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Hydroperoxide Isomerase

A NEW ENZYME OF LIPID METABOLISM

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ABSTRACT

An enzyme has been isolated from flaxseed (*Linum usitatissimum*) which utilizes the product of lipoxidase for its substrate. The enzyme, termed hydroperoxide isomerase, converts the conjugated diene hydroperoxide of linoleic acid to the corresponding monoenoic ketohydroxy fatty acid. The structure of the latter has been determined by ultraviolet, infrared, and nuclear magnetic resonance spectroscopy; periodate and permangate oxidation; gas chromatography; and thin layer chromatography. Hydroperoxide isomerase activity has also been demonstrated in crude extracts from barley (*Hordeum vulgare*), wheat germ (*Triticum aestivum*), mung beans (*Phaseolus aureus*), and corn (*Zea mays*) and from partially purified extracts of soybean (*Glycine max*).

The hydroperoxide isomerase enzyme from flaxseed has a pH optimum of 7.0. The enzyme was not inhibited by nordihydroguairetic acid, p-chloromercuribenzoic acid, or cyanide, but it was inhibited by cupric ion. One hundred per cent of the activity was lost by heating the enzyme for 1 minute at 68 C. Both linoleic and linolenic acid hydroperoxides can serve as substrates for the enzyme. The pH optimum for the hydroperoxide isomerase enzyme from barley is 6.2; from wheat germ, 6.1; and from soybean, 6.1.

The identification of the hydroperoxide isomerase enzyme clarifies the role of lipoxidase in plant tissue and suggests a participation of lipid in the electron transport system.

In 1954, Franke and Frehse reported that flaxseed extracts catalyzed the reduction of certain dyes, Bindschedler's Green and 2,6-dichlorophenolindophenol, in the presence of linoleic and linolenic acids (7). The authors believed that the reaction involved a direct dehydrogenation between the dye and the fatty acid chain. Purification of the enzyme or enzymes was not attempted, nor were the products identified. While repeating this work, we observed that either endogenous flaxseed lipoxidase or added soybean lipoxidase was necessary for dye reduction. The hydroperoxides resulting from lipoxidase action on linoleic or linolenic acids did not reduce the dyes. Thus, it appeared that the product of lipoxidase activity was serving as a substrate for another enzyme. This paper reports the isolation of an enzyme from flaxseed and certain other plant sources. It catalyzes the isomerization of an unsaturated hydroperoxide to a ketohydroxy compound and is named hydroperoxide isomerase. The identification of this enzyme and its reaction product provides a new insight into this area of lipid metabolism. A preliminary report appeared in 1966 (2).

MATERIALS AND METHODS

Preparation of Extracts. Ungerminated flaxseed (CI No. 980) was ground with acetone in a VirTis 45 homogenizer[2] at full speed for 20 sec. The supernatant was decanted and filtered with suction. The residue was rehomogenized with acetone for 10 sec, decanted, and filtered. The combined precipitates were rinsed with acetone, diethyl ether, and then dried under vacuum. The powder has produced active extracts when stored for over 2 years at −12 C. One gram of acetone powder was extracted with 10 ml of 0.1 M potassium phosphate buffer, pH 7.0, for 1 hr in an ice bath. The mixture was centrifuged at 27,000*g* for 15 min, and the clear supernatant was decanted. This crude extract was treated with liquid ammonium sulfate, and the protein fraction precipitating between 42 and 50% saturation was collected. This fraction contained hydroperoxide isomerase activity but no lipoxidase activity.

Barley seed (variety Dixon) was ground in a Wiley mill through a 20-mesh screen. Three grams of ground barley were extracted with 30 ml of 0.1 M potassium phosphate buffer, pH 7.0, for 1 hr in an ice bath. The mixture was centrifuged at 12,000*g* for 20 min, and the supernatant was made 50% saturated with ammonium sulfate. The precipitate was redissolved in 5 ml of 0.02 M phosphate buffer, pH 7.4. This fraction still contained lipoxidase activity along with hydroperoxide isomerase activity.

Commercial wheat germ (obtained locally) was extracted with 0.1 M phosphate buffer, pH 7.0, centrifuged at 12,000*g*, and fractionated with ammonium sulfate. The fraction that precipitated at 0 to 37% saturation possessed hydroperoxide isomerase activity but no lipoxidase activity.

Fifty grams of soybeans (variety Crest) were crushed in a mortar and pestle and then homogenized in the VirTis for 3 min at full speed with acetone. The mixture was filtered, washed with acetone, diethyl ether, then dried under vacuum at 25 C. Soybean acetone powder was extracted with 0.1 M phosphate buffer, pH 7.4, and centrifuged at 500 *g* for 20 min. The supernatant was treated with ammonium sulfate, and the fraction precipitating between 30 and 40% saturation was collected. This

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fraction possessed both lipoxidase and hydroperoxide isomerase activity.

An acetone powder of oriental-type mung beans and corn was prepared in the same way as with soybeans. Extraction of the acetone powders with 0.1 M phosphate buffer, pH 7.0, for 1 hr provided extracts which possessed both lipoxidase and hydroperoxide isomerase activities.

Mung beans were germinated in moist paper towelling at 25 C in the dark and in the light, 1200 ft-c, 16-hr photoperiod for 9 days. They were harvested; separated into root, hypocotyl, epicotyl, and leaves; frozen in liquid nitrogen; crushed; and extracted with 0.1 M phosphate buffer, pH 7.4.

Assay Procedures. Fatty acid substrates were emulsified with Tween 20, according to Surrey (23). Linoleic and linolenic acids were purchased from the Hormel Institute and soybean lipoxidase (10,000 units/mg) from Sigma Chemical Co. Lipoxidase and hydroperoxide isomerase activities were determined by measuring the conjugated diene absorption at 234 nm in a recording spectrophotometer equipped with a constant temperature cell holder at 25 C. The reaction mixture contained 2.9 ml of buffer, 0.02 ml of enzyme extract (about 0.2 mg of protein), and 0.02 ml of linoleic or linolenic acid substrate solution (7.9 μmoles/ml) in the order given. The reaction mixture for determining hydroperoxidase isomerase activity was 2.45 ml of buffer, 0.53 ml of hydroperoxide substrate, and 0.02 ml of enzyme extract. The hydroperoxide substrate solution was prepared by incubating 0.4 ml of soybean lipoxidase solution (1 mg/10 ml of borate buffer, pH 9.0), 0.2 ml of linoleic acid substrate, and 10 ml of water for 15 min.

For analysis of the product of flaxseed hydroperoxide isomerase activity, a larger reaction mixture was used. Three liters of 0.1 N phosphate buffer, pH 7.4; 120 ml of linoleic acid substrate; and 30 ml of soybean lipoxidase solution were incubated for 15 min. Then, 60 ml of the 42 to 50% ammonium sulfate fraction of a flaxseed extract was added. After 15 min, the mixture was acidified to pH 1.5 with HCl and extracted with petroleum ether (b.p. 30–60 C) and concentrated. All operations after the incubation were conducted under nitrogen to prevent autoxidation. The extract was esterified with diazomethane, and the methyl esters were placed on a column packed with 40 ml of acid-treated Florisil or Adsorbosil-CAB. The column was eluted with 40 ml of petroleum ether, 160 ml of petroleum ether-diethyl ether (95:5, v/v), 160 ml of petroleum ether-diethyl ether (90:10, v/v), and 160 ml of petroleum ether-diethyl ether (85:15, v/v). Ten-milliliter fractions were collected and assayed by thin layer chromatography on Silica Gel H with petroleum ether-diethyl ether-acetic acid (60:40:1, v/v) solvent. Spray reagents used were a saturated alcoholic solution of 2,4-dinitrophenylhydrazine for keto compounds, *N*,*N*-dimethyl-*p*-phenylenediamine for hydroperoxides (25), and a 50% (v/v) sulfuric acid spray followed by charring for other organic compounds. Column fractions containing the keto compound were combined and concentrated under nitrogen.

The assay of radioactive linoleic acid was performed as follows. Linoleic acid-1-^{14}C, 2.3 μc, was added to 0.3 ml of linoleic acid substrate solution. This was added to 6.0 ml of water, saturated with O_2, containing 0.2 ml soybean lipoxidase solution. After 1 hr at 25 C, 6.0 ml of 0.1 M phosphate buffer, pH 7.0, were added; and 9.0 ml of this solution were incubated with 0.06 ml of flax extract, 30 to 42% ammonium sulfate fraction. Heat-denatured extract, 0.02 ml, was added to the remaining 3.0 ml of the mixture as a control. After 1 hr, both solutions were acidified to pH 3 and extracted three times with diethyl ether. The ether solution was dried over anhydrous sodium sulfate, concentrated under nitrogen, and spotted on a thin layer of silica gel coated on a plastic sheet. After developing the chromatogram with petroleum ether-diethyl ether-acetic acid (60:40:1, v/v), the various regions of the chromatogram were cut out and placed in liquid scintillation vials with 15 ml of counting solution (5 g of PPO, 0.5 g of dimethyl-POPOP per liter of toluene). Vials were counted and efficiency was determined by the channels ratio method.

Product Analysis. Infrared spectra were determined with the samples placed in carbon disulfide in microliquid cells with 0.5-mm path length. For nuclear magnetic resonance studies, the sample was placed in carbon disulfide solution and scanned on a Varian A-60A spectrometer at 36 C. Periodate oxidations were conducted on a microscale according to the procedure of Dixon and Lipkin (4).

The position of the keto and of the hydroxyl groups in the fatty acid molecule was determined by catalytic reduction of the double bond followed by permanganate oxidation and analysis of the products by gas chromatography. Palladium oxide, 5 mg, in methanol was reduced with hydrogen gas at 25 C for 15 min. About 1 mg of the ketohydroxy compound was added, and hydrogen was bubbled through the solution for 60 min. Water was added, and the mixture was extracted with petroleum ether. The ether solution was evaporated under nitrogen and oxidized with 4.3 mg of potassium permanganate, according to the procedure of Tinoco and Miljanich (24). Gas chromatographic analysis of the oxidation products was accomplished by use of a 6-ft column packed with 15% (w/w) diethylene glycol succinate on Gas Chrom W, in conjunction with a hydrogen flame detector. The column temperature was programmed as follows: 12 min at 70 C, followed by a 7.5 C/min increase up to a final temperature of 190 C. Retention times were determined for the methyl esters of known monocarboxylic acids C_6 through C_{20} the C_9 dicarboxylic acid, the C_{12} dicarboxylic acid, and the C_9 aldehyde-acid.

Chlorophyll Bleaching. Chlorophyll was obtained by extracting endive leaves with 80% aqueous acetone in the dark. The solution was concentrated, extracted with petroleum ether, and purified on a cellulose column with 3% (v/v) diethyl ether in petroleum ether as the eluting solvent. The fractions containing chlorophyll were combined; the solvent was evaporated and replaced with diethyl ether. The ether solution contained 3.33 mg/liter of total chlorophyll (72.7% chlorophyll *a* and 27.3% chlorophyll *b*). The bleaching of chlorophyll was followed by measuring the decrease in absorbance at 431 nm. The reaction mixture contained 2.9 ml of 0.02 M phosphate buffer, pH 5.8 (barley) or pH 7.4 (flax); 0.01 ml of linoleic acid substrate; 0.02 ml of chlorophyll solution; and 0.02 ml of enzyme extract.

RESULTS

Initial experiments confirmed the reduction of either Bindschedler's Green or DCPIP[3] by a crude flaxseed extract in the presence of linoleic or linolenic acid. This dye reduction did not occur with purified soybean lipoxidase; or with a flax extract which had been heated at 100 C for 5 min; or when the endogenous flaxseed lipoxidase was inhibited by nordihydroguairetic acid. These observations indicated that the product of lipoxidase, the conjugated diene hydroperoxide of linoleic or linolenic acid, was necessary for dye reduction. This was substantiated by a loss of dye reduction when the hydroperoxide was converted to a hydroxyl group by stannous chloride.

The addition of DCPIP to the reaction mixture caused a significant decrease in the conjugated diene absorption at 234 nm. The absorption at 234 nm after adding linoleic acid to a crude flaxseed extract is shown in Figure 1A. The conjugated diene increased rapidly, because of lipoxidase activity, and then decreased, because of a subsequent enzymatic reaction. The decrease in conjugated diene absorption was accompanied by a

[3] Abbreviation: DCPIP: dichlorophenolindophenol.

loss in peroxide content, as determined by the colorimetric method of Koch *et al.* (18). The enzyme that caused the loss in diene absorption (Fig. 1B) is named hydroperoxide isomerase and could be separated from lipoxidase activity by ammonium sulfate fractionation of the crude extract (Table I). Direct analysis of the products of the isomerase activity by gas chromatography or mass spectrometry was not possible, owing to instability of the compounds at high temperatures. Several attempts were made by using different columns and conditions, but in each case the chromatograms were too complex for meaningful interpretation.

Analysis of the reaction mixture on thin layer chromatography showed that the major product (>70%) had an R_F of 0.28, and a minor product had an R_F of 0.17 (Fig. 2). Linoleic acid-1-^{14}C and linoleic acid-U-^{14}C were incubated with soybean lipoxidase to form the hydroperoxide and then with the 30 to 42% fraction from flaxseed. After thin layer chromatography, individual fractions were removed from the plate and counted for activity in a liquid scintillation counter. The results are shown in Table II. Approximately 78% of the decrease in hydroperoxide was accounted for by increase in the ketohydroxy compound (R_F 0.28) and the remainder in the R_F 0.17 material. Total yield of the radioactivity was 32%, and recovery from the thin layer chromatogram was 87%. Radioautographs showed that the distribution of radioactivity was the same with the carboxyl-^{14}C linoleic acid as with the uniformly labeled linoleic acid, and that there had been no decarboxylation or cleavage of the fatty acid molecule.

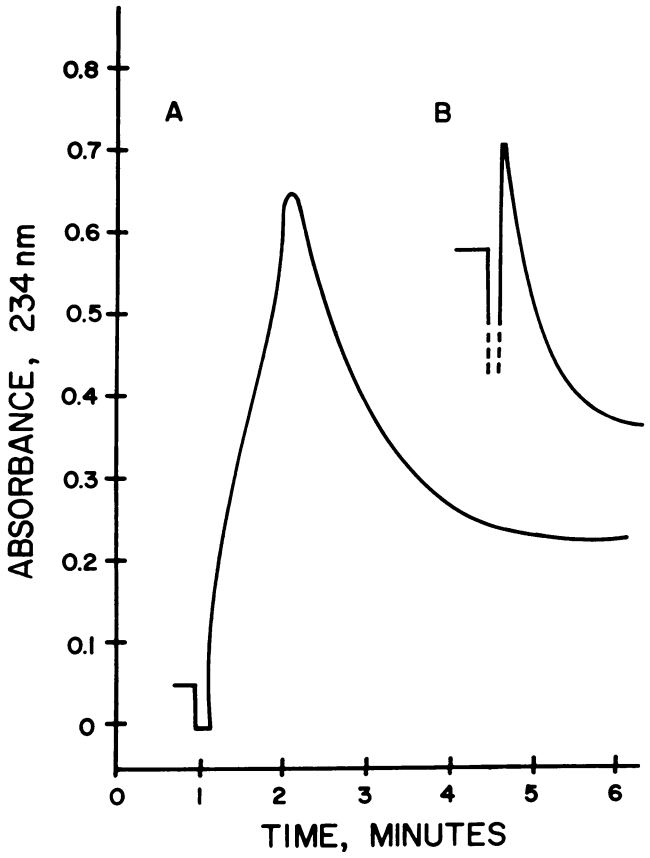


FIG. 1. Lipoxidase and hydroperoxide isomerase activity as evidenced by absorption at 234 nm. Reaction mixture: A: 2.9 ml of 0.05 M potassium phosphate buffer (pH 6.6), 0.02 ml of crude flax extract, and 0.03 ml of linoleic acid substrate solution; B: 2.45 ml of 0.05 M potassium phosphate buffer (pH 6.6), 0.53 ml of hydroperoxide substrate solution, 0.01 ml of flax extract, 42% to 50% saturated ammonium sulfate fraction.

The essentiality of the hydroperoxide group in the reaction was demonstrated in an experiment in which it was reduced to a hydroxyl group by stannous chloride. The resulting hydroxy-conjugated diene compound did not react with flaxseed hydroperoxide isomerase. Also, the reaction did not utilize molecular oxygen, as measured with the Gilson oxygraph. Infrared spectra were determined on the free acid and methyl ester of the keto compound (R_F 0.28) in carbon disulfide solution. Figure 3 shows the spectrum of the latter compound. The significant structural characteristics which can be identified from the spectrum are a secondary hydroxyl group (3500 cm^{-1}), C-H of a *cis* double bond (3015 cm^{-1}), an ester carbonyl group (1745 cm^{-1}), a ketone carbonyl (1720 cm^{-1}), and a secondary hydroxyl with α-unsaturation (1075 cm^{-1}) (9). The absence of any indication of a *trans* double bond at 952 cm^{-1} or 990 cm^{-1} was most significant. Since the product of soybean lipoxidase is predominantly 13-hydroperoxyoctadeca-*cis*-9-*trans*-11-dienoic acid (5, 11), the disappearance of the *trans* double bond established the point at which isomerization occurred in the hydroperoxide molecule, that is at carbons 12 and 13. Based on the ratio of absorbance values at 3010 cm^{-1} and 2920 cm^{-1}, there was only one *cis* double bond present (20), indicating that there was no reaction at the double bond in the 9, 10 position.

The methyl ester of the keto compound was also examined by nuclear magnetic resonance spectroscopy and the results are shown in Figure 4. All of the groupings indicated by the infrared data were confirmed by the nuclear magnetic resonance spectrum. The spectrum indicated two ethylenic protons (4.45 PPM, τ), four α-carbonyl, α-olefinic protons (6.95), one methine proton (5.95), and one hydroxyl proton (2.80). The two protons at 6.95 PPM (τ) indicate that the keto group is at carbon 12 and not at carbon 13.

The ultraviolet absorption spectra of the keto methyl ester indicated a λ_{max} 275 nm with an inflection at 230 nm. An estimate of the molar absorptivity in methanol was ϵ_{275} 5270.

Periodate oxidation was used to determine whether the keto

Table I. *Ammonium Sulfate Fractionation of a Flaxseed Extract*

Ammonium Sulfate Fraction	Total Protein	Specific Hydroperoxide Isomerase	Activity Lipoxidase	Total Hydroperoxide Isomerase	Activity Lipoxidase	Yield	
						Hydroperoxide Isomerase	Lipoxidase
%	*mg*	$-A_{234}/min \cdot mg$	$+A_{234}/min$	$-A_{234}/min \cdot mg$	$+A_{234}/min$	%	%
Crude	789.6	8.06	1.47	6360	1158	100	100
30	31.2	60.90	8.65	1900	270	29.9	23.3
42	96.6	23.29	3.52	2250	340	35.4	29.4
50	109.4	2.47	0	270	0	4.2	0
60	57.5	2.35	0	135	0	2.1	0
70	72.2	0	0	0	0	0	0

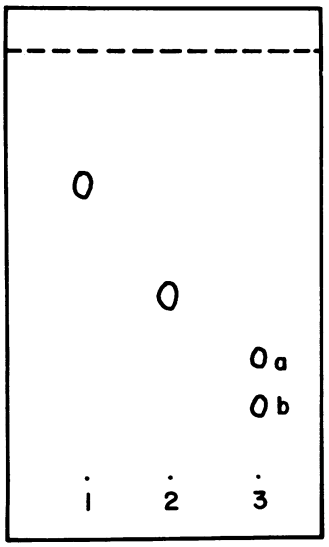


FIG. 2. Thin layer chromatogram of reaction compounds spotted on silica gel H, developed in petroleum ether-diethyl ether-acetic acid (60:40:1, v/v). 1: Linoleic acid; 2: hydroperoxide of linoleic acid; 3a: ketohydroxycompound formed from linoleic acid; 3b: unknown.

Table II. *Reaction of Hydroperoxide Isomerase with Linoleic Acid Hydroperoxide-1-^{14}C*

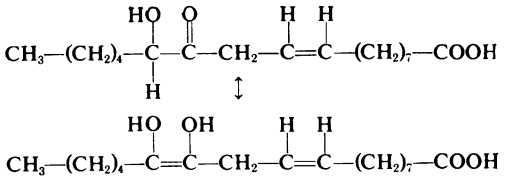
Region of Thin Layer Chromatogram	Control[1]	Flax Extract	Net Change
	nc	*nc*	*nc*
Linoleic acid	1.50	1.25	−0.25
Hydroperoxide	6.01	2.04	−3.97
Ketohydroxy	2.23	5.34	+3.11
Unknown	0.43	1.55	+1.12

[1] Extract of reaction mixture which contained linoleic acid-1-^{14}C, soybean lipoxidase and heat-denatured flax extract, 30 to 42% saturated ammonium sulfate fraction.

and hydroxyl groups were on adjacent carbon atoms (4). The results (Fig. 5) showed that the product of isomerase activity was oxidized at the same rate as 9,10-dihydroxystearic acid, indicating that the keto and hydroxyl groups were indeed adjacent to each other. The position of the two groups within the fatty acid molecule was determined by oxidative cleavage followed by identification of the products by gas chromatography. Chromatography of the reaction mixture on a column of Adsorbosil-CAB yielded two keto-containing compounds which had about the same R_F on a thin layer plate. The ratio of the two compounds was 95% isomer A and 5% isomer B. Their infrared spectra were identical. The two isomers were oxidized separately by permanganate, and the products were characterized. Isomer A yielded a C_6 monocarboxylic acid and a C_{12} dicarboxylic acid, indicating cleavage between carbons 12 and 13. Isomer B yielded a C_9 monocarboxylic acid and a C_9 aldehyde-acid, indicating cleavage between carbons 9 and 10. The exact amounts of the oxidized products were not determined, but the ratio of the peak size on the gas chromatogram was close to 1:1. The small amount of isomer B which formed is probably the result of autoxidation of linoleic acid during the experiment. It should be noted that the position of the hydroperoxide group is determined by the specificity of the soybean lipoxidase used in these experiments. The results of other workers (5, 11) and experiments in our own laboratory (unpublished data) indicate that the product of soybean lipoxidase and linoleic acid is 13-hydroperoxyoctadeca-9-*cis*-11-*trans*-dienoic acid. Consequently, the location of the keto and hydroxyl groups on carbon 12 and 13 is consistent with the isomerization of the hydroperoxide to 12-keto-13-hydroxy-octadec-*cis*-9-enoic acid by the flaxseed hydroperoxide isomerase enzyme.

Recent experiments (28) have shown that the specificity of flaxseed lipoxidase is identical to that of soybean lipoxidase when linoleic and linolenic acids are substrates, *i.e.*, 13-hydroperoxy-octadeca-9-11-dienoic and 13-hydroperoxyoctadeca-9-11,15-trienoic acids, respectively (28). Consequently, the products of flaxseed hydroperoxide isomerase are 12-keto-13-hydroxyoctadec-9-enoic and 12-keto-13-hydroxyoctadeca-9,15-dienoic acids, respectively.

Thin layer chromatography of the reaction mixture had indicated the presence of a second product, R_F 0.17 (Fig. 2). Analysis of this compound has not been completed, but some data have been obtained. The infrared spectrum indicated the loss of the keto group along with the formation of a rather broad but weak hydroxyl absorption. Acetylation produced a change in R_F value, indicating the presence of a hydroxyl group or groups. It is our belief that this compound is probably the enediol form of the ketohydroxy compound, and that it may have formed during the isolation procedure.



The hydroperoxide isomerase enzyme from flaxseed has not been completely purified. However, several properties of the enzyme are known. All of its activity was destroyed by heating for 1 min at 68 C or for 5.5 min at 55 C. All activity was lost when the assay was conducted in 4 M urea. Hydroperoxide isomerase of flaxseed was unaffected by 10^{-3} M potassium cyanide, 10^{-3} M EDTA, 10^{-3} M iodoacetamide, 2×10^{-4} M nordihydroguairetic acid, 10^{-4} M *p*-chloromercuribenzoic acid. However, it was inhibited 100% by 5×10^{-4} M cupric chloride and about 50% by 5×10^{-4} M cuprous chloride. The only other inhibitor of the isomerase enzyme observed in our laboratory was linoleic acid. A crude flax extract (0.18 mg of protein) showed no isomerase activity with 1.26×10^{-4} M linoleic acid, but it was very active at 5.13×10^{-5} M concentration. This inhibition was not observed with linolenic acid at these same concentrations. Varying the ratio of linoleic acid to Tween 20 from 1:0 to 1:2 (v/v) in the substrate solution had no effect on the pH optimum but did depress the activity at a ratio of 1:2 (v/v). The same effect was observed by Surrey for lipoxidase activity from gram (Cicer sp.) flour (23). The pH optima for flaxseed hydroperoxide isomerase and lipoxidase are shown in Figure 6. The optimum for isomerase activity was pH 7.0, and lipoxidase showed two optima, one at pH 5.4 and another at pH 6.5. It is not yet clear whether there are two separate lipoxidase enzymes in flaxseed or a single enzyme with two optima.

Assays could be run in either 0.1 M or 0.05 M phosphate buffer with equivalent activity, but at 0.01 M concentration there was very little activity. The ionic strength has a direct effect on the shape or size of lipid micelles, which can affect enzyme activity (27). The amount of hydroperoxide present in the substrate varied with its preparation from 0.23 to 1.09%. The higher values were obtained with linolenic acid.

The effect of flax seedling age on lipoxidase and hydroperoxide isomerase activities was determined during 10 days germination. It was observed that acetone treatment of the germinated tissue destroyed the lipoxidase activity completely and reduced the

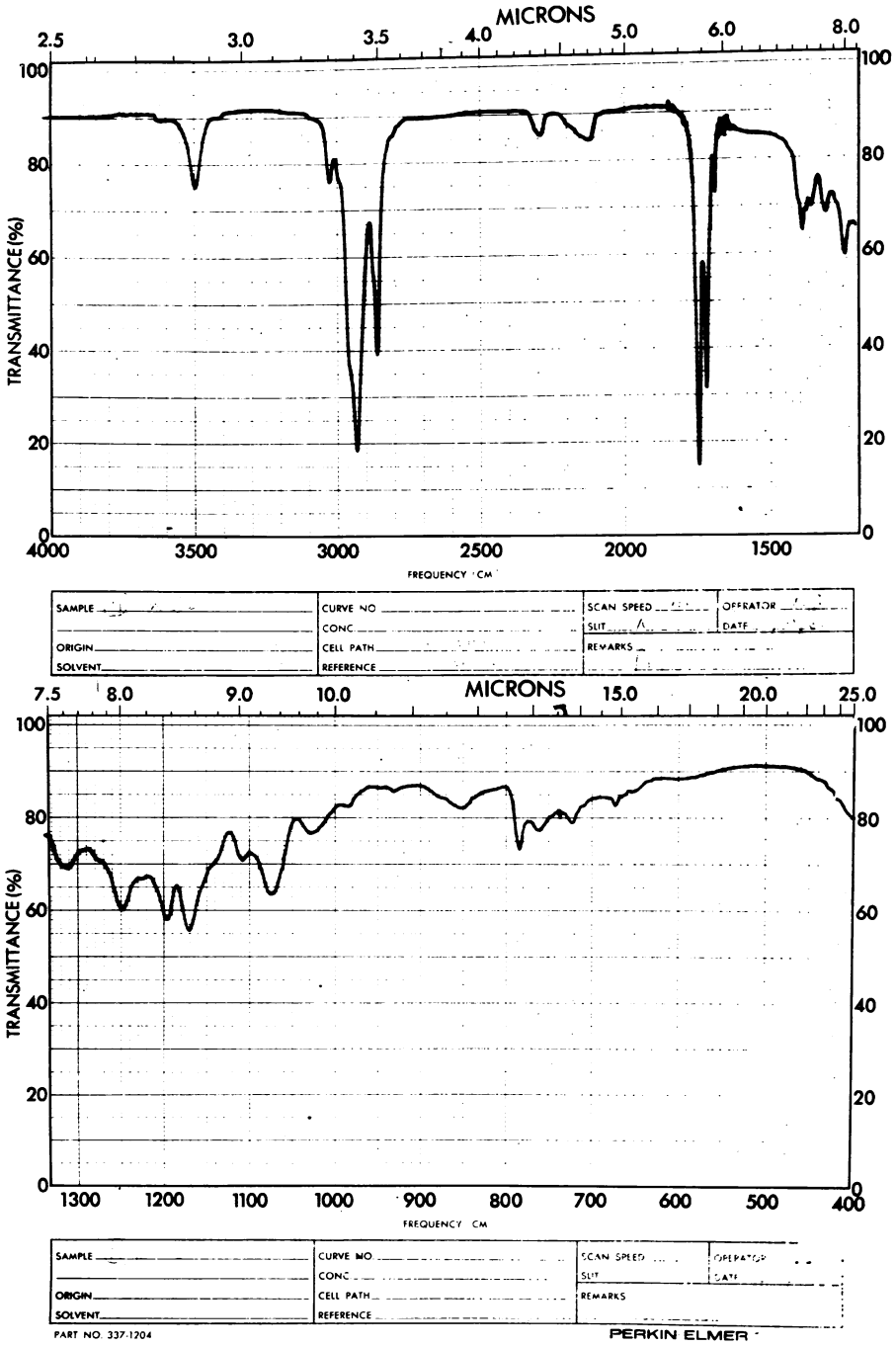


FIG. 3. Infrared spectrum of ketohydroxy compound.

isomerase activity to 20% of that obtained with aqueous extracts. This acetone sensitivity may reflect two locations in the seedling tissue, one site being the original cotyledons (insensitive to acetone) and the other in newly formed cells (sensitive to acetone). Because of this acetone sensitivity, we used aqueous extracts during the germination experiments. The results are shown in Table III. While the total lipoxidase activity decreased to about 1/10 that of the 2-day value, the hydroperoxide isomerase activity only decreased to 1/3. Since these values reflect activity of the whole seedling, 7-day-old flax seedlings were separated into root, hypocotyl, and cotyledons, and the enzyme activity in each section was determined. The results, Table IV, show that lipoxidase appeared only in the cotyledons, whereas the isomerase was present in each section with a significantly higher specific activity in the hypocotyl. Although the total activity of the hydroperoxide isomerase enzyme decreased during germination, its presence in the hypocotyl indicates the formation of new enzyme.

The subcellular location of lipoxidase and hydroperoxide isomerase was determined by separating the cotyledons and hypocotyls from 10-day-old flax seedlings into chloroplasts, mitochondria, ribosomes and soluble fractions. Lipoxidase activity was present only in the soluble fraction (105,000*g* supernatant) of the cotyledons. Isomerase activity was present predominantly in the soluble fraction of both the cotyledon and hypocotyl. Preliminary experiments have indicated that hydro-

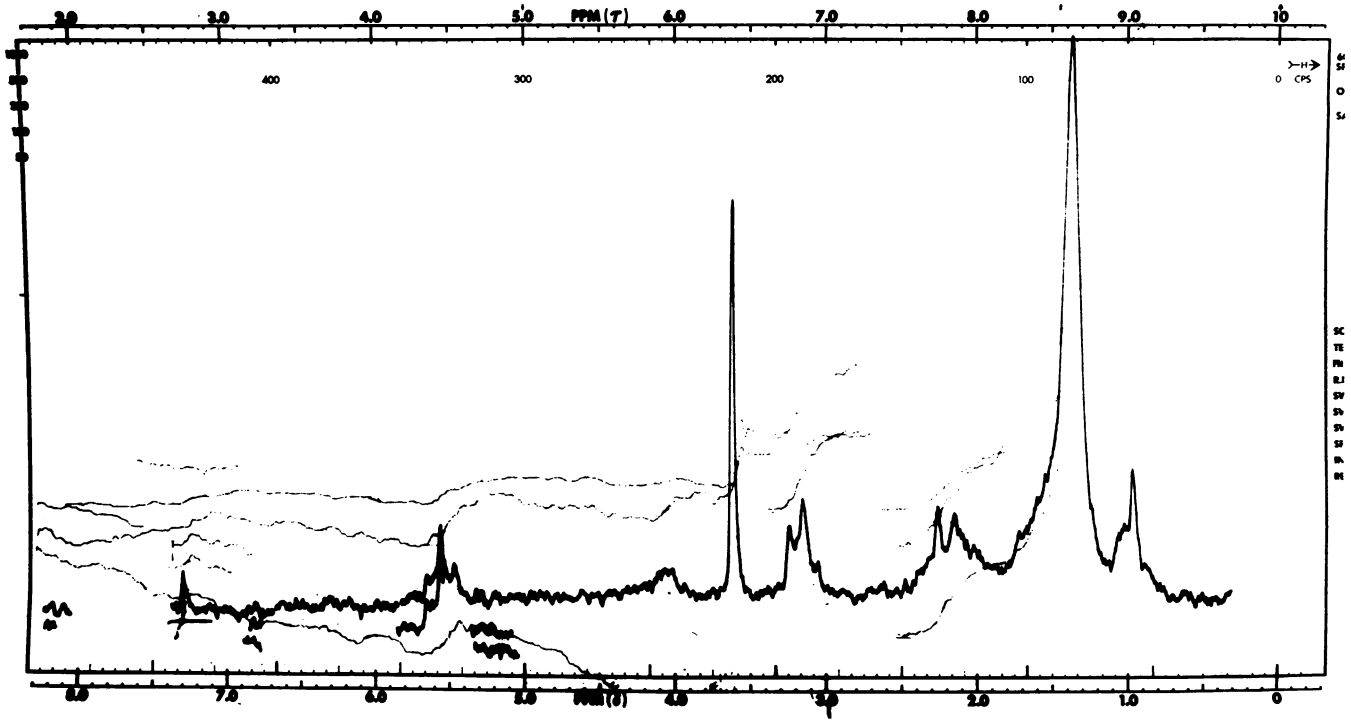


FIG. 4. Nuclear magnetic resonance spectrum of ketohydroxy compound.

peroxide isomerease is also present in leaves from 21-day-old flax plants.

In addition to flaxseed, other seed sources known to possess high lipoxidase activity were tested for the presence of hydroperoxide isomerase. A comparison of the activities of crude extracts is shown in Table V. Crude barley extracts showed isomerase activity as evidenced both by decreasing 234 nm absorption and by reduction of DCPIP. Much higher isomerase activity could be demonstrated by making the 12,000g supernatant 50% saturated with ammonium sulfate and redissolving the precipitate in 0.02 M phosphate buffer. The barley isomerase enzyme was also inhibited by increasing linoleic acid concentrations. The pH optimum was 6.2 (Fig. 7).

Several attempts were made to demonstrate hydroperoxide isomerase activity in wheat seeds. The attempts with crude extracts were unsuccessful, and extracts fractionated with ammonium sulfate were not tested. However, extracts of wheat germ (embryo) showed both lipoxidase and hydroperoxide isomerase activities. Fractionation with ammonium sulfate showed that the isomerase activity was highest in the 30 to 40% fraction. Treatment with 10^{-4} M p-chloromercuribenzoic acid caused a 50% inhibition of hydroperoxide isomerase activity, indicating that a sulfhydryl group may be required for activity. The pH optimum for the lipoxidase enzyme was 5.65 and that for the hydroperoxide isomerase, 6.1. Analysis of the reaction mixture by thin layer chromatography indicated that the product had the same R_F as that from the flaxseed enzyme.

Crude extracts of soybeans or a soybean acetone powder showed only a trace of hydroperoxide isomerease activity. However, when an extract of soybean acetone powder was treated with ammonium sulfate, the fraction precipitating between 30 and 40% saturation indicated significant hydroperoxide isomerase activity. There was a loss in diene absorption at 234 nm and a reduction of DCPIP. The pH optimum for isomerase activity in 0.02 M phosphate buffer was 6.1.

A crude extract of mung bean acetone powder showed both lipoxidase and hydroperoxide isomerase activities. The two activities were readily separated by ammonium sulfate fractionation with the lipoxidase concentrated in the 40 to 50% fraction and the isomerase predominantly in the 0 to 30% fraction. The pH optimum was not determined. Other seeds were tested for the presence of hydroperoxide isomerase enzyme. There was no activity in jack beans, and the results with green peas and alfalfa were inconclusive.

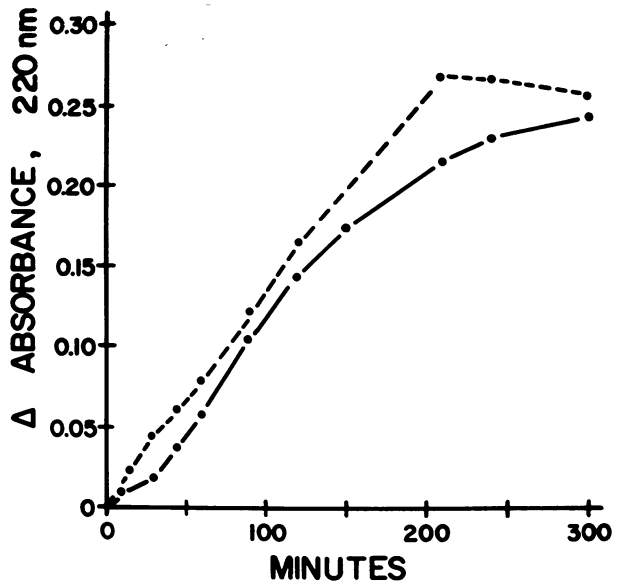


FIG. 5. Periodate oxidation of threo-9,10-dihydroxy stearic acid (——) and 12-keto-13-hydroxyoctadec-*cis*-9-enoic acid (- -).

The activities of lipoxidase and hydroperoxide isomerase in the various tissues of light- and dark-grown mung bean seedlings were determined. The results (Table VI) showed there was considerable lipoxidase activity in the hypocotyl, root, and leaves of light-grown seedlings and in the hypocotyl and leaves of dark-

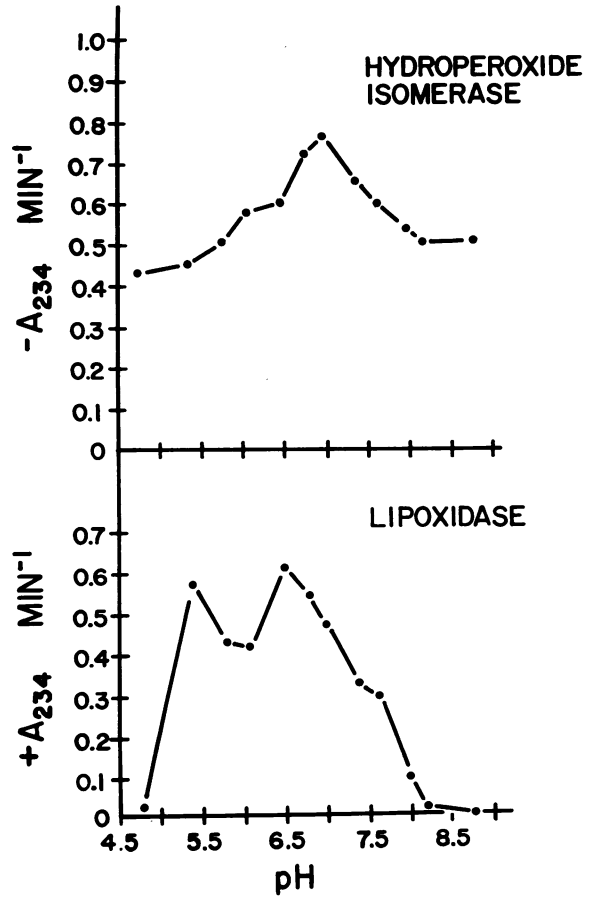


FIG. 6. pH optima for flaxseed hydroperoxide isomerase and lipoxidase. Isomerase assay: 2.9 ml of 0.05 M phosphate buffer, 0.011 μmole of linoleic acid hydroperoxide (from soybean lipoxidase), 0.001 ml of flax extract, pH 6.5 (0–30% ammonium sulfate fraction). Lipoxidase assay: 2.9 ml of 0.10 M phosphate buffer, 0.03 ml of linoleic acid substrate solution, 0.01 ml of flax extract, pH 6.5, diluted 1:10 (0–30% ammonium sulfate fraction).

Table III. *Lipoxidase and Hydroperoxide Isomerase Activity during Flaxseed Germination*

Age	Lipoxidase Specific Activity	Hydroperoxide Isomerase Specific Activity	Protein Concn	Total Protein	Lipoxidase Total Activity	Hydroperoxide Isomerase Total Activity
days	*ΔA/min · mg*		*mg/ml*	*mg*	*ΔA/min*	
2	0.606	−0.972	19.3	77.2	46.7	−75.0
4	0.269	−0.839	15.6	62.4	16.8	−52.4
6	1.80	−1.88	5.2	20.8	37.4	−39.1
8	0.672	−1.45	6.4	25.6	17.2	−37.1
10	0.224	−1.51	4.6	18.4	4.1	−27.8

grown seedlings. There was also hydroperoxide isomerase activity in both the light- and dark-grown seedlings, predominantly in the hypocotyl. The most striking effect of the light was a 450% increase in the hydroperoxide isomerase specific activity of the root as compared to that of the corresponding dark activity. The activity in the hypocotyl increased about 190% when grown in the light.

The enzymatic bleaching of chlorophyll by long chain fatty acids with a number of legume seed extracts was reported by

Table IV. *Distribution of Enzyme Activities in 7-day-old Flax Seedling*

Plant Section	Lipoxidase Specific Activity	Hydroperoxide Isomerase Specific Activity
	ΔA/min · mg	
Root	...	−0.115
Hypocotyl	...	−0.859
Cotyledon	0.070	−0.476

Table V. *Hydroperoxide Isomerase Activities of Various Seed Sources*

Source[1]	Hydroperoxide Isomerase Specific Activity	
	pH 7.0	pH 6.5
	ΔA_{234}/min·mg	
Flax	−9.542	−7.550
Corn	−4.021	−3.517
Barley	−0.715	−1.014
Wheat germ	−0.291	−0.391
Mung bean	−0.031	−0.031
Soybean	−0.019	−0.026

[1] All were acetone powders prepared from the seeds except for barley, which was gound in a Wiley mill through a 60-mesh screen. All powders were extracted with 10 volumes of buffer.

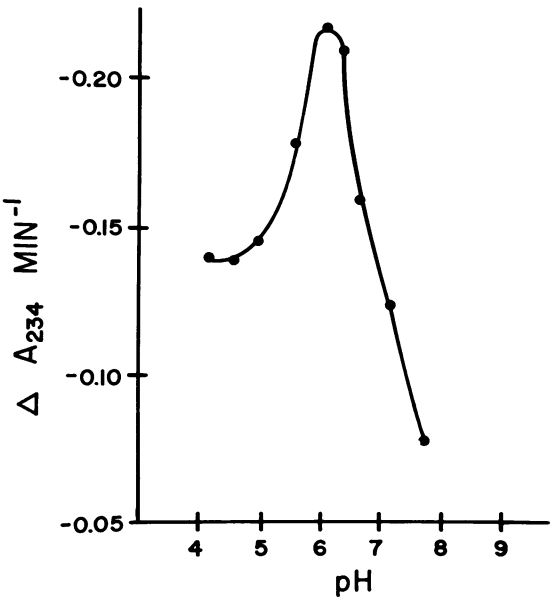


FIG. 7. Barley hydroperoxide isomerase activity in 0.02 M phosphate or citrate-phosphate buffer at different pH levels.

Holden (12). Although the extracts possessed lipoxidase, purified soybean lipoxidase did not bleach chlorophyll. These results were confirmed in our laboratory in that neither soybean lipoxidase nor flax lipoxidase would bleach chlorophyll in the presence of linoleic acid. However, barley, flax, or soybean extract fractions which contained hydroperoxide isomerase activity did bleach chlorophyll, as evidenced by a decrease in absorbance at 431 nm (Fig. 8). The bleaching occurred only in the presence of linoleic acid, lipoxidase activity, and hydroperoxide isomerase activity. When the isomerase activity was inactivated by heating the ex-

Table VI. *Lipoxidase and Hydroperoxide Isomerase Activity in Light- and Dark-grown Mung Bean Seedlings*

	Specific Activity		Total Activity	
	Lipoxidase	Hydroperoxide isomerase	Lipoxidase	Hydroperoxide isomerase
	$+A_{234}/min \cdot mg$	$-A_{234}/min \cdot mg$	$+A_{234}/min$	$-A_{234}/min$
Light-grown[1]				
Root	0.068	0.027	20.7	8.2
Hypocotyl	0.067	0.044	26.2	17.3
Epicotyl	0.055	0.032	4.2	2.4
Leaves	0.230	0.019	77.5	6.4
Dark-grown[2]				
Root	0.041	0.006	9.4	1.4
Hypocotyl	0.055	0.023	21.2	8.9
Epicotyl	0.036	0.021	4.7	2.7
Leaves	0.213	0.019	52.6	4.7

[1] Two hundred twelve plants grown at 25 C, 1200 ft-c, 16-hr photoperiod for 9 days, extracted and assayed in 0.1 M phosphate buffer, pH 7.4.

[2] Two hundred twelve plants grown at 25 C in the dark for 10 days, extracted and assayed in 0.1 M phosphate buffer, pH 7.4.

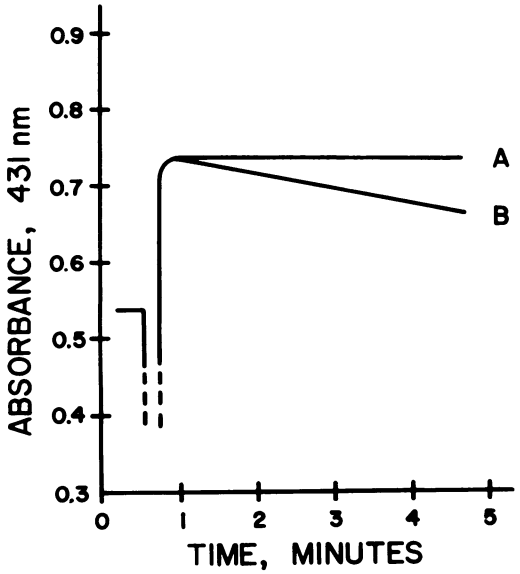
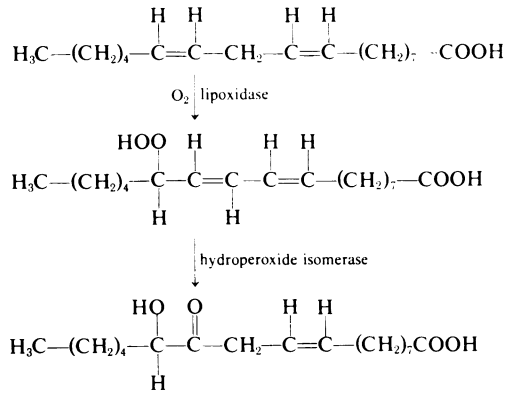


FIG. 8. Chlorophyll bleaching as evidenced by decrease in absorption at 431 nm. Reaction mixture: 2.6 ml of 0.02 M phosphate buffer (pH 6.2), 0.3 ml of chlorophyll solution, 0.02 ml of barley extract, 50% saturated ammonium sulfate fraction. A: Heat-denatured barley extract or without linoleic acid; B: with 0.04 ml of linoleic acid substrate solution.

tract at 100 C for 5 min, there was no bleaching of chlorophyl. The nature of the bleaching reaction has not been determined, but the visible absorption spectra of chlorophyll before and after bleaching indicated a general decrease in absorption over the whole range, rather than in any particular region of the spectrum. It would appear that an oxidation-reduction reaction between the keto-hydroxy fatty acid and some particular portion of the extensive conjugated bond system of the chlorophyll molecule is responsible for the decreased absorption.

DISCUSSION

The various experiments on the structure of the product of flaxseed hydroperoxide isomerase and linoleic acid showed that the compound contained a keto group, a hydroxyl group, and one *cis* double bond. The location of the hydroxyl and keto groups was fixed at positions 12 and 13 and the NMR spectrum indicated the placement of the keto group in the 12 position. Combined with the known specificity of flaxseed lipoxidase and linoleic acid (28), the reaction catalyzed by the flaxseed hydroperoxide isomerase is shown below:



The redox properties of the ketohydroxyl groups account for the reduction of DCPIP. The enediol form of the molecule is very similar to the active center of ascorbic acid. The redox potential of the ketohydroxy fatty acid is different from that of ascorbic acid, however, since the ketohydroxy compound will reduce DCPIP at pH 7.4 but not at pH 4.3. The presence of the hydroperoxide isomerase enzyme in various seeds and tissues was based on three factors: the ability to reduce DCPIP, the decrease in absorption at 234 nm, and loss of activity by heating at 100 C for 5 min.

Recognition of the hydroperoxide isomerase enzyme and its reaction product can help us understand the unexplained results of several previous studies on lipoxidase. A number of workers have reported the disappearance of linoleate hydroperoxide catalyzed by crude soybean extracts (1–3, 9). In each case, the description of the observed activity matches very closely that of the hydroperoxide isomerase observed in this study. Surrey (22) postulated the presence of an unsaturated fat oxidase in mung beans to explain the changes in 234 nm absorption which he observed with crude extracts. From our work with fractionated mung bean extracts it is clear that the absorption changes were due to hydroperoxide isomerase. Navy bean extracts have also shown a loss of hydroperoxide content (17). Irvine and Anderson (14) observed two distinct phases of the lipoxidase reaction obtained with durum wheat semolinas. Since the observations were based on oxygen uptake, there was no evidence of a loss of hydroperoxide.

The bleaching of carotene in the presence of polyunsaturated fatty acids has been associated with lipoxidase activity for many years. In 1940, Sumner and Sumner (21) showed that lipoxidase and carotene oxidase were identical. However, Kies (15) observed that carotene oxidase could be destroyed by heating at 70 C for 2 min, without affecting the lipoxidase activity as measured by conjugated diene absorption at 234 nm. In a more recent article, Kies *et al.* (16) reported that crystalline lipoxidase does not bleach carotene when methyl linoleate is the primary substrate.

The destruction of chlorophyll by legume seed extracts has also been attributed to lipoxidase activity. In 1965, Holden (12) reported on an extensive study of this phenomenon. She found that

although the bleaching activity in soybeans bore some relation to lipoxidase activity, it did not parallel it exactly. The pH optimum was different for bleaching than for peroxidation and purified lipoxidase did not bleach chlorophyll under the conditions used for testing seed extracts. Holden concluded that the chlorophyll was bleached by co-oxidation during a chain reaction involving peroxidation of fatty acid and the breakdown of hydroperoxide by a heat-labile factor. The experiments on chlorophyll bleaching reported here show that the hydroperoxide isomerase enzyme is required for bleaching. This strongly suggests that the heat-labile factor described by Holden is the hydroperoxide isomerase enzyme.

In 1965, Gardner reported that alfalfa seedling extracts possessed a catalytic factor which decomposed linoleate hydroperoxide (8). The product was postulated to contain a keto group, an epoxide ring, and a *trans* double bond. A second product in which the epoxide ring was hydrolyzed to a dihydroxy compound was also suggested. On the basis of the reported structures, the products were different from the ketohydroxy compounds observed in this study. Gardner did not report whether there was dye reduction or chlorophyll bleaching by alfalfa extracts. Vioque and Holman characterized two ketodiene compounds resulting from the oxidation of ethyl linoleate by a crude soybean extract (25). Although the products were attributed to the action of lipoxidase, this is not certain, since a crude, unpurified extract was used. In the experiments that we conducted with soybean extracts, there was no evidence of ketodiene formation on thin layer chromatography.

The location of lipoxidase and hydroperoxide isomerase in the various tissues of a plant is of particular interest in relation to metabolic function of these enzymes. In flax seedlings, we have observed lipoxidase activity only in the cotyledons, whereas we found hydroperoxide isomerase in root tissue, the hypocotyl. cotyledons, and primary leaves. In mung bean seedlings, we found both enzymes present in the root, hypocotyl, epicotyl, and leaves. The amount of hydroperoxide isomerase in the root and hypocotyl tissues showed a marked increase in response to light.

The presence of lipoxidase in roots and leaves of 18-day-old soybean seedlings was observed by Holman (13), of 9½-day-old barley seedlings by Franke and Frehse (6), and of 7-day-old wheat seedlings by Guss *et al.* (10). The distribution of lipoxidase and hydroperoxide isomerase in tissues other than seed tissues suggests a broad role for these enzymes in plant cell metabolism. The oxidation-reduction properties of the ketohydroxy fatty acid could indicate participation in an electron transport system. These properties are under further investigation.

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