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Pathways of Fatty Acid Hydroperoxide Metabolism in Spinach Leaf Chloroplasts

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ABSTRACT

The metabolism of 13-hydroperoxylinolenic acid was examined in protoplasts and homogenates prepared from mature leaves of spinach (*Spinacia oleracea* L.). Chloroplast membranes were the principal site for metabolism of the compound by at least two highly hydrophobic enzyme systems, hydroperoxide lyase and hydroperoxide dehydrase, the new name for an enzyme system formerly known as hydroperoxide isomerase and hydroperoxide cyclase. Hydroperoxide lyase was most active above pH 7 and could be separated from hydroperoxide dehydrase by anion exchange chromatography. Hydroperoxide dehydrase, measured by the formation of both α-ketol product and 12-oxo-phytodienoic acid, had its optimum activity in the range of pH 5 to 7. Lyase was more active than dehydrase activity when the enzymes were extracted by homogenization. The reverse was true when the enzyme activities were measured in protoplasts, which are isolated by gentle extraction methods. The variation in enzyme activity ratios with extraction methods suggests that hydroperoxide lyase is activated by plant injury and thus may function in a wound response. In the absence of injury, the normal pathway of fatty acid hydroperoxide metabolism is probably by hydroperoxide dehydrase activity. The molecular weights of both the lyase and dehydrase were approximately 220,000, as estimated by gel filtration.

Fatty acid hydroperoxides are produced in plants from polyunsaturated fatty acids and oxygen in a reaction catalyzed by lipoxygenase. Linoleic and linolenic acids are the most abundant polyunsaturated fatty acids in plants, and therefore are the most likely substrates for lipoxygenase catalysis. Two major pathways for the metabolism of fatty acid hydroperoxides have been characterized in plants (Fig. 1). In one pathway, HL[1] catalyzes the cleavage of 13-hydroperoxylinolenic acid into 12-oxo-dodecenoic acid and hexenal. In the other pathway, HD transforms its substrate into a short-lived allene oxide (8) that quickly undergoes hydrolysis to form α- and γ-ketols or rearranges to form the cyclic compound 12-oxo-PDA. Prior to the characterization of the allene oxide intermediate by Hamberg (8), the enzyme system responsible for the formation of ketols was referred to as hydroperoxide isomerase (22), and the system that formed 12-oxo-PDA was called hydroperoxide cyclase (24). The latter product is a key intermediate in the biosynthesis of jasmonic acid, a compound that has growth regulating properties.

Our recent investigations have concentrated on the role of the jasmonic acid pathway in plants (19). As a part of these studies, this paper will show that a key enzyme of the jasmonic acid pathway, HD, is localized in the chloroplasts of spinach leaves. The study also shows that spinach chloroplasts possess both the HL and HD pathways of fatty acid hydroperoxide metabolism. These results supplement and clarify earlier investigations of fatty acid hydroperoxide metabolism in plants. In the past, investigators of hydroperoxide metabolism have usually focused on only one of the above pathways in the study of a particular plant, and have not dealt with the possibility that both pathways have the potential to function simultaneously.

MATERIALS AND METHODS

Chemicals. Linoleic and linolenic acids were purchased from NuChek Prep, Inc.,[2] Elysian, MN. Soybean lipoxygenase, Percoll, and Triton X-100R (reduced form of Triton X-100) were from Sigma Chemical Co. Cellulysin and macerase were purchased from Calbiochem. Wacker silicone oils AR20 and AR200 were obtained from SWS Silicones Corp., Adrian, MI. BCA protein assay reagent was a product of Pierce Chemical Co. Normal phase HPTLC plates and $LKC_{18}F$ TLC plates were from Whatman, Inc.

Plant Material and Extraction Procedures. Spinach bunches (*Spinacia oleracea* L., California-grown) were purchased at a local supermarket. Spinach extracts for column chromatography were prepared by first removing the midvein and then cutting the leaves into small pieces with a razor blade. A Brinkmann PT10/35 Polytron homogenizer was used to homogenize 10 g of leaves in 30 ml of 0.2 M K-phosphate (pH 7.5), containing 0.1% Triton X-100R. After filtration through 70 μm nylon mesh, the extract was centrifuged twice at 12,000*g* to remove cellular debris. For studies of enzyme pH optimum, the extraction was conducted at pH 7.0 and the ratio was 10 ml of extraction buffer for each g of leaves.

Spinach chloroplasts from 35 g of leaves were prepared in low cation medium by the method of Nakatani and Barber (12). The chloroplasts were resuspended in 4 ml of the low cation medium and then further purified on linear Percoll gradients by a procedure similar to that described by Haas *et al.* (7). The gradients were prepared from 14 ml of 0.33 M sorbitol in Percoll adjusted to pH 7.6 and 14 ml of low cation medium, which consisted of 0.33 M sorbitol adjusted to pH 7.6 with 0.5 mM Tris base. After centrifugation for 20 min at 3300*g*, the gradient was separated into 2-ml fractions by pumping from the bottom of the gradient, and the fractions were assayed for various enzymes. The intact chloroplasts present in the lower band were then resuspended in

[1] Abbreviations: HL, hydroperoxide lyase; 12-oxo-PDA, 12-oxo-*cis*,*cis*-10,15-phytodienoic acid; *t*OPC-8:0, (1*R*,2*R*)3-oxo-2-(2′-pentenyl)cyclopentaneoctanoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HD, hydroperoxide dehydrase.

[2] Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by the United States Department of Agriculture.

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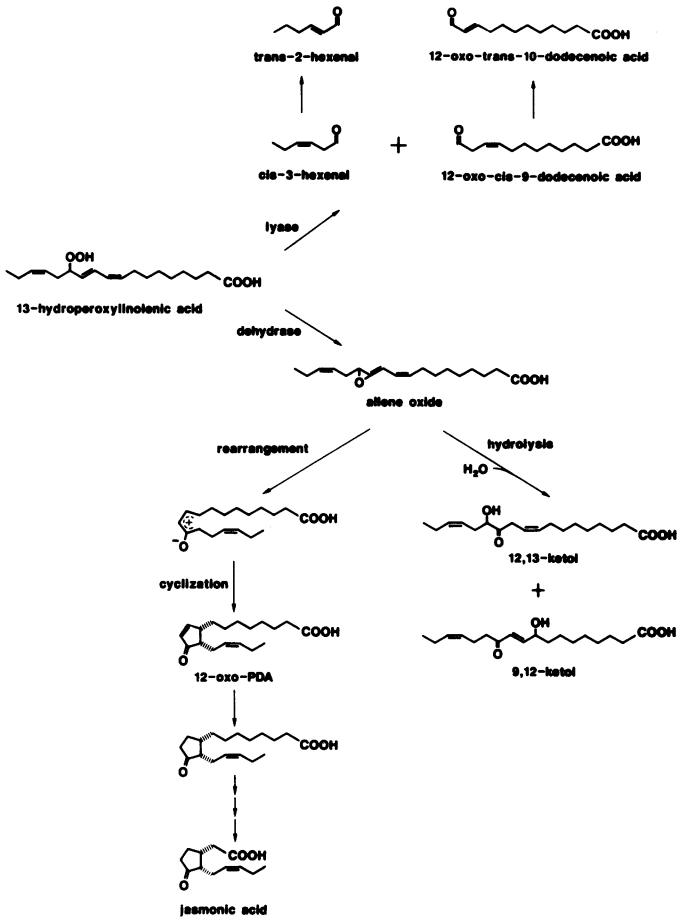

FIG. 1. Pathways of 13-hydroperoxylinolenic acid metabolism.

20 ml of low cation medium and centrifuged at 2200*g* for 20 s. After the supernatant was decanted, the chloroplasts were ruptured by resuspending them in 4 ml of 100 mM Tris (pH 7.5) containing 1 mM DTT, and then recentrifuged on a Percoll gradient identical to the one described above.

Spinach leaf protoplasts were prepared by removing the midvein of the leaf and then slicing the leaf into 1 mm strips. The strips were floated for 4 h in a solution containing 1% Cellulysin and 0.5% Macerase in 0.5 M sorbitol, 10 mM calcium chloride, and 5 mM Mes (pH 6.0). The strips were then swirled and the brei filtered through 70 μm nylon mesh. After centrifugation for 5 min at 150*g* the sedimented protoplasts were purified by the procedure described by Edwards *et al.* (5). The rapid separation of chloroplasts from protoplasts was accomplished by silicone oil centrifugation according to Robinson and Walker (13). The protoplasts, resuspended in 100 to 150 μl of low cation medium, were placed in a reservoir on top of a 400 μl tube. A 20 μm nylon mesh formed the bottom support of the reservoir. The lower portion of the tube contained 50 μl of 0.4 M sucrose as the bottom phase, 100 μl of silicone oil (19 parts AR200 to 1 part AR20) as the middle phase, and 50 μl of 0.4 M sorbitol as the upper phase. The protoplasts were centrifuged for 6 s in a Beckman Microfuge B. After passing through the nylon mesh, the components of the broken protoplasts distributed into the lower or upper phases of sucrose or sorbitol.

Gel Filtration and Ion Exchange Chromatography. HL and HD were partially purified by gel filtration and anion exchange chromatography. A spinach extract (6 ml) was applied to a Sephacryl S-300 column (2.5 × 29 cm) and eluted with 0.2 M K-phosphate (pH 7.5) containing 0.1% (w/v) Triton X-100R. The flow rate was 1.9 ml/min and 2.5-ml fractions were collected. The mol wt of the fatty acid hydroperoxide-metabolizing enzymes were estimated by their elution volumes on this column. The mol wt standards were ferritin (440,000), β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (67,000), and ovalbumin (43,000).

Fractions that actively metabolized fatty acid hydroperoxides were combined and applied to a DEAE Sepharose CL-6B column (2.5 × 21 cm). Proteins were eluted with 300 ml of a gradient ranging from zero to 0.2 M NaCl in 50 mM K-phosphate (pH 7.0), containing 0.1% (w/v) Triton X-100R. The flow rate was 3.2 ml/min and 4.2-ml fractions were collected.

Enzyme Assays. 13-Hydroperoxylinolenic acid substrate was prepared by reacting 1 ml of 8 mM linolenic acid solution prepared according to Surrey (14) with 32 ml of a solution containing 2 mg of soybean lipoxygenase in 0.6 mM borate (pH 9). After 20 min at room temperature, the hydroperoxide substrate was ready for use. The concentration of the substrate solution was about 230 μM (estimated from a molar absorptivity of 25000 L mol^{-1} cm^{-1}).

HL and HD activities were measured simultaneously in a GC assay with the use of an internal standard. The reaction mixture contained about 35 μM 13-hydroperoxylinolenic acid substrate (prepared as described above), 0.5 to 2.0 ml of enzyme solution, and 50 mM K-phosphate (pH 7.0) in a final volume of 10 ml. After 1 min the reaction was stopped by the addition of 1 ml of 1 N HCl containing 10 nmol of *t*OPC-8:0 (20) as an internal standard. The lipid products were collected by passing the reaction mixture through a preconditioned C_{18} solid phase extraction column and eluting with diethyl ether. To remove the small amount of water present in the ether, the solution was briefly centrifuged and the ether layer carefully decanted to a clean test tube. The products were esterified with diazomethane and the ether evaporated under a stream of N_2. To remove Triton X-100R introduced with the enzyme, the sample was dissolved in 1 ml of hexane, applied to a silica extraction column, and eluted with 5 ml of diethyl ether. Under these conditions the oxygenated fatty acid metabolites were eluted, whereas the Triton X-100R remained on the column. The solvent was evaporated and the products were redissolved in 30 μl of hexane. The amount of products formed was determined by the injection of an aliquot of the sample into a Hewlett-Packard 5880 gas chromatograph equipped with a 25 m × 0.31 mm (i.d.) methyl silicone column (Hewlett-Packard). The gas chromatograph was operated in the splitless mode and the temperature was programmed from 90 to 270°C at 10°C/min. Hydroperoxide lyase activity was measured by the use of the internal standard to calculate the amount of 12-oxo-*trans*-10-dodecenoic acid formed. This product possesses an α,β-unsaturated aldehyde group that reacts in varying degrees with diazomethane to form a cyclopropane ring at the double bond position (2). Therefore, this esterification by-product was included in the calculation of the lyase activity. Hydroperoxide dehydrase activity was similarly determined by measuring the amount of α-ketol or 12-oxo-PDA products that resulted from the hydrolysis or rearrangement, respectively, of the allene oxide.

$NADP^+$-dependent GAPDH was measured according to the method reported by Heber *et al.* (10). The relative protein concentration of fractions from the gel filtration and anion exchange columns was estimated by their absorption at 280 nm. For the specific activity determinations at varying pH, protein was assayed with Pierce BCA protein assay reagent, with BSA as the standard. Chl was estimated by its absorption at 652 nm (1).

Characterization of Metabolites. Metabolites were prepared by the reaction of 13-hydroperoxylinolenic acid with a spinach enzyme preparation that had been partially purified on a Sephacryl S-300 column. The reaction mixture contained 70 μM 13-hydroperoxylinolenic acid, 12 ml of partially pure enzyme, and 50 mM K-phosphate (pH 5.3; the low pH was used to reduce the

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activity of HL). After 1 h the mixture was adjusted to pH 3 and the solution was passed through a C_{18} solid phase extraction column. The metabolites were eluted with diethyl ether and esterified with diazomethane. The products were reacted overnight with 0.1 ml of 2% (w/v) methoxyamine-HCl in pyridine to prepare the methoxime derivatives. Water (5 ml) was added, the mixture was adjusted to pH 3, and then passed through another C_{18} solid phase extraction column. After elution with diethyl ether, the solution was dried with anhydrous sodium sulfate and concentrated to 20 μl. The sample was analyzed with a Hewlett-Packard 5992 gas chromatograph-mass spectrometer that had a cool, on-column injector and a 25 m × 0.31 mm i.d. cross-linked methyl silicone column (Hewlett-Packard). The temperature was programmed from 120 to 250°C at 10°C/min.

RESULTS

Intracellular Location of Enzymes of Fatty Acid Hydroperoxide Metabolism. Purified spinach protoplasts were subjected to centrifugation through a zone of silicone oil positioned between a layer of 0.4 M sucrose below it and a layer of 0.4 M sorbitol above it. With this technique, the protoplasts were ruptured as they passed through a 20 μm nylon mesh, and the cellular contents then moved into the upper sorbitol phase. According to Robinson and Walker (13), only the chloroplasts move through the zone of silicone oil and into the lower sucrose phase. Table I shows the results of a typical experiment in which approximately 750,000 protoplasts were subjected to silicone oil centrifugation. The majority of the activity (67%) of the chloroplast marker enzyme, NADP$^+$-GAPDH was found in the lower sucrose phase. Likewise, HL and HD activities were predominantly in the chloroplast fraction. The somewhat higher percent of recorded HL (81%) can probably be attributed to increased experimental variability from interfering chromatographic peaks that become more significant at low levels of enzyme activity.

We noted that in some silicone oil centrifugation experiments there was minor contamination of the lower phase with catalase and Cyt *c* oxidase, raising the possibility that peroxisomes or mitochondria may also possess enzymes that metabolize fatty acid hydroperoxides. However, additional experiments in which spinach leaf organelles were separated on a discontinuous Percoll gradient indicated that there was no metabolism of fatty acid hydroperoxides in peroxisomes or mitochondria (data not shown). The results suggest that the spinach enzymes that metabolize fatty acid hydroperoxides reside principally in the chloroplasts.

An additional experiment was conducted to determine whether the enzymes of fatty acid hydroperoxide metabolism were present in the stroma or in the membranes of the chloroplast. Spinach chloroplasts that were prepared by differential centrifugation were further purified on a Percoll gradient. Figure 2 shows that HL and HD enzymes were present both in the lower band of intact chloroplasts and in the upper band consisting primarily of broken chloroplast membranes. This result suggested that the enzymes are associated with the membranes of the chloroplasts. In contrast, the chloroplast marker enzyme NADP$^+$-GAPDH was only active in the intact chloroplasts, as expected for a stromal enzyme. The association of the enzymes of fatty acid hydroperoxide metabolism with the chloroplast membrane was confirmed in an additional experiment in which the intact chloroplasts were ruptured by osmotic shock and then repurified on an identical Percoll gradient (Fig. 3). This time the activity of the three enzymes was located in the lighter band of broken membranes. Except for a small amount of α-ketol formation, no enzymes of hydroperoxide metabolism were evident in the upper region of the gradient which contained soluble enzymes of the stroma, such as NADP$^+$-GAPDH.

We routinely noticed that when chloroplasts were prepared by homogenization and purification on Percoll gradients, high amounts of lyase products were synthesized relative to 12-oxo-PDA (Fig. 2). Conversely, 12-oxo-PDA synthesis was consistently higher than the formation of lyase products in chloroplasts isolated from protoplasts (Table I).

Partial Purification of Enzymes and Molecular Weight Determination. Elution of a spinach extract from a Sephacryl S-300 column failed to separate the HL and HD enzyme activities (Fig.

Table I. *Distribution of Enzyme Activities between Chloroplast and Cytoplasm Fractions of Spinach Leaf Protoplasts*

Spinach leaf protoplasts were subjected to a silicone oil centrifugation procedure (13) in which chloroplasts were separated from the cytoplasm and cytoplasmic organelles. Details of the procedure are described in "Materials and Methods."

Enzyme	Total Enzyme Activity in Protoplast Fractions			
	Upper, sorbitol phase (cytoplasm)		Lower, sucrose phase (chloroplasts)	
	nmol/min	%	*nmol/min*	%
GAPDH	32.4	(33)	66.5	(67)
HL	1.6	(19)	7.0	(81)
HD (12-oxo-PDA)	5.4	(35)	10.1	(65)
HD (α-ketol)	0.6	(26)	1.7	(74)

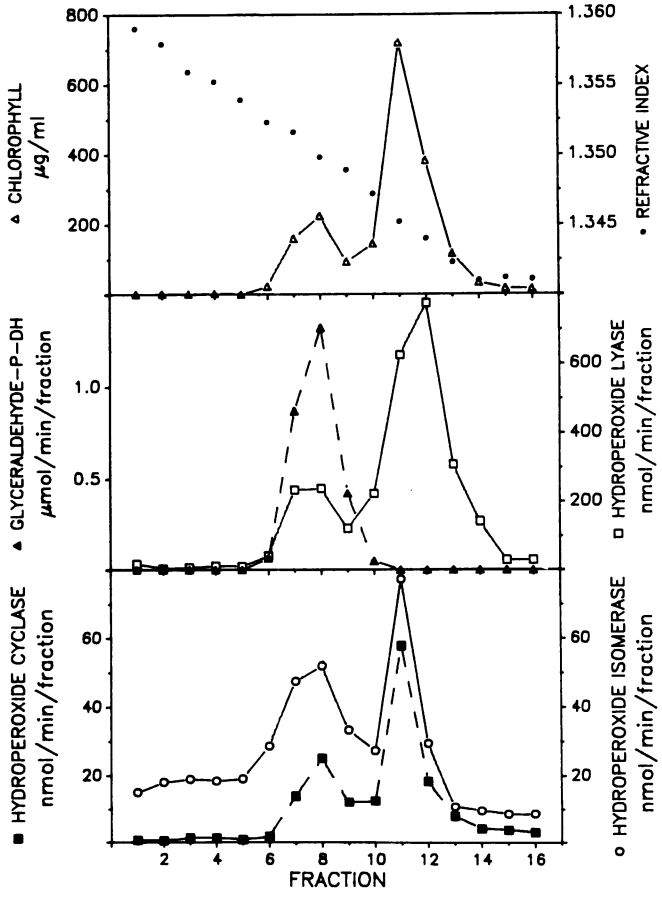

FIG. 2. Percoll gradient of spinach chloroplasts isolated by differential centrifugation in low cation buffer. The lower band consists of intact chloroplasts, and the upper band is composed of broken chloroplast membranes. (△), Chl; (▲), GAPDH, chloroplast marker enzyme; (□), HL; (○), HD (α-ketol formation); (■), HD (12-oxo-PDA formation); (●), refractive index.

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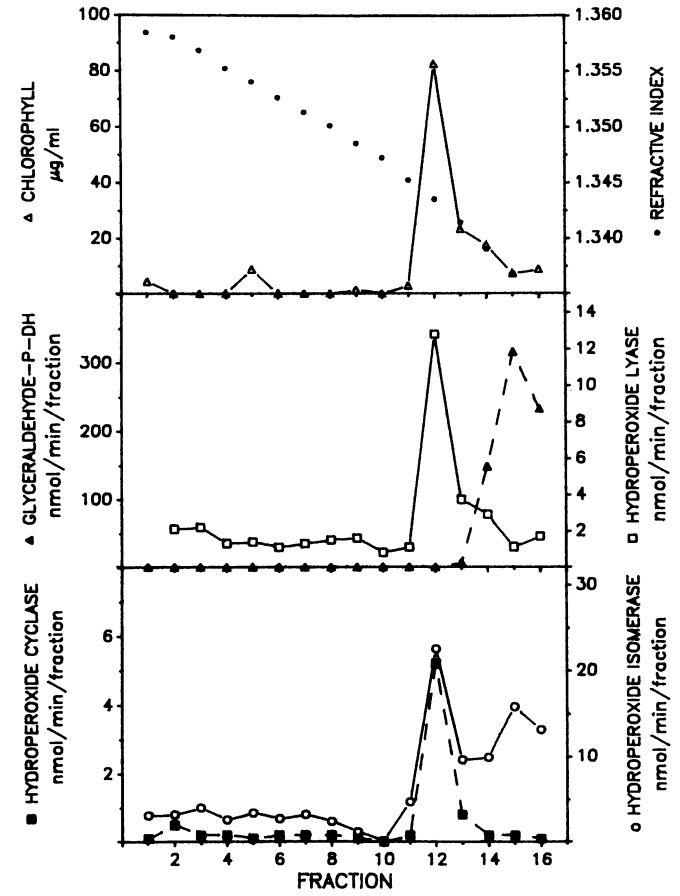

FIG. 3. Percoll gradient of chloroplast membranes obtained by the breakage of intact chloroplasts (lower band, Fig. 1) through osmotic shock. (△), Chl; (▲), GAPDH, chloroplast marker enzyme; (□), HL; (○), HD (α-ketol formation); (■), HD (12-oxo-PDA formation); (●), refractive index.

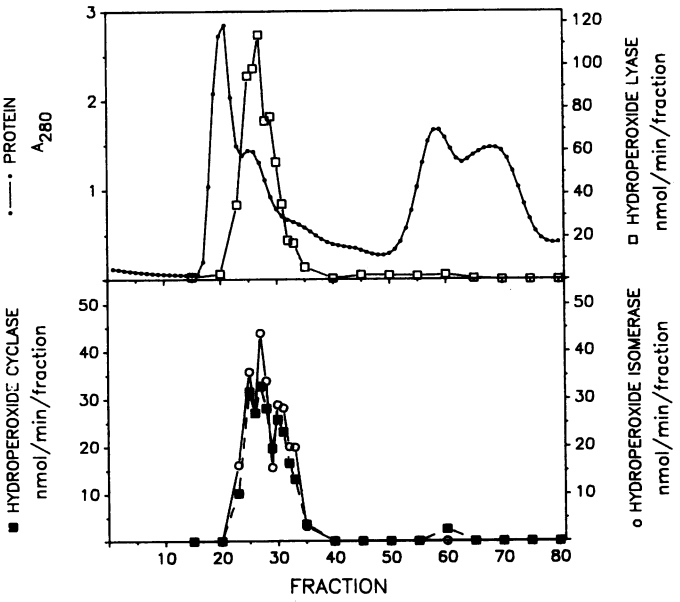

FIG. 4. Separation of enzymes of fatty acid hydroperoxide metabolism on a Sephacryl S-300 column (2.5 × 29 cm). (□), HL; (○), HD (α-ketol formation); (■), HD (12-oxo-PDA formation); (●), protein.

4). The mol wt of the two enzymes was estimated at 220,000 by comparison of their elution volumes with those of known mol wt standards. When Triton X-100R was removed from the elution buffer, the activities eluted with the void volume. This suggests that the enzymes are highly hydrophobic and, in the absence of a detergent, aggregate to form high mol wt complexes. Such a degree of hydrophobicity would be expected from enzymes associated with the chloroplast membranes.

The fractions from the Sephacryl S-300 column that metabolized fatty acid hydroperoxides were combined and applied to a DEAE Sepharose CL-6B anion exchange column. When Triton X-100R was not present with the enzyme and the elution buffer, the enzymes adsorbed strongly to the DEAE Sepharose CL-6B column and could not be eluted even in the presence of 1 M NaCl. However, when Triton X-100R was added to the buffer, the enzymes showed very weak adsorption to the anion exchanger (Fig. 5). The strong adsorption of hydrophobic proteins to Sephadex and Sepharose columns in the absence of detergent has been observed by others and may be due to a strong interaction with the hydrophobic ether portion of the resin (11). Chromatography on the DEAE Sepharose CL-6B column showed that, although not completely separated, there is a clear distinction between HL and HD (Fig. 5). This was not true with every sample of spinach tested. With many samples of spinach that were examined, the enzymes of fatty acid hydroperoxide metabolism could not be separated by the DEAE Sepharose CL-6B column. Whether these inconsistencies were due to varietal, environmental, or procedural differences is not known.

Effect of pH on Enzymes of Fatty Acid Hydroperoxide Metabolism. There was a marked difference between the pH activity profile of HL and HD (Fig. 6). The activity of HL decreased sharply below pH 7, but was still high even at pH 9, the highest pH tested. In contrast, the formation of 12-oxo-PDA (Fig. 6B) and α-ketol (Fig. 6C) as a result of HD activity had similar pH activity profiles in which both processes were most active in the range of pH 5 to 7.

Characterization of Metabolites. When 13-hydroperoxylinolenic acid was reacted with a spinach enzyme preparation that had been partially purified on a Sephacryl S-300 column, three major products were identified in the reaction mixture. The HL product (R_t = 6.85 min), was identified by its methoxime, methyl ester derivative as 12-oxo-*trans*-10-dodecenoic acid by the characteristic ions at m/z 255 $[M]^+$ and 224 $[M\text{-}OCH_3]^+$, as earlier reported by Zimmerman and Coudron (23). The second product (R_t = 12.01 min) was identified as 12-oxo-PDA, the rearrangement product of the allene oxide. Mass fragments of m/z 335 $[M]^+$, 304 $[M\text{-}OCH_3]^+$, and 266 $[M\text{-}C_5H_9 + H]^+$ were characteristic of the methoxime, methyl ester of 12-oxo-PDA (18). The third product (R_t = 12.56 min) was characterized as the α-ketol hydrolysis product of the allene oxide. Distinguishing mass fragments of the methoxime, methyl ester derivative of 13-hydroxy-12-oxo-*cis*,*cis*-9,15-octadecadienoic acid were m/z 322 $[M\text{-}OCH_3]^+$, 306 $[322\text{-}H_2O]^+$, 284 $[M\text{-}C_5H_9]^+$, 252 $[284\text{-}CH_3OH]^+$, and 192 $[M\text{-}C_6H_{10} - (OCH_3)_2]^+$.

DISCUSSION

Hamberg (8) has recently characterized an intermediate in the enzymic conversion of 13-hydroperoxylinoleic acid to α-ketol. The compound, 12,13-(*S*)-oxido-9(*Z*),11-octadecadienoic acid, has a half-life of about 33 s at 0°C. This product is formed by the enzyme-catalyzed loss of water from the hydroperoxide. A similar allene oxide intermediate would be expected in the reaction with 13-hydroperoxylinolenic acid (Fig. 1). Nonenzymic hydrolysis of the allene oxide intermediate would lead to the formation of α- and γ-ketols. In previous literature this process was attributed to a hydroperoxide isomerase enzyme. Alternately, the allene oxide can undergo nonenzymic rearrangement

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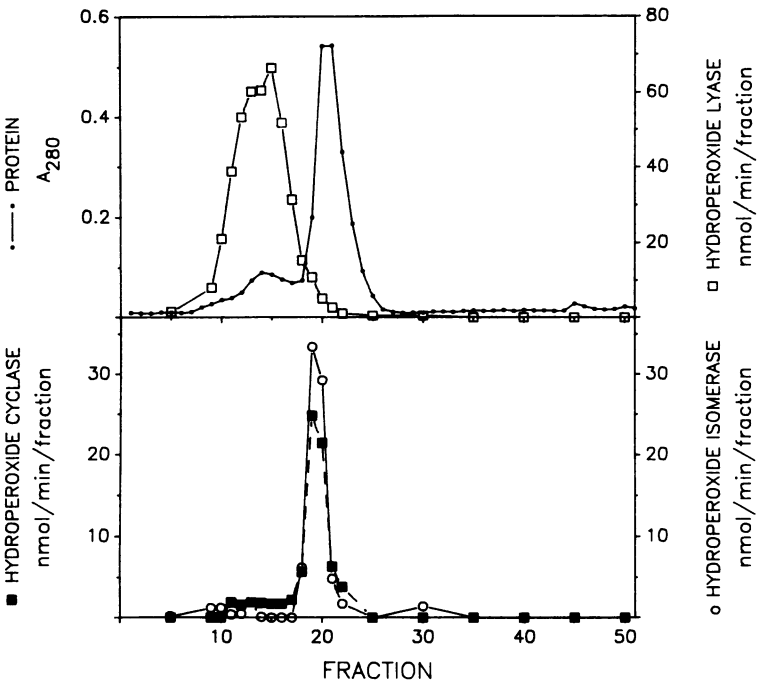

FIG. 5. Separation of enzymes of fatty acid hydroperoxide metabolism on a DEAE Sepharose CL-6B column (2.5 × 21 cm). (□), HL; (○), HD (α-ketol formation); (■), HD (12-oxo-PDA formation); (●), protein.

with ring closure to form 12-oxo-PDA, a process previously credited to a hydroperoxide cyclase enzyme. The rearrangement reaction of the allene oxide most likely occurs through the formation of an oxidopentadienyl cation with antarafacial ring closure. The same mechanism has been suggested for the synthesis of similar cyclic compounds in the coral *Clavularia viridis* (4). Thus, Hamberg has proposed that the names 'hydroperoxide isomerase' and 'hydroperoxide cyclase' be discontinued and replaced by the term 'hydroperoxide dehydrase.' According to the proposed scheme, both ketols and 12-oxo-PDA are products of the hydroperoxide dehydrase reaction. However, only the first step, the formation of the allene oxide, is enzymic. Because the hydroperoxide dehydrase enzyme can give rise to two products in some kind of nonrandom manner, we would like to suggest that a parenthetical expression be added to the name to indicate the specific route of allene oxide transformation under discussion. For example, the cyclic rearrangement path would be referred to as hydroperoxide dehydrase (cyclo), or HD (cyclo); the hydrolysis path would be hydroperoxide dehydrase (ketol), or HD (ketol).

The reaction mechanism proposed by Hamberg is consistent with observations by us in this report and previous reports concerning the properties of hydroperoxide isomerase and hydroperoxide cyclase. These two activities always co-elute on gel filtration and anion exchange columns (16, 17, 20). The two activities have never been separated, and this property prompted speculation that both activities were associated with the same protein. The similarity in pH activity profiles (Fig. 6, B and C) also supports the supposition that the two enzyme activities are attributable to the same protein.

Past investigations into fatty acid hydroperoxide metabolism have typically focused on either the HL system or HD system. This may have led to the incorrect impression that the two systems are mutually exclusive in plants. The results presented here demonstrate that both systems are potentially functional within a given plant tissue. In spinach leaves the principal intracellular site for the two systems is the chloroplast.

The identification of chloroplasts as a site of metabolism of fatty acid hydroperoxides is not new. Chloroplasts from cucumber peel (21), tea leaf (9), and endive (6) have previously been shown to have high HL activity. However, the demonstration of HD activity in chloroplasts has not been previously reported. It is now clear that in spinach leaves the HL enzyme and the HD enzyme coexist in the chloroplast, thus creating two divergent pathways for fatty acid hydroperoxide metabolism.

Enzyme assays with purified, broken chloroplasts indicated that both enzyme systems are predominantly associated with the membranes. The results do not specifically exclude the chloroplast envelope as a site for hydroperoxide metabolism, but we believe, as others have concluded (6, 9), that the thylakoid membranes are the principal site for metabolism of fatty acid hydroperoxides in photosynthetic tissue. As would be expected for proteins bound to membranes, both HL and HD are highly hydrophobic enzymes. This was evident from the tendency of the enzymes to aggregate in the absence of detergent. It was also apparent from the strong binding observed when the enzymes were applied to a DEAE Sepharose CL-6B column. This binding was clearly due to hydrophobic interactions because the enzymes were not eluted by high ionic strength eluents, but were quickly eluted by the addition of Triton X-100R detergent.

A curious feature of this research was our inability to demonstrate lipoxygenase activity in spinach leaf extracts. Lipoxygenase could not be detected by either spectrophotometric assays or polarographic (O_2 consumption) assays at varying pHs. Whether lipoxygenase was truly absent or inhibitors were present is not known. It is unlikely that spinach leaves have no lipoxygenase activity because that would restrict HL and HD to substrates that arise from autoxidation or photochemical oxidation. In contrast to our work, Takagi *et al.* (15) have reported the purification of lipoxygenase from the chloroplasts of spinach leaves. The enzyme was located on the internal surface of the chloroplast, and not on the outer surface. It is possible that lipoxygenase inhibitors such as Chl (3) or carotenoids (15) may have been responsible for the lack of lipoxygenase activity in our spinach extracts.

The presence of two pathways for fatty acid hydroperoxide metabolism suggests that certain physiological controls exist for directing the hydroperoxide to the HL or the HD pathway. One possible mechanism could be through the alteration of pH in the microenvironment of the HL or HD enzymes. At low pH ranges,

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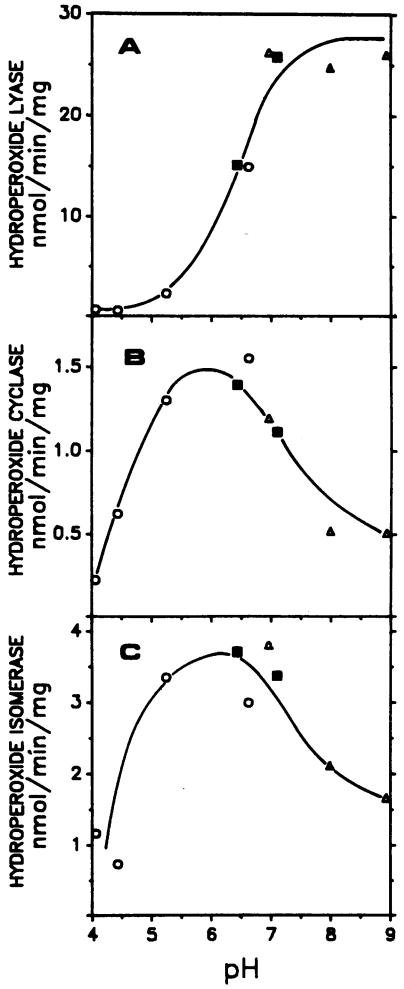

FIG. 6. Effect of pH on the activity of (A) HL, (B) HD 12-oxo-PDA formation), and (C) HD (α-ketol formation). (○), 0.1 M Na-acetate; (■), 0.1 M K-phosphate; (△), 0.1 M Tris.

like those expected inside the thylakoid lumen during the light reaction of photosynthesis, HL would be relatively inactive (Fig. 6) and HD would predominate. In regions of high pH, HL would be more active. Another factor relevant to the divergence of the HL *versus* HD pathways was observed in the experiments with the spinach protoplasts. HL activity was low in relation to 12-oxo-PDA synthesis when the enzymes originated from protoplasts (Table I). But when the enzymes were prepared by homogenization of spinach leaves, lyase products were formed more rapidly than 12-oxo-PDA (Figs. 2–6). This suggests that HL activity is stimulated by harsh extraction techniques. *In vivo* this could mean that HL is activated when the plant tissue is wounded or subjected to mechanical stress. Under conditions of low mechanical stress the normal pathway of fatty acid hydroperoxide metabolism is probably the HD pathway.

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