# THE EFFECT OF PEROXIDASE ON MODEL SYSTEMS OF LIPOXIDASE AND LINOLEIC ACID

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
KERT F. IVIE
1973

# ABSTRACT

## THE EFFECT OF PEROXIDASE ON MODEL SYSTEMS OF LIPOXIDASE AND LINOLEIC ACID

BY

Kert F. Ivie

Model systems containing linoleic acid, lipoxidase (E. C. 1.13.1.13 linoleate oxygen oxidoreductase) and peroxidase (Donor: $H_2O_2$ oxidoreductase, E. C. 1.11.1.7) were used to study the effect of peroxidase on the lipoxidase catalyzed oxidation of linoleic acid. Changes in the model system were followed by the use of oxygen uptake determinations, conjugated diene formation and conjugated triene formation. The ultraviolet spectra of the linoleic acid oxidation products were also determined and the effect of bisulfite on the spectra of these products was observed. Thin layer chromatography was used to determine changes in products formed with the addition of peroxidase in the lipoxidase-linoleic acid system.

A mechanism is proposed which shows the positions in the lipoxidase-linoleic acid reaction where peroxidase exerts an influence. The results showed that peroxidase was capable of acting in two ways in the system. The first function was that of a hydroperoxide breakdown factor. The

second function is that of a stimulation effect on the lipoxidase and linoleic acid reaction which was reflected in oxygen uptake, conjugated dienes and conjugated trienes.

The lag period reported for the lipoxidase-linoleic acid reaction was verified and shown to be related to a changeover from conjugated triene production to conjugated triene destruction as well as a change in the bisulfite reactable component. Thin layer chromatography revealed that there were no new major products formed during the reaction.

Lipoxidase and peroxidase activity was shown in spinach and this indicates that both enzymes are present in natural products and therefore the possibility of interactions to affect the quality of food systems is quite pertinent.

# THE EFFECT OF PEROXIDASE ON MODEL SYSTEMS OF LIPOXIDASE AND LINOLEIC ACID

By

Kert F. Ivie

A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1973

[illegible]

**Dedicated to**

**Mary and Shawn**

# ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. L. R. Dugan, Jr. for his advice and guidance during this study and for his critical review of this manuscript. Gratitude is also expressed to Dr. R. F. McFeeters and Dr. S. D. Aust for their guidance in the preparation of this manuscript.

The author also wishes to thank his wife and family for their patience and understanding throughout the academic program.

# TABLE OF CONTENTS

## LIST OF TABLES

# LIST OF FIGURES

# INTRODUCTION

Lipid oxidation and reactions coupled to lipid oxidation have been a problem in the maintenance of fresh and frozen vegetable quality which have not been fully explained. It is known that both enzymatic and non-enzymatic oxidations are involved in product deterioration (42, 44, 83) and loss of quality. Lipoxidase has been indicated to be widespread in nature (15, 30, 32) and is therefore one of the enzymes most frequently involved in the oxidation of lipids. Wagenknecht et al. (83) showed lipoxidase to be involved in the production of off-flavors in peas. Lipoxidase has been shown by several workers (10, 58, 59) to catalyze the coupled oxidation of linoleic acid and pigment degradations as well as the production of off-flavors.

The heme proteins have been implicated by Tappel et al. (71) to be involved in lipid oxidation. One of the heme proteins involved is peroxidase which is abundant in many plants (37, 44, 45), and is a catalyst of lipid oxidation (2, 53, 71). The peroxidase enzyme is of interest in blanched, frozen vegetables because it can regenerate after some blanching procedures and regain its activity (86). Maier et al. (54) showed that peroxidase activity involves a free radical mechanism. Ben-Aziz et al. (2) compared its action to lipoxidase and indicated the action of peroxidase was different, but did not proceed to determine if the enzymes had any affect on each other.

In this study, the effect of peroxidase on lipoxidase catalyzed linoleic acid oxidation was studied in order to gain a further insight into the action of both lipoxidase and peroxidase catalyzed oxidation of unsaturated fatty acids. One purpose of this investigation was to verify and expand upon one of the several mechanisms proposed for the lipoxidase catalyzed oxidation of linoleic acid (18, 26, 66, 68, 75).

Lipoxidase has been reported by Gardner *et al*. (24) to have the hydroperoxides formed in the reaction used by a hydroperoxide isomerase and possibly by an acetylating enzyme. Other investigators have reported a hydroperoxide decomposing enzyme which follows lipoxidase (6, 8, 92). Peroxidase inhibitors have an effect on the hydroperoxide breakdown factor, therefore, it has been proposed to be a peroxidase type enzyme (29). The study which follows yielded information as to the nature of the hydroperoxide breakdown factor which is of a peroxidase nature.

As mentioned earlier, both lipoxidase and peroxidase have been shown to be present in many plants. However, lipoxidase activity has not been reported in spinach. This study attempted to show that lipoxidase, along with peroxidase, is present in spinach. Lipoxidase and peroxidase presence in the same tissues and their known wide distribution in nature provide evidence that lipoxidase, peroxidase interactions are possible in many types of plant material.

In showing the interaction of lipoxidase and peroxidase and the effect of peroxidase on the system, a model system similar to the one used by Theorell *et al*. (78) was used. This model system has been used by other investigators and shown to be an indicator of enzymatic oxidation. The conjugated dienes measured in the model system have been shown to be linearly related to thiobarbituric acid (TBA) values (14) which have long been used as a means of measuring the oxidation of fats in some systems.

The overall goal of this work is the elucidation of some of the reactions involved in lipid oxidation of plant material during storage by using a model system which has components found in natural systems. The aim was to further clarify the mechanisms involved in the enzymatic oxidation of lipids and show areas which need further investigation in order for the natural system to be better understood.

# LITERATURE REVIEW

Many studies have been conducted on both lipoxidase and peroxidase in model systems, but there are no complete explanations as to their effect on the total system or their modes of action. The following study was concerned with elucidating some of the possible interactions of lipoxidase and peroxidase rather than in covering all of their possible modes of action. Since the scope of the work was limited to interactions of these two enzymes and their possible interactions in food systems, the following literature review will be limited to: 1) Lipoxidase properties and reactions of importance, 2) hydroperoxide breakdown factors, 3) peroxidase properties and reactions of interest, and 4) presence of peroxidase and lipoxidase in natural systems.

## Lipoxidase Properties and Reactions of Importance

Crystalline soybean lipoxidase (E. C. 1.13.1.13 linoleate oxygen oxidoreductase) was first prepared by Theorell *et al.* (78, 79). The enzyme was found to have a molecular weight of 108,000 by Stevens *et al.* (69) and an isoelectric point of pH 5.65 by Catsimpoolas *et al.* (11). Investigations of the amino acid content revealed that lipoxidase was low in sulphur containing amino acids and abundant in valine (69), leucine and isoleucine (41, 69).

The pH optimum for soybean lipoxidase was shown to be pH 9.0 for linoleic acid by Ben-Aziz *et al.* (2) and above 7.0 for free fatty acids (39). Other lipoxidases exhibit similar characteristics, with navy bean lipoxidase having a pH optimum of 7.5, green pea lipoxidase a pH optimum of 7.5, peanut lipoxidase a pH optimum of 8.1 and small red bean lipoxidase a pH optimum of 7.0 for linoleic acid (15). It was also reported that these lipoxidase preparations had activity on trilinoleate with a secondary pH optimum for trilinoleate at the linoleic acid optimum. The major pH optimum was around pH 5.5 for the trilinoleate substrate.

The differing pH optimums for linoleic acid and trilinoleate were found by Koch *et al.* (49) to be caused by the presence of two lipoxidases; one a linoleic acid lipoxidase and the other a triglyceride lipoxidase. Other investigators have reported the presence of isoenzymes of lipoxidase in soybeans, wheat and peas (16, 32, 34, 48).

Lipoxidase is competitively inhibited by detergents such as Tween 20 under certain conditions (2). It has also been proven that acetylenic compounds such as eicosatetraynoic acid are competitive inhibitors (7). Dillard *et al.* (16) and Grossman *et al.* (31) have shown nordihydroguaiaretic acid (NDGA) and other antioxidents are inhibitors of lipoxidase. The natural antioxident, αtocopherol, has been shown to be inhibitory to lipoxidase (76). This was elucidated by Tappel *et al.* (75) to be the oxidation of one mole of αtocopherol oxidized per mole of linoleic acid which is not oxidized. Siddiqi *et al*. (66) exhibited, through the use of inhibitors, that the thiol groups are not involved and there are no prosthetic groups or easily dissociable metal ions involved.

Hirano *et al*. (38) indicated methemoglobin can act as an inhibitor to lipoxidase under certain conditions, but will not decrease the level of oxygen uptake. Alcohols inhibit lipoxidase through hydrophobic bonding with long, straight chain alcohols having the greatest inhibitory effect (56). This was used as evidence for a non-polar area being involved in the enzyme's active site.

Lipoxidase has been shown to be active in both a homogeneous and heterogeneous system by Tappel *et al*. (72). The activity in the heterogeneous or colloidal system is somewhat reduced (71). Koch *et al*. (48) noted the enzyme was activated by $Ca^{++}$ while $Mg^{++}$ did not act as an activating ion. The calcium ion also acts on the enzyme to increase its substrate specificity (40). Balls *et al*. (1) noted that a peptide activator was required for enzyme activity.

Substrates for lipoxidase action have been exhibited to contain a cis, cis 1,4 pentadiene group with a methylene group located at $\omega 3$ (36). It was proven by Privett *et al*. (60) that the bonds have to be in the cis, cis configuration for activity. The points of attack have been demonstrated to be at the $\omega 6$ or $\omega 9$ position (35, 40). The ninth carbon atom from the carboxyl end is the second best site of attack, provided it begins the diene system (35). The lipoxidase forms hydroperoxides at the 9 and 13 position on linoleic acid, as elucidated by Eriksson _et al_. (20). Beare-Rogers _et al_. (3) have indicated that only one hydroperoxide is found per molecule of fat, even if there are multiple double bonds. The 9-hydroperoxide formed is in the D-configuration (3) while the 13-hydroperoxide is in the L form (36). The new bond formed in making the hydroperoxide has been shown to be in the trans configuration (18, 36). The percent of 9 hydroperoxy 10, 12 octadecadienoic acid and 13 hydroperoxy 9, 11 octadecadienoic acid produced by lipoxidase has been reported to vary for different systems and sources of enzyme. Dolev _et al_. (18) noted that the 13 isomer is formed exclusively by soybean lipoxidase. Dolev _et al_. (19) also illustrated that $O_2$ comes from the atmosphere. Alfalfa lipoxidase was reported by Chang _et al_. (12) to produce 50 percent 9 isomer and 50 percent 13 isomer. They also reported 70 percent 13 isomer and 30 percent 9 isomer for lipoxidase from soybeans. Corn lipoxidase was reported by Gardner (25) to produce 85 percent of the 13 hydroperoxy and 15 percent 9 hydroperoxy. He also gave proof of the trans 11 double bond. Lipoxidase isolated from flax seed has been shown to produce 80 percent 13 hydroperoxy and 20 percent 9 hydroperoxy octadecadienoic acid by Zimmerman _et al_. (93). These discrepancies indicate that the system may be able to act in slightly different modes to produce various amounts of the 9 and 13 hydroperoxides, and that enzymes from different sources vary slightly in their specificity.

In the production of hydroperoxides by lipoxidase it has been reported that free radicals are produced (23). This was confirmed in both the aerobic and anaerobic system by Walker *et al*. (85) and to involve the formation of a peroxy radical (26). It has been demonstrated that there is an induction period which can be reduced by linoleate oxidation products (67, 68). The abolition of the induction period was reported by Smith *et al*. (68) to be due to linoleate hydroperoxide.

Lipoxidase was reported to catalyze the anaerobic destruction of peroxides in such a manner that dienes do not decrease (6). Smith *et al*. (68) stated that hydroperoxides were necessary for lipoxidase to catalyze the formation of hydroperoxides. It was not stated where these initial hydroperoxides originated. Hydroperoxide decomposition by lipoxidase has also been reported to be involved in chlorophyll bleaching (10) with the main hydroperoxide involved being the 9 hydroperoxide (21).

Lipoxidase has been shown to be involved in several coupled reactions. Buckle (10) has demonstrated lipoxidase to be involved in chlorophyll bleaching and Orthoefer (59) found this to be coupled to the oxidation of linoleic acid catalyzed by lipoxidase. Pheophytin *a* was discovered to be one of the end products (84). Lipoxidase is also the catalyst of a coupled oxidation of carotene and linoleic acid (80) and to a system in which glutathion oxidation is coupled to linoleic acid oxidation (55). The presence of alcohol was found to activate the latter oxidation. Mapson _et al_. (55) reported lipoxidase to be located in the soluble portion of the cytoplasm where oxygen and substrates are available.

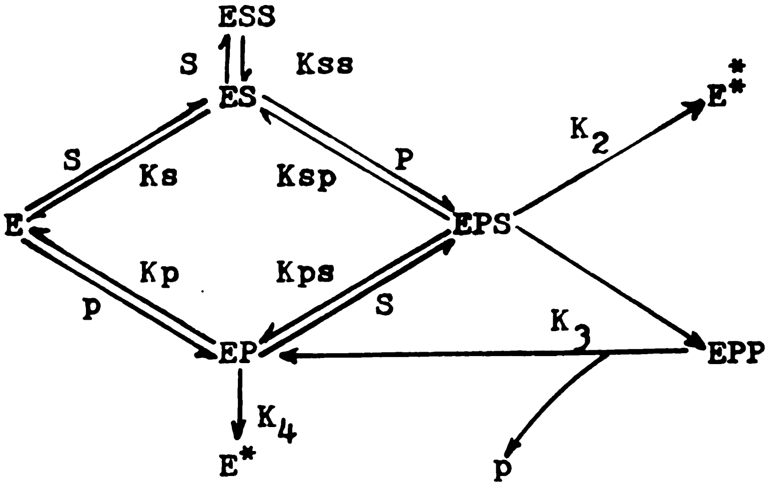
Some of the end products of the lipoxidase reaction are brought about by autoxidation of hydroperoxides. Lipoxidase end products have been reported to be alcohols in bread dough (30) due to the unstable nature of the hydroperoxides formed. It has been reported by Johnston _et al_. (42) that one mole of oxygen absorbed by a linoleate hydroperoxide destroys one mole of cis, trans conjugated diene, one-half mole peroxide group and one mole linoleate hydroperoxide. Dimers of varying polarity, scission acids, and isolated trans bonds are formed by autoxidation. The cis, trans diene formed by lipoxidase (60) can be further oxidized to produce ketodienes and also aldehydes (26, 82). Dahle _et al_. (14) reported that conjugated dienes produced during the reaction are linearly related to TBA values which are used to measure fat oxidation. Other products are possibly due to autoxidation and enzymatic action.

Many mechanisms have been proposed for the action of lipoxidase. Tappel _et al_. (75) proposed that a complex of lipoxidase oxygen and substrate is formed, followed by the transfer of one electron and the hydrogen ions to the oxygen, which forms a biradical. The biradical reacts to give a conjugated peroxide which then dissociates from the enzyme. They proposed that lipoxidase functions in the stabilization of the biradical. They did not account for the specificity of the enzymatic reaction.

Dolev *et al*. (18) proposed a mechanism for lipoxidase which accounts for the specificity. The enzyme is activated by oxygen, forming an enzyme oxygen radical, which adds to linoleic acid at the carbon 13 position and is held there by either the double bond at carbon 9 or the carboxyl group. A series of one electron shifts in this complex establish a new trans double bond at carbon 11 which transfers the hydrogen to oxygen, forming the hydroperoxide and liberating the enzyme. The enzyme could be a free radical which could start the reaction again.

Siddiqi *et al*. (66) proposed a mechanism in which the initial step is the formation of an enzyme substrate complex where oxygen is held close to the linoleate molecule attached by the α methylene group. Secondly, a free radical is formed at the methylene group in which the hydrogen goes to the media or the protein. Thirdly, the double bonds are isomerized, yielding the trans form in a cis, trans peroxyradical which exhibits resonance so that an asymmetric center is created by addition of biradical oxygen. The hydroperoxide is formed by the peroxy radical oxygen, receiving an electron from lipoxidase and hydrogen from the media or a hydrogen radical from the enzyme. The hydroperoxide is then released. They also state the peroxyl group could abstract hydrogen from other linoleic acid molecules, thus allowing a lipoxidase modulated chain reaction.

Smith *et al.* (67, 68) showed that lipoxidase is capable of self inactivation during the reaction sequence. They proposed the following kinetic formulation of lipoxidase action.



E = Lipoxidase  P = Product  S = Substrate

$E^{**}$ and $E^{*}$ are inactive forms of lipoxidase

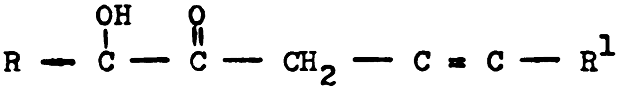
The above is based on a binding site for product and substrate, and oxygen which must also be bound to the enzyme for activity. From this model they propose the following mechanism: an enzyme substrate, oxygen and hydroperoxide complex is first formed in which the hydroperoxide reacts with oxygen to form a tetroxide transition state which goes to a perepoxide intermediate. Next the perepoxide breaks down to hydroperoxide by stereospecific removal of the L-n-8 hydrogen from the adjacent methylene carbon, forming the trans double bond and the hydroperoxide on the substrate molecule. The complex could then release the new hydroperoxide and begin again. They also proposed that the self inactivation of lipoxidase is due to interactions of the highly reactive intermediates with portions of the enzyme. The results also indicate how lipoxidase is capable of being involved in coupled reactions due to the highly reactive intermediates present in the reaction.

Tappel (74) has demonstrated that the Km value for the oxygen requirement of lipoxidase is dependent on the substrate concentration and that there is competition for the oxygen binding site between the oxygen and substrate.

## Hydroperoxide Breakdown Factors

A hydroperoxide breakdown factor has been associated with lipoxidase activity. Blain *et al.* (6) showed this factor was enzymatic, heat labile and non-dialyzing. The lipoperoxidase was shown to be different from hematin activity due to differing pH activity curves (8). Heme proteins were shown by Tappel (73) to break down hydroperoxides. Gini *et al.* (29) illustrated that the hydroperoxide breakdown factor was partially inhibited by potassium cyanide (KCN), indicating that a peroxidase type enzyme was involved.

Gardner (24) has indicated that there is a linoleate hydroperoxide isomerase in corn which utilizes the hydroperoxides formed by lipoxidase. He also showed that the hydroperoxides were only present in trace amounts in the lipoxidase reactions. A hydroperoxide isomerase was also shown in flax seed (93) with the production of



$$R - \overset{\overset{OH}{|}}{C} - \overset{\overset{O}{\|}}{C} - CH_2 - C = C - R^1$$

being proposed as one of the products (92).

## Peroxidase Properties and Reactions of Interest

Peroxidase (Donor: $H_2O_2$ oxidoreductase E. C. 1.11.1.7) is a heme protein with a molecular weight of 40,000 (51, 86). The isoelectric point has been reported to be 7.2 and the optimum pH at 7.0 by Maehly et al. (51). Maehly et al. (52) have shown the substrate to be hydrogen peroxide, methyl peroxide and ethyl peroxide. Peroxidase also requires the presence of a hydrogen donor. Bjorksten (4) reported that thyroxine could act as the donor and Fox et al. (22) demonstrated that indol-3-acetic acid could also be used. Theorell (77) showed that at a concentration of $10^{-5}$ molar both cyanide and sulfide act as a reversible inhibitor.

Peroxidase has been reported by several investigators to contain 6 to 7 isoenzymes (47, 64, 70, 86) which contain protohemin IX. Apoprotein, as well as the heme group, is involved in the reaction (87) on normal substrates. Peroxidase has been found by Ben-Aziz et al. (2) to be involved in lipid oxidation. The pH optimum was found to be pH 4.5 with a secondary maximum at pH 8.5. The activity of peroxidase and other heme enzymes was reported to be much less than that of lipoxidase. Tappel (73) showed that the heme proteins are capable of causing homolytic scission of hydroperoxides. Maier et al. (53, 54) illustrated the homolytic cleavage of the hydroperoxide to be similar to that caused by iron at higher concentrations.

The enzyme is inhibited by hydrogen peroxide at levels greater than one molar, while at levels lower than one molar there is very little inhibition (87). The heme group and protein were both shown to be involved in the phenomena. The peroxidase heme iron can exist in many oxidation states which affect the enzyme properties. Blumberg et al. (9) indicated that a ferric protein (peroxidase) and a hydroperoxide could form a radical called compound I and that this, if reduced by one electron, can produce compound II. They also showed that ferroperoxidase is diamagnetic. Both compound II and compound I were shown to be in the iron plus 4 state. However, for compound I the second oxidizing equivalent is the porphyrin ring.

Ferroperoxidase and hydrogen peroxide have been demonstrated by Bjorksten et al. (5) to be capable of forming oxyperoxidase or compound III. They reported evidence of interenzymatic reactions which allowed peroxidase to change states of the iron in the porphyrin ring. Wittenberg et al. (88) showed ferrous horseradish peroxidase reacting with one equivalent of oxygen to produce oxyperoxidase. The oxyperoxidase was found to contain all four oxidizing equivalents of oxygen and to be able to accept electrons. George (27) demonstrated that horseradish peroxidase in the presence of peroxidase goes to compound I while excess hydrogen peroxide forces the formation of compound III from compound II. If hydrogen peroxide is then lost, compound III will revert back to compound II.

Peroxidase was shown by Yokota _et al._ (91) to catalyze the aerobic oxidation of reduced nicotinamide-adenine dinucleotide (NADH) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH). In this reaction, free radical forms of NADH and NADPH are active intermediates in the formation of compound III and the reduction of peroxidase. The peroxidase is reduced in this reaction if $H_2O_2$ or $O_2$ is present. There was also a peroxidase cycle shown in which peroxidase and $H_2O_2$ goes to compound I, followed by a one electron reduction to create compound II. Compound II then has a one electron reduction to peroxidase and the cycle can begin again. The electrons came from the NADH or NADPH.

## Lipoxidase and Peroxidase in Foods

Lipoxidase activity has been exhibited in a variety of foods. Dillard _et al._ (15) reported lipoxidase to be present and active in navy beans, peanuts, green peas, baby lima beans and small red beans. Gardner (25) demonstrated lipoxidase activity in corn, along with the hydroperoxide-decomposing enzyme. Grossman _et al._ (31) reported lipoxidase to be present in egg plant while Hale _et al._ (34) showed lipoxidase to be in green peas and their seeds and in green beans and their seeds. Theorell _et al._ (78)

isolated lipoxidase from soybeans. Lipoxidase was also shown in flax seed (92, 93) and wheat (32).

Peroxidase has been reported in many plant products. Joslyn et al. (45, 46) reported activity in asparagus and artichokes. Joslyn et al. (43, 44) also reported activity in spinach, peas, pea pods and lima beans. Other investigators have shown peroxidase in other plant material. It is now presumed that peroxidase is common to all plant materials.

The role of these two widespread enzymes in food products is not fully understood. In the case of lipoxidase, its biological function has not been clearly established. One function proposed is mobilization of energy in germinating seeds (58). Wagenknecht et al. (83) have demonstrated that both lipoxidase and peroxidase are involved in the production of off-flavors in frozen peas. Wagenknecht et al. (83, 84) also reported lipoxidase to be involved in color loss and that lipase liberates the free fatty acid substrate for this reaction. Rackis et al. (62) have shown the presence of both peroxidase and lipoxidase and that lipoxidase is related to off-flavors. They also noted peroxidase to be capable of breaking down linoleate hydroperoxides. Purr (61) showed lipoxidase to be involved in the production of carbonyls in low water content foods. Rhee et al. (63) proposed that in frozen peas the lipoxidase action did not contribute significantly to off-flavor. They did note the production of off-flavor compound by lipoxidase at low levels.

# EXPERIMENTAL

## Reagents and Purifications

### Linoleic Acid

High purity linoleic acid obtained from the Hormel Institute had a final purity of greater than 99% by GLC and TLC. The linoleic acid used for the determination of lag time was prepared by a procedure reported by Gardner (24) and was modified as follows: a 2 cm X 22 cm column length was packed with 125 mesh silicic acid in benzene slurry. The column was then washed with 100 ml of benzene and 1 g of Sigma high purity linoleic acid was added to the column with 5 ml of benzene. The following elution sequence was followed.

100 ml of 100% benzene
50 ml, 90% benzene, 10% anhydrous diethyl ether
50 ml, 80% benzene, 20% anhydrous diethyl ether
50 ml, 60% benzene, 40% anhydrous diethyl ether
25 ml, 50% benzene, 40% anhydrous diethyl ether, 10% methanol
25 ml, 40% benzene, 40% anhydrous diethyl ether, 20% methanol

The column was then washed with 100 ml of 100% methanol, followed by 100 ml benzene and reused. The fractions collected were examined by thin layer chromatography and those with pure linoleic acid were pooled and concentrated under nitrogen. The entire procedure was conducted under a stream of nitrogen.

The thin layer chromatography procedure used was that of Gardner (24). The solvent system was isooctane, diethyl ether and acetic acid (50:50:1) by volume, with the use of 70% $H_2SO_4$ and saturated $CrO_3$ spray and charring for visualization.

## Enzymes

The enzymes used were obtained from Worthington Biochemical Corporation. The lipoxidase (code Lx) was of soybean origin and of the fatty acid type with an activity of 2372 Worthington units per mg. The peroxidase (code HPOD) was obtained from horseradish with an activity of 600 Worthington units per mg. All other reagents were obtained from commercial sources. One Worthington lipoxidase unit is equal to the amount of enzyme necessary to cause a 0.001 optical density change in one minute at 25° C. One peroxidase unit equals the amount of enzyme required to decompose one micromole of peroxide per minute using the Worthington assay.

## Preparation of Lipoxidase From Spinach

Three hundred grams of fresh spinach were macerated with 600 ml of 0.05 molar, pH 6.8, sodium phosphate buffer for 5 minutes in a Waring blender on high speed at 5° C. The slurry was then centrifuged at 4° C for 40 minutes at

$1.5 \times 10^4$ g. The supernatant was decanted and 50 g of activated charcoal added. This was filtered through number six Whatman paper with celite #545 overlaid as a filter aid. This step, conducted in a cold room, was repeated until the color was removed. The filtrate was then dialyzed against deionized water to remove the buffer. The protein solution was then frozen and freeze dried. The resultant powder was taken up in a 10 ml 0.1 molar borate buffer, pH 9.0 and centrifuged at $3.9 \times 10^4$ g for 60 minutes to remove nonsoluble material. The supernatant was then used for enzyme assays.

### Preparation of Peroxidase From Spinach

Four hundred grams of spinach were blended for 3 minutes in a Waring blender with 300 ml of pH 6.8 sodium phosphate buffer 0.05 molar. The homogenate was then centrifuged at $2.2 \times 10^4$ g for 70 minutes and the supernatant collected and recentrifuged at $3.5 \times 10^4$ g for 45 minutes. The supernatant was then filtered through glass wool and used as a crude enzyme preparation. An alternate procedure was to take 60 g of spinach and blend for 5 minutes in pH 6.8 phosphate buffer 0.05 molar and then centrifuge at $3.9 \times 10^4$ g for 70 minutes at $4^\circ$ C. The supernatant was used as the crude enzyme preparation.

## Assay Procedures

### Oxygen Uptake

Oxygen uptake studies were performed using standard procedures described in the Gilson Respirometer Manual (28).

This procedure is based on standard Warburg manometric procedures and was used by Tappel et al. (78) as an assay method for lipoxidase.

The reaction system used consisted of the following: Linoleic acid was at a final concentration of 1 mg/ml. Both peroxidase and lipoxidase were used at a final concentration of 0.1 mg/ml which equals 237 units of lipoxidase and 60 units of peroxidase. The buffer used consisted of 0.1 molar sodium borate buffer pH 9.0 with a final concentration of 0.5% ethanol. The final reaction mixture had a volume of 5 ml. The center well of the reaction vessel contained three potassium hydroxide pellets to absorb $CO_2$. The temperature was maintained at 30° C.

The following combinations were used: 1) Linoleic acid in buffer. 2) Lipoxidase and linoleic acid in buffer. 3) Lipoxidase, peroxidase and linoleic acid in buffer. 4) Peroxidase and linoleic acid. 5) Lipoxidase and linoleic acid were reacted for 10 minutes and peroxidase added. At this time the oxygen uptake was determined. 6) Peroxidase and linoleic acid were reacted for 10 minutes, lipoxidase was then added and the oxygen uptake determined.

The enzymes being tested were added at time zero from a side arm flask. The system was equilibrated for 5 minutes before the addition of any enzymes.

### Conjugated Dienes

Conjugated diene determinations at 234 nm were performed through the use of the method originally described by Theorell *et al*. (74) and modified by Tappel *et al*. (71). The substrate was prepared and used as described by Worthington (86). The final reaction mixture consisted of the following: Linoleic acid was at a final concentration of 0.11 mg/ml. The enzymes used were in a concentration of 6.66 µg/ml for both lipoxidase and peroxidase, which equals 158 units of lipoxidase and 40 units of peroxidase. The total reaction volume was 3 ml with the buffer being 0.1 molar sodium borate buffer at a pH of 9.0 with 0.55% ethanol. The increase in absorbancy at 234 nm was measured on a Beckman DU at 15 second intervals and on a Beckman DU equipped with a recorder. The initial rate after the induction period was used as the reaction rate. The molar extinction coefficient for conjugated dienes has been reported to be 28,000 at 234 nm (34). The following systems were used: 1) Linoleic acid and buffer. 2) Lipoxidase and linoleic acid. 3) Peroxidase and linoleic acid. 4) Lipoxidase, peroxidase and linoleic acid. 5) Lipoxidase and linoleic acid reacted 1.5 minutes, then peroxidase added. 6) Peroxidase and linoleic acid reacted 1.5 minutes, then lipoxidase added.

The enzymes being tested were added at time zero from a micropipette. Reactions were permitted to proceed at ambient temperature.

## Lag Time Assay

The lag time for the reaction was determined by using the conjugated diene procedure and varying the lipoxidase enzyme concentration (33). The systems were the same as those in the conjugated diene procedure, except for the following concentrations of lipoxidase which were used: 1.66 µg/ml, 3.33 µg/ml, 6.66 µg/ml and 0 µg/ml. The lag period was the time necessary for the reaction rate to become linear. This was determined by reading the absorbancy at 234 nm at 15 second intervals on a Beckman DU or recording the absorbancy changes on a Beckman DU with recorder. The enzymes were added at time zero.

## Conjugated Triene Assay

Conjugated trienes were determined by a modification of the conjugated diene procedure given in the Worthington Enzyme Manual (90). The same systems were used, but the change in the absorption was read at 270 nm. The change in absorption was monitored at 15 second intervals on a Beckman ACTA spectrophotometer and the resulting curves drawn in. The model systems used were the same as those in the conjugated diene procedure. The reaction was started by the addition of the enzyme from a micropipette at time zero. The enzyme activity was the same as that used in the conjugated diene procedure.

## Ultraviolet Spectroscopy

Ultraviolet spectra (scans) of model systems were made by a modification of the conjugated diene procedure previously described. The reaction mixture consisted of the following: 0.166 mg/ml linoleic acid was reacted with 10 μg/ml lipoxidase and or peroxidase in 0.2 molar borate buffer pH 9.0 with 0.83% ethanol, and a final volume of 2 ml at the end of the desired reaction time. One ml of 100% ethanol was added by pipette to stop the reaction at the desired time in the reaction and the absorbancy scanned on a Beckman DK 2A or ACTA from 300 nm to 200 nm. The final reaction mixture at the time of scanning consisted of the same components as the conjugated diene assay systems, except the alcohol was at 34.16%. The blank consisted of everything in the system except lipid. Buffer was added to make the volume 3 ml.

The effect of bisulfite on the ultraviolet spectra was determined by using the procedure for determining the ultraviolet spectra of the reaction products. This was done by the addition of 20 μl of a saturated bisulfite solution at the same time the ethanol was added and then scanning to obtain the spectra. The blank was the same as in the ultraviolet spectra studies with bisulfite added, if it was included in the reaction mixture.

## Enzyme Inactivation

Two methods of enzyme inactivation were used. For peroxidase, the enzyme was inactivated by either heat or potassium cyanide. Five ml of peroxidase stock solution was placed in a 22 X 150 mm test tube, which was then placed in a boiling water bath for 5 minutes as a heat treatment. The cyanide inhibition was carried out by adding 1 ml of a 0.01 molar KCN solution to 1 ml of a 2 mg/ml peroxidase solution. The procedure used for assaying the effects was that of assaying conjugated diene formation and peroxidase activity. For the inhibition of lipoxidase, 5 ml of enzyme stock solution was placed in a 22 X 150 mm test tube which was placed in a boiling water bath for 3 minutes as a heat treatment. The conjugated diene and triene assay procedures previously reported were used to determine the effects on the model systems. The systems used were the same as those in the conjugated diene assay procedure. A heated linoleic acid system was also used. To heat treat the linoleic acid a sample of stock solution was placed in a 50 ml volumetric flask which was then immersed for 3 minutes in a boiling water bath. Nitrogen was bubbled through the solution and filled the headspace for the removal of oxygen prior to the heat treatment. The heat treated linoleic acid was then used in the assay procedures in the same manner as linoleic acid which was not heat stressed.

### Peroxidase Assay

The determination of peroxidase activity was accomplished by using the procedure given in the Worthington Enzyme Manual (86). In this procedure, the rate of hydrogen peroxide decomposition by peroxidase with a o-dianisidine as hydrogen donor was measured by the rate of color development at 460 nm. The reaction mixture used was the same as in the Worthington Enzyme Manual except for the crude extract, in which case 5 µl or an amount necessary to give a measurable rate of absorption at 460 nm was used.

### Thin Layer Chromatography

Thin layer chromatography of the reaction products was performed as reported in the section on preparation of linoleic acid. The following solvent systems were used: diethyl ether, hexane and acetic acid (30:70:0.5) by volume, and isooctane, diethyl ether, and acetic acid (50:50:1) by volume. The visualization of compounds was performed using the reagents given by Vioque _et al_. (82). One percent N, N dimethyl-p-phenylenediamine in chloroform, acetic acid and water (5:5:1) by volume was used to visualize peroxides and some aldehydes, followed by 50% $H_2SO_4$ and charring to visualize other spots.

### Statistics

Statistics were calculated using the following equations given by Chase and Rabinowitz (13) in which:

$$S = \sqrt{\frac{1}{N-1} \quad (n-\bar{n})^2} \qquad \text{and} \qquad \bar{S} = \frac{S}{\sqrt{N}}$$

The $\bar{S}$ value was then doubled and used to determine if the differences were still present at the $2\sigma$ level.

Statistical variance analyses were also used to determine if the differences reported were significant at the 95% level. The method used was that described by Kramer *et al*. (50). This method showed data which was significant at the 99% confidence level as well as the 95% confidence level. The method was programmed on a Wang computer.

# RESULTS AND DISCUSSION

In studying the effect of peroxidase on the lipoxidase catalyzed oxidation of linoleic acid, the model systems used were designed to establish various features of the system.

1) Linoleic acid was used as the oxidizable substrate and to determine the autoxidation which was taking place in the other systems. Linoleic acid also served as a control.

2) Lipoxidase and linoleic acid were reacted to determine the standard rate of the reaction for the model system used.

3) Peroxidase and linoleic acid were reacted so that heme catalyzed oxidation could be detected and compared to the other model systems.

4) Three systems were used to determine the effect of peroxidase on the lipoxidase-linoleic acid system.

   a. When lipoxidase and peroxidase were added at the same time, the effect of peroxidase on the lipoxidase reaction was determined.

   b. When peroxidase was reacted with linoleic acid before the lipoxidase was added, the result being studied was peroxidase effect on the substrate and the resulting effect on the lipoxidase reaction.

c. When lipoxidase was reacted with linoleic acid and then peroxidase added, the result being studied was peroxidase effect on reaction products already formed and the resultant effect on the lipoxidase reaction.

5) Model systems in which the peroxidase was inhibited by KCN were used to determine whether the heme iron was involved in the reactions of the previously described model systems.

6) Model systems in which peroxidase and lipoxidase were inactivated by heat were used to determine if the enzyme had to be in the active protein conformation for activity to occur.

7) Systems in which bisulfite was added determined whether conjugated triene absorption in the ultraviolet region was due to ketones or aldehydes. This was then correlated to changes occurring in the systems.

## Oxygen Uptake Determinations

In determining oxygen uptake the operational sequence used in the procedure had to be standardized in order to obtain reproducible results. The pH of the system proved important since at a pH of 6.8 the author found it difficult to obtain reproducible results, while at a pH of 9.0 reproducibility became easier. This may be a result of the lipid being incompletely suspended at pH 6.8. At a pH of 9.0 the lipid was in a better suspension as evidenced by less turbidity. A more uniform lipid concentration around the enzyme may have occurred due to a smaller, micelle size or better suspension.

Table 1 gives the results of the oxygen uptake experiments and Figure 1 shows the graph of oxygen uptake in the model systems described. These results inidicate that if peroxidase is added at the same time or before lipoxidase, the oxygen uptake will be stimulated by 12.5% and 12.6% respectively. The rate of oxygen uptake of the lipoxidase-linoleic acid reaction when measured from 10 to 15 minutes after the addition of lipoxidase, will be less when compared to the oxygen uptake rate measured immediately after the addition of lipoxidase. If peroxidase is present in the system the rate of oxygen uptake is also decreased by 10 minutes after the initiation of the reaction. This rate of oxygen uptake decrease with peroxidase present in the system first is 9.5% faster than found in the lipoxidase and linoleic acid reaction. If peroxidase is added at the same time, the rate of oxygen uptake also decreased slightly faster. This is a relative rate change and may not be significant since by this time substrate is becoming a limiting factor as shown by the curved line. If peroxidase is added after the lipoxidase has had 10 minutes to interact with linoleic acid, the addition will bring about an increase of 70% in oxygen uptake. This was found by comparing the initial rate of the lipoxidase-linoleic acid reaction, with peroxidase added late, to the rate measured

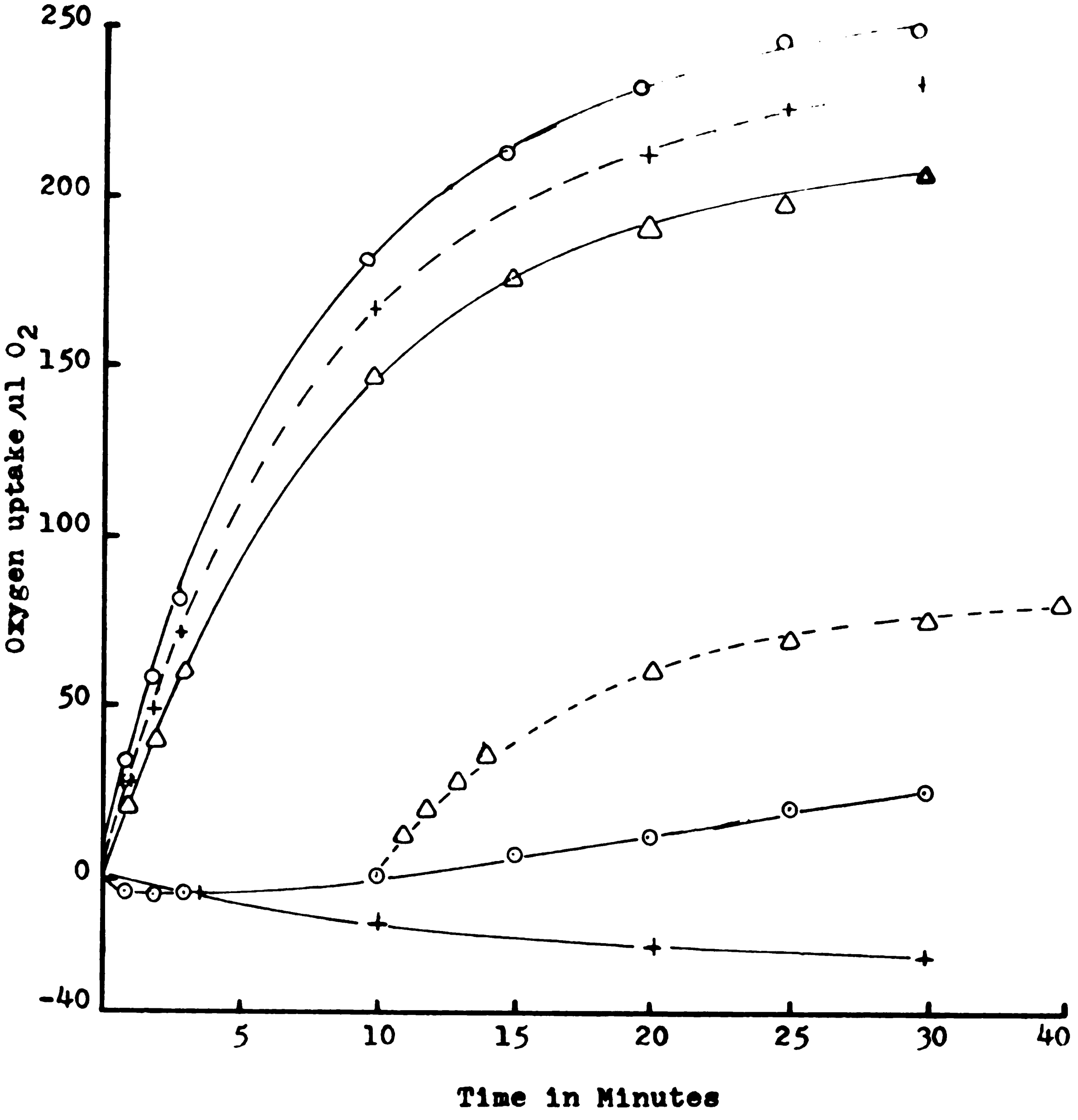
Table 1: Results of oxygen uptake studies in model systems containing lipoxidase, peroxidase and linoleic acid in various combinations.

| | Oxygen Uptake | |
|---|---|---|
| System Components[2] | Initial Rate[1] | Rate Measured From 10 to 15 Minutes After Initiation of the Reaction |
| Linoleic acid (control) | -0.065 | -0.065 |
| Lipoxidase and linoleic acid | 0.662 | 0.203 |
| Peroxidase and linoleic acid | decreasing | 0.037 |
| Lipoxidase, peroxidase and linoleic acid | 0.827 | 0.237 |
| Peroxidase added after lipoxidase and linoleic acid were reacted for 10 minutes | 0.346 | 0.286 |
| Lipoxidase added after peroxidase and linoleic acid were reacted for 10 minutes | 0.836 | 0.196 |

---

[1] Rates are reported as µM $O_2$/ minute and the above differences are significant at a 2 $\bar{s}$ level and a 95% significance level.

[2] Reaction concentrations for the above systems were equivalent. Linoleic acid concentrations, 1 mg/ml; peroxidase concentrations, 1 mg/ml; lipoxidase concentrations, 1 mg/ml. Final volume was 5 ml, 0.2 molar pH 9.0 borate buffer used in all preparations and stock solutions.



Lipoxidase, peroxidase and linoleic acid ——○——
Lipoxidase and linoleic acid ——△——
Peroxidase and linoleic acid reacted 10 minutes, then peroxidase – – + – –
Linoleic acid —— + ——
Peroxidase and linoleic acid ——⊙——
Lipoxidase and linoleic acid reacted 10 minutes, then peroxidase – – △ – –

Figure 1: Results of oxygen uptake studies in model systems containing lipoxidase, peroxidase and linoleic acid in various combinations.

from 10 to 15 minutes in the lipoxidase and linoleic acid reaction. This was the actual rate of the system peroxidase was added to. It is also apparent that the reaction rate from 10 to 15 minutes after the addition of peroxidase was 0.286 µM $O_2$ uptake per minute. In contrast, the rate in the linoleic acid and lipoxidase system had dropped to approximately zero at 30 minutes, which corresponds with the 15 minute rate in the system to which peroxidase was added late.

The preceding indicates that peroxidase has a greater stimulation effect on oxygen uptake when added after lipoxidase than in the systems in which it is added first or at the same time lipoxidase was added. One explanation is that peroxidase uses oxygen to break down products which have accumulated in the system. These products may have acted as inhibitors to lipoxidase based on the Smith *et al.* (68) model. Therefore the destruction could result in higher oxygen uptake due to less lipoxidase inhibition. If peroxidase is added first or at the same time lipoxidase is added, the rate could be increased by the destruction of end products. The loss of end products could shift the equilibrium toward the formation of more products and the utilization of more oxygen. Lipoxidase could still be self inactivated since the peroxides would be produced faster and a greater number of radical enzyme interactions could occur. This is supported by peroxidase having a slightly greater stimulating effect on oxygen uptake if added first. This is followed by a faster reaction rate decrease when measured from 10 to 15 minutes after the reaction was initiated. The results show that there are 4 moles of oxygen consumed per mole of linoleic acid oxidized, if the reaction is allowed to go to completion. This indicates that 3 moles of the oxygen consumed are used to further oxidize the hydroperoxide initially formed, or its decomposition products. This also indicates that the initially formed hydroperoxide is further oxidized and does not accumulate to be the final product.

## Conjugated Diene Determination

In studying the reaction of lipoxidase and linoleic acid, the use of conjugated diene absorption was not reproducible at a pH of 6.8 due to cloudiness of the lipid solution. For this reason the higher pH of 9.0 was used in the determination. This is the pH optimum for lipoxidase action on linoleic acid (2). Heme proteins have a small pH optimum for lipid oxidation at around pH 9.0 (2). The conjugated diene measured is formed by the lipoxidase catalyzed oxidation reaction, forming a cis, trans diene structure in making the linoleate hydroperoxide (18). The breakdown products of the hydroperoxide formed are also capable of having conjugated diene structures which absorb at the wavelength used and contribute to the results reported.

Table 2 and Figure 2 give a summary of the conjugated diene results. They show that if peroxidase is added to

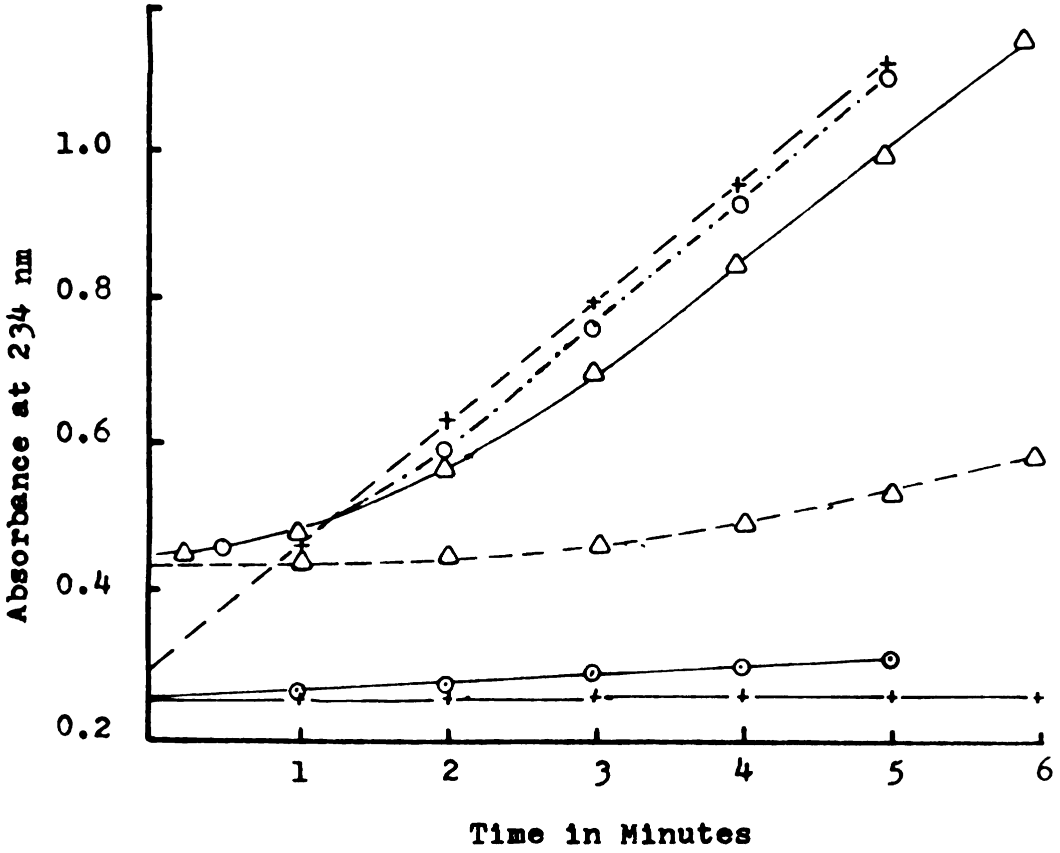
Table 2: Conjugated diene formation in model systems containing lipoxidase, peroxidase and linoleic acid in various combinations.

| System Components | Rate of[1] Conjugated Dienes Formed (µM/minute) |
|---|---|
| Linoleic acid (control) | 0.035 |
| Lipoxidase and linoleic acid | 5.46 |
| Peroxidase and linoleic acid | 0.143 |
| Lipoxidase, peroxidase and linoleic acid | 6.03 |
| Peroxidase added after lipoxidase and linoleic acid were reacted for 1.5 minutes | 5.89 |
| Lipoxidase added after peroxidase and linoleic acid were reacted for 1.5 minutes | 3.64[2] |

---

[1] All systems contained the same concentration of lipid and enzyme in a total of 3 ml, 0.2 molar pH 9.0 borate buffer. Lipoxidase and peroxidase concentration, 6.66 µg/ml; linoleic acid concentration 0.11 mg/ml and 0.55% ethanol.

[2] Based on average of the inconsistent reaction rates.



Peroxidase and linoleic acid ——⊙——
Linoleic acid ——+——
Lipoxidase and linoleic acid ——△——
Lipoxidase, linoleic acid and peroxidase — • — o — • —
Lipoxidase and linoleic acid 1.5 minutes, then peroxidase - - + - -
Peroxidase and linoleic acid 1.5 minutes, then lipoxidase – –△– –

Figure 2: Conjugated diene formation in model systems containing lipoxidase, peroxidase and linoleic acid in various combinations.

the system at the same time lipoxidase is added, there will be an increase of 10% in conjugated diene formation. If peroxidase is added after the lipoxidase, there is an 8% increase in conjugated diene formation. The addition of peroxidase 1.5 minutes prior to the addition of lipoxidase results in a system which exhibits variability. One-half of the reactions proceed at a rate comparable to that of the system containing lipoxidase and linoleic acid. The other one-half of the reactions have 67% fewer conjugated dienes formed. This variability was also found in conjugated triene determinations reported later.

Possible explanations for the effect of peroxidase follow. Peroxidase destroys a factor which is capable of inactivating lipoxidase. By keeping lipoxidase in the active state the reaction rate would be increased. Another explanation is that the presence of peroxidase causes a faster breakdown of hydroperoxides. The breakdown of hydroperoxides may kinetically shift the lipoxidase reaction toward the production of more hydroperoxides. The oxidized hydroperoxides or their products could still have a conjugated diene structure which would account for the increased activity. Both explanations are feasible since it is known that hydroperoxides are necessary for the lipoxidase to react. The destruction of hydroperoxides could be an explanation of why the peroxidase, if added first, inhibits the reaction. If added at the same time or later the rate is increased since the lipoxidase has some hydroperoxide present from autoxidation which it will use to initiate the reaction. The loss of hydroperoxides could aid in the prevention of product inhibition.

The rate may also be higher due to peroxidase breaking down hydroperoxides which could potentially inactivate the lipoxidase as reported by Smith et al. (64). This is possible since hydroperoxide is competitive with linoleic acid for the substrate binding site on lipoxidase in Smith's model.

## Ultraviolet Spectroscopy

The conjugated diene results led to an interest in the ultraviolet spectra and the effect of peroxidase on the spectra. Both conjugated dienes and trienes absorb in the ultraviolet region. Ultraviolet spectra were therefore determined to determine the spectral properties of the reaction products. Figure 3 shows typical spectra obtained during this procedure. The results indicate that the conjugated diene activity results were correct, and that lipoxidase was providing for increased conjugated trienes for at least 3 minutes. However, after 5 minutes conjugated trienes were decreasing. It appeared that peroxidase had an affect on the amount of trienes produced. This is reported later. There were no apparent shifts in the position of the product peaks. There was a shift in the products being formed as evidenced by a change in the ratio of absorption between the 280 nm and 270 nm peaks. From

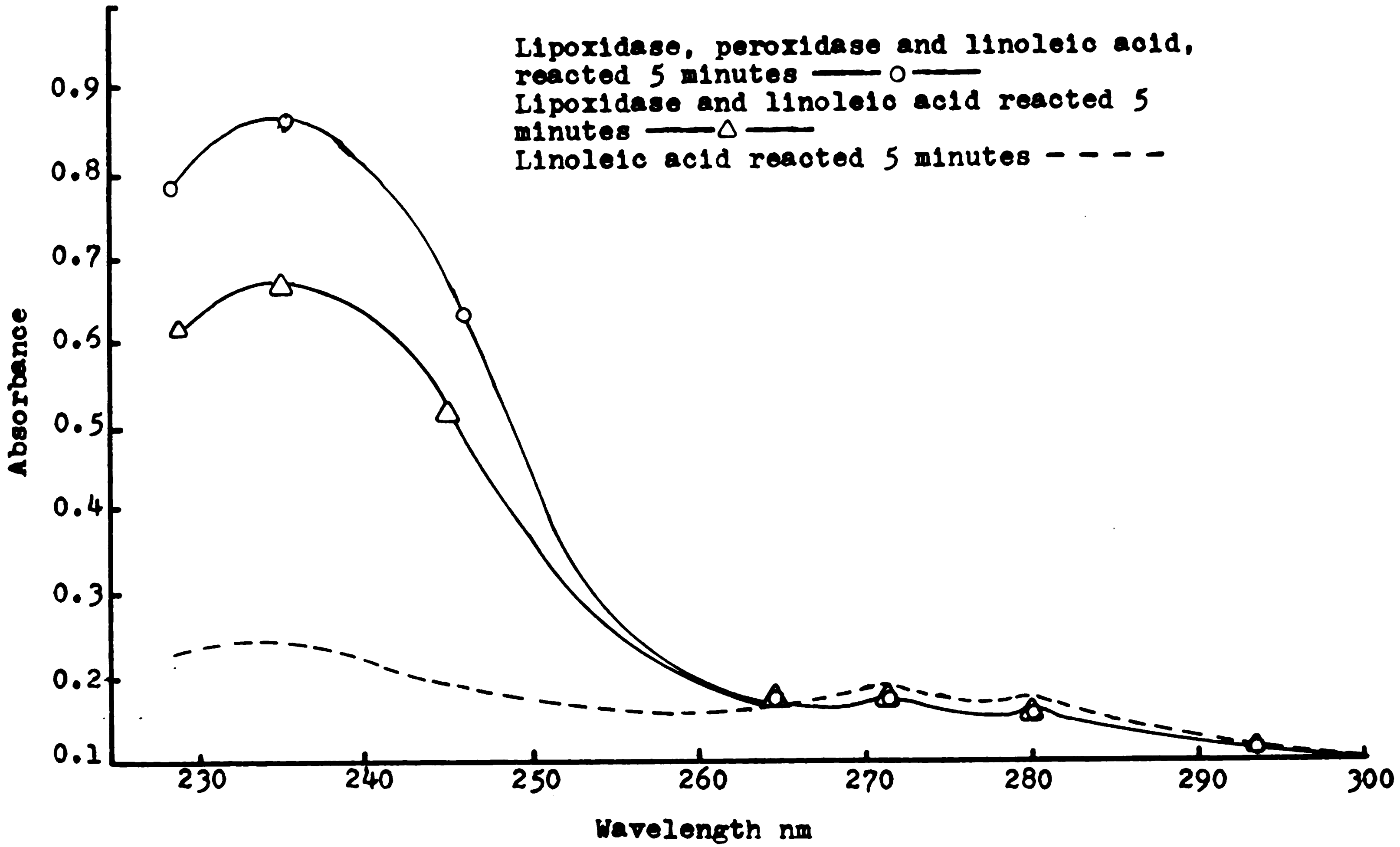


Figure 3: Ultraviolet spectra of model systems of lipoxidase, peroxidase and linoleic acid.

this it appears that if peroxidase is added at the same time as lipoxidase, there is a slight increase in the 280 nm absorption area of 3% over the 270 nm product. If the lipoxidase acts first, there is 10% difference in the ratio and the product present has a higher 270 nm absorption if peroxidase is present.

The data was used to calculate an extinction coefficient at 270 nm for conjugated trienes using the $E_{278}$ of 22,040 given by Vioque *et al.* (82) for the ketodiene they isolated. The following equation was used:

$$\frac{22{,}040}{\text{absorption at 278 nm}} = \frac{\text{molar extinction coefficient at 270 nm}}{\text{absorption at 270 nm}}$$

The calculated value was 25,617 with a standard deviation of the mean of 702. This was the value used in the conjugated triene data reported later. The molar extinction coefficient for trienes was calculated at 270 nm so that the maximum absorption change could be used and still be quantitated. This resulted in relative changes which may not reflect the true amount of product being formed at 270 nm since the absorption is the result of a mixture of products. The changes of the 280 nm to 270 nm ratio accounted for the variation expressed in the standard deviation of the mean.

Change in the 280 nm and 270 nm absorption ratio could be the result of a change from ketodiene or a similar structure which absorbs strongly at 280 nm, to an aldehyde or conjugated triene structure which absorbs strongly at 270 nm.

If the preceding hypothesis is true, the presence of peroxidase before the addition of lipoxidase would cause a decrease in the ratio of ketodiene to 270 nm conjugated triene. However, if added at the same time, the ketodiene would be increased slightly over that found in the system of lipoxidase and linoleic acid alone.

The preceding indicates that if peroxidase is in the system first, the components which absorb strongly at 270 nm are converted to some other compound causing a higher proportion of 280 nm absorption component. This observation was later verified by conjugated triene determination, since peroxidase and linoleic acid have fewer conjugated trienes formed per minute than were formed in linoleic acid alone. This is of importance since the bisulfite reactable component reported later is of the aldehyde nature and does not appear in the reaction until the lag period ends.

### Lag Time Determinations

In studying conjugated dienes, it was noted that there was an initiation time or lag time before the reaction became linear. This was also reported by other authors (63, 64). The lag time was determined at three different enzyme concentrations. Table 3 shows the effect of enzyme concentration on lag time in the lipoxidase-linoleic acid reaction.

Table 3: Effect of lipoxidase concentration on lag time in the lipoxidase-linoleic acid reaction.

| Enzyme Concentration µg Lipoxidase/ml Assay Mixture | Time at End of Lag Period (minutes) |
|---|---|
| 0.00 | |
| 1.66 | 4.50 |
| 3.33 | 3.75 |
| 6.66 | 2.95 |

---

Buffer - pH 9.0, 0.2 molar borate buffer, 0.5% alcohol

3 ml final volume in all reactions

Lipid - 0.11 mg/ml linoleic acid

The concentration, when plotted against lag time on semi-log paper, forms a straight line between the concentrations used (Figure 4). The initial rate for reactions in the conjugated diene determinations was determined by using the straight line portion after the lag period had occurred. The assay mixture of 6.66 µg lipoxidase/ml was chosen since it gave a reasonably short but measurable lag period with an increasing absorbance rate which was measurable on a Beckman DU.

The lipid used for the lag time experiments was purified by the column chromatography procedure previously reported. The lag times found using this preparation were the same as those found with Hormel's high purity linoleic acid. The thin layer chromatography of the column cleaned linoleic acid gave only one spot with an $R_f$ of 0.70. The Hormel linoleic acid obtained after this work was completed had an $R_f$ of 0.70 under the same system. Unpurified linoleic acid had three spots, at $R_f$ 0.70, 0.56 and 0.42. Although the column system used cleans up the linoleic acid, it is not recommended because it is time consuming and great care must be taken to avoid autoxidation.

## Conjugated Triene Determinations

In determining conjugated trienes, the initial rate was measured from the time the enzyme was added. This facilitated the determination of changes in the system. Table 4 and Figure 5 show the results of the determinations.

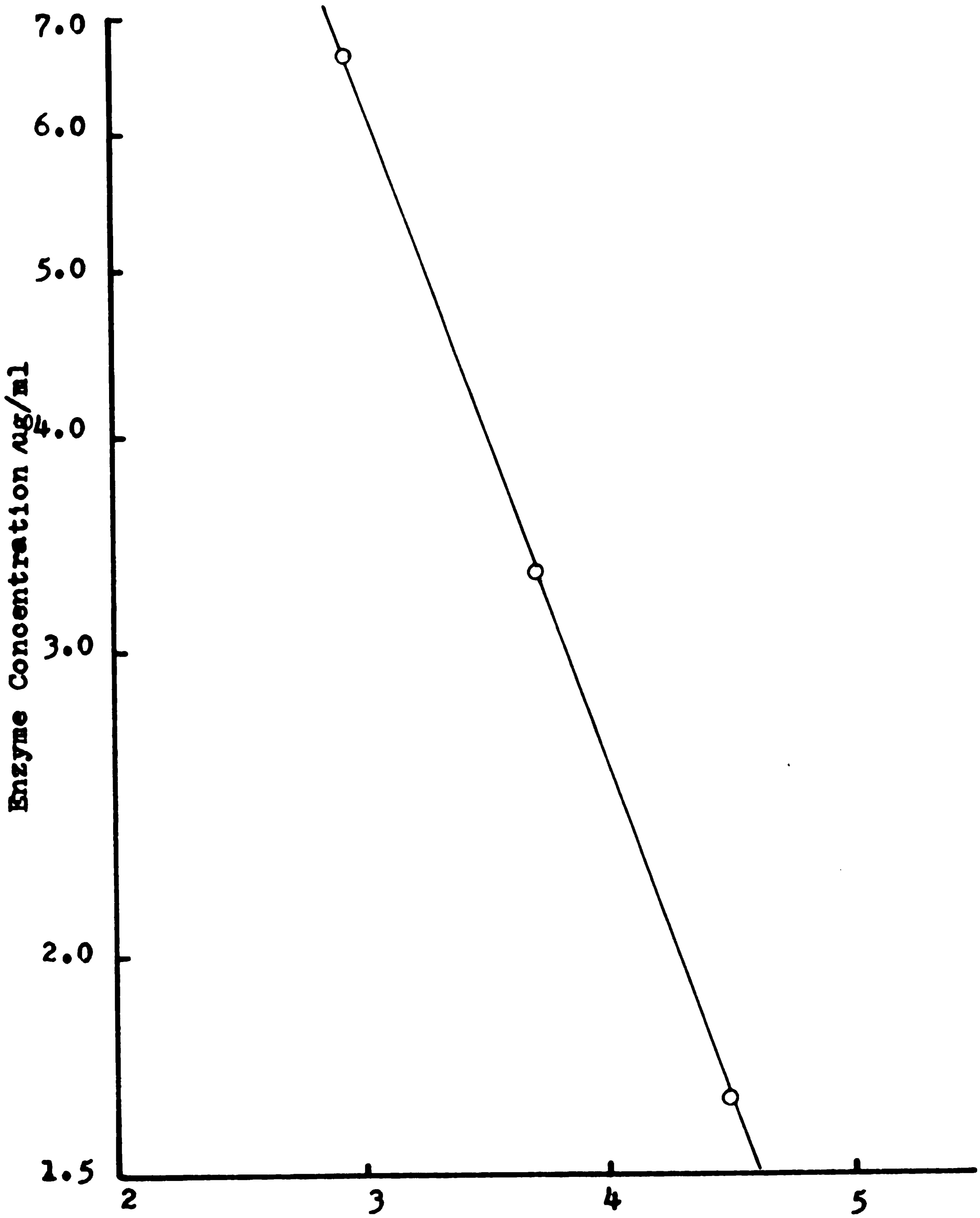


Figure 4: Effect of lipoxidase concentration on lag time in the lipoxidase-linoleic acid reaction.

Table 4: Conjugated triene formation and destruction in model systems containing lipoxidase, peroxidase and linoleic acid.

| System[2] Components | Initial Rate of Conjugated Triene Formation (µM/minute) | Sustained Rate of Conjugated Triene Formation (µM/minute) | Rate of Conjugated Triene Loss (µM/minute) | Minutes for Changeover From Production to Destruction |
|---|---|---|---|---|
| Linoleic acid | 0.625 | 0.625 | ----- | ---- |
| Peroxidase and linoleic acid | 0.566 | 0.566 | ----- | ---- |
| Lipoxidase and linoleic acid | 0.586 | ----- | 0.390 | 3.00 |
| Peroxidase, lipoxidase and linoleic acid | 0.773 | ----- | 0.453 | 3.66 |
| Peroxidase and linoleic acid 1.5 minutes, then lipoxidase added | 0.742 | 0.722[1] | 0.351[1] | 6.00 |
| Lipoxidase and linoleic 1.5 minutes, then peroxidase | 0.742 | ----- | 0.453 | 5 + 1.5 for reaction of lipoxidase and linoleic acid |

[1] The system was inconsistent as half remained constant and half changed over to destruction.

[2] Concentrations were equivalent in all systems.

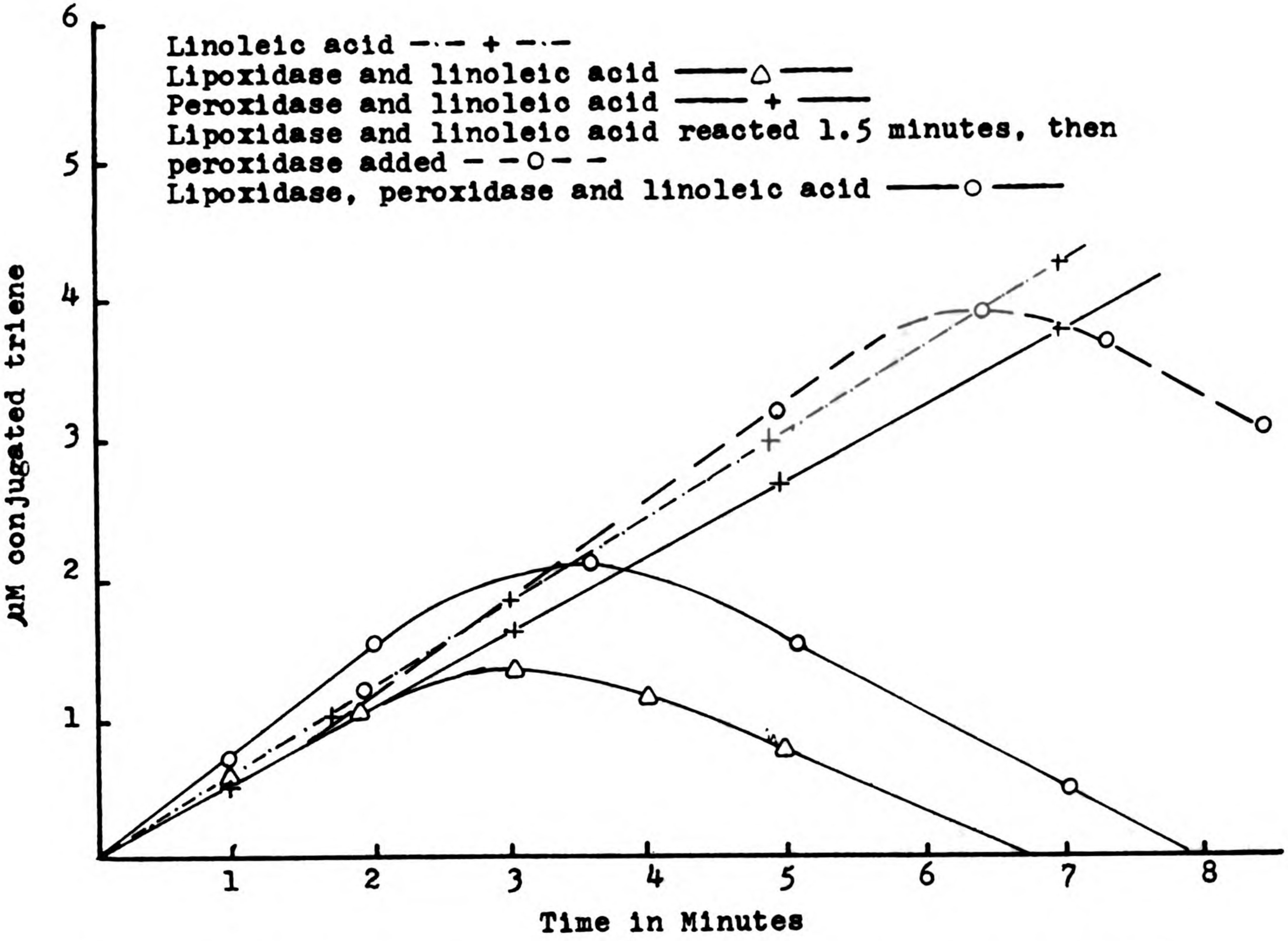


Figure 5: Conjugated triene formation and destruction in model systems containing lipoxidase, peroxidase and linoleic acid.

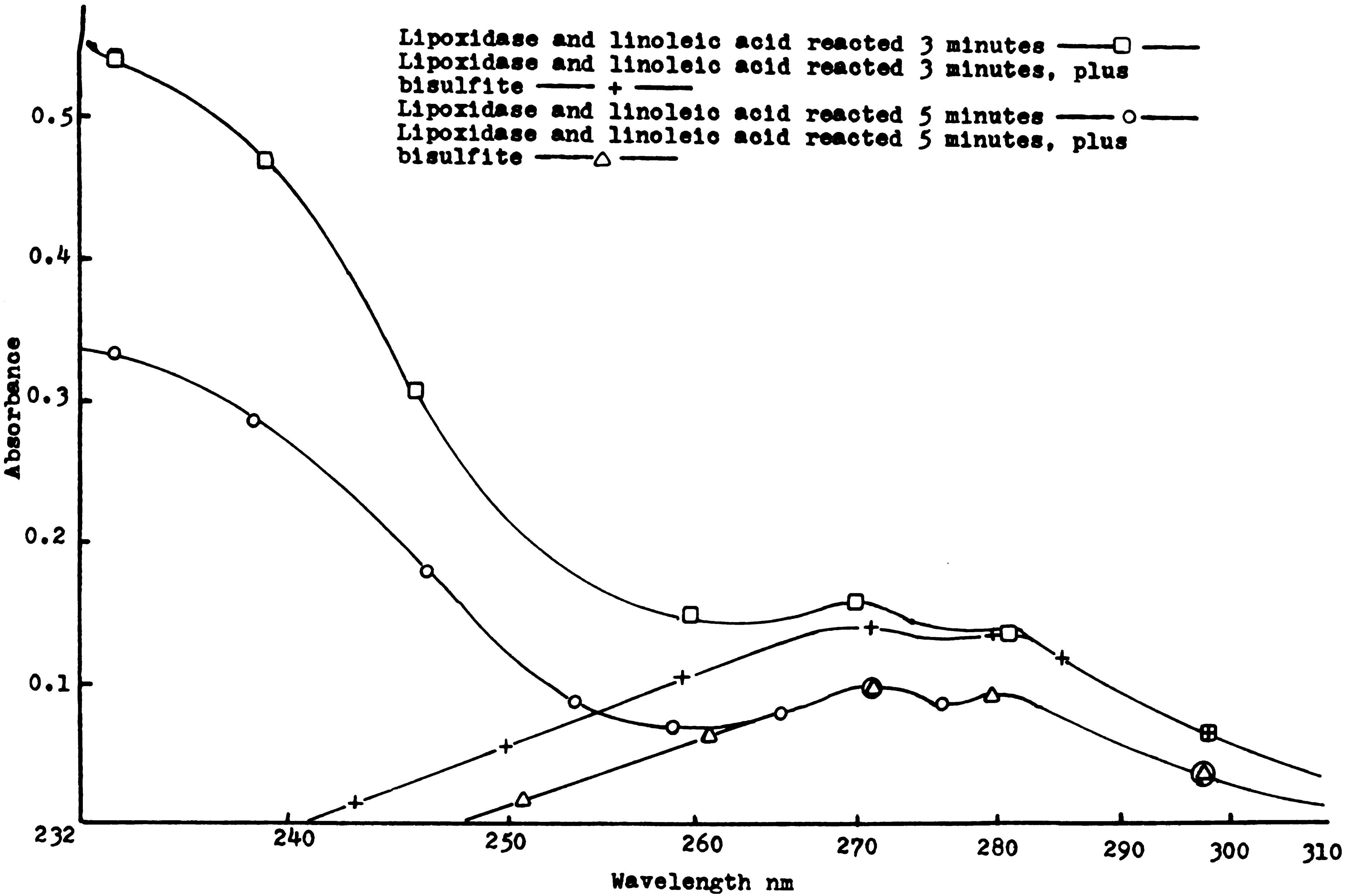
The results indicate that peroxidase does affect the system. The system containing peroxidase and linoleic acid had 9% fewer conjugated trienes formed than were formed in the linoleic acid system. The same is true of the system containing lipoxidase and linoleic acid in which 93.5% of the linoleic acid autoxidation rate is found. If both lipoxidase and peroxidase were present at the beginning of the reaction, the rate was increased by 12% over that of the linoleic acid autoxidation rate and 19% faster than the lipoxidase and linoleic acid reaction rate. The results show that an increase in changeover time, caused by peroxidase, causes an increase in the triene fraction necessary for lipoxidase to change from triene formation to triene destruction. The time period for changeover from triene formation to triene destruction for lipoxidase and linoleic acid corresponds to that found for the lag time. It is apparent that if peroxidase was added after the lipoxidase and linoleic acid reaction had proceeded for a period of 1.5 minutes, the system can be regenerated to that providing for conjugated triene formation for a 3.5 minute additional time period. This is a total of 6.5 minutes of conjugated triene formation instead of the normal 3 minute period. If peroxidase was reacted for 1.5 minutes before the addition of lipoxidase, it seemed to destabilize the system in that one-half the reactions would change over to destruction at a time of 6 minutes from the time of lipoxidase addition.

These results indicate that if peroxidase is in the system, the enzyme system is activated to the formation of conjugated trienes. Normally both enzymes use conjugated trienes as evidenced by the slight decrease from the autoxidation rate. The peroxidase keeps the system in its initial state of conjugated triene production longer, as evidenced by the longer turnover from production to destruction. The destruction of conjugated trienes was increased after the turnover point, which could be due to a concentration effect caused by the increased production period and product build-up time. The variability of the system in which peroxidase was added first was similar to that encountered in measuring the conjugated diene in that the system only reacts the same one-half of the time. This may be the result of hydroperoxide destruction which causes the lipoxidase to begin the reaction more slowly and to give variation in the data.

## Spectra of Systems With Bisulfite

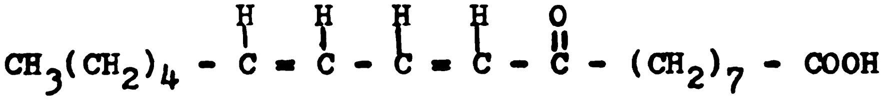
The ability of bisulfite to react with aldehydes and not with ketones was used to find the nature of the conjugated triene structure and what occurs in this area of the spectra during the reaction. The lipoxidase and linoleic acid reaction was used for this study. The scans obtained are shown in Figure 6. It can be seen that for the first 2 minutes there are no bisulfite reactable components from 285 nm to 265 nm. At three minutes there is a bisulfite

Figure 6: The effect of bisulfite on the spectra of model systems containing lipoxidase and linoleic acid


Lipoxidase and linoleic acid reacted 3 minutes —□—
Lipoxidase and linoleic acid reacted 3 minutes, plus bisulfite —+—
Lipoxidase and linoleic acid reacted 5 minutes —○—
Lipoxidase and linoleic acid reacted 5 minutes, plus bisulfite —△—
Absorbance
0.5
0.4
0.3
0.2
0.1
232
240
250
260
270
280
290
300
310
Wavelength nm

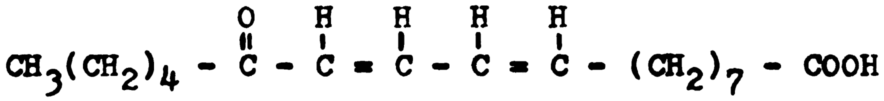
reactable component. This implies that during the lag period ketodienes or non-bisulfite products are built up. However, when the lag period ends and the conjugated triene destruction begins there is a conjugated triene present which can be reacted with bisulfite. The structure of some compounds can be hypothesized from the dismutation of the hydroperoxides formed.

The ketodiene or bisulfite stable product could have the following structure, depending upon whether the hydroperoxidase is at the 9 or 13 position.



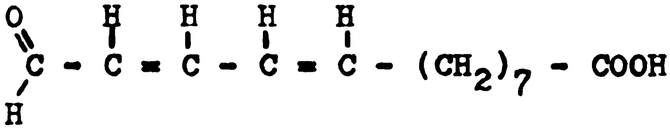
$$CH_3(CH_2)_4 - \overset{H}{\overset{|}{C}} = \overset{H}{\overset{|}{C}} - \overset{H}{\overset{|}{C}} = \overset{H}{\overset{|}{C}} - \overset{O}{\overset{\|}{C}} - (CH_2)_7 - COOH$$

OR



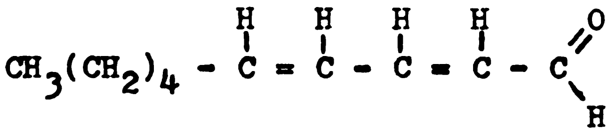
$$CH_3(CH_2)_4 - \overset{O}{\overset{\|}{C}} - \overset{H}{\overset{|}{C}} = \overset{H}{\overset{|}{C}} - \overset{H}{\overset{|}{C}} = \overset{H}{\overset{|}{C}} - (CH_2)_7 - COOH$$

The aldehyde portion or bisulfite reactable product could have the following structure, depending on the dismutation of the 9 or 13 hydroperoxide.



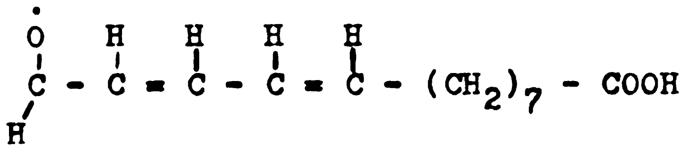
$$\overset{O}{\overset{\|}{\underset{H}{C}}} - \overset{H}{\overset{|}{C}} = \overset{H}{\overset{|}{C}} - \overset{H}{\overset{|}{C}} = \overset{H}{\overset{|}{C}} - (CH_2)_7 - COOH$$

OR

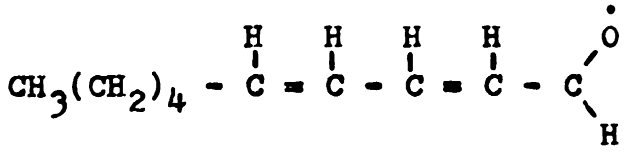


$$CH_3(CH_2)_4 - \overset{H}{\overset{|}{C}} = \overset{H}{\overset{|}{C}} - \overset{H}{\overset{|}{C}} = \overset{H}{\overset{|}{C}} - \overset{O}{\overset{\|}{\underset{H}{C}}}$$

The scans also revealed that the absorption between 265 nm and 235 nm was decreased. This could be due to the interaction of bisulfite with compounds which are intermediates between conjugated dienes and trienes, such as the free radicals hypothesized below.



OR



Free radicals such as those hypothesized have been suspected by other authors in the non-dismutated form and it is possible to have them after dismutation. The presence of free radicals in the lipoxidase reaction has been shown by Fridovich *et al*. (23).

The conjugated triene and diene data can be correlated to the bisulfite results since the time of changeover from strictly a non-bisulfite reactable product to one which reacts with bisulfite is at 3 minutes. This is the same as the time for changeover from triene formation to triene destruction and linearity in the diene determination. Peroxidase therefore may keep the system producing keto or non-bisulfite reactable products. They may influence the lipoxidase reaction toward a faster rate by binding more substrate and fewer end products to the substrate binding site.

## Heat Inhibition Studies

The use of heat inactivation on various components of the system yielded the results shown on Table 5. From this it can be seen that heating lipoxidase will result in a 93% decrease in the rate of conjugated diene production and an 11% increase in the rate of conjugated triene production over that found in the lipoxidase-linoleic acid reaction. If peroxidase is added in the system with the inactivated lipoxidase, there will be no further effect on the system. If these same experiments are conducted using heated linoleic acid in the system, there is the same 93% decrease of conjugated diene formation, but there is a 63% decrease of conjugated triene formation. When peroxidase is added to this system, there is a further decrease of 50% in the rate of conjugated diene production and a 91.2% decrease of conjugated triene production rate.

Peroxidase and heated linoleic acid will give an 80% reduction in the formation of conjugated dienes. However there is no effect on the rate of conjugated trienes formed when compared to the system of peroxidase and linoleic acid which has not been heated.

These results show that, if lipoxidase is heat denatured, activity for the production of conjugated dienes is lost and the depression of conjugated triene production is also lost. This indicates that lipoxidase must be in the active state for peroxidase to have a stimulating effect on the formation of conjugated dienes and on the repression of

Table 5: Effect of heat inactivation on diene and triene conjugation in model systems containing lipoxidase, peroxidase and linoleic acid.

| System Components[1] | Rate of Conjugated Dienes Formed (µM/minute) | Rate of Conjugated Trienes Formed (µM/minute) |
|---|---|---|
| Linoleic acid | 0.125 | 0.137 |
| Heated linoleic acid | 0.118 | 0.129 |
| Peroxidase and non-heated linoleic acid | 0.176 | 0.039 |
| Peroxidase and heated linoleic acid | 0.036 | 0.039 |
| Lipoxidase and linoleic acid | 5.036 | 0.351 |
| Heated lipoxidase and heated linoleic acid | 0.366 | 0.129 |
| Heated lipoxidase and non-heated linoleic acid | 0.357 | 0.390 |
| Peroxidase, heated lipoxidase, and heated linoleic acid | 0.176 | 0.013 |
| Peroxidase, linoleic acid and heated lipoxidase | 0.357 | 0.390 |

---

[1] Linoleic acid concentration - 0.11 ug/ml
Lipoxidase concentration - 6.66 µg/ml
Peroxidase concentration - 6.66 µg/ml
Buffer 0.2 molar borate buffer pH 9.0
Heat treatments were as follows:

lipoxidase, 3 minutes at 100° C
linoleic acid, 3 minutes at 100° C

conjugated trienes. This is evidenced by the lack of change between the inactivated lipoxidase, non-heat stressed linoleic acid system and the same system containing peroxidase. The results indicate that if the linoleic acid is heat stressed, peroxidase is then capable of causing a reduction in the rate of both conjugated diene and triene formation. This is implied by the further decrease in rates between the inactivated lipoxidase stressed linoleic acid system and the same system with peroxidase. This same type of effect is noted in the peroxidase controls.

The previously discussed results indicate that peroxidase is capable of acting at two points in the system. One point is in the destruction of conjugated dienes and trienes. This destruction does not depend on lipoxidase as evidenced by the results with heat inactivated lipoxidase and heat activated linoleic acid in which peroxidase caused a decrease in the production rate. The other function is that of diene and triene stimulation found in the previous experiments. This phenomenon was not found when the lipoxidase was in the heat denatured state and the linoleic acid had not been heat stressed so hydroperoxides were formed for peroxidase substrate. The peroxidase had no effect on this system which indicates the need for active lipoxidase.

It was discovered that if the linoleic acid was heated under nitrogen or atmospheric conditions there was no difference in the results. This was probably due to the heat starting some chain mechanisms with the residual oxygen in the nitrogen flushed system. This could form substrate for the peroxidase reaction.

Peroxidase Inhibition

Potassium cyanide is a competitive inhibitor of peroxidase at $10^{-5}$ molar (77). It interacts with the heme iron so that it cannot function in the normal reaction sequence. KCN inhibition was used to determine the role of heme iron in the model system under study. Heat denaturation of peroxidase would show if the protein must be in the active state for stimulation of the lipoxidase-linoleic acid reaction to occur. The results obtained are presented in Table 6.

From Table 6 it is evident that the presence of peroxidase in the active form gave an 11% increase in the formation of conjugated dienes. When KCN was added to the system, the conjugated diene production rate decreased by 7% from that of the lipoxidase-linoleic acid system. When the peroxidase was heat inactivated the decrease was 4% less than the lipoxidase-linoleic acid system. These differences were significant at the 99% confidence level. These results indicate that both the heme iron and the protein portion of peroxidase are necessary for the stimulation effect to occur. This and previous evidence that peroxidase has two roles indicates that the action is enzymatic. It also substantiates the previous work in that the necessity of both enzymes being in the active state is mandatory for the stimulation of the lipoxidase reaction to occur.

Table 6: Effect of peroxidase inactivation on model systems containing lipoxidase, peroxidase and linoleic acid.

| System Components[1] | Rate of Conjugated Diene Formation (µM/minute) |
|---|---|
| Lipoxidase and linoleic acid | 7.45 |
| Lipoxidase, linoleic acid and KCN | 7.45 |
| Lipoxidase, linoleic acid and peroxidase | 8.11 |
| Lipoxidase, linoleic acid and KCN treated peroxidase | 6.939 |
| Lipoxidase, linoleic acid and heated peroxidase | 7.171 |

---

[1] Lipid and enzyme concentrations were constant for all systems.

### Thin Layer Chromatography

The chromatograms indicated that the major products of the lipoxidase-peroxidase-linoleic acid system were the same as those from the lipoxidase-linoleic acid system. Both systems gave three spots on the iso-octane diethyl ether acetic acid system in addition to that for linoleic acid. These spots had the following $R_f$ values: 0.66, 0.56 and 0.42. The spot at $R_f$ 0.42 was weak for both systems and visualized by the use of N, N dimethyl-p-phenylenediamine spray, indicating the presence of hydroperoxides. This spot cannot be found in either system after a 30 minute reaction time. The linoleic acid had an $R_f$ of 0.70 which compares well with the earlier work. Autoxidation for the same time period resulted in only one peak other than that for linoleic acid and it had an $R_f$ of 0.66.

The peroxides were not visible in the hexane-ether-acetic acid system. However, the linoleic acid had an $R_f$ of 0.50 and its product an $R_f$ of 0.42. The products of the enzymatic reaction were found at $R_f$ 0.45 and $R_f$ 0.36. Again this indicates that the two model systems have the same major end products.

### Lipoxidase Activity in Spinach

Activity of lipoxidase in spinach was found at low levels which were significant at the 5% level. The activity found was 100 Worthington units per gram of spinach (One Worthington unit equals 0.001 $\Delta$OD/minute (89) ).

The percent recovery is not known since activity assays prior to final concentrations were not possible due to color interference, hydroperoxide breakdown factors and dilution problems. It was discovered that upon addition of the pH 9.0 buffer several proteins appeared to become insoluble and were centrifuged out.

### Peroxidase Activity in Spinach

Peroxidase activity in spinach was found to be 0.8 Worthington units per gram of spinach using the first extraction procedure and 0.88 Worthington units per gram using the second procedure. The addition of KCN to the assay mixture completely inhibited the reaction, indicating that peroxidase was the enzyme activity being measured in both extractions. One Worthington unit equals the amount of enzyme required to decompose one micromole of peroxide per minute (90). In assaying the crude peroxidase extract for lipoxidase activity by the conjugated diene procedure, it was found there was an initial increase in conjugated dienes after a one minute lag time. The reaction ran for one minute and was followed by a decrease in conjugated dienes, indicating that a hydroperoxide and conjugated diene decomposing factor was present in the extract.

### General Discussion

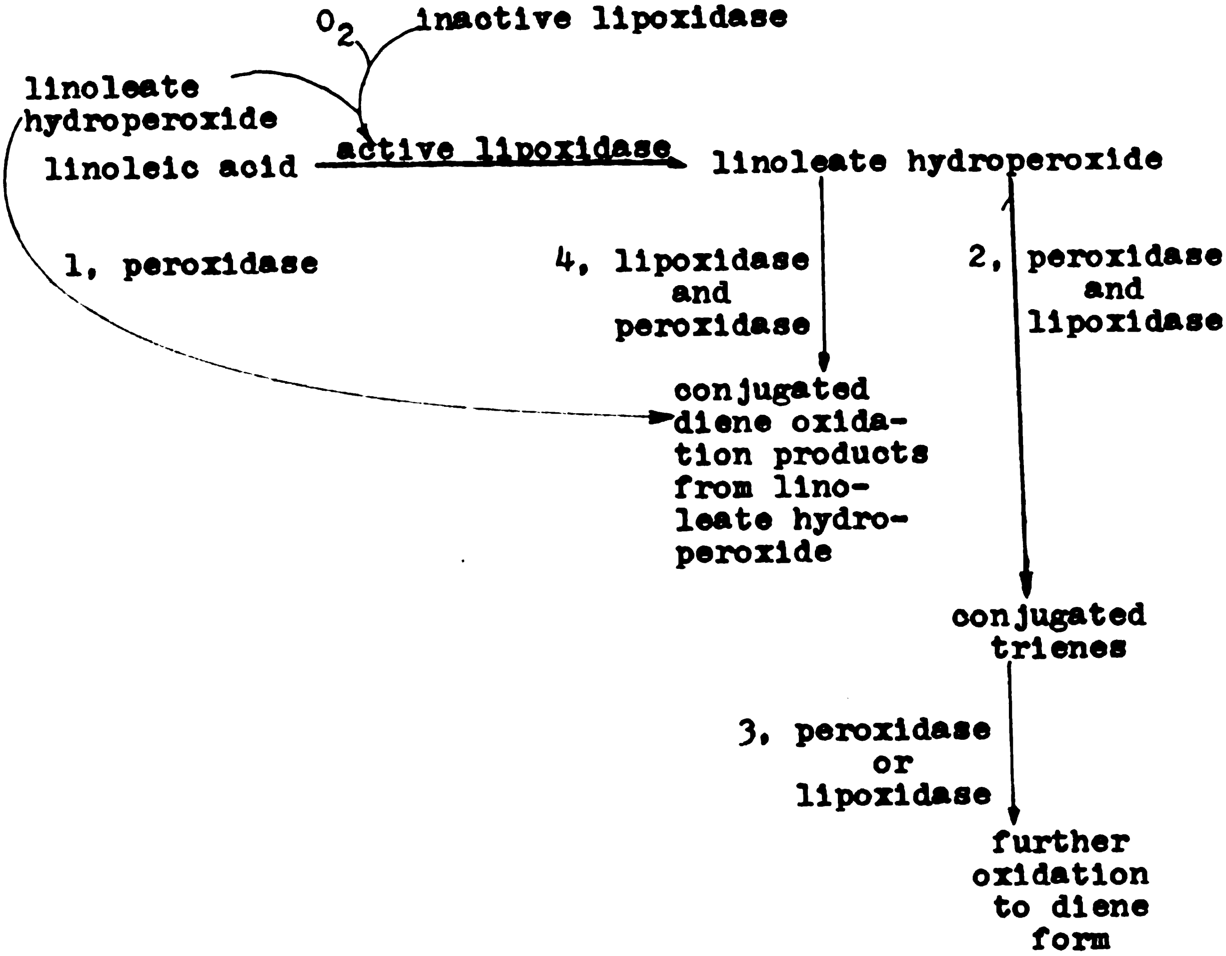
The following model for the effect of peroxidase on the lipoxidase-linoleic acid system is proposed. The system uses the mechanism proposed by Smith *et al*. (68)

for the linoleate hydroperoxide requirement in the lipoxidase reaction.

Figure 7 is a diagramatic representation of the lipoxidase-linoleic acid reaction showing the points of peroxidase action.

At position 1 in Figure 7 peroxidase oxidized the linoleate hydroperoxide necessary for the activation of lipoxidase into a conjugated diene type structure which does not activate lipoxidase. This results in a decrease in lipoxidase activity and is seen when the peroxidase is in the system before lipoxidase. The inhibition is not complete since peroxidase forms some peroxides which allow partial activation of lipoxidase. This effect is not noticeable if peroxidase is added at the same time or after lipoxidase, indicating a reaction rate slower than that of lipoxidase but similar to that reported for heme iron oxidation of hydroperoxides. This result supports the need for linoleate hydroperoxide in lipoxidase activation as reported by Smith _et al_. (68). The action at position 1 and 4 in the scheme are of the same nature with position 1 utilizing initial peroxides as substrates while position 4 uses peroxides made in the lipoxidase reaction as substrates. The initial peroxides can result from autoxidation or heme catalyzed oxidation and need not be the same as those produced in the lipoxidase-linoleic acid reaction.

Positions 2 and 3 in the proposed mechanism indicate the effect of peroxidase in the system when it is present



Numbers 1, 2, 3 and 4 represent points at which peroxidase has an effect. They are discussed in the text.

Figure 7: Proposed mechanism for lipoxidase-peroxidase interactions.

at the same time as lipoxidase. If only lipoxidase or peroxidase is present the enzyme action will be greater at position 3 and conjugated triene production will decrease from that of autoxidation as indicated by a lower triene production rate. If both enzymes are present in the system, the enzyme actions are faster at position 2, since there is a stimulation in the rate of triene production. This stimulation effect is only temporary and related to the lipoxidase lag time which also corresponds to the changeover from triene formation to triene destruction. Peroxidase presence in the system lengthens the period of changeover time and therefore conjugated trienes are produced for a longer period of time as well as at a faster rate.

From the preceding results, it can be hypothesized that the lag period corresponds to the time necessary for lipoxidase to become saturated with linoleate hydroperoxide. This is suggested by the ability of peroxidase to decrease activity if added before lipoxidase. This loss of activity could be due to a loss of peroxide necessary for lipoxidase activation. The changeover from net triene formation to destruction would then occur when the lipoxidase is saturated with hydroperoxide. This enzyme saturation would normally occur at the end of the lag period.

It is apparent that both enzymes must be in the active state for the increased production of conjugated dienes and trienes by lipoxidase action. The increased rate of conjugated diene production is dependent on the two enzymes being active, as the inhibition studies show. For this reason it can be concluded that the action of both enzymes is enzymatic in the combined system and not a heme iron effect caused by peroxidase. This means that for increased conjugated diene production and increased conjugated triene production the effect is not that of iron induced oxidation by peroxidase. In fact, both the iron and the protein of peroxidase are involved which supports the work of Weinryb (87). This phenomenon is occurring at position 4 in the proposed mechanism and is directly related to the changes in the system at 2 and 3. This is suggested by the results which show stimulation of conjugated diene and triene production which parallel each other. If one system was stimulated for dienes it would also be stimulated for trienes, and vice versa. Both lipoxidase and peroxidase have been shown to be involved in the breakdown of linoleate hydroperoxides (66, 73). The combined enzymes could speed up hydroperoxide breakdown and the production of final products. This could kinetically shift the lipoxidase effect toward the production of more hydroperoxide.

The oxygen uptake data support this since the same stimulation effects are shown. The data show that oxygen is used in the breakdown of hydroperoxides since a total of 4 moles of oxygen are used to oxidize one mole of linoleic acid to its final end products in the system containing lipoxidase, peroxidase and linoleic acid. The use of oxygen is also found in the lipoxidase breakdown of hydroperoxides at about the same 4 to 1 ratio. The increased oxygen uptake rate with peroxidase present indicates a stimulation of the lipoxidase activity requiring oxygen along with the oxygen used by peroxidase in the system where peroxidase was added.

The preceding indicates that lipoxidase, with the necessary substrates, acts first to form a hydroperoxide and then the peroxidase and lipoxidase act on the products. The overall result of the peroxidase action is the control of lipoxidase action and the direction of product formation. This is based upon the increased oxygen consumption, diene formation and changes in rates of triene formation and destruction, and the nature of trienes produced. This control is exerted through a kinetic effect on lipoxidase caused by the oxidation of hydroperoxides and possibly other products in the reaction.

The proposed mechanism supports the work of other investigators. The mechanism which is most closely followed is that proposed by Smith *et al*. (68). In that system hydroperoxide is necessary in catalytic amounts for lipoxidase action, as is oxygen and substrate. The net result is the formation of new hydroperoxides. The loss by peroxidase action of hydroperoxides formed in the lipoxidase reaction can result in an increase in activity or a condition in which lipoxidase activity is maintained for a longer period of time.

The work also supports the theories on the induction period (67, 68) and its dependence on substrate peroxide to shorten this as evidenced by the decreased lipoxidase activity when peroxidase is added early.

Blain _et al_. (6) reported that lipoxidase destroys hydroperoxides without decreasing the amount of conjugated dienes. This was indicated in this work by the small peroxide spots evidenced on TLC without decrease of conjugated dienes. Rather these were increased by both lipoxidase and peroxidase.

The ability of peroxidase to act upon hydroperoxides was shown in the system where peroxidase was added first and lipoxidase action was decreased, indicating a loss of the initiating hydroperoxide. This is in agreement with the suggestion of Gini _et al_. (29) who proposed the hydroperoxide breakdown factor was of the peroxidase enzyme type. The work reported shows that the peroxidase has effects other than hydroperoxide breakdown in the system and may not be the hydroperoxide decomposing enzyme which decreased conjugated dienes as reported by others (6, 24) since the conjugated dienes are increased and not decreased. The ability of peroxidase to break down hydroperoxides has also been reported by Tappel _et al_. (76) but was not linked to the hydroperoxide breakdown factor. This work shows that active enzyme is involved in the stimulation of the lipoxidase reaction. The need for both protein and iron in peroxidase action has been reported for other processes (87, 91).

This work has shown the presence of lipoxidase and peroxidase in spinach. Other investigators (15, 25, 34, 46) have reported the presence of the two enzymes in other plant tissue.

Lipoxidase has been demonstrated to be supplied with substrate from lipase action in a report by Wagenknecht *et al*. (79) who showed further that both lipoxidase and peroxidase could produce off-flavors. The fact that both enzymes are present in foods and that there is substrate available for lipoxidase action implies that the reactions elucidated in this work may be found in many vegetables including spinach. The reactions could also be involved in the production of off-flavors as the breakdown products have been reported to be aldehydes, ketones and alcohols (30, 42, 93).

# CONCLUSION

Lipoxidase and peroxidase activities were found in spinach. Their presence together is evidence that the reactions described below possibly can occur in food systems.

Peroxidase was found to have two roles in the lipoxidase-linoleic acid reaction. The first role is that of a hydroperoxide decomposing factor which is capable of destroying the hydroperoxide necessary to activate the lipoxidase. The second role is enzymatic in nature and results in a change of lipoxidase activity, in which the reaction rate is increased and the time for various changes in the system is lengthened by peroxidase presence. This is seen in the increase of oxygen uptake, conjugated diene formation and conjugated triene formation. The production of conjugated trienes with the peroxidase present was accompanied by an increase in the time for changeover from triene production to triene destruction compared to the lipoxidase-linoleic acid system. This changeover is accompanied by an increase in the rate of triene destruction.

The formation of a bisulfite reactable product at the end of the lag period has been shown and this may be the product which is utilized by both lipoxidase and peroxidase as a substrate for conjugated triene destruction. The ability of lipoxidase and peroxidase to decrease conjugated triene formation from that of the linoleic acid autoxidation rate was demonstrated, which indicates conjugated triene destruction is enzymatic in nature. Ultraviolet spectroscopy revealed that in both systems the products are of the same nature. This was verified by thin layer chromatography. This implies that peroxidase is exerting a controlling influence on the lipoxidase reaction in its ability to increase production of dienes and trienes even though the products measured do not change in nature. This control is provided through kinetic effects on the lipoxidase reaction caused by peroxidase removing products which the lipoxidase has made or needs for activation.

The enzymatic effect reported requires that both lipoxidase and peroxidase must be in the active state for the increase in activity to occur. It was shown that both the heme iron and protein are necessary for peroxidase to increase the lipoxidase-linoleic acid reaction rate. Inhibition and inactivation studies on peroxidase and lipoxidase led to this conclusion since if either enzyme were inactive, the increased activity was not found. Thin layer chromatography revealed that the hydroperoxides formed by lipoxidase were not present in large quantities which supports Gardner (24) who found the hydroperoxides to be present at low levels.

From the preceding results it can be concluded that in natural systems where both enzymes are present lipoxidase is capable of oxidizing linoleic acid to hydroperoxides which can then be broken down by peroxidase. The action of peroxidase causes lipoxidase action to be increased. This is assuming the model system approaches that found in nature, which has not yet been established. The possibility of these interactions does exist however, and their role in the metabolism of the cell could be important with respect to off-flavor production in frozen or improperly blanched vegetables since some of the breakdown products are aldehydes and ketones.

# FURTHER RESEARCH

Studies of the mechanism of peroxidase action on the substrates provided by lipoxidase are needed to further elucidate the system. Research is needed on the products produced in the lipoxidase reaction and the kinetics of the two enzyme systems.

Another project which could expand the knowledge of the two enzyme systems is the study of the oxidation state of the iron in the peroxidase enzyme. Also a study of free radicals produced in both reactions could lead to an understanding of the two system interaction.

There is a need for research on this combined enzyme system in the natural plant system so that the interactions can be compared to physical changes in the plant material upon storage.

# BIBLIOGRAPHY

# BIBLIOGRAPHY

1. Balls, A. K., Axelrod, B., and Kies, M. W. 1943. Soybean lipoxidase. J. Biol. Chem. 149, 491.

2. Ben-Aziz, A., Grossman, S., Ascarelli, I., and Budowski, P. 1970. Linoleate oxidation induced by lipoxygenase and heme proteins: A direct spectraphotometric assay. Anal. Biochem. 34, 88-100.

3. Beare-Rogers, J. L., and Ackman, R. G. 1969. Reaction of lipoxidase with polyenoic acids in marine oils. Lipids 4, 441-443.

4. Bjorksten, F. 1966. The peroxidase catalyzed oxidation of thyroxine. Acta Chem. Scand. 20, 1438.

5. Bjorksten, F. 1968. Participation of horseradish oxyperoxidase (compound III) in interenzymic reaction steps. Biochem. Biophy. Acta 151, 309.

6. Blain, J. A., and Barr, T. 1961. Destruction of linoleate hydroperoxides by soya extracts. Nature 190, 538-539.

7. Blain, J. A., and Shearer, G. 1965. Inhibition of soya lipoxidase. J. Sci, Food Agr. 16, 373.

8. Blain, J. A., and Styles, E. C. C. 1959. A lipoperoxidase factor in soya extracts. Nature 184, #2, 1141.

9. Blumberg, W. E., Peisach, J., Wittenberg, B. A., and Wittenberg, J. B. 1968. An electron paramagnetic resonance and optical study of horseradish peroxidase and its derivatives. J. Biol. Chem. 243, 1854.

10. Buckle, K. A., and Edwards, R. A. 1970. Chlorophyll degradation and lipid oxidation of frozen unblanched peas. J. Sci. Food Agr. 21, 307.

11. Catsimpoolas, N. 1969. Isolation of soybean lipoxidase by isoelectric focusing. Arch. Biochem. Biophys. 131, 185.

12. Chang, C. C., Esselman, W. J., Clagett, C. O. 1971. The isolation and specificity of alfalfa lipoxygenase. Lipids 6, 100-106.

13. Chase, G. D., and Rabinowitz, J. L. 1968. Principles of Radioisotope Methodology, p. 75-108. Burgess Publishing Company, Minneapolis, Minnesota.

14. Dahle, L. K., Hill, E. G., and Holman, R. T. 1962. The thiobarbituric acid reaction and the autoxidations of polyunsaturated fatty acid methyl esters. Arch. Biochem. Biophys. 98, 253-261.

15. Dillard, M. G., Henick, A. S., and Koch, R. B. 1960. Unsaturated triglyceride and fatty acid lipoxidase activities of navy beans, small red beans, peanuts, green peas and lima beans. Food Res. 25, (4), 544.

16. Dillard, M. G., Henick, A. S., and Koch, R. B. 1961. Differences in reactivity of legume lipoxidases. J. Biol. Chem. 236, #1, 37-40.

17. Dolev, A., Rohwedder, W. K., and Dutton, H. J. 1966. Quantitative separation of methyl 9-hydroxystearate from methyl 13-hydroxystearate by column chromatography of silica gel. Lipids 1, 231.

18. Dolev, A., Rohwedder, W. K., and Dutton, H. J. 1967. Mechanism of lipoxidase reaction. I. Specificity of hydroperoxidation of linoleic acid. Lipids 2, 28.

19. Dolev, A., Rohwedder, W. K., Mounts, T. L., and Dutton, H. J. 1967. Mechanism of lipoxidase reaction. II. Origin of the oxygen incorporated into linoleate hydroperoxide. Lipids 2, 33.

20. Eriksson, C. E., and Leu, K. 1971. Gas chromatography separation of linoleic acid hydroperoxides as trimethylsilyl ethers of methyl hydroxystearates. Lipids 6, 144.

21. Fedeli, E., Camurati, F., and Jacini, G. 1971. Structure of monohydroperoxides formed by chlorophyll photo-sensitized oxidation of methyl linoleate. J. Am. Oil Chemist's Soc. 48, 787.

22. Fox, L. R., Purves, W. K., and Nakada, H. I. 1965. The role of horseradish peroxidase in indole-3-acetic acid oxidation. Biochem. 4, 2754.

23. Fridovich, I., and Handler, P. 1961. Detection of free radicals generated during enzymic oxidations by the initiation of sulfite oxidation. J. Biol. Chem. 236, 1836-1840.

24. Gardner, H. W. 1970. Sequential enzymes of linoleic acid oxidation of corn germ: lipoxygenase and linoleate hydroperoxide isomerase. J. Lipid Res. 2, 311-321.

25. Gardner, H. W., and Weisner, D. 1972. Hydroperoxides from oxidation of linoleic and linoleine acids by soybean lipoxygenase. Proof of the trans-11 double bond. Lipids 7, #3, 191-193.

26. Garssen, G. J., Vliegenthart, J. F. G., and Boldingh, J. 1971. An anaerobic reaction between lipoxygenase, linoleic acid and its hydroperoxides. Biochem. J. 122, 327.

27. George, P. 1953. The third intermediate compound of horseradish peroxidase and hydrogen peroxide. J. Biol. Chem. 201, 427.

28. Gilson Respirometer. Directions for operation. Gilson Medical Electronics. Middleton, Wisconsin.

29. Gini, B., and Koch, R. B. 1961. Study of lipohydroperoxide breakdown factor in soy extracts. J. Food Sci. 26, 359.

30. Graveland, A. 1971. Enzymatic oxidations of linoleic acid and glycerol-1-monolinoleate in doughs and flour-water suspension. J. Am. Oil Chemist's Soc. 47, 352-361.

31. Grossman, S., Trop, M., Artulion, R., and Pinsk, A. 1972. Egg plant lipoxygenase: Isolation and partial characterization. Lipids 7, 467.

32. Guss, P. L., Richardson, T., and Stahmann, M. A. 1967. The oxidation-reduction enzymes of wheat. III. Isoenzymes of lipoxidase in wheat fractions and soybeans. Cereal Chem. 44, 607.

33. Haining, J. L., and Axelrod, B. 1958. Induction period in the lipoxidase-catalyzed oxidation of linoleic acid and its abolition by substrate peroxide. J. Biol. Chem. 232, 193.

34. Hale, S. A., Richardson, T., Von Elbe, J. H., and Hagedorn, D. J. 1969. Isoenzymes of lipoxidase. Lipids 4, 209.

35. Hamberg, M., and Samuelsson, B. 1965. On the specificity of the lipoxidase catalyzed oxygenation of unsaturated fatty acids. Biochem. Biophys. Res. Comm. 21, 531.

36. Hamberg, M., and Samuelsson, B. 1967. On the specificity of the oxygenation of unsaturated fatty acids catalyzed by soybean lipoxidase. J. Biol. Chem. 242, 5329-5335.

37. Henick, A. S., Benca, M. F., and Mitchell, J. H., Jr. 1954. Estimating carbonyl compounds in rancid fats and foods. J. Am. Oil Chemist's Soc. 31, 88-91.

38. Hirano, Y., and Olcott, H. S. 1971. Effect of heme compounds on lipid oxidation. J. Am. Oil Chemist's Soc. 48, 523.

39. Holman, R. T. 1947. Crystalline lipoxidase. II. Lipoxidase activity. Arch. Biochem. 15, 403.

40. Holman, R. T., Egwim, P. O., and Christie, W. W. 1969. Substrate specificity of soybean lipoxidase. J. Biol. Chem. 244, 1149.

41. Holman, R. T., Panzer, F., Schweigert, B. S., and Ames, S. R. 1950. Crystalline lipoxidase. III. Amino acid composition. Arch. Biochem. 26, 199.

42. Johnston, A. E., Zilch, K. T., Selke, E., and Dutton, H. J. 1961. Analysis of fat acid oxidation products by countercurrent distribution methods. V. Low-temperature decomposition of methyl linoleate hydroperoxide. J. Am. Oil Chemist's Soc. 38, 367.

43. Joslyn, M. A. 1950. Report on peroxidase in frozen vegetables. J. of Assoc. Official Agric. Chemist's 504.

44. Joslyn, M. A. 1953. Report on peroxidase in frozen vegetables. J. of Assoc. Official Agric. Chemist's 36, 161.

45. Joslyn, M. A., and Bedford, C. L. 1940. Enzyme activity in frozen vegetables - asparagus. Indus. Eng. Chem. 32, 702.

46. Joslyn, M. A., Bedford, C. L., and Marsh, G. L. 1938. Enzyme activity in frozen vegetables - artichoke hearts. Indus, Eng. Chem. 30, 1068.

47. Kay, E., Shannon, L. M., and Lew, J. Y. 1967. Peroxidase isozymes from horseradish roots. II. Catalytic properties. J. Biol. Chem. 242, 2470.

48. Koch, R. B. 1968. Calcium ion activation of lipoxidase. Arch. Biochem. Biophys. 125, 303-307.

49. Koch, R. B., Stern, B., and Ferrari, C. G. 1958. Linoleic acid and trilinolein as substrates for soybean lipoxidase(s). Arch. Biochem. Biophys. 78, 165-179.

50. Kramer. A., and Twigg, B. A. 1966. Fundamentals of Quality Control for the Food Industry, p. 486. AVI Publishing Co., Inc. Westport, Connecticut.

51. Maehly, A. C. 1955. Plant peroxidase, Methods in Enzymology, Volume II, P. 807. Colowick, S. P., and Kaplan, N. O., eds. Academic Press, Inc., New York.

52. Maehly, A. C., and Chance, B. 1954. The assay of catalases and peroxidases. Methods of Biochemical Analysis, Volume I, p. 357. Glick, D., ed. Interscience Pub., Inc., New York.

53. Maier, V. P., and Tappel, A. L. 1959. Rate studies of unsaturated fatty acid oxidation catalyzed by hematin compounds. J. Am. Oil Chemist's Soc. 36, 8.

54. Maier, V. P., and Tappel, A. L. 1959. Products of unsaturated fatty acid oxidation catalyzed by hematin compounds. J. Am. Oil Chemist's Soc. 36, 12.

55. Mapson, L. W., and Moustafa, E. M. 1955. The oxidation of glutathione by a lipoxidase enzyme from pea seeds. Biochem. J. 60, 71.

56. Mitsuda, H., Yasumuto, K., and Yamamoto, A. 1967. Inhibition of lipoxygenase by saturated monohydric alcohols through hydrophobic bondings. Arch. Biochem. Biophys. 118, 664-669.

57. Moss, T. H., Ehrenberg, A., and Bearden, A. J. 1969. Mossbauer spectroscopic evidence for the electronic configuration of iron in horseradish peroxidase and its peroxidase derivatives. Biochem. 8, 4159-4162.

58. Ohmura, T., and Howell, R. W. 1962. Respiration of developing and germinating soybean seeds. Physiol. Plant. 15, 341.

59. Orthoefer, F. T. 1969. Chlorophyll A bleaching by a coupled oxidation with linoleic acid catalyzed by lipoxidase. Doctoral thesis, Michigan State Univ.

60. Privett, O. S., Nickell, C., Lundberg, W. O., and Boyer, P. D. 1955. Products of the lipoxidase-catalyzed oxidation of sodium linoleate. J. Am. Oil Chemist's Soc. 32, 505.

61. Purr, A. 1970. Chemical changes in food-stuffs having low water content. II. Experiments with lipoxygenase as well as mixture of enzymatically active principles and their impact on autoxidative deterioration of fats in dry products as a function of equilibrium moisture content. Fette Seifen Anstrickmittel 72, 725.

62. Rackis, J. J., Honig, D. H., Sessa, D. J., and Moser, H. A. 1972. Lipoxygenase and peroxidase activities of soybeans as related to the flavor profile during maturation. Cereal Chem. 49, 586.

63. Rhee, K. S., and Watts, B. M. 1966. Lipid oxidation in frozen vegetables in relation to flavor change. J. Food Sci. 31, 675.

64. Shannon, L. M., Kay, E., and Lew, J. Y. 1966. Peroxidase isozymes from horseradish roots. I. Isolation and physical properties. J. Biol. Chem. 241, 2166.

65. Siddiqi, A. M., and Tappel, A. L. 1956. Catalysis of linoleate oxidation by pea lipoxidase. Arch. Biochem. Biophys. 60, 90.

66. Siddiqi, A. M., and Tappel, A. L. 1957. Comparison of some lipoxidases and their mechanism of action. J. Am. Oil Chemist's Soc. 34, 529.

67. Smith, W. L., and Lands, W. E. M. 1970. The self-catalyzed destruction of lipoxygenase. Biochem. Biophys. Res. Comm. 47, 846.

68. Smith, W. L., and Lands, W. E. M. 1972. Oxygenation of unsaturated fatty acids by soybean lipoxygenase. J. Biochem. 247, 1038-1047.

69. Stevens, F. C., Brown, D. M., and Smith, E. L. 1970. Some properties of soybean lipoxygenase. Arch. Biochem. Biophys. 136, 413.

70. Strickland, E., Hardin, E. K., Shannon, L. M., and Horwitz, J. 1968. Peroxidase isoenzymes from horseradish roots. III. Circular dichroism of isoenzymes and apoisoenzymes. J. Biol. Chem. 243, 3560.

71. Tappel, A. L. 1952. Linoleate oxidation catalyzed by hog muscle and adipose tissue extracts. Food Res. 17, 550.

72. Tappel, A. L. 1953. Linoleate oxidation catalysts occurring in animal tissue. Food Res. 18, 104.

73. Tappel, A. L. 1961. Autoxidation and Antioxidents. Volume I, p. 325. Lundber, W. D., ed. Interscience, New York.

74. Tappel, A. L. 1963. Lipoxidase. The Enzymes, 2nd edition, Volume 8, p. 275-284. Boyer, P. D., Lardy, H., and Myrbach, K., eds. Academic Press, New York.

75. Tappel, A. L., Boyer, P. D., and Lundberg, W. O. 1952. The reaction mechanism of soybean lipoxidase. J. Biol. Chem. 199, 267.

76. Tappel, A. L., Lundberg, W. O., and Boyer, P. D. 1953. Effect of temperature and antioxidents upon lipoxidase catalyzed oxidation of sodium linoleate. J. Biol. Chem. 201, 427.

77. Theorell, H. 1951. The iron containing enzymes. B. Catalases and peroxidases. "Hydroperoxidases." The Enzymes, Volume II, Part I, p. 397. Sumner, J. B., and Myrbach, K., eds. Academic Press, Inc., New York.

78. Theorell, H., Bergstrom, S., and Akeson, A. 1943. Activity determination and further purification of lipoxidase. Pharm. Acta Helvet. 149, 491.

79. Theorell, H., Holman, R. T., and Akeson, A. 1947. A note on the preparation of crystalline soy bean lipoxidase. Arch. Biochem. 14, 250.

80. Tookey, H. L., Wilson, R. G., Lohmar, R. L., and Dutton, H. J. 1958. Coupled oxidation of carotene and linoleate catalyzed by lipoxidase. J. Biol. Chem. 230, 65-72.

81. Veldink, G. A., Vliegenthart, J. F. G., and Boldingh, J. 1970. Proof of the enzymatic formation of 9-hydroperoxy-10-trans, 12 cis octadecadienoic acid from linoleic acid by soya lipoxygenase. Biochem. Biophys. Acta 202, 198-199.

82. Vioque, E., and Holman, R. T. 1962. Characterization of ketodienes formed in the oxidation of linoleate by lipoxidase. Arch. Biochem. Biophys. 99, 522-528.

83. Wagenknecht, A. C., and Lee, L. A. 1958. Enzyme action and off-flavor in frozen peas. Food Res. 23, 25.

84. Wagenknecht, A. C., Lee, L. A., and Boyle, F. P. 1952. The loss of chlorophyll in green peas during frozen storage and analysis. Food Res. 17, 343.

85. Walker, G. C. 1963. The formation of free radicals during the reaction of soy bean lipoxidase. Biochem. Biophys. Res. Comm. 13, 431.

86. Wang, S. S., and Dimarco, G. R. 1972. Isolation and characterization of the native, thermally inactivated and regenerated horseradish peroxidase isozymes. J. Food Sci. 37, 574.

87. Weinryb, I. 1966. The behavior of horseradish peroxidase at high hydrogen peroxide concentrations. Biochem. 5, 2003.

88. Wittenberg, J. B., Noble, R. W., Wittenberg, B. A., Antonini, E., Brunori, M., and Wyman, J. 1967. Studies on the equilibria and kinetics of the reactions of peroxidase with ligands. II. The reaction of ferroperoxidase with oxygen. J. Biol. Chem. 242, 626.

89. Worthington Enzymes Manual. 1972. Lipoxidase 1.99.2.1, p. 25. Worthington Biochemical Corporation, Freehold, New Jersey.

90. Worthington Enzymes Manual. 1972. Peroxidase 1.11.1.7, p. 43. Worthington Biochemical Corporation, Freehold, New Jersey.

91. Yokota, K., and Yamazaki, I. 1965. Reaction of peroxidase with reduced nicotinamide-adenine dinucleotide and reduced nicotinamide-adenine dinucleotide phosphate. Biochem. Biophys. Acta 105, 301-312.

92. Zimmerman, D. C. 1966. A new product of linoleic acid oxidation by a flaxseed enzyme. Biochem. Biophys. Res. Comm. 23, 398.

93. Zimmerman, D. C., and Vick, B. A. 1970. Specificity of flaxseed lipoxidase. Lipids 5, 392-397.