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The Occurrence of Abscisic Acid and Abscisyl-β-D-Glucopyranoside in Developing and Mature Citrus Fruit as Determined by Enzyme Immunoassay[1]

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ABSTRACT

The contents of (+)-*cis*-abscisic acid (ABA) and alkaline-hydrolyzable ABA-conjugate(s) were analyzed by means of enzyme immunoassay in partially purified extracts of developing and mature sweet orange fruit (*Citrus sinensis* [L.] Osbeck cv Washington navel). A relatively small increase in ABA was observed in the fruit exocarp during the natural color transition from green to orange. At the same time, the ABA-conjugate level increased approximately 12-fold in this tissue. The contents of ABA and ABA-conjugate equaled 15.0 ± 0.7 and 107.8 ± 2.1 nanomoles per gram fresh weight, respectively, in the exocarp at harvest. Other tissues also contained considerable quantities of these compounds. Whereas the highest ABA content was observed in the exocarp, the highest ABA-conjugate content was observed in the central vascular axis of the fruit and equaled 187.0 ± 10.3 nanomoles per gram fresh weight. The only immunoreactive conjugate found in significant quantity in mature fruit was identified as abscisyl-β-D-glucopyranoside (ABA-GE) based on (a) immunological cross-reactivity, (b) thin layer chromatography co-chromatography with authentic standards in two solvent systems, (c) susceptibility to both chemical and enzymic degradation, and (d) mass spectroscopy.

ABA and water-soluble ABA-conjugates are important components of the growth inhibitor complex of many plants (1). The levels of these compounds have been reported to increase during fruit maturation and this observation has given rise to speculation on their role in fruit ripening and other related developmental phenomena (16). Increases in ABA and ABA-conjugates in citrus fruit have been related to exocarp senescence and the transition of chloroplasts to chromoplasts that occurs in this tissue (6, 7, 23). Accumulation of ABA and possibly the 2,*trans*-isomer of ABA in fruit has been postulated to influence bud dormancy and contributed to biennial-bearing which is common in many citrus varieties (5, 10).

Numerous reports on the endogenous level of growth inhibitors in citrus fruit have appeared in the literature (see Ref. 4 for review). For the most part, these reports have relied upon relatively nonspecific bioassays for the estimation of ABA. Although reports using GC or GC-MS have also appeared, the requirements for sample prepurification make routine analyses difficult by these methods. Furthermore, the variable sample loss incurred during purification may explain in part the wide variation in ABA and ABA-conjugate contents that have been reported for citrus using these methods (7, 22).

Several recent advances in immunological methods have provided attractive alternatives to the traditional assays for these compounds (12, 21, 24–27). These assays minimize the need for purification and allow for rapid analyses of many samples using little plant material. For the present study, a modification of the EIA[3] described by Weiler (27) has been employed to evaluate changes in endogenous levels of ABA and ABA-conjugate which occur during fruit maturation. Since information is unreported on the distribution of these compounds within citrus fruit, various tissues from orange fruit have been analyzed for their endogenous ABA and ABA-conjugate contents.

In most studies, conjugated ABA refers to the ABA liberated by hydrolysis following solvent partitioning. This definition has been adopted in the present study for comparative purposes. In citrus, the conjugate has been assumed to be glucosyl ester of ABA, although little evidence has been provided to support this assumption. In this study, the identity of the major ABA-conjugate in citrus fruit has been confirmed by immunoassay, TLC, and MS.

MATERIALS AND METHODS

Chemicals. All chemicals, unless otherwise specified, were from the Sigma Chemical Co. and were of the highest purity available. [2-^{14}C]-(±)-*cis*-ABA (947 MBq mmol^{-1}) and [G-^{3}H]-(±)-*cis*-ABA (1.4 TBq mmol^{-1}) were from Amersham Corp. (−)-ABA was a generous gift of E. Weiler, Universität Osnabrück, FRG. Phaseic acid and (±)-abscisyl-β-D-glucopyranoside were gifts from J. Zeevaart, Michigan State University, East Lansing, MI. The *cis*-diol of methyl abscisate was prepared from (+)-ABA after methylation with ethereal diazomethane by sodium borohydride reduction (3). Dihydrophaseic acid was prepared from phaseic acid by sodium borohydride reduction (25). (±)-Abscisyl-(2,3,4,6-*O*-tetraacetyl)-β-D-glucopyranoside was prepared with acetic anhydride-pyridine reagent and purified by TLC (Silica GF, 250 μm; solvent system: chloroform:methanol, 96:4 [v/v]; R_F=0.55) (1). For quantitation, the UV spectra in ethanol (absorption maximum = 273 nm; molar extinction coefficient = 20,000 M^{-1} cm^{-1} was used (11).

Immunogen Synthesis. (±)-ABA was conjugated through the —COOH group to BSA by a mixed anhydride method described by Weiler (27); the conjugate was lyophilized and stored at −26°C. Epitope density was determined by UV spectrophoto-

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[3] Abbreviations: EIA, enzyme immunoassay; RIA, radioimmunoassay; TBS, Tris-buffered saline; BHT, 2,6-di-*tert*-butyl-4-methylphenol; AP, alkaline phosphatase.

metric analyses and isotopic recovery in [^{14}C]ABA-labeled samples. Densities determined by the two methods were in reasonable agreement and equaled 16.9 and 20.2 mol ABA/mol BSA, respectively.

Immunization and Isolation of Immunoglobulins. Randomly bred 12-week-old rabbits were immunized through a combined intramuscular/intradermal route. Five mg of conjugate were dissolved in 2 ml of isotonic phosphate-buffered saline (pH 7.4) and emulsified with an equal volume of Freund's complete adjuvant (Difco). Each animal received primary intramuscular injections of 0.2 ml (0.25 mg conjugate) at four hindquarter locations. After 1 month and at 2-week intervals thereafter for a period of 6 weeks, equivalent intradermal booster injections in incomplete adjuvant were made at four locations on the back of each animal.

Blood was collected by venous puncture 10 d after the final booster injection and allowed to coagulate at 23°C for 2 h. After overnight sedimentation at 4°C, antiserum was separated from coagulate by low speed centrifugation and titer was determined. Titer was defined as that dilution of antiserum which bound 50% of a fixed mass [^{3}H]-(±)-ABA (26). The IgG fraction of sera exhibiting high titers was isolated by rivanol-ammonium sulfate precipitation (9), lyophilized and stored at −26°C. Sera from all animals had demonstrable ABA binding capacity; however, serum from only one animal was used for the studies presented herein.

Synthesis of AP-Labeled Tracer (ABA-AP). (+)-*cis*-ABA was conjugated to AP (Sigma type VII-S) with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl (27). The tracer was stored in 30% glycerol (v/v) in TBS (50 mM Tris, 1 mM $MgCl_2$, and 10 mM NaCl, pH 7.4) at −26°C with no loss in enzymic activity or immunoreactivity over a 2-year period.

EIA Procedure. EIA was done according to the methods of Weiler (26) with some modifications. Flat-bottom, 96-well, microtiter plates (Immulon 1; Flow Labs, McLean, VA) were filled with 200 μl/well of a 4 μg ml^{-1} solution of IgG in carbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6) and incubated at 4°C for 24 h. The coating solution was decanted and the wells were rinsed (4 times) with distilled H_2O. To each coated well was then added 100 μl TBS and 50 μl of (+)-ABA standard or sample. After 30 min at 4°C, 50 μl of diluted tracer (1500-fold in TBS) was added and the plates were incubated for an additional 2 h. The contents of the wells were then discarded and each well was rinsed (4 times) with 10 mM NaCl. Bound tracer activity was determined by adding 200 μl *p*-nitrophenyl phosphate (1 mg ml^{-1}) in diethanolamine buffer (1 M diethanolamine and 0.5 mM $MgCl_2$, pH 9.8) to each well. The plates were incubated at 37°C and the colored reaction product was allowed to develop for 90 min. Reaction progress was stopped by the addition of 50 μl of 3 N NaOH and absorbance at 405 nm was determined with an EIA plate reader (model 2550; Bio-Rad Labs, Richmond, CA). The assay tracer dilution was selected such that absorbance in the absence of standard or sample equaled 1.0. Unspecific binding of tracer was determined either in wells coated with nonspecific IgG, or with an excess (100 pmol) of (+)-ABA. In both cases, unspecific binding equaled less than 5%. Standard curve logit-log transformation and interpolation of unknowns was by computer program (20).

Plant Material. Sweet orange fruit (*Citrus sinensis* [L.] Osbeck cv Washington navel) were harvested from 25-year-old field-grown trees that were budded to 'Troyer' citrange rootstocks (*Poncirus trifoliata* [L.] Raf. × *C. sinensis*). The samples were collected at midday during the weeks of September 1, October 15, and January 7. On these dates, the fruit were near fully expanded and green, at colorbreak, and at maturity, respectively. Fruit were dissected when harvested (Fig. 1), frozen in liquid N_2, and lyophilized. Lyophilized material was stored at −26°C until extracted.

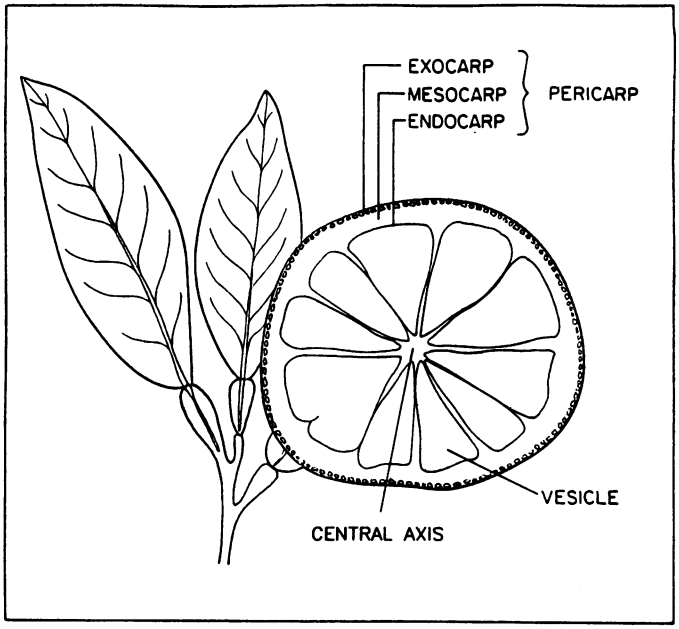

FIG. 1. Fruit tissues that were excised for ABA determination.

Glassware. All extraction and partitioning was performed in glassware that had been treated for 10 min with 5% (v/v) dimethyldichlorosilane in hexane, successively rinsed with hexane, methanol, and hexane, and dried at 100°C for 24 h (R Morrison, personal communication).

Extraction of Samples. Lyophilized fruit tissue was powdered using a mortar and pestle and samples (10 mg) were placed in 5-ml tubes and extracted with 80% aqueous acetone (1 ml) containing 100 mg L^{-1} of BHT. The samples were placed on a wrist-action shaker in the dark at 4°C and periodically flushed with N_2. After 24 h, the samples were centrifuged (6000*g*, 10 min) and the supernatants were decanted. The pelleted debris was resuspended in extraction medium (1 ml) and extracted for an additional 6 h. The samples were then centrifuged as before, and the supernatants decanted and combined with those from the initial centrifugation. The remaining unpigmented debris was discarded.

To each extract was added 10^5 dpm of [^{3}H]-(±)-ABA (equal to 0.52 pmol of immunoreactive material) as internal standard. In some cases, (+)-ABA standards were added to parallel extracts to determine potential losses due to isomerization to *trans*-ABA which might have occurred during extraction and assay. The acetone was then removed under N_2 at 35°C and the aqueous extracts were acidified to pH 2.5 with 0.1 N HCl and partitioned (3 times) against water-saturated methylene chloride. The organic phase was then reduced to dryness and the residue was dissolved in acetone and subjected to TLC or methylated with ethereal diazomethane, suitably diluted in TBS, and assayed. The aqueous phase was adjusted to pH 11.0 with 0.5 N NaOH and heated for 1 h at 60°C. After cooling to room temperature, the extract was acidified to pH 2.5 with 1.0 N HCl, respiked with [^{3}H]-(±)-ABA, and was partitioned and treated as previously described.

Sample aliquots were removed at various stages of purification and subjected to TLC. These samples were dried under N_2 at 35°C, redissolved in acetone, and applied as thin bands on Silica GF plates which had been washed by development in chloroform:methanol (90:10, v/v) and oven-dried at 110°C for 30 min (250 μm; solvent system I: chloroform:methanol:water, 75:22:3 [v/v]; ABA, R_F=0.63; ABA-GE, R_F=0.40). Distribution of immunoreactive material was determined by scraping 0.5-cm bands from the plates. The material was eluted with acetone and treated

as previously described. Authentic standards were applied as spots on the same plates in a lane separated by removing a 0.25-cm band of silica. Standards were visualized under UV illumination after covering the sample side of the chromatogram with aluminum foil.

Characterization of Putative ABA-Conjugate(s). Ten g of lyophilized and powdered mature fruit exocarp was extracted at 4°C in the dark in 1 L of 80% acetone containing 100 mg L^{-1} BHT. After 48 h, the homogenate was filtered through a glass-fiber filter and the residue was resuspended in an additional 500 ml medium. After 24 h, the homogenate was filtered as before and the filtrates combined. Acetone was removed in a rotary evaporator at 35°C and the aqueous solution was adjusted to pH 2.5 with 6 N HCl and partitioned (3 times) against an equal volume of water-saturated methylene chloride. The aqueous phase was then loaded on a charcoal-Celite column (1) and washed with water containing 1% acetic acid and eluted in 30% acetone. Acetone was evaporated and the aqueous solution lyophilized. The dry residue was resuspended in a small volume of acetone and applied as thin bands on preparative Silica GF plates (500 μm, solvent system I). The distribution of immunoreactive material was then determined as described above.

The eluted immunoreactive material was subjected to enzymic hydrolysis with β-glucosidase or acetylation with acetic anhydride-pyridine (1). For hydrolysis, samples (equal to approximately 1 g tissue) were dissolved in 0.1 M (pH 4.6) sodium acetate buffer and incubated at room temperature for 48 h with an excess of almond β-glucosidase (Worthington Biochemicals). Reaction progress was assessed by observing the change in immunoreactivity at the R_F corresponding to ABA by TLC (Silica GF 250 μm, solvent system I). Control samples were run in parallel with an equivalent amount of BSA alone.

For acetylation, samples were reacted with acetic anhydride-pyridine (1:1, v/v) for 24 h at room temperature. Reagent was removed *in vacuo* and the samples applied as thin bands to Silica GF plates (250 μm, solvent system II: chloroform:methanol, 96:4 [v/v]). Distribution of immunoreactive material on the plates was determined as described above. Identity of the acetylated material was confirmed by electron-impact spectra from a VG ZAB mass spectrometer linked to a VG Data System 11/250 (direct probe: 70 eV, resolution 2000, scan 3 s $decade^{-1}$, source 200°C).

RESULTS AND DISCUSSION

ABA-Assay Characteristics. In the assay described in the present study, antiserum raised against (±)-ABA was used in conjunction with (+)-ABA-AP as a tracer and (+)-ABA as a reference standard. Antisera raised in this manner have been reported to have a differential affinity for the enantiomers; this was investigated in preliminary studies. RIA, done according to the method of Weiler (26), indicated that the naturally occurring (+)-form of ABA was ineffective in displacing the (−)-form when commercially obtained [^{3}H]-(±)-ABA was used as the tracer (Fig. 2). This condition was in accordance with other reports (24, 25) and resulted in a diminished RIA sensitivity and precision. Accordingly, the present EIA was developed to circumvent the problems associated with stereochemistry. In this regard, the assay was analogous to a recently reported RIA for ABA (12) that employed similarly raised antiserum in conjunction with radiolabeled [^{125}I]-(+)-ABA as an effective tracer.

Standard curves for the two enantiomers and their corresponding racemate are presented in Figure 3. It can be seen that the (+)-ABA displaces more than 90% of the tracer over a concentration range of 0.05 to 75 pmol; whereas, the (−)-enantiomer, over the same range, is much less effective. It can also be seen that the apparent cross-reactivity (%CR = 100 x/y, where x = (+)-ABA concentration required to displace 50% of the tracer

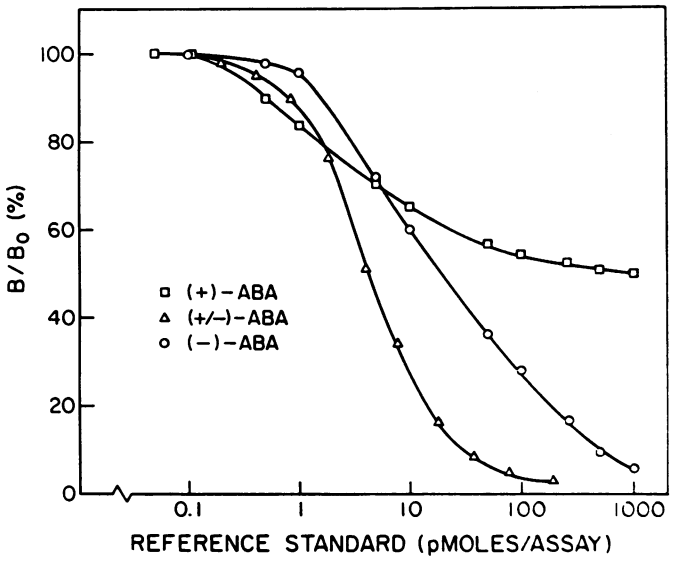

FIG. 2. RIA standard curves for ABA enantiomers (as the methyl esters) and corresponding racemate. Tracer: [^{3}H-G]-(±)-ABA-methyl ester. B/B_0 is the relative binding (%) of tracer in the presence of (B) as compared to the absence (B_0) of respective standards.

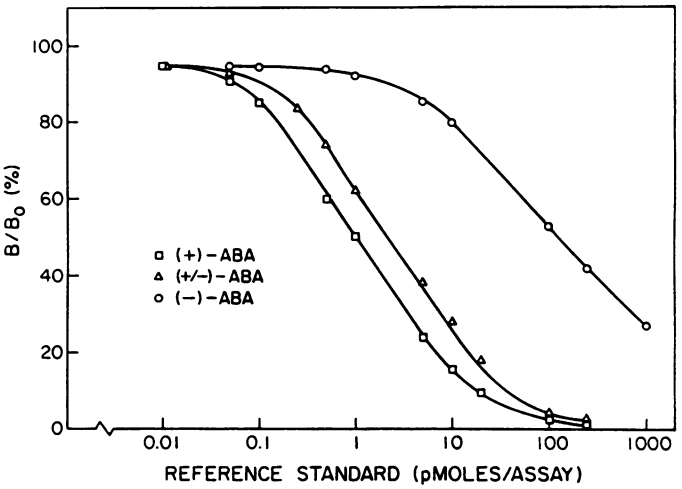

FIG. 3. Enzyme immunoassay standard curves for ABA enantiomers (as the methyl esters) and corresponding racemate. Tracer: (±)-ABA-AP. B/B_0 is the relative binding (%) of tracer in the presence of (B) as compared to the absence (B_0) of respective standards.

and y = cross-reactant concentration required to produce the same effect) of the racemate is approximately 50% (Table I), as expected from the reactivity of the two enantiomers.

In contrast to the ABA-EIA described by Weiler (27), methylation of samples resulted in a significant increase in assay sensitivity (>10-fold); consequently, all standards and unknowns were methylated in parallel prior to assay. A linear logit-log plot of the ABA-methyl ester standard curve is presented in Figure 4. The measuring range of the assay compares favorably with other published immunoassays (13, 20, 23–26) from 0.1 to 50 pmol of (+)-ABA. Slope of the logit-log plot, an indicator of the assay precision, equals −0.69, a value lower than that for RIA (26), but comparable to the EIA reported by Weiler (27). Assay ED_{50}, or the dosage which is effective in displacing 50% of the tracer, equals 0.81 pmol when done according to the methods described. The coefficient of variation for triplicate analyses of standards throughout the linear range of the curve equaled 4.53 ± 3.26. Variation in unknown samples did not exceed this value.

Assay specificity was tested by examining the %CR of several

Table I. *Cross-Reactivities of Various Compounds with Anti-ABA Serum*

Test Compound	%CR[a]
(+)-*cis-ABA*	100
(−)-*cis*-ABA	0.7
(±)-*cis*-ABA	48.6
(±)-*trans*-ABA	<0.05
(+)-*cis*-ABA-*cis*-diol	4.6
(±)-*cis*-ABA-β-D-glucopyranoside[b]	28.5
(±)-*cis*-ABA-(2,3,4,6-*O*-tetraacetyl)-gluco-pyranoside[b]	29.6
Phaseic acid[c]	<0.05
4′-Dihydrophaseic acid[c]	<0.05
Xanthoxin	<0.05
Farnesol, mixed isomers	<0.05

[a] %CR = 100 x/y, where x = (+)-ABA concentration required to displace 50% of the tracer and y = cross-reactant concentration required to produce the same effect. [b] %CR calculated for (+) enantiomer only since the (−) enantiomer is only slightly immunoreactive. [c] Enantiomeric mixture unknown.

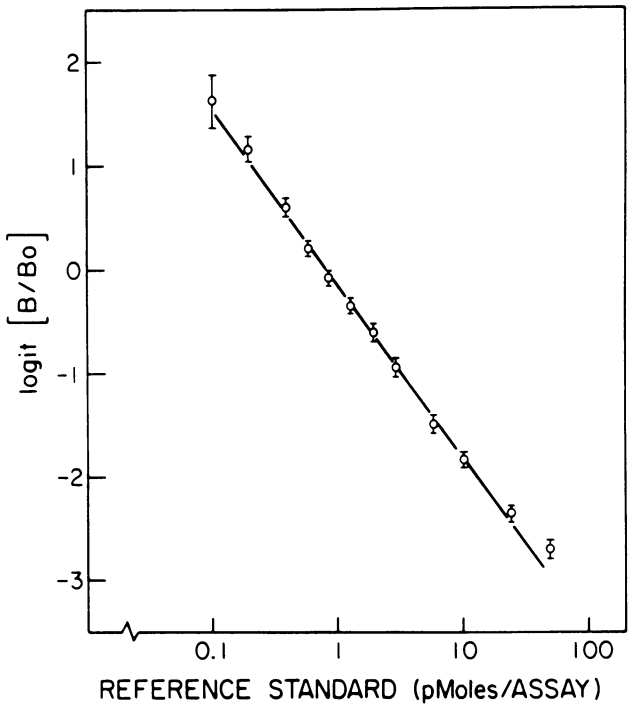

FIG. 4. Linearized standard curves for (+)-ABA (as the methyl ester) enzyme immunoassay (mean ± SE; n = 6). B/B_0 is the relative binding (%) of tracer in the presence (B) as compared to the absence (B_0) of standards. Logit B/B_0 = ln $[(B/B_0)/(100 - B/B_0)]$.

potentially interfering compounds (Table I). As previously noted, the (−)-enantiomer of ABA was relatively nonreactive. The 2,*trans*-isomer of ABA which has been reported in some plants, notably *Citrus* (10), was nonreactive. ABA metabolites and dihydrophaseic and phaseic acids were equally nonreactive, as were the ABA-related compounds, xanthoxin and farnesol. On the other hand, the naturally occurring conjugate, abscisyl-β-D-glucopyranoside, was reactive, as was the acetylated derivative. Presumably other, as yet unidentified, C_1-conjugates of ABA will be equally reactive.

Sample Preparation and Assay. Based on the observed cross-reactivities, it was apparent that some plant extract prepurification would be required in order to assay both ABA and ABA-conjugate. In the present study, solvent partitioning was employed to separate these compounds. The results discussed below were obtained from fruit exocarp extracts, although similar results were obtained from all citrus tissue sources. The efficacy of purification was verified by examining the distribution of immunoreactive material on TLC.

TLC of crude acetone extracts revealed the presence of two major immunoreactive materials (Fig. 5A). These materials were putatively identified as ABA (R_F = 0.63) and ABA-GE (R_F = 0.40) based on co-chromatography with authentic standards. After removing the acetone, the ABA was partitioned into methylene chloride from acidified (pH 2.5) aqueous extracts. When this fraction was chromatographed as before, a single immunoreactive material co-migrating with authentic standard was observed (Fig. 5B). The quantity of ABA thus obtained was equivalent to that observed in the original acetone extracts (compare 14.3 ± 0.9 and 15.0 ± 0.7 nmol g^{-1} fresh weight in Table II). Between 90 and 100% of the [^{3}H]ABA added as an internal standard was recovered in the methylene chloride fraction. Of this label, 76% ± 11% could be recovered in methylated TLC eluates. The data are corrected for this variable recovery.

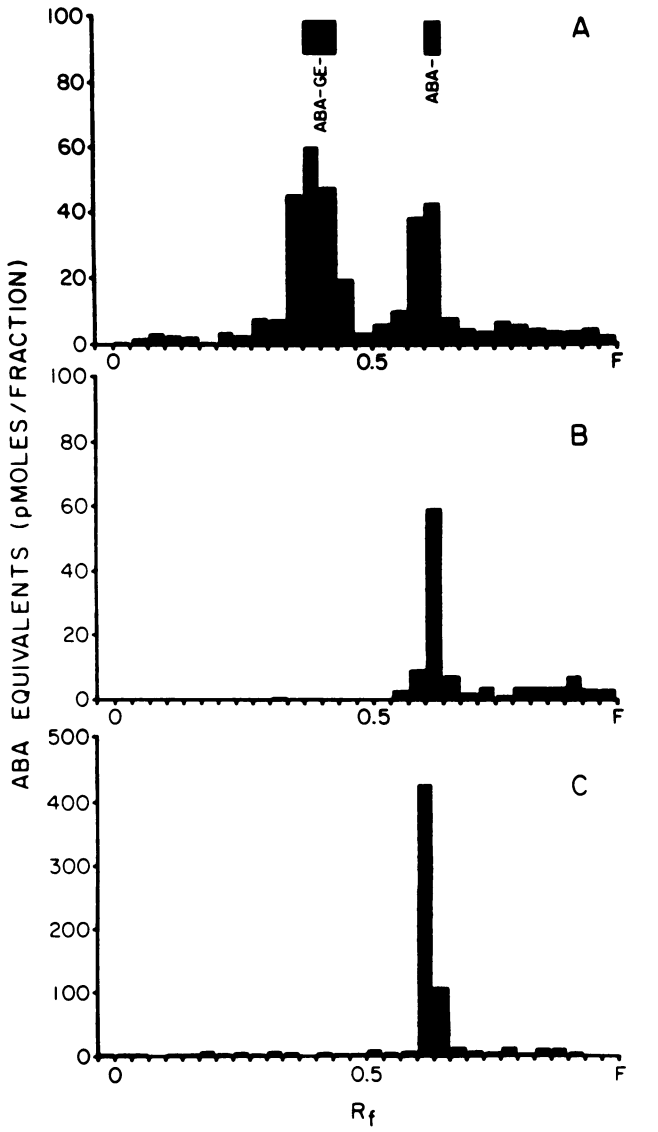

FIG. 5. Distribution of immunoreactive material by TLC: A, Total crude 80% acetone extract; B, the acidic methylene chloride fraction; C, alkaline-hydrolyzed fraction. See "Materials and Methods" for added information. For comparative estimates of the levels of ABA in each fraction confer Table II.

Table II. *ABA and ABA-Conjugate Content of the Exocarp (Flavedo) as Determined in TLC Eluates and by Direct Extract Analysis*

Extract Used[a]	TLC Eluates		Direct Analysis
	ABA	ABA-conjugate	
	nmol g^{-1} fresh wt ± SE		
Total	14.3 ± 0.9	102.3 ± 1.1[b]	
Free acid	15.0 ± 0.7	ND[c]	17.3 ± 0.2
Alkali-labile	ND	107.8 ± 2.1	101.3 ± 7.1

[a] See "Materials and Methods." [b] Corrected for cross-reactivity assuming ABA-GE is the principle immunoreactive compound. [c] Not detected.

The residual aqueous phase remaining after solvent partitioning contained the more polar material originally observed in the acetone extracts. When hydrolyzed (pH 11.0, 60°C, 1 h) and partitioned as before, this material co-migrated as a single immunoreactive band with authentic ABA standard (Fig. 5C). A comparison of ABA content in TLC eluates of the acetone extracts (corrected for percent cross-reactivity assuming ABA-GE as the predominant component) and the partitioned extracts after hydrolysis were in close agreement (compare 102.3 ± 1.1 and 107.8 ± 2.1 nmol g^{-1} fresh weight in Table II). In lieu of a specific radiolabeled standard for the alkali-labile immunoreactive material, [^{3}H]ABA was added after hydrolysis and prior to partitioning to estimate recovery. The isotope recovery was comparable with that observed earlier and the data from the samples were thus corrected. Analyses of the residual aqueous fractions that remained after partitioning revealed little or no immunoreactive material (<1% of that observed in 80% acetone extracts).

In preliminary studies, diethyl ether was used as a solvent for partitioning. However, chromatography of fractions obtained in this manner revealed the presence of immunoreactive materials which were previously unobserved in 80% acetone extracts. These materials were assumed to represent either degradation products or reaction products attributable to ether peroxides. Similar problems apparently linked with the use of diethyl ether in RIA have also been noted (24). The choice of methylene chloride as an alternative solvent was based on its comparably high partition coefficient for ABA (24) and low partition coefficient for ABA-GE (18) under the conditions described. The anomalous materials originally observed with either partitioning were not observed when methylene chloride was used.

Results from the TLC analyses of solvent-partitioned extracts indicated that these fractions might be accurately analyzed without the need for chromatographic separation. Table II shows estimates of both ABA and ABA-conjugate content in exocarp extracts determined without chromatographic purification were indeed in close agreement with those obtained with TLC eluates (compare 17.3 ± 0.2 with 14.3 ± 0.9 and 15.0 ± 0.7 nmol g^{-1} fresh weight for ABA). An overestimation of ABA due to cross-reacting nonpolar compounds (R_F = 0.90; Fig. 5B) was consistently small in all tissues (the exocarp extracts representing the most extreme case); consequently, the ABA content of partitioned extracts was routinely determined without any additional purification.

Assay accuracy was confirmed by previously outlined methods (27). Absence of interfering compounds was demonstrated by showing parallelism of extract dilution curves with the standard curve and by internal standardization. With internal standardization, a constant volume of extract was assayed with and without the addition of known amounts of unlabeled (+)-ABA. The amount of (+)-ABA determined by EIA was then plotted as a function of the amount of (+)-ABA added. In the absence of interfering compounds, this plot yields a straight line with a slope of unity and a *y*-intercept equal to the amount of (+)-ABA contributed by the extract (19). Results of one experiment are presented in Figure 6. Unknown samples showing a slope deviating from unity by more than 15% were rejected.

Quantitative Changes during Fruit Development. The ABA and ABA-conjugate content of the exocarp was measured during maturation (Table III). A relatively small increase in ABA was observed in fruit that were sampled progressively later in the year. In contrast, the level of ABA-conjugate increased by more than 12-fold in the same fruit. That these changes were associated with maturation and not due simply to differences in prevailing climatic or edaphic conditions was strengthened by data obtained at colorbreak. At this time, the distal end of the fruit was typically more advanced in coloration when compared to the proximal end. The higher ABA levels observed in this fruit portion was consistent with a general conclusion that ABA, particularly in the conjugated form, increases during fruit maturation. A similar accumulation of ABA and ABA-conjugate during the natural and ethylene-induced senescence of citrus peel has been reported elsewhere (2, 7).

ABA in Fruit and Leaf Tissues. Mature fruit were dissected to determine the relative distribution of ABA in various tissues (Table IV). Although ABA and ABA-conjugate were detected in all tissues, discernible differences were apparent. As reported by others (6–8), the exocarp contained a high level of both ABA

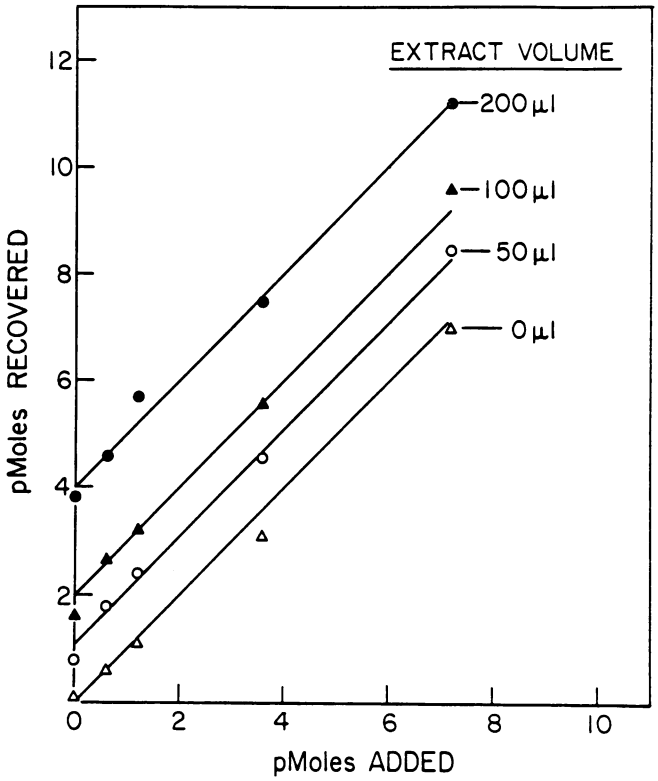

FIG. 6. Internal standard curves at various levels of sample dilution to demonstrate the absence of interfering compounds (tissue analyzed: mature fruit exocarp).

Table III. *ABA and ABA-Conjugate Content of the Exocarp (Flavedo) during Fruit Development*

Stage of Development	ABA	ABA-Conjugate
	nmol g^{-1} fresh wt ± SE	
Immature green fruit	15.2 ± 0.5	8.1 ± 0.6
Color break		
Proximal hemisphere	18.4 ± 1.4	22.7 ± 2.3
Distal hemisphere	19.4 ± 0.9	31.1 ± 2.7
Mature orange fruit	17.3 ± 0.2	101.3 ± 7.1

Table IV. *ABA and ABA-Conjugate Content and the Percent Dry Weight of Mature Fruit and Vegetative Tissue*

Tissue Examined	ABA	ABA-Conjugate	Dry Weight
	nmol g^{-1} fresh wt ± SE		%
Fruit tissue			
Pericarp			
Exocarp (flavedo)	17.3 ± 0.2	101.3 ± 7.1	25.4 ± 1.4
Mesocarp (albedo)	4.7 ± 0.5	61.7 ± 10.2	27.7 ± 2.4
Endocarp	10.1 ± 0.2	72.6 ± 8.7	20.4 ± 0.9
Vesicles	6.3 ± 0.2	40.8 ± 7.8	15.2 ± 1.3
Central axis	13.6 ± 1.2	187.0 ± 10.3	26.4 ± 1.7
Peduncle	7.6 ± 0.1	89.9 ± 18.6	47.9 ± 4.2
Leaf tissue	5.3 ± 0.7	25.1 ± 1.1	43.6 ± 4.4

and ABA-conjugate; however, similarly high ABA levels were also observed in other tissues, most notably, tissue of the central axis. The conjugate level in this tissue was nearly 2-fold that observed in the exocarp.

The leaves subtending fruit at harvest contained 5.3 ± 0.7 and 25.1 ± 1.1 nmol g^{-1} fresh weight of ABA and ABA-conjugate, respectively. These values contrast with those determined earlier in the season for newly expanded leaves which contained only 4.5 ± 0.4 and 1.7 ± 0.1 nmol g^{-1} fresh weight, respectively. These results suggest that ABA, particularly as the conjugate, accumulates in maturing leaves, as well as fruit.

Identification of ABA-Conjugate(s). As previously noted, TLC of crude 80% acetone plant extracts revealed the presence of at least two immunoreactive materials (Fig. 5A) in all tissues examined. The more polar of these materials was assumed to be the ABA-conjugate, ABA-β-D-glucopyranoside, based on cochromatography with authentic standard. With the report that the 1′-*O*-glucoside of ABA is also a naturally occurring compound (16) and the suspected occurrence of other ABA-conjugates (14), this assignment was further evaluated by additional experiments. Identification was supported by the following observations: (a) if the material was subjected to either alkaline (pH 11.0, 60°C, 1 h) or enzymic (β-glucosidase, pH 4.6, 23°C, 24 h) hydrolysis, ABA was liberated (as determined by TLC) in an amount that could quantitatively account for the original material present (the other hydrolysis product(s) was not identified); (b) if acetylated, the material co-migrated as a single immunoreactive band on TLC with authentic ABA-(2,3,4,6-*O*-tetraacetyl)-l)-glucopyranoside; and (c) mass spectra of the acetylated material were in close agreement with published data for this compound (1, 18). Major ions were observed at the following m/z values (percent relative abundance): 538 (0.1%), 441 (1%), 331 (36%), 271 (3%), 247 (10%), 190 (21%), 169 (100%), 145 (10%), 127 (18%), 109 (49%). Although these results do not preclude the existence of other, as yet unidentified, ABA-conjugates, they do suggest that only one conjugate, *viz.* ABA-β-D-glucopyranoside, is present in significant quantity in mature citrus fruit.

CONCLUSIONS

A primary objective of this study was to develop methodology for measurement of ABA and ABA-conjugate by EIA in relatively small tissue samples. It was desirable to minimize the prepurification of extracts so that a large number of samples could be processed with little procedural effort and correspondingly small sample loss. Solvent partitioning with methylene chloride proved to be well-suited for this purpose in citrus extracts which contained only two major immunoreactive compounds, ABA and ABA-β-D-glucopyranoside. Different plant materials may require additional fractionation and the efficacy of purification should be confirmed by TLC or other suitable methods. The presence of a high level of highly polar cross-reacting materials similar to that seen in ether-partitioned *Phaseolus vulgaris* extracts (12) was not observed with the present assay.

In general, the ABA levels in citrus fruit determined by EIA are higher than those reported by others using different techniques (2, 5–8, 21). However, comparisons are obscured by potential varietal and horticultural differences, as well as differences attributable to the quantitation methodology. The present estimates are in good agreement with those made using GC-selected ion monitoring-MS together with specific ABA and ABA-GE tracers (18). In that report, peel extracts (mesocarp plus exocarp) of mature fruit contained 4.1 and 94.5 nmol g^{-1} fresh weight of ABA and ABA-GE, respectively, as compared to 4.7 and 61.7, respectively, for the mesocarp and 17.3 and 101.3, respectively, for the exocarp in the present study.

Mature citrus fruit accumulate considerable quantities of both ABA and ABA-GE. For comparison, immature fruitlets at the time of flower petal abscission contain only 1.6 and 1.5 nmol g^{-1} fresh weight of ABA and ABA-GE, respectively (MJ Harris, WM Dugger, unpublished data). Whether this accumulation originates from synthesis or import is unknown; however, its concurrence with color break suggests a relationship with carotenoid biosynthesis and the transition from chloroplasts to chromoplasts that occurs at this time (2, 23). That the fruit possess a high inherent capacity for ABA synthesis is indicated by the ethylene-induced accumulation of ABA and ABA-conjugate that can occur in immature fruit following detachment (7).

In contrast to the ABA-GE content which increases more than 10-fold, ABA content increases only slightly during the color transition from green to orange fruit. This observation suggests that a steady-state level of ABA is maintained in part through glucosylation. Assuming ABA is localized primarily in the chloroplasts or associated plastids (15), the observation that little of the inhibitor complex of the mature exocarp (as determined by bioassay) is associated with the plastid fraction (2) indicates that ABA and ABA-GE are located in differing intracellular compartments and/or that ABA-biosynthesis and glucosylation are compartmentally separated.

That various tissues of the orange fruit accumulate differing amounts of ABA and ABA-GE is not surprising in view of fruit complexity and differences in tissue metabolism. The significance of these differences and their role in the overall inhibitor balance within the fruit is unknown and merits further study.

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