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OXYGENATION OF POLYUNSATURATED FATTY ACIDS BY SHEEP VESICULAR GLAND AND SOYBEAN DIOXYGENASES

by

William Lee Smith

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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TABLE OF CONTENTS

	Page
DEDICATION	ii
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	vii
LIST OF FIGURES	ix
NOMENCLATURE AND ABBREVIATIONS	xii
INTRODUCTION	1

PART I

Studies on Soybean Lipoxygenase

EXPERIMENTAL METHODS	10
Materials	10
Determination of Lipoxygenase Activity	10
Determination of GSH-peroxidase Activity	11
Purification of Lipoxygenase	12
Purification of GSH-peroxidase	13
Amino Acid Analysis	14
RESULTS	15
Inactivation of Lipoxygenase during the Oxygenation Reaction	15
Effect of GSH-peroxidase on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Soybean Lipoxygenase	36
The Lag Phase in the Oxygenation of Fatty Acid Substrates by Lipoxygenase and the Effect of Product and Enzyme Concentrations on the Length of the Lag	44

Page

The Effect of Fatty Acid Substrate Concentration on the Length of the Lag Period in the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase..... 56

DISCUSSION..... 60

Self-catalyzed Destruction of Lipoxygenase..... 60

Product Activation of Lipoxygenase..... 65

Kinetic Mechanism for Lipoxygenase..... 69

SUMMARY..... 70

PART II

Studies on Sheep Vesicular Gland Dioxygenases

EXPERIMENTAL METHODS..... 80

Materials..... 80

Determination of GSH-peroxidase Activity..... 80

Determination of VG Dioxygenase Activity by Radioisotope Assays..... 81

Determination of VG Dioxygenase Activity by Oxygen Absorption Measurements..... 83

Purification of GSH-peroxidase..... 84

Preparation of VG Dioxygenase..... 84

RESULTS..... 88

The Effect of GSH-peroxidase on the Oxygenation of Polyenoic Acids by Sheep Vesicular Gland Preparations..... 88

The Effect of Phenol on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder..... 97

The Self-catalyzed Inactivation of VG Acetone Powder During Oxygenation of Fatty Acid Substrates..... 102

Page

Loss in Phenol-stimulatible Activity during the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Untreated VG Acetone Powder..... 109

Preservation of Phenol-stimulatible Activity during Self-destruction of Phenol-insensitive Activity in the Presence of 5,8,11,14-eicosatetraenoic Acid and GSH-peroxidase..... 118

The Effect of Fatty Acid Substrate Concentration on the Activity of the Phenol-treated VG Acetone Powder..... 124

The Effect of NaCN and Metal Chelating Agents on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder and Phenol-activated VG Acetone Powder.... 124

DISCUSSION..... 135

Product Activation of the Vesicular Gland Dioxygenases.... 135

Self-catalyzed Destruction of the Vesicular Gland Dioxygenases..... 137

Overall Kinetic Mechanisms for Vesicular Gland Dioxygenases..... 139

A Possible Role for Pseudo-singlet State Oxygen in Fatty Acid Dioxygenases..... 146

Phenol Activation and the Role of Metals in VG Dioxygenases E_2 Activity..... 151

The Role of Metal in VG Dioxygenase E_1..... 154

SUMMARY..... 157

BIBLIOGRAPHY..... 159

LIST OF TABLES

Table		Page
I	Rates and Extents of Oxygenation of Different Fatty Acids by Lipoxygenase	26
II	Summary of Rate Constants for the Inactivation of Lipoxygenase by Various Processes	40
III	Amino Acid Composition of Lipoxygenase and of Lipoxygenase Inactivated During Oxygenation of 5,8,11,14,17,20-docosahexaenoic Acid	41
IV	Levels of GSH-peroxidase Necessary to Inhibit the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Different Concentrations of Lipoxygenase	45
V	The Effect of Hydrogen Peroxide and Alkyl Hydroperoxides on the Lag Period in the Oxygenation of 9,12-octadecadienoic and 5,8,11,14-eicosatetraenoic Acids by Lipoxygenase	52
VI	The Effect of the Nature of the Hydroperoxide Product on the Rate of Oxygenation of 9,12-octadecadienoic and 5,8,11,14-eicosatetraenoic Acids by Lipoxygenase	54
VII	The Effect of Lipoxygenase Concentration on the Lag Period in the Oxygenation of 5,8,11,14-eicosatetraenoic Acid	57
VIII	The Effect of Fatty Acid Substrate Concentration on the Lag Period in the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase	58
IX	The Effect of Fatty Acid Substrate Concentration on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase in the Presence and Absence of Hydroperoxide Product	59
X	Prediction of the Maximum Velocities from k_2 Values in Lipoxygenase Reaction Mixtures Incompletely Inhibited by GSH-peroxidase	66
XI	Calculation of a Constant V_{max} for Lipoxygenase From the Kinetic Equation and a Set of Dissociation Constants	75

Table		Page
XII	Effect of GSH-peroxidase on Prostaglandin Synthesis by VG 3000 g.min Supernatant	89
XIII	The Effect of GSH on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG 3000 g.min Supernatant	91
XIV	The Effect of GSH-peroxidase on the Oxygenation of 8,11,14-eicosatrienoic Acid by VG Acetone Powder	98
XV	The Effect of GSH-peroxidase on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Phenol-treated VG Acetone Powder	99
XVI	Inactivation of Phenol-sensitive and Phenol-insensitive VG Dioxygenase Activities during Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder in the Presence of GSH-peroxidase	121
XVII	The Effect of Substrate Concentration on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Phenol-treated VG Acetone Powder	125
XVIII	The Effect of Bathocuproine Sulfonate, o-phenanthroline and Diethyldithiocarbamate on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder and Phenol-treated VG Acetone Powder	131
XIX	Calculation of the Observed Velocities for the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Phenol-activated VG Acetone Powder from the Kinetic Equation for VG Dioxygenases, E_1 and E_2	143
XX	Comparison of the Predicted and Observed Rates of Hydroperoxide Product Formation and Distribution during Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Phenol-treated VG Acetone Powder in the Presence of GSH-peroxidase	150

LIST OF FIGURES

Figure		Page
1	Prostaglandin Biosynthesis from 8,11,14-eicosatrienoic Acid	2
2	Oxygenation of 8,11,14-eicosatrienoic Acid by Soybean Lipoxygenase	6
3	Product Formation versus Time for the Oxygenation of 9,12-octadecadienoic and 8,11,14-eicosatrienoic Acids by Lipoxygenase	16
4	Velocity versus Substrate Concentration for the Oxygenation of 9,12-octadecadienoic, 9,12,15-octadecatrienoic, 8,11,14-eicosatrienoic, 5,8,11,14-eicosatetraenoic, 5,8,11,14,17-eicosapentaenoic and 5,8,11,14,17,20-docosahexaenoic Acids by Lipoxygenase	18
5	Log Initial Velocity versus Time for the Aerobic Inactivation of Lipoxygenase by Hydroperoxide Products	28
6	Log Initial Velocity versus Preincubation Time for the Anaerobic Incubation of Lipoxygenase with 9,12,15-octadecatrienoic Acid in the Presence and Absence of 13-hydroperoxy-9,11,15-octadecatrienoic Acid	37
7	The Effect of GSH-peroxidase on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase in the Presence of Various Sulfhydryl Compounds	43
8	GSH Consumption with Different Levels of GSH-peroxidase in a Lipoxygenase Reaction System	46
9	The Effect of NEM on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase	48
10	Measurement of the Kinetic Lag Period in the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase	50
11	Kinetic Model for the Self-catalyzed Destruction of Lipoxygenase	61
12	Common Structural Features of Lipoxygenase Product Activators	68

Figure		Page
13	Mechanism for Removal of the (n-8) Hydrogen Atom from 9,12-octadecadienoic Acid by an Enzyme-bound 13-hydroperoxy-9,11-octadecadienoic Acid Radical	69
14	Kinetic Mechanism for Lipoxygenase Activity	70
15	Thin-layer Chromatographic System to Separate Products for the Radioisotope Assay of Prostaglandin Synthesis	82
16	Comparison of the Radioisotope and Oxygen Absorption Assays for Prostaglandin Synthesis	85
17	The Effect of NEM on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG 3000 g.min Supernatant	93
18	The Effect of NEM on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG 3000 g.min Supernatant	95
19	The Effect of Preincubation of VG Acetone Powder with Phenol on the Rate of Oxygenation of 5,8,11,14-eicosatetraenoic Acid	100
20	The Effect of VG Acetone Powder Concentration on the Rate of Phenol Activation	103
21	The Effect of the Phenol Concentration on the Rate of Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder	105
22	Deactivation of the Phenol-activated VG Acetone Powder	107
23	Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder	108
24	Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder with GSH in the Presence and Absence of GSH-peroxidase	110
25	Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14-eicosatetraenoic by Phenol-treated VG Acetone Powder in the Presence and Absence of GSH	112

Figure		Page
26	Velocity versus Substrate Concentration for the Oxygenation of 8,11,14-eicosatrienoic Acid by Phenol-treated VG Acetone Powder	114
27	The Loss of Phenol-sensitive VG Dioxygenase Activity during the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder	116
28	Product Formation versus Time for the Inactivation of Phenol-sensitive and Phenol-insensitive VG Dioxygenase Activities in the Presence of GSH-peroxidase	119
29	K_M Determination for 5,8,11,14-eicosatetraenoic Acid with VG Acetone Powder and Phenol-treated VG Acetone Powder	123
30	The Effect of NaCN on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder	126
31	The Effect of Tiron and o-phenanthroline on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder	129
32	The Effect of o-phenanthroline and Diethyldithiocarbamate on the Phenol-activation of VG Acetone Powder	133
33	A Mechanism for VG Dioxygenase E_2 Involving Pseudo-singlet State Oxygen	147
34	Possible Mechanism for the Oxygenation of Polyenoic Acid by Vesicular Gland Dioxygenase E_2	155
35	Possible Mechanism for the Oxygenation of Polyenoic Acids by Vesicular Gland Dioxygenase E_1	156

NOMENCLATURE AND ABBREVIATIONS

Free fatty acids and hydroperoxy fatty acids are designated by IUPAC nomenclature in all cases in the text. In some tables, free fatty acids are designated by number of carbons, number and position of double bonds. For example, 18:2 (n-6) signifies an acid having 18 carbons and 2 double bonds. The first double bond is on the sixth carbon from the methyl terminal end. (The double bonds are separated by a single methylene group in all cases encountered in this study.)

In Part I, the double bond on the seventh carbon from the methyl end of all hydroperoxy fatty acids is trans and is conjugated with the cis double bond on the ninth carbon from the methyl terminal end. In Part II, the double bond on the eighth carbon from the methyl end of all hydroperoxy acids is trans and is conjugated with the cis double bond on the eighth carbon from the methyl terminal end.

A.P.	Acetone powder
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetate
FFA	free fatty acid
GSH	reduced glutathione
NEM	N-ethylmaleimide
OA-FA	hydroxy fatty acids
Santoquin	1,2-dihydro-6-ethoxy-2,2,4-trimethyl quinoline

INTRODUCTION

Studies on the group of biologically active acidic lipids called prostaglandins have been particularly intense since the determination of the structure of these compounds by Bergström and coworkers in the early 1960's. Comprehensive reviews exist on the structure (1,2), biosynthesis (3,4), metabolism (2,5) and biological activities (6,7) of the prostaglandins.

The biosynthesis of PGE_1 (8), $PGF_{1\alpha}$ (8,9), 11-dehydro-$PGF_{1\alpha}$ (10), 12-hydroxy-trans, trans-8,10-heptadecadienoic acid (11) and a twenty carbon monohydroxy fatty acid (10,11,12,13) from esterified 8,11,14-eicosatrienoic acid is outlined in Fig. 1. These conversions are catalyzed by preparations of sheep vesicular gland.

Three major processes can be delineated in the biosynthesis of prostaglandins. The first is release of certain esterified polyenoic acids from precursor lipids. The hydrolytic release of fatty acids from lipid appears to be a property of many tissues (as is prostaglandin formation itself (14)), but that activity has seldom been shown quantitatively to any appreciable extent. A principal reservoir of polyunsaturated acids in the tissues is phosphoglycerides. For instance, the renal medulla contains large amounts of 5,8,11,14-eicosatetraenoic acid in its phospholipids, and it produces PGE_2 rather than PGE_1 (15). On the other hand, the vesicular gland of sheep contains little 5,8,11,14-eicosatetraenoic acid but large amounts of 8,11,14-eicosatrienoic acid in its phospholipids and

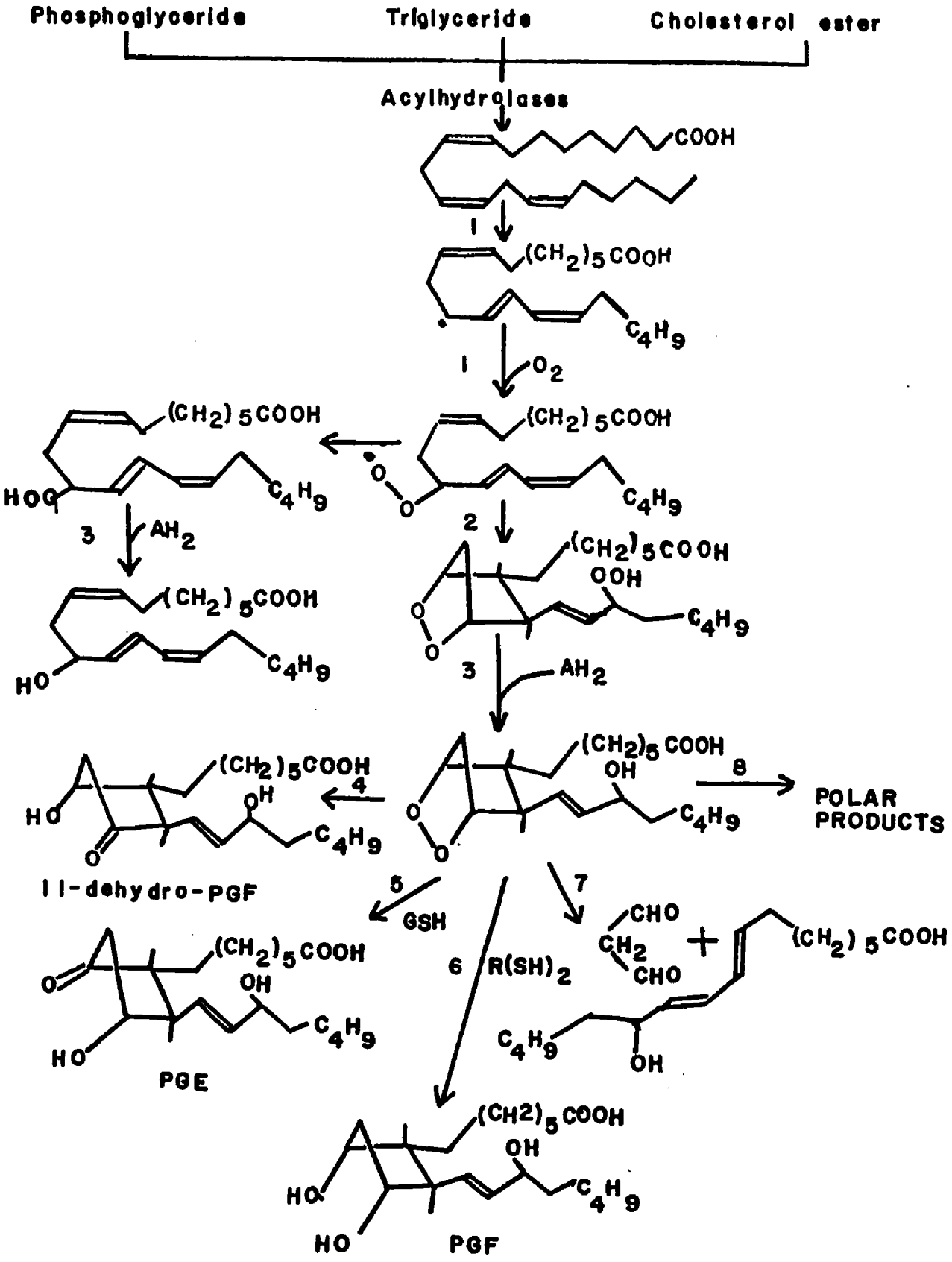


Fig. 1. Prostaglandin Biosynthesis from 8,11,14-eicosatrienoic Acid.

produces PGE_1 and $PGF_{1\alpha}$. Thus a tenable hypothesis would be that the factors regulating the fatty acid composition at the 2-position of the tissue phospholipids are also indirectly regulating the nature of the prostaglandins produced. The triglycerides and cholesterol esters provide additional reservoirs of polyunsaturated acids and must also be considered as possible sources of prostaglandin precursors. At the present time it is reasonable to speculate that the enzymic hydrolysis of precursor acids from an esterified form may be under some hormonal control possibly via cyclic AMP regulation.

The second major process involved in the biosynthesis of prostaglandins is that of oxidizing the acid and forming the cyclic endoperoxide intermediate (Reactions 1 and 2; Fig. 1.) and will be the main focus of this introduction. The third process in prostaglandin biosynthesis is the conversion of the endoperoxide intermediate to the various prostaglandins (Reactions 4, 5, 6, 7 and 8; Fig. 1.). Although PGE and PGF have different physiological effects, no clear pattern emerges as to which is produced by a given tissue at any given time. Once the endoperoxide intermediate for prostaglandin formation is formed, its selective conversion to either PGE, 11-dehydro-PGF or PGF may be regulated by the supply of appropriate cofactors. The long-recognized need for glutathione to stimulate PGE formation (5) may be modulated <u>in vivo</u> by the action of glutathione reductase and glutathione peroxidase. An additional electron-donor system, needed to produce PGF, provides another means of controlling the amount of particular prostaglandin produced. The donor may be a reduced lipoamide group (16). If so, regulation of PGF synthesis <u>in vivo</u> may be controlled

by reduction of the lipoamide group.

Samuelsson (17) showed that the two oxygen atoms in the cyclopentane ring portion of PGE_1 were derived from the same oxygen molecule. This finding indicates the functioning of a dioxygenase in PGE formation. The dioxygenase apparently catalyzes the addition of oxygen to the n-10 carbon of the fatty acid substrate since 11,14-eicosadienoic acid is rapidly oxidized by sheep vesicular gland preparations to 11-hydroxy-12,14-eicosadienoic acid (5). This observation along with the fact that 15-hydroperoxy-8,11,13-eicosatrienoic acid is not converted to prostaglandin products by sheep vesicular gland (8) indicates that addition of oxygen to the n-10 carbon of the substrate is the first of the two oxygen addition reactions involved in PGE formation. Enrichment of ^{3}H in the precursor acid is observed if [13-L-^{3}H, 2-^{14}C]-8,11,14-eicosatrienoic acid is incubated with sheep vesicular gland homogenates indicating that the rate-determining step in the formation of prostaglandins from precursor acid is removal of the 13-L-hydrogen atom (18). The rate of removal of this hydrogen and subsequent addition of an oxygen molecule to the n-10 carbon of fatty acid substrate by the vesicular gland (VG) dioxygenase is thus the initial and rate limiting process in the overall formation of prostaglandins from free polyenoic acids. Whether this oxygenation step is rate limiting in the formation of prostaglandins from esterified precursor acids is not known.

The catalytic properties of the VG dioxygenase are poorly defined. The enzyme has not been purified to date although several attempts have been reported (4,19). The prostaglandin synthesizing system of sheep vesicular gland has been located in particle

preparations sedimenting as liver microsomes (13); however, some evidence exists which suggests that the enzyme system may occur in a fraction more dense than typical liver microsomes (16).

Much of the earlier work on prostaglandin biosynthesis focused on the rate of formation of the PGE compounds (3). Stimulation or inhibition of PGE formation is not necessarily attributable to stimulation or inhibition of the VG dioxygenase since formation of PGE is dependent on the competing rates of utilization of cyclic peroxide intermediate by reactions 4,5,6,7 and 8 (Fig. 1.). For example, Cu^{++} actually stimulates the utilization of fatty acid substrate (16) whereas Cu^{++} suppresses PGE formation (13). VG dioxygenase activity must therefore be measured by analysis of the rate of formation of oxygenated fatty acid or by the rate of absorption of oxygen.

Using the oxygen absorption procedure, Yoshimoto et al. (19) have shown that both hemin and hydroquinone stimulate oxygen uptake by acetone powder preparations from bovine seminal vesicles. The stimulatory effect of phenolic antioxidants on PGE formation by VG microsomal systems had previously been demonstrated by Van Dorp and coworkers (3).

A number of polyenoic acid isomers will serve as substrates for PGE formation. Three cis-methylene interrupted double bonds are required (3). However, only two double bonds seem to be required for the VG dioxygenase to act (5,11).

The difficulties inherent in purifying and assaying the water-insoluble VG dioxygenase have been partially circumvented by the finding of Hamberg and Samuelsson (20) that a soluble fatty acid

dioxygenase (21,22) from soybeans, lipoxygenase [E.C. 1.99.2.1], catalyzes the formation of fatty acid hydroperoxides in an oxygenation reaction similar to that catalyzed by the VG dioxygenase (8). The rate-determining step in both processes is abstraction of the 13-L-hydrogen of 8,11,14-eicosatrienoic acid. However, the position of oxygen insertion in the oxygenation of 8,11,14-eicosatrienoic (and 5,8,11,14-eicosatetraenoic) acid by lipoxygenase is the n-6 carbon atom (Fig. 2); whereas with the VG dioxygenase, insertion occurs at the n-10 carbon.

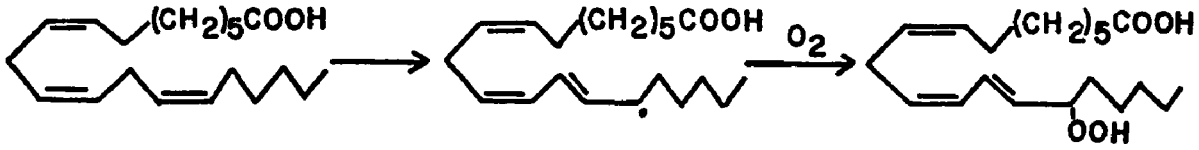


Fig. 2. Oxygenation of 8,11,14-eicosatrienoic Acid by Soybean Lipoxygenase.

The most active substrates of lipoxygenase are polyenoic acids containing cis-methylene interrupted double bond systems beginning at the n-6 carbon atom and extending toward the carboxyl end of the acid (20,23,24). The enzyme is reversibly inhibited by conjugated linoleic acid (25) and irreversibly inactivated by 5,8,11,14-eicosatetraynoic acid (26).

Lipoxygenases have been studied extensively because of their importance in the processing and storage of plant oils. Earlier reports of soybean lipoxygenase activities such as the "carotene oxidase" activity of Sumner and Sumner (27) are difficult to evaluate

because at least three lipoxygenase activities have subsequently been shown to be present in soybean extracts (28,29,30,31). Lipoxygenase-1, first purified by Theorell et al. (32), has a pH optimum of 8.5 (28,33) and is more active toward free polyenoic acids than polyenoic acids esterified to glycerol (28). The opposite substrate specificity is observed for lipoxygenase-2, which was recently purified by Christopher and Axelrod (28). The latter enzyme has a pH optimum of 6.8. Another lipoxygenase activity similar to lipoxygenase-1 in its specificity for polyenoic acids but differing in its sensitivity to calcium ion activation is found in soybean extracts (31).

Of the soybean lipoxygenase isozymes, lipoxygenase-1, which will now be referred to as simply lipoxygenase, has been the subject of the most extensive studies and several reviews are available (34,35,36). The enzyme is not affected by iron, calcium or copper chelating agents making lipoxygenase singular among known dioxygenases (37). In addition, the purified enzyme does not appear to have any known prosthetic groups or dialyzable cofactors and is unaffected by sulfhydryl reagents (36). Lipoxygenase is inhibited by antioxidants (38)--a finding consistent with the involvement of free radicals in the enzyme activity (39).

A number of conflicting reports have appeared on the kinetic lag period with lipoxygenase (29,31,40,41); however, most can be reconciled with the earlier report of product activation which indicated that the lag time could be virtually eliminated with high enzyme concentrations (40).

The mechanism for lipoxygenase formulated by Tappel et al. (41) and modified by the studies of Hamberg and Samuelsson (20) is shown in Fig. 2.

This report presents the results of studies designed to establish the catalytic properties of the sheep vesicular gland dioxygenase. The problem was approached by using a detailed kinetic investigation of the easily-assayed soybean lipoxygenase to suggest corollary experiments with the VG dioxygenase.

Part I deals with three features of the soybean enzyme which had not been previously studied in detail: (a) self-catalyzed destruction; (b) product activation; and (c) substrate inhibition. These studies have allowed us to propose a kinetic mechanism for the action of soybean lipoxygenase.

Part II concerns itself directly with the VG dioxygenase using the studies with soybean lipoxygenase as a model system. Besides the self-catalyzed destruction, product activation and substrate inhibition phenomena, two other features of the VG dioxygenase which are not properties of the soybean dioxygenase were investigated. One was the activation of the VG dioxygenase by phenol, and the other was the involvement of metal ions in the activity of the vesicular gland enzyme. These studies have allowed us to gain some insight into both the possible regulation of prostaglandin formation by the sheep vesicular gland dioxygenase and certain properties of polyenoic acid dioxygenases in general.

PART I

STUDIES ON SOYBEAN LIPOXYGENASE

EXPERIMENTAL METHODS

Materials

All fatty acids were high purity grade obtained from the Hormel Institute except 8,11,14-eicosatrienoic acid which was a gift of the Upjohn Company. The fatty acids were dissolved in benzene containing Santoquin (0.3% w/w) and stored at -20°. 1-octyl-hydroperoxide was generously donated by Dr. Rodney Boyer. The soybean lipoxygenase used in most experiments was purchased from Sigma Chemical Company and used without further purification. Soybean lipoxygenase obtained from Worthington Biochemical Corporation was used, after partial purification, for amino acid analyses.

All other chemicals were reagent grade obtained from common commercial sources. Deionized water was used for the preparation of all solutions.

Determination of Lipoxygenase Activity

The oxygenation reactions were initiated by the addition of aliquots of solutions of soybean lipoxygenase (0.2-1.0 mg/ml) prepared daily in 0.1 M TrisHCl (pH 9.0) to 1.0 ml suspensions of fatty acid (30-260 μM) in 0.1 M TrisHCl (pH 9.0). Assays of lipoxygenase were performed at room temperature in cuvettes exposed to air by continuously monitoring the absorbance at 234 mμ due to the formation of cis-trans conjugated diene on a Beckman DU spectrophotometer equipped with a Gilford Model 2000 Multiple Sample Absorbence Recorder. A molar extinction coefficient of 28,000 1/mole-cm (41) was used

convert absorbance readings to moles of product. The commercial enzyme preparation (Sigma) catalyzed the oxygenation of 1.52 μmoles of acid per minute per mg protein in an assay mixture containing 5-15 μg lipoxygenase and 100 nmoles of 9,12-octadecadienoic acid in 1.0 ml of 0.1 M TrisHCl (pH 9.0). One unit of lipoxygenase is defined as that amount of enzyme which will catalyze the oxygenation of one μmole of 5,8,11,14-eicosatetraenoic acid per minute at room temperature in air.

Determination of GSH-peroxidase Activity

GSH-peroxidase activity was measured colorimetrically by a modification of the procedure of Jocelyn (42). Aliquots (0.3 ml) of 10 mM DTNB were added after different times of incubation of samples containing 690 nmoles GSH, 50-80 μM 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid and 5-20 μl aliquots of the GSH-peroxidase preparation in 4.0 ml of 0.1 M TrisHCl (pH 7.4 or pH 9.0) at room temperature. When assays were performed at pH 9.0, the pH was adjusted to 7.4 with 0.1 M HCl immediately before addition of DTNB. Controls containing no enzyme were run with all samples. The absorbance at 413 mμ of the assay solutions was determined on either a Beckman Model B or Model DU spectrophotometer. The readings were converted to nmoles hydroperoxide consumed using an extinction coefficient of 13,600 ℓ/mole·cm for thionitro-benzoate anion (43) and the observation that GSH-peroxidase catalyzes the oxidation of 2 molar equivalents of GSH in reducing one equivalent of hydroperoxide (44). One unit of GSH-peroxidase activity is defined as the amount of GSH-peroxidase which will catalyze the reduction of one nmole of hydroperoxide per min under the standard assay conditions. A K_m of approximately 1.0 mM for GSH was determined

using 0.1 mM 1-octyl-hydroperoxide and 0.1 M TrisHCl at pH 8.5, the pH optimum reported for the rat liver enzyme (45).

Purification of Lipoxygenase

Soybean lipoxygenase purchased from Worthington Biochemical Corporation was partially purified by chromatography on DEAE-Sephadex (A-50) according to a modification of the procedure of Allen (33). All procedures were performed at 0-4°. The commercial enzyme (150-250 mg) was dissolved in 10-20 ml of 0.12 M NaCl in 0.09 M TrisHCl (pH 8.5) and dialyzed overnight against two or three 500 ml portions of 0.12 M NaCl in 0.09 M TrisHCl (pH 8.5). The dialyzed enzyme solution was applied to a column of DEAE-Sephadex (2.2 x 30 cm). The DEAE-sephadex had been previously washed, defined and equilibrated with 0.12 M NaCl in 0.09 M TrisHCl (pH 8.5). The column was washed using a linear gradient (total volume = 300 ml; flow rate = 15-20 ml/hr.) from 0.12 M NaCl in 0.09 M TrisHCl (pH 8.5) to 0.18 M NaCl in 0.09 M TrisHCl (pH 8.5); 8-15 ml fractions were collected. Lipoxygenase was eluted at approximately the middle of the gradient. Fractions with high lipoxygenase activity were pooled and protein precipitated with 75% $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation at 15,000 x g for 15 min and resuspended in 0.1 M TrisHCl (pH 8.5). This procedure afforded a 2-4 fold purification of the dialyzed lipoxygenase with a 50% recovery of activity. The $(NH_4)_2SO_4$ precipitate, after dialysis against 0.1 M TrisHCl (pH 8.5), showed one major and two minor protein staining bands (46) on disc gel electrophoresis performed essentially according to the procedure of Brewer and Ashworth (47). The major band contained all of the

lipoxygenase activity on the gel; however only 50% of the units could be eluted from the gel after electrophoresis.

Purification of GSH-peroxidase

GSH-peroxidase was partially purified from rat liver by a procedure similar to that of Little and O'Brien (45). Either male or female Sprague Dawley rats (175 to 200 g) were faster 18-24 hrs and sacrificed by decapitation. The livers were perfused according to the method of Eibl et al. (48). All subsequent procedures were carried out at 0-4° unless otherwise indicated. The livers were homogenized in either 0.25 M sucrose containing 1 mM EDTA (pH 7.4) or in 0.1 M TrisHCl (pH 7.4) using a Dounce ball-type homogenizer. The pH was adjusted to 7.4 when necessary by the dropwise addition of a 1% solution of Tris. The homogenate was centrifuged at 600 x g for 5 min (3000 g.min). Either the 3000 g.min supernatant or the supernatant derived from a 90 min centrifugation of the 3000 g.min supernatant at 100,000 x g was added to two volumes of absolute ethanol at -20° in a -25° dry ice-ethanol-water bath. The suspension was allowed to stand for 30 min at -20° and then centrifuged at 10,000 x g for 15 min. The resulting pellet was extracted for 1 hour with 2-4 ml of 0.1 M TrisHCl (pH 7.4). The suspension was again centrifuged at 10,000 x g for 15 min. The resulting supernatant was stored at -20°. This procedure yielded a four-fold purification of GSH-peroxidase from the 3000 g.min supernatant.

Amino Acid Analysis

Amino acid analyses were performed on a Beckman 120 C amino acid analyzer operated as described by Spackman et al (49). The protein preparations to be analyzed were hydrolyzed at 110° in 6 N HCl in constricted ignition tubes previously washed with HNO_3, HCl, and water to remove trace metals. Prior to sealing and hydrolysis, the samples were flushed with nitrogen and then degassed twice by freezing and thawing with evacuation by a mechanical pump to less than 0.1 mm mercury pressure. After hydrolysis, the HCl was removed at 37° under a vacuum with a rotatory evaporator. The samples were dissolved in 0.2 M sodium citrate buffer (pH 2.2) and stored frozen at -15° until submitted to analysis.

RESULTS

Inactivation of Lipoxygenase During the Oxygenation Reaction

Our preliminary studies with lipoxygenase and 5,8,11,14-eicosatetraenoic acid indicated that the reaction velocity decreased to zero before all the fatty acid appeared to be oxidized. Further addition of substrate with concomittant dilution of the reaction mixture produced no further extent of reaction. However, if lipoxygenase was added to the reaction mixture, product formation occurred at a rate which (at saturating substrate levels) was directly proportional to the amount of fresh enzyme added. This phenomenon is illustrated in Fig. 3 for 9,12-octadecadienoic and 8,11,14-eicosatrienoic acids. It should be noted that no kinetic lag period appears in Fig. 3. To achieve consistent kinetic results over the wide range of substrate concentrations used in these studies, lipid hydroperoxide was added as indicated to eliminate the lag period and ensure fully activated enzyme in the assay systems (40). The addition of heat-treated lipoxygenase (80° for 10 min) or bovine serum albumin (50 μg) either to the initial substrate solution or to the reaction mixture after the rate had dropped to zero did not result in further reaction.

A preliminary kinetic analysis was provided by measuring instantaneous velocity for many time points during the reaction and plotting them against the substrate concentration at that time as shown in Fig. 4. The decreased rate with decreasing substrate does not follow the

Fig. 3. Product Formation versus Time for the Oxygenation of 9,12-Octadecadienoic and 8,11,14-eicosatrienoic Acids by Lipoxygenase.

Curve A: The sample initially contained 76 nmoles of 9,12-octadecadienoic acid and 4 nmoles of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid in 1.0 ml of 0.1 M TrisHCl (pH 9.0). The reaction was initiated by the addition of 2.5 µl of a solution of soybean lipoxygenase (0.4 mg/ml). After no further change in absorbance occurred, lipoxygenase was added as indicated. Curve B: The sample contained 37.5 nmoles of 8,11,14-eicosatrienoic acid and 4 nmoles of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid. The reaction was initiated with 5.0 µl of a lipoxygenase solution (0.4 mg/ml). Further additions were made as indicated.

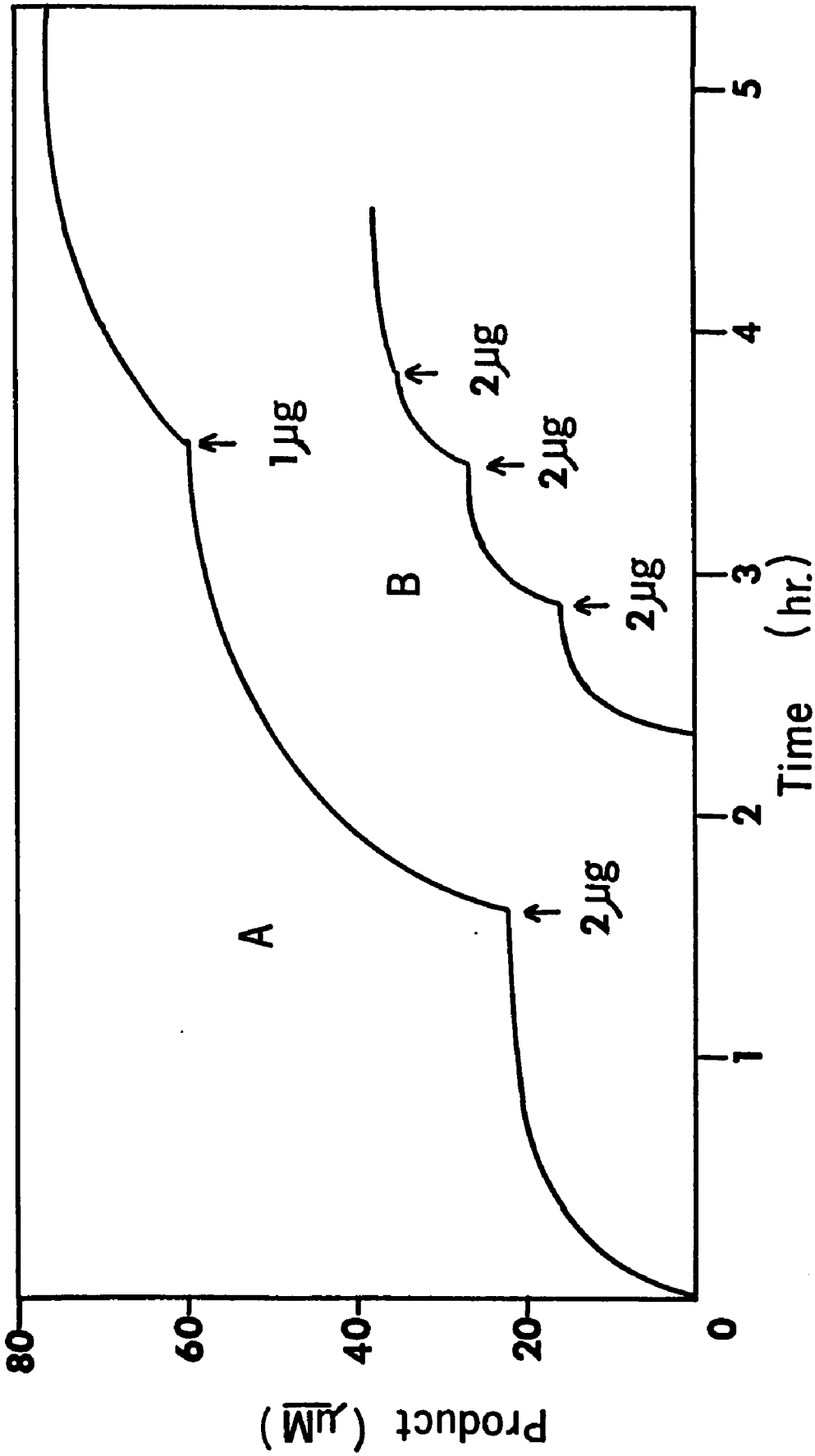


Fig. 3. Product Formation versus Time for the Oxygenation of 9,12-octadecadienoic and 8,11,14-eicosatrienoic Acids by Lipoxygenase.

Fig. 4. Velocity versus Substrate Concentration for the Oxygenation of 9,12-octadecadienoic, 9,12,15-octadecatrienoic, 8,11,14-eicosatrienoic, 5,8,11,14-eicosatetraenoic, 5,8,11,14,17-eicosapentaenoic and 5,8,11,14,17,20-docosahexaenoic Acids by Lipoxygenase.

The instantaneous rate at various substrate concentrations was determined from Gilford tracings similar to those depicted in Fig. 3. All points are from samples which contained hydroperoxide product initially (3-60 μM) and exhibited maximal velocity upon initiating the reaction with 1-5 μg lipoxygenase. The velocity is expressed as μmole/min per assay and the substrate concentration as μM.

Figure: (A) 18:2 (n-6); (B) 18:3 (n-3); (C) 20:3 (n-6); (D) 20:4 (n-6); (E) 20:5 (n-3); and (F) 22:6 (n-3).

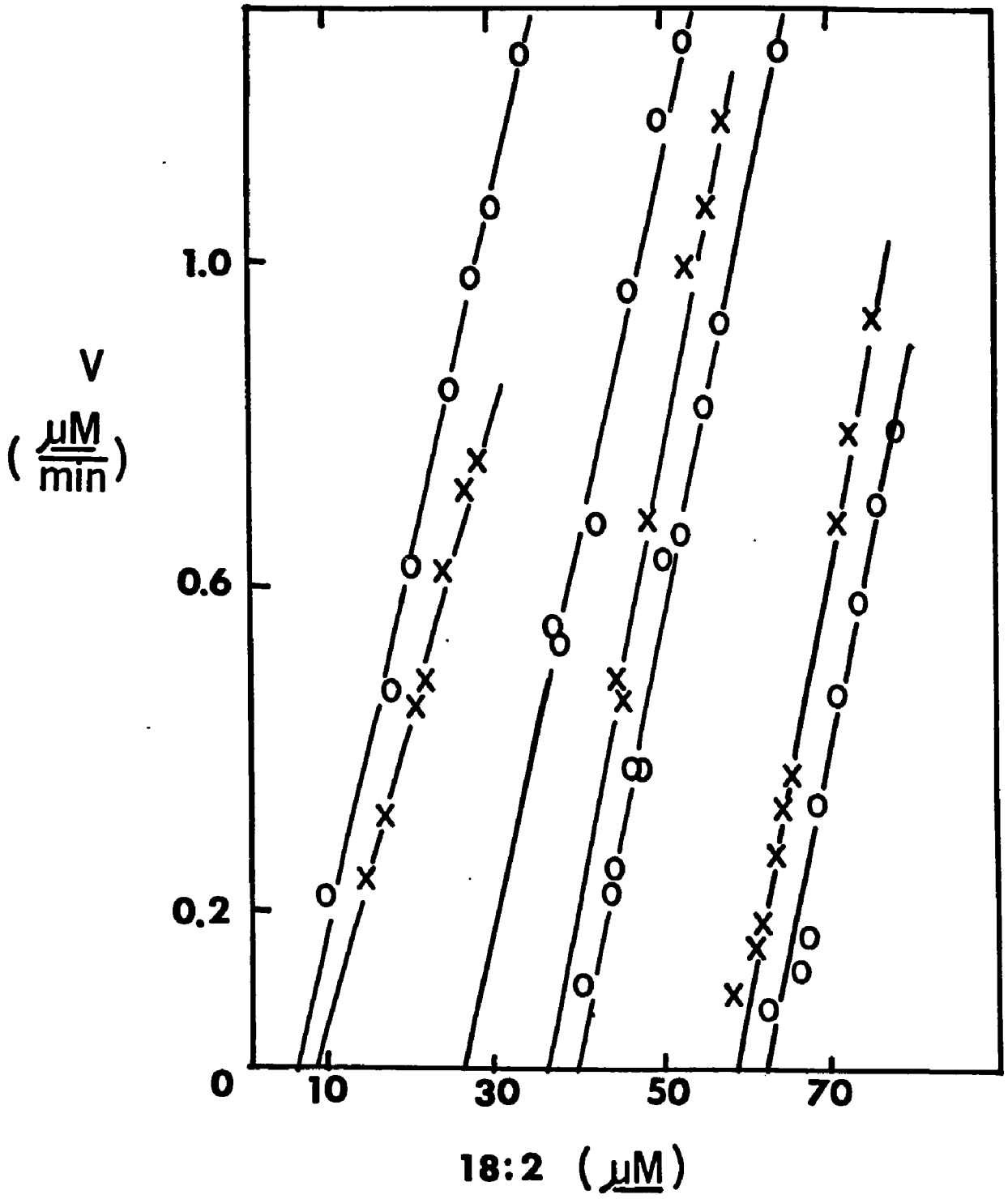


Fig. 4A. Velocity versus Substrate Concentration for the Oxygenation of 9,12-octadecadienoic Acid by Lipoxygenase.

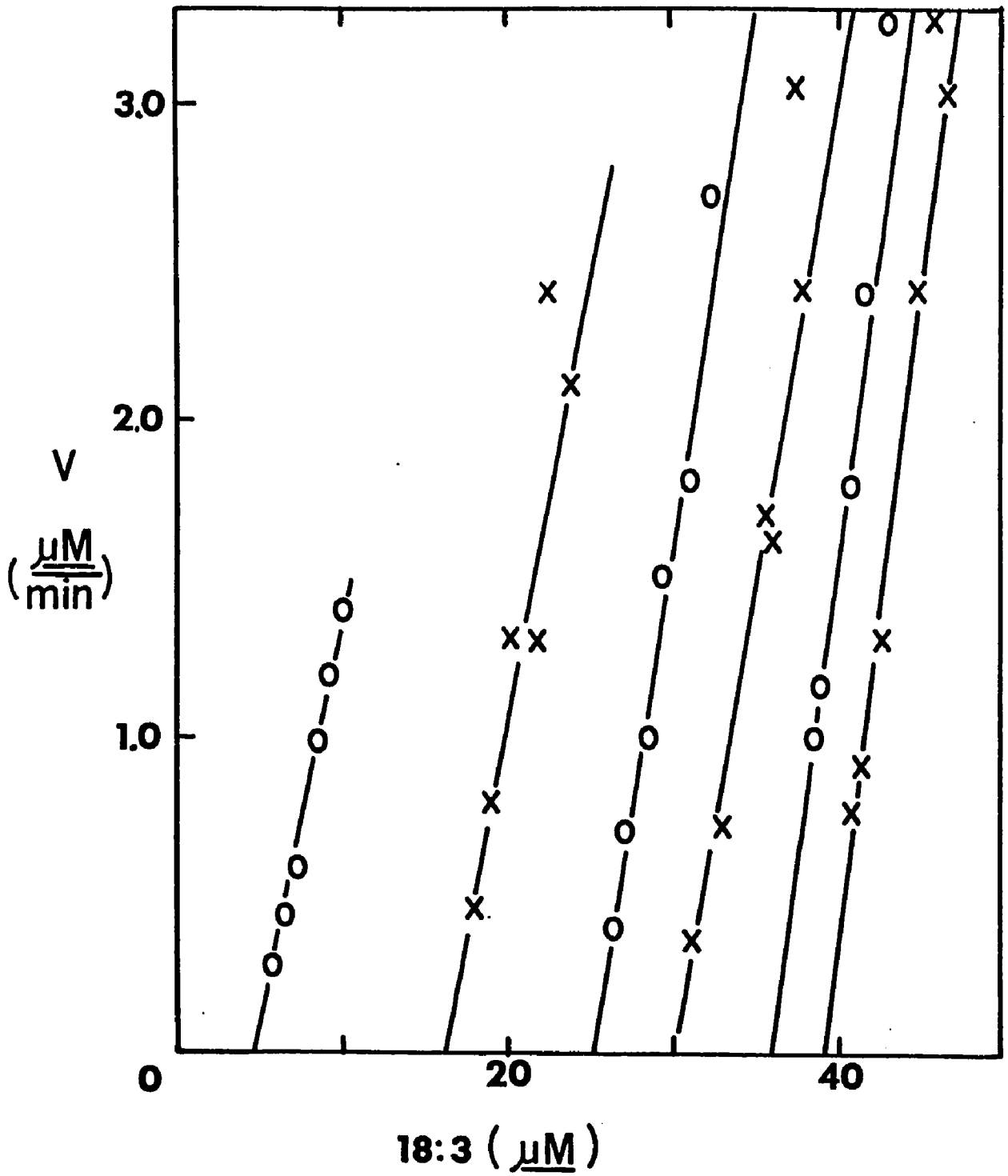


Fig. 4B. Velocity versus Substrate Concentration for the Oxygenation of 9,12,15-octadecatrienoic Acid by Lipoxygenase.

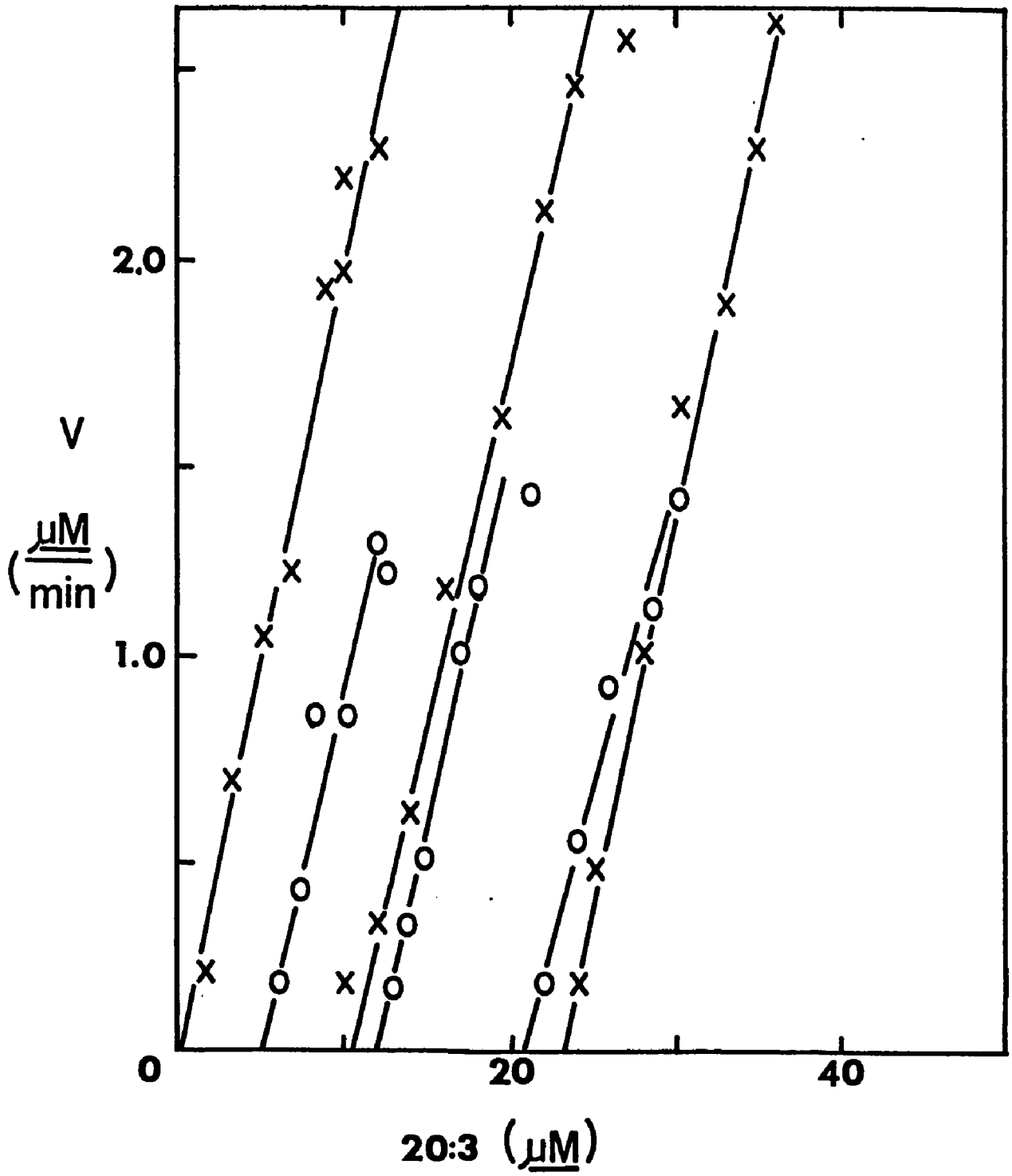


Fig. 4C. Velocity versus Substrate Concentration for the Oxygenation of 8,11,14-eicosatrienoic Acid by Lipoxygenase.

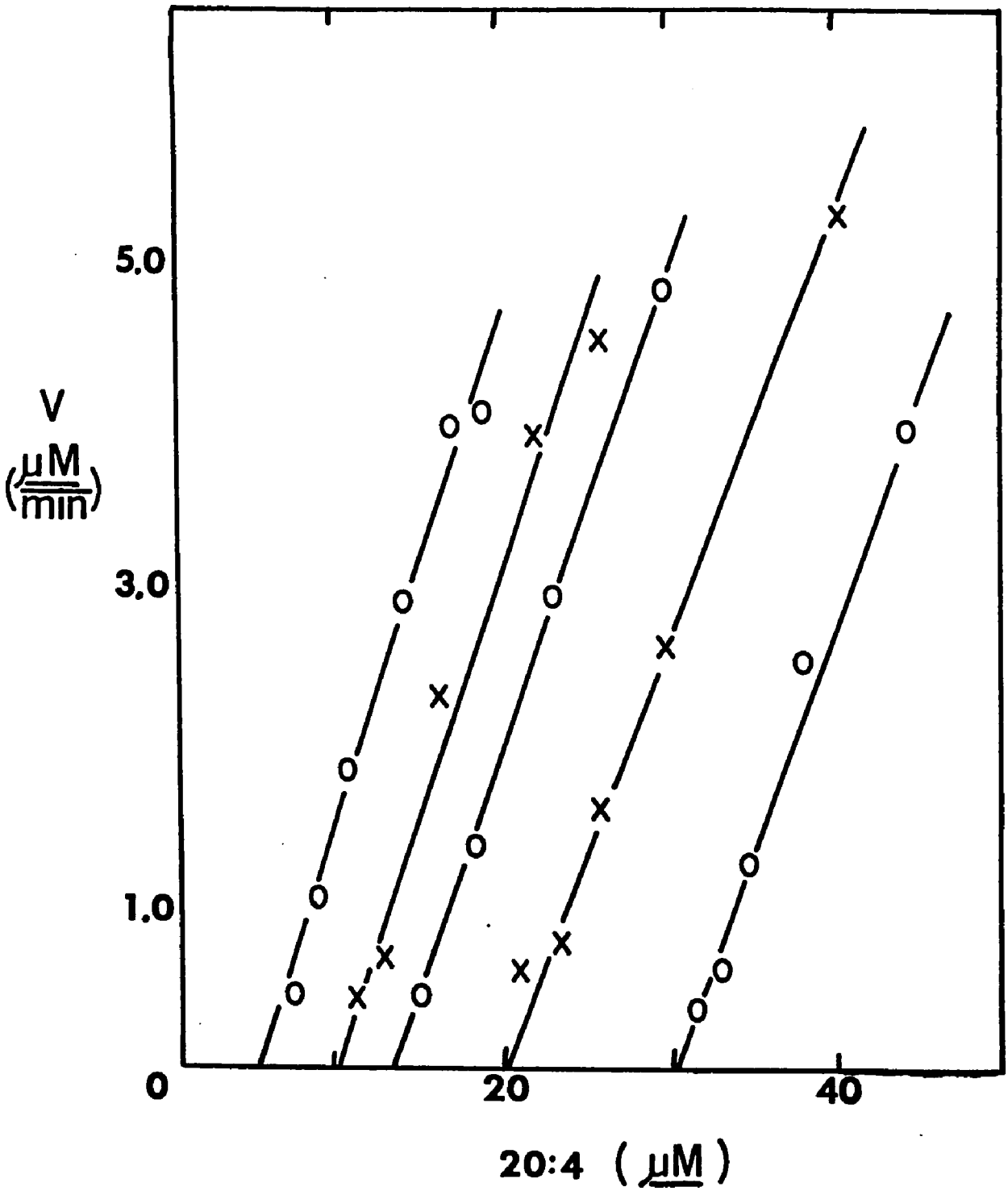


Fig. 4D. Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase.

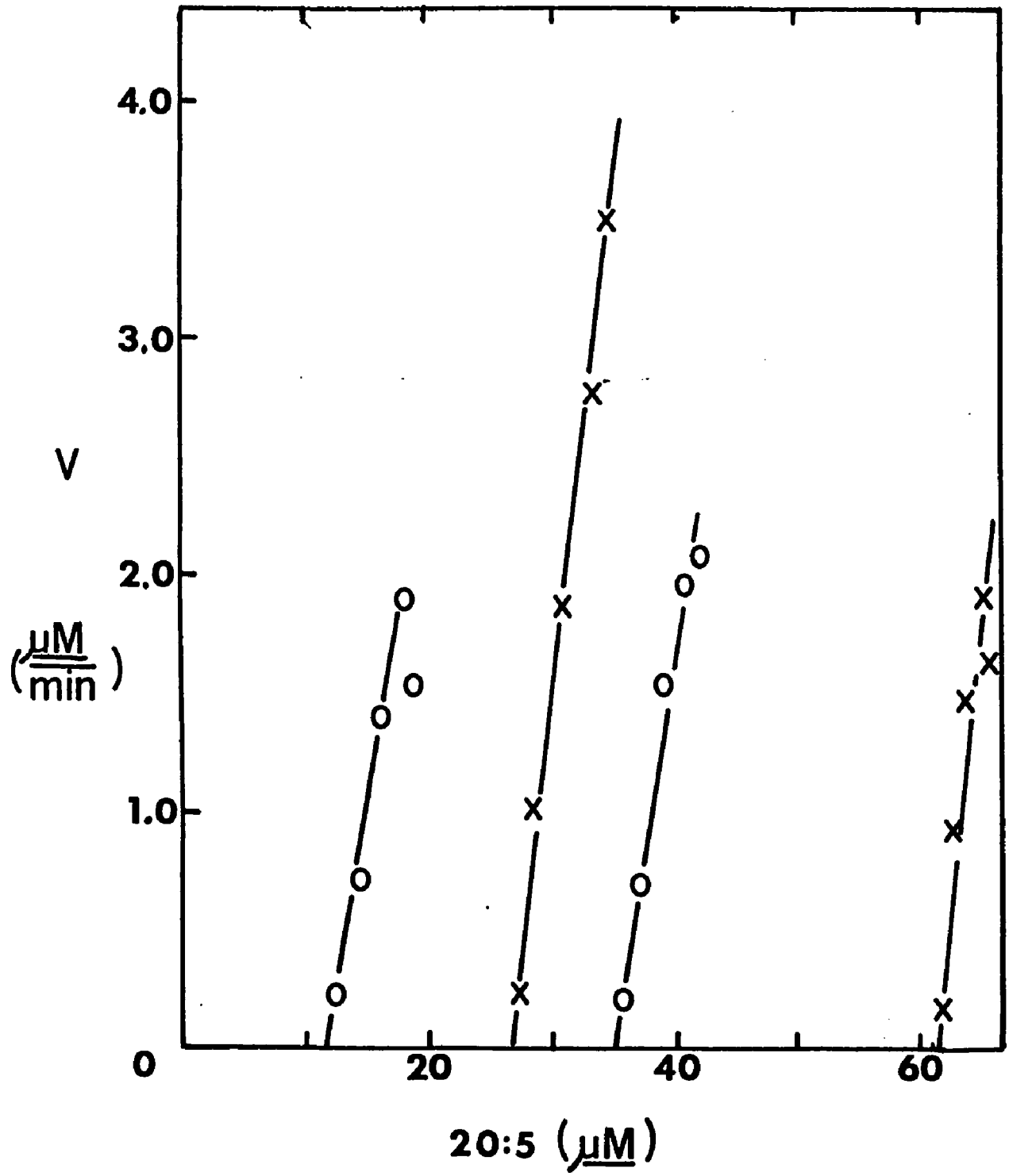


Fig. 4E. Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14,17-eicosapentaenoic Acid by Lipoxygenase.

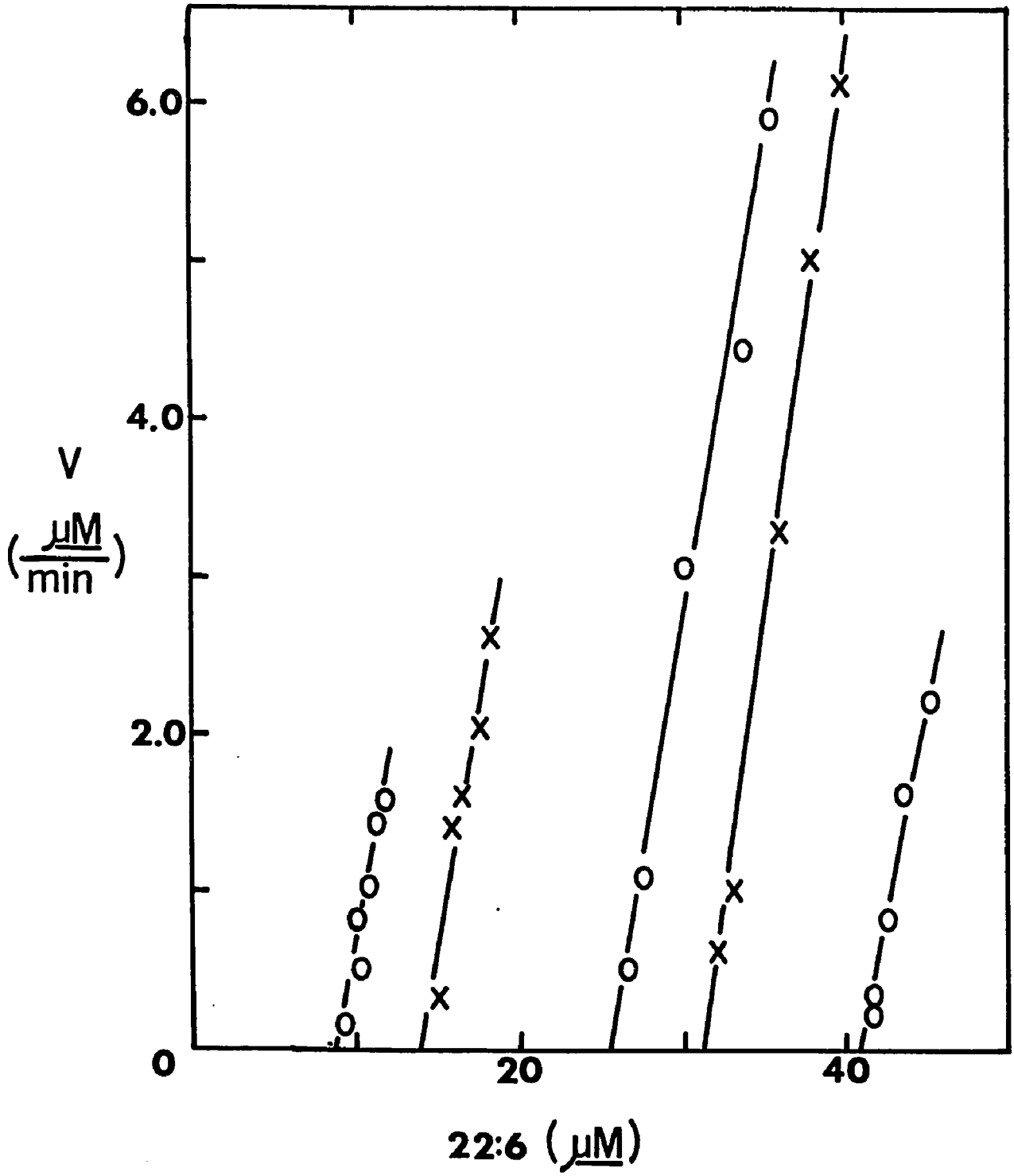


Fig. 4F. Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14,17,20-docosahexaenoic Acid by Lipoxygenase.

classical rectangular hyperbola but appears to be quite linear during the course of the reaction. The families of parallel lines indicate that the rate by which the velocity decreases with respect to substrate has a characteristic value for each fatty acid substrate. The average of the slopes (k_2) for the different fatty acids are summarized in Table II. A ten fold range in k_2 values was observed among the different substrates with the more highly unsaturated acids producing much less product per observed unit of catalytic activity. Also shown in Table II for comparison are the average ratios of initial rate to final extent of reaction for the experiments in Fig. 4. These values are seen to agree closely with the values of the slopes (k_2) derived from Fig. 4. However, for experiments in which no product was present in the initial reaction mixture the initial rate to final extent ratios are seen to be significantly lower for all fatty acid substrates (Table I).

Fig. 5 indicates the results obtained when a series of polyunsaturated fatty acid hydroperoxides were preincubated with lipoxygenase aerobically in the absence of fatty acid substrate. Although the rates of inactivation of lipoxygenase under these conditions do not appear to be true first order processes with respect to enzyme, the decrease in the log of the velocities allowed us to estimate a half-life for lipoxygenase inactivation. First order rate constants (k_4) for this inactivation system were calculated from the estimated half-lives ($k_4 = 0.69/t_{1/2}$) and are summarized in Table II. The k_4 values are much less than the corresponding k_2 values for all substrates except 9,12-octadecadienoic and 5,8,11,14,17,20-docosahexaenoic acids.

TABLE I

Rates and Extents of Oxygenation of

Different Fatty Acids by Lipoxygenase

All assays were performed as described in Methods. Lipoxygenase was added to each of the substrate solutions shown in the table (containing no hydroperoxide) to initiate reactions and the resulting maximum rate measured. After no further change in the absorbance at 234 mμ was observed, more substrate was added to verify that no enzyme activity remained. In no case did the reaction proceed until another aliquot of enzyme was added, and in all cases the reaction then proceeded.

TABLE I

Acid	Enzyme (μg)	Substrate (μM)	Rate (μM/min)	Extent (μM)	Rate / Extent (min^{-1})	Avg. (min^{-1})
	1	46	0.72	39	0.018	
18:2(n-6)	1	92	0.72	38	0.019	0.020
	2	92	1.84	76	0.023	
	2	42	0.48	28	0.017	
20:2(n-6)	2	84	0.44	24	0.018	0.019
	4	84	0.96	45	0.021	
	2	45	1.9	32	0.059	
18:3(n-3)	2	90	2.0	36	0.056	0.062
	4	90	4.3	61	0.070	
	2	72	0.32	6.0	0.053	
18:3(n-6)	2	144	0.24	6.4	0.038	0.048
	4	144	0.64	12.0	0.053	
	10	37	2.3	26	0.089	
20:3(n-3)	10	74	2.3	27	0.085	0.095
	20	74	5.5	49	0.11	
	2	47	0.36	5.6	0.064	
20:3(n-6)	2	94	0.24	6.0	0.040	0.059
	4	94	0.88	12	0.073	
	10	50	0.95	8.5	0.11	
20:4(n-6)	20	50	2.4	16	0.15	0.13
	20	100	1.6	12	0.13	
	10	24	1.9	11	0.17	
20:5(n-3)	20	24	4.7	23	0.20	0.19
	20	48	5.2	25	0.21	
	3	32	2.3	10	0.23	
22:6(n-3)	6	32	4.9	18	0.27	0.23
	6	64	5.7	31	0.18	

Fig. 5. Log Initial Velocity versus Time for the Aerobic Inactivation of Lipoxygenase by Hydroperoxide Products

All assays were performed as described in Methods. Hydroperoxide was generated *in situ* by the additions of lipoxygenase to fatty acid substrates. After this initial quantity of substrate was consumed, fatty acid (40-80 μM) was again added (0 min) to the reaction mixture and the initial velocity measured. Subsequent substrate additions were made to identical samples at the indicated times after the 0 min sample and initial velocities measured.

Figure 5	Acid	Legend	Lipoxygenase Added Initially (µg)	Hydroperoxide Product (µM)
A	18:2(n-6)	0	5	20
		Δ	10	40
B	18:3(n-3)	0	20	0
		Δ	20	16
		X	20	54
		●	20	28
C	20:3(n-6)	0	15	37
		Δ	10	21
		X	10	20
		●	10	30
D	20:4(n-6)	0	10	36
		Δ	10	18
		X	10	36
		●	10	24
		θ	10	0
E	20:5(n-3)	0	60	40
		X	30	20
F	22:6(n-3)	0	100	40
		Δ	60	40
		X	30	20
		●	20	20

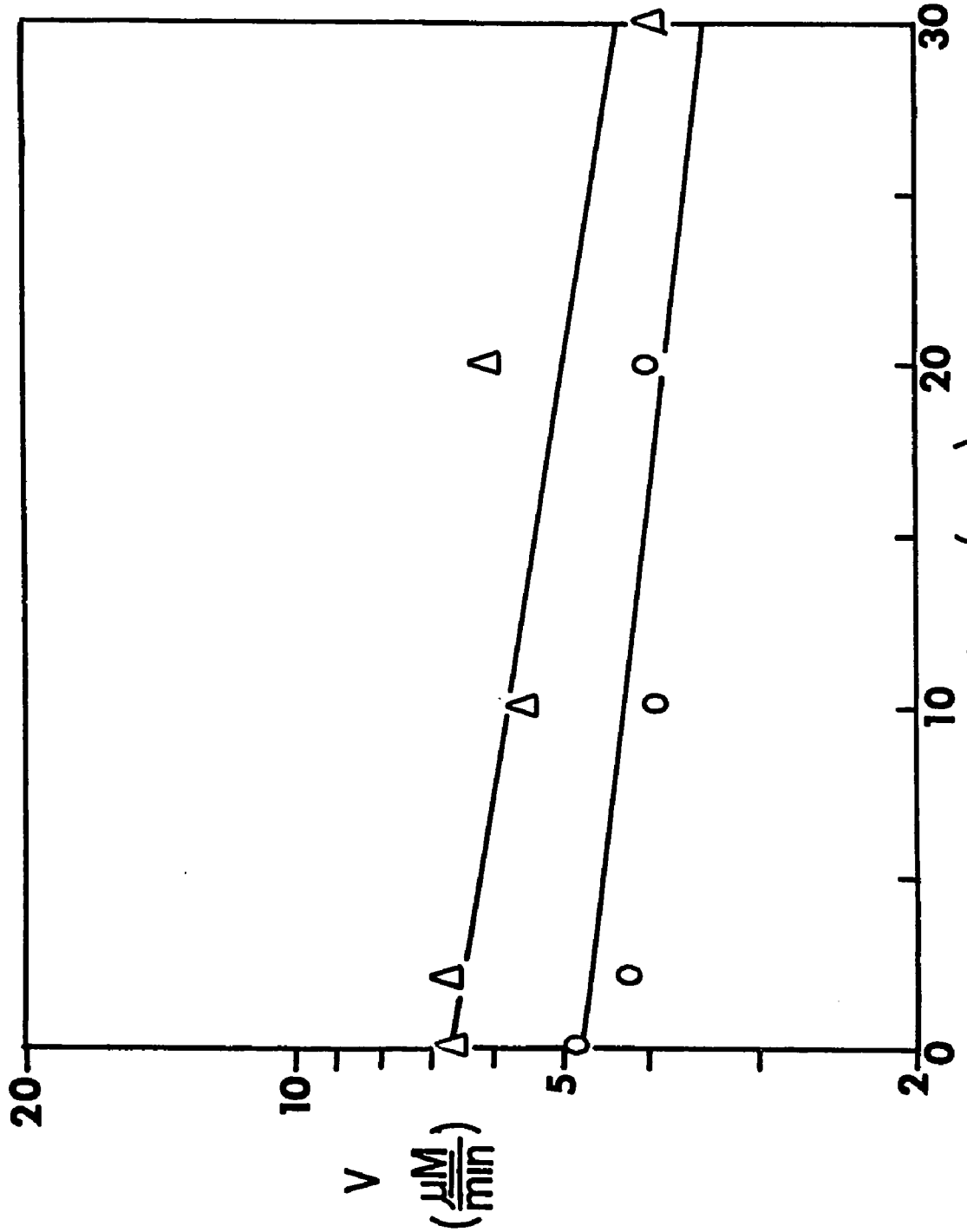


Fig. 5A. Log Initial Velocity versus Time for the Aerobic Inactivation of Lipoxygenase by 13-hydroperoxy-9,11-octadecadienoic Acid.

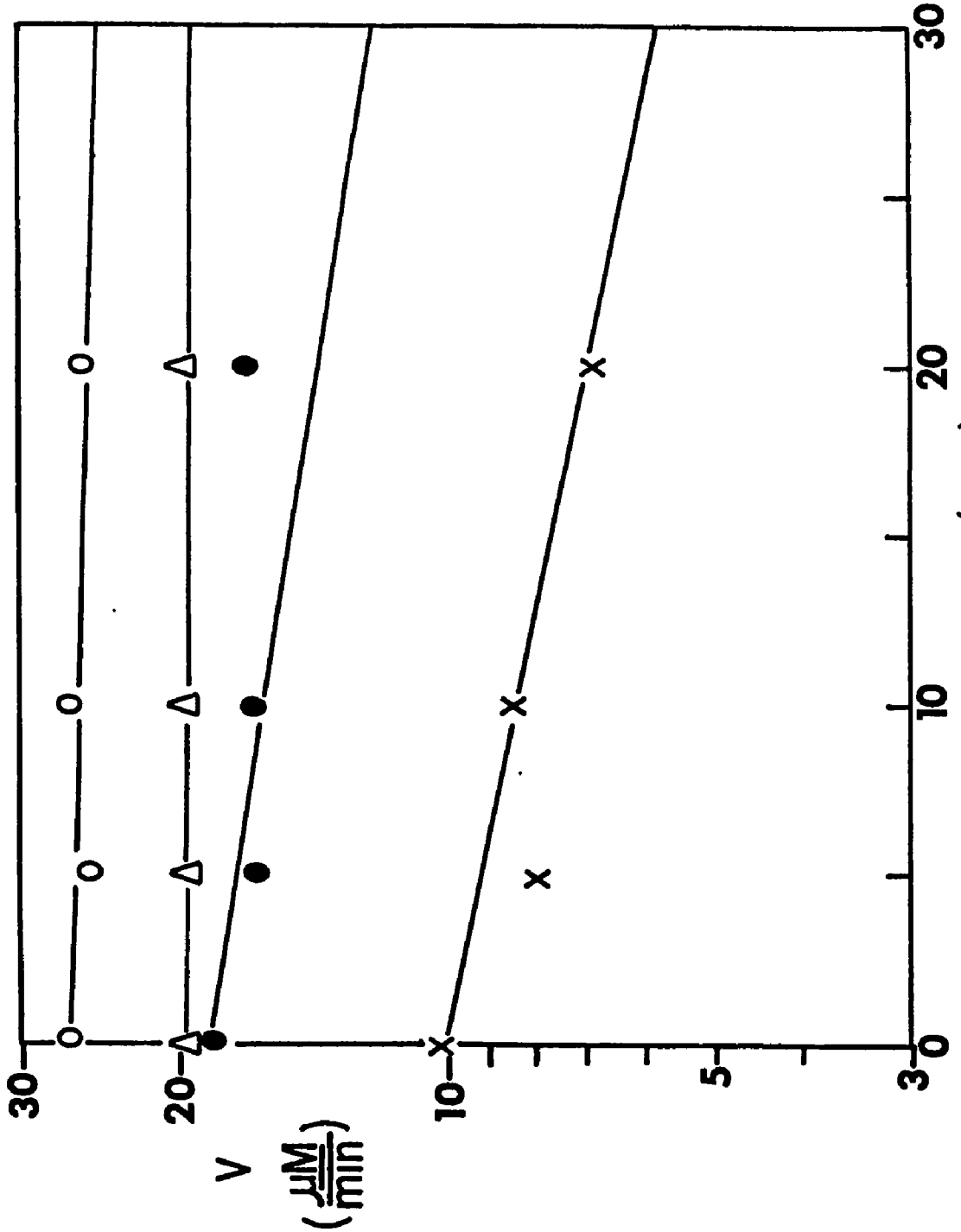


Fig. 5B. Log Initial Velocity versus Time for the Aerobic Inactivation of Lipoxygenase by 13-hydroperoxy-9,11,15-octadecatrienoic Acid.

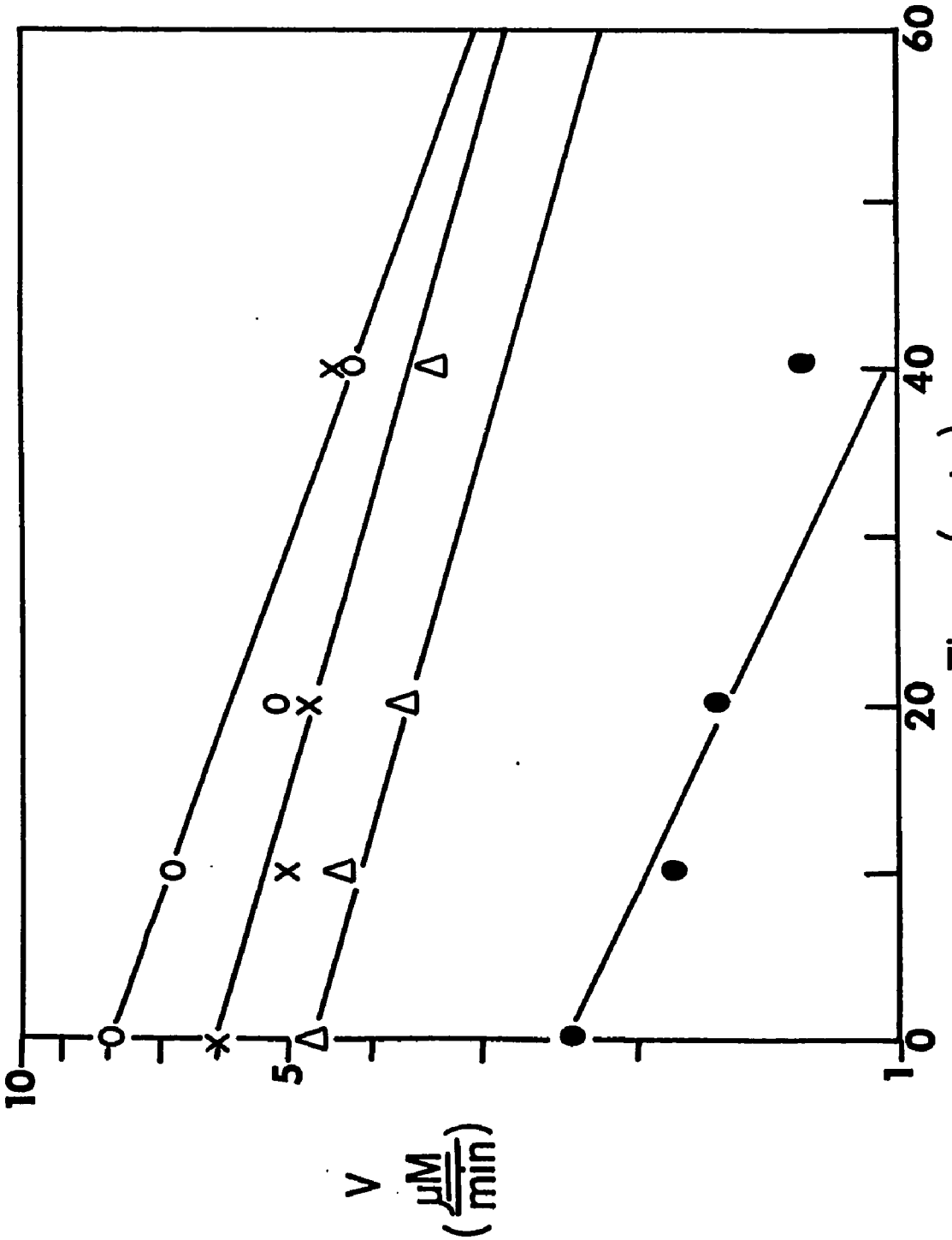


Fig. 5C. Log Initial Velocity versus Time for the Aerobic Inactivation of Lipoxygenase by 15-hydroperoxy-8,11-13-eicosatrienoic Acid.

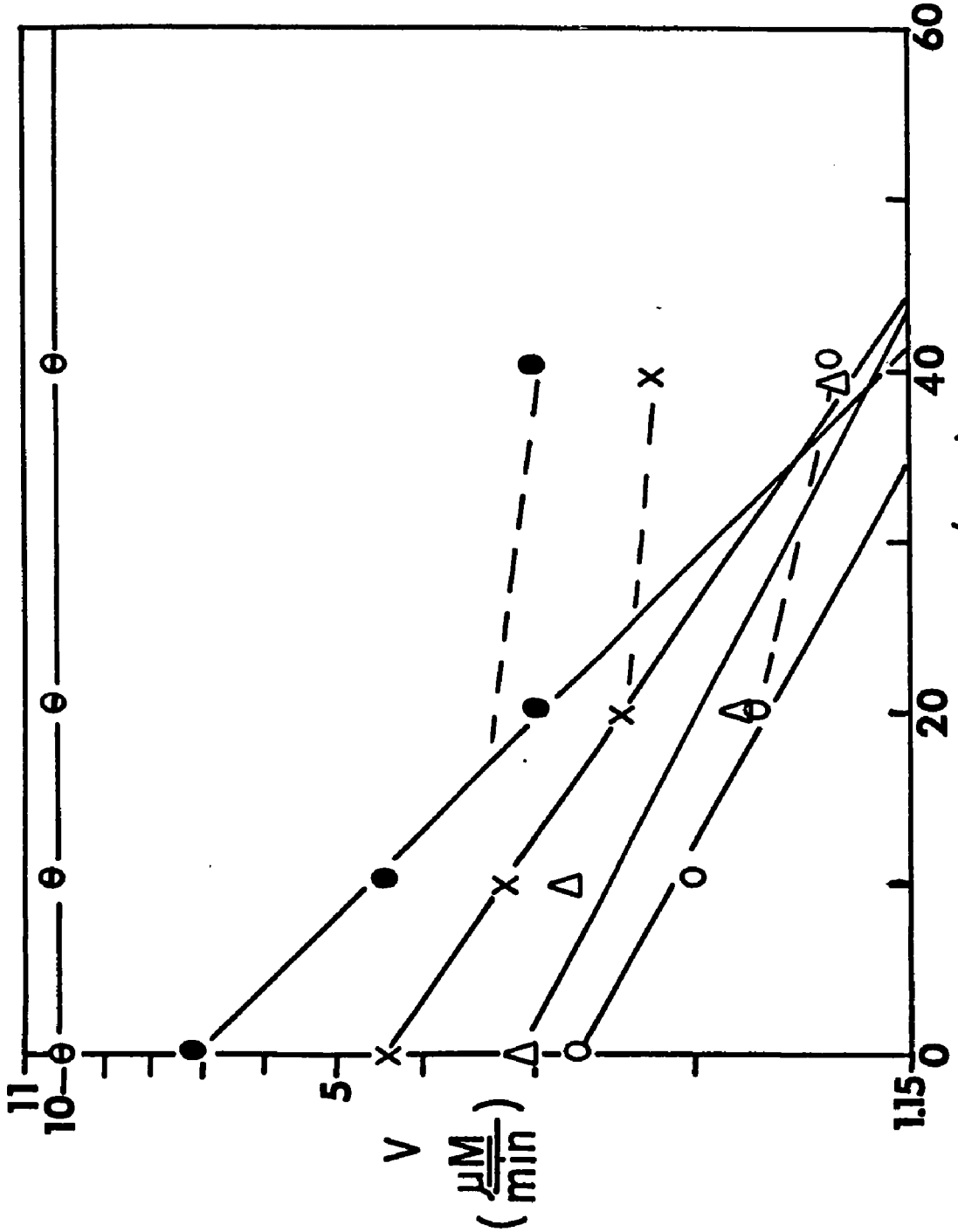


Fig. 5D. Log Initial Velocity versus Time for the Aerobic Inactivation of Lipoxygenase by 15-hydroperoxy-5,8,11,13-eicosatetraenoic Acid.

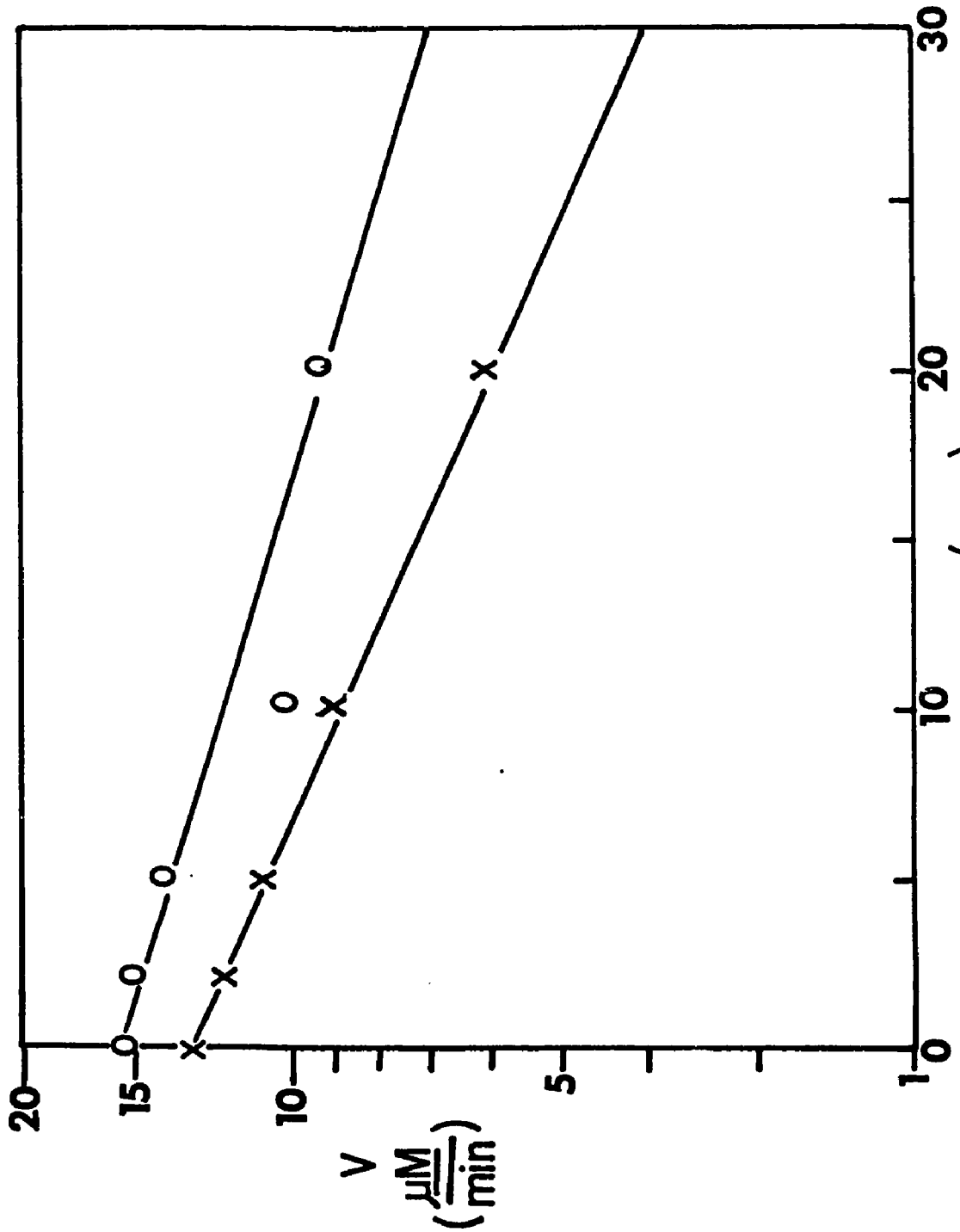


Fig. 5E. Log Initial Velocity versus Time for the Aerobic Inactivation of Lipoxygenase by 15-hydroperoxy-5,8,11,13,17-eicosapentaenoic Acid.

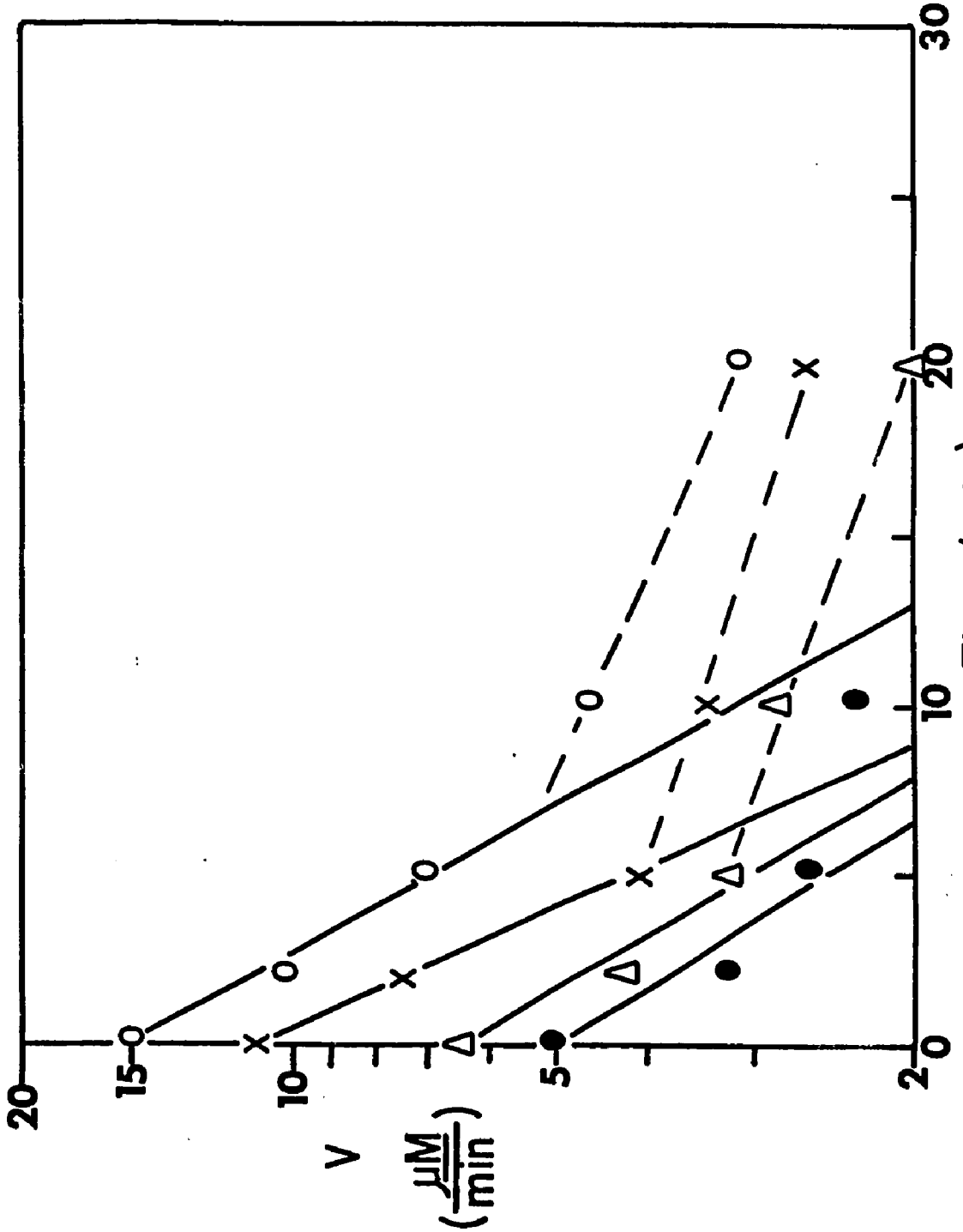


Fig. 5F. Log Initial Velocity versus Time for the Aerobic Inactivation of Lipoxygenase by 17-hydroperoxy-5,8,11,14,16,20-docosahexaenoic Acid.

To determine if lipoxygenase was rapidly inactivated anaerobically in the presence of fatty acid or fatty acid and hydroperoxide, 9,12,15-octadecatrienoic acid was incubated in the presence and absence of its corresponding hydroperoxide product. Fig. 6 shows the results of these experiments. The first order rate constants for these inactivation processes (0.046 min^{-1} with substrate plus product; 0.012 min^{-1} with substrate alone) were in both cases much lower than the corresponding k_2 value (0.40 min^{-1}) for 9,12,15-octadecatrienoic acid.

We attempted to study the effect of inactivation on the amino acid composition of lipoxygenase hoping that these studies would provide us with information about residues involved in the active site of this enzyme. However, we were unable to detect a loss in any amino acid residue after inactivation during the oxygenation of 5,8,11,14,17,20-docosahexaenoic acid (Table III).

Effect of GSH-peroxidase on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Soybean Lipoxygenase

In experiments to help elucidate the mechanism by which the sheep vesicular gland dioxygenase catalyzes the addition of oxygen to unsaturated fatty acid substrate, we noted that this enzyme was inhibited by partially purified preparations of rat liver GSH-peroxidase in the presence of GSH. We then observed that soybean lipoxygenase was also inhibited by the peroxidase plus GSH. As shown in Fig. 7 the inhibition of lipoxygenation of 5,8,11,14-eicosatetraenoic acid by GSH-peroxidase occurs in the presence of GSH, dithiothreitol, or cysteine but not in the absence of RSH. Ascorbate (0.16 mM) will not replace thiols in this system. Heat treatment (85° for 15 min) of the GSH-peroxidase preparation completely abolished

Fig. 6. Log Initial Velocity versus Preincubation Time for the Anaerobic Incubation of Lipoxygenase with 9,12,15-octadecatrienoic Acid in the Presence and Absence of 13-hydroperoxy-9,11,15-octadecatrienoic Acid.

Lipoxygenase (2-4 μg) was added where indicated to excess 9,12,15-octadecatrienoic acid in the bottom of a Thunberg tube and the reaction allowed to proceed until the enzyme was completely inactivated. The tube was then closed with the side arm containing lipoxygenase (30-50 μg). The air was removed by aspirating for 1 min and the tube flushed with N_2 which had been passed through a solution of propyl gallate in 0.1 M NaOH. This degassing procedure was repeated 3 times on all samples. At time 0 min the contents of the tubes were mixed thoroughly. After the indicated preincubation times the tubes were opened and the contents transferred quickly to a cuvette which was shaken thoroughly. Initial rates were determined spectrophotometrically as described in Methods. The table below indicates the initial concentrations of hydroperoxide product and substrate in the bottom of the Thunberg tube and the quantity of lipoxygenase in the sidearm at the time of mixing. The volume of each sample before the beginning of the aspiration procedure was 0.75 ml of 0.1 M TrisHCl (pH 9.0) in both the bottom and sidearm of the Thunberg tube. The first order rate constant for the anaerobic inactivation of lipoxygenase in the presence of substrate and product is 0.046 min^{-1} (average of four experiments); and for anaerobic inactivation in the presence of substrate, 0.012 min^{-1} (average of three experiments).

Legend	13-hydroperoxy-9,11,15-octadecatrienoic Acid (μM)	9,12,15-octadecatrienoic Acid (μM)	Lipoxygenase (μg)
▼	0	82	50
●	30	55	40
0	0	55	30
X	23	50	40
Δ	35	50	40
▲	45	39	40

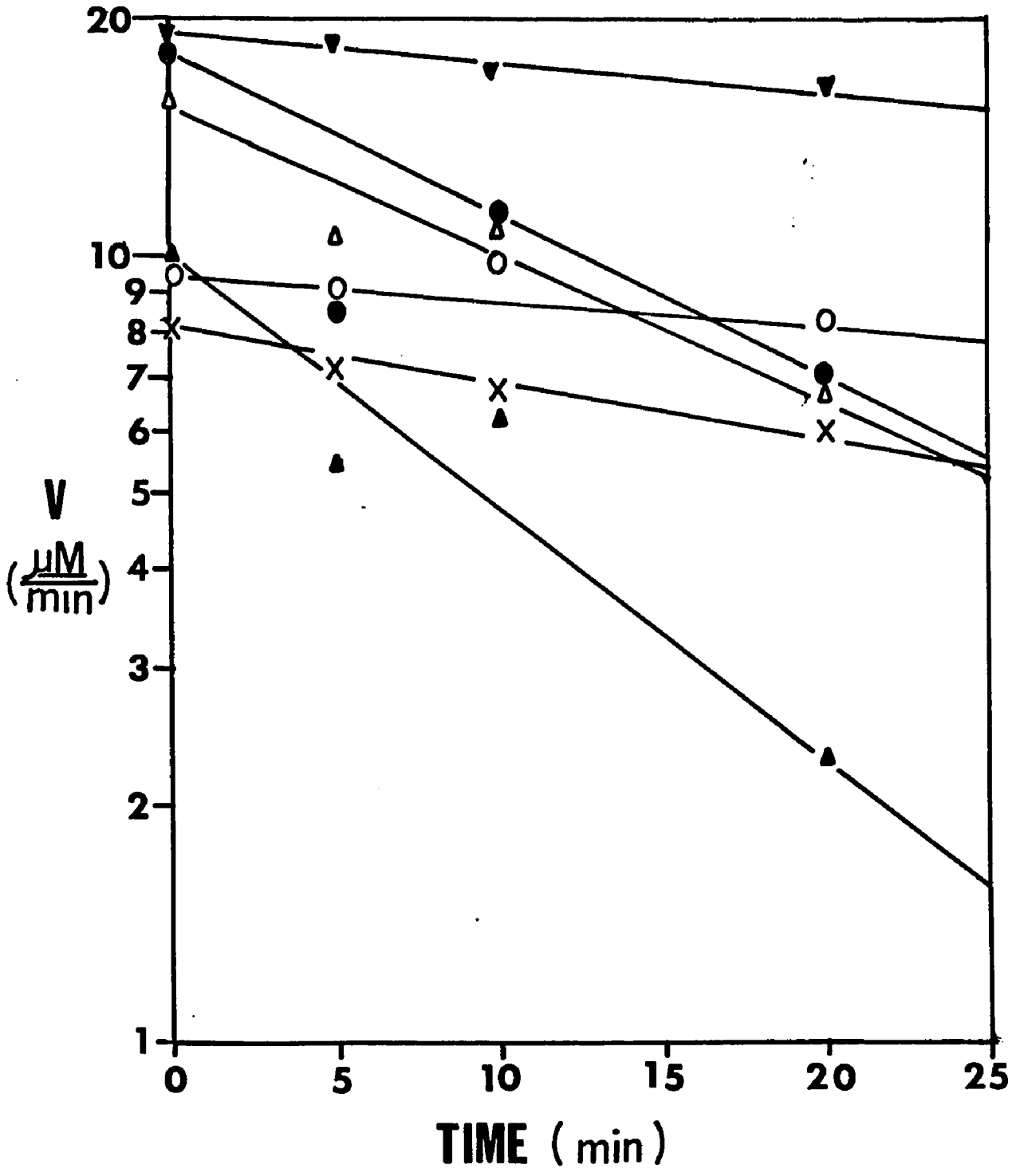


Fig. 6. Log Initial Velocity versus Preincubation Time for the Anaerobic Incubation of Lipoxygenase with 9,12,15-octadecatrienoic Acid in the Presence and Absence of 13-hydroperoxy-9,11,15-octadecatrienoic Acid.

TABLE II

Summary of Rate Constants for the Inactivation of Lipoxygenase by Various Processes

Acid	$k_2(min^{-1})$	$\frac{Rate}{Extent}(min^{-1})$	$k_4(min^{-1})$
18:2(n-6)	0.050 (7)	0.041 (7)	<0.023 (2)
18:3(n-3)	0.40 (5)	0.43 (5)	0.017 (3)
20:3(n-6)	0.17 (8)	0.15 (8)	0.016 (6)
20:4(n-6)	0.32 (5)	0.24 (5)	0.027 (6)
20:5(n-3)	0.74 (7)	0.70 (7)	0.029 (2)
22:6(n-3)	0.69 (9)	0.65 (9)	0.15 (6)

All values are an average of the number of separate determinations indicated in parentheses. Values of k_2 and rate/extent are from experiments such as those shown in Fig. 4; k_4 values are from experiments such as those shown in Fig. 5.

TABLE III

Amino Acid Composition of Lipoxygenase and of Lipoxygenase Inactivated During Oxygenation of 5,8,11,14,17,20-docosahexaenoic Acid

Lipoxygenase (20 units, 4.8 mg) partially purified as described in Methods was added to a suspension of 5,8,11,14,17,20-docosahexaenoic acid (100 μmoles) to give a final volume of 8.0 ml of 0.1 M TrisHCl (pH 8.5). Aliquots were withdrawn at various times and assayed for remaining lipoxygenase activity. After no activity remained (15-20 min), the reaction mixture was cooled to 0° and 1.0 mmole of $NaBH_4$ added to reduce hydroperoxides. The sample was allowed to stand in ice for 30 min and then at room temperature for approximately 5 hours. The sample was then exhaustively dialyzed against (1) 90% ethanol, (2) 1.0 mM EDTA and (3) deionized water. Following the dialysis procedures, the resulting protein suspension was divided into aliquots and subjected to hydrolysis (24 and 48 hr.) and amino acid analysis as described in Methods. Control values were obtained by incubating lipoxygenase with 9-octadecaenoic acid and carrying this sample through an identical procedure to that outlined above. Results are presented as moles of amino acid per mole of enzyme calculated on the basis of a molecular weight for lipoxygenase of 100,000 daltons (36) and an average amino acid residue weight of 127 daltons. Each set of values has been corrected for serine and threonine destruction. Tryptophan and cysteine were not included in these calculations.

TABLE III

Amino Acid	Treatment			
	18:1(n-9)		22:6(n-3)	
	24 hr. (moles/mole)	48 hr. (moles/mole)	24 hr. (moles/mole)	48 hr. (moles/mole)
Lys	46	47	47	44
His	22	19	18	20
Arg	35	33	36	33
Asp	94	92	95	112
Thr	104	106	107	102
Ser	73	74	75	72
Glu	86	82	87	83
Pro	45	45	47	46
Gly	46	51	45	43
Ala	51	52	52	50
Val	36	36	33	35
Met	15	17	16	14
Ile	33	34	31	31
Leu	54	55	54	53
Tyr	26	26	25	25
Phe	22	23	22	22

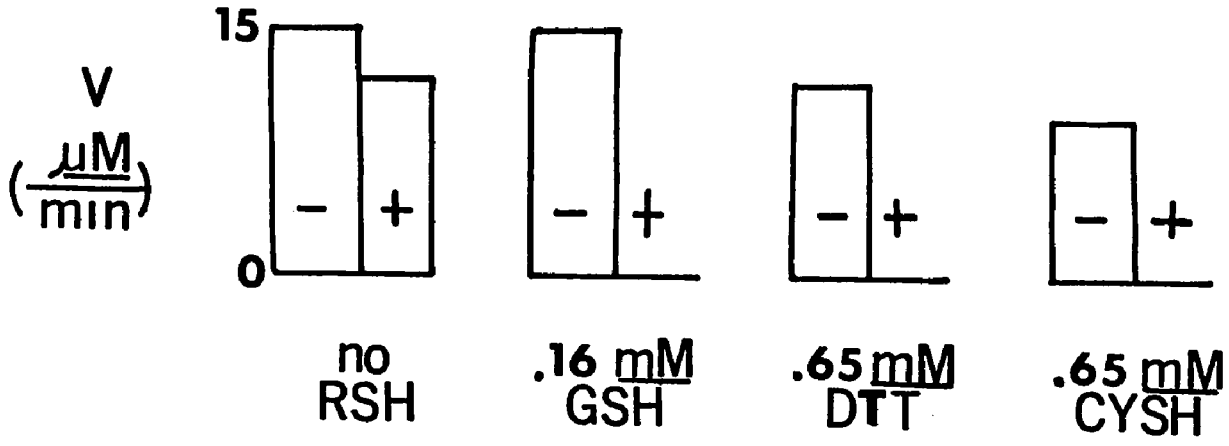


Fig. 7. The Effect of GSH-peroxidase on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase in the Presence of Various Sulfhydryl Compounds.

All lipoxygenase assays were performed as described in Methods using 100 nmoles of 5,8,11,14-eicosatetraenoic acid in 1.0 ml of 0.1 M TrisHCl (pH 9.0) containing the indicated concentrations of GSH, dithiothreitol (DDT) or cysteine; and 21 units of GSH-peroxidase. The reactions were initiated by addition of 10 μg lipoxygenase. GSH-peroxidase activity was determined at pH 9.0 by the assay procedure outlined in Methods. RSH is alkyl hydroperoxide.

the GSH dependent inhibition of lipoxygenase. Table IV indicates the levels of GSH-peroxidase required to inhibit oxygenation with different quantities of soybean lipoxygenase. Quantities of GSH-peroxidase which do not completely prevent oxygenation cause a decrease in the specific activity of the lipoxygenase and an increase in the kinetic lag period. Measurement of the absorbance at 413 mμ after addition of DTNB to aliquots of an oxygenation reaction mixture completely inhibited by GSH-peroxidase indicated that no sulfhydryl consumption was occurring (Fig. 8); however GSH was consumed in systems incompletely inhibited by the peroxidase. In the latter system the rate of GSH consumption at the beginning of the reaction was twelve times faster than the rate of product formation resulting in the oxidation of 2.6 moles GSH for each mole of hydroperoxide formed at the end of the reaction.

The inhibition of lipoxygenase by GSH-peroxidase may be reversed by addition of NEM to an inhibited system containing 5,8,11,14-eicosatetraenoic acid, GSH, lipoxygenase and GSH-peroxidase (Fig. 9). A 25% lower rate of oxygenation is observed after addition of NEM than at the beginning of the reaction.

<u>The Lag Phase in the Oxygenation of Fatty Acid Substrates by Soybean Lipoxygenase and the Effect of Product and Enzyme Concentrations on the Length of the Lag</u>

The incomplete inhibition of lipoxygenase by GSH-peroxidase caused a marked increase in the length of the kinetic lag phase. Haining and Axelrod (40) had reported earlier that addition of hydroperoxide product abolished the lag phase which they observed using 9,12-octadecadienoic acid as a substrate for lipoxygenase. We

TABLE IV

Levels of GSH-peroxidase Necessary to Inhibit the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Different Concentrations of Lipoxygenase

All samples (except #4) contained 0.16 μmoles of GSH, 100 nmoles of 5,8,11,14-eicosatetraenoic acid and the indicated units of GSH-peroxidase. Reactions were initiated by the addition of lipoxygenase to yield a final volume of 1.0 ml of 0.1 M TrisHCl (pH 9.0). Lag times, specific activities and the concentration of product at which maximum rate was attained were measured as described in the legend to Fig. 10.

Experiment	GSH-peroxidase (units)	Lipoxygenase (μg)	Sp. Activity (μM/min/10μg)	Lag Time (min)	Product (μM)
1	0	15	11.3	0.8	4
2	13	15	9.0	2.1	10
3	21	15	0	∞	-
4	42	20	12.1	0.46	3
5	13	20	9.9	1.5	6
6	21	20	0	∞	-
7	0	25	15.0	0.34	3
8	13	25	12.6	0.80	8
9	21	25	9.5	4.2	20
10	42	25	0	∞	-

Fig. 8. GSH Consumption with Different Levels of GSH-peroxidase in a Lipoxygenase Reaction System

Each sample contained initially 600 nmoles of 5,8,11,14-eicosatetraenoic acid, the indicated levels of GSH-peroxidase (assayed at pH 9.0) and GSH. Reactions were initiated by the addition of 200 µg of lipoxygenase to give a total volume of 10.0 ml of 0.1 M TrisHCl (pH 9.0). After mixing, a 1.0 ml aliquot was withdrawn and the absorbance at 234 mµ followed continuously with time as indicated in Methods. Aliquots (0.5 ml) of the remaining solution were withdrawn at the indicated time intervals and added to 3.3 ml of 1 mM DTNB in 0.1 M TrisHCl (pH 7.4). The absorbance of these solutions were determined at 413 mµ and GSH consumption calculated as described in Methods.

Part A. The 10 ml reaction mixture contained 3750 units of GSH-peroxidase and 1.56 µmoles of GSH.

Part B: The 10 ml reaction mixture contained 1550 units of GSH-peroxidase and 1.78 µmoles of GSH.

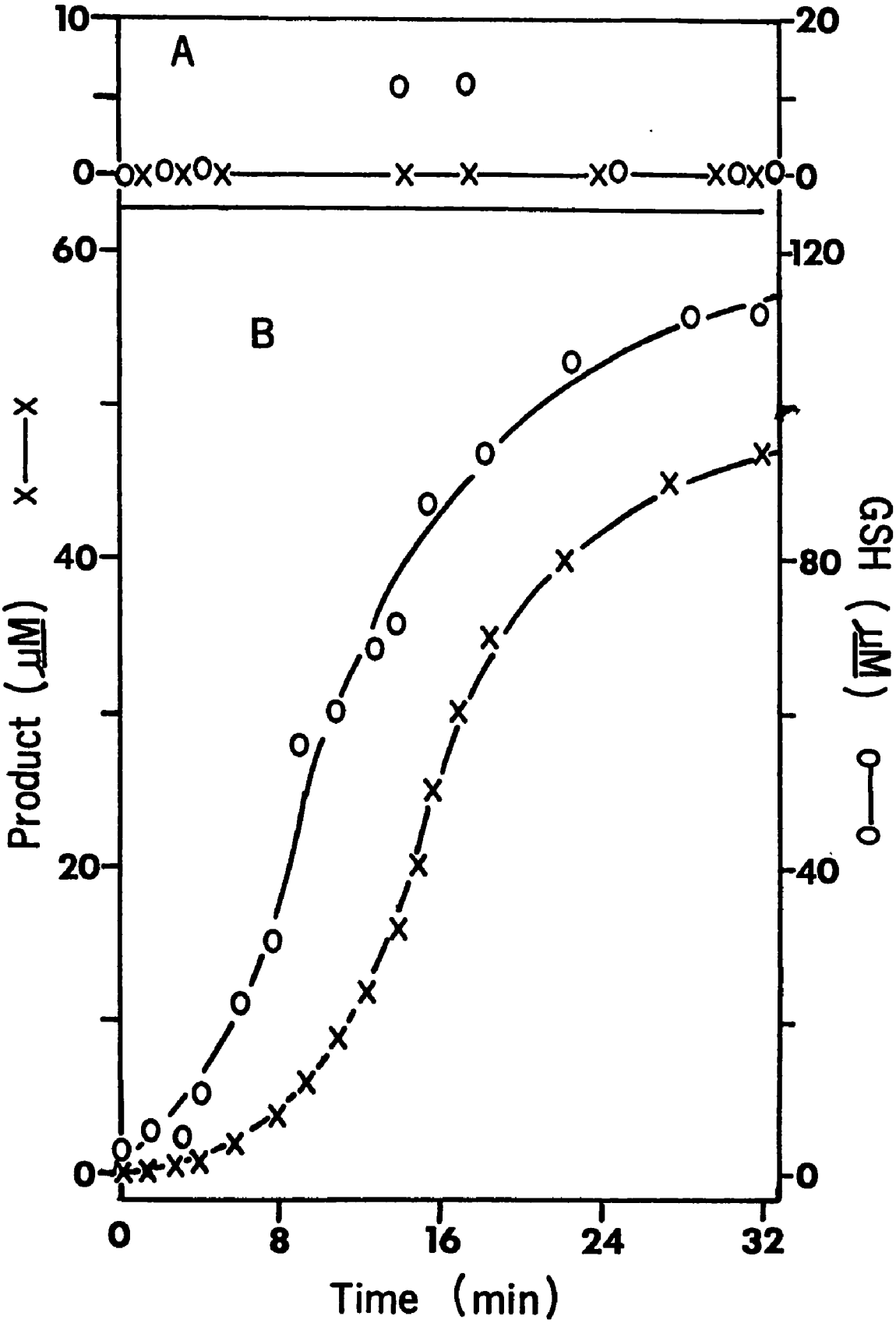


Fig. 8. GSH Consumption with Different Levels of GSH-peroxidase in a Lipoxygenase Reaction System.

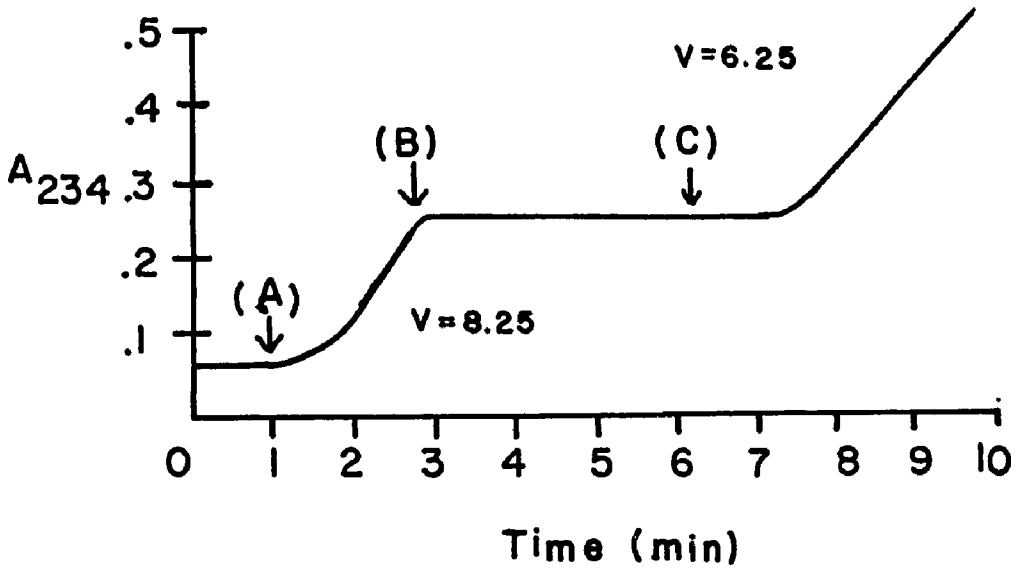


Fig. 9. The Effect of NEM on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic by Lipoxygenase.

Measurements of the absorbance at 234 mμ were performed on a Cary recording spectrophotometer using the 0-0.5 slidewire. Initially the sample cell contained 160 nmoles GSH and 100 nmoles 5,8,11,14-eicosatetraenoic acid in 0.9 ml of 0.1 M TrisHCl (pH 9.0). The reference cell contained 160 nmoles GSH in 0.9 ml of 0.1 M TrisHCl (pH 9.0). Additions were made to both reference and sample cells as indicated in the figure: (A) 10 μg of lipoxygenase; (B) 21 units of GSH-peroxidase (units measured at pH 9.0 as described in Methods); and (C) 500 nmoles of NEM (0.050 ml of 10 mM NEM in 0.1 M TrisHCl (pH 9.0)). Maximum velocities in μM/min after additions (A) and (C) are indicated in the figure.

therefore set out to study the nature of the lag phase and the effect of hydroperoxide product on the length of this lag.

Fig. 10 shows a tracing of the kinetic lag phenomenon and indicates how the lag time was measured in these experiments. We noted that the rate becomes independent of product concentration above a product concentration of approximately 3 μM. Table V shows that the lag observed in the oxygenation of 5,8,11,14-eicosatetraenoic acid is essentially abolished if 13-hydroperoxy-9,11-octadecadienoic acid or 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid are included in the reaction mixture at a concentration of 5 μM; in addition, either of these hydroperoxides will abolish the lag observed in the oxygenation of 9,12-octadecadienoic acid. However, inclusion of 1-octyl hydroperoxide (20 μM) does not abolish the lag with either fatty acid substrate. Hydrogen peroxide (50 μM) does not remove the lag with 5,8,11,14-eicosatetraenoic acid.

The initial rate of reaction in the presence of a level of product hydroperoxide necessary to remove the lag is independent of the nature of the product hydroperoxide but depends on the nature of the fatty acid substrate as shown in Table VI. The ratio of the rate of oxygenation of 9,12-octadecadienoic acid to the rate of oxygenation of 5,8,11,14-eicosatetraenoic acid is the same in the presence of levels of either 13-hydroperoxy-9,11-octadecadienoic acid or 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid approximately ten times in excess of those required to abolish the lag with their respective substrates. This ratio is also the same when low levels of hydroperoxide (4-5 μM) are used to activate the enzyme toward each

Fig. 10. Measurement of the Kinetic Lag Period in the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase.

All assays were performed as described in Methods. Lipoxygenase was added at zero min to initiate the reaction. Lag time is measured from time zero to the time at which a line tangent to the maximum velocity intersects the "time-axis". Initial concentrations of reactants are: (A) 66 μM 20:4 (n-6), 10 μg/ml enzyme; (B) 132 μM 20:4 (n-6), 10 μg/ml enzyme; (C) 66 μM 20:4 (n-6), 5 μg/ml enzyme; and (D) 132 μM 20:4 (n-6), 5 μg/ml enzyme. The final reaction volume is 1.0 ml of 0.1 M TrisHCl (pH 9.0). Each curve is derived from at least two individual experiments.

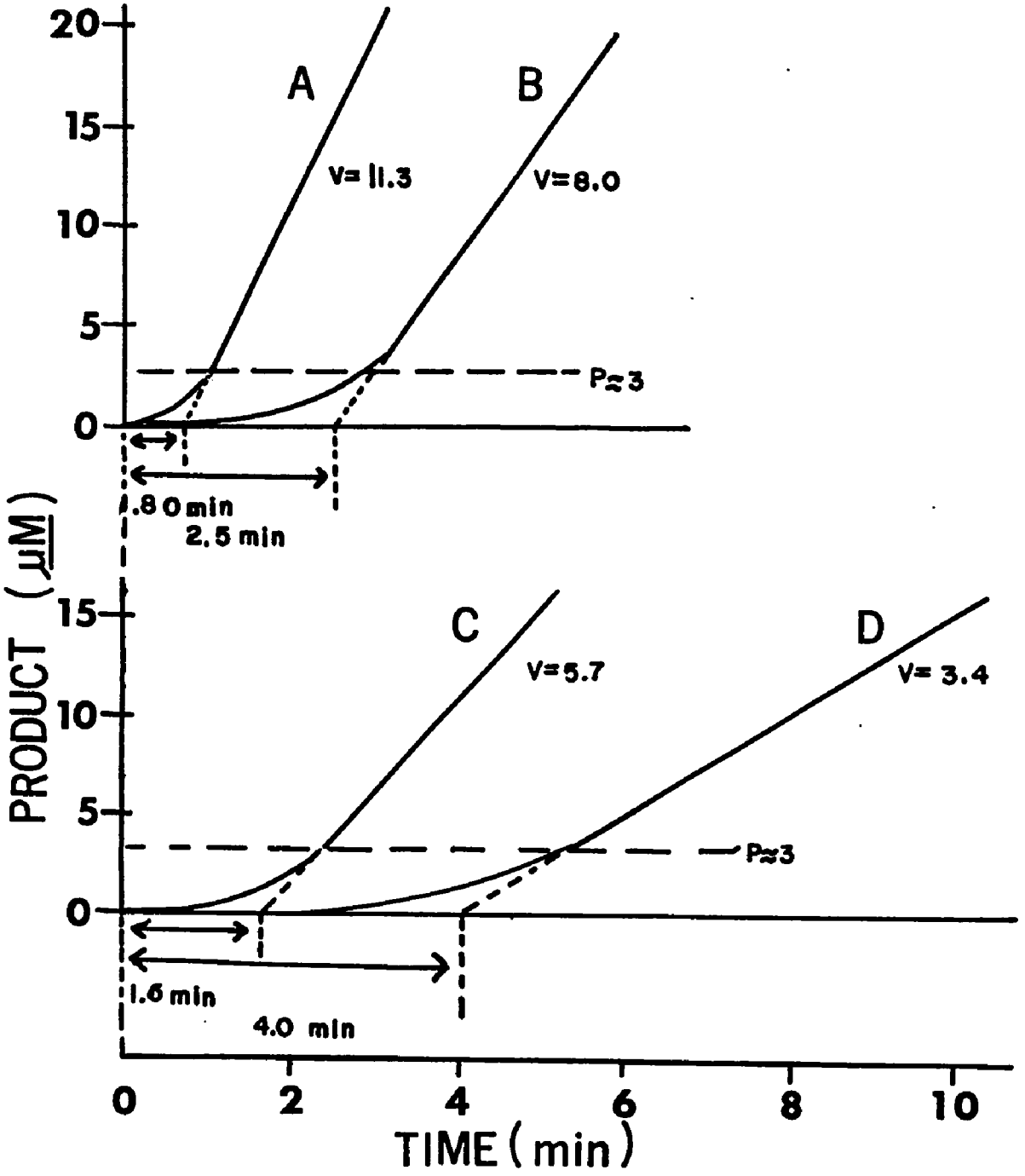


Fig. 10. Measurement of the Kinetic Lag Period in the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase.

TABLE V

The Effect of Hydrogen Peroxide and Alkyl Hydroperoxides on the Lag Period in the Oxygenation of 9,12-octadecadienoic and 5,8,11,14-eicosatetraenoic Acids by Lipoxygenase

Hydrogen peroxide and 1-octyl-hydroperoxide were added at the indicated concentrations to a solution containing either 60 μM 9,12-octadecadienoic acid or 66 μM 5,8,11,14-eicosatetraenoic acid. Lipoxygenase (3 μg) was then added to initiate the reaction and lag time and maximum velocities determined as indicated in Fig. 10. For experiments with product hydroperoxides, the hydroperoxides were generated *in situ* with lipoxygenase (3 μg) and substrate was then added to initiate the oxygenation. All experiments were performed in a final reaction volume of 1.0 ml of 0.1 M TrisHCl (pH 9.0).

TABLE V

Sample	H_2O_2 (μM)	1-octyl hydroperoxide (μM)	18:2[a] ROOH (μM)	20:4[b] ROOH (μM)	Lipoxygenase (μg)	18:2 (n-6) (μM)	20:4 (n-6) (μM)	Lag (min)	Maximal Velocity (μM/min)
1	0	0	0	0	3	60	0	1.8	3.7
2	0	0	5	0	3	60	0	0.20	3.5
3	0	5	0	0	3	60	0	1.5	3.4
4	0	10	0	0	3	60	0	1.7	3.5
5	0	20	0	0	3	60	0	1.5	3.1
6	0	40	0	0	3	60	0	0.88	0.92
7	0	60	0	0	3	60	0	∞	0
8	0	0	0	0	3	0	66	2.3	2.5
9	0	0	0	5	3	0	66	0.11	2.6
10	0	20	0	0	3	0	66	2.9	1.7
11	0	40	0	0	3	0	66	1.6	0.52
12	0	0	0	0	3	0	66	2.0	2.8
13	10	0	0	0	3	0	66	1.7	2.5
14	20	0	0	0	3	0	66	1.7	2.5
15	50	0	0	0	3	0	66	2.2	2.4

[a] 13-hydroperoxy-9,11-octadecadienoic acid

[b] 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid

TABLE VI

The Effect of the Nature of the Hydroperoxide Product on the Rate of Oxygenation of 9,12-octadecadienoic and 5,8,11,14-eicosatetraenoic Acids by Lipoxygenase

13-hydroperoxy-9,11-octadecadienoic and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acids were generated *in situ* with the indicated level of lipoxygenase. After no further change in the absorbance at 234 mμ was observed, either 9,12-octadecadienoic or 5,8,11,14-eicosatetraenoic acid was added to initiate the reaction. Lag times and maximum velocities were measured as indicated in Fig. 10. The final volume of all samples was 1.0 ml of 0.1 M TrisHCl (pH 9.0).

TABLE VI

Sample	20:4 ROOH (μM)	18:2 ROOH (μM)	Lipoxygenase (μg)	20:4 (n-6) (μM)	18:2 (n-6) (μM)	Lag time (min)	Velocities (maximum) (μM/min)	Velocity A / Velocity B
1A	26	0	10	0	30	0.16	4.7	0.67
B	27	0	10	33	0	0.16	7.0	
2A	0	35	4	0	30	0.24	3.7	0.66
B	0	35	4	33		0.16	5.6	
3A	5.3	0	5	0	60	0.12	4.7	0.66
B	5.7	0	5	66	0	0.08	6.7	
4A	0	4.2	5	0	60	0	1.9	0.70
B	0	4.2	5	66	0	0.08	2.9	
5A	0	1.6	6	0	30	0.19	4.8	0.62
B	3.0	0	6	33	0	0.24	7.8	
6A	0	1.6	3	0	30	0.24	2.4	0.80
B	3.0	0	3	33	0	0.48	3.0	

respective substrate. However the ratios of rates are seen to increase with decreasing enzyme concentration when 1.6-3.0 μM hydroperoxide product is present initially. This increasing ratio with decreasing enzyme is a result of a decrease in the specific activity of lipoxygenase with 5,8,11,14-eicosatetraenoic acid while the specific activity of lipoxygenase with 9,12-octadecadienoic acid remains essentially constant. As shown in Table VII this decrease in specific activity with decreasing enzyme concentrations occurs with a gradual increase in the length of the lag time.

The Effect of Fatty Acid Substrate Concentration on the Length of the Lag Period in the Oxgenation 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase

Table VIII shows that the length of the lag period increases when constant quantities of lipoxygenase are added to samples containing increasing concentrations of 5,8,11,14-eicosatetraenoic acid in the absence of hydroperoxide product. Table IX shows the identical phenomenon and indicates that the initial velocity decreases with increasing substrate concentration. After the initial enzyme has been completely inactivated the rate of oxygenation upon further lipoxygenase addition is independent of the concentration of the remaining fatty acid; in addition, no lag period is observed.

TABLE VII

The Effect of Lipoxygenase Concentration on the Lag Period in the Oxygenation of 5,8,11,14-eicosatetraenoic Acid

All assays were performed as described in Methods. All samples contained 66 μM 5,8,11,14-eicosatetraenoic acid in a final volume of 1.0 ml of 0.1 M TrisHCl (pH 9.0). The lag time and maximum velocities were measured as indicated in Fig. 10.

Experiment	Lipoxygenase (μg)	Lag Time (min)	Maximum Velocity (μM/min)	Sp. Activity (μM/min/μg enzyme)
1	1	6.1	0.4	0.40
2	2	2.9	1.3	0.65
3	3	2.6	3.0	1.0
4	4	2.1	4.3	1.1
5	6	1.3	7.7	1.3
6	8	0.95	11.5	1.4
7	9	0.75	12.9	1.4
8	12	0.52	25.6	2.1
9	15	0.28	28.5	1.9
10	18	0.18	30.8	1.7
11	21	0.16	37.3	1.8

TABLE VIII

The Effect of Fatty Acid Substrate Concentration on the Lag Period in the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase

All assays were performed as described in Methods. All reactions were initiated by the addition of 3 µg of lipoxygenase to give a final volume of 1.0 ml of 0.1 M TrisHCl (pH 9.0) containing the indicated concentrations of 5,8,11,14-eicosatetraenoic acid. The lag times were measured as indicated in Fig. 10.

Experiment	[20:4(n-6)] (µM)	Lag Time (min)
1	25	0.24
2	50	1.20
3	66	1.60
4	75	1.84
5	132	4.16
6	264	∞

TABLE IX

The Effect of Fatty Acid Substrate Concentration on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Lipoxygenase in the Presence and Absence of Hydroperoxide Product

All assays were performed as described in Methods. The Lag times and maximum velocities were measured as indicated in Fig. 10. Lipoxygenase (3 µg) was added to initiate the reactions. After the indicated amounts of product were formed and no change in absorbance at 234 mµ occurred with time, another aliquot of enzyme (3 µg) was added. The final volume in all assays was 1.0 ml of 0.1 M TrisHCl (pH 9.0).

[20:4(n-6)] (µM)	1st E ↓ Lag Time (min)	Maximum Velocity (µM/min)	Product formed (µM)	[20:4(n-6)] (µM)	2nd E ↓ Lag Time (min)	Maximum Velocity (µM/min)
55	1.5	2.7	15	40	0	4.1
70	1.8	2.5	15	55	0	3.9
94	3.4	2.1	14	80	0	4.0
110	4.4	1.9	14	96	0	3.9

DISCUSSION

Self-Catalyzed Destruction of Lipoxygenase

Soybean lipoxygenase is irreversibly inactivated during oxygenation of fatty acid substrates, an observation first made by Bergström (50). We noted that the velocity of the reaction decreases as a linear function of substrate utilization with all substrates tested. The inactivation observed during oxygenation of 9,12,15-octadecatrienoic, 8,11,14-eicosatrienoic, 5,8,11,14-eicosatetraenoic and 5,8,11,14,17-eicosapentaenoic acids is most easily explained as a deleterious breakdown of an active complex resulting from the interaction of enzyme, product, fatty acid and oxygen (Fig. 11). The involvement of fatty acid in the formation of this complex is suggested by the fact that the rate of decrease in lipoxygenase activity observed in the presence of product, fatty acid and oxygen cannot be accounted for by the much slower rate of inactivation which occurs when enzyme is incubated aerobically with product. In addition, the relatively slow inactivation of lipoxygenase which occurs during the anaerobic incubation of 13-hydroperoxy-9,11,15-octadecatrienoic and 9,12,15-octadecatrienoic acids with the soybean enzyme suggests that oxygen is necessary for formation of the active complex. The involvement of product in formation of this complex and the concept of a product binding site are discussed in detail below in "Product Activation of Lipoxygenase."

The pseudo first order inactivation of lipoxygenase by 17-hydroperoxy-5,8,11,14,16,20-docosahexaenoic and 13-hydroperoxy-

9,11-octadecadienoic acids is rapid enough to account for destruction of the enzyme by product alone during oxygenation of 5,8,11,14,17,20-docosahexaenoic and 9,12-octadecadienoic acids, respectively. However, inactivation by the products of the dienoic and hexaenoic acids, as well as those of the other substrates tested, may not be occurring during the oxygenation process at high fatty acid concentrations unless these products inactivate the enzyme by modifying some site on the molecule not involved with the binding of product, fatty acid or oxygen (see equations (19-22) in "Kinetic Mechanism for Lipoxygenase").

$$E + P + S + O_2 \rightleftharpoons [E_aS'] \begin{cases} \xrightarrow{k_2} E^* \\ \xrightarrow{k_3} E + P \end{cases}$$

Fig. 11 Kinetic Model for the Self-Catalyzed Destruction of Lipoxygenase.

In the situation depicted in Fig. 11, each molecule of complex has a certain probability of either forming product (k_3) or destroying itself (k_2). At any time,

$$\text{velocity} = v = d[P]/dt = k_3[E_aS'] \tag{1}$$

and

$$d[E^*]/dt = k_2[E_aS'] \tag{2}$$

so

$$d[E^*]/dt = (k_2/k_3)(d[P]/dt) \tag{3a}$$

Integration of both sides of equation (3a) from zero time to time, t, gives:

$$[E^*]_t = (k_2/k_3)[P]_t \tag{3b}$$

From the steady state expression for the above kinetic formulation and since $[E] = [E_o - E^*]$:

$$V_t = \frac{k_3[E_o - E^*]_t}{\frac{K_M}{[S]_t} + 1} = \frac{k_3[E_o - \frac{k_2}{k_3} P]_t}{\frac{K_M}{[S]_t} + 1} \qquad (4)$$

Since $P_t = S_o - S_t$:

$$V_t = \frac{k_3[E_o] - k_2[S_o]}{\frac{K_M}{[S]_t} + 1} + \frac{k_2[S]_t}{\frac{K_M}{[S]_t} + 1} \qquad (5)$$

When $[S]_t >> K_M$

$$V_t = k_3[E_o] - k_2[S_o] + k_2[S]_t = K' + k_2[S]_t \qquad (6)$$

Equation (6) forms the basis for plotting instantaneous velocities versus substrate concentration during oxygenation of fatty acid at high fatty acid concentrations. The slopes derived from such plots therefore represent a measurement of the first order rate constants (k_2) for the self-destruction of lipoxygenase.

Theoretically this k_2 value may be approximated by dividing the initial oxygenation rate by the final extent of product formation since:

$$\frac{\text{rate}}{\text{extent}} = \frac{v}{P_{t=\infty}} = \frac{k_3[E_o]/(\frac{K_M}{[S_o]} + 1)}{k_3[E^*]_{t=\infty}/k_2} = \frac{k_2}{\frac{K_M}{[S_o]} + 1} \simeq k_2 \qquad (7)$$

We noted that the rate/extent measurement for k_2 agreed well with the slopes determined from velocity versus substrate concentration plots in systems where hydroperoxide product was included initially in the reaction mixture. The maximal velocity of oxygenation for a given acid is consistently lower when no product is initially present in the reaction mixture than when initial low concentrations of product are present. Therefore the rate/extent values determined in systems containing no product initially were always lower than rate/extent values determined in systems containing product initially.

The chemical nature of this self-destruction phenomenon observed with lipoxygenase is not clear. Comparison of the amino acid composition of active and inactivated enzyme did not allow us to draw any conclusions about the destruction of any particular residue. Based on our analyses with partially purified enzyme, methionine is the only residue of which there are less than twenty per lipoxygenase molecule. Detection of a loss of one residue in twenty requires a resolution of less than 5% which we did not achieve. However, it seems reasonable that a deleterious modification of at least one active site amino acid residue does occur. A potentially related inactivation of lipoxygenase by 5,8,11,14-eicosatetraynoic acid has been reported by Downing (26) who suggested that modification of an active site histidine residue resulted in loss of lipoxygenase activity. The pH-activity profile for oxygenation of 9,12-octadecadienoic acid by lipoxygenase indicates a catalytically active group with pH = 6.5 - 6.8 (33) which could be due to a histidine residue.

Homolytic cleavage of the oxygen-oxygen bond in hydroperoxides occurs easily and yields the highly reactive hydroxyl radical (51).

In addition, nonenzymic formation of malondialdehyde from polyunsaturated hydroperoxides (52) provides another reactive species which along with the hydroxyl radical is capable of reacting with a number of amino acids (51,53,54,55). With a constant rate of formation of malondialdehyde and/or hydroxyl radicals, a decreasing rate of dissociation of new product from the lipoxygenase substrate site would increase the chances of some deleterious modification of an amino acid at that site. The proposed homolytic cleavage of the oxygen-oxygen bond would be expected to yield keto-fatty acid derivatives during lipoxygenase action as has been demonstrated (56). Such derivatives appear to form at lower oxygen concentrations (57) and formation is enzyme dependent (58).

Lipoxygenase is easily inactivated in the presence of hydrogen peroxide but is stable in the presence of a competitive inhibitor of the enzyme (25) and hydrogen peroxide (59). This suggests that small hydroperoxide molecules can gain access to the substrate site to cause modifications and that the modification caused by hydrogen peroxide may be the same as that occurring during oxygenation of fatty acid substrates. The high k_2/k_4 ratio observed with most fatty acid substrates suggests that the product hydroperoxy grouping only interacts with the easily-modified site on the lipoxygenase molecule during the oxygenation process; and therefore, that enzyme destruction may be caused by the hydroperoxy grouping of newly formed product.

Kinetically similar self-catalyzed enzyme destructions occur in the case of citrate lyase (60), threonine deaminase (61) and 3-hydroxyanthranilate oxidase (62). In this last-named instance the self-destruction seems to be due to oxidation of Fe^{2+} to Fe^{3+} during the reaction.

Product Activation of Lipoxygenase

The oxygenation of 5,8,11,14-eicosatetraenoic acid by lipoxygenase may be completely and reversibly inhibited by GSH-peroxidase in a process requiring the presence of mercaptans. No inhibition occurs when the GSH-peroxidase is heated.

This inhibition may be caused by a GSH-dependent binding of some catalytically active grouping on the lipoxygenase molecule by the peroxidase. However, inhibition by a protein-protein interaction is inconsistent with the existence of a threshold level for GSH-peroxidase below which essentially no inhibition is observed and above which complete inhibition occurs. The existence of this threshold is indicated by the relatively small difference in the levels of GSH-peroxidase required to give partial (20-50%) versus complete reduction in the maximum rate (Table IV). Furthermore, as shown in Table X, the reduced maximum rate that we noted in the presence of intermediate levels of GSH-peroxidase is closely predicted by equation (6) on the basis of the observed substrate consumption and therefore may be attributed to loss of lipoxygenase by the process noted by k_2.

The concept of a threshold level for GSH-peroxidase inhibition is consistent with a delicate balance in rates of hydroperoxide formation and destruction. Such a balance would explain the fact that GSH-peroxidase causes a marked increase in the kinetic lag period and the fact that GSH consumption occurs in systems incompletely inhibited by GSH-peroxidase but does not occur with higher levels of the peroxidase.

Whether the first few molecules of hydroperoxide required for lipoxygenase activity are produced by a very slow product-independent enzymic process or by an autooxidative process is unknown.

TABLE X

Prediction of the Maximum Velocities from k_2 Values in Lipoxygenase Reaction Mixtures Incompletely Inhibited by GSH-peroxidase

Experiment	GSH-peroxidase (units)	S_o (μM)	S_t (μM)	$V_{initial}$ (μM/min)	k_3E_o (μM/min)	V_t (μM/min)	$V_{predicted}$ (μM/min)
1	13	100	90	11.3	12.3	9.1	9.1
2	13	100	94	12.1	13.1	9.9	11.2
3	13	100	92	15.0	16.0	12.6	13.4
4	21	100	80	15.0	16.0	9.5	9.8
5	21	100	92.5	8.25	9.25	6.25	6.6

The numbers for experiments 1-4 are from Table IV and for experiment 5 from Fig. 9. $V_{initial}$ values are derived from controls containing no GSH-peroxidase. The values for k_3E_o are calculated from equation (6) using the indicated $V_{initial}$ value, S_o = 100 μM, S_t = 97 μM and k_2 = 0.32 min^{-1}. Values of $V_{predicted}$ are calculated from the data in the Table using equation (6) and k_2 = 0.32 min^{-1} (Table II).

Equation (6):

$$V_t = k_3[E_o] - k_2[S_o] + k_2[S]_t$$

Non-stereospecific autooxidation of polyenoic acids (63,64) by traces of hydroperoxide present in fatty acid solutions (65) may account for initial production of the required hydroperoxide in the absence of GSH-peroxidase. However, after hydroperoxide is apparently removed by GSH-peroxidase, the oxygenation of fatty acid by lipoxygenase is able to proceed following NEM treatment. But with a K_M of 10^{-6} M for hydroperoxide (66) the peroxidase may be working at too low an efficiency during the brief inhibition period before NEM treatment to remove the last traces of hydroperoxide.

We have found, in agreement with others (40,67), that the only hydroperoxides tested which abolish the kinetic lag period are products of the lipoxygenase oxygenation process. The apparent obligatory role of product in the activity of lipoxygenase, the marked increase in the kinetic lag time caused by GSH-peroxidase and a similarity in structures of the hydroperoxides which eliminate the kinetic lag period all suggest that a binding site exists for hydroperoxide product. The concept of such a binding site is compatible with the results of Mitsuda et al (59). The common structural feature of all hydroperoxy acids which have been shown to reduce the kinetic lag time is an (n-7)-trans-(n-9)-cis diene grouping (Fig. 12). The hydroperoxy grouping must contribute significantly to the binding of product to the enzyme since hydroxy acid, with an identical carbon structure, does not seem to affect the velocity of the reaction at concentrations of 10^{-5} M (Table X).

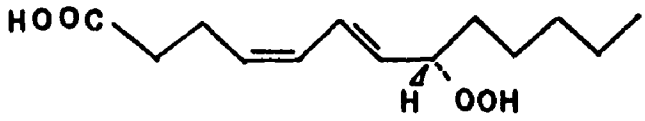


Fig. 12 Common Structural Features of Lipoxygenase Product Activators.

Both 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid and 13-hydroperoxy-9,11-octadecadienoic acid will abolish the lag with either 5,8,11,14-eicosatetraenoic acid or 9,12-octadecadienoic acid. However, in product activated systems the maximum velocity was independent of the nature of the hydroperoxide product but depended only on the nature of the substrate. The rate-determining step in the oxygenation of 8,11,14-eicosatrienoic acid by lipoxygenase is the removal of the 13-L hydrogen (20). If hydroperoxide product, possibly as a hydroperoxy radical as has been suggested (35), abstracts this hydrogen atom, our results suggest that the ease of abstraction may be a function of the methylene hydrogen atom of the substrate and its position with respect to a peroxy radical fixed in both selectivity and position (68). We have found that lipoxygenase is about one and a half times more active toward 5,8,11,14-eicosatetraenoic acid than 9,12-octadecadienoic acid which is qualitatively consistent with the stability of a resonance stabilized free radical intermediate. However, Holman et al (24) found, in contrast to our results, that lipoxygenase was more active toward 9,12-octadecadienoic than 5,8,11,14-eicosatetraenoic acid. The reasons for this discrepancy are not clear although these authors either may not have been working in product-activated systems or were working with a different isozyme (31).

A model for the possible interaction of an enzyme-bound

hydroperoxy radical, a fatty acid substrate and oxygen is diagrammed in Fig. 13. The important features of this model are: (A) the overlap of the (n-6) cis double bond of the 9,12-octadecadienoic acid substrate and the (n-7) trans double bond of 13-hydroperoxy-9,11-octadecadienoic acid product; (B) the close approach of the (n-8) L-hydrogen of the substrate to the hydroperoxyl radical of the product; and (C) the antiparallel positioning of the carbon chains to prevent interaction of the negatively charged carboxyl groups present in both the substrate and product.

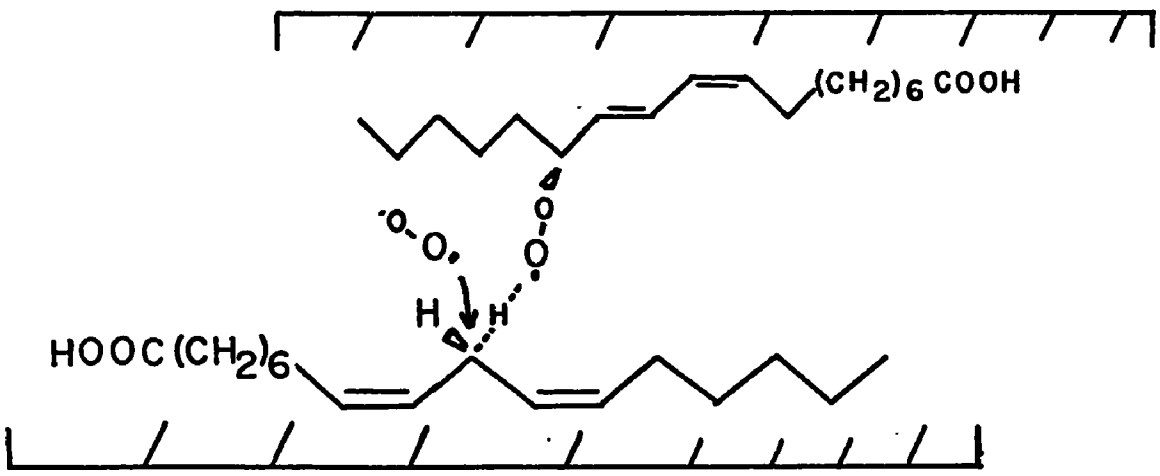


Fig. 13 Mechanism for Removal of the (n-8) Hydrogen Atom from 9,12-octadecadienoic Acid by an Enzyme-bound 13-hydroperoxy-9,11-octadecadienoic Acid Radical.

Kinetic Mechanism for Lipoxygenase

We noted that the specific activity of lipoxygenase was reduced and the length of the kinetic lag period increased as the concentration of fatty acid was increased. These effects of high fatty acid substrate concentrations were qualitatively similar to the effects of

GSH-peroxidase on lipoxygenase and suggested that high concentrations of fatty acid caused a decrease in the level of the enzyme-product complex required for product formation. One explanation was that substrate prevented formation of the enzyme-product complex by being bound at the product binding site. Using this concept of substrate inhibition we formulated a kinetic mechanism for soybean lipoxygenase activity (Fig. 14).

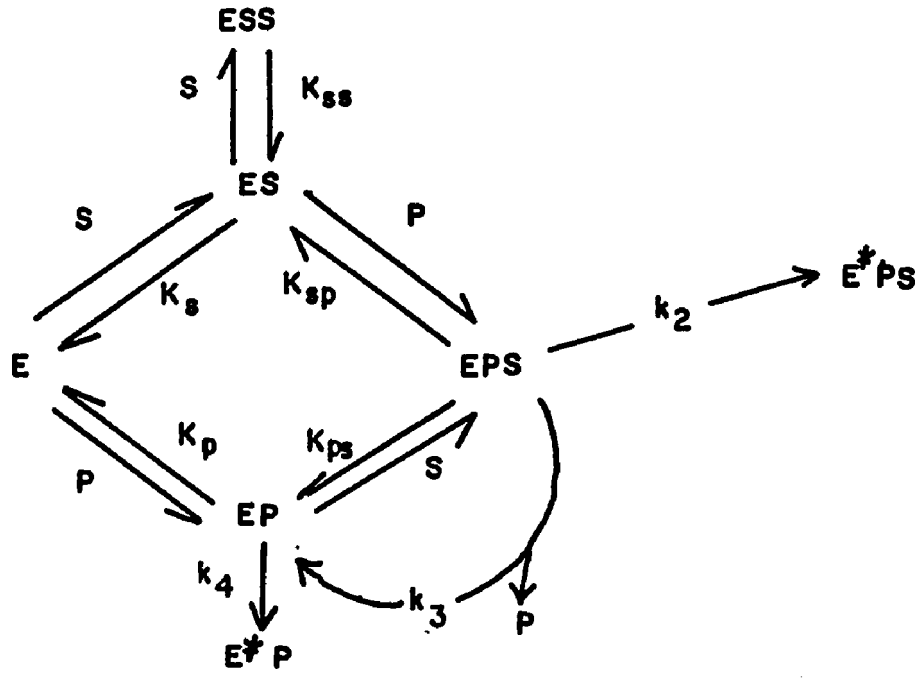


Fig. 14 Kinetic Mechanism for Lipoxygenase Activity.

This formulation supposes that: (A) product (P) and substrate (S) binding sites exist; (B) binding of S to the substrate site may occur in the absence of P; (C) binding of P to the product site may occur in the absence of S; (D) S may bind to the product site; (E) P may not bind to the substrate site; (F) the affinity of S for the substrate

site is higher than the affinity of S for the product site; and (G) all species are in equilibrium with one another. Using these assumptions we can calculate a rate equation for the above model.

$$\text{velocity} = v = k_3[EPS] \tag{8}$$

$$K_s = \frac{[E][S]}{[ES]} \tag{9a}$$

$$K_p = \frac{[E][P]}{[EP]} \tag{9b}$$

$$K_{ps} = \frac{[EP][S]}{[EPS]} \tag{9c}$$

$$K_{sp} = \frac{[ES][P]}{[EPS]} \tag{9d}$$

$$K_{ss} = \frac{[ES][S]}{[ESS]} \tag{9e}$$

From the equilibrium equations (9a-e):

$$[ES] = \frac{[E][S]}{K_s} \tag{10a}$$

$$[EP] = \frac{[E][P]}{K_p} \tag{10b}$$

$$[ESS] = \frac{[ES][S]}{K_{ss}} = \frac{[E][S]^2}{K_s K_{ss}} \tag{10c}$$

$$[EPS] = \frac{[EP][S]}{K_{ps}} = \frac{[ES][P]}{K_{sp}} = \frac{[E][P][S]}{K_{ps}K_p} = \frac{[E][P][S]}{K_s K_{sp}} \tag{10d}$$

Since E_o = total enzyme and $[E^*] = [E^*_{k_4}] + [E^*_{k_2}]$:

$$[E_o] = [E] + \frac{[E][S]}{K_s} + \frac{[E][P]}{K_p} + \frac{[E][P][S]}{K_s K_{sp}} + \frac{[E][S]^2}{K_s K_{ss}} + [E^*] \tag{11a}$$

so

$$[E_o - E^*] = [E]\left\{1 + \frac{[S]}{K_s} + \frac{[P]}{K_p} + \frac{[P][S]}{K_s K_{sp}} + \frac{[S]^2}{K_s K_{ss}}\right\} \tag{11b}$$

and

$$[E] = [E_o - E^*] / \left(1 + \frac{[S]}{K_s} + \frac{[P]}{K_p} + \frac{[P][S]}{K_s K_{sp}} + \frac{[S]^2}{K_s K_{ss}}\right) \qquad (11c)$$

From equations (8) and (10d):

$$v = k_3[EPS] = \frac{k_3[E][P][S]}{K_s K_{sp}} \qquad (12)$$

From equations (11c) and (12):

$$v = \frac{k_3[E_o - E^*][P][S]}{K_s K_{sp}\left(1 + \frac{[S]}{K_s} + \frac{[P]}{K_p} + \frac{[P][S]}{K_s K_{sp}} + \frac{[S]^2}{K_s K_{ss}}\right)} \qquad (13)$$

Dividing both numerator and denominator of equation (13) by ([P][S]):

$$v = \frac{k_3[E_o - E^*]}{\frac{K_s K_{sp}}{[P][S]} + \frac{K_{sp}}{[P]} + \frac{K_s K_{sp}}{K_p[S]} + \frac{K_{sp}[S]}{K_{ss}[P]} + 1} \qquad (14)$$

From equations (9a-d):

$$K_s = \frac{K_p K_{ps}}{K_{sp}} \qquad (15a) \qquad K_s K_{sp} = K_p K_{ps} \qquad (15b)$$

$$\frac{K_s K_{sp}}{K_p} = K_{ps} \qquad (15c) \qquad K_{sp} = \frac{K_p K_{ps}}{K_s} \qquad (15d)$$

From equations (14) and (15c):

$$v = \frac{k_3[E_o - E^*]}{\frac{K_p K_{ps}}{[P][S]} + \frac{K_{sp}}{[P]} + \frac{K_{ps}}{[S]} + \frac{K_{sp}[S]}{K_{ss}[P]} + 1} \qquad (16a)$$

or

$$v = \frac{k_3[E_o - E^*k_4 - E^*k_2]}{\frac{K_{ps}}{[S]}\left(1 + \frac{K_p}{[P]}\right) + \frac{K_{sp}}{[P]}\left(1 + \frac{[S]}{K_{ss}}\right) + 1} \qquad (16b)$$

From equation (4) and (16b):

$$v = \frac{k_3[E_o - E^*k_4] - k_2[P^*]}{\frac{K_{ps}}{[S]}\left(1 + \frac{K_p}{[P]}\right) + \frac{K_{sp}}{[P]}\left(1 + \frac{[S]}{K_{ss}}\right) + 1} \qquad (16c)$$

Rate equation (16c) predicts that no velocity is observed if P is zero since the denominator of the equation is infinite in this situation. This prediction is consistent with the results discussed in "Product Activation of Lipoxygenase."

We set out to estimate values for the various dissociation constants in Equation (16c) for the oxygenation of 5,8,11,14-eicosatetraenoic acid in the following manner. Equation (16c) indicates that the velocity will become independent of the concentration of P when P is much greater than K_{sp} and K_p. In such a case equation (16c) may be simplified to equation (17).

$$v = \frac{k_3[E_o - E^*k_4] - k_2[P^*]}{\frac{K_{ps}}{[S]} + 1} \qquad (17)$$

In this situation the K_{ps} term would be the same as the apparent K_M. We determined an apparent K_M of approximately 5 μM for 5,8,11,14-eicosatetraenoic acid and thus according to our previous assumptions and equation (17), K_{ps} must also be on the order of 5 μM.

With various concentrations of 5,8,11,14-eicosatetraenoic acid used in our assay system, it was observed that the velocity is independent of the product concentration when the concentration of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid is greater than approximately 2-5 μM. The slightly higher threshold levels of product are required with the higher substrate concentrations than with the lower substrate concentrations. We estimated that the values for K_p and K_{sp} must be no greater than 1 μM so that equation (16c) would be reduced to equation (17) when the concentration of hydroperoxide product is greater than 3 μM. The order of magnitude of K_{ss} was difficult to estimate and our original predictions were as high as 200 μM.

During the early part of the oxygenation of 5,8,11,14-eicosatetraenoic acid by lipoxygenase, E* (k_2) and E* (k_4) are essentially zero. Using this initial velocity restriction, equation (16c) may be rearranged to give:

$$v\left[\frac{K_{ps}}{[S]}\left(1+\frac{K_p}{[P]}\right)+1+\frac{K_{sp}}{[P]}\left(1+\frac{[S]}{K_{ss}}\right)\right]=k_3[E_o]=V_{max} \qquad (18)$$

Using equation (18) and data from a series of experiments at constant enzyme concentration in which v, [S] and [P] were known, we attempted to determine a constant value for V_{max} using a series of successive approximations of K_p, K_{ps} and K_{ss}. Our requirements were that V_{max} should be equal or greater than v and values of the dissociation constants should be on the same order of magnitude which we qualitatively predicted as discussed above. One set of estimates which allowed a close fit to both equation (18) and our qualitative observations was $K_{ps} = K_p = K_{sp} = 1$ μM and $K_{ss} = 50$ μM. K_s was then calculated from

equation (15a) to be 1 μM. Table XI summarizes the results of the set of calculations made with the latter dissociation constants.

The contribution of the term, $E^*(k_4)$, to equation (16c) may be estimated in the following manner from equation (10b):

$$\frac{d[E^*_{k_4}]}{dt} = k_4[EP] = \frac{k_4[E][P]}{K_p} \qquad (19)$$

From equations (11c) and (19) and since $[E_o-E^*] = [E_o-[E^*(k_4)]-[E^*(k_2)]$:

$$\frac{d[E^*(k_4)]}{dt} = \frac{k_4[E_o-E^*_{k_4}-E^*_{k_2}][P]}{K_p\left(1+\frac{[S]}{K_s}+\frac{[P]}{K_p}+\frac{[P][S]}{K_sK_{sp}}+\frac{[S]^2}{K_sK_{ss}}\right)} \qquad (20)$$

Dividing both numerator and denominator of equation (20) by [P]:

$$\frac{d[E^*(k_4)]}{dt} = \frac{k_4[E_o-E^*_{k_4}-E^*_{k_2}]}{\frac{K_p}{[P]}+\frac{K_p[S]}{K_s[P]}+\frac{K_p[S]}{K_sK_{sp}}+\frac{K_p[S]^2}{K_sK_{ss}[P]}+1} \qquad (21a)$$

or

$$\frac{d[E^*(k_4)]}{dt} = \frac{k_4[E_o-E^*_{k_4}-E^*_{k_2}]}{\frac{K_p}{[P]}\left(1+\frac{[E]}{K_s}\right)+K_p[S]\left(\frac{1}{K_sK_{sp}}+\frac{[S]}{K_sK_{ss}[P]}\right)+1} \qquad (21b)$$

Using the values of the dissociation constants estimated above and the k_4 value for 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid from Table II of Results:

TABLE XI

Calculation of a Constant V_{max} for Lipoxygenase from the Kinetic Equation and a Set of Dissociation Constants

Experiment	[S] (μM)	[P] (μM)	v (μM/min)	V_{max} (μM/min)
1	55	2	2.7	5.70
2	110	2	1.9	4.98
3	40	15	4.1	4.71
4	94	15	3.9	4.68

Values of [S], [P] and v were taken from Table IX. V_{max} was calculated as described in the Discussion from equation (16c) using $K_{ps} = K_p = K_{sp} = 1$ μM and $K_{ss} = 50$ μM.

$$\frac{d[E^*_{k_4}]}{dt} = \frac{0.027\ [E_o - E^*_{k_4} - E^*_{k_2}]}{\frac{1}{[P]}\left(1 + [S] + \frac{[S]^2}{50}\right) + [S] + 1} \qquad (22)$$

Therefore even with a relatively low concentration of 5,8,11,14-eicosatetraenoic acid (20 μM) and a reasonably high concentration of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (50 μM), formation of $E^*_{k_4}$ will be slow:

$$\frac{d[E^*_{k_4}]}{dt} = 5.1 \times 10^{-4}\ [E_o - E^*_{k_4} - E^*_{k_2}] \qquad (23)$$

This indicates that the inactivation of lipoxygenase by a k_4 process is relatively insignificant during the oxygenation of fatty acid substrate.

SUMMARY

Lipoxygenase catalyzes its own destruction during oxygenation of fatty acid substrates. The destruction is first order with respect to enzyme. The rate constant for this inactivation (k_2) has a characteristic value for each fatty acid. The values are greater for acids having correspondingly higher degrees of unsaturation. In the presence of product, the enzyme loses activity ten times faster with both substrates present than with either alone.

The lipoxygenase-catalyzed oxygenation of fatty acids may be completely and reversibly inhibited by rat liver GSH-peroxidase in the presence of mercaptans. This inhibition appears to be due to the removal of a hydroperoxide product which plays an obligatory role in the lipoxygenase mechanism.

I have confirmed that oxygenation of fatty acids by lipoxygenase occurs with a kinetic lag period which can be abolished by adding product hydroperoxides. I have further shown that the lag period may be extended as a result of inhibition of the enzyme by polyenoic acid substrates.

These findings as well as those of others may be accommodated by a kinetic model which supposes that distinct sites exist for product and fatty acid substrate; product may bind only at the product site whereas fatty acid substrate may bind at either site. The equilibrium rate equation for this mechanism is:

$$v = k_3[E_o]/\{\frac{K_{sp}}{[P]}(1 + \frac{K_p}{[P]}) + 1 + \frac{K_{ps}}{[S]}(1 + \frac{[S]}{K_{ss}})\}$$

For 5,8,11,14-eicosatetraenoic acid, the values of the constants are: $K_p = K_{sp} = K_{ps} = K_s = 1$ μM; $K_{ss} = 50$ μM; $k_2 = 0.32$ min^{-1}; and $k_3 = 100$ min^{-1}.

PART II

STUDIES ON SHEEP VESICULAR GLAND DIOXYGENASES

EXPERIMENTAL METHODS

Materials

All solvents were reagent grade and were used without further purification. Silica Gel G was obtained from Brinkman Industries.

[1-^{14}C]-5,8,11,14-eicosatetraenoic (52 mc/mM) and [5,6,8,9,11,12,14,15-^{3}H]-5,8,11,14-eicosatetraenoic (5c/mM) acids were purchased from Applied Science Laboratories and New England Nuclear Corporation, respectively. 5,8,11,14-eicosatetraenoic acid (high purity grade) was obtained from the Hormel Institute; 8,11,14-eicosatrienoic acid was generously donated by the Upjohn Company. The fatty acids were dissolved in benzene containing Santoquin* (0.3% w/w) and stored at -20°.

Reduced glutathione, sodium diethyldithiocarbamate, 1,10-phenanthroline and Bathocuproine (sodium salt, disulfonate) were purchased from Sigma Chemical Company. Tiron (4,5-dihydroxy-m-benzene-disulfonic acid, disodium salt) was a product of Eastman Organic Chemicals.

Sheep vesicular gland, vesicular gland acetone powder, PGE_1 and $PGF_{1\alpha}$ were generously donated by the Upjohn Company. All other chemicals were reagent grade obtained from common commercial sources.

Determination of GSH-peroxidase Activity

GSH-peroxidase was assayed as described in Methods (Part I).

Determination of VG Dioxygenase Activity

by Radioisotope Assays

In the radioisotope procedure for measuring vesicular gland dioxygenase activity, reactions were initiated by addition of 0.05 to 0.2 ml of vesicular gland preparations (1-5 mg) to incubation mixtures containing either [5,6,8,9,11,12,14,15-^{3}H]- or [1-^{14}C]-5,8,11,14-eicosatetraenoic acid in 0.1 M TrisHCl (pH 7.4, 8.5 or 9.0), or 0.25 M sucrose (pH 7.4). The final volume of all incubation mixtures was 1.0 ml. Assays were performed at room temperature with occasional shaking. The incubations were stopped by the addition of 7.0 ml of chloroform:methanol (1:1). The suspension was centrifuged to remove the precipitated protein, and the supernatant was poured into tubes containing 3.0 ml of chloroform and 1.6 ml of 0.88% formic acid (v/v) and mixed. The resultant lower phase was evaporated to dryness with the aid of a stream of nitrogen, the residue dissolved in a small volume of chloroform and quantitatively transferred to a Silica Gel G thin layer plate. Thin layer plates (20 x 20 cm) were spread 0.37 mm thick with a slurry prepared by mixing Silica Gel G with water, air dried 2-4 hours and activated for 90 min at 115°. The plate was developed to 15 cm in benzene:dioxane:acetic acid:formic acid (82:14:1:1) and air dried for 15 minutes. A line was then scratched across the layer of silica gel 7.5 cm above the origin and the plate developed to that line with acetone:methylene chloride (60:40). Oleic, ricinoleic (12-hydroxy-oleic), cholic and deoxycholic acids were routinely used as reference compounds. The standards were visualized with the aid of iodine vapor, and the plate was sectioned (Fig. 15) into regions corresponding to free fatty acid, hydroxy acids, PGE, and PGF with

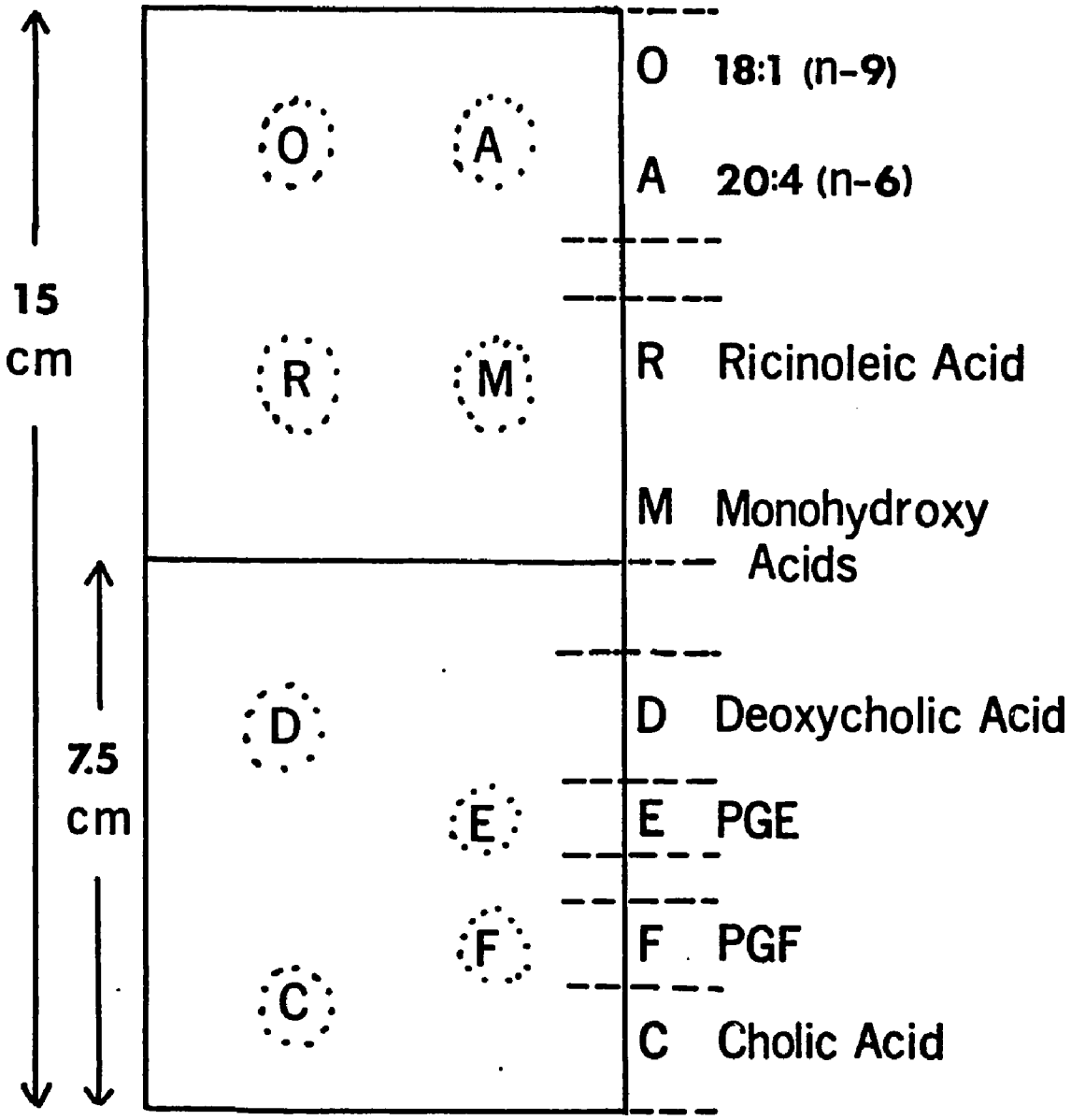


Fig. 15. Thin-layer Chromatographic System to Separate Products for the Radioisotope Assay of Prostaglandin Synthesis.

Horizontal dotted lines indicate sections which were scraped into vials and counted.

appropriate unoccupied intermediate bands. After spontaneous loss of the absorbed iodine, the sections were scraped into scintillation vials, suspended in 6 ml of toluene scintillation fluid (69) and assayed for radioactivity in a Packard series 2000 scintillation spectrometer. The amounts of radioactive material produced were estimated after subtracting the experimental results from the values obtained in control incubations without enzyme.

Determination of VG Dioxygenase Activity by Oxygen Absorption Measurements

All oxygen absorption measurements were performed on a Yellow Springs Instrument Company Model 53 Oxygen Monitor equipped with a Model 5301 Bath Assembly. The monitor was attached to a Beckman Model 100502 Linear-Log Ten-Inch Potentiometric Recorder, and all readings were made using the % transmittance setting of the recorder. A constant temperature of 29.5 ± 0.5° was maintained with a Haake Type F Thermoregulated circulator bath. Oxygen uptake measurements were routinely made using the "air" position of the oxygen monitor and a 100 mV recorder setting. To double the sensitivity, the "O_2" position of the oxygen monitor and a 10 mV recorder setting were employed using a voltage offset attached to the recorder. In most experiments, the total final volume of the reaction mixture in the sample chamber was 3.0 ml. Additions were made through the side of the electrode holder. The maximum volume of any addition made at one time was 0.25 ml (less than 10% of the total sample volume).

To determine if oxygen uptake assayed on the oxygen monitor was a valid measure of prostaglandin synthesis, an experiment was

performed monitoring oxygen uptake using [^{3}H]-5,8,11,14-eicosatetraenoic acid as substrate. Fig. 16 shows the correspondence between the radioisotope assay and oxygen electrode assay with two concentrations of acetone powder treated with phenol and one concentration of untreated acetone powder.

Purification of GSH-peroxidase

GSH-peroxidase was partially purified from rat liver as described in Methods (Part I).

Preparation of VG Dioxygenase

Two fractions were prepared from sheep vesicular gland which contained the VG dioxygenase activity: (A) 3000 g.min supernatant; and (B) VG acetone powder. The 3000 g.min supernatant was prepared by homogenizing the vesicular gland in 1-3 volumes of 0.1 M TrisHCl (pH 7.4 or pH 9.0) or 0.25 M sucrose (pH 7.4) per g of gland in a Dounce ball-type homogenizer using a loose pestle. The pH was adjusted to 7.4 with a 1% Tris solution. Homogenization and all other procedures were carried out at 0-4°. The homogenate was centrifuged at 600 x g for 5 min to yield the VG 3000 g.min supernatant.

Protein concentrations were estimated using a nomograph based on the extinction coefficients for enolase and nucleic acid given by Warburg and Christian (70). The spectrophotometric measurements were performed on a Beckman DB Spectrophotometer using protein solubilized with 0.5% deoxycholate. The average of at least two spectrophotometric readings were used.

Two similar VG acetone powder preparations were used in this work. One was prepared by Dr. Robert E. Lee according to the following

Fig. 16. Comparison of the Radioisotope and Oxygen Absorption Assays for Prostaglandin Synthesis.

All reactions were performed in 0.1 M TrisHCl (pH 8.5) containing 3.3 μmoles GSH in a final total volume of 3.5 ml. Reactions were initiated by the addition of 142 nmoles of ^{3}H-20:4 (n-6) to the reaction mixture 2 min after the addition of acetone powder. At the indicated times the electrode was withdrawn from the sample and a 1.0 ml aliquot added to 7.0 ml of $CHCl_3$:MeOH (1:1) and product formation measured by the radioisotope assay outlined in Methods. Percent reaction for the O_2 electrode measurements was calculated by dividing the scale deflection at the time of withdrawal of the O_2 electrode by two times the scale deflection obtained with an equivalent amount of substrate treated with excess lipoxygenase. Percent reactions for the radioisotope assays were obtained by calculating the percent of counts in the FFA region of the TLC place in the zero time control which subsequently chromatographed with lower R_f values after incubations were performed. These calculated % values were multiplied by 142 nmoles 20:4 (n-6) present initially to give the nmoles of product formed. Curve: (A) 4 mg acetone powder; 2 μmoles phenol; (B) 2 mg acetone powder; 2 μmoles phenol; and (C) 4 mg acetone powder; no phenol. Product formation calculated from oxygen uptake (x-x) and fatty acid consumption (o-o).

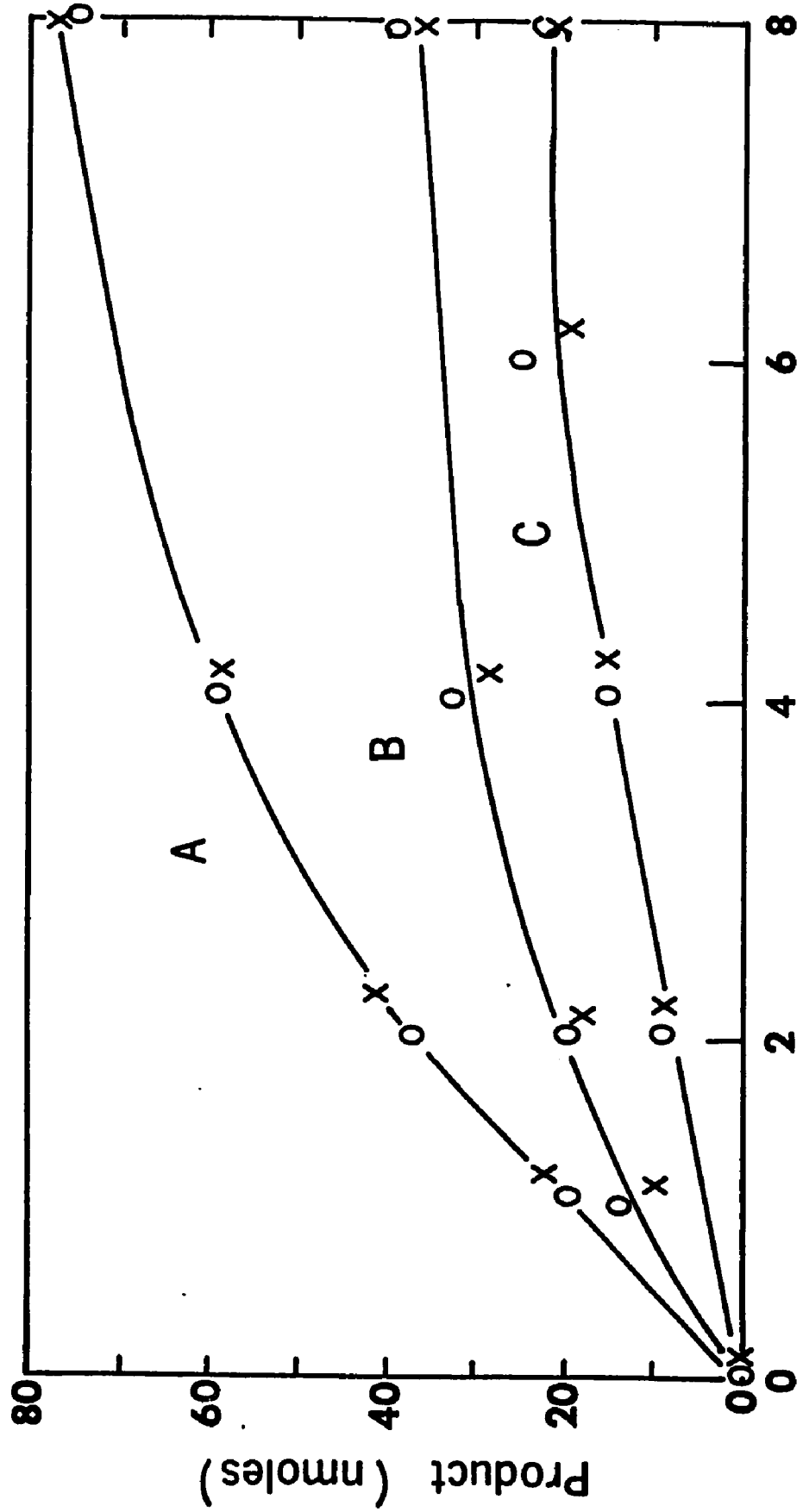


Fig. 16. Comparison of the Radioisotope and Oxygen Absorption Assays for Prostaglandin Synthesis .

procedure. All procedures were carried out at 0-4°. A homogenate of sheep vesicular gland was prepared by homogenizing 20 g of minced tissue with a Dounce ball-type homogenizer in 30 ml of 0.25 M sucrose solution, pH 7.4, which was 1 mM in EDTA. The pH of the homogenate was adjusted to 7.4 with dropwise additions of a solution of 1% Tris and centrifuged at 7000 x g for 10 min. Ascorbate (1 mM) was added to the supernatant, and the pH was adjusted to 5.2 with 0.5 M citric acid. This supernatant was centrifuged at 25,000 x g for 15 min and the supernatant discarded. The pellet was added with trituration and agitation to 500 ml of anhydrous acetone which had been cooled to -20 to -30 °C. The suspension was filtered and washed with three 200 ml portions of cold (-20°) acetone. The solid material was immediately dried in vacuo over P_2O_5 in a precooled desiccator. The yield of acetone powder varied from 300 to 500 mg from 20 g of tissue. The acetone powder preparation used in many experiments was a generous gift of the Upjohn Company and was prepared by a procedure similar to that described above for the acetone powder prepared by Dr. Lee. Powder was routinely suspended to a concentration of 20-50 mg/ml in 0.1 M TrisHCl (pH 7.4, 8.5 or 9.0) using a small Dounce ball-type homogenizer. The 3000 g.min supernatant and acetone powder suspensions were used within 24 hours of preparation.

RESULTS

The Effect of GSH-peroxidase on the Oxygenation of Polyenoic Acids by Sheep Vesicular Gland Preparations

Rat liver GSH-peroxidase catalyzes the nucleophilic cleavage of a wide variety of hydroperoxides with a variety of mercaptans (44). In an attempt to study the vesicular gland dioxygenase in isolation from the subsequent enzymic steps in the pathway leading to prostaglandins, preparations of GSH-peroxidase were added to incubation mixtures to accelerate hydroperoxide reduction and hopefully to produce exclusively twenty carbon hydroxy fatty acids. However, when partially purified rat liver GSH-peroxidase was added to samples containing vesicular gland 3000 g.min supernatant, [^{14}C]-5,8,11,14-eicosatetraenoic acid and glutathione, it did not cause an accumulation of hydroxy fatty acids, but rather inhibited all oxygenation of substrate (Table XII). Inhibition of VG low-speed supernatant activity (2.5 mg protein/ml) in the presence of 0.65 mM GSH was 50% complete with 120 units/ml of GSH-peroxidase. Table XIII shows that the inhibition of the oxygenation of 5,8,11,14-eicosatetraenoic acid by GSH-peroxidase is dependent upon the presence of GSH in the reaction mixture.

The partial inhibition by GSH-peroxidase of the oxygenation of 5,8,11,14-eicosatetraenoic acid by sheep vesicular gland supernatant may be reversed by the addition of NEM to the GSH-peroxidase-inhibited reaction mixture (Fig. 17 and Fig. 18).

TABLE XII

Effect of GSH-peroxidase on Prostaglandin Synthesis by VG 3000 g.min Supernatant

Reactions in this experiment were initiated by the addition of 2.5 mg of protein from a sheep vesicular gland 3000 g.min supernatant prepared as described in Methods in 0.1 M TrisHCl (pH 7.4). The incubation mixtures contained [1-^{14}C]-5,8,11,14-eicosatetraenoic acid, 0.65 mM GSH and GSH-peroxidase as indicated. The GSH-peroxidase preparation used in this experiment was assayed at pH 7.4 as described in Methods. After 6 min incubation times at room temperature, the reactions were stopped with $CHCl_3$:MeOH (1:1), lipids extracted and thin layer chromatography done as described in Methods. The table shows the total counts in each fraction of the TLC plate along with the percent reaction calculated by subtracting the percentage of total counts in the FFA region in each sample from the percentage of total counts in the FFA region of the 0' control.

Sample

TABLE XII	0' Control	1	2	3	4	Product
GSH-Peroxidase (units)	--	0	24	120	600	
[GSH] (mM)	--	0.65	0.65	0.65	0.65	
	13963	8984	10026	11893	13045	FFA
	127	71	102	175	84	
	549	1393	1225	850	600	OH-FA
	276	1048	817	686	630	11-dehydro-PGF
^{14}C counts per 2 min	22	200	155	138	104	
	63	2611	2002	1175	505	PGE
	2	431	300	241	67	
	28	189	228	358	133	PGF
	170	292	334	260	374	
Total counts	15200	15219	14919	15776	15542	
% total counts in FFA fraction	91.9	59.0	67.2	75.4	84.2	
% Reaction	0	32.9	24.7	16.5	7.7	

TABLE XIII

The Effect of GSH on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG 3000 g.min Supernatant

Reactions in this experiment were initiated by the addition of 2.5 mg of protein from a sheep vesicular gland 3000 g.min supernatant prepared as described in Methods in 0.1 M TrisHCl (pH 7.4). The incubation mixtures contained [1-^{14}C]-5,8,11,14-eicosatetraenoic acid, GSH and GSH-peroxidase as indicated in the table. The GSH-peroxidase preparation used in this experiment was assayed at pH 7.4. After 6 min incubation times at room temperature, the reactions were stopped with $CHCl_3$:MeOH (1:1), lipids extracted and thin layer chromatography performed as described in Methods. The percent reaction is calculated by subtracting the percentage of total counts in the FFA region in each sample from the percentage of total counts in the FFA region of the 0' control.

Sample

TABLE XIII	0' Control	1	2	3	4	5	6
GSH-peroxidase (units)	--	0	0	12	60	180	180
[GSH] (mM)	--	0.65	0	0.65	0.65	0.65	0
^{14}C counts per 2 min in FFA fraction	15080	11407	10591	11170	11968	13506	11783
Total counts	16339	15821	14946	14645	15175	16022	15879
% total counts in FFA fraction	92.4	72.1	70.9	76.3	78.8	84.3	74.2
% Reaction	0	20.3	21.5	16.1	13.6	8.1	18.2

Fig. 17. The Effect of NEM on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG 3000 g.min Supernatant.

After the preincubations described below were performed, aliquots of the protein solutions (0.4 ml) were added to the respective substrate solutions (0.6 ml) as indicated in the figure. Incubations were performed and VG 3000 g.min supernatant was prepared as described in Methods; GSH-peroxidase was assayed at pH 7.4.

Protein Solutions: Solution A: Vesicular gland 3000 g.min supernatant (100 mg) was allowed to stand at 0°, with 1300 nmoles GSH and 2500 units GSH-peroxidase for 5 min in a final volume of 2.65 ml of 0.1 M TrisHCl (pH 7.4). Solution A_1: 1.0 ml of Solution A was added to 0.2 ml of 10 mM NEM and allowed to stand 3 min at room temperature; Solution A_2: 1.0 ml of Solution A was added to 0.2 ml of 0.1 M TrisHCl (pH 7.4) and allowed to stand 3 min at room temperature. Solutions B, B_1 and B_2 were prepared in a manner identical to Solutions A, A_1 and A_2 respectively except no GSH-peroxidase was present in the B solutions.

Substrate Solutions: Substrate solutions 1,2,3 and 4 each contained 330 nmoles GSH, 1500 units GSH-peroxidase and 5 nmoles of ^{14}C-20:4(n-6) in a final volume of 0.50 ml of 0.1 M TrisHCl (pH 7.4). After allowing the samples to stand 5 min at 25°, 0.1 ml of 10 mM NEM was added to samples 1 and 2; and 0.1 ml of 0.1 M TrisHCl (pH 7.4) was added to samples 3 and 4. The substrate solutions were then allowed to stand 3 min at room temperature before the oxygenation reactions were started. Substrate solutions 5,6,7 and 8 were treated as substrate solutions 1,2,3 and 4 respectively except no GSH-peroxidase was added.

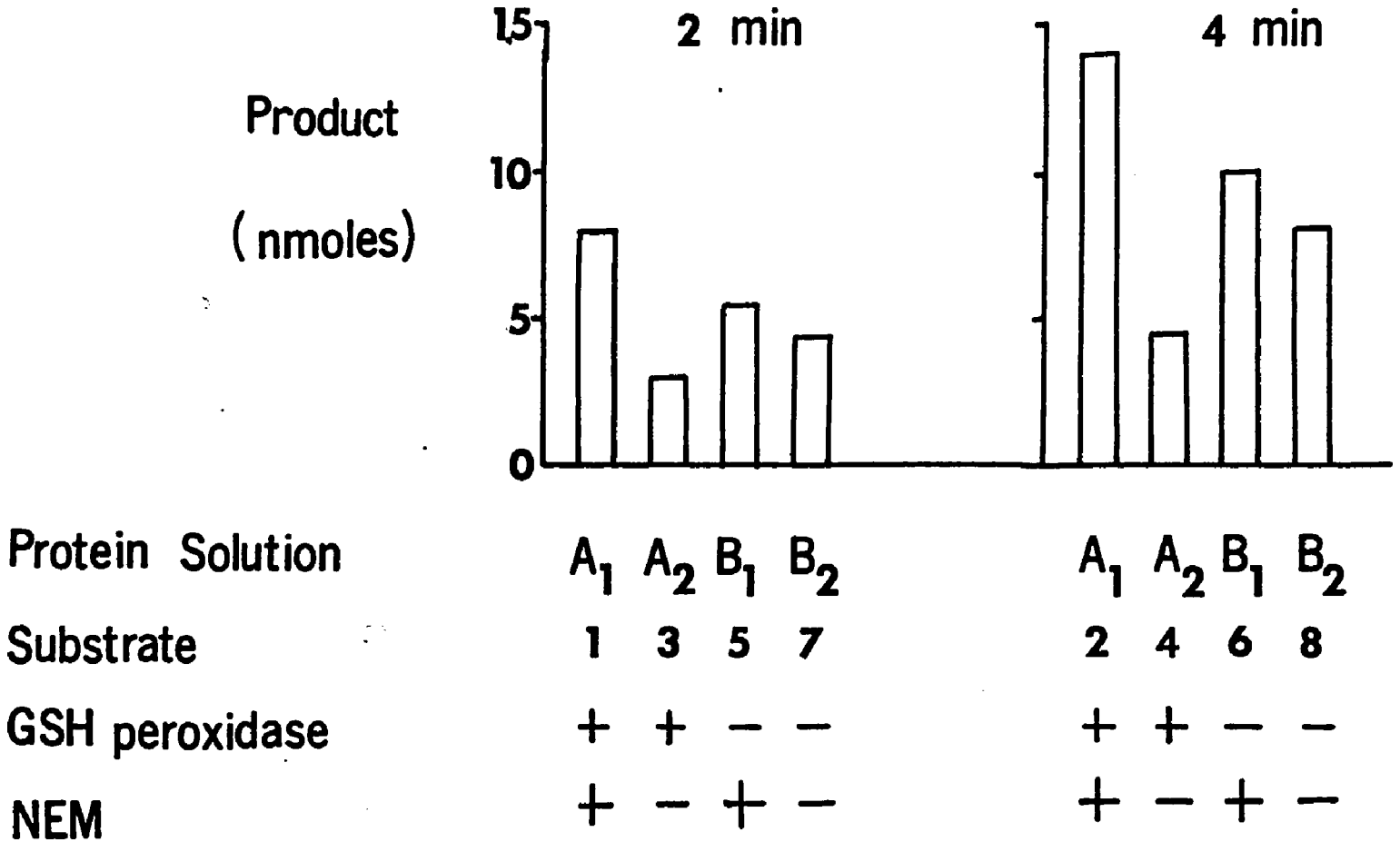


Fig. 17. The Effect of NEM on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG 3000 g.min Supernatant.

Fig. 18. The Effect of NEM on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG 3000 g.min Supernatant.

After the preincubations described below were performed, aliquots of the protein solutions (0.4 ml) were added to the respective substrate solutions (0.6 ml) as indicated in the figure. Incubations were performed and VG 3000 g.min supernatant was prepared as described in Methods; GSH-peroxidase was assayed at pH 7.4.

Protein Solutions: Solution A: VG 3000 g.min supernatant (100 mg) was allowed to stand at room temperature, with 1600 nmoles GSH and 2500 units GSH-peroxidase for 3 min in a final volume of 2.65 ml of 0.1 M TrisHCl (pH 7.4). Solution A_1: 1.0 ml of Solution A was added to 0.25 ml of 10 mM NEM and allowed to stand 3 min at room temperature. Solution A_2: 1.0 ml of Solution A was added to 0.25 ml of 0.1 M TrisHCl (pH 7.4) and allowed to stand 3 min at room temperature. Solutions B, B_1 and B_2 were prepared in a manner identical to Solutions A, A_1 and A_2 respectively except no GSH-peroxidase was present in the B solutions.

Substrate Solutions: Substrate solutions 1,2,3 and 4 each contained 650 nmoles GSH, 1500 units of GSH-peroxidase and 10 nmoles ^{14}C-20:4(n-6) in a final volume of 0.4 ml of 0.1 M TrisHCl (pH 7.4). After allowing the samples to stand 3 min at room temperature, 0.2 ml of 10 mM NEM was added to substrate solutions 1 and 2; and 0.2 ml of 0.1 M TrisHCl (pH 7.4) was added to samples 3 and 4. The solutions were then allowed to stand 3 min at room temperature before the oxygenation reactions were initiated. Substrate solutions 5,6,7 and 8 were treated as substrate solutions 1,2,3 and 4 respectively except no GSH-peroxidase was added.

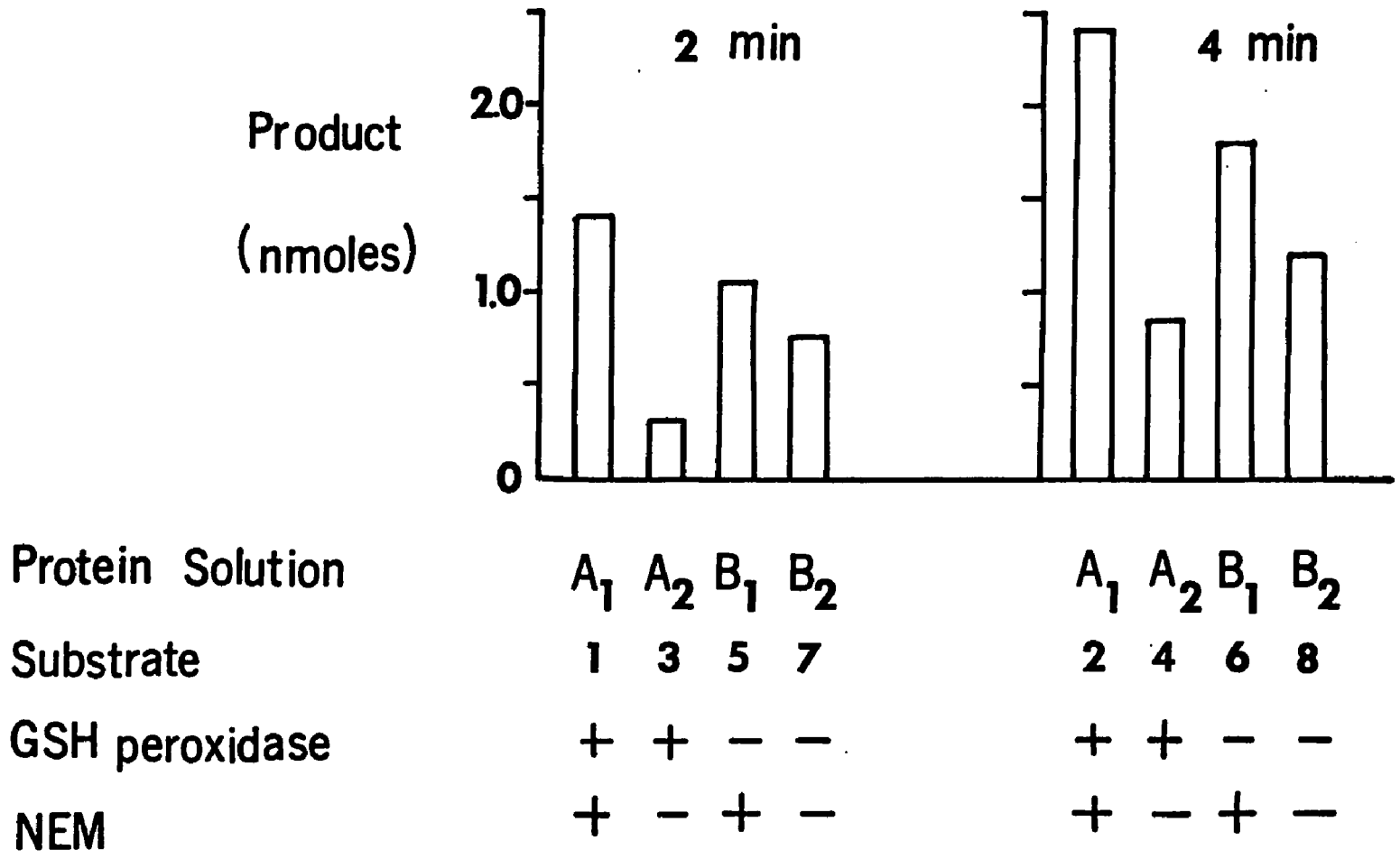


Fig. 18. The Effect of NEM on the GSH-peroxidase Inhibition of the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG 3000 g.min Supernatant.

Although GSH-peroxidase inhibits the oxygenation of 5,8,11,14-eicosatetraenoic acid catalyzed by VG 3000 g.min supernatant, no inhibition of the oxygenation of 8,11,14-eicosatrienoic acid catalyzed by VG acetone powder was observed with 12,500 units/ml of GSH-peroxidase (Table XIV). Sensitivity to GSH-peroxidase inhibition appeared to be lost during preparation of VG acetone powder.

We found, however, as shown in Table XV that the more active phenol-treated acetone powder is inhibited by GSH-peroxidase. This inhibition was 50% complete with phenol-treated VG Powder (0.16 mg/ml) in the presence of 2100 units/ml of GSH-peroxidase and 1.1 mM GSH.

The Effect of Phenol on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder

Previous workers had shown that phenolic antioxidants cause an increase in the rate of prostaglandin synthesis with microsomal preparations of sheep vesicular gland (3) and acetone powders of bovine seminal vesicles (19). Because the phenol-treated acetone powder showed an inhibition by GSH-peroxidase similar to that observed with the native VG 3000 g.min supernatant, we decided to study the nature of the phenol activation process and the kinetic properties of the phenol-treated enzyme.

Fig. 19 shows that a slight instantaneous increase followed by a sigmoidal increase in the initial rate of the oxygenation reaction is observed when acetone powder (0.66 mg/ml) was preincubated for different times with 0.66 mM phenol before addition of fatty acid substrate to the reaction chamber. The data indicate that activation

TABLE XIV

The Effect of GSH-peroxidase on the Oxygenation of 8,11,14-eicosatrienoic Acid by VG Acetone Powder

Oxygen uptake was measured as described in Methods and fatty acid substrate consumption calculated as in Fig. 16. Lag time was measured as shown in Fig. 10. All samples contained 5.0 mg Upjohn acetone powder, 3.3 μmoles GSH and the indicated levels GSH-peroxidase. GSH-peroxidase used in this experiment was assayed at pH 8.5. After a two min preincubation, reactions were initiated by the addition of 100 nmoles of 8,11,14-eicosatrienoic acid to the sample chamber to yield a final volume of 3.0 ml of 0.1 M TrisHCl (pH 8.5).

Sample	GSH-peroxidase (units)	Lag Time (min)	Velocity (μM20:3/min)
1	0	0.40	2.5
2	12,500	0.36	2.2
3	20,000	0.42	3.2
4	25,000	0.42	2.8
5	37,500	—	5.6

TABLE XV

The Effect of GSH-peroxidase on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Phenol-treated VG Acetone Powder

Oxygen uptake was measured as described in Methods and fatty acid substrate consumption calculated as in Fig. 16. Upjohn VG acetone powder (20 mg) was homogenized in 0.1 M TrisHCl (pH 8.5) containing 0.66 mM phenol. The homogenate was allowed to stand at room temperature for 30 min to allow complete activation and then placed in ice. Aliquots of the cooled homogenate were added to solutions containing 0.66 mM phenol, 1.1 mM GSH, 60 μM 5,8,11,14-eicosatetraenoic acid and the indicated units of GSH-peroxidase to yield a total volume of 3.0 ml of 0.1 M TrisHCl (pH 8.5). The GSH-peroxidase preparation used in this experiment was assayed at pH 8.5.

Sample	GSH-peroxidase (units)	Phenol-treated VG Powder (mg)	Velocity (μM20:4/min)	% Inhibition
1	0	0.5	2.9	0
2	6300	0.5	1.45	50
3	0	1.0	4.5	0
4	6300	1.0	2.6	42
5	0	2.0	7.6	0
6	6300	2.0	5.2	32
7	0	2.0	7.1	0
8	12,500	2.0	4.4	38

Fig. 19. The Effect of Preincubation of VG Acetone Powder with Phenol on the Rate of Oxygenation of 5,8,11,14-eicosatetraenoic Acid.

All rates were obtained by measurements of oxygen uptake as outlined in Methods and Fig. 16. For measurements of phenol activation, Upjohn VG acetone powder (0.83 mg/ml) was incubated for a total of five min in the reaction chamber at 30°. Curve A: Phenol (0.66 mM, final concentration) was added to the sample at various times before addition of 5,8,11,14-eicosatetraenoic acid (60 μM, final concentration) to initiate the reaction as indicated in the figure. Curve B was determined by subtracting the activity of untreated acetone powder after 5 min preincubation from Curve A. Final volume in the reaction mixture was 3.0 ml 0.1 M TrisHCl (pH 8.5). Measurement of the activity of untreated powder was performed by adding the powder to the sample chamber at a final concentration of 0.83 mg/ml at the indicated times before addition of fatty acid substrate (Curve C).

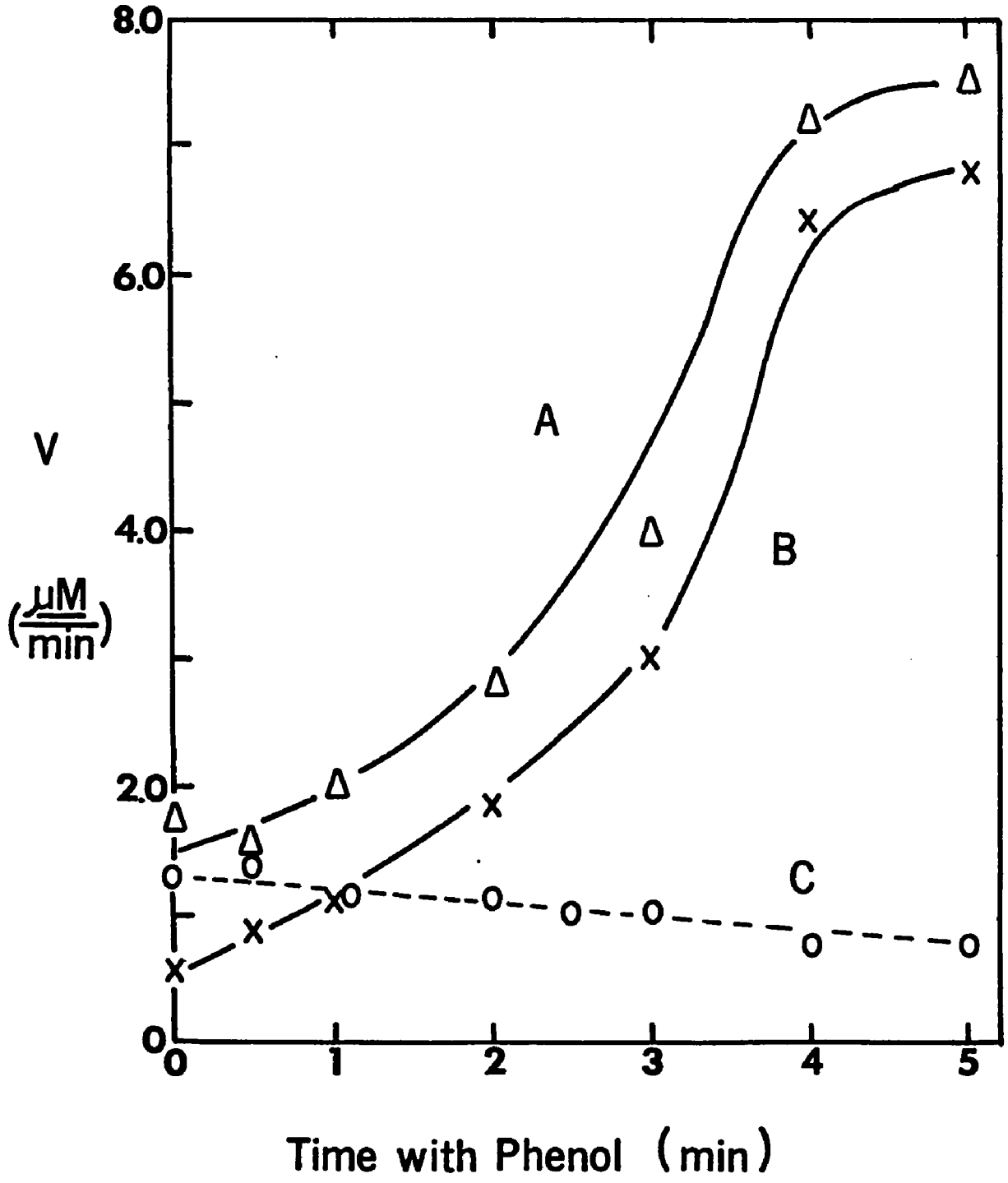


Fig. 19. The Effect of Preincubation of VG Acetone Powder with Phenol on the Rate of Oxygenation of 5,8,11,14-eicosatetraenoic Acid.

by phenol was complete in 5 min in this system. Also shown for comparison is the initial rate of oxygenation observed when acetone powder (0.66 mg/ml) was preincubated in the absence of phenol. Fig. 20 shows that the time required to reach half maximal activity in 0.66 mM phenol is independent of the powder concentration over a four fold range of VG acetone powder concentrations. A phenol concentration of 0.66 mM was chosen for the activation experiments since we observed that the rate of oxygenation in the presence of phenol without preincubation with phenol was maximal at this concentration (Fig. 21). If acetone powder is incubated with phenol to achieve maximal activation, and phenol is then essentially removed by dilution, the activity of the VG powder rapidly falls to its original level of activity (Fig. 22). This deactivated VG powder may again be reactivated by phenol to 85% of its original activity with the same time dependence shown in Fig. 19.

The Self-catalyzed Inactivation of VG Acetone Powder During Oxygenation of Fatty Acid Substrates

If 5,8,11,14-eicosatetraenoic acid is combined with acetone powder in the absence of phenol, the reaction velocity decreases to zero before all the fatty acid is oxidized; if more acetone powder is then added to the reaction mixture, product formation occurs at a rate which (at saturating substrate levels) is directly proportional to the amount of fresh enzyme added. If the instantaneous velocity for many time points during the reaction are plotted against the concentration of 5,8,11,14-eicosatetraenoic acid at that time, a linear decrease in velocity with substrate concentration is observed (Fig. 23). A similar linear decrease in velocity with substrate concentration occurs if GSH

Fig. 20. The Effect of VG Acetone Powder Concentration on the Rate of Phenol Activation.

All rates were obtained by measurements of oxygen uptake as outlined in Methods and Fig. 16. Upjohn VG acetone powder was incubated for a total of five min in the reaction chamber at 30°. Phenol (0.66 mM, final concentration) was added to the sample at the times indicated in the figure before addition of 5,8,11,14-eicosatetraenoic acid (60 μM, final concentration) to initiate the reaction. The rates of oxygenation by untreated VG powder (at equivalent concentrations) after 5 min preincubations are subtracted from all rates. Curve: (A) 1.33 mg/ml; (B) 0.66 mg/ml; and (C) 0.33 mg/ml. Final reaction volume was 3.0 ml of 0.1 M TrisHCl (pH 8.5).

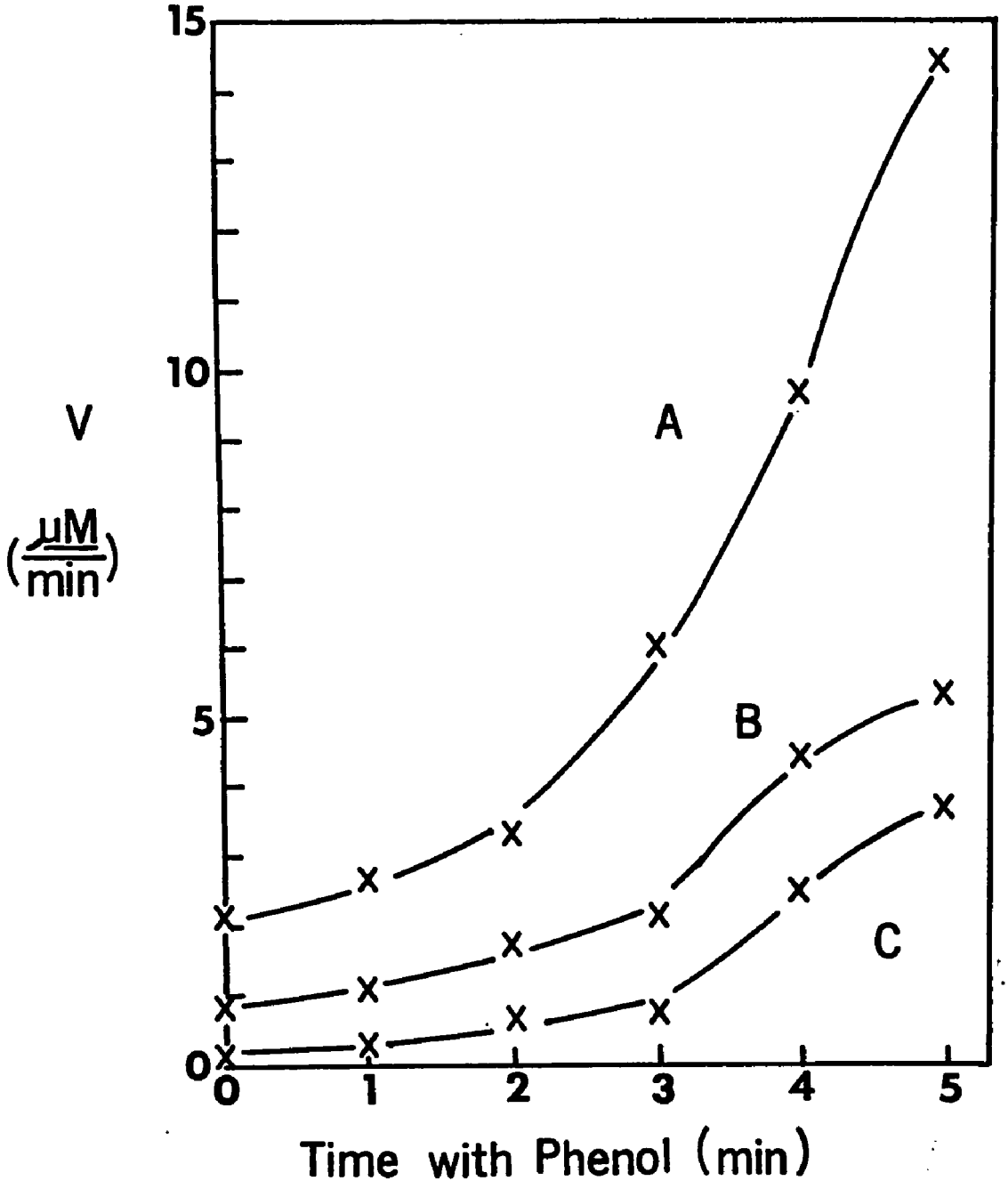


Fig. 20. The Effect of VG Acetone Powder Concentration on the Rate of Phenol Activation.

Fig. 21. The Effect of the Phenol Concentration on the Rate of Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder.

All rates were obtained by measurements of oxygen uptake as outlined in Methods and Fig. 16. Upjohn VG powder (0.83 mg/ml, final concentration) was added to the reaction chamber containing 5,8,11,14-eicosatetraenoic acid (60 μM) and the indicated phenol concentration to initiate oxygen uptake. Curve: (A) observed initial rates; and (B) rate of oxygen uptake in the absence of phenol subtracted from all points on Curve A.

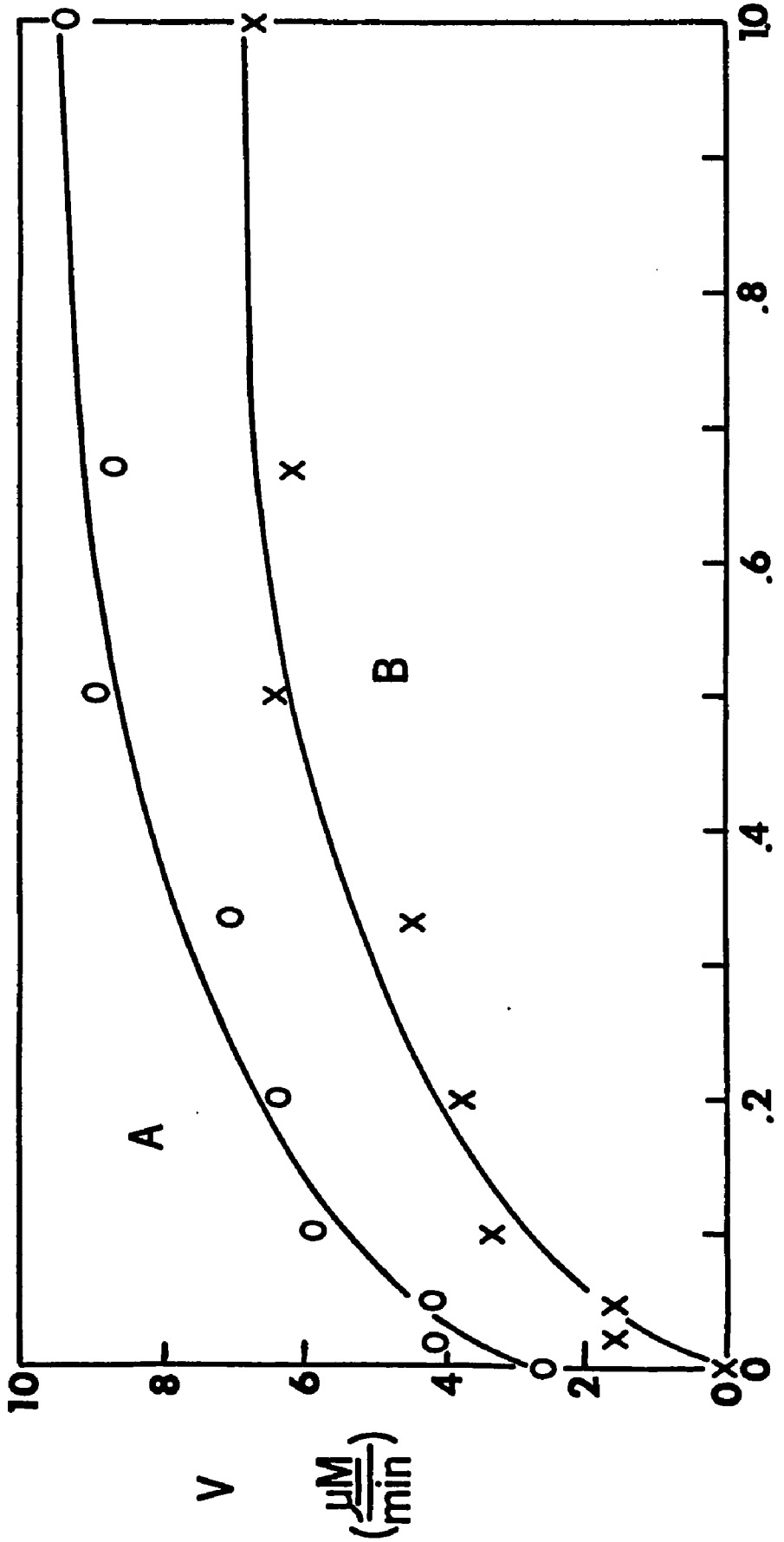


Fig. 21. The Effect of the Phenol Concentration on the Rate of Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder.

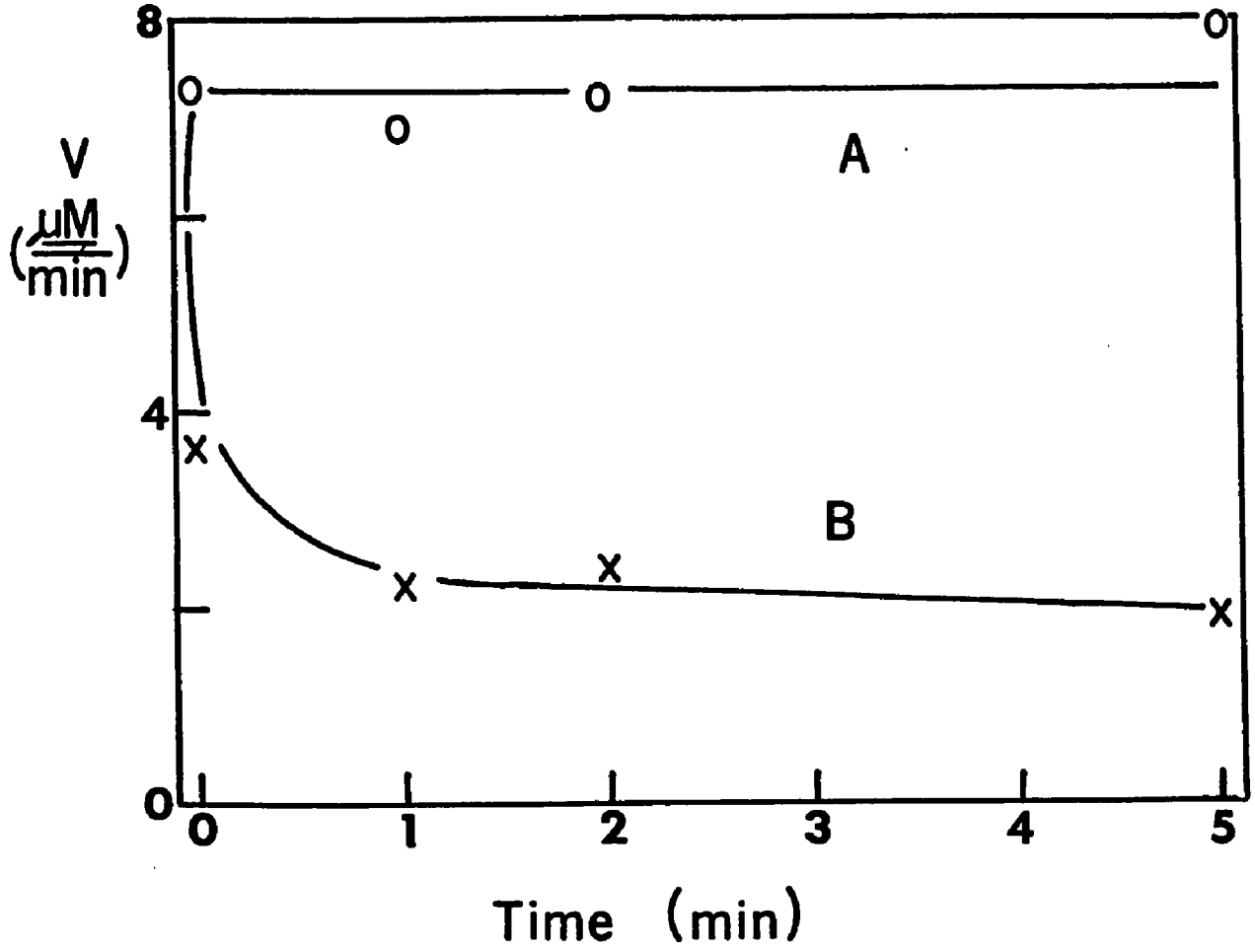


Fig. 22. Deactivation of the Phenol-Activated VG Acetone Powder.

All rates were obtained by measurements of oxygen absorption as outlined in Methods and Fig. 16. Upjohn VG acetone powder was treated with 0.66 mM phenol for 30 min at room temperature and then kept at 0-4° before use. Aliquots of the activated enzyme were added to the assay mixtures and 5,8,11,14-eicosatetraenoic acid (60 μM, final concentration) was added after the times indicated to initiate the reactions. The final volume in all assays was 3.0 ml of 0.1 M TrisHCl (pH 8.5). Curve: (A) assay mixture contained 0.66 mM phenol; and (B) assay mixture contained 0.022 mM phenol.

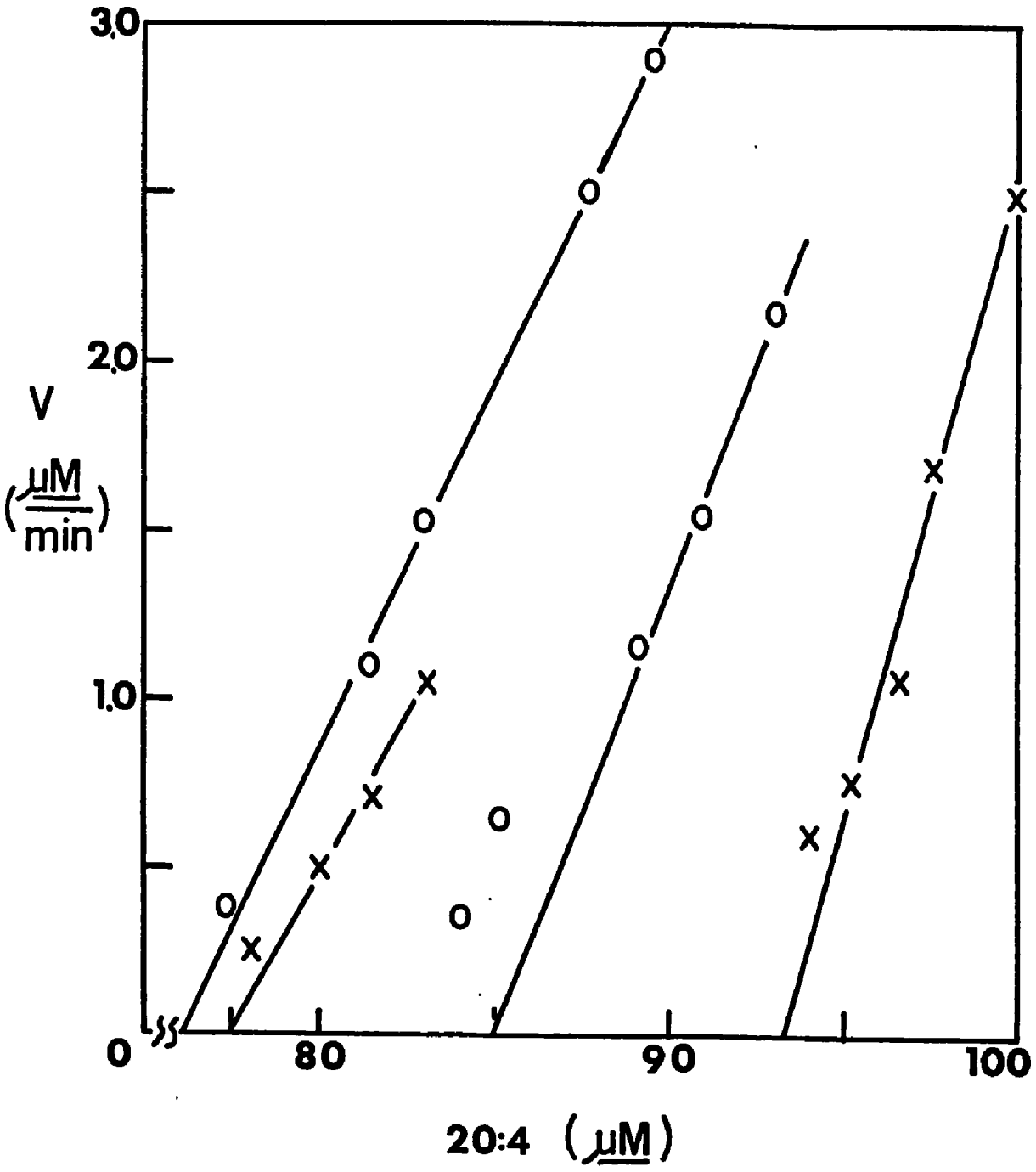


Fig. 23. Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder.

The instantaneous rate of substrate consumption at various concentrations of 5,8,11,14-eicosatetraenoic acid was determined from oxygen uptake tracings similar to Gilford tracings shown in Fig. 3. Final volume in all samples was 3.0 ml.

and GSH-peroxidase are included in the reaction mixture to remove hydroperoxide (Fig. 24).

The phenol-treated VG powder is also inactivated during the oxygenation process. This is shown for the phenol-treated acetone powder with 5,8,11,14-eicosatetraenoic acid (Fig. 25) and with 8,11,14-eicosatrienoic acid (Fig. 26). The rate constant for inactivation with 5,8,11,14-eicosatetraenoic acid in the phenol-treated system (0.55 min^{-1}) seems to be decreased slightly (0.36 min^{-1}) if GSH is included in the reaction mixture (Fig. 25). However, the untreated acetone powder exhibits a lower rate of inactivation (0.24 min^{-1}) with 5,8,11,14-eicosatetraenoic acid than the phenol-treated powder.

Loss in Phenol-stimulatible Activity during the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Untreated VG Acetone Powder

Phenol caused a stimulation in rate when added during the first three minutes of reaction to a mixture containing untreated acetone powder and 5,8,11,14-eicosatetraenoic acid (Fig. 27A). After three minutes no stimulation was observed upon phenol addition although the rate of oxygenation of fatty acid had not yet dropped to zero. The pseudo first order rate constant for the loss in phenol-stimulatible activity was approximately 1.1 min^{-1} (Fig. 27B) which is four times greater than the rate constant for self-destruction of the untreated acetone powder (approximately 0.25 min^{-1}). The results suggested that two VG dioxygenases were present in the VG acetone powder preparation and that one of these enzymes, stimulatible by phenol, was rapidly inactivated in the presence of hydroperoxide product formed during

Fig. 24. Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder with GSH in the Presence and Absence of GSH-peroxidase.

The instantaneous rate of substrate consumption at various concentrations of 5,8,11,14-eicosatetraenoic acid was determined as described in the legend to Fig. 22 from oxygen uptake tracings. Reactions were initiated by the addition of 5 mg of Upjohn acetone powder to samples containing GSH (1.1 mM) and 5,8,11,14-eicosatetraenoic acid (60 μM). Curve: (A) 6250 units GSH-peroxidase; and (B) no GSH-peroxidase. The GSH-peroxidase preparation used in this experiment was assayed at pH 8.5. The final volume in both assays was 3.0 ml of 0.1 M TrisHCl (pH 8.5).

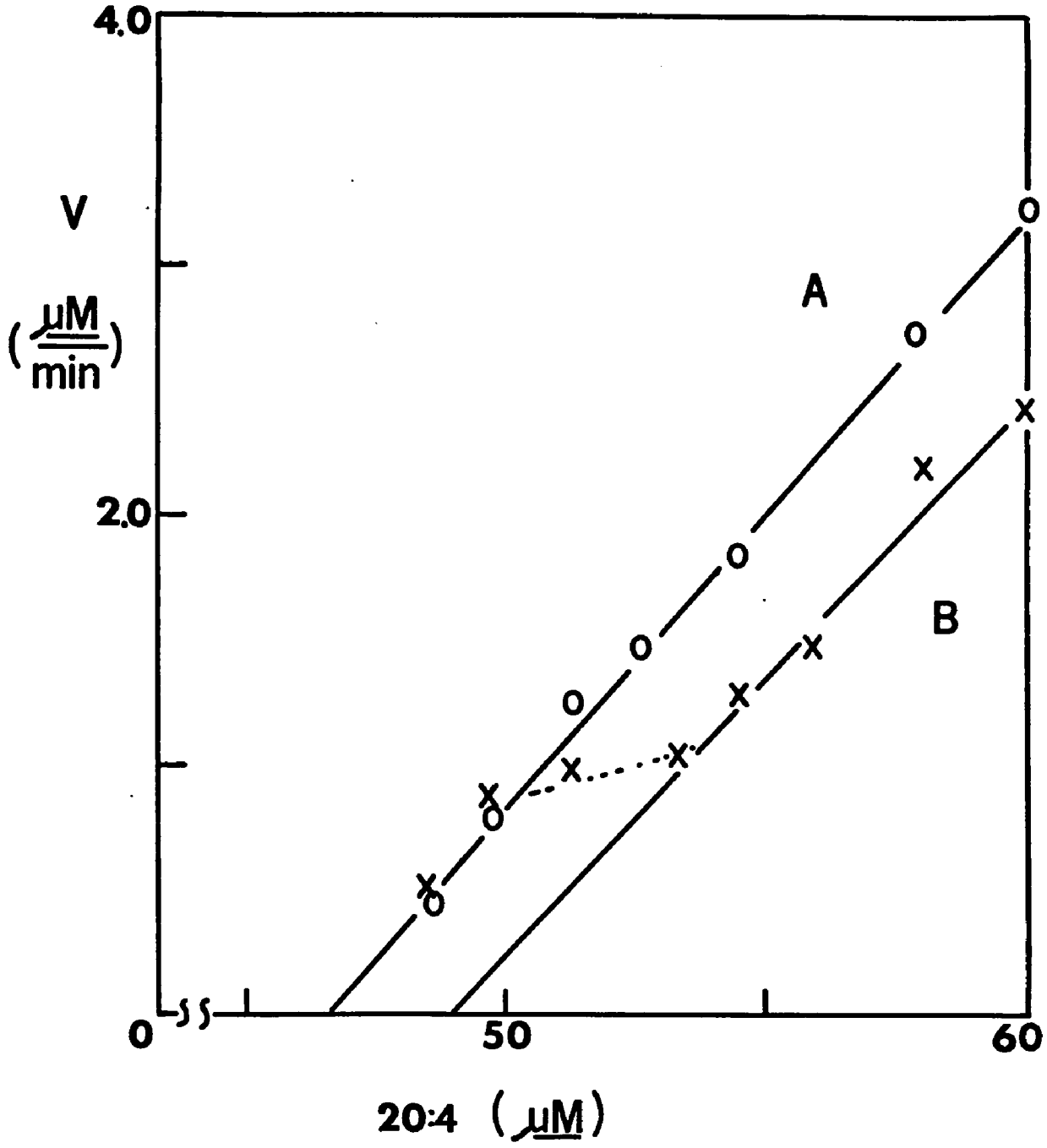


Fig. 24. Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder with GSH in the Presence and Absence of GSH-peroxidase.

Fig. 25. Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14-eicosatetraenoic by Phenol-treated VG Acetone Powder in the Presence and Absence of GSH.

The instantaneous rate at various concentrations of 5,8,11,14-eicosatetraenoic acid was determined from oxygen uptake tracings as described in the legend to Fig. 22. Reactions were initiated by the addition of 5,8,11,14-eicosatetraenoic acid to samples containing acetone powder (A.P.) which had been preincubated two min in 0.66 mM phenol. Initial concentrations of reactants are curve: (A) 0.71 mg/ml A.P., 0.93 mM GSH; (B) 1.4 mg/ml A.P., 0.93 mM GSH; (C) 0.83 mg/ml A.P.; (D) 0.83 mg/ml A.P.; (E) 0.83 mg/ml A.P.; (F) 1.66 mg/ml A.P.

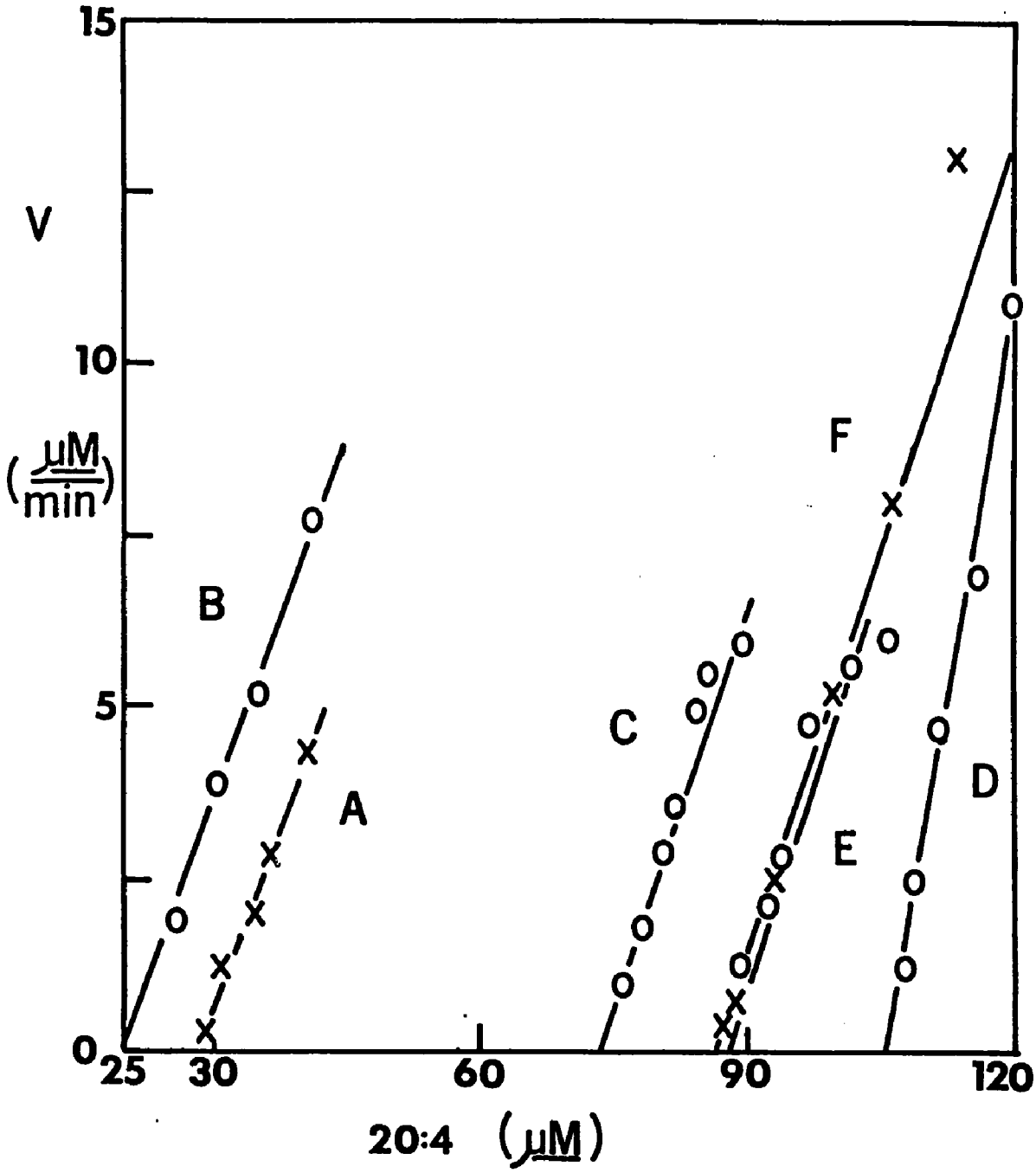


Fig. 25. Velocity versus Substrate Concentration for the Oxygenation of 5,8,11,14-eicosatetraenoic by Phenol-treated VG Acetone Powder in the Presence and Absence of GSH.

Fig. 26. Velocity versus Substrate Concentration for the Oxygenation of 8,11,14-eicosatrienoic Acid by Phenol-treated VG Acetone Powder.

The instantaneous rate at various concentrations of 8,11,14-eicosatrienoic acid was determined from oxygen uptake tracings as described in the legend to Fig. 22. Reactions were initiated by the addition of 8,11,14-eicosatrienoic acid to samples containing acetone powder (A.P.) which had been preincubated for two min in 0.66 mM phenol. Initial protein concentrations are curve: (A) 0.66 mg/ml A.P.; (B) 1.33 mg/ml A.P.; and (C) 0.66 mg/ml A.P.

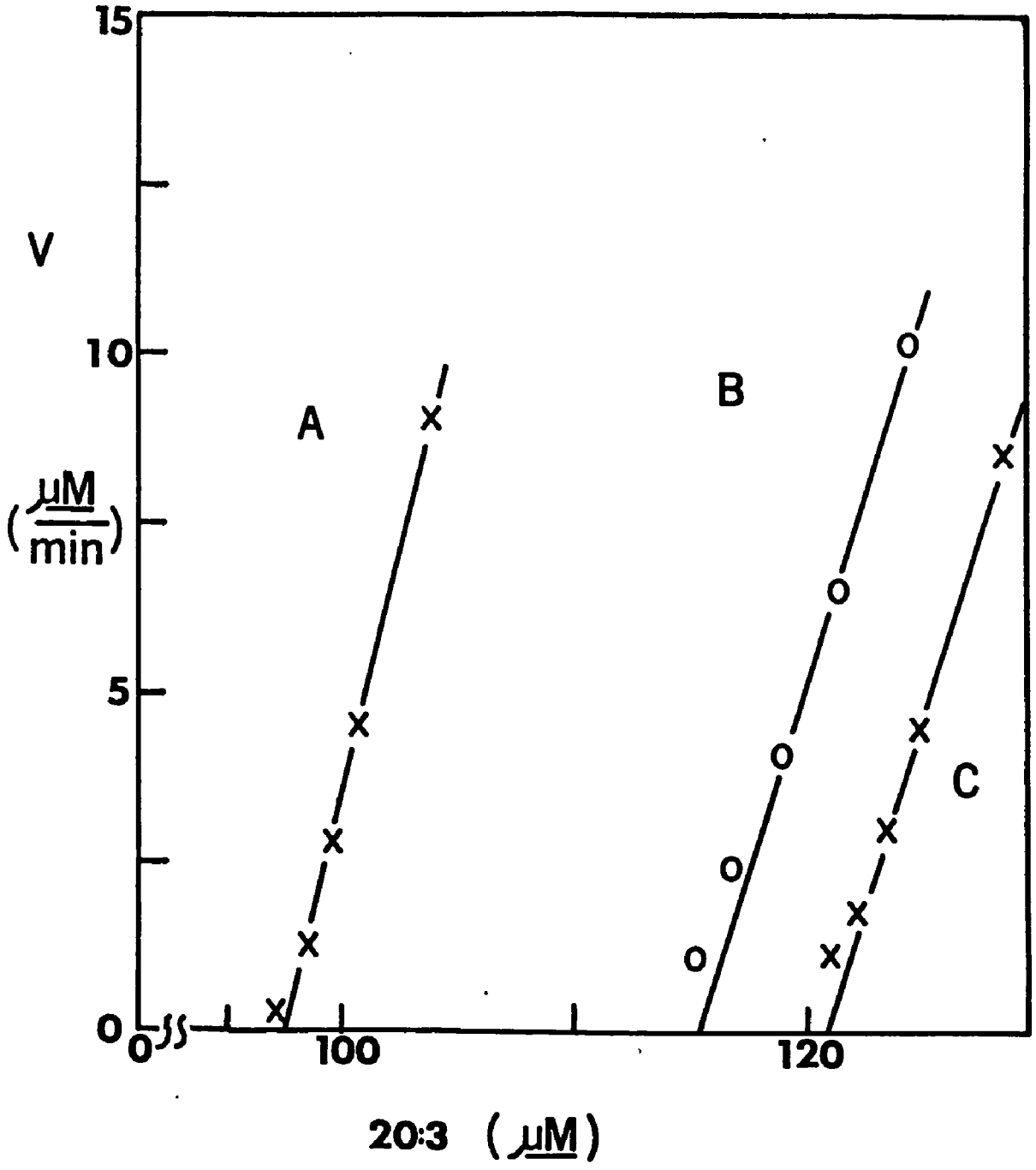


Fig. 26. Velocity versus Substrate Concentration for the Oxygenation of 8,11,14-eicosatrienoic Acid by Phenol-treated VG Acetone Powder.

Fig. 27. The Loss of Phenol-sensitive VG Dioxygenase Activity during the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder.

Part A: Assays were performed as described in Methods. The final volume of all solutions was 3.0 ml of 0.1 M TrisHCl (pH 8.5). Acetone powder (80 µl, 4 mg) was added to the sample chamber and 2 min after the acetone powder addition, 5,8,11,14-eicosatetraenoic acid was added to yield a final concentration of 60 µM. At 0.8, 1.0, 1.5, 2.5 and 3.0 min after this substrate addition, the solution was made to 0.66 mM in phenol and the velocity determined (Curve A). Curve B shows the instantaneous velocity of fatty acid substrate utilization just prior to the time of phenol addition.

Part B: The log of the difference between the velocities from Curves A and B of Part A are plotted as a function of time.

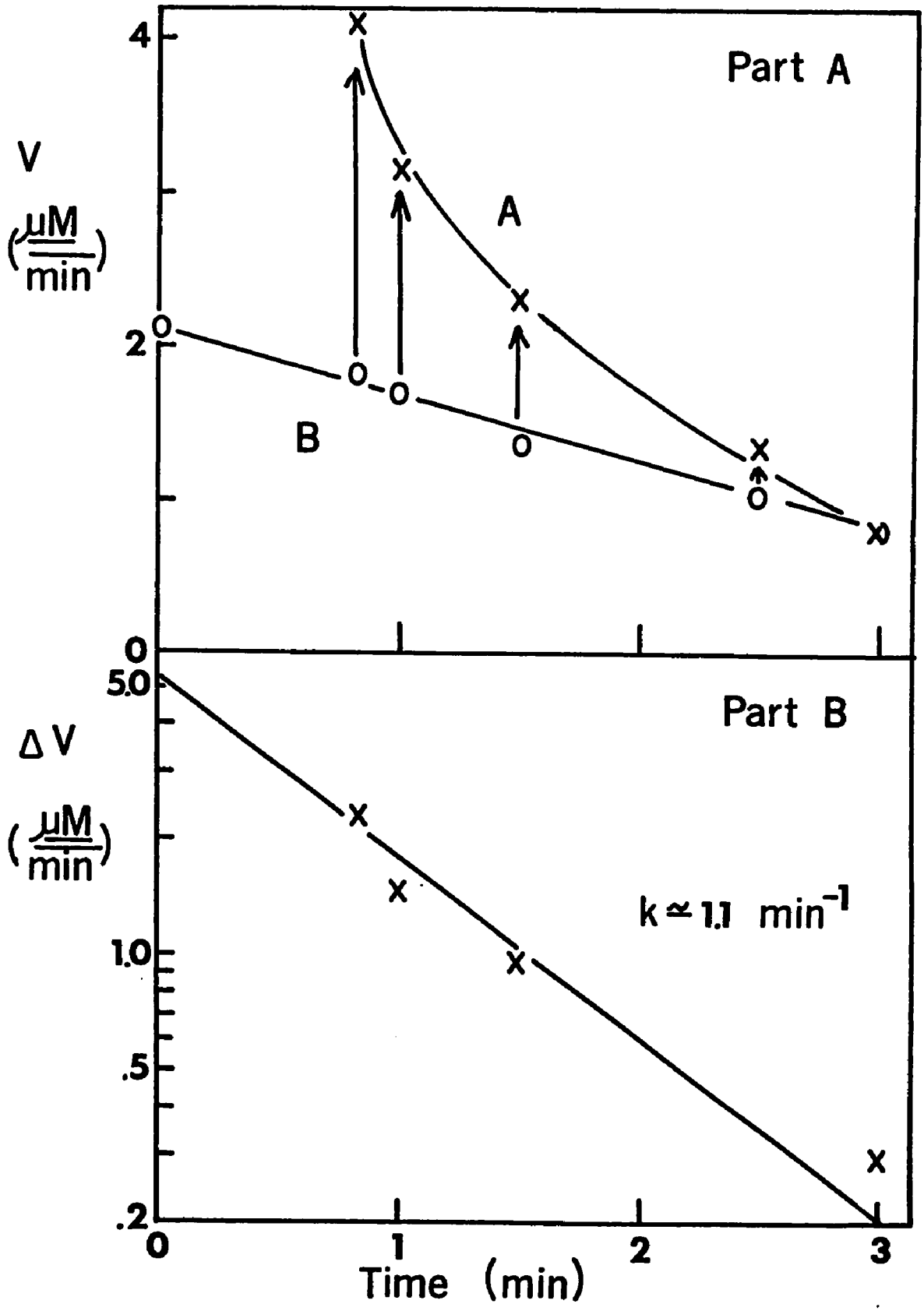


Fig. 27. The Loss of Phenol-sensitive VG Dioxygenase Activity during the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder.

oxygenation in the absence of phenol.

Preservation of Phenol-stimulatible Activity during Self-destruction of Phenol-insensitive Activity in the Presence of 5,8,11,14-eicosatetraenoic Acid and GSH-peroxidase

We reasoned that if hydroperoxide were continually removed during the oxygenation of fatty acid by untreated VG acetone powder, we might be able to prevent the loss of the phenol-stimulatible activity while inactivating the phenol-insensitive enzyme by a self-destruction process. Therefore the VG powder was incubated in the presence of 5,8,11,14-eicosatetraenoic acid, GSH and GSH-peroxidase for various times (4-18 min), the reaction mixture treated with phenol for 5 min and NEM added to remove GSH. Fig. 28 shows the type of observation made in these experiments. Table XVI shows the rate of oxygenation initially and at the time of phenol addition along with the initial rate after phenol and NEM additions. After eighteen minutes of reaction in the presence of either 1330 or 6750 units of GSH-peroxidase, the rate of oxygenation of fatty acid by untreated acetone powder had dropped to zero; however, phenol-stimulatible activity still remained in the sample containing the higher level of peroxidase. The percent remaining activities of the phenol-stimulatible enzyme shown in Table XVI were determined from the difference between the V_{max} of the untreated VG powder and the V_{max} of the phenol-treated VG powder (Fig. 29). These calculated remaining activities indicate that in the absence of phenol and the presence of GSH-peroxidase, phenol-insensitive activity is lost more rapidly than the phenol-stimulatible activity.

Fig. 28. Product Formation versus Time for the Inactivation of Phenol-sensitive and Phenol-insensitive VG Dioxygenase Activities in the Presence of GSH-peroxidase.

The reaction was initiated by the addition of Upjohn acetone powder (5 mg) at zero min to a sample containing 180 nmoles of 5,8,11,14-eicosatetraenoic acid, 3.3 μmoles of GSH and 12,500 units of GSH-peroxidase. The volume of the sample after addition of powder was 3.0 ml of 0.1 M TrisHCl (pH 8.5). Further additions to the sample chamber: (A) 2.0 μmoles phenol (0.20 ml) and (B) 0.9 mmoles NEM (0.10 ml) five min after the phenol addition. The GSH-peroxidase preparation used in this experiment was assayed in 0.1 M TrisHCl (pH 8.5). Product formation was calculated as described in the legend to Fig. 16.

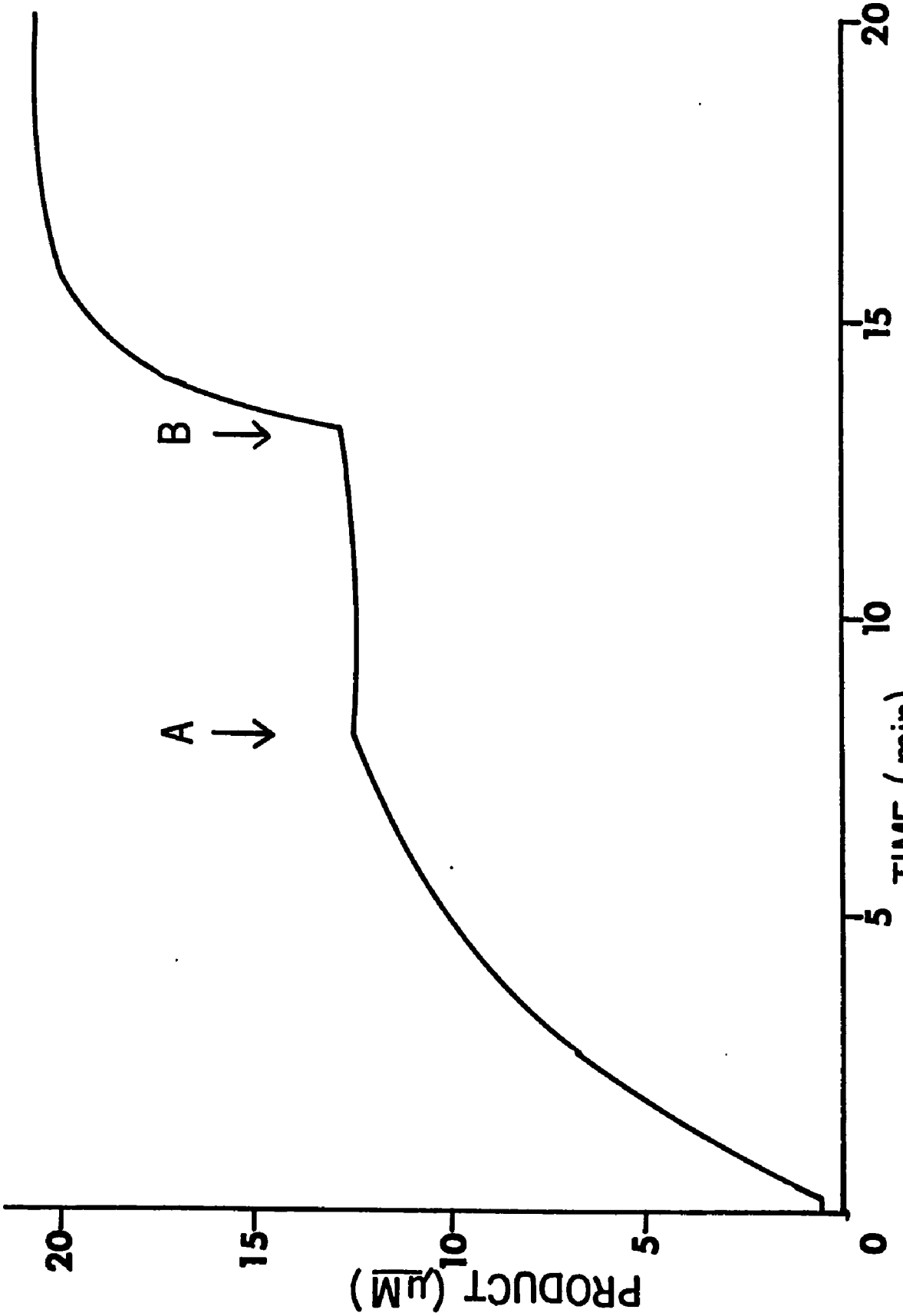


Fig. 28. Product Formation versus Time for the Inactivation of Phenol-sensitive and Phenol-insensitive VG Dioxygenase Activities in the Presence of GSH-peroxidase.

TABLE XVI

Inactivation of Phenol-sensitive and Phenol-insensitive VG Dioxygenase Activities during Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder in the Presence of GSH-peroxidase

All experiments were performed as described in the legend to Fig. 28. Remaining phenol-insensitive activity was determined from the ratio of the velocity at the time of phenol addition to the initial velocity. Remaining phenol-sensitive activity was determined by dividing the velocity after NEM addition by the initial velocity multiplied by the ratio of the V_{max} values for the untreated and phenol-treated acetone powders (4.7) as determined in Fig. 29.

TABLE XVI

Experiment	GSH-Peroxidase (units)	Initial Velocity (μM20:4/min)	Velocity at the time of phenol addition (μM20:4/min)	Velocity after NEM addition (μM20:4/min)	Time of phenol addition (min)	% Remaining Activity	
						Phenol Insensitive	Phenol Sensitive
1	1,330	2.5	0	0	19	0	0
2	6,750	3.1	0	2.2	19	0	16
3	12,500	2.9	0.38	7.8	8	13	57
4	12,500	2.9	1.7	10	4	58	73

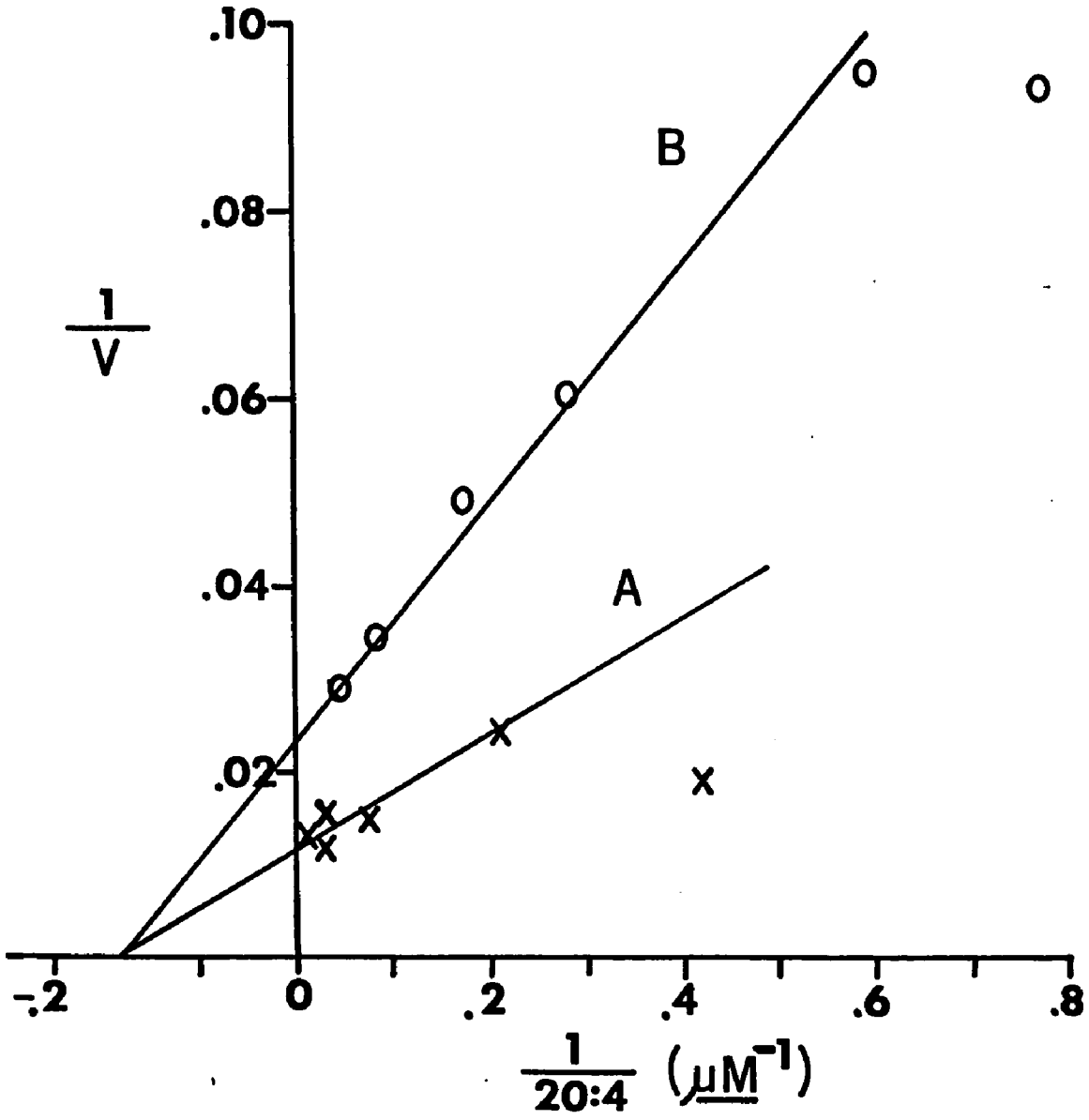


Fig. 29. K_m Determination for 5,8,11,14-eicosatetraenoic Acid with VG Acetone Powder and Phenol-treated VG Acetone Powder.

All oxygen uptake measurements were made as described in Methods. Reactions were initiated by adding the indicated concentrations of 5,8,11,14-eicosatetraenoic acid to reaction mixtures containing (A) 5.0 mg Upjohn acetone powder which had been incubated 2 min at 30°; and (B) 2.5 mg acetone powder which had been preincubated 2 min with 0.66 mM phenol at 30°. The final volume in all assays was 3.0 ml of 0.1 M TrisHCl (pH 8.5).

The Effect of Fatty Acid Substrate Concentration on the Activity of the Phenol-treated VG Acetone Powder

Oxygenation by phenol-treated VG powder is partially inhibited by high concentrations of 5,8,11,14-eicosatetraenoic acid. The degree of inhibition is similar in systems in which acetone powder was pre-incubated with phenol before addition of substrate and in systems in which acetone powder was added to the sample containing both phenol and 5,8,11,14-eicosatetraenoic acid (Table XVII). A variable lag phase is observed over a 20-fold range of substrate concentrations. The apparent lag times shown in Table XVII decrease slightly with increasing substrate, and the O_2 uptake curve does not show a gradual increase in rate during the lag like that observed with lipoxygenase but rather a sharp transition between zero O_2 uptake and maximum O_2 uptake.

The Effect of NaCN and Metal Chelating Agents on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder and Phenol-activated VG Acetone Powder

When acetone powder was homogenized in 1.0 M NaCN and diluted one to ten for assay with [1-^{14}C]-5,8,11,14-eicosatetraenoic acid, the oxygenation of substrate was almost completely inhibited (Fig. 30). Fifty percent inhibition was observed with 0.01 M NaCN. However, removal of the NaCN by collection of the pellet at 9×10^6 g.min yielded 60 - 80% recovery of the activity present initially in the untreated acetone powder.

Inhibition by NaCN suggested that the phenol-insensitive VG dioxygenase might require a metal ion for activity. We further tested

TABLE XVII

The Effect of Substrate Concentration on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Phenol-treated VG Acetone Powder

All oxygen absorption measurements were made as described in Methods. Lag times were measured as described in Fig. 10. The final volume in all samples was 3.0 ml of 0.1 TrisHCl (pH 8.5).

Part: (A) Upjohn A.P. (2.5 mg) was added to a solution containing 0.66 mM phenol and the indicated concentration of 5,8,11,14-eicosatetraenoic acid to initiate the oxygenation reaction; and

(B) Upjohn A.P. (2.5 mg) was incubated for 2 min at 30° with 0.66 mM phenol and the indicated concentrations of 5,8,11,14-eicosatetraenoic acid were added to initiate the reaction.

Part A			Part B		
20:4(n-6) (μM)	Velocity (μM20:4/min)	Lag Time (min)	20:4(n-6) (μM)	Velocity (μM20:4/min)	Lag Time (min)
12	14	0.45	12	17	0.22
30	16	0.45	30	23	0.16
60	14	0.45	60	24	0.079
120	11	0.42	120	22	0.079
180	9.0	0.37	180	16	0.059
240	8.5	0.35	240	15	0.020

Fig. 30. The Effect of NaCN on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder.

All samples contained [1-^{14}C]-5,8,11,14-eicosatetraenoic acid. Incubations were initiated by the addition of 1 mg acetone powder (prepared by Dr. Lee) to the substrate solutions to yield a final volume of 1.0 ml. Incubations were stopped after the indicated times and products separated as described in Methods. Results were calculated as described in the legend to Table XII.

Part A: VG powder homogenized in (A) 0.1 M TrisHCl (pH 9.0); (B) 0.1 M TrisHCl (pH 7.4); (C) 0.1 M TrisHCl (pH 9.0) containing 1 M NaCN; and (D) 0.1 M TrisHCl (pH 7.4) containing 1 M NaCN.

Part B: VG powder homogenized in (A) 0.1 M TrisHCl (pH 9.0); (B) 0.1 M TrisHCl (pH 9.0) containing 0.1 M NaCN; (C) Pellet from 9 x 10^6 g.min centrifugation of (A) resuspended in 0.1 M TrisHCl (pH 9.0); and (D) Pellet from 9 x 10^6 g.min centrifugation of (B) resuspended in 0.1 M TrisHCl (pH 9.0).

Part C: VG powder homogenized in (A) 0.1 M TrisHCl (pH 9.0); (B) 0.1 M TrisHCl (pH 9.0) containing 1 M NaCN; (C) Pellet from 9 x 10^6 g.min centrifugation of (A) resuspended in 0.1 M TrisHCl (pH 9.0); and (D) Pellet from 9 x 10^6 x g.min centrifugation of (B) resuspended in 0.1 M TrisHCl (pH 9.0).

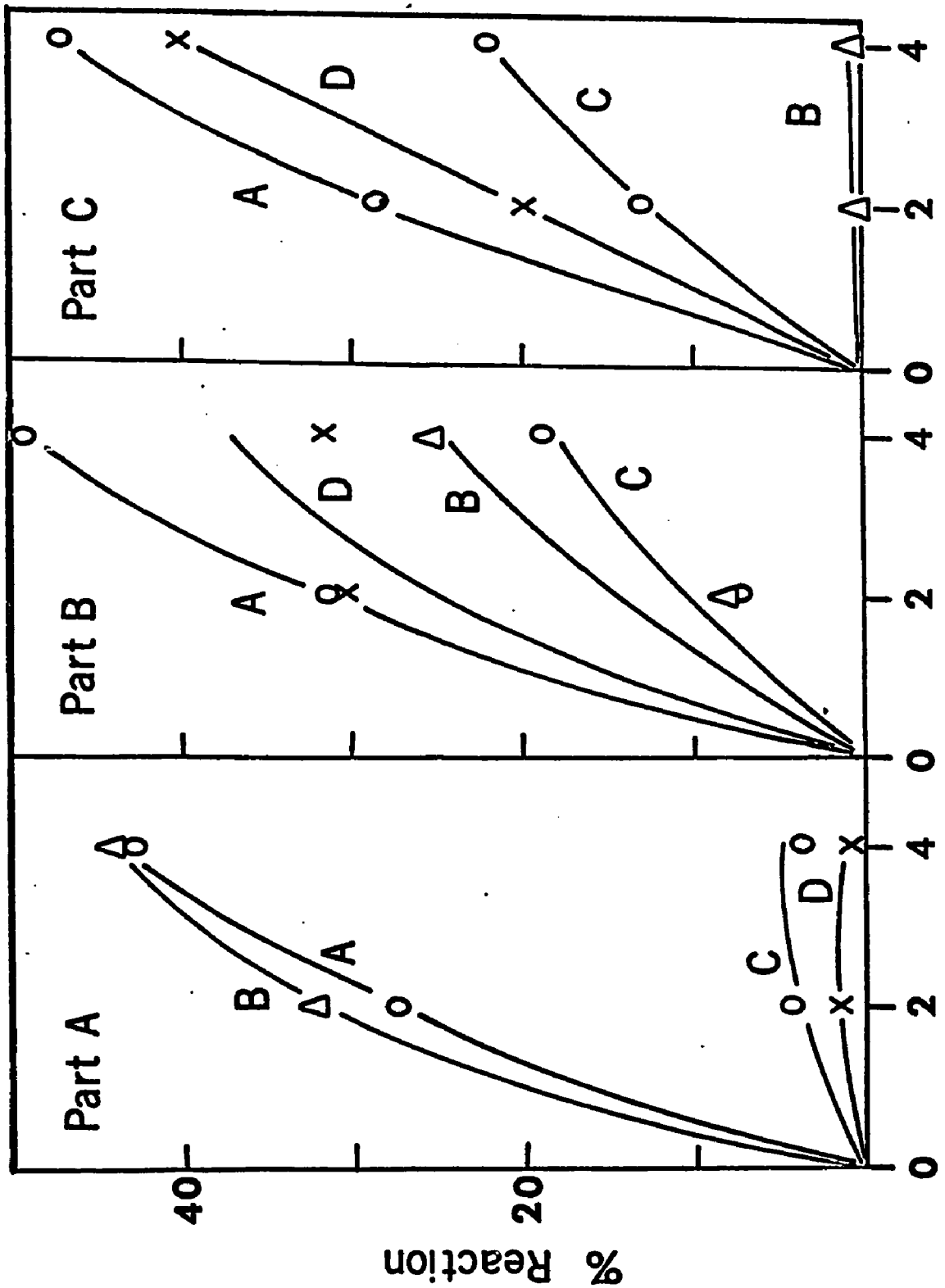


Fig. 30. The Effect of NaCN on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder.

this hypothesis using Tiron, an Fe (III) chelating agent, and o-phenanthroline, an Fe (II) chelating agent. Fig. 31 shows that preincubation of a VG acetone powder preparation with 5 mM o-phenanthroline strongly inhibits oxygenation of eicosatetraenoic acid; however, prolonged incubation of the enzyme with Tiron actually causes a slight increase in the rate of oxygenation.

Table XVIII indicates that bathocuproine does not inhibit the oxygenation of fatty acid by untreated VG powder. However, both bathocuproine and o-phenanthroline inhibit fatty acid oxygenation catalyzed by phenol-treated powder. Fig. 32 shows that o-phenanthroline prevents activation by phenol while diethyldithiocarbamate slows the activation process but does not prevent it.

Fig. 31. The Effect of Tiron and o-phenanthroline on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder.

VG acetone powder (prepared by Dr. Lee) was homogenized in 0.1 M TrisHCl (pH 9.0) to give a protein concentration of 40 mg/ml. This solution was diluted to yield the following protein solutions (20 mg/ml) in: (A) 0.1 M TrisHCl (pH 9.0); (B) 5 mM o-phenanthroline in 0.1 M TrisHCl (pH 9.0); and (C) 5 mM Tiron in 0.1 M TrisHCl (pH 9.0).

These solutions were incubated at 0-4° for the times indicated in the figure and 0.4 ml of the respective protein solutions added as indicated in the figure to samples containing 5 nmoles [^{14}C]-5,8,11,14-eicosatetraenoic acid in 0.6 ml of: (1) 0.1 M TrisHCl (pH 9.0); (2) 5 mM o-phenanthroline in 0.1 M TrisHCl (pH 9.0); and (3) 5 mM Tiron in 0.1 M TrisHCl (pH 9.0). Reactions were performed at room temperature and stopped after 5 min. Products were separated as described in Methods. Oxygenation is expressed as nmoles arachidonic acid disappearing from the fatty acid region of the thin-layer plate in a 5 min reaction time.

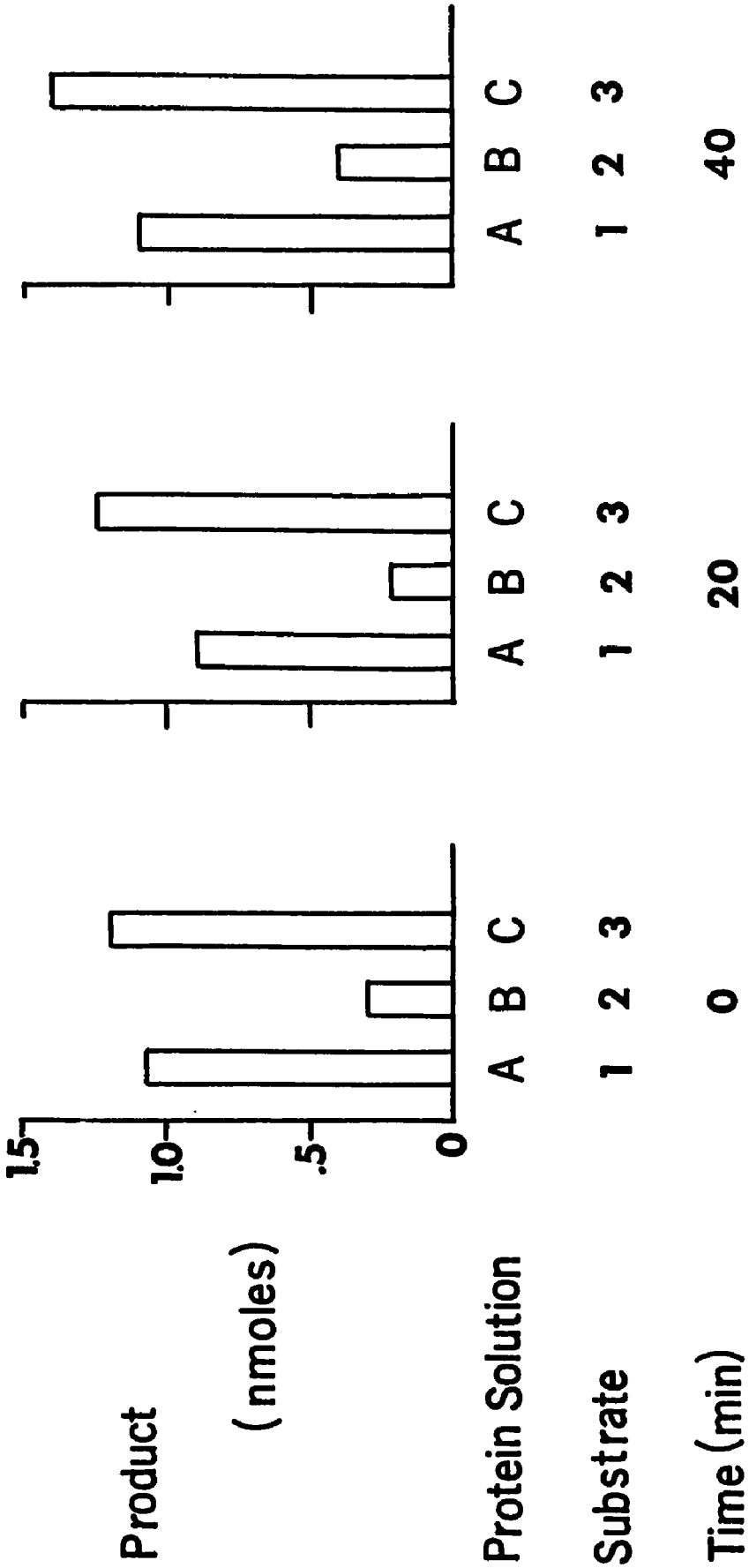


Fig. 31. The Effect of Tiron and o-phenanthroline on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder.

TABLE XVIII

The Effect of Bathocuproine Sulfonate, o-phenanthroline and Diethyldithiocarbamate on the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by VG Acetone Powder and Phenol-treated VG Acetone Powder

Oxygen absorption measurements were used to determine rates of fatty acid oxygenation as described in Methods and the legend to Fig. 16. All samples contained 5,8,11,14-eicosatetraenoic acid at a concentration of 60 μM. Concentrations of other reactants in the assay mixture are indicated in the table. Reactions were initiated by the addition of phenol-treated acetone powder (A.P.) which had been preincubated with 0.66 mM phenol for 30 min at room temperature; or by the addition of untreated acetone powder. The final volume in all experiments was 3.0 ml of 0.1 M TrisHCl (pH 8.5).

TABLE XVIII

Experiment	o-phenanthroline (mM)	Diethyldithiocarbamate (mM)	Bathocuproine sulfonate (mM)	Phenol (mM)	Phenol-treated A.P. (mg/ml)	Untreated A.P. (mg/ml)	Velocity (μM20:4/min)	% Remaining Activity
1	0	0	0	0.66	0.66	0	6.0	101
2	0	0	5	0.66	0.66	0	2.95	52
3	0	0	10	0.66	0.66	0	2.75	49
4	0	0	1	0.66	0.66	0	5.0	88
5	10	0	0	0.66	0.66	0	0.66	12
6	5	0	0	0.66	0.66	0	3.20	57
7	1	0	0	0.66	0.66	0	7.20	127
8	0	10	0	0.66	0.66	0	0.60	11
9	0	5	0	0.66	0.66	0	1.93	34
10	0	0	0	0.66	0.66	0	5.6	99
11	0	0	0	0	0	0.84	0.89	102
12	0	0	0	0	0	1.66	1.70	97
13	0	0	5	0	0	1.66	1.80	103
14	0	0	10	0	0	1.66	1.50	86
15	10	0	0	0	0	1.66	0.82	47

Fig. 32. The Effect of o-phenanthroline and Diethyldithiocarbamate on the Phenol-activation of VG Acetone Powder.

Oxygen absorption measurements were made as described in Methods and rates of fatty acid consumption calculated as indicated in the legend to Fig. 16. VG acetone powder was homogenized and diluted to 12.5 mg/ml to yield the protein solutions indicated below. These solutions were incubated at 21° for the times shown in the figure before 0.1 ml aliquots were assayed in samples containing 0.66 mM phenol and 60 μM 5,8,11,14-eicosatetraenoic acid in a final volume of 3.0 ml of 0.1 M TrisHCl (pH 8.5).

Protein Solution	Diethyldithio-carbamate(mM)	o-phenan-throline(mM)	Phenol (mM)
θ - θ	0	0	0.66
X - X	0	12.5	0.66
Δ - Δ	0	0	0
0 - 0	12.5	0	0.66

The velocities of phenol-activated acetone powder (0.42 mg/ml) in the presence of 0.42 mM o-phenanthroline and 0.42 mM diethyldithiocarbamate (the concentrations of inhibitors after dilution for assay) were 5.6 and 3.4 μM 20:4 (n-6)/min respectively. The velocity of untreated acetone powder (0.42 mg/ml) in the absence of phenol was 0.51 μM 20:4 (n-6)/min.

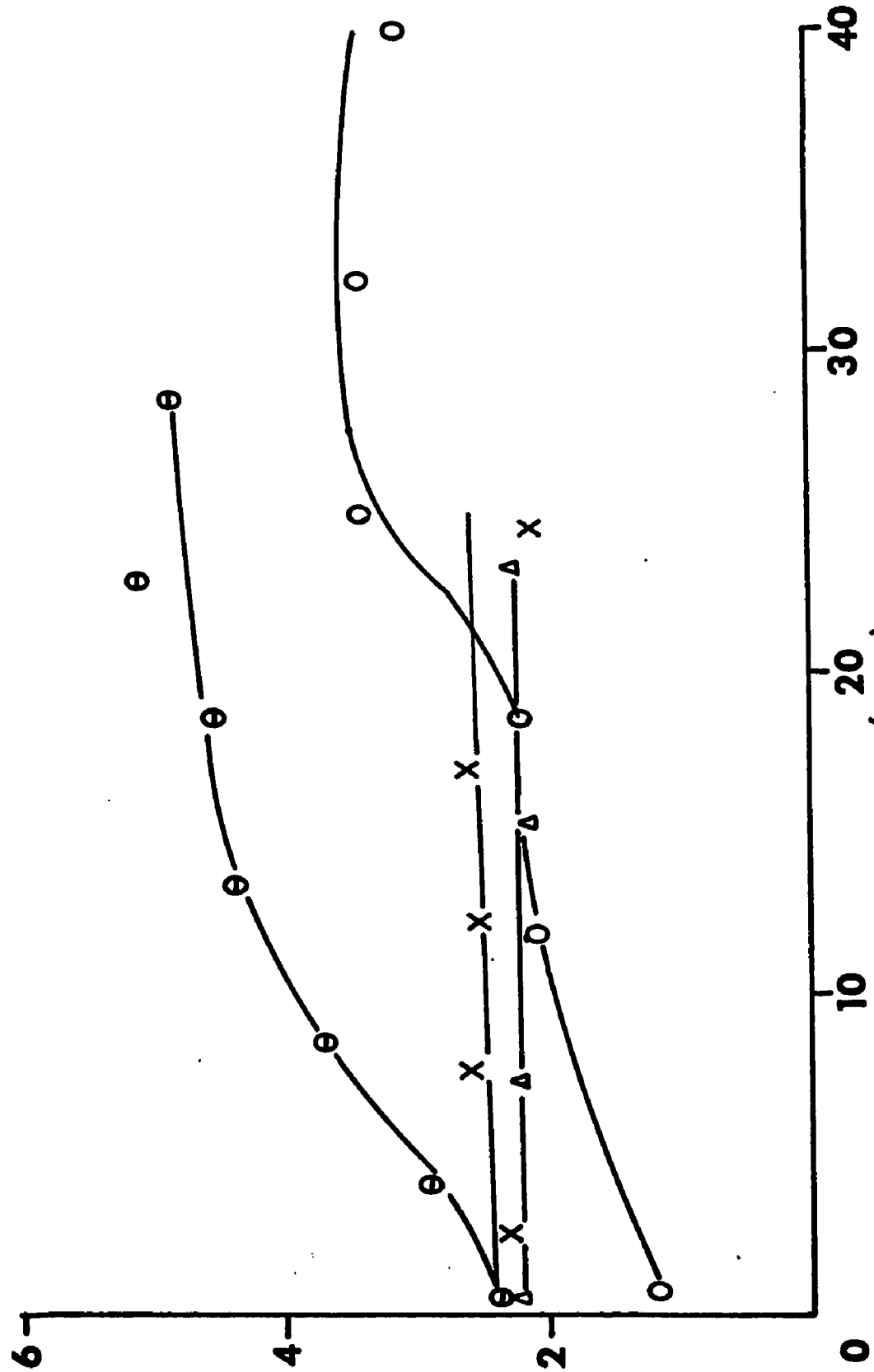


Fig. 32. The Effect of o-phenanthroline and Diethlydithiocarbamate on the Phenol-activation of VG Acetone Powder.

DISCUSSION

Product Activation of the Vesicular Gland Dioxygenases

We observed that GSH-peroxidase caused a 70% inhibition of the rate of oxygenation of 5,8,11,14-eicosatetraenoic acid catalyzed by vesicular gland homogenates. This inhibition requires the presence of GSH and may be reversed by adding NEM. These results suggest the presence, in the vesicular gland homogenate, of a dioxygenase which requires hydroperoxide for activity.

No evidence could be found for a product-dependent dioxygenase in untreated VG acetone powder. GSH-peroxidase, at a level sufficient to catalyze the destruction of 186 μmoles hydroperoxide per min at saturating concentrations of hydroperoxide does not inhibit the oxygenation catalyzed by untreated acetone powder. This indicates that this activity (E_1) is not activated by hydroperoxide or, if it is, it requires only extremely low concentrations of product for activity:

$$E_1 + S + O_2 \rightleftharpoons [E_1SO_2] \xrightarrow{k_{13}} E_1 + P$$

Treatment of the acetone powder with phenol increases the overall activity and restores the sensitivity of the powder to GSH-peroxidase. Oxygenation catalyzed by phenol-treated VG acetone powder may be inhibited 50% by GSH-peroxidase in the presence of GSH. This result suggests that the phenol-treated VG acetone powder contains a dioxygenase which requires hydroperoxide for activity. Therefore, the phenol-treated powder is more similar than the untreated powder to the enzyme

system found in vesicular gland homogenates. However, no rigorous comparison can be made between the homogenate which was assayed by the radioisotope technique and the phenol-treated acetone powder which was assayed by the oxygen absorption technique. This is because endogenous substrates in the vesicular gland homogenate cause an unknown dilution of isotopically labeled fatty acid.

Untreated VG acetone powder becomes completely inactivated during oxygenation of fatty acid in the presence of GSH-peroxidase. When this occurs, no further activity appears after treatment of the apparently inactivated acetone powder with phenol. However, following the removal of the GSH with NEM, another dioxygenase activity (E_2) appears. This E_2 activity must have been completely suppressed by the functioning of the GSH-peroxidase system. Therefore, the phenol-stimulatible dioxygenase, E_2, like soybean lipoxygenase may require hydroperoxide as an obligatory intermediate:

$$E_2 + P \quad [E_2P] \overset{S+O_2}{\rightleftharpoons} [E_2PSO_2] \xrightarrow{k_{23}} E_2P + P$$

E_1 activity is lost more rapidly (0.25 min^{-1}) than the latent E_2 activity during acetone powder-catalyzed oxygenation in the absence of phenol and in the presence of GSH and GSH-peroxidase. However, the capacity to stimulate E_2 with phenol is lost more rapidly (1.1 min^{-1}) than E_1 activity (0.25 min^{-1}) during oxygenation in the absence of phenol, GSH and GSH-peroxidase. Since E_2 is inactivated at different rates in the presence and absence of GSH and GSH-peroxidase whereas E_1 is inactivated at the same rate in both systems, E_1 and E_2 must be two distinct dioxygenases. Furthermore, E_2 is apparently rapidly

inactivated in the presence of the hydroperoxides produced by the E_1-catalyzed reaction.

Comparison of the radioisotope assay to the oxygen absorption assay showed that both the untreated (E_1) and phenol-treated (E_1 and E_2) acetone powder consumed two moles of oxygen per mole of fatty acid. This result indicates that E_1 and E_2 may both function as a first enzyme in the formation of prostaglandins from free fatty acid substrate.

We have no direct evidence for the existence of a specific threshold level for the inhibition of VG dioxygenase E_2 by GSH-peroxidase such as presented in Part I for lipoxygenase. However, as will be shown in "Kinetic Mechanisms for VG Dioxygenases," the inhibition of E_2 appears to be due to removal of a hydroperoxide required for E_2 activity.

Self-catalyzed Destruction of the Vesicular Gland Dioxygenases

VG dioxygenase E_1 is inactivated during the oxygenation of fatty acid. The loss in activity is a linear function of substrate consumption and is therefore kinetically analogous to the self-destruction of lipoxygenase. At relatively high substrate concentrations, the untreated acetone powder is inactivated at similar rates in either the presence or absence of GSH-peroxidase. Since the level of GSH-peroxidase added was sufficient to destroy 32 μmoles hydroperoxide per minute under maximum conditions, it seems reasonable that hydroperoxide was not causing the inactivation of E_1 at an appreciable rate during the oxygenation reaction. Therefore the inactivation of E_1 observed during the oxygenation of fatty acid is probably due to a

destructive side-reaction of the enzyme-substrate complex. Nevertheless, we cannot rule out the possibility that very low levels of product which might saturate the activation process might also saturate the inactivation process.

$$E_1 + S + O_2 \rightleftharpoons [E_1SO_2] \begin{cases} \xrightarrow{k_{12}} E_1^* \\ \xrightarrow{k_{13}} E_1 + P \end{cases}$$

VG dioxygenase E_2 is inactivated during oxygenation of fatty acid. This process, like that observed with E_1, occurs with a linear loss in velocity with substrate consumption. The rate constant for loss in latent phenol-stimulatible activity in the presence of hydroperoxide (1.1 min^{-1}) is larger than the rate constant for inactivation of E_2 during oxygenation of fatty acid (0.50 min^{-1}). This suggests that the presence of substrate fatty acid protects E_2 activity against inactivation by product. However, if inactivation of E_2 were caused by product, independent of substrate, the loss of E_2 activity would be expected to increase during the oxygenation reaction as the ratio of product to substrate concentration increased. Since the rate of inactivation remained constant during reaction, some loss of E_2 must occur by a self-destruction process:

$$E_2 + P \rightleftharpoons [E_2P] \overset{S+O_2}{\rightleftharpoons} [E_2PSO_2] \begin{cases} \xrightarrow{k_{22}} E_2^* \\ \xrightarrow{k_{23}} E_2P + P \end{cases}$$

GSH, which will spontaneously reduce hydroperoxides (45), causes a small but reproducible drop (from 0.50 to 0.36 min^{-1}) in the rate of loss in activity of the phenol-treated acetone powder during

fatty acid oxygenation. This observation, along with the fact that hydroperoxide rapidly destroys E_2 in the absence of phenol, suggests that product alone can cause appreciable inactivation (k_{24}) of E_2 during the oxygenation reaction:

$$E_2 + P \rightleftharpoons [E_2P] \overset{S+O_2}{\rightleftharpoons} [E_2PSO_2] \begin{cases} \xrightarrow{k_{22}} E_2^* \\ \xrightarrow{k_{23}} E_2P + P \end{cases}$$

$$[E_2P] \xrightarrow{k_{24}} E_2^*$$

Pace-Asciak and Wolfe (71) reported that 9,12-octadecadienoic acid and 6,9,12-octadecatrienoic irreversibly inhibited the oxygenation of 5,8,11,14-eicosatetraenoic acid by hydroquinone-treated VG acetone powder. Since fatty acids containing two or more double bonds will serve as substrates for the VG dioxygenase (5,11), this irreversible inhibition could have been due to inactivation of the dioxygenase by either the self-destruction process (k_{22}) or the substrate-independent process (k_{24}) described here.

Overall Kinetic Mechanisms for Vesicular Gland Dioxygenases

E_1 appears to catalyze the oxygenation of fatty acid substrate in a product-independent reaction according to the following formulation:

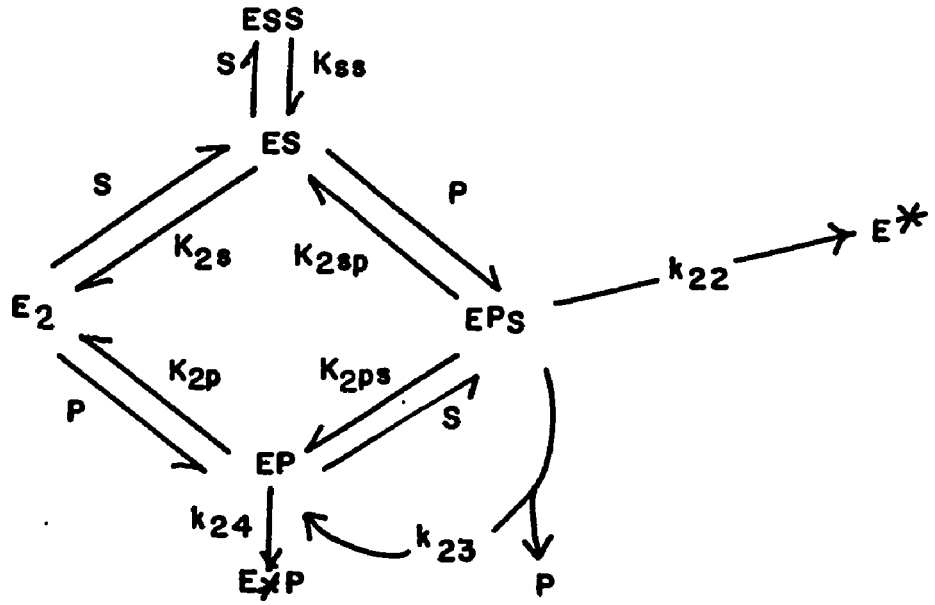
$$E_1 + S + O_2 \rightleftharpoons [E_1SO_2] \begin{cases} \xrightarrow{k_{12}} E_1^* \\ \xrightarrow{k_{13}} E_1 + P \end{cases}$$

This formulation is described by a steady state rate equation similar to equation (4) of Part I:

$$V_1 = \frac{k_{13}[E_1^o] - k_{12}[P]}{\frac{K_{1M}}{[S]} + 1} \qquad (24)$$

We determined from a Lineweaver-Burk plot (Fig. 29) that for untreated VG acetone powder (E_1), K_{1M} is 5.5 μM and k_{13} (V_{max}/E_1^o) is 3.4 min^{-1} (nmoles 20:4/mg protein). A k_{12} value of 0.25 min^{-1} was determined from the slopes of plots of instantaneous velocity versus substrate concentration.

The fully activated form of E_2 appears to catalyze the oxygenation of polyunsaturated fatty acids by a product-dependent mechanism identical to that observed for soybean lipoxygenase:



Equation (25) is the equilibrium rate expression for this kinetic mechanism:

$$V = \frac{k_{23}[E_2^o - E_2^*{}_{(k_{24})}] - k_{22}[P]}{\frac{K_{2ps}}{[S]}\left(1 + \frac{K_{2p}}{[P]}\right) + \frac{K_{2sp}}{[S]}\left(1 + \frac{[S]}{K_{2ss}}\right) + 1} \qquad (25)$$

Constants for equation (25) were estimated in the following manner. The K_{2ps} value was estimated from the K_M of the phenol-treated VG enzyme since with high concentrations of product, $K_M \simeq K_{2ps}$. For 5,8,11,14-eicosatetraenoic acid the K_M is on the order of 5 μM. In order to simplify the calculations, K_{2ss} was assigned an infinite value. This was done since we observed only 30% inhibition of the rate of oxygenation of 5,8,11,14-eicosatetraenoic acid by phenol-treated VG powder with a fatty acid concentration of 240 μM whereas complete inhibition of soybean lipoxygenase occurs with a fatty acid concentration of 264 μM. The value for k_{22} (0.50 min^{-1}) was determined from the slopes of plots of instantaneous velocities versus remaining substrate concentration. Lineweaver-Burk plots were used to estimate k_{23} (12.5 min^{-1} (nmoles 20:4/mg protein)) since for phenol-treated powder, $V_{max} \simeq k_{13}[E_1^o] + k_{23}[E_2^o]$ while for the untreated powder $V_{max} \simeq k_{13}[E_1^o]$.

Since we observed no product-dependent lag in initial velocity in the oxygenation of 5,8,11,14-eicosatetraenoic acid by phenol-treated VG acetone powder, at a measureable product concentration (> 1 μM), we estimated that the VG dioxygenase E_2 must be operating at maximum velocity at that product concentration. Therefore we estimated that K_{2p} was no greater than 0.1 μM. Our initial velocity determinations were obtained from sections of the kinetic curves where the apparent product concentrations were no larger than 1 μM; this upper limit of 1 μM for [P] was then used in our subsequent calculations.

In phenol-treated acetone powder systems, both E_1 and E_2 are active and therefore the total rate of oxygenation is represented by

the sum of equations (24) and (25):

$$V_{total} = V_1 + V_2 =$$

$$\frac{k_{13}[E_1^o] - k_{12}[P]}{\frac{K_{1M}}{[S]} + 1} + \frac{k_{23}[E_2^o - E_2^*(k_{24})] - k_{22}[P]}{\frac{K_{2ps}}{[S]}\left(1 + \frac{K_{2p}}{[P]}\right) + 1 + \frac{K_{2sp}}{[P]}\left(1 + \frac{[S]}{K_{2ss}}\right)} \qquad (26)$$

At a product concentration ([P]) of 1 μM, enzyme concentrations ($[E_1^o] = [E_2^o]$) of 0.84 mg/ml and using K_{2p} = 0.1 μM; $K_{2ss} = \infty$; k_{22} = 0.5 min^{-1}; k_{12} = 0.3 min^{-1}; k_{23} = 12.5 min^{-1} (nmoles 20:4/mg); k_{13} = 3.4 min^{-1} (nmoles 20:4/mg); and K_{1M} = 5.5 μM, we calculated values of K_{2ps} and K_{2sp} which when substituted into equation (26) with the above constants would predict the observed velocity for the oxygenation of different concentrations of 5,8,11,14-eicosatetraenoic by phenol-treated VG acetone powder. The values of K_{2ps} and K_{2sp} which allowed a reasonable prediction of the observed velocities under these conditions were 5 μM and 0.03 μM respectively. The value of K_{2s} was subsequently calculated (equation (15a)) to be 17 μM. Table XIX indicates the correspondence between the observed and calculated velocities for several incubations.

Using phenol-treated acetone powder, Nugteren (72) observed a K_i of 0.12 μM for the apparent competitive inhibition of the oxygenation of 8,11,14-eicosatrienoic acid by 8-*cis*-12-*trans*-14-*cis*-eicosatrienoic acid. This K_i value was more than 300 times smaller than the apparent K_M for the 8-*cis*-11-*cis*-14-*cis*-eicosatrienoic acid substrate (40 μM). We found that the K_{2p} value (0.1 μM) was 50 times less than the apparent

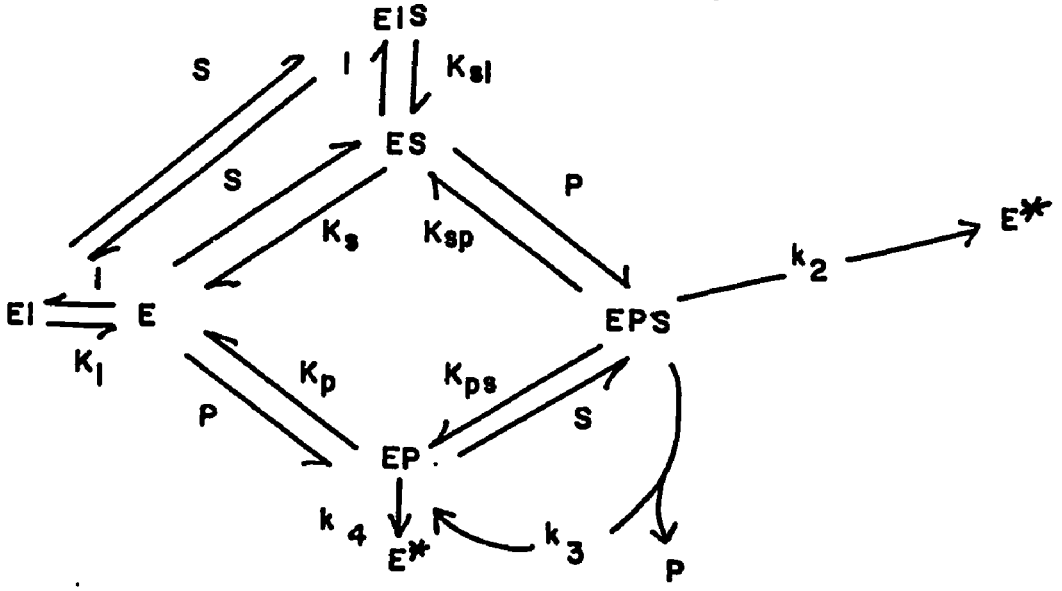
TABLE XIX

Calculation of the Observed Velocities for the Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Phenol-activated VG Acetone Powder from the Kinetic Equation for VG Dioxygenases, E_1 and E_2

Experiment	$[E_o]$ (mg/ml)	[P] (μM)	[S] (μM)	$V_{observed}$ (μM20:4(n-6)/min)	$V_{predicted}$ (μM20:4(n-6)/min)
1	0.84	1	3.6	5.2	5.2
2	0.84	1	6.0	6.4	6.9
3	0.84	1	12.0	9.4	9.0
4	0.84	1	24.0	11.0	10.6

Values of $[E_o]$, [S], and $V_{observed}$ are from Fig. 29B. [P] is the estimated product concentration at which $V_{observed}$ is determined. $V_{predicted}$ is calculated from equation (26) and the necessary constants indicated in the text.

K_M (K_{2ps}) for 5,8,11,14-eicosatetraenoic acid. In addition, the trans-inhibitor is structurally similar to 15-hydroperoxy-8-cis-12-trans-14-cis-eicosatrienoic acid, one of the hydroperoxides produced in the oxygenation catalyzed by sheep vesicular gland. We believe that the inhibition observed by Nugteren may be due to the binding of the trans fatty acid isomer to the product binding site of VG dioxygenase E_2; in this situation competitive inhibition may be observed. Using the reaction scheme shown below which shows the trans-inhibitor (I) binding to the product site, we derived an equilibrium rate equation (27) in a manner strictly analogous to the derivation for equation (25):



$$V = \frac{k_3[E_o - E^*(k_4)] - k_2P}{\frac{K_{ps}}{[S]}\left(1 + \frac{K_p}{[P]} + \frac{K_p}{[P]}\left(\frac{[I]}{K_I}\right)\right) + 1 + \frac{K_{sp}}{[P]}\left(1 + \frac{[S]}{K_{ss}} + \frac{[I]}{K_{sI}}\right)} \qquad (27)$$

Using our estimated dissociation constants for VG dioxygenase E_2 and [P] = 1 μM, equation (27) becomes:

$$V = \frac{k_3[E_o - E^*(k_4)] - k_2}{\frac{5.0}{[S]}\left(1.1 + 0.1\,\frac{[I]}{K_I}\right) + 1 + 0.03\left(\frac{[I]}{K_{sI}}\right)} \qquad (28)$$

With a high ratio for $[I]/K_{sI}$ we would expect non-competitive inhibition; however, if $[I]/K_{sI}$ is low (i.e. < 5-10)

$$V \simeq \frac{k_3[E_o - E^*(k_4)]}{\frac{5.0}{[S]}(1.1 + 0.1\frac{[I]}{K_I}) + 1} \qquad (29)$$

Equation (29) is analogous to the classical rate equation for competition enzyme inhibition; raising the concentration of substrate at constant [I] gives:

$$V \simeq k_3[E_o] = V_{max} \qquad (30)$$

Therefore it seems reasonable to infer that Nugteren observed competitive inhibition although his trans-isomer was not competing with substrate in the classical sense, but rather was actually competing with product for the product binding site.

In addition to finding that 8-cis-12-trans-14-cis-eicosatrienoic acid inhibited the VG dioxygenase, Nugteren also noted that this acid did not inhibit soybean lipoxygenase. However, we would anticipate that another isomer, 8-cis-11-cis-13-trans-eicosatrienoic acid would be an effective competitive inhibitor of lipoxygenase. This latter acid has a carbon structure similar to the lipoxygenase product, 15-hydroperoxy-5-cis-8-cis-11-cis-13-trans-eicosatetraenoic acid which we showed (Part I) would activate the soybean enzyme. Lipoxygenase catalyzes oxygen insertion at the (n-6) carbon atom of the substrate whereas the VG dioxygenase catalyzes oxygen insertion at the (n-10) position. The position of this insertion may be effected by the corresponding products of the two enzymes--15-hydroperoxy-8-cis-11-cis-13-trans-eicosatrienoic

acid for lipoxygenase and 11-hydroperoxy-8-cis-12-trans-14-cis-eicosatrienoic acid for VG dioxygenase E_2.

A Possible Role for Pseudo-singlet State Oxygen in Fatty Acid Dioxygenases

A mechanism was proposed in Part I to account for the product activation of lipoxygenase. This mechanism indicated that an enzyme-bound hydroperoxy radical abstracted a hydrogen atom from the fatty acid substrate. A similar mechanism will be proposed later in this discussion for the product-activated VG dioxygenase E_2. Recently, Chan (73) has introduced evidence which suggests that singlet state oxygen is produced in the oxygenation of ethyl-9,2-octadecadienoate by lipoxygenase. Another mechanism may account for the product-dependency observed in the reactions catalyzed by lipoxygenase and VG dioxygenase E_2 (Fig. 33). In this alternate formulation shown for VG dioxygenase E_2, product reacts with oxygen to form a tetroxide transition state (I) (68). Formation of the transition state (I) is reversible to hydroperoxide and triplet ground state oxygen, $^3\Sigma_g$. In the transition state (I) "the four oxygen atoms must all lie in a plane with the carbon and hydrogen out of the plane if the oxygen is formed in its first excited state" (68). The excited oxygen, $^1\Delta_g$, is 22 kcal above its ground state energy in transition state (I) indicating that a substantial amount of energy would be required to form this transition state. The double bond of an enzyme-bound substrate in the plane of the oxygen atoms could conceivably supply some of this energy leading to production of a perepoxide metastable intermediate (II) without free singlet state oxygen actually being formed. The perepoxide intermediate has been proposed as a preferred intermediate for the formation of

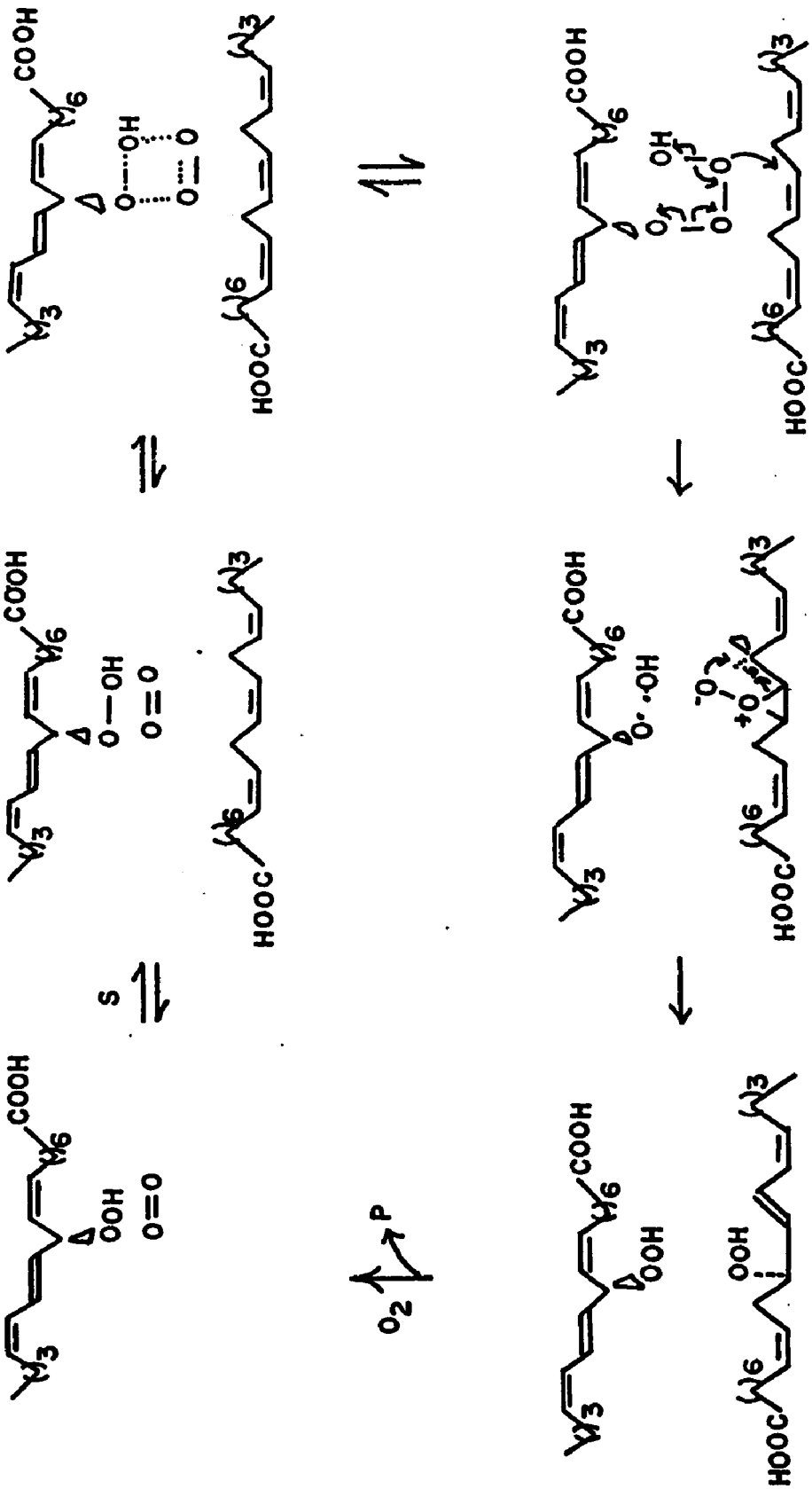


Fig. 33. A Mechanism for VG Dioxygenase E_2 Involving Pseudo-singlet State Oxygen.

hydroperoxides from alkenes and hydroperoxides (74).

Breakdown of intermediate (II) to hydroperoxide occurs with a stereospecific removal of the hydrogen from a carbon next to the double bond of the substrate. This results in the formation of a new trans double bond.

The enzymes, lipoxygenase or VG dioxygenase E_2, would function in this mechanism by positioning the appropriate double-bond on the substrate in such a way that the driving force for formation of intermediate (II) would be available. With lipoxygenase, this double bond would be at the (n-6) position whereas with the VG dioxygenase E_2 the double bond would be at the (n-9) position. Perepoxide formation across either double bond could result in the eventual abstraction of the L-(n-8) hydrogen atom of the substrate. The enzymes may make cleavage of this particular carbon-hydrogen bond more favorable by stabilization of an incipient free radical at that position.

As was discussed in Part I, the alkoxy and hydroxy radicals shown in intermediate (II) could account for the lipoxygenase self-destruction process. Both this pseudo-singlet state oxygen mechanism and the free radical mechanism would involve free radicals and therefore could not be distinguished on the basis of the occurrence of a free radical epr signal. However, intermediate (II) may be susceptible to attack by an appropriate nucleophile such as N_3^- (74). Excited singlet state oxygen has been suggested as an intermediate in the action of quercitinase, a dioxygenase from Aspergillus flavus (75,76).

The inhibition of VG dioxygenase E_2 by GSH-peroxidase may be due to the enzymic removal of a hydroperoxide required for E_2 activity. If so, the overall rate of product formation with phenol-treated VG

acetone powder in the presence of GSH-peroxidase should be predicted by the difference between the rate of product formation (V^{+ROOH}) according to equation (26) and the rate of product destruction by (V^{-ROOH}) by GSH-peroxidase. Porcine blood GSH-peroxidase (E_G) catalyzes the reduction of hydroperoxides by a ping-pong mechanism (66). The rate equation for this mechanism is of the form (77):

$$V^{-ROOH} = -\frac{d[P]}{dt} = \frac{k_G E_G}{(1 + \frac{K_{GSH}}{[GSH]})(1 + \frac{K_{GP}}{[P]})} \qquad (31)$$

For the GSH-peroxidase preparation used in the inhibition experiments with phenol-treated VG acetone powder, k_G is 42 μM ROOH/min/ml GSH-peroxidase (E_G). We have determined a K_M for GSH at pH 8.5 of 1.0 mM for the rat liver GSH-peroxidase. This value is in agreement with the K_{GSH} value found by Little et al (66) for porcine blood GSH-peroxidase. In addition, these workers determined a K_{GP} of approximately 1.0 μM.

Table XX shows the observed and calculated values for product formation and destruction during oxygenation of 5,8,11,14-eicosatetraenoic acid by phenol-treated VG acetone powder in the presence of rat liver GSH-peroxidase.

The variability between the calculated and observed rates does not allow us to distinguish whether or not the inhibition by GSH-peroxidase is due to a binding of some active grouping of the VG dioxygenase E_2 or an enzymic removal of hydroperoxide activity.

The similarity between the effect of GSH-peroxidase on lipoxygenase, which requires hydroperoxide for activity, and VG dioxygenase, E_2, makes us believe that E_2 requires hydroperoxide for activity. In

TABLE XX

Comparison of the Predicted and Observed Rates of Hydroperoxide Product Formation and Destruction During Oxygenation of 5,8,11,14-eicosatetraenoic Acid by Phenol-treated VG Acetone Powder in the Presence of GSH-peroxidase

Experiment	$[E_o]$ (mg/ml)	GSH-peroxidase (E_G) (ml)	$V^{+ROOH}_{predicted}$ (μM/min)	$V^{+ROOH}_{observed}$ (μM/min)	$V^{-ROOH}_{predicted}$ (μM/min)	$V^{-ROOH}_{estimated}$ (μM/min)	$V^{\pm ROOH}_{observed}$ (μM/min)
1	0.167	0.25	2.4	2.9	2.8	1.45	1.45
2	0.33	0.25	4.8	4.5	2.8	2.2	2.3
3	0.66	0.25	9.5	7.6	2.8	2.4	5.2
4	0.66	0.50	9.5	7.1	5.5	2.7	4.4

All data are taken from experiments shown in Table XV. $V^{+ROOH}_{predicted}$ is calculated from equation (26) using the constants indicated in the text, [P] = 1 μM, [S] = 60 μM and the indicated $[E_o]$. $V^{+ROOH}_{observed}$ is the rate observed in control incubations containing no GSH-peroxidase. $V^{-ROOH}_{predicted}$ is calculated from equation (31) using the constants indicated in the text, [P] = 1 μM, [GSH] = 1.1 mM and the indicated E_G. $V^{-ROOH}_{estimated}$ is determined by the difference between $V^{+ROOH}_{observed}$ and $V^{\pm ROOH}_{observed}$. The $V^{\pm ROOH}_{observed}$ value is the rate of oxygenation in the presence of GSH-peroxidase.

addition our kinetic calculations are compatible with the existence of a product binding site for VG dioxygenase E_2.

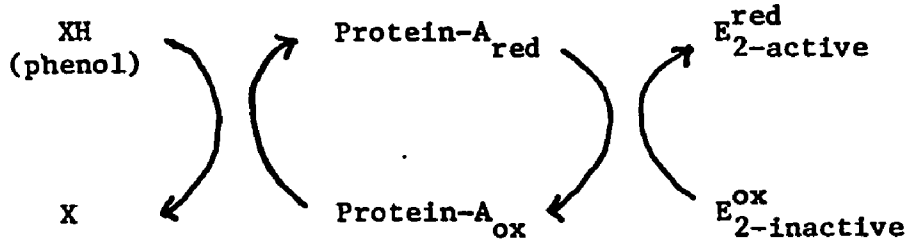
Phenol Activation and the Role of Metals in VG Dioxygenase E_2 Activity

The results of our studies of the effects of metal chelating agents on the action of VG dioxygenase E_2 suggest that both copper and iron (Fe (II)) may in some way be involved in the catalytic activity of this enzyme. Bathocuproine, diethyldithiocarbamate and o-phenanthroline each (at a concentration of 10 mM) inhibit oxygenation catalyzed by phenol-treated VG acetone powder by more than 50%. Since these inhibitory effects cannot be due solely to inhibition of VG dioxygenase E_1, which accounts for only 20% of the activity of phenol-treated acetone powder, the metal chelating agents must all inhibit VG dioxygenase E_2.

The effects of metal chelating agents on E_2 activity must, however, be interpreted with caution. Bathocuproine and diethyldithiocarbamate are normally considered to be specific inhibitors of copper-containing proteins (78,79) whereas o-phenanthroline is usually considered specific for proteins requiring Fe(II) for activity (80). However, o-phenanthroline inhibits salicylate hydroxylase (81) and bathocuproine inhibits imidazoleacetate monooxygenase (82) although neither enzyme contains a metal ion (83). In addition, it has been suggested that o-phenanthroline inhibits yeast alcohol dehydrogenase (84,85) and pseudomonad metapyrocatechase (86) by a hydrophobic interaction with the enzyme proteins. o-Phenanthroline will also inhibit the activity of copper-containing proteins (87).

Initially, phenol treatment of VG acetone powder causes a small

but rapid increase in the rate of oxygenation of fatty acid substrate. This is followed by a 2-3 min period in which no appreciable increase in rate occurs and then by another rapid increase in dioxygenase rate to almost five times that observed in the absence of phenol. The initial rapid increase is saturable with an apparent K_M for phenol of 10^{-4} M. Therefore, the overall activation appears to be due to the action of phenol at some limiting site. The half-life for activation (approximately 3.5 min at 30°) is independent of protein concentration. If the sigmoidal shape of the activation curve were due to an autocatalytic production of an activator (such as seen in the product activation of lipoxygenase) or removal of an inhibitor, the half-life for maximal activation would decrease with increasing acetone powder concentrations. Therefore, the phenol-activation is not autocatalytic. A model which accounts for the kinetics of the activation process is shown below:

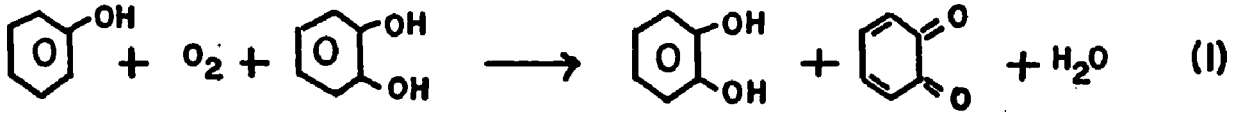


In this situation some group A_{ox} on endogenous protein would be reduced by phenol causing the reduction of active enzyme. As the level of protein-A_{red} increased, the concentration of $E^{red}_{2\text{-active}}$ would also increase.

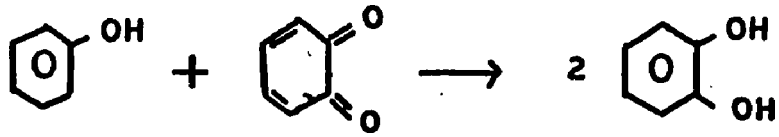
We found it necessary to have phenol in our assay mixture because the activated enzyme is rapidly deactivated by the removal of phenol even in the presence of substrate. This suggested that phenol was being continually consumed both during activation and during

oxygenation by the fully activated enzyme. This would be expected if either $E^{red}_{2\text{-active}}$ or A^{red} were rapidly oxidized. We detected no oxygen consumption during the activation process; however, oxidation of $E^{red}_{2\text{-active}}$ by molecular oxygen could be occurring but is immeasureable with the nanomolar concentrations of enzyme used in our assay system. We would also be unable to detect an aerobic reduction of protein-A^{ox} by phenol. Therefore, phenol may be acting by either an anaerobic or aerobic reduction process.

Because o-phenanthroline inhibits the activation, A^{red} may be Fe(II); o-phenanthroline could cause inhibition of the reduction of $E^{ox}_{2\text{-inactive}}$ by Fe(II) of protein-A^{red}. However, we have no fully plausible explanation for reduction of iron to Fe(II) by phenol. Direct one electron reduction of Fe(III) may occur with hydroquinone and other dihydroxybenzene compounds (80), but is energetically unfavorable with phenol. Production of a dihydroxy benzene (catechol) from phenol may occur in the case of phenolase (36) by reaction (1); reaction (2) has been reported to occur spontaneously (88):



(1)



(2)

However, this explanation seems unlikely since phenolase is inhibited by diethyldithiocarbamate (78) and the phenol activation is not prevented with this agent.

One electron reductions of compounds I and II of horse radish peroxidase by substituted phenols occur with concommittant production of substituted biphenyl and polyphenyl derivatives (36) which also may be capable of reducing Fe(III) to Fe(II). However, formation of compounds I and II from an endogenous hematin peroxidase would require hydrogen peroxide. We have no evidence for or against hydrogen peroxide formation in our reaction mixtures.

An anaerobic reduction of iron to Fe(II) which is potentially related to the VG dioxygenase occurs with tryptophan oxygenase. Knox and coworkers have shown that tryptophan oxygenase is activated by a two step process (89,90,91): (A) conjugation of hematin to the apoenzyme; and (B) one electron reduction of the hematin by ascorbate to yield the active holoenzyme. The fact that a tryptophan oxygenase has been reported which contains both a hematin prosthetic group and a catalytically active copper moeity (92) makes this protein of additional interest since VG dioxygenase E_2 is inhibited by both iron and copper chelating agents.

Fig. 34 indicates a possible mechanism for the product-dependent oxygenation of fatty acid by VG dioxygenase E_2 similar to that postulated earlier for lipoxygenase. The E_2 mechanism is consistent with our findings on both the phenol activation and the inhibition of E_2 by metal chelating agents.

The Role of Metal in VG Dioxygenase E_1

Based on the inhibition of untreated VG acetone powder by o-phenanthroline but not bathocuproine sulfonate or Tiron, oxygenation catalyzed by VG dioxygenase E_1 seems to require Fe(II) but not Fe(III)

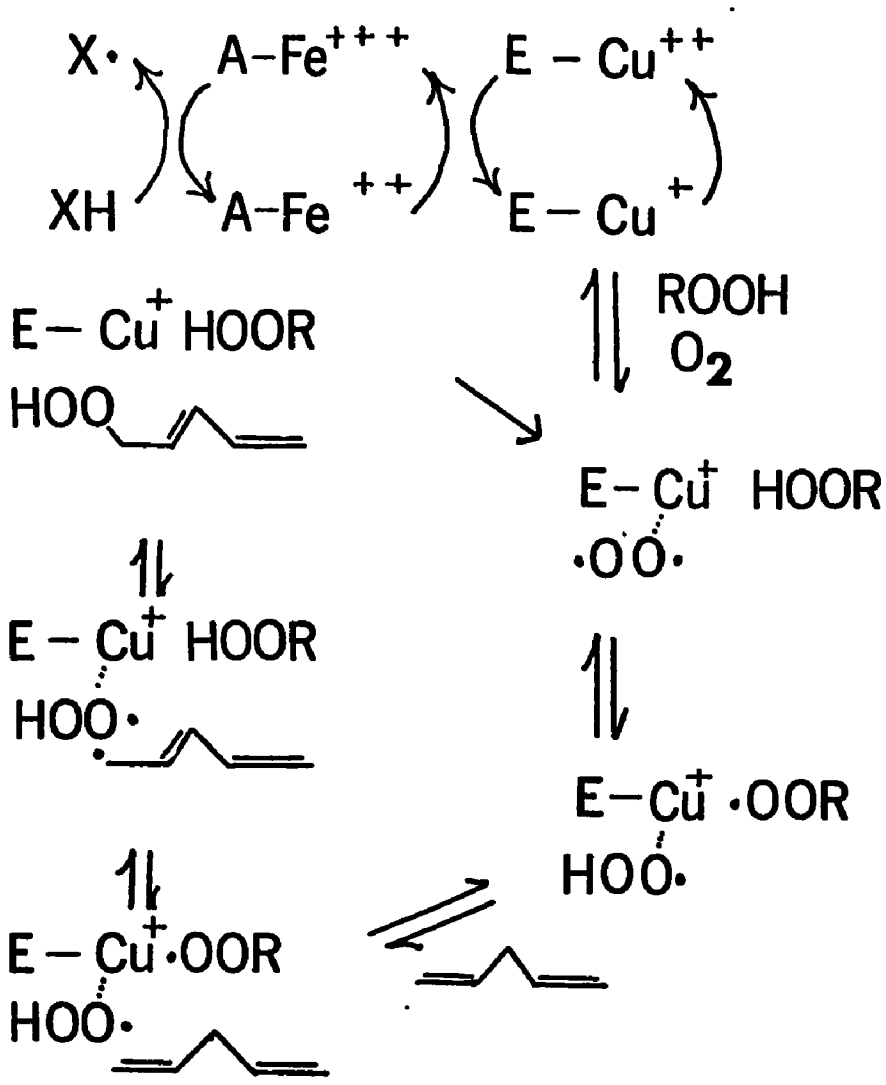


Fig. 34. Possible Mechanism for the Oxygenation of Polyenoic Acids by Vesicular Gland Dioxygenase E_2.

or copper. The mechanism of hydroperoxy acid formation by VG dioxygenase E_1 may involve an oxygen activation similar to that postulated for Fe(II) dioxygenases (37) (Fig. 35):

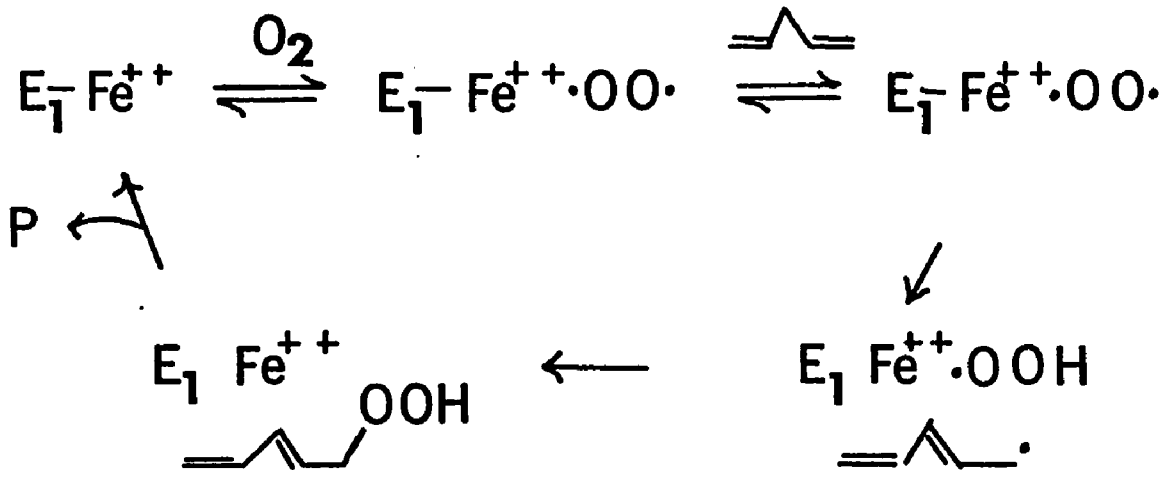


Fig. 35. Possible Mechanism for the Oxygenation of Polyenoic Acids by Vesicular Gland Dioxygenase E_1.

Since product does not appear to be involved in this mechanism, we would expect the trans-isomer of Nugteren to have no effect on the rate of oxygenation catalyzed by untreated acetone powder. Inactivation of E_1 could result from production of alkoxy and hydroxyl radicals from an enzyme-bound product. Alternatively, inactivation of E_1 may be caused by a slow oxidation of Fe(II) such as that which occurs with certain other Fe(II) dioxygenases (62,93). In this case, the inactivation would be expected to be reversible. Although GSH, which is a mild reductant, does not prevent inactivation, a stronger reductant such as formamidine sulfonic acid might reverse the inactivation of VG dioxygenase E_1 (94).

SUMMARY

GSH-peroxidase in the presence of GSH partially inhibits the oxygenation of fatty acids catalyzed by both sheep vesicular gland homogenate and phenol-treated vesicular gland acetone powder. These results suggest the presence in both preparations of a fatty acid dioxygenase that requires hydroperoxide as an obligatory intermediate.

We found that two distinct fatty acid dioxygenases are present in phenol-treated VG acetone powder. One enzyme (E_2) requires phenol for activity and may be completely suppressed by the functioning of GSH-peroxidase. Therefore, E_2 seems to have an obligatory requirement for hydroperoxide. The other less active dioxygenase (E_1) is not affected by GSH-peroxidase and therefore probably does not require hydroperoxide.

E_1 is inactivated in the presence of both substrates, fatty acid and oxygen, by a process which is first order with respect to enzyme concentration. E_2 is inactivated by a kinetically similar process; however, the latter enzyme also loses activity at a significant rate in the presence of hydroperoxide by a substrate-independent process.

These findings as well as those of others may be accommodated by a kinetic model which supposes that distinct sites exist for product and fatty acid substrate on the VG dioxygenase E_2; product may bind only at the product site whereas fatty acid substrate may bind at either site. E_1 appears to have only a substrate binding site. The total rate of oxygenation of fatty acid by phenol-treated acetone powder is

represented by the sum of the steady state rate equation for $E_1(v_1)$ and the equilibrium rate expression for $E_2(v_2)$:

$$V_{total} = V_1 + V_2 =$$

$$\frac{k_{13}[E_1^o] - k_{12}[P]}{\frac{K_{1M}}{[S]} + 1} + \frac{k_{23}[E_2^o - E_2^* (k_4)] - k_{22}[P]}{\frac{K_{2ps}}{[S]} (1 + \frac{K_{2p}}{[P]}) + 1 + \frac{K_{2sp}}{[P]} (1 + \frac{[S]}{K_{2ss}})}$$

For 5,8,11,14-eicosatetraenoic acid, the values of the constants are: K_{1M} = 5.5 μM; K_{2ps} = 5 μM; K_{2p} = 0.1 μM; K_{2sp} = 0.03 μM; $K_{2ss} = \infty$; k_{12} = 0.30 min^{-1}; k_{22} = 0.5 min^{-1}; k_{13} = 3.4 min^{-1} (nmoles 20:4/mg); and k_{23} = 12.5 min^{-1} (nmoles 20:4/mg).

Activation of E_2 by phenol occurs with a sigmoidal increase of dioxygenase activity with time; the half-life for activation is independent of protein concentration. The activation is inhibited by o-phenanthroline suggesting that Fe(II) is needed for the activation process.

Inhibition of phenol-treated acetone powder by o-phenanthroline, diethyldithiocarbamate and bathocuproine suggests that both iron and copper are involved in E_2 activity. Inhibition of untreated VG acetone powder by o-phenanthroline but not Tiron or bathocuproine sulfonate suggests that only Fe(II) is necessary for E_1 activity.

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