Plant Physiol. (1976) 58, 790-795

Abscisic Acid Metabolism by a Cell-free Preparation from *Echinocystis lobata* Liquid Endosperm[1]

Received for publication May 7, 1976 and in revised form July 20, 1976

DOUGLAS F. GILLARD AND DANIEL C. WALTON
Biochemistry-Physiology Group, State University of New York College of Environmental Science and Forestry, Syracuse, New York 13210

ABSTRACT

A cell-free enzyme system capable of metabolizing abscisic acid has been obtained from Eastern Wild Cucumber (*Echinocystis lobata* Michx.) liquid endosperm. The reaction products were determined to be phaseic acid (PA) and dihydrophaseic acid (DPA) by co-chromatography on thin layer chromatograms as the free acids, methyl esters, and their respective oxidation or reduction products. The crude enzyme preparation was separated by centrifugation into a particulate abscisic acid (ABA)-hydroxylating activity and a soluble PA-reducing activity. The particulate ABA-hydroxylating enzyme showed a requirement for O_2 and NADPH, inhibition by CO, and high substrate specificity for (+)-ABA. Acetylation of short term incubation mixtures gave evidence for the presence of 6'-hydroxymethyl-ABA as an intermediate in PA formation. Determinations of endogenous ABA and DPA concentrations suggest that the ABA-hydroxylating and PA-reducing enzymes are extensively metabolizing ABA in the intact *E. lobata* seed.

The *in vivo* feeding of 2-^{14}C-ABA to embryonic bean axes (*Phaseolus vulgaris* L.) has established PA[2] and DPA as products in one ABA metabolic pathway (21, 23). The accumulation of DPA in bean seeds (24), as well as the continuous formation of PA and DPA in water-stressed leaves (5), indicates that this pathway has an active role in regulating total ABA concentration within the plant. The decreased activity of PA in bioassay systems for growth inhibition (9, 22), abscission promotion (1, 4), and stomatal closure (7) suggests that the DPA pathway has a role in regulating the hormonal activity of ABA. The recent observation that PA inhibits photosynthesis, while ABA does not, indicates that the DPA pathway may also play a role in the activation of physiological activities (7). The actual role which the DPA pathway plays in regulating ABA levels requires the investigation of the enzymes involved. The goal of the present investigation was to establish a cell-free enzyme system capable of metabolizing ABA by the DPA pathway.

MATERIALS AND METHODS

Preparation of Seed Tissue. Immature fruit of Eastern Wild cucumber (*Echinocystis lobata* Michx.) was collected along creeks, drainage ditches, and compost piles within a 20-mile radius of Syracuse, N. Y. The fruit was collected from late August until the middle of October (first frost). The fully expanded green fruit contained four to six seeds of about 5 × 15 mm. The seeds were judged suitable for use if they were pale green, contained no brown pigments, and were soft in texture. At this stage in development, the cotyledons were 1 to 12 mm in length. The seed's contents were removed by nicking the seed's apical tip with a razor blade and squeezing the liquid contents into a centrifuge tube. Each seed yielded an average of 0.2 ml of semifluid endosperm with a pH of 6.4.

Crude Enzyme and Enzyme Concentrate Preparation. Initial enzyme preparations were prepared by homogenizing the liquid endosperm in an equal volume of 0.1 M HEPES-NaOH buffer, pH 7.5. This homogenate, filtered through Miracloth, is referred to as the crude enzyme preparation (Fig. 1). Later work showed that the enzyme activities could be concentrated by centrifuging the liquid endosperm prior to homogenization. The enzyme concentrate was prepared by centrifuging the endosperm at 40,000*g* for 15 min. The supernatant of the centrifuged endosperm was discarded while the pellet was resuspended in $^1/_{10}$ its precentrifugation volume of 0.05 M HEPES buffer, pH 7.5. The resuspended pellet was homogenized with a glass homogenizer and centrifuged at 1,000*g* for 5 min. The resulting supernatant is referred to as the enzyme concentrate preparation and contained 1 to 2 mg/ml protein as determined by the biuret procedure (8). The preparation could be stored by freezing at −20 C, retaining 20% of the ABA-hydroxylating enzyme activity after 2 months.

ABA-hydroxylating Enzyme Preparation. The ABA-hydroxylating activity was separated from the PA-reducing activity by centrifuging the enzyme concentrate preparation at high speeds. Most of the ABA-hydroxylating activity was obtained by centrifuging the enzyme concentrate preparation at 100,000*g* for 60 min. The PA-reducing activity was routinely removed from the ABA-hydroxylating enzyme by centrifuging the enzyme concentrate preparation at 45,000*g* for 15 min. The supernatant obtained is referred to as the PA-reducing enzyme preparation. The pellet was resuspended in its original volume of 0.05 M HEPES buffer, pH 7.5, and is referred to as the ABA-hydroxylating enzyme preparation (Fig. 1).

General Incubation Conditions. The incubation mixture consisted of 0.45 ml of enzyme preparation, 6.3 nmol of 2-^{14}C-(±)-ABA (10.3 mCi/mmol), and 0.05 ml of 0.05 M HEPES buffer, pH 7.5, containing 0.5 μmol NADPH. ABA dissolved in acetone was added to a 25-ml Erlenmeyer flask and the solvent was evaporated with a stream of N_2. The enzyme solution was added to the ABA and the reaction initiated with the addition of the NADPH solution. The reaction was carried out at 26 C for 2 hr with slow shaking in a Dubnoff metabolic incubator. The reaction was stopped by the addition of 0.1 ml of 1 N HCl.

Extraction and Quantitation of Reaction Products. The acidic reaction mixture was extracted three times with equal volumes of water-saturated 2-butanone. The 2-butanone extracts were combined and the 2-butanone was removed under a stream of N_2 at 45 C. The residue was taken up in acetone and spotted on a 0.25-mm thick Merck precoated Silica Gel F-254 TLC plate

[1] This work was supported by McIntyre-Stennis funds and by NSF National Science Foundation Grant GB29428.

[2] Abbreviations: PA: Phaseic acid; DPA: dihydrophaseic acid.

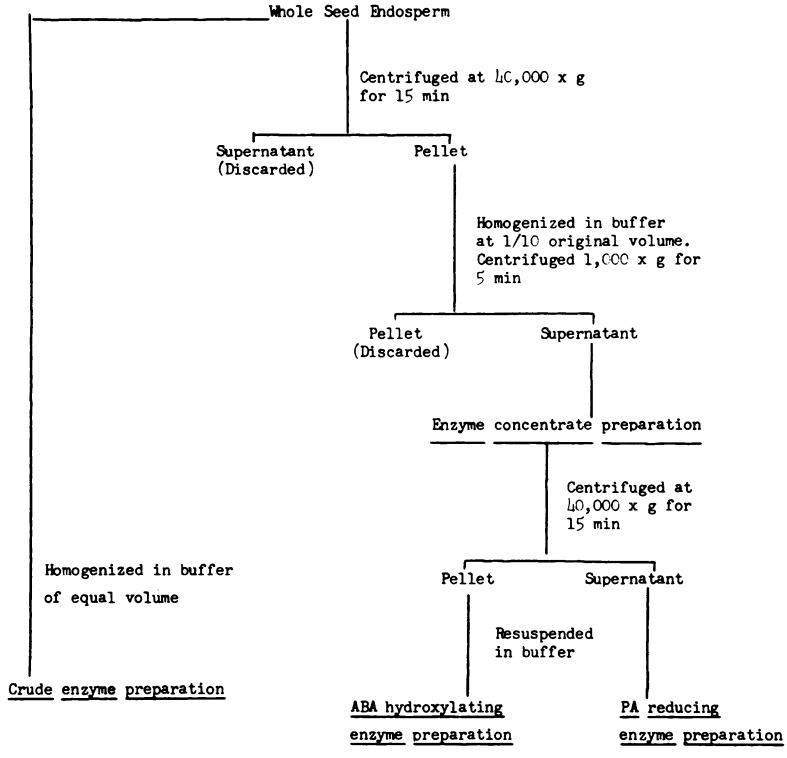

FIG. 1. Flow sheet of enzyme preparations.

which was developed continuously for 2.75 hr in benzene-ethyl acetate-acetic acid (50:15:6). The various compounds were located by autoradiography or by co-chromatography with authentic ABA, PA, and DPA. The quantity of isotope in each compound was determined by liquid scintillation counting. Silica gel from the radioactive area was scraped into a counting vial, 0.5 ml of 0.05 N NaOH was added, followed by 10 ml of dioxane containing 0.1 g naphthalene and 0.08 g Omnifluor (New England Nuclear). The percentage of radioactivity in each product was calculated by dividing its radioactivity by the total radioactivity recovered from the plate. The amount of each product formed was determined by multiplying its percentage of radioactivity by the total amount of 2-^{14}C-ABA added to the reaction mixture.

Product Characterization. The putative PA and DPA formed by the crude enzyme preparation were partially purified by TLC on 0.25-mm Silica Gel F-254 plates with chloroform-methanol-water (75:22:3), and benzene-ethyl acetate-acetic acid (50:15:6) as the developing solvents. The partially purified products were methylated with diazomethane (18) and were further purified by TLC in hexane-ethyl acetate (1:1). The putative and authentic DPA methyl esters were oxidized overnight in excess CrO_3-pyridine reagent prepared by slowly dissolving 100 mg chromic anhydride in 100 ml of ice-cold anhydrous pyridine. The oxidation products were compared to authentic PA methyl ester by TLC with hexane-ethyl acetate (1:1) as the developing solvent. The putative PA methyl ester, authentic PA methyl ester, and DPA oxidation products were reduced by dissolving them in 0.3 ml water-methanol (1:2) and adding a few crystals of $NaBH_4$. The reduction was carried out for 1 hr on ice and stopped by the addition of acetone. The acetone-extracted reduction products were compared with authentic DPA methyl ester by TLC in hexane-ethyl acetate (1:1).

Oxygen and Carbon Monoxide Studies. The enzyme preparation was placed in one arm of a two-arm reaction flask, while the other arm contained NADPH and ABA. The reaction flask, while being vigorously shaken, was evacuated and filled with the desired gas mixture five successive times. Mixtures of gases were obtained by using a gas manifold equipped to measure the volume of N_2, O_2, or CO entering the evacuated system. The reaction was initiated by tipping the enzyme solution into the arm containing the NADPH and ABA. The incubation conditions and work-up were the same as those previously described.

Acetylation. Short term incubation mixtures were frozen in a Dry Ice acetone bath and lyophilized to dryness. The residue was mixed immediately with 0.5 ml of acetic anhydride-pyridine (1:1) and allowed to stand overnight. The acetylation was stopped by adding methanol and the mixture was evaporated to dryness *in vacuo* at 35 C. The residue was dissolved in 1 ml of 0.1 M HEPES, pH 7.5, and the pH adjusted to 2 by the addition of 1 N HCl. Extraction and quantitation of reaction products were the same as previously described.

Substrate Specificity. The ABA derivatives *t*, *t*-2-^{14}C-(±)-ABA, and 2-^{14}C-(−)-ABA were available from previous preparations (19). The 2-^{14}C-(±)-ABA methyl ester was prepared by methylating 2-^{14}C-(±)-ABA with diazomethane (18). The 1′,4′-diols of 2-^{14}C-(±)-ABA were prepared by $NaBH_4$ reduction of the 2-^{14}C-(±)-ABA methyl ester, followed by base hydrolysis (3). The incubation conditions and extractions were the same as described for (±)-ABA. Reaction products were located by autoradiography after TLC and quantitated by liquid scintillation counting.

Determination of Endogenous Concentrations of ABA and DPA. The two sources of material used in these determinations were: (*a*) the cellular and particulate portion of the liquid endosperm from immature *E. lobata* seeds after centrifugation at 40,000*g* for 15 min; and (*b*) the fully developed embryo from the previous season's mature *E. lobata* seeds. The material in 5-g portions was extracted overnight in 100 ml of acetone-water (9:1). For recovery determinations, 2×10^3 dpm of 2-^{14}C-ABA (10.3 mCi/mmol) or 9×10^3 dpm of 4′-^{3}H-DPA (30.2 mCi/mmol) were added to their respective extraction mixtures prior to further purification steps. The supernatants from the centrifuged acetone extracts were concentrated *in vacuo* and chromat-

ographed by TLC with water-saturated chloroform-acetic acid (98:2) and benzene-ethyl acetate-acetic acid (50:15:6) as developing solvents for the ABA and DPA extracts, respectively. The partially purified ABA and DPA were methylated (18) and further purified by TLC in hexane-ethyl acetate (1:1), and in 2-propanol-hexane (1:4) for ABA and DPA methyl esters, respectively. The ABA and DPA were quantified by gas chromatography as previously described (5).

Preparation of 4'-^{3}H-DPA. Unimbibed bean seeds (*P. vulgaris* var. Red Kidney) were ground to a fine powder using a Wiley mill. The bean powder in 1-kg lots was extracted overnight in 5 liters of acetone-water (9:1). The acetone was removed from the filtered extract *in vacuo* at 35 C. At this stage, approximately 2.5 × 10^5 dpm of 2-^{14}C-DPA were added to the aqueous solution to act as a DPA marker. The radioactive DPA had been isolated from bean seed previously incubated with 2-^{14}C-ABA (10 mCi/mmol). The aqueous solution was adjusted to pH 8 with sodium bicarbonate and then extracted twice with equal volumes of water-saturated 2-butanone which was discarded. The aqueous solution was adjusted to pH 2 with HCl and extracted three times with equal volumes of water-saturated 2-butanone. The 2-butanone extracts were combined and the 2-butanone was removed *in vacuo* at 35 C. The aqueous residue was adjusted to pH 7, added to a column (2.5 × 30 cm) of washed PVP (Sigma), and eluted from the column with 0.1 M sodium phosphate buffer, pH 7. The fractions containing radioactivity were combined and added to charcoal-Celite 545 (1:1) column (2.5 × 30 cm). The charcoal column was washed with water followed by the elution of DPA with acetone-water (4:6). The fractions containing radioactivity were combined and evaporated to dryness. The residue was taken up in acetone-methanol (3:1) and streaked on a 2-mm thick Merck precoated Silica Gel F-254 TLC plate. The plate was developed continuously with benzene-ethyl acetate-acetic acid (50:15:6) for 3 hr. The DPA was located by co-chromatography with authentic DPA and removed from the silica with acetone-water (9:1) in a Soxhlet extractor. The DPA was methylated with diazomethane (18) and chromatographed on 0.25-mm thick TLC plates of previous description, using hexane-2-propanol (80:20) as the developing solvent. The DPA methyl ester was located and eluted from the TLC plates as previously described.

To the partially purified DPA methyl ester, 0.2 ml of CrO_3-pyridine-oxidizing reagent was added and the mixture allowed to stand overnight in the dark. The oxidizing reagent was prepared by slowly adding 100 mg of finely ground chromic anhydride to 3 ml of ice-cold anhydrous pyridine. The ethyl acetate extract of the oxidizing mixture was evaporated to dryness and the residue was chromatographed on 0.25-mm thick TLC plates developed in hexane-ethyl acetate (1:1). The PA methyl ester was located by co-chromatography with authentic PA and eluted from the silica with acetone.

A 1-mg sample of the partially purified PA methyl ester, as determined by UV absorption at 258 nm (ε 14,500), was dissolved in 0.3 ml of water-methanol (1:2) and placed on ice. The PA methyl ester solution was reacted with 25 mCi NaB^3H_4 (185 mCi/mmol [New England Nuclear]) for 1 hr at 0 C. Caution was used because tritium gas was evolved. The reaction was stopped by adding acetone and labile tritium removed by repeated additions and evaporations of acetone. The 4'-^{3}H-DPA methyl ester epimer was separated from 4'-^{3}H-DPA methyl ester by spotting the reduction product on a 0.25-mm TLC plate and developing the plate three times in hexane-ethyl acetate (1:1). The slower moving 4'-^{3}H-DPA (12, 21) was located by co-chromatography with authentic DPA and eluted from the plate with acetone. A specific radioactivity of 30.2 mCi/mmol was determined for the 4'-^{3}H-DPA by quantification at 267 nm (ε 19,900) and liquid scintillation counting. The ^{3}H-DPA methyl ester was hydrolyzed to the acid by allowing it to stand overnight in a 10% NaOH-methanol (1:1) solution. The acid was further purified by TLC in benzene-ethyl acetate-acetic acid (50:15:6).

RESULTS

The homogenization of the liquid endosperm of *E. lobata* seed in an equal volume of 0.05 M HEPES buffer, pH 7.5, resulted in a crude enzyme preparation which, in the presence of NADPH, converted 2-^{14}C-ABA to two radioactive products that co-chromatographed with PA and DPA (Fig. 2). Further TLC characterization of these products also suggests their identities to be PA and DPA (Table I). Oxidation of the putative DPA methyl ester and authentic DPA methyl ester in CrO_3-pyridine yielded products which cochromatographed with authentic PA methyl ester. The $NaBH_4$ reduction of the DPA oxidation products, putative PA methyl ester and authentic PA methyl ester, yielded in each case two products. One of the reduction products co-chromatographed with authentic DPA methyl ester and the other reduction product co-chromatographed with epi-DPA (12, 21). PA and DPA appeared to be the only products formed by the crude enzyme system as determined by autoradiography.

The conversion of ABA to PA and DPA in the crude enzyme preparation was unaffected by pH changes between 6.8 and 7.8. At pH 7.5, however, the total conversion was greater in HEPES buffer than in sodium phosphate, TES, or tris buffers. The enzymes were thus routinely prepared in 0.05 M HEPES buffer, pH 7.5. No metal ions were added to the enzyme preparation since preliminary studies showed no enhancement of PA formation by their addition.

Each immature seed yielded about 0.2 ml of liquid endosperm consisting of a clear fluid and a gelatinous cellular portion. The cellular material, which contained the ABA-metabolizing enzymes, could be sedimented by centrifuging the liquid endosperm at 250*g* for 10 min. To aid in decanting, however, a firmer particulate fraction was routinely prepared by centrifuging the liquid endosperm at 40,000*g* for 15 min. Sedimentation of the cellular material afforded a concentrating technique since the inactive supernatant comprised 90% of the liquid endosperm.

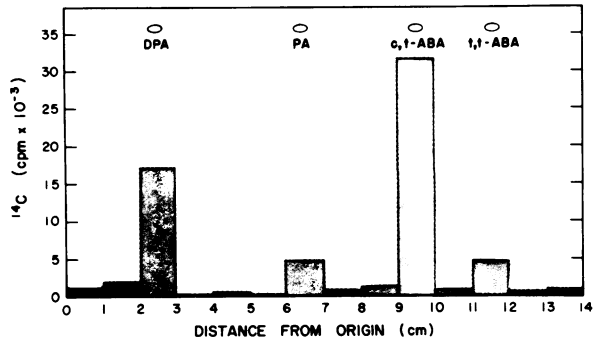

FIG. 2. Distribution of ^{14}C on TLC plates after incubation of 2-^{14}C-(±)-ABA with crude enzyme preparation.

Table I. Chemical Characterization and Chromatographic Behavior of Products Formed by the Crude Enzyme Preparation

Treatment	Putative PA	Putative DPA	Authentic PA	Authentic DPA
	R_f Values			
Untreated[1]	0.41	0.14	0.41	0.14
Untreated[2]	0.43	0.31	0.43	0.31
Diazomethane[3]	0.28	0.07	0.28	0.07
CrO_3[3,4]	NR[5]	0.28	NR	0.28
CrO_3, $NaBH_4$[3,4]	----	0.07,0.09	----	0.07,0.09
$NaBH_4$[3,4]	0.07,0.09	NR	0.07,0.09	NR

[1] TLC on silica gel in chloroform:methanol:water (75:22:3)

[2] TLC on silica gel in benzene:ethyl acetate:acetic acid (50:15:6) continuously for 2.75 hr

[3] TLC on silica gel in hexane:ethyle acetate (1:1)

[4] Methyl ester treated

[5] No reaction

Concentration by centrifugation has the added advantage of partially stabilizing the enzymes for storage by freezing. Freezing of whole fruit, whole seed, or unhomogenized endosperm resulted in total loss of enzyme activity. Freezing of the enzyme concentrate preparation, however, resulted in a 20% retention of enzyme activity after a 2-month period. In view of the short fruiting season for the wild *E. lobata*, much of the work reported here was performed on the frozen enzyme preparation.

In the enzyme concentrate preparation, the presence of two enzymes acting in sequence is suggested by the kinetics of PA and DPA formation (Fig. 3). The initial conversion of 2-^{14}C-ABA to PA followed by a rise in DPA levels is in accord with *in vivo* studies suggesting the pathway as ABA → PA → DPA (19). The separation of the two enzyme activities was accomplished by centrifuging the enzyme concentrate preparation at 100,000*g* for 1 hr (Table II). The 100,000*g* pellet resuspended in 0.05 M HEPES, pH 7.5, converted ABA only as far as PA, while the supernatant showed no reaction with ABA. The resuspension of the high speed pellet in its own supernatant, however, resulted in both PA and DPA formation. These data suggest that the ABA-hydroxylating enzyme is particulate. The PA-reducing activity requires a soluble factor in addition to NADPH. The particulate enzyme was further characterized by fractionation of the enzyme concentrate preparation into a 12,000*g* pellet and supernatant. The ABA-hydroxylating enzyme activity was partitioned between the particulate and supernatant fraction whether or not the preparations contained 0.25 M sucrose (Table II).

The omission of NADPH or O_2 from the incubation mixtures resulted in essentially a complete loss of enzyme activity. The cofactor NADH is a poor substitute for NADPH in that a 7-fold decrease in enzyme activity was observed when 1 nM NADH was used instead of 1 nM NADPH. The presence of the enzyme activity in the particulate fraction and the requirements for NADPH and O_2 suggest that the enzymic oxidation of ABA to PA is of the external monooxygenase type described extensively in mammalian systems (14). A distinguishing characteristic of such a system is the presence of a Cyt P-450 electron transport system which is inhibited competitively by CO. A CO to O_2 ratio of 4:1 did inhibit the crude ABA-metabolizing system by 50%. Incubation of the crude enzyme preparation in a 80% N_2-20% O_2 atmosphere yielded 0.89 nmol of ABA converted to PA plus DPA, whereas in an 80% CO-20% O_2 atmosphere, only 0.45 nmol of ABA was converted to the two products.

A high degree of substrate specificity is shown by the particulate enzyme (Table III). Reactivity of the enzyme toward the various substrates was determined from autoradiographs of reaction mixtures chromatographed by TLC. In the case of *t*, *t*-(±)-ABA, (±)-ABA methyl ester, and the 1',4'-diols of (±)-ABA, no reaction products were detected. The enzyme does convert (−)-ABA to PA but to a sharply reduced extent. The drop in enzymic activity for the (−)-ABA is even more dramatic if (+) contamination, as determined by ORD, is considered. Using an 11% (+) contamination correction factor, the (−)-ABA is only $^1/_{10}$ as effective a substrate as the naturally occurring (+)-ABA.

Structural considerations of the PA molecule indicated that it is not formed directly but probably through an intermediate with a 6'-hydroxymethyl group (9). This compound, which contains a primary and relatively unhindered alcohol group, will be acetylated if it exists as a free intermediate. In order to test this possibility, reaction mixtures were lyophillized after 10- and 20-min incubation periods and then acetylated with an acetic anhydride-pyridine mixture. The tertiary alcohol present in ABA and all of the metabolites is not acetylatable under these conditions (20). A new radioactive compound appeared only in acetylated incubation mixtures and not in unacetylated incubation mixtures or boiled acetylated controls (Table IV). Hydrolysis of the acetylated metabolite yielded a compound which co-chromatographed with PA. The ratio of acetylated compound to PA after 10 min is 3.2:1, whereas after 20 min, the ratio has dropped to 1.2:1, which suggests that the acetylatable compound is formed first. The data in Table IV also suggest that in the unacetylated reaction mixtures, the acetylatable compound was unstable and formed PA during the extraction and purification procedure.

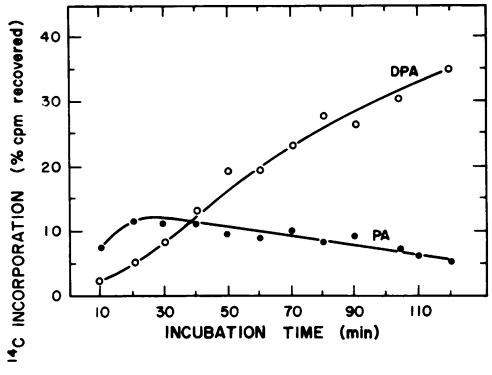

FIG. 3. Distribution of PA and DPA at increasing incubation times of 2-^{14}C-(±)-ABA with the enzyme concentrate preparation. (●——●): PA; (○——○): DPA.

Table II. Fractionation of Enzyme Activity by Centrifugation

The enzyme concentrate preparation was prepared as described in Materials and Methods and centrifuged at designated gravimetric force. The supernatants were used directly as an enzyme source while the pellets were resuspended in an original volume of 0.05 M HEPES buffer, pH 7.5. In experiment 2, the enzyme concentrate preparation and buffer were 0.25 M in sucrose. The liquid endosperm for experiments 1 and 2 were derived from different seeds. Incubation conditions were as described in Materials and Methods.

Fraction	PA	DPA	PA + DPA
	nmoles		
Experiment 1			
- Sucrose			
1,000g supernatant	0.59	1.57	2.16
1,000-12,000g pellet	0.76	0.02	0.78
12,000g supernat	0.49	0.38	0.87
1,000-100,000g pellet	1.27	0.07	1.34
100,000g supernat	0.03	0.02	0.05
pellet + supernat	0.55	1.49	2.04
Experiment 2			
+ Sucrose			
1,000g supernatant	0.26	0.91	1.18
1,000-12,000g pellet	0.26	0.04	0.30
12,000g supernat	0.41	0.16	0.57
1,000-100,000g pellet	0.45	0.02	0.47
100,000g supernat	0.04	0.03	0.07

Table III. Substrate Specificity for ABA Hydroxylating Enzyme Activity

The ABA hydroxylating enzyme preparation and incubation conditions were as described in Materials and Methods.

Substrate	Product Formed
	nmoles
(±)-ABA	1.13[1]
(±)-ABA	1.29[1]
(-)-ABA	0.32[1]
2-t(±)-ABA	NO[2]
(±)ABA methyl ester	NO
(±) 1', 4' cis ABA diol	NO
(±) 1', 4' trans ABA diol	NO

[1] PA

[2] No Product Observed

Table IV. Acetylation of ABA Hydroxylating Enzyme Reaction Mixtures after Short Term Incubations

The ABA hydroxylating enzyme preparation, incubation conditions and acetylation were as described in Materials and Methods.

Incubation Conditions	Acetylation Product	PA Formed	Total ABA Converted
	nmoles		
10 Min Incubation			
+ Acetylation	0.13	0.04	0.17
- Acetylation	NO[1]	0.13	0.13
20 Min Incubation			
+ Acetylation	0.14	0.11	0.25
- Acetylation	NO	0.24	0.24
Boiled Control			
+ Acetylation	NO	NO	----
- Acetylation	NO	NO	----

[1] No Product Observed

The sum of acetylatable compound plus PA in the acetylated incubation mixtures equals approximately the PA formed in the unacetylated incubation mixtures.

To determine whether the isolated cell-free system has an *in vivo* role in *E. lobata* seed, 2-^{14}C-(±)-ABA feedings were performed and endogenous concentrations of ABA and DPA were determined in immature and mature seeds. In the feeding studies, ABA was actively metabolized by both mature and immature seeds to predominantly PA and DPA. The presence of endogenous ABA and DPA as determined by gas chromatography also suggested that ABA is metabolized by the DPA pathway in the intact seeds. The DPA concentration increased from 450 ng/g fresh weight in the liquid endosperm to 3,900 ng/g fresh weight in the mature seed, whereas the ABA concentration dropped from 260 to 60 ng/g fresh weight.

DISCUSSION

Using the liquid endosperm from immature *E. lobata* seeds, we have established a cell-free system capable of metabolizing ABA. The *E. lobata* liquid endosperm was chosen because of the successful isolation of a kaurene hydroxylase from *E. macrocarpa* liquid endosperm (13). Among the sources showing a high degree of *in vivo* ABA metabolism as demonstrated by 2-^{14}C-ABA feeding experiments, but which have produced inactive cell-free systems, are immature and mature bean seeds, bean leaves, stems and roots, immature pea seeds, *E. lobata* leaves, and coconut liquid endosperm. Preliminary experiments suggest that the liquid endosperm of maize and squash may be a feasible enzyme source. Some probable causes for problems in isolating the enzymes from material other than *E. lobata* endosperm are dilution effects, presence of inhibitors, and enzyme instability. Our attempts to obtain a particulate ABA-hydroxylating activity from bean leaves were unsuccessful. The bean leaf homogenates inhibited *E. lobata* enzyme activity, however, suggesting the presence of inhibitors in the leaf extracts. Attempts to remove possible inhibitors from bean leaf extracts by using PVP, BSA, anion resins, exclusion chromatography desalting, and reducing agents also proved unsuccessful. The use of the reducing agent, dithiothreitol, had the added complication of nonenzymatically reacting with ABA to yield a compound with a TLC polarity similar to DPA.

The requirements for O_2 and NADPH and the particulate nature of the ABA-hydroxylating enzyme are characteristics of NADPH-requiring monooxygenases. This class of enzymes, as characterized in several animal systems, inserts 1 atom of oxygen into the substrate while the other oxygen is reduced to water using the NADPH-reducing potential. A distinguishing feature of these animal monooxygenases is the involvement of Cyt P-450 (14). The involvement of P-450 is less well defined for plant systems, but the action spectra for reversal of CO inhibition for cinnamic acid 4′-hydroxylase and kaurene hydroxylase are similar to spectra shown by animal P-450 systems (6, 13, 16). The ABA-hydroxylating enzyme has characteristics similar to those of cinnamic acid 4′-hydroxylase and the kaurene hydroxylase in that it is particulate, requires O_2 and NADPH, and is inhibited by CO. The plant monooxygenase isolated from bean seedlings which converts gibberellin A_1 to gibberellin A_8, however, appears quite different from the ABA-hydroxylating enzyme and established P-450 monooxygenases since it is soluble and shows no CO inhibition (15).

Unlike many of the rat liver microsomal monooxygenases, the ABA-hydroxylating enzyme has a high substrate specificity for (+)-ABA. The cell-free system confirms the results of previous 2-^{14}C-ABA feeding studies (22) in that *t-t* (±)-ABA, (±)-ABA methyl ester, and 1′,4′-ABA diols were not metabolized to their respective analogs. These derivatives also have a greatly reduced inhibitory effect on bean axes growth (22). It thus appears that structural features such as geometry of the side chain, and the presence of a free carboxylic acid and 4′-keto group can have a profound effect on both metabolism and hormonal reactivity of ABA. The change in asymmetry to (−)-ABA had a dramatic effect on metabolism but not one of total inhibition. The effect of ABA asymmetry on hormonal activity is more difficult to establish and is currently disputed (11, 17, 19).

Using the cell-free system from *E. lobata,* we have further verified the sequence: ABA → 6′-hydroxymethyl-ABA → PA → DPA. This sequence, when first proposed, was based in part on the rate of PA and DPA formation during 2-^{14}C-ABA feeding studies (20, 23). Experiments using the cell-free system showed the same pattern with the initial appearance of PA followed by accumulation of DPA. The cell-free system has an added advantage in that the formation of PA and DPA can be separated by centrifugation. The formation of PA alone by the 100,000*g* particulate enzyme preparation and its subsequent conversion to DPA when the supernatant is added back, are consistent with the ABA → PA → DPA pathway. Further evidence supporting the proposed pathway is the inability of the cell-free system to metabolize the 1′,4′-ABA diols. These results suggest that 4′-dihydro-ABA is not an intermediate in DPA formation (24).

The presence of 6′-hydroxymethyl-ABA as an intermediate is supported by results obtained with the cell-free system. Its participation in the pathway was initially proposed because of structural considerations (9). The tetrahydrofuran ring of PA is probably derived from the addition of the 6′-hydroxyl group to the C-2′ position which is part of an α-β unsaturated carbonyl system. The 6′-hydroxymethyl-ABA has been isolated once from tomato shoots but rearranged to PA before full characterization (9). Subsequent attempts to isolate the compound have been unsuccessful (10). The intramolecular nature of the reaction probably allows it to proceed readily, thus making isolation of the compound with the free hydroxyl group difficult. Trapping of the hydroxyl group, however, is possible by acetylation (9). Acetylation of short term incubation mixtures containing the particulate ABA-hydroxylating activity results in an acetylated product. Kinetic analysis of product appearance suggests that the acetylatable compound is formed before PA, and hydrolysis of the acetylated compound yields a compound tentatively identified as PA. These data are in agreement with 6′-hydroxymethyl-ABA as an intermediate between ABA and PA, since we would expect hydrolysis of acetylated 6′-hydroxymethyl-ABA to yield PA on TLC. Whether the ring closure is enzymic or spontaneous is still subject to further investigation. The use of 6′-hydroxymethyl-ABA as a precursor in the cell-free system has not been possible because of our inability to isolate the free 6′-hydroxymethyl-ABA. Our attempts to retard ring closure by extracting and purifying in the absence of acid resulted only in PA isolation.

The presence of DPA in the immature and mature *E. lobata* seeds suggests that the enzymes observed in the cell-free system are actively metabolizing ABA in the intact seed. The turnover of ABA to DPA appears to be a continuous process since DPA is present in both immature and mature seeds and its concentration far exceeds ABA in the mature seed. DPA also occurs endogenously in *Echinocystis macrocarpa* endosperm (2), and *P. vulgaris* leaves (5) and seeds (24), suggesting that metabolism plays a role in regulating the total level of ABA in these tissues as well as in *E. lobata*. We believe that the demonstration of a cell-free system is the first step in understanding the specific way in which ABA is metabolized.

Acknowledgment—We thank C. West for the gift of immature *E. macrocarpa seeds*.

LITERATURE CITED

1. Addicott, F. T. and J. L. Lyon. 1969. Physiology of abscisic acid and related substances. Annu. Rev. Plant Physiol. 20: 139-164.
2. Beeley, L. J., P. Gaskin, and J. MacMillan. 1975. Gibberellin A_{45} and other terpenes in endosperm of *Echinocystis macrocarpa*. Phytochemistry 14: 779-783.
3. Cornforth, J. W., W. Draber, B. V. Milborrow, and G. Ryback. 1967. Absolute

stereochemistry of (+)-abscisin II. Chem. Commun. 114–116.
4. Davis, L. A., J. L. Lyon, and F. T. Addicott. 1972. Phaseic acid: occurrence in cotton fruit, acceleration of abscission. Planta 102: 294–301.
5. Harrison, M. A. and D. C. Walton. 1975. ABA metabolism in water-stressed bean leaves. Plant Physiol. 56: 250–254.
6. Hill, A. C. and M. J. C. Ross. 1975. The properties of cinnamic acid 4′-hydroxylase of aged swede root disks. Phytochemistry 14: 2387–2391.
7. Kriedemann, P. E., B. R. Loveys, and W. J. S. Downton. 1975. Internal control of stomatal physiology and photosynthesis. II. Photosynthetic responses to phaseic acid. Aust. J. Plant Physiol. 2: 553–567.
8. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol. 3: 447–454.
9. Milborrow, B. V. 1969. Identification of metabolite "C" from abscisic acid and a new structure for phaseic acid. Chem. Commun. 966–967.
10. Milborrow, B. V. 1970. The biosynthesis and degradation of abscisic acid. *In:* D. J. Carr, ed., Plant Growth Substances. Springer-Verlag, New York. pp. 281–290.
11. Milborrow, B. V. 1974. The chemistry and physiology of abscisic acid. Annu. Rev. Plant Physiol. 25: 259–307.
12. Milborrow, B. V. 1975. Absolute configuration of phaseic and dihydrophaseic acid. Phytochemistry 14: 1045–1053.
13. Murphy, P. and C. A. West. 1969. The role of mixed function oxidases in kaurene metabolism in *Echinocystis macrocarpa* Greene endosperm. Arch. Biochem. Biophys. 133: 395–407.
14. Orrenius, S. and L. Ernster. 1974. Microsomal cytochrome P-450-linked monooxygenase systems in mammalian tissue. *In:* O. Hayaishi, ed., Molecular Mechanisms of Oxygen Activation. Academic Press, New York. pp. 215–244.
15. Patterson, R. and L. Rappaport. 1975. Characterization of an enzyme from *Phaseolus vulgaris* seeds which hydroxylates GA_1 to GA_8. Phytochemistry 14: 363–368.
16. Pott, J. R. and R. Weklych. 1974. The hydroxylation of cinnamic acid by *Sorghum* microsomes and the requirement for cytochrome P-450. J. Biol. Chem. 249: 5019–5026.
17. Raschke, K. 1975. Stomatal action. Annu. Rev. Plant Physiol. 26: 309–340.
18. Schlenk, H. and J. K. Gellerman. 1960. Esterification of fatty acids with diazomethane on a small scale. Anal. Chem. 32: 1412–1414.
19. Sondheimer, E., E. C. Galson, Y. P. Chang, and D. C. Walton. 1971. Asymmetry, its importance to the action and metabolism of abscisic acid. Science. 174: 829–831.
20. Sondheimer, E., E. C. Galson, E. Tinelli, and D. C. Walton. 1974. The metabolism of hormones during seed germination and dormancy. IV. The metabolism of (S)-2-^{14}C-abscisic acid in ash seed. Plant Physiol. 54: 803–808.
21. Tinelli, E. T., E. Sondheimer, D. C. Walton, P. Gaskin, and J. MacMillan. 1973. Metabolites of 2-^{14}C-abscisic acid. Tetrahedron Lett. 139–140.
22. Walton, D. C. and E. Sondheimer. 1972. Activity and metabolism of ^{14}C-(±)-abscisic acid derivatives. Plant Physiol. 49: 290–292.
23. Walton, D. C. and E. Sondheimer. 1972. Metabolism of 2-^{14}C-(±)-abscisic acid in excised bean axes. Plant Physiol. 49: 285–289.
24. Walton, D. C., B. Dorn, and J. Fey. 1973. The isolation of an abscisic acid metabolite, 4′-dihydrophaseic acid, from non-imbibed *Phaseolus vulgaris* seed. Planta 112: 87–90.