THE REACTION MECHANISM OF SOY BEAN LIPOXIDASE*

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Based on their observations that the lipoxidase-catalyzed oxidation of linoleate, like autoxidation, gave primarily conjugated diene peroxides (1, 2) and caused a coupled oxidation of some easily oxidizable substances, such as carotenoids (3) and antioxidants (4), Bergström and Holman proposed a reaction mechanism for lipoxidase catalysis. They suggested (4) that lipoxidase could cause initiation of a free radical chain reaction similar to that operative in the autoxidation of methyl linoleate (5). Because the lipoxidase reaction yields peroxides whose molecular extinction coefficient is considerably greater than the molecular extinction coefficient of peroxides produced in autoxidation (4) under the same conditions, Lundberg (6) suggested that lipoxidase may not function merely to initiate a chain reaction like that prevailing in autoxidation but may form a complex with each linoleate molecule and thus influence the structure of the resulting peroxides. More recently Kunkel (7) reported results that were interpreted as being in accord with a chain reaction mechanism for lipoxidase catalysis. He found that the rate of oxidation of methyl linoleate measured by bixin decolorization was proportional to the square root of the chain initiator (lipoxidase) concentration and, therefore, conformed to the same kinetic relationships that had previously been reported for the autoxidation of ethyl linoleate (5). Direct proportionality between reaction velocity and the square root of the enzyme concentration would in general be expected if a chain mechanism is operative (8). Kunkel also reported data on the inhibition of lipoxidase by α- and γ-tocopherols, from which he calculated that the minimum chain length in his system was 12 molecules.

An understanding of lipoxidase catalysis is of interest in relation to proposals that some enzyme reactions may proceed by chain reactions (9–12). Other than for lipoxidase, speculations on possible chain mechanisms are largely without experimental support. It was the purpose of

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the studies reported herein to examine some of the experimental work on which the proposed chain reaction mechanism for lipoxidase has been based and to obtain further information which would aid in clarifying the nature of lipoxidase action. On the basis of the results obtained, a new mechanism for lipoxidase catalysis is proposed.

**Materials and Methods**

*Lipoxidase Preparations*—The crude lipoxidase preparation used in most studies was obtained by aqueous extraction of soy bean meal defatted by petroleum ether extraction. Mature soy beans were ground, defatted, and suspended in 10 parts by weight of distilled water. After centrifuging, the supernatant was stored in small portions at $-18^\circ$ until used.

The purified lipoxidase used was prepared several years ago and was kindly supplied by Dr. R. T. Holman.

For comparative purposes, the potency of the lipoxidase preparations is expressed in units according to the assay of Bergström and Holman (4).

*Preparation of Linoleate*—Highly purified linoleic acid and methyl linoleate were prepared from recrystallized tetrabromostearic acid and were known to contain more than 99 per cent of 9,12-octadecadienoate, of which approximately 91 per cent was the cis-cis form. Linoleic acid neutralized with sodium hydroxide and dissolved in 0.1 N ammonium hydroxide-ammonium chloride buffer at pH 9.0 was used as substrate for most of the studies. The linoleate substrate was protected from oxidation during preparation and storage by an atmosphere of nitrogen from which oxygen had been removed by passage over hot copper.

*Direct Spectrophotometric Method of Enzyme Assay*—In order to determine accurately initial reaction rates, lipoxidase-catalyzed oxidations of linoleate were carried out in the silica cells of a Beckman DU quartz spectrophotometer that was equipped with a temperature-controlled cell compartment.

For measurements of the initial reaction rate, 3 ml. of sodium linoleate substrate at the desired temperature were saturated with pure oxygen, introduced into a silica cell, and allowed to equilibrate to the temperature of the cell compartment. At zero time, 0.3 ml. of diluted lipoxidase was added, the contents were mixed, and measurements of the optical density at 232.5 m$\mu$ were made every 15 seconds by simultaneously balancing the photoelectric circuit and observing a stop-watch.

*Variation in Oxygen Concentration*—Lipoxidase-catalyzed oxidations of linoleate in which the oxygen concentration was a variable were carried out in Thunberg tubes. 5 ml. of buffered sodium linoleate were placed in the main compartment and 0.2 ml. of diluted lipoxidase was placed in the side arm. Each tube was evacuated and gassed four times with oxygen-nitrogen mixtures supplied from a gas burette. The tubes were equilibrated
10 minutes in a constant temperature bath before tipping. The reaction was stopped after 5 minutes by addition of 5 ml. of ethyl alcohol (distilled from potassium hydroxide and zinc dust) and a 3 ml. aliquot was taken for readings of the spectral absorption at 232.5 m\textmu. In each experiment it was established that the decrease in reaction rate with time was sufficiently small so that accurate initial velocities could be obtained.

**Peroxide Determinations**—The determination of the peroxide concentrations followed a modification of the iodometric method of Lundberg and Chipault (13). The free fatty acids and peroxides were liberated from the sodium soaps by acidification and quantitatively extracted with three 20 ml. portions of peroxide-free ethyl ether. The extract was washed and dried, the ether was removed by evaporation, and the residue was used for the peroxide determination.

**Oxygen Absorption Measurements**—Oxygen absorption measurements were made by conventional techniques with a standard Warburg apparatus. The main compartment of the vessels contained the sodium linoleate substrate and other additions to give a final total liquid volume in the flask of 3.0 ml. Diluted lipoxidase (0.2 ml.) was placed in the side arm and mixed with the contents of the main compartment after temperature equilibration.

**Nordihydroguaiaretic Acid Oxidation**—Nordihydroguaiaretic acid (NDGA) when oxidized by the lipoxidase system forms a product with an absorption maximum at 415 m\textmu, which in dilute solution appears yellow-brown. A product with an absorption maximum at 415 m\textmu was also obtained by oxidation of NDGA with iodine, Tollens' reagent, or with air at pH 9. The product reacted with the o-phenylenediamine to give a crystalline derivative. The mode of formation and properties suggested that the product absorbing at 415 m\textmu is an o-quinone. The appearance of absorption at 415 m\textmu was used for estimation of the amount of NDGA oxidized. This procedure may be regarded as giving only an approximation of the amount of NDGA oxidation, since other oxidation products might be formed or the yellow-brown oxidation product may undergo further changes.

**EXPERIMENTAL AND DISCUSSION**

**Effect of Lipoxidase Concentration**—Most proposed methods of assay for lipoxidase have been based upon the observation that the rate of linoleate oxidation was directly proportional to the lipoxidase concentration (4). In our studies the rate of sodium linoleate oxidation measured either by oxygen absorption or the direct spectrophotometric method likewise was directly proportional to the lipoxidase concentration.1 In contrast, Kunkel

found that the rate of methyl linoleate oxidation as measured by bixin destruction was proportional to the square root of the enzyme concentration (7). Because of the importance of the relation between enzyme concentration and activity with reference to the lipoxidase mechanism, further studies on the rate of methyl linoleate oxidation were undertaken. In particular it was felt that use of methyl linoleate might offer some difficulty in kinetic studies because methyl linoleate emulsions when used as substrates may show non-specific activator effects upon addition of proteins or surface-active agents. To test this possibility the experimental assay conditions of Kunkel (7) were duplicated with the exception that a less pure enzyme preparation was used and the increase in light absorption at 232.5 μm was taken as a measurement of the rate of methyl linoleate oxidation in preference to the indirect assay based on bixin destruction. If the action of lipoxidase is that of producing chain-initiating free radicals, then from the nature of such oxidations the following relationships should apply.

\[ \frac{d(O_2)}{dt} = \frac{d(\text{absorption at 232.5 μm})}{dt} = (\text{enzyme})^{1} \times (\text{linoleate}) \]

The rate of methyl linoleate oxidation as a function of enzyme concentration under assay conditions as used by Kunkel is shown in the lower curve of Fig. 1. In the upper curve are given results obtained under the same conditions but with addition of Tween 612 to the reaction mixture. This addition resulted in a linear relationship between the extent of methyl linoleate oxidation and the enzyme concentration. With lipoxidase concentrations above 1 unit the same linear relationship was obtained in the presence or absence of Tween 61. These data suggest that deviations observed from a linear relationship between rate of methyl linoleate oxidation and enzyme concentrations arise because of the inadequate dispersion of the methyl linoleate and may offer an explanation of the results obtained by Kunkel (7). The sharp increase in reaction rate noted in the lower curve of Fig. 1 at a lipoxidase concentration above 0.5 unit probably arises as a result of better dispersion and availability of the substrate resulting from interaction between the substrate and non-enzymic material in the crude lipoxidase preparation. With the more highly purified enzyme preparation used by Kunkel, the tendency for a decline of the rate in relation to the increase in enzyme concentration as noted at low enzyme concentration (Fig. 1) may have been more pronounced and extended over a wider range of enzyme concentrations, leading to the reported linear relationship between rate of bixin destruction and (enzyme)\(^{1}\) (7). Such a possibility is also in harmony with enzyme concentration-velocity relationships and

A polyoxyethylene sorbitan monostearate supplied by the Atlas Powder Company.
lipoxidase activation effects of crude and purified lipoxidase, as reported by Holman (2).

Effect of Linoleate Concentration—The relationship obtained between the initial reaction velocity and the concentration of sodium linoleate did not conform well to theoretical curves corresponding to the Michaelis-Menten equation, $v = \frac{VS}{(K_m + S)}$ where $v$ = observed velocity, $V$ = theoretical maximum velocity, $S$ = substrate concentration, and $K_m$ = the Michaelis constant. Typical results are shown in Fig. 2. Velocities at substrate concentrations below $3 \times 10^{-4} \text{ M}$ could be accurately determined and results of such measurements are shown in Fig. 3. An approximation of the $K_m$ in the usual manner (14), based on separate experiments with both crude and purified lipoxidase at substrate concentration below $3 \times 10^{-4} \text{ M}$, gave a value of $2 \times 10^{-5} \text{ M}$. The approximately linear increase in

![Graph](https://example.com/graph.png)

**Fig. 1.** The relation between the rate of methyl linoleate oxidation and the lipoxidase concentration. The reaction was carried out in Thunberg tubes containing 0.6 ml. of ethyl alcohol, 5 mg. of methyl linoleate, 3.0 ml. of 0.05 M phosphate buffer, pH 7.0, and 0.2 ml. of the crude lipoxidase preparation. The gas phase was oxygen, temperature 20°, and the time 5 minutes. The reaction was carried out with and without 0.05 mg. of Tween 61 per tube, as indicated. The values for the optical density represent the increase during the 5 minute period subsequent to addition of the amounts of enzyme as indicated.
FIG. 2. The relation between the initial velocity of the lipoxidase reaction and the concentration of linoleate. The direct spectrophotometric assay was used, as described in the text. Each silica cell contained 0.033 unit of lipoxidase, 0.1 M ammonia-ammonium chloride buffer, pH 9.0, and the concentration of linoleate, as indicated, in a total volume of 3.0 ml. The temperature was 20°. The velocity is expressed as increase in optical density at 232.5 mμ per minute.

FIG. 3. The relation between the initial velocity of the lipoxidase reaction and the concentration of linoleate at low linoleate concentrations. The assay procedure and conditions were as in Fig. 2.
reaction velocity between linoleate concentrations of $3 \times 10^{-4} \text{ M}$ and $3 \times 10^{-3} \text{ M}$ may explain in part the relatively high value for the $K_m$ previously reported (2).

The cause of the apparently anomalous increase in reaction velocities with increase in substrate concentration beyond $3 \times 10^{-4} \text{ M}$ is not known. It may be related to association of substrate molecules such as leads to micelle formation in soap solutions. Another suggestion, which is discussed later, is the possible occurrence of part of the oxidation as a non-enzymic chain reaction at the higher substrate levels. The inhibition of the reaction velocity noted with substrate concentrations above $3.5 \times 10^{-3} \text{ M}$ is comparable to results frequently obtained with other enzyme systems.

Effect of Oxygen Concentration—In studies of reaction velocity at various sodium linoleate levels the concentration of linoleate at which inhibition became apparent was found to be decreased at lower oxygen tensions. This suggested that oxygen might competitively reduce the inhibition of higher sodium linoleate concentrations. The results of experiments which show that the concentration of oxygen necessary for maximum velocity is dependent upon the linoleate concentration are presented in Fig. 4. The apparent $K_m$ for oxygen in this system is thus a function of the linoleate concentration; the values calculated from the data of Fig. 4 in the usual manner are $0.30 \times 10^{-4} \text{ M}$ and $2.9 \times 10^{-4} \text{ M}$ at linoleate concentrations of $0.36 \times 10^{-3}$ and $7.2 \times 10^{-4} \text{ M}$ respectively. The $K_m$ for oxygen at pH 9.0, 20°, in the absence of linoleate inhibition is thus probably equal to or

![Graph showing the relation between initial velocity of lipoxidase reaction and concentration of oxygen.](http://www.jbc.org/)

**Fig. 4.** The relation between the initial velocity of the lipoxidase reaction and the concentration of oxygen. *A*, $0.36 \times 10^{-3} \text{ M}$ linoleate, 0.065 unit of lipoxidase; *B*, $7.2 \times 10^{-3} \text{ M}$ linoleate, 0.042 unit of lipoxidase. The reaction was carried out in Thunberg tubes containing 5 ml. of linoleate substrate, pH 9.0, prepared as described in the text. The gas phase contained known mixtures of oxygen and nitrogen at a total pressure of 740 mm. The temperature was 20°, and the linoleate and lipoxidase concentrations were as indicated. The values for the molarity of oxygen were calculated from the partial pressure of oxygen in the tube and the solubility of oxygen in water at 20°. The velocities are expressed as increase in optical density at 232.5 nm per minute.
somewhat less than $0.39 \times 10^{-4}$ M, corresponding to an oxygen partial pressure of 21.3 mm. At $0^\circ$ and $7 \times 10^{-3}$ M linoleate, Holman reported the $K_m$ as equal to 40 mm. of oxygen partial pressure (2).

The inhibition caused by high linoleate concentrations could be explained if, with increase in linoleate concentration, additional linoleate combines with the enzyme so as to block the oxygen combination competitively. This might result from the combination of the linoleate with the site needed for oxygen combination; such a mechanism has been considered for lactic dehydrogenase inhibition by diphosphopyridine nucleotide, which is overcome by increase in the lactate concentration (15). With regard to the formation of complex of enzyme, oxygen, and linoleate, an obligatory combination of the enzyme with oxygen prior to combination with linoleate is not feasible if the linoleate inhibition is the result of the blocking of the oxygen absorption site.

Relation between Peroxide Formation and Extinction Coefficient—To substantiate further that the products of lipoxidase catalysis have a relatively high extinction coefficient, the spectral absorption at 232.5 m$\mu$ was correlated with the oxygen uptake and peroxide determinations. The results of the oxygen uptake-spectral absorption correlations were in essential agreement with the data of Holman (2). The extinction coefficient was high at $0^\circ$, $\epsilon_M = 27,400$, and decreased to $\epsilon_M = 23,000$ at $37^\circ$. The lower value of $37^\circ$ was probably the result of more extraneous reactions at this temperature.

The result of the correlation between spectral absorption and peroxide concentration shown in Fig. 5 may be more indicative of the true extinction coefficient of the products of the lipoxidase-catalyzed oxidation than the spectral absorption-oxygen uptake correlation because extraneous oxygen uptake should not cause interference. Here again the molar extinction coefficient of 27,500 indicates that the peroxides produced show a greater absorption at 232.5 m$\mu$ than the corresponding products produced in auto-oxidation.

Inhibition of Linoleate Oxidation by Antioxidants—Various results obtained suggested that the action of antioxidants in the lipoxidase system was not primarily the result of their inhibition of a chain reaction. Some antioxidants inhibited linoleate oxidation completely or considerably without a corresponding depression in the rate of oxygen absorption, as shown by the effects of propyl gallate and hydroquinone in Table I. If the principal action of antioxidants was the breaking of reaction chains with chain lengths of the order of 12 or more, as suggested by Kunkel (7), then the decrease in oxygen absorption should be nearly as great as the decrease in linoleate oxidation. In some experiments with $\alpha$-tocopherol, in which the amounts of linoleate oxidation and $\alpha$-tocopherol oxidation were compared, the oxidation of 1 mole of $\alpha$-tocopherol was found to prevent
the oxidation of only 0.3 to 0.4 mole of linoleate. If a chain mechanism were operative, the oxidation of 1 mole of \( \alpha \)-tocopherol should prevent the oxidation of considerably more than 1 mole of linoleate.

The most definitive results pertinent to the mechanism of lipoxidase

![Graph](image)

**Fig. 5.** The relation between spectral absorption and peroxide concentration of products from the lipoxidase-catalyzed oxidation of linoleate. Peroxide determinations were made as described in the text. The reaction was carried out in flasks which were shaken in a constant temperature bath. Each flask contained 10 ml. of 0.025 M linoleate, pH 9.0, and 5 ml. of a diluted crude lipoxidase preparation. The reaction was stopped by addition of 10 ml. of ethyl alcohol. A 2 ml. aliquot was taken for a determination of the spectral absorption and a 20 ml. aliquot was used for the peroxide determination.

**Table I**

*Comparison of Inhibition of Linoleate Oxidation and Total Oxidation Due to Presence of Various Antioxidants at 30*°

The sodium linoleate concentration was 0.0036 M and the phosphate buffer 0.05 M, pH 7.0. Linoleate oxidation was determined by the direct spectrophotometric method, and total oxidation was determined by oxygen absorption, as described in the text.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Concentration</th>
<th>Inhibition of linoleate oxidation</th>
<th>Inhibition of total oxidation</th>
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<td></td>
<td>M</td>
<td>per cent</td>
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<tr>
<td>Propyl gallate</td>
<td>(1 \times 10^{-4})</td>
<td>100</td>
<td>73</td>
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<tr>
<td>(\alpha)-Tocopherol</td>
<td>(2 \times 10^{-4})</td>
<td>39</td>
<td>19</td>
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<td>Hydroquinone</td>
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<td>21</td>
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<td>(2 \times 10^{-3})</td>
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were obtained with NDGA. NDGA is a potent inhibitor of the oxidation of linoleate by lipoxidase, and the extent of its oxidation can be readily approximated by spectrophotometric methods. Fig. 6 shows the rates of linoleate oxidation and oxygen uptake and the approximate rate of NDGA oxidation as a function of increasing NDGA concentration. At concentrations of NDGA above $1 \times 10^{-4}$ M, the rate of linoleate oxidation was zero within experimental error. At the concentrations of $1 \times 10^{-4}$ M to $2 \times 10^{-4}$ M NDGA, the NDGA oxidation rate remained comparatively high in the absence of net linoleate oxidation, as measured by diene conjugation and peroxide formation. However, without linoleate the oxidation of NDGA did not occur. At concentrations about $3 \times 10^{-4}$ M NDGA all oxidation was stopped. At low NDGA concentrations (Fig. 6) the total moles of linoleate oxidized plus approximate moles of the oxidation product formed from NDGA oxidation (measured by absorption at 415 mμ) are less than the moles of oxygen taken up. This is probably the result of reaction of more than 1 mole of oxygen with each mole of NDGA to form products which do not absorb at 415 mμ. Above $1 \times 10^{-4}$ M NDGA, the amount of the NDGA oxidation product formed is only slightly less than the oxygen uptake. The inhibition of lipoxidase by NDGA was reversible, as demon-
skated by the presence of lipoxidase activity after dilution of solutions containing lipoxidase and NDGA. The time required for recovery of full activity increased from 0 to 10 minutes when a solution containing $5 \times 10^{-4}$ M NDGA was diluted 33.3 or 6.6 times respectively.

These data show two important effects of NDGA. First, higher concentrations of NDGA can completely inhibit lipoxidase catalysis. Secondly, NDGA can be oxidized at a rapid rate in the presence of lipoxidase and linoleate, even when there is not net linoleate oxidation. Clearly this oxidation cannot be the result of the interruption of linoleate oxidation chains by NDGA. The formation of a linoleate or linoleate peroxide-free radical not associated with the enzyme as required by a chain mechanism would lead to conjugated products, as noted in autoxidation (16). Such products would be measured as representing oxidation of linoleate in the assay used.

**Possible Induction Period**—Although Holman (17) found that the oxidation of bixin in the lipoxidase system had an induction period, measurement of the initial rate of linoleate oxidation by the direct spectrophotometric method in these studies did not show the presence of an induction period. The rate of product formation was linear with time within the limits of the experimental method from the instant lipoxidase was introduced into the substrate. The induction period found by Holman may have been the result of some property of the bixin oxidation system.

**Evidence against Chain Reaction Mechanism of Lipoxidase**—The experimental evidence reported herein, together with results of some previous investigations, offers the following arguments against a chain reaction as the principal mechanism for lipoxidase catalysis.

1. The initial rate of the linoleate oxidation under valid assay conditions is directly proportional to the lipoxidase concentration and not to the square root of the lipoxidase concentration.

2. The formation by lipoxidase action of peroxides with a higher molecular extinction coefficient than that found in peroxides formed by autoxidation is most readily explained on the basis that the oxidation of each linoleate molecule occurs under the directive influence of the enzyme. Recent evidence\(^*\) indicates that this difference in the molecular extinction coefficients is at least in part, if not entirely, due to differences in the geometric configuration of the double bond systems rather than to differences in the degree of conjugation, as previously assumed (6). In either case the peroxides are structurally different, thus ruling out the possibility that the lipoxidase-catalyzed oxidation proceeds by a chain reaction that differs from the autoxidation only in the chain-initiating step.

3. The action of antioxidants in the lipoxidase catalysis is readily ex-

\(^*\) Privett, O. S., and Lundberg, W. O., unpublished data.
plainable by mechanisms not involving chain reactions, and oxidation of antioxidants under some conditions cannot be the result of interruption of oxidation chains. This is well illustrated by the data on NDGA oxidation presented herein and supported by other data on α-tocopherol oxidation.\textsuperscript{1} Further, calculations from Kunkel's data ((7), Fig. 4) show that 1 mole of α-tocopherol wasoxidized per mole of linoleate oxidation prevented. If the lipoxidase-catalyzed oxidation of methyl linoleate in his experiments was a chain reaction with a minimum chain length of 12 as suggested (7), then oxidation of 1 mole of α-tocopherol should have prevented oxidation of considerably more than 1 and possibly as many as 24 moles of linoleate.

4. With increase in sodium linoleate concentration, the lipoxidase-catalyzed reaction approaches a maximum velocity, in harmony with other enzymic reactions. While it is possible on the basis of the chain reaction theory to propose mechanisms which would account for such observations (9), the concept of an obligatory formation of an enzyme-substrate complex for each reacting molecule of substrate is a simpler explanation of the data. In addition, in the autoxidation of ethyl linoleate, which is a chain reaction, the oxidation rate was found to be directly proportional to the linoleate concentration at high oxygen tensions (16).

5. The lipoxidase reaction does not show an induction period. Occurrence of an induction period has been cited as evidence in favor of chain reaction mechanisms for enzyme reactions (9).

\textbf{Plausible Reaction Mechanism for Lipoxidase Action—}A simple mechanism for lipoxidase catalysis which is in accord with experimental observations is presented in Fig. 7. The initial step is visualized as the formation of a complex linoleate, oxygen, and lipoxidase, followed by a transfer of an electron and hydrogen ion from the linoleate to the oxygen to form a biradical on the enzyme surface (Step 2). The biradical then reacts to give a conjugated peroxide (Step 3) while still under the directive influence of the enzyme, and the peroxide dissociates from the enzyme (Step 4).

The action of lipoxidase may involve stabilization of the biradical. Stabilization of free radicals has been suggested by Michaelis (18) as one explanation of the promotion of oxidation by enzymes. The formation of a biradical might occur through a direct transfer of a hydrogen atom, or through transfer of an electron with discharge and uptake of a hydrogen ion from the medium. The presence of oxygen is probably necessary as an electron acceptor for the enzyme to form a radical from linoleate. If radical formation would occur without oxygen, then lipoxidase might be expected to catalyze conjugation of linoleate anaerobically. This does not occur. Further, lipoxidase does not contain any known prosthetic group which might serve in electron transfer (4).

The oxidation of NDGA and other antioxidants in the presence of lino-
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leate and lipoxidase could logically result through reaction of the antioxidant with the biradical formed, as depicted in Fig. 8. An antioxidant is represented by \( \text{AH}^* \). The reactive biradical formed in Step 2 could readily react with the antioxidant abstracting 1 hydrogen at a time, forming first a free radical and then the oxidized antioxidant (Steps 3 and 4). In this process the free radical of linoleate might accept a hydrogen to become linoleate again, or the \( \cdot \text{OOH} \) radical might accept a hydrogen to form hydrogen peroxide. An alternative mechanism is possible in which the antioxidants could transfer hydrogens only to one of the radicals and the other radical could obtain a hydrogen by an intermolecular rearrangement.

Whether hydrogen peroxide is formed in the oxidation of antioxidants, as indicated by Step 5, is not known, but reduction of oxygen to the hydrogen peroxide level by the enzyme would seem more probable than complete reduction to water. If hydrogen peroxide were formed, it could react non-enzymically with additional antioxidant.

The lack of formation of conjugated products from linoleate in the presence of sufficient NDGA might result if the linoleate which originally forms a radical does not dissociate measurably from the enzyme prior to reacting again with oxygen, as shown in Fig. 8. Alternatively the linoleate radical with a free electron on carbon atom 11 may be stabilized by the
enzyme and react with the antioxidant. Shift of the free electron to the carbon 9 or 13 position in the absence of excess antioxidant might result in a rapid reaction with the OOH radical, giving a conjugated peroxide.

The reaction of a linoleate molecule in solution with a biradical formed from oxygen and linoleate on the enzyme surface might also occur. This would be analogous to the postulated reaction of antioxidants, and could under favorable conditions give rise to oxidation of some of the linoleate by a chain mechanism. Such a reaction would account for the decrease in the extinction coefficient of the hydroperoxides formed at higher temperatures, and for the continued anomalous increase in reaction rate with linoleate concentration shown by the data in Fig. 2. That enzyme reactions which may involve free radical intermediates can initiate chain reactions was shown by Parravano (12).

An important aspect of this mechanism is that it offers an explanation for oxidation of antioxidants without net oxidation of the linoleate but dependent on the presence of both linoleate and lipoxidase. In this type of oxidation the function of linoleate is analogous to that of a coenzyme. This raises the interesting question of the possible biological significance of such oxidation.

SUMMARY

Results of experiments directed toward increased understanding of lipoxidase catalysis are reported. With sodium linoleate as a substrate, under suitable conditions the reaction velocity was linear with respect to the enzyme concentration and the reaction did not show an induction period. With methyl linoleate the reaction velocity was not directly proportional to enzyme concentration unless a Tween preparation was added. Measurements of initial reaction velocities at sodium linoleate concentrations below $3 \times 10^{-4}$ M, pH 9.0, 20°, gave results in good confirmation of the Michaelis-Menten equation, $K_m$ equal to $2 \times 10^{-8}$ M. However, with substrate concentrations between $3 \times 10^{-4}$ M and $4 \times 10^{-4}$ M, an apparently anomalous increase in reaction velocity was noted and discussed.

The value for the $K_m$ for oxygen increased with increase in the linoleate concentration, possibly as a result of interference by linoleate with the combination of oxygen and the enzyme. The $K_m$ for oxygen at pH 9.0, 20°, in the absence of linoleate inhibition is probably equal to or slightly less than $0.39 \times 10^{-4}$ M.

Spectral absorption measurements at 232.5 m\m were correlated with both oxygen uptake and peroxide formation for the lipoxidase-catalyzed oxidation of sodium linoleate. The results further confirm the higher value of the extinction coefficient for peroxides formed in presence of lipoxidase as contrasted to those formed in autoxidation.
Antioxidants inhibited linoleate oxidation as a result of a direct effect on lipoxidase and of a preferential oxidation of the antioxidant. Rapid oxidation of nordihydroguaiaretic acid under suitable conditions was obtained in the absence of net linoleate oxidation, the linoleate having a function analogous to that of a coenzyme.

The strong evidence obtained against a chain reaction mechanism for lipoxidase is discussed. A simple reaction mechanism is postulated involving formation of a biradical from linoleate and oxygen on the enzyme surface. The biradical may accept electrons from antioxidants or may react to give the conjugated linoleate peroxide.

BIBLIOGRAPHY

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