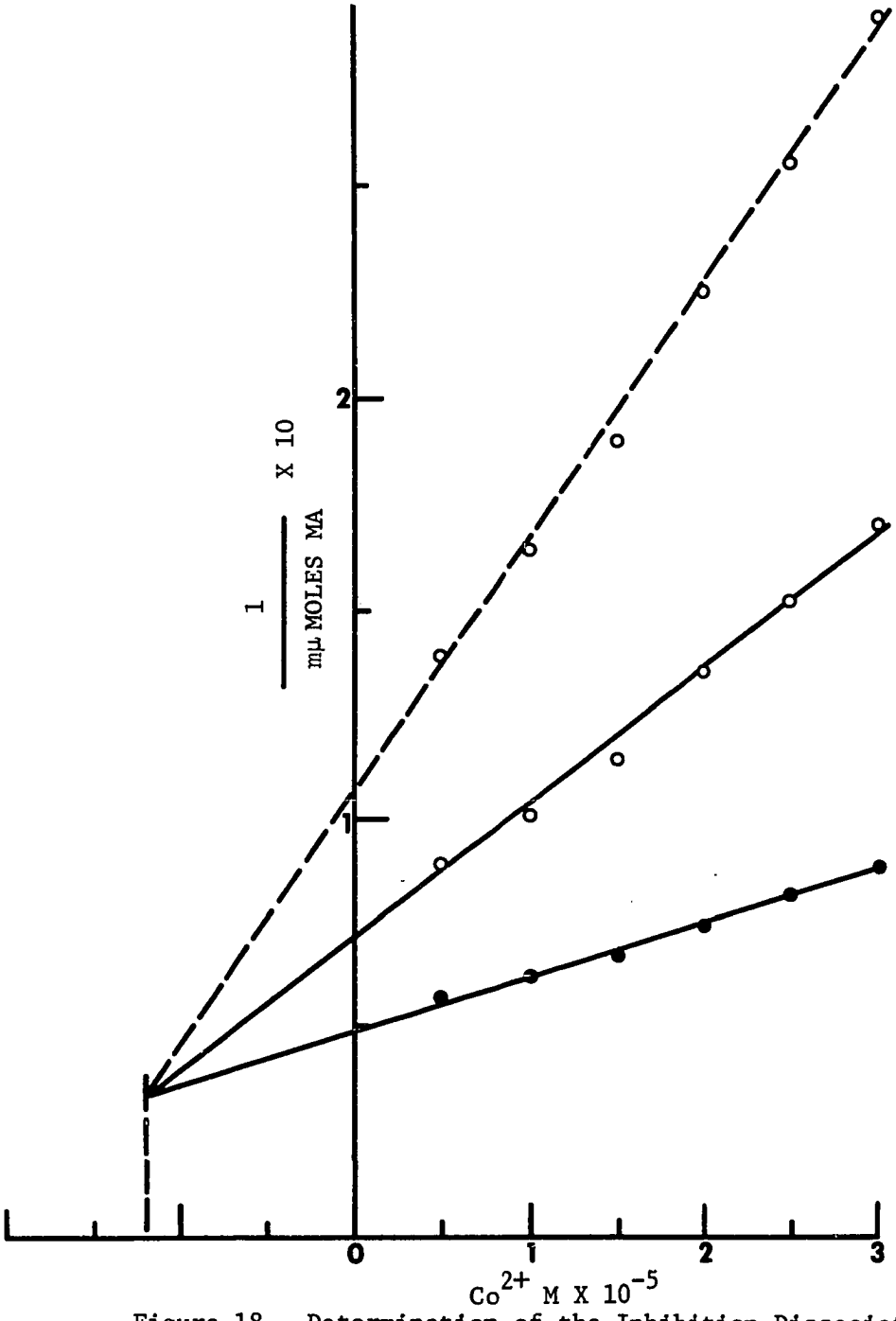


Figure 18. Determination of the Inhibition Dissociation Constant, K_I, for Co^{2+} Using 10^{-3} M Ascorbic Acid as Substrate. Incubation time 2.5 minutes.

●——● 6 X 10^{-6} M Fe^{3+}
o——o 3 X 10^{-6} M Fe^{3+}
o--o 1.5 X 10^{-6} M Fe^{3+}

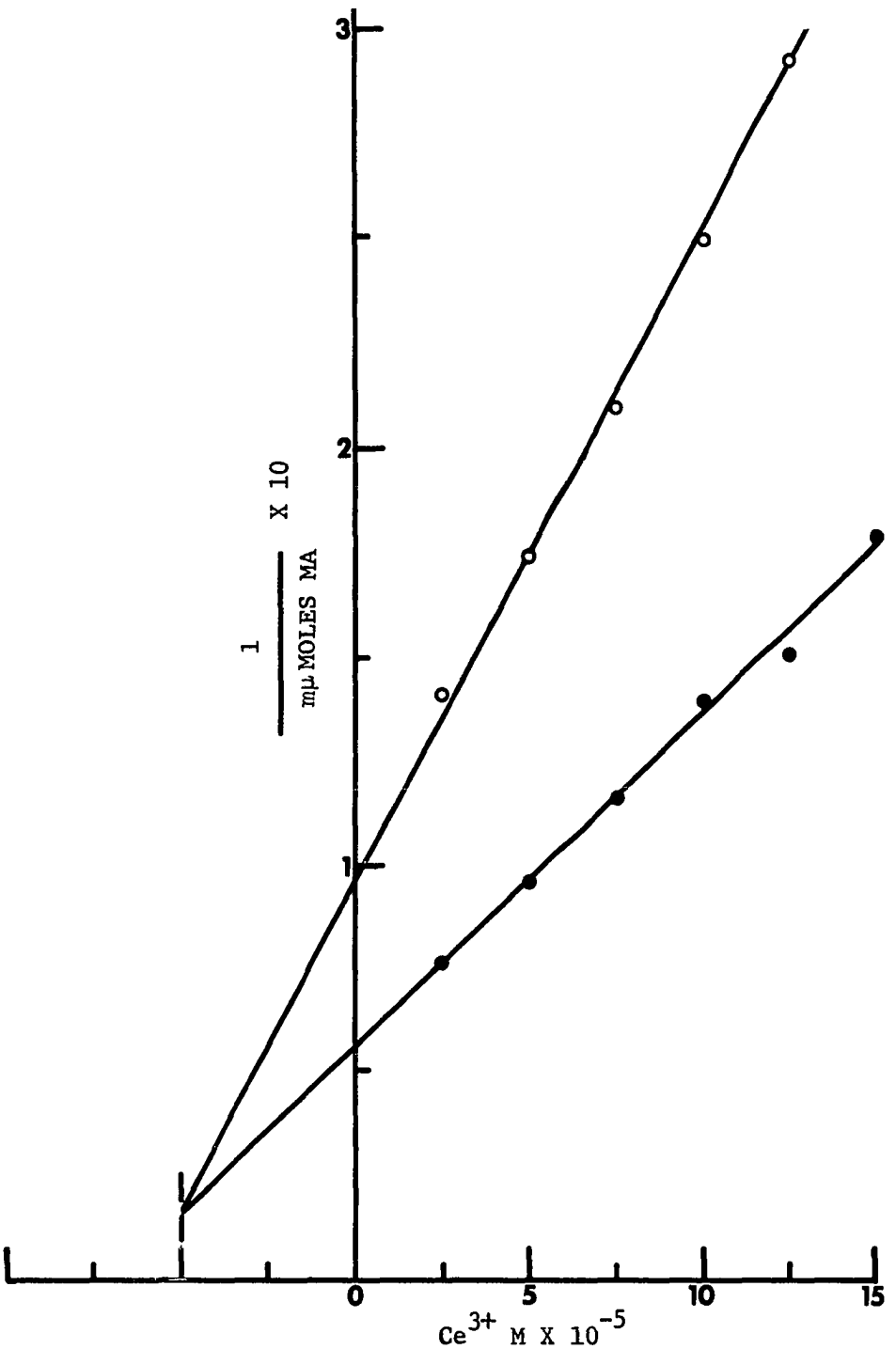


Figure 19. Determination of the Inhibition Dissociation Constant, K_I, for Ce^{3+} Using 0.3 mM NADPH as Substrate. Incubation time 2.5 minutes.

●——● 6 X 10^{-6} M Fe^{3+}
o——o 3 X 10^{-6} M Fe^{3+}

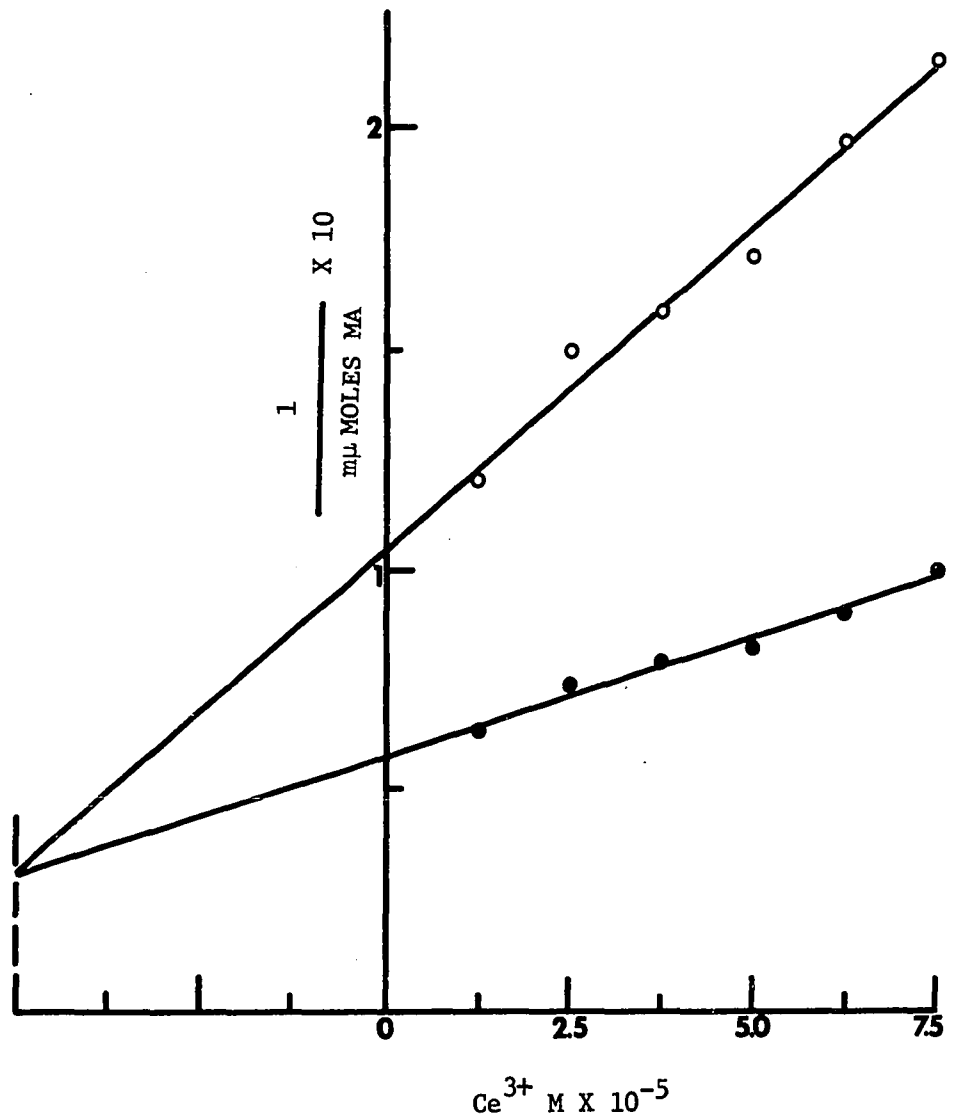


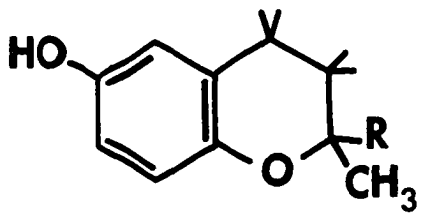
Figure 20. Determination of the Inhibition Dissociation Constant, K_I, for Ce^{3+} Using 10^{-3} M Ascorbic Acid as Substrate. Incubation time 2.5 minutes.

●——● 6 X 10^{-6} M Fe^{3+}
o——o 3 X 10^{-6} M Fe^{3+}

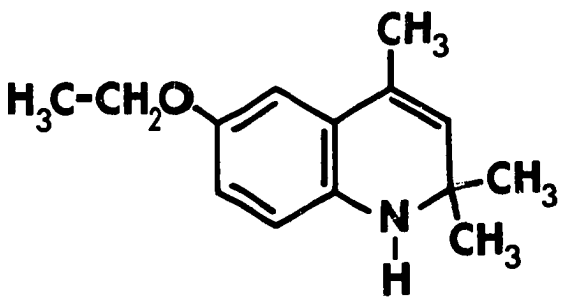
capable of acting as "anti-oxidants" or "free radical traps" i.e. are capable of reacting with a radical species to produce two nonradical products. A number of such compounds capable of reacting with free radicals were investigated to determine their effect on microsomal enzymic lipid peroxidation. These compounds were of two types: (1) those which react to produce nonradical products; α-tocopherol, diphenyl-p-phenylenediamine, santoquin (see Figure 21), and ascorbic acid, and (2) aromatic amines which react with free radicals in the presence of O_2 to form semi-stable nitric oxide (N-O·) radicals (1) as products, such as aniline, N-methyl aniline, and diphenyl amine. Since sulfite ion has been shown to react with free radicals formed in other enzyme systems (10) thereby initiating the aerobic oxidation of the sulfite ion, a study of the sulfite oxidation during microsomal lipid peroxidation was done.

The two types of free radical-trapping compounds were tested with the stable free radical, diphenylpicrylhydrazyl (Figure 21), to determine a general pattern of reactivity. The compounds reacting with free radicals to form nonradical products of the type (1) class gave fast reactions with 10^{-3} M diphenylpicrylhydrazyl (DPPH) and 2 X 10^{-3} M free radical-trapping agent in chloroform or methanol solution (fast being less than 15 seconds for complete reaction to form the completely "reduced" diphenylpicrylhydrazine, measured by the loss of the DPPH absorption peak at 528 mμ). Compounds of the type (2) class reacted slowly with the DPPH radical (10 to 30 minutes). Mercaptoacetic acid was also in the type (2) class "slow" category.

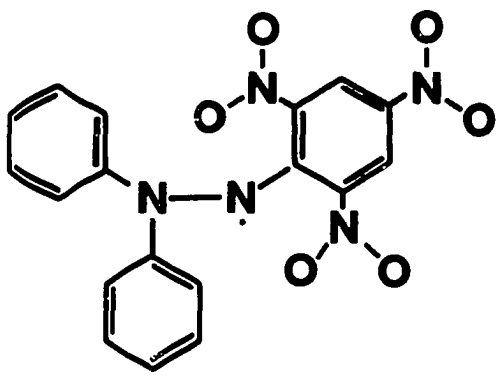
Because the two nonenzymic substrates, ascorbic acid and



α-tocopherol R = 4, 8, 12-trimethyltridecyl (phytyl) group



Santoquin (2,2,4-trimethyl-6-ethoxy-1,2-dihydroquinoline)



Diphenylpicryl hydrazyl free radical (DPPH)

Figure 21

2-mercaptoacetic acid, are both capable of reacting with free radicals, an experiment was done to determine if microsomal lipid peroxidation was dependent upon the concentration of the substrate added. Figure 22 shows that for both ascorbic acid and 2-mercaptoacetic acid there is an optimum concentration at which maximum malonaldehyde is formed, and that concentrations of substrate above this inhibit the formation of malonaldehyde. Ascorbic acid at these higher concentrations also inhibits the formation of malonaldehyde in the enzymic NADPH-oxidase system as well.

The effect of these free radical-trapping compounds on microsomal lipid peroxidation was determined by measuring their effect on O_2 consumption and concomitant malonaldehyde formation, using both the enzymic NADPH-oxidase system and the nonenzymic ascorbic acid system, to ensure that some ancillary effect on the enzyme was not involved. The results are shown in Table 6. Because dietary α-tocopherol was known to inhibit the microsomal enzymic lipid peroxidation, it was somewhat surprising that the added α-tocopherol, added as a suspension, did not inhibit malonaldehyde formation and O_2 consumption. Therefore the dietary effect on these two parameters was investigated for the three radical-trapping compounds diphenyl-para-phenylenediamine, santoquin, and α-tocopherol. It was found for the dietary situation that only santoquin (Table 6) was now not effective after being fed in the diet for 14 days.

The addition of 40 μmoles/ml incubation system of sulfite ion to the NADPH and ascorbic acid incubation systems caused an increase in the consumption of O_2 (Table 6). However, it was somewhat surprising that the malonaldehyde formation was inhibited in the case of the NADPH-oxidase system but was not inhibited for the ascorbic acid system.

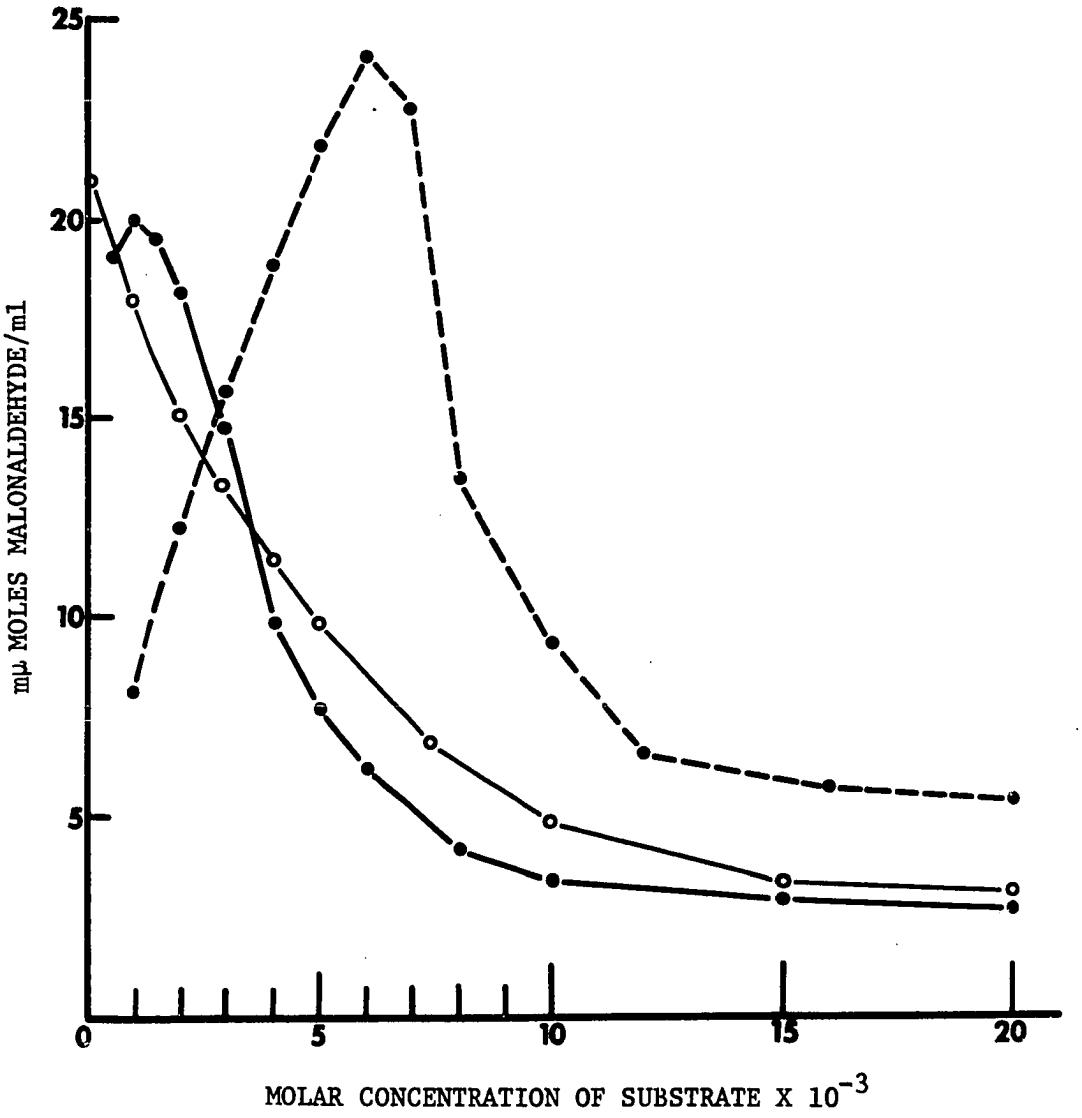


Figure 22. The Effect of the Concentration of Ascorbic Acid and 2-mercaptoacetic Acid on Malonaldehyde Formation. Incubation time 2.5 minutes.

o——o Ascorbic acid incubation system
o——o 0.3 mM NADPH incubation system + ascorbic acid
o- -o 2-mercaptoacetic acid incubation system

TABLE 6

THE EFFECT OF RADICAL TRAPS ON O_2 CONSUMPTION AND MALONALDEHYDE FORMATION

Additions to Incubation System	Substrate NADPH		Substrate Ascorbic Acid	
	µmoles O_2/ml	mµmoles MA/ml	µmoles O_2/ml	mµmoles MA/ml
None	1.73	83.1	1.77	71.7
40 µmoles $SO_3^{=}$ /ml	2.89	41.4	2.62	69.0
Aniline (5×10^{-3} M)	0.05	0.3	0.31	0.3
N-methyl aniline (10^{-3} M)	0	0.2	0.31	0.2
Diphenylamine (10^{-3} M)	0	0.3	0.38	0.2
DPPD (10^{-3} M suspension)	0	0.2	0.32	0.2
Santoquin (10^{-3} M emulsion)	0.1	0.2	0.32	0.2
α-tocopherol (10^{-3} M emulsion)	1.39	82.9	1.50	80.6
Dietary Additions (14 day diet)				
None (α-tocopherol free diet)	1.44	78.4	1.84	91.8
DPPD (0.1% of diet)	0.11	0.1	0.05	0.2
Santoquin (0.13% of diet)	1.32	78.0	1.71	87.4
α-tocopherol acetate (90 mg/100 grams diet)	0.16	0.3	0.07	0.4

Effect of Fe^{3+} on O_2 Consumption and Malonaldehyde Formation

A study was made to determine the effect of Fe^{3+} on O_2 utilization and malonaldehyde formation with concomitant lipid peroxidation. Both the enzymic NADPH-oxidase and the nonenzymic ascorbic acid lipid peroxidizing systems were studied. Figure 23 and Table 7 show the composite results of the effect of Fe^{3+} on O_2 utilization and malonaldehyde formation for the NADPH-oxidase system. When microsomes prepared in a 0.15 M potassium phosphate buffer low in Fe^{3+} (5.5×10^{-8} M) were incubated in the absence of added Fe^{3+}, O_2 utilization and malonaldehyde formation were very low. Microsomes prepared in the regular 0.15 M potassium phosphate buffer containing about 2×10^{-6} M Fe^{3+} had a little more than one-half the activity when no Fe^{3+} was added, as compared to a system with added Fe^{3+}. When an NADPH regenerating system (glucose-6-phosphate + glucose-6-phosphate dehydrogenase) was added, the O_2 utilization and malonaldehyde formation were increased to almost that of the standard incubation system which contains added Fe^{3+}. When the NADPH regenerating system was added to the standard incubation system containing Fe^{3+} an increase in O_2 utilization and malonaldehyde production was observed.

Similar results were obtained for the nonenzymic ascorbic acid incubation system (Figure 24). When microsomes prepared in a 0.15 M potassium phosphate buffer low in Fe^{3+} were incubated with 10^{-3} M ascorbic acid in the absence of added Fe^{3+}, O_2 utilization and malonaldehyde formation were almost completely absent. When microsomes prepared in the regular stock 0.15 M phosphate buffer containing about 2×10^{-6} M Fe^{3+} were incubated in the absence of added Fe^{3+} with ascorbic acid, 90

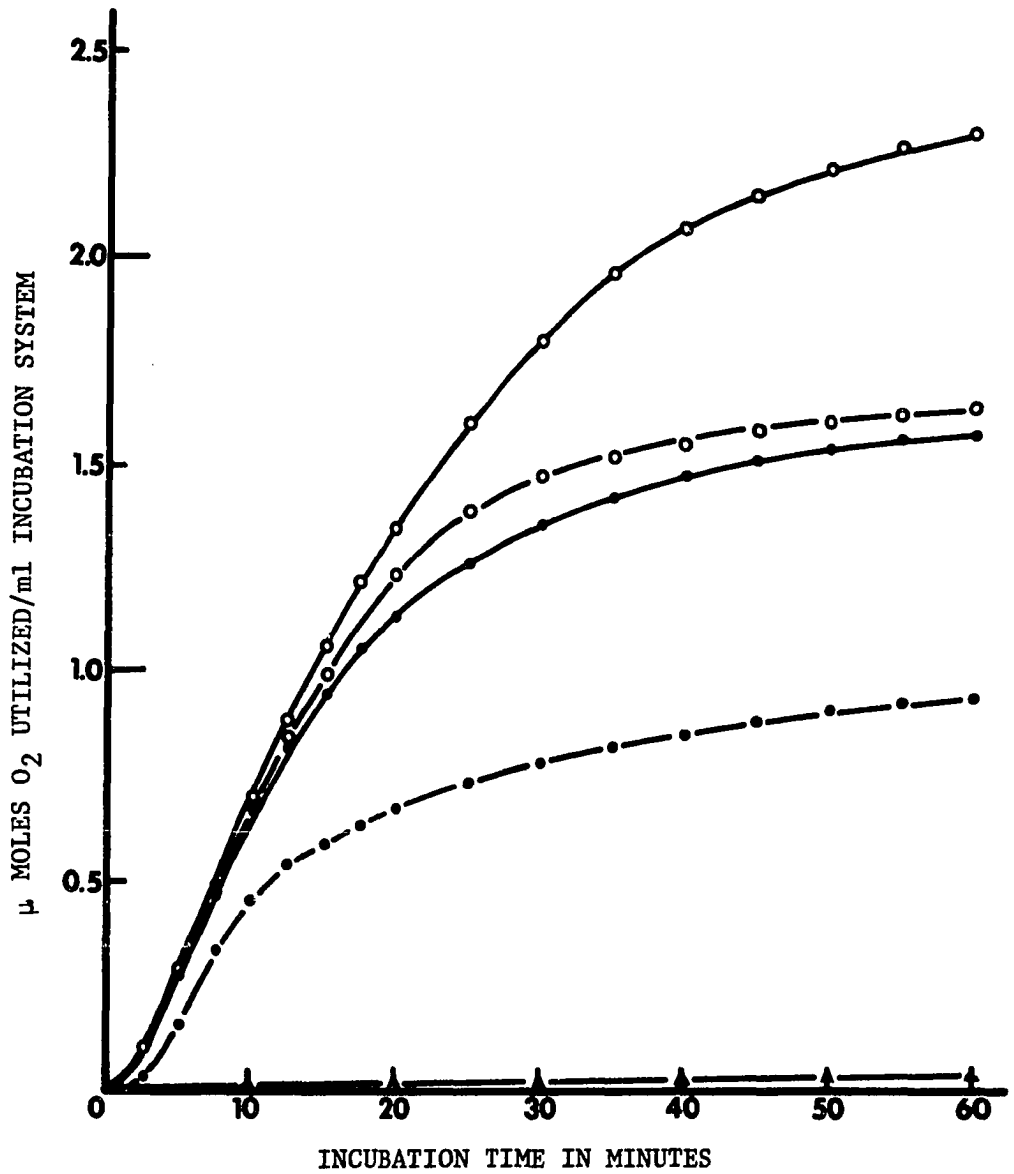


Figure 23. The Dependence of O_2 Utilization of Fe^{3+}. All incubations performed in the presence of 0.3 mM NADPH.

▲——▲ Fe^{3+}-free microsomes + NADPH
●——● Regular 0.15 M phosphate prepared microsomes + NADPH
o——o Regular 0.15 M phosphate prepared microsomes + NADPH + Gen[a]
●——● Regular 0.15 M phosphate prepared microsomes + NADPH + Fe^{3+}
o——o Regular 0.15 M phosphate prepared microsomes + NADPH + Fe^{3+} + Gen

[a]A NADPH regenerating system containing G-6-P and G-6-P dehydrogenase

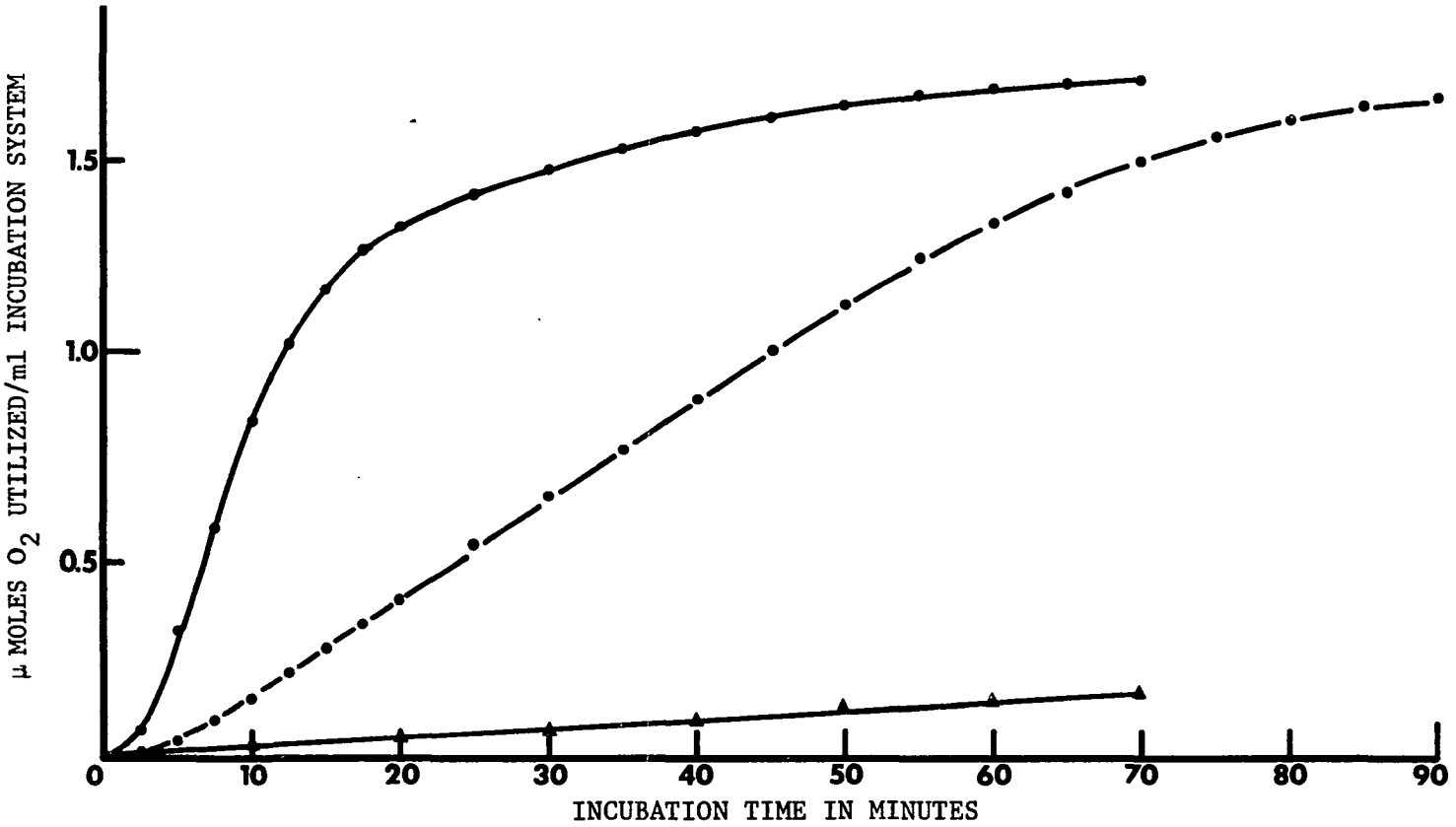


Figure 24. The Dependence of O_2 Utilization on Fe^{3+}. All incubations performed in the presence of 10^{-3} M ascorbic acid.

Fe^{3+}-free microsomes[a] + ascorbic acid
Regular 0.15 M phosphate prepared microsomes + ascorbic acid
Regular 0.15 M phosphate prepared microsomes + Fe^{3+} + ascorbic acid

[a]Microsomes prepared in a 0.15 M phosphate buffer low in Fe^{3+} (5×10^{-8} M)

minutes were required to accomplish as complete O_2 utilization and malonaldehyde formation as is accomplished for the same system to which Fe^{3+} has been added. These results are compiled in Table 7 and indicate the complete dependence of both the enzymic NADPH-oxidase and the non-enzymic ascorbic acid lipid peroxidation on Fe^{3+}.

TABLE 7

THE DEPENDENCE ON Fe^{3+} FOR O_2 CONSUMPTION AND MALONALDEHYDE FORMATION

Incubation System	Incubation Time (Minutes)	µmoles O_2/ml Consumed	mµmoles MA Formed
Fe^{3+}-free Microsomes[a] + NADPH[c]	60	0.06	2.2
Microsomes[b] + NADPH	60	0.94	52.0
Microsomes[b] + Gen.[d] + NADPH	60	1.64	85.6
Microsomes[b] + Fe^{3+} + NADPH	60	1.59	89.1
Microsomes[b] + Fe^{3+} + Gen + NADPH	60	2.31	116.5
Fe^{3+}-free Microsomes[a] + Ascorbic acid	70	0.16	2.4
Microsomes[b] + Ascorbic acid	90	1.64	85.6
Microsomes[b] + Fe^{3+} + Ascorbic acid	70	1.69	78.4

a) Microsomes prepared in 0.15 M phosphate buffer low in Fe^{3+}.
b) Microsomes prepared in 0.15 M phosphate buffer containing about 2 X 10^{-6} M Fe^{3+}.
c) 0.3 m M
d) Glucose-6-phosphate + Glucose-6-phosphate dehydrogenase.

Comparative Reactivity of Thiol Compounds

Since the thiol compound 2-mercaptoacetic acid was found to be active as a nonenzymic substrate for lipid peroxidation, it was of interest to determine what other, if any, thiol compounds might also be

active, and to what quantitative extent. The relative reactivities with respect to malonaldehyde formation for the thiol compounds 2-mercapto-acetic acid, 2-mercaptoethanol, 2-mercaptoethylamine, D and L-cysteine, N-acetyl cysteine, cysteine methyl ester, and reduced glutathione are shown in figure 25. The only thiol compounds of this group which were appreciably active in addition to 2-mercaptoacetic acid were the D and 1 isomers of cysteine, the two isomers being identical in reactivity.

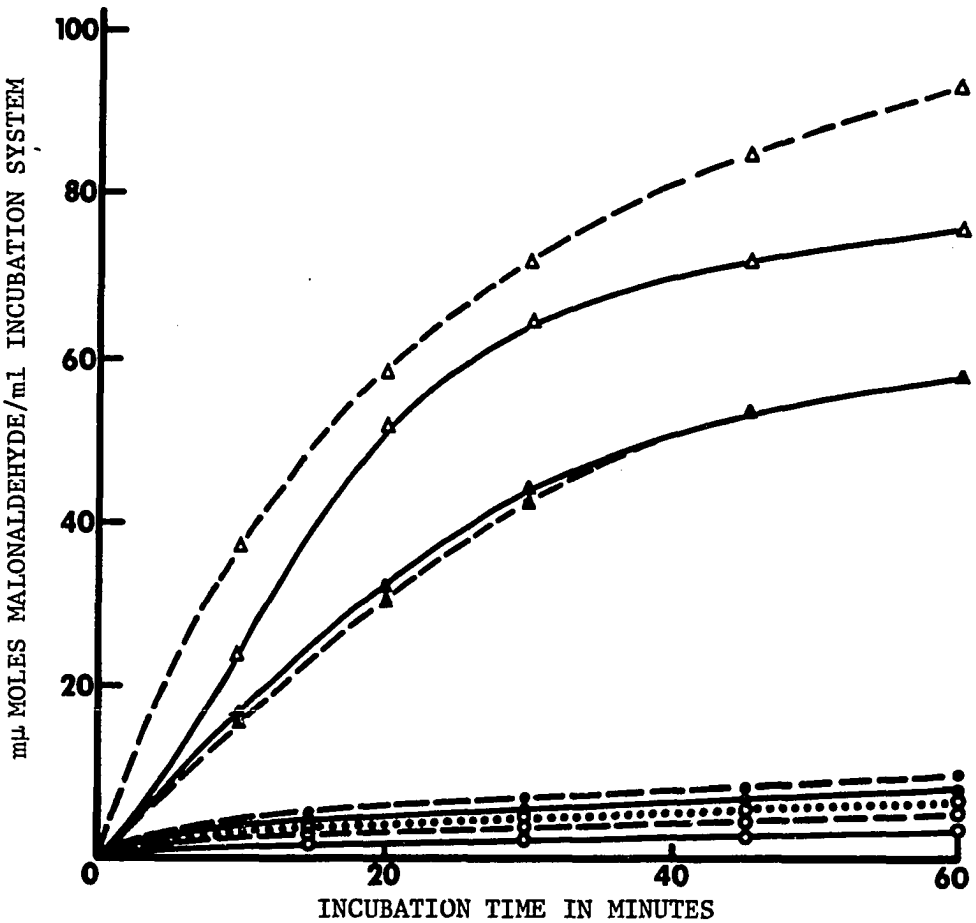


Figure 25. Comparative Reactivity of Thiol Compounds. All substrates incubated at 4 mM concentration unless otherwise noted.

△——△ 2-mercaptoacetic acid
●——● 2-mercaptoethanol
o——o 2-mercaptoethylamine
●——● N-acety cysteine
o•••••o Cysteine methyl ester
o——o Reduced glutathione
▲——▲ D-cysteine 2 mM
▲——▲ L-cysteine 2 mM
△——△ D-cysteine 4 mM

CHAPTER IV

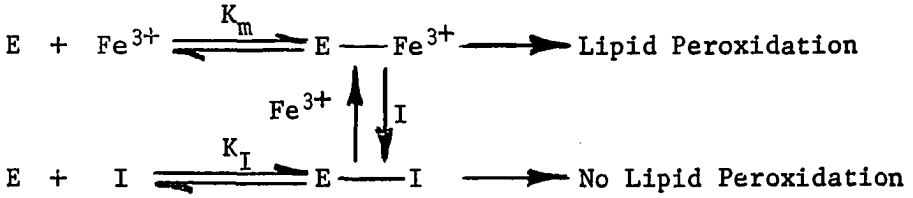
DISCUSSION

The results of the attempts to demonstrate that malonaldehyde is the one molecular species produced by the microsomal NADPH-oxidase lipid peroxidizing system, which reacts with TBA to form a 532 mμ absorbing chromogen, are regarded as sufficient evidence that malonaldehyde is indeed the only compound produced which forms a TBA chromogen. The three criteria used to establish the identity of malonaldehyde: (1) the identical rates of reaction of malonaldehyde and the product of lipid peroxidation with TBA, (2) a comparison of the results of the p-nitroaniline spectrophotometric method for malonaldehyde determination with the ethyl-p-aminobenzoate and 4,4'-sulfonyldianiline spectrofluorometric methods for the estimation of malonaldehyde and the product which forms a chromogen with TBA, and (3) the identity of elution behavior at two different pH values on a Sephadex G-10 column for both malonaldehyde and the quantitative elution of all of the TBA chromogen forming material in one peak.

It was necessary to establish that the lipid peroxidation product reacted with TBA to form a chromogen, while absorbed at 532 mμ, since the determination of this product was to be used for studies where it was important that there be as little ambiguity as possible in the

reaction product determined, and used as an index of the microsomal lipid peroxidation (27, 28). The Lineweaver-Burk (double reciprocal) plots of Mn^{2+}, and Co^{2+} ions as inhibitors of both the enzymic NADPH-oxidase and the nonenzymic ascorbic acid formation of malonaldehyde indicate competitive inhibition of Mn^{2+} and Co^{2+} for Fe^{3+} for a common binding site. Additional evidence was obtained for competitive inhibition when inhibitor dissociation constants (K_I values) were determined using the method described by Dixon (6), for the inhibitory ions Mn^{2+}, Co^{2+}, and Ce^{3+}. As an additional test for competitive inhibition for the case of NADPH used as substrate, a plot of V_o/V_i was made, where V_o is the amount of malonaldehyde formed when no inhibitory metal ion is added, and V_i is the amount of malonaldehyde formed at various arbitrarily selected inhibitory metal ion concentrations. If the slopes of the two such plots are different, using two different Fe^{3+} concentrations, then it is competitive inhibition (Dawes) (5). The slopes are clearly different at two different Fe^{3+} concentrations for both Mn^{2+} and Co^{2+} ions using NADPH as substrate. The same condition was also found using the Ce^{3+} ion as inhibitor, although the results are not shown.

This competitive inhibition may be schematically represented as:



where I is the inhibitory metal ion
K_m is the dissociation constant of the E Fe^{3+} complex
K_I is the dissociation constant of the E I complex

for both the enzymic NADPH-oxidase system and the nonenzymic ascorbic

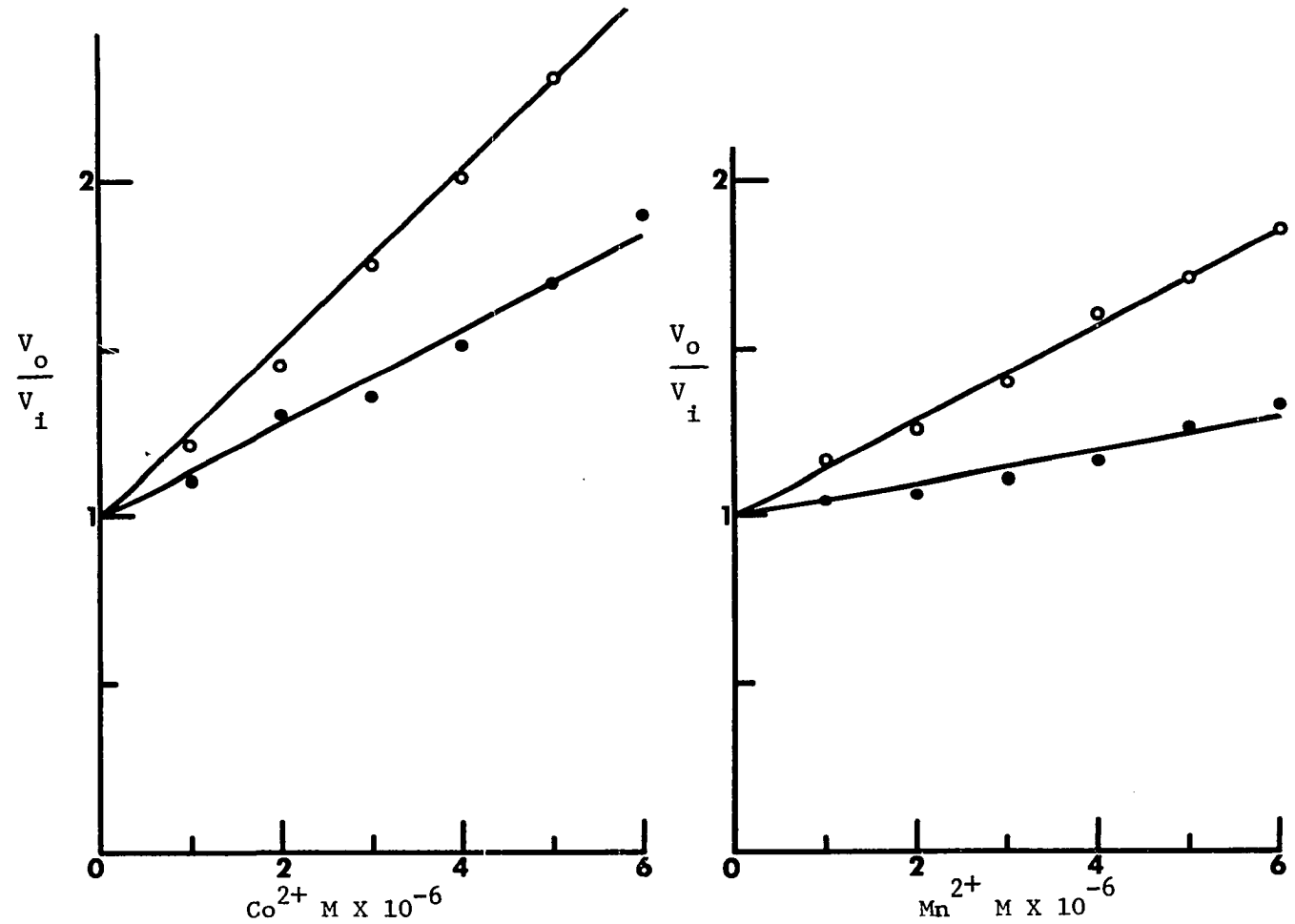


Figure 26. Determination of Competitive Inhibition From a Plot of the Ratio of V_o/V_i Against the Concentration of Inhibitory Ion, for Both Co^{2+} and Mn^{2+} Ions.

● 6 X 10^{-6} M Fe^{3+}
○ 3 X 10^{-6} M Fe^{3+}

acid and 2-mercaptoacetic acid systems.

Perhaps the most interesting result of the determination of the competitive inhibitor dissociation constants (K_I) for Mn^{2+}, Co^{2+}, and Ce^{3+} ions using NADPH, ascorbic acid, and 2-mercaptoacetic acid as substrates was that for Mn^{2+} the K_I values were all basically the same (allowing for a ± 20% experimental error) for the three substrates NADPH, ascorbic acid, and 2-mercaprtoacetic acid. Similarly for the Co^{2+} ion the K_I values were the same for both NADPH and ascorbic acid substrates. Again, the K_I values for Ce^{3+} were the same for both NADPH and ascorbic acid substrates. The similarity of the K_I values for any of the three inhibitory ions Mn^{2+}, Co^{2+}, or Ce^{3+} with the substrate NADPH and ascorbic acid is regarded as not a coincidental situation, but indicating that there is one common binding site on the microsomal protein for Mn^{2+}, Fe^{3+}, Co^{2+} and Ce^{3+}.

The results of the data from Figures 12 and 13 indicate a dependency of malonaldehyde formation on the concentration of Fe^{3+} present in the incubation system. It is interesting to note that the K_m values for Fe^{3+} for ascorbic acid and 2-mercaptoacetic acid were almost identical, but differed from the K_m value obtained for NADPH as substrate. Also, when the microsomes were heated at 65° C for one minute, so that all enzymic NADPH lipid peroxidizing activity was lost (denatured) the nonenzymic ascorbic acid and 2-mercaptoacetic acid malonaldehyde formation activity was retained, and the K_m values for the two nonenzymic substrates were again almost identical, but now shifted to lower values. This shift to a lower K_m value would seem to indicate that the binding site has been altered and that its affinity for Fe^{3+} has now increased.

This, however, may not be the case. It would seem more likely that the heat treatment results in an unwrapping or unfolding of the protein binding the Fe^{3+}, which exposes more Fe^{3+} ligand binding sites and increases the number of binding sites available for ascorbic acid or 2-mercaptoacetic acid binding, thus increasing the rate of reaction and giving an apparent lower K_m value for Fe^{3+}. It is probably only coincidental that the Fe^{3+} K_m values for ascorbic acid and 2-mercaptoacetic acid using heated microsomes are the same as the K_m value for the unheated enzymic NADPH-oxidase system.

As previously described, microsomal lipid peroxidation is inhibited by certain compounds capable of functioning as free radical trapping agents. The effect of these free radical trapping compounds on microsomal lipid peroxidation was determined by observing the effect on O_2 consumption and malonaldehyde formation, using both the enzymic NADPH oxidase and the nonenzymic ascorbic acid systems. All of the organic compounds capable of functioning as free radical traps which were added to the incubation systems inhibited both O_2 utilization and malonaldehyde formation, with the exception of α-tocopherol. In comparison, all of the free radical trapping compounds introduced as dietary additions (14 day diet), except santoquin, inhibited both O_2 utilization and malonaldehyde formation. This seeming discrepancy may be only a physical phenomenon in which the α-tocopherol suspension added to the incubation system is not incorporated into the proper locus of the microsomal membrane so that it can function as a free radical trap. The dietary santoquin is either similarly not incorporated into the proper locus in vivo, or it is metabolized by the animal and the metabolic product(s)

excreted without being incorporated into the proper locus of the microsomal membrane. Apparently other qualities are required to inhibit microsomal lipid peroxidation than simply that of the capability of functioning as a free radical trapping compound.

The free radical initiation of aerobic oxidation of sulfite ion is thought to take place according to the reactions:

$$X\cdot + SO_3^{-2} \longrightarrow X^- + SO_3^-\cdot$$

$$SO_3^-\cdot + O_2 \longrightarrow SO_3 + O_2^-\cdot$$

$$O_2^-\cdot + H^+ \longrightarrow HOO\cdot$$

$$HOO\cdot + SO_3^{-2} \longrightarrow SO_3^-\cdot + HOO^-$$

As shown in Table 6 the addition of 40 μmoles/ml incubation system of sulfite ion caused an increase in the consumption of O_2. However, formation of malonaldehyde was somewhat inhibited for the NADPH-oxidase system, but was not inhibited for the ascorbic acid system. This is somewhat surprising if the same radical species is responsible for the aerobic initiation of sulfite oxidation. Apparently there is some effect on the enzyme other than just the initiation of sulfite oxidation by a free radical species, which is not observed in the case of the nonenzymic ascorbic acid system. The inhibition of both O_2 utilization and malonaldehyde formation for both the enzymic NADPH-oxidase and the nonenzymic ascorbic acid system by the addition of free radical trapping compounds both in vitro and in vivo (diet) indicates that a free radical species is formed in the process of microsomal lipid peroxidation.

The demonstration of the dependence on Fe^{3+} of both the enzymic NADPH-oxidase and the nonenzymic ascorbic acid and 2-mercaptoacetic acid catalized lipid peroxidation for O_2 utilization and malonaldehyde formation, together with the fact that Fe^{3+} alters the redox potential of chelated sulfhydryl compounds from a value of about -0.3 to about 0, and the known flavoprotein nature of the NADPH-oxidase enzyme prompted us to consider the hypothesis that there might be an enzymic complex, such as that depicted in Figure 27 A, which contained a reducible sulfhydryl group bound to the protein (represented by the rectangular bar) and which might also have some other ligand, such as a carboxylate group (Figure 27 B),capable of binding Fe^{3+}. Fe^{3+} bound to the sulphydryl of the enzyme could conceivably alter the redox potential of the reduced enzyme, causing the formation of some reduced oxygen free radical species (Figure 27 B), similarly to the case for xanthine oxidase, and leading further to radical attack on the polyunsaturated fatty acids of the microsomal membrane, causing the observed lipid peroxidation and malonaldehyde formation. The Fe^{3+}-protein would then be cyclically reduced by NADPH and oxidized by molecular O_2.

However, an alternate pathway involving the reduction of the Fe^{3+} to Fe^{2+} with the formation of the $\cdot O_2^-$ radical must also be considered, as shown in Figure 28. In the presence of H^+ ions the $\cdot O_2^-$ radical would be converted to the HOO· radical.

The nonenzymic ascorbic acid and 2-mercaptoacetic acid-catalyzed lipid peroxidation could then be visualized as taking place from the production of free radicals formed by the oxidation of Fe^{3+}-protein - substrate complexes, as shown in Figure 29. When other thiol compounds

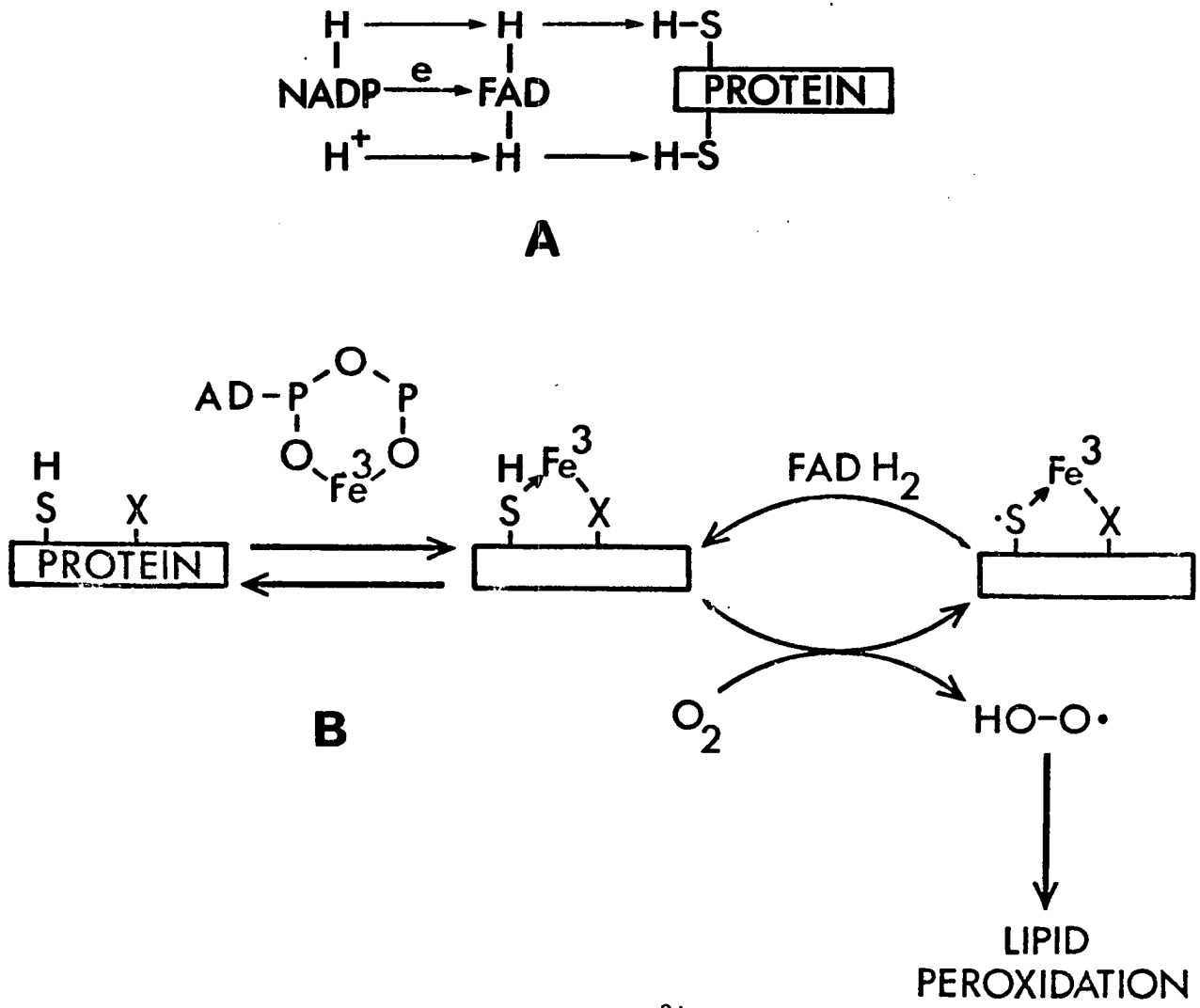


Figure 27. Structure of NADPH-oxidase-Fe^{3+} Complex and Mechanism for Lipid Peroxidation.

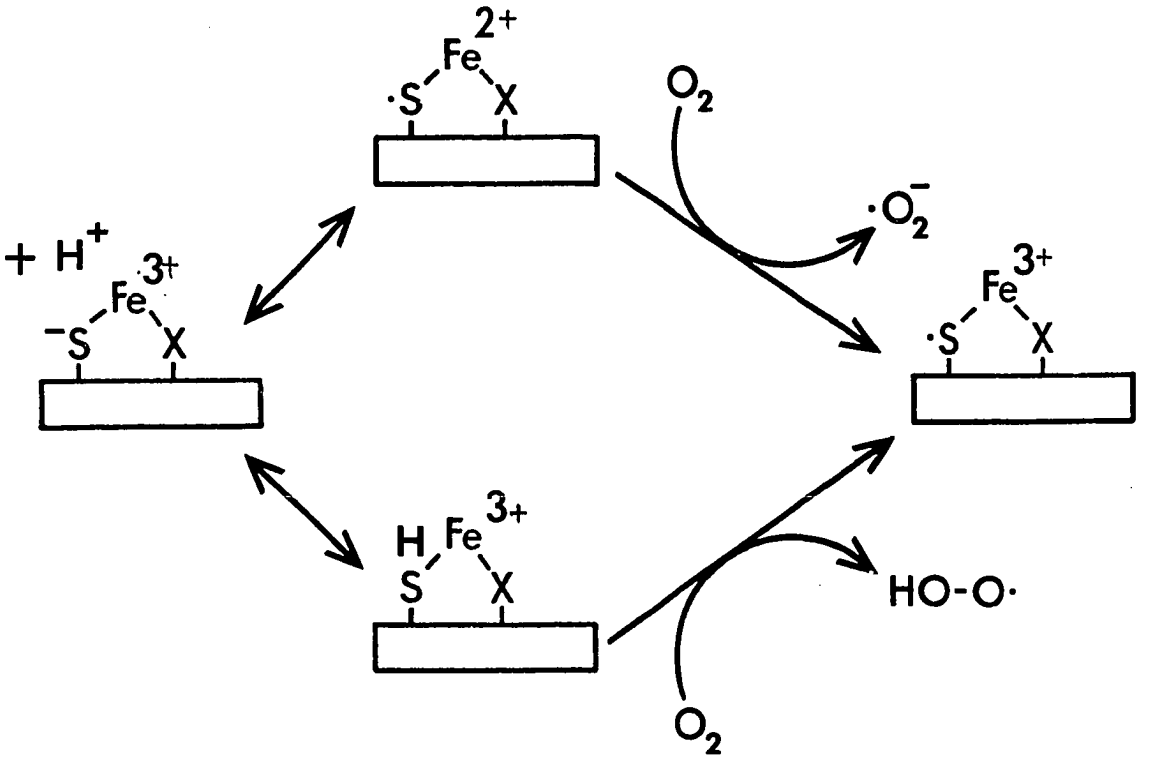


Figure 28. Alternate Mechanism for Free Radical Formation.

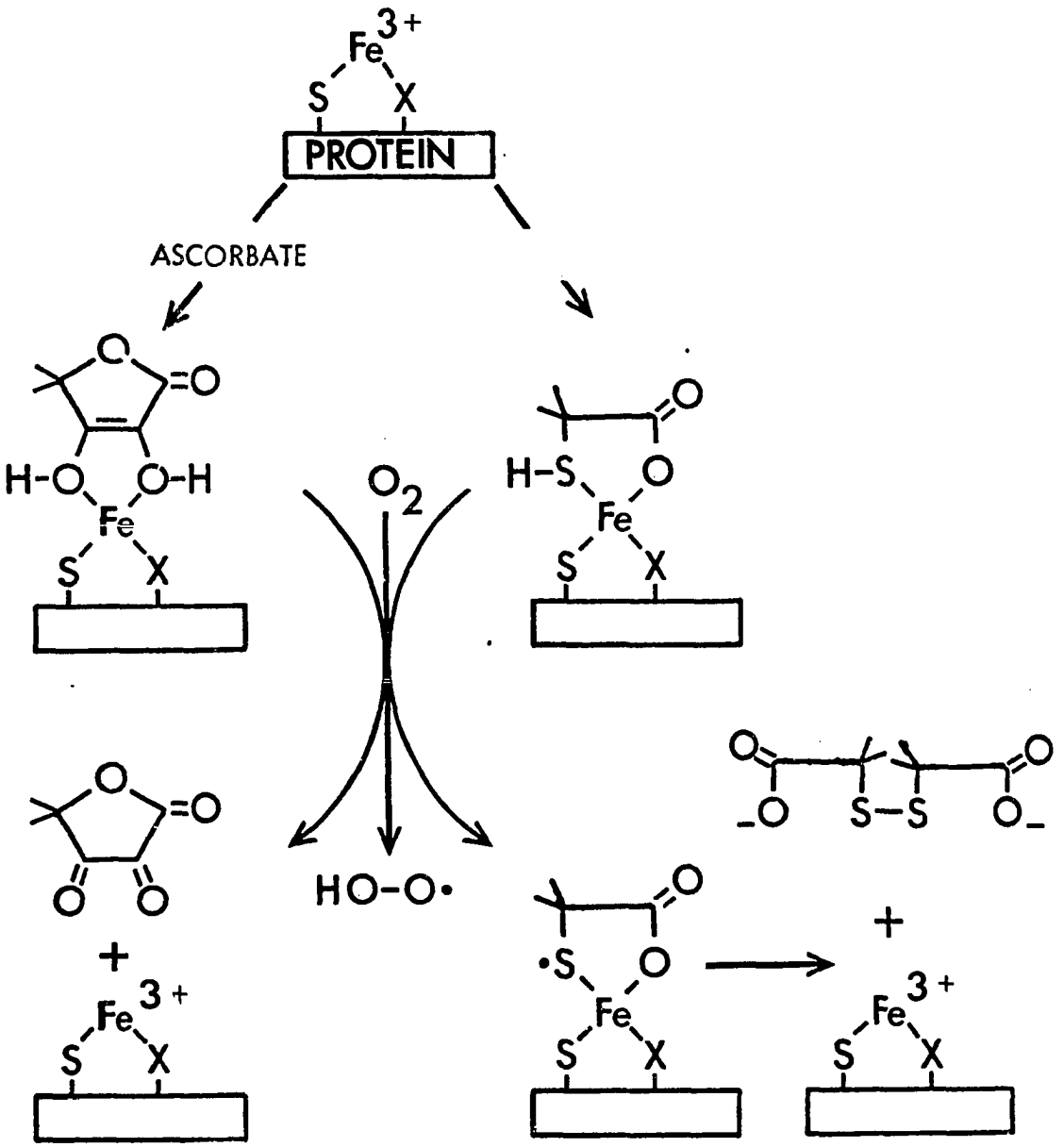


Figure 29. Mechanism for Non-Enzymic Lipid Peroxidation.

were tested for nonenzymic lipid peroxidation activity, it was found that cysteine was an active substrate, but that the N-acetyl and methoxy ester derivatives were not active. This would indicate that all three of the ligands of cysteine ($-COO^-$, $-NH_2$, and -SH) were required for binding to the Fe^{3+}-protein complex for lipid peroxidation activity. When the structure of cysteine is appropriately depicted (Figure 30 B) it can be seen that the three ligand groups are capable of having a stereo-configuration favorable for the simultaneous binding of all three ligands to a Fe^{3+} atom. Thus the N-acetyl and methoxy ester derivatives of cysteine, and reduced glutathione, would have one ligand each blocked and incapable of complexing with the Fe^{3+} atom.

This suggests that the protein-bound Fe^{3+} has at least three weakly bound ligands (Figure 30 A) where X, Y and Z are the three easily displaced ligands and the two arcs represent the protein bound to the Fe^{3+}, which are easily displaced by the three cysteine ligands. Apparently an ionic group such as carboxylate is also required, since 2-mercaptoacetic acid is a very active substrate for nonenzymic lipid peroxidation, whereas 2-mercaptoethanol and 2-mercaptoethylamine have little activity. The mechanism for radical formation from an Fe^{3+}-protein complex having 3 easily replaceable ligands, for ascorbid acid, 2-mercaptoacetic acid and cysteine is shown in Figure 31. The inhibition of lipid peroxidation by the metal ions Mn^{2+}, Co^{2+}, and Ce^{3+} would then involve the formation of the nonreactive octahedral Mn^{2+}, Co^{2+}, and Ce^{3+} complexes depicted in Figure 30 C.

A three-dimensional representation of the sulfhydryl protein-Fe^{3+} complex as it might function to produce free radicals in the

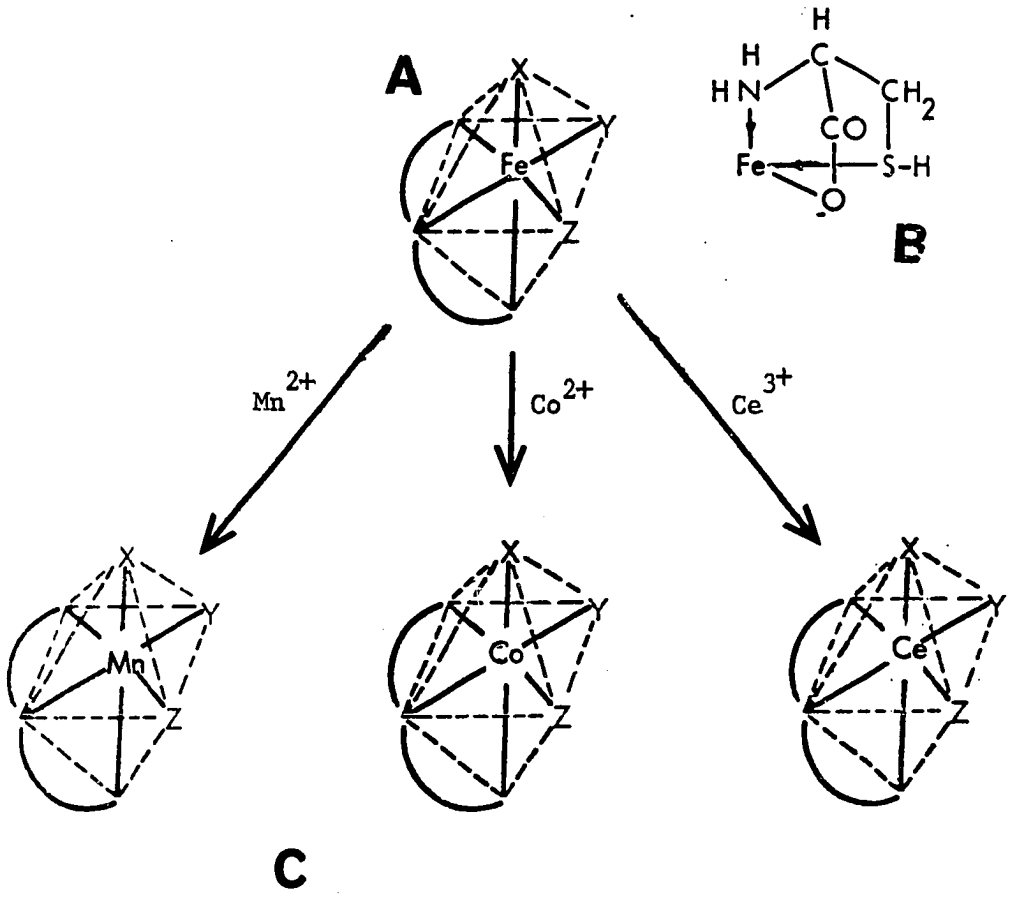


Figure 30. 3-Dimensional Cysteine and Protein-Metal Ion Structures.

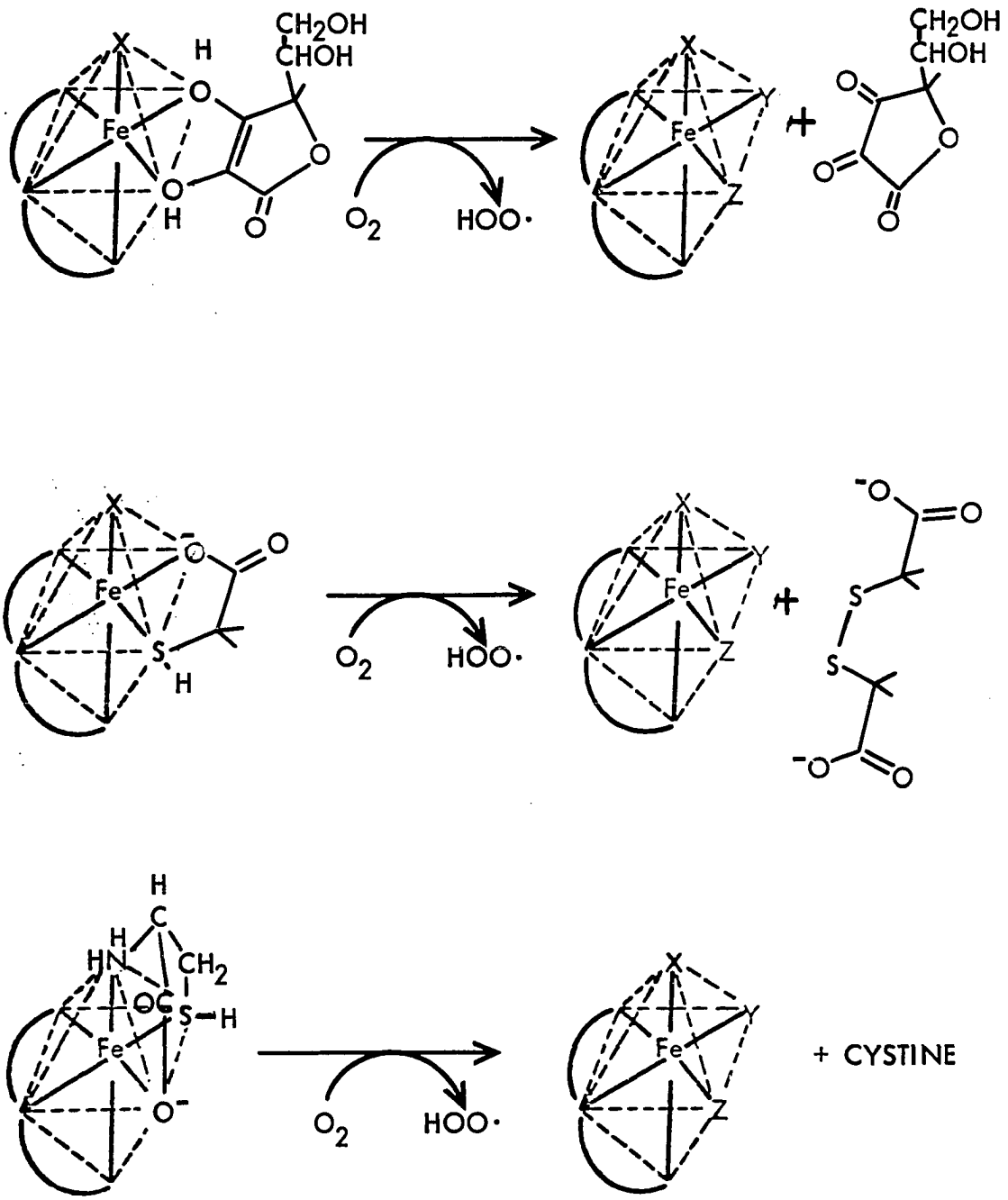


Figure 31. 3-Dimensional Mechanism for Non-Enzymic Lipid Peroxidation.

enzymic NADPH-oxidase system is shown in Figure 32, which is analogous to the two-dimensional representation of Figure 28.

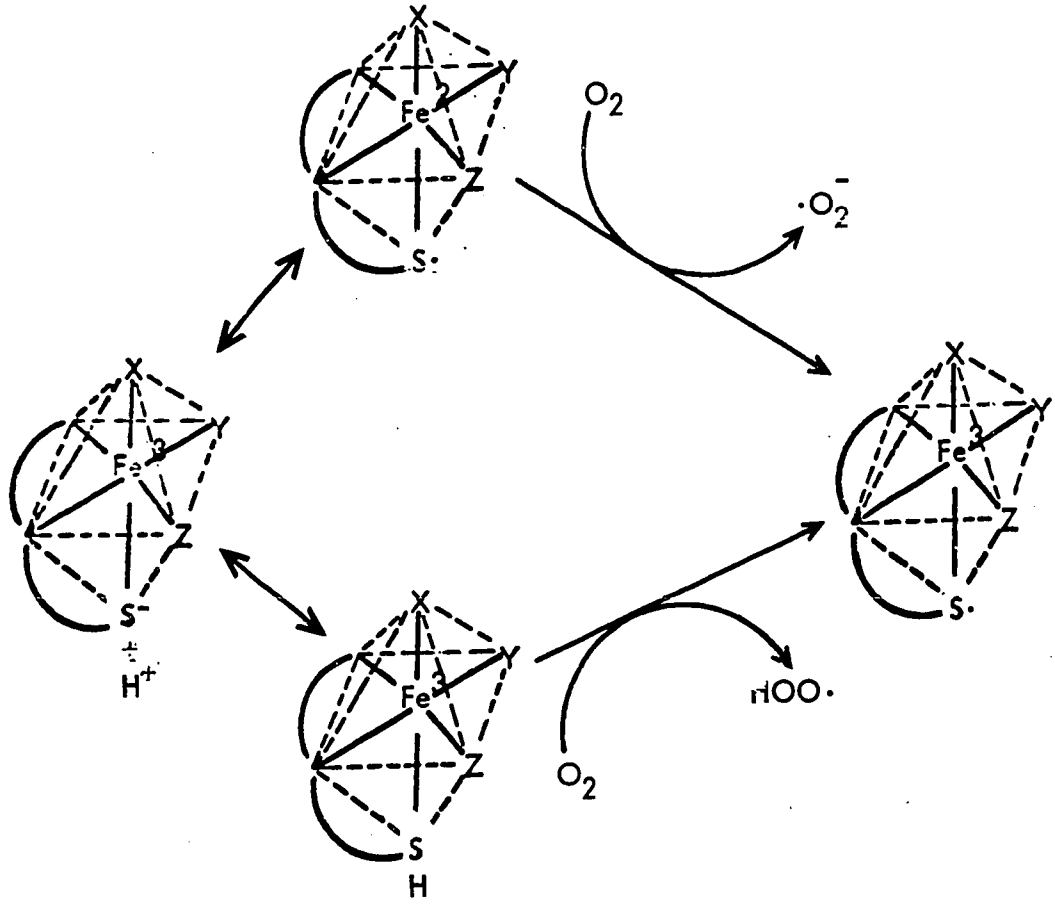


Figure 32. 3-Dimensional Mechanism for Enzymic Free Radical Formation.

CHAPTER V

SUMMARY

A product reacting with 2-thiobarbituric acid, and formed by the microsomal electron transport system requiring $NADPH_2$ and O_2, is associated with a concomitant peroxidation of microsomal membrane lipids. The identity of this product was established in this study to be malonaldehyde and its mechanism of formation was studied. It was determined that malonaldehyde was the only one of the molecular species produced during enzymic NADPH-oxidase catalyzed lipid peroxidation which can react with 2-thiobarbituric acid to form a chromogen absorbing at 532 mμ, and which could be used as an index of lipid peroxidation in this study. This conclusion was based on three criteria: (1) a comparison of the p-nitroaniline spectrophotometric assay and (2) the ethyl-p-aminobenzoate and 4,4'-sulfonyldianiline spectrofluorometric methods for malonaldehyde and the NADPH-oxidase catalyzed lipid peroxidation product, and (3) the identity of the elution behavior at two different pH values on a Sephadex G-10 column for both malonaldehyde and the NADPH-oxidase catalyzed lipid peroxidation 2-thiobarbituric acid-reacting product.

A kinetic study of the effect of the inhibitory metal ions Mn^{2+}, Co^{2+} and Ce^{3+} on both the enzymic NADPH and nonenzymic ascorbic acid-catalyzed lipid peroxidation gave Lineweaver-Burk plots indicating

these metal ions are competitive with Fe^{3+} in these systems. The inhibitory dissociation constants, K_I values, were the same for both the NADPH and ascorbic acid as substrates, with any one of the above inhibitory ions, which indicates a common binding site for Fe^{3+} for both the enzymic and nonenzymic lipid peroxidation. The dissociation constants, K_m, for Fe^{3+} was determined for NADPH, ascorbic acid, and 2-mercaptoacetic acid used as substrates, using microsomes prepared in a phosphate buffer low in Fe^{3+} content (5.5×10^{-8} M). Both the enzymic NADPH-oxidase and the nonenzymic ascorbic acid-catalyzed lipid peroxidation were inhibited by the free radical trapping compounds aniline, N-methyl aniline, diphenylamine, and diphenylphenylenediamine. Microsomes prepared in phosphate buffer low in Fe^{3+} were inactive with respect to both enzymic NADPH and nonenzymic ascorbic acid catalyzed lipid peroxidation.

An octahedral protein-bound Fe^{3+} complex, the Fe^{3+} bound by an -SH group and some other protein ligand, is proposed as the catalytic site of microsomal lipid peroxidation. This complex has three easily replaced, weakly bound ligands, as demonstrated by the activity of cysteine, but the lack of activity of the N-acetyl and methoxy ester derivatives of cysteine for lipid peroxidation. An octahedral protein-inhibitor complex is proposed for the three inhibitory metal ions Mn^{2+}, Co^{2+}, and Ce^{3+}. Either the HOO· or $\cdot O_2^-$ radical is proposed as the reactive lipid peroxidation-initiating free radical species, and alternate mechanisms are proposed for both the enzymic and nonenzymic formation of these free radicals.

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