Seasonal Changes in Citrus Auxin and 2 Auxin Antagonists as Related to Fruit Development [1, 2]

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The natural existence of such varied growth regulators as auxins, gibberellins, kinins, and inhibitors, suggests that growth is a function of a complex balance of such regulators and not a function of just one. Our objective was to study the correlations between fruit development of the parthenocarpic navel orange, *Citrus sinensis* (L.) Osbeck, and the existence of auxins and auxin antagonists (5) within the tissue.

The auxin of particular interest in this study is *Citrus* auxin (11). This compound is active in the *Avena* curvature test and in basic paper chromatography has an R_F very close to IAA. In other respects the chemical and physical properties of *Citrus* auxin and IAA are different (12) (table I).

Although the *Avena* curvature test is a classic assay, activity in this test does not mean that the compound is important as an auxin to the source fruit. If some correlation could be established between the concentration of this compound and important stages of growth of the source fruit, this would strengthen the possibility that *Citrus* auxin functions as a true auxin in citrus fruit.

Materials and Methods

The Washington variety of the navel orange was used in this study. This is a parthenocarpic fruit requiring no pollination or fertilization for fruit set. The fruit were grown on full size trees in Riverside, California. A block of trees was divided into 4 replications of 36 trees each; care was taken at each harvest date to take an equal portion of the sample from each of the 36 trees. Samples were harvested at approximately 2-week intervals from anthesis on April 18 to September 5. Actually April 18 was about the middle of a 10-day period of 60 % full bloom. A minimum of 36 fruit or 100 g, whichever gave the larger sample, was taken from each replication. Early in the season 100 g represented over 600 fruit while at the end of the season 36 fruit weighed almost 2 kg. Fresh weight, dry weight and fruit diameter were determined throughout the experiment.

The entire fruit was frozen in dry ice at harvest time, ground with a Waring Blendor, and freeze dried. The chemical compounds were extracted from the dried tissue by shaking this tissue in a mixture of peroxide-free ether and water (80:20, v/v) for 2 hours. The ether extract was evaporated to dryness in vacuo. The residue was dissolved in acetonitrile and the resulting solution washed with hexane until fresh hexane appeared clear. The acetonitrile was dried in vacuo and the residue dissolved in 5 % sodium bicarbonate solution (pH 8.5). Remaining pigments and lipids were removed by washing the bicarbonate solution with peroxide-free ether until fresh ether remained clear. Adjusting the pH of the remaining bicarbonate extract to 3.0 with HCl and shaking with peroxide-free ether resulted in an ether solution of the acidic fraction of the original plant extract (12,13). This fraction was purified further by 2-dimensional descending paper chromatography on Whatmann 3 MM 18 x 22 inch paper with *n*-butanol : ammonium hydroxide : water (4:1:1, v/v) in the first direction, and isopropanol : ammonium hydroxide : water (10:1:1, v/v) in the second. Fluorescent spots were detected by scanning the dried chromatograms with ultraviolet light of primary 253 mμ wavelength.

The pertinent spots were eluted with 15 ml of distilled water and the maximum excitation wavelength, the maximum fluorescent wavelength, and the relative fluorescent intensity (RFI) were determined. By chromatographing in the ammonia, salts of our acidic unknowns were formed. As a result all eluates from basic chromatography had a pH of about 7.0. pH is an important consideration in fluorometry as it may effect both quantitation and the absorption-fluorescent characteristics (3,9, 20,23).

The excitation maximum was found by holding the fluorescent monochromator constant and scanning with the excitation monochromator. The opposite was done to find the fluorescent maximum. RFI is a measure of the relative light energy emitted by the fluorescent light as recorded on the photomultiplier. Since RFI depends on the properties of each compound (10,22), it cannot be used to compare concentrations of different compounds but only concentrations of 1 compound.

Following fluorescent characterization, the biological properties of the compounds were determined by various bioassays. The coleoptile curvature test

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with *Avena sativa* (13) was used to detect the activity of auxin and auxin antagonists.

The 15 ml chromatographic eluate was prepared for the bioassay by acidifying to pH 2.8 and extracting with diethyl ether. The ether was taken to dryness and the residue containing the auxin dissolved in 0.15 ml of 1.5 % agar containing 0.002 M $CaCl_2$ and 0.01 M citrate, pH 6.0. The final agar block is 0.10 ml in volume. Therefore only 10 of the original 15 ml are considered in the bioassay. All subsequent quantitative values will therefore be based on 10 ml of solution.

Auxin antagonists were bioassayed as described above except that the agar block also contained IAA or citrus auxin.

Little Marvel peas, *Pisum sativum*, the d_5 dwarf corn mutant, *Zea mays* (19), and the London cucumber, *Cucumis sativus* (2) were used to check for gibberellin activity.

Thin layer chromatography was used to distinguish citrus auxin from IAA and to further characterize the auxin; it was not used in the above procedures for quantitation. The adsorbent was a silica gel layer 0.25 mm thick and the developing solvent was *di*-isopropyl ether: acetic acid (95/5, v/v) and benzene: acetic acid: water (8/3/5, v/v). This latter solvent requires overnight equilibration in the vapors of the developing solvent (16). Since the compounds involved in this study had the same excitation and fluorescent spectra between pH 3.5 and 7.5 no pH adjustment was needed for qualitative comparisons. Quantitative comparisons with the eluates from the basic chromatograms would require adjustment of the pH to 7.0.

Results

Fruit Development. The ratio of fresh weight to dry weight did not vary greatly during this study, so growth is shown in dry weight since concentrations of the hormones are based on this measurement. Using either the cube of diameter to represent volume or the dry weight value as the ordinate resulted in curves of almost identical slopes. Bain (1) reported the similarity of growth curves based on diameter and volume for *Citrus sinensis*. Apparently weight is equally reliable.

A semi-log plot of fruit dry weight versus harvest dates (fig 1) showed 3 different growth rates. From anthesis on April 18 to May 16 the growth rate was highest. From May 16 to August 1 the growth rate was slower and appeared to be exponential. An arithmetic plot of this period showed a sigmoid curve. From August 1 on, the rate of growth again seemed to drop.

The first 2 weeks in June was the period of maximum fruit drop often referred to as "June drop."

Orange fruit color developed in mid-November and the fruit was legally mature in January. Therefore, the month of August was approximately halfway between anthesis and maturation.

Auxin-like Compound. The chromatographic and spectrofluorometric characteristics of *Citrus* auxin are compared with those of IAA in table I. The activity of *Citrus* auxin in the *Avena* curvature test for various RFI and similar values for IAA are shown in table II. When the auxin content of 10 ml of a solution with RFI of 15 was applied to 12 plants, the maximum curvature was about 17°.

Table I. *Chromatographic, Fluorometric, and Biological Characteristics of 1 Auxin and 2 Auxin Antagonists Present in Parthenocarpic Navel Orange Fruit*

Test	IAA	Citrus Auxin	Inhibitor I	Inhibitor II
R_{F1}*	0.25–0.31	0.26–0.32	0.78–0.87	0.91–0.98
R_{F2}	0.29–0.34	0.33–0.40	0.79–0.88	0.92–0.98
R_{F3}	0.83	0.32	0.88	1.00
R_{F4}	0.16	0.10	0.33	1.00
Color in UV	Ash	Bluish white	Light blue	Purple
Color in visible light after TLC** with acetic acid solvents	Pink	None	None	None
Color in visible light after H_2SO_4 spray of TLC plate	Brown	None	Pink	Dark green
Excitation maximum (mμ)	290	350	330	280
Fluorescence maximum (mμ)	360	460	410	330
Biology activity	Auxin	Auxin	Auxin antagonist	Auxin antagonist

* The solvents used for these R_F values are as follows: R_{F1} – *n*-butanol, NH_3, H_2O (4:1:2, v/v), paper; R_{F2} – isopropanol, NH_3, H_2O, (10:1:1, v/v), paper; R_{F3} – *di*-isopropyl ether, acetic acid (95:5, v/v), TLC; R_{F4} – benzene, acetic acid, H_2O (8:3:5, v/v), TLC.

** TLC – thin layer chromatography.

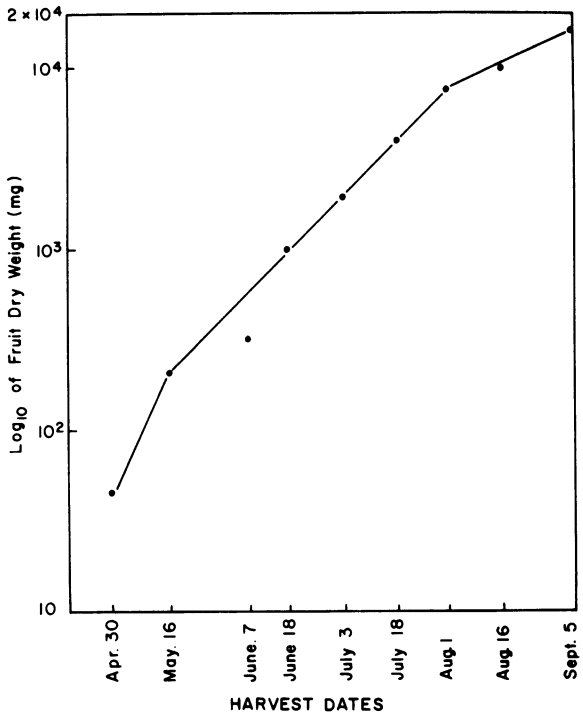

FIG. 1. Growth rate of fruit on dry weight basis.

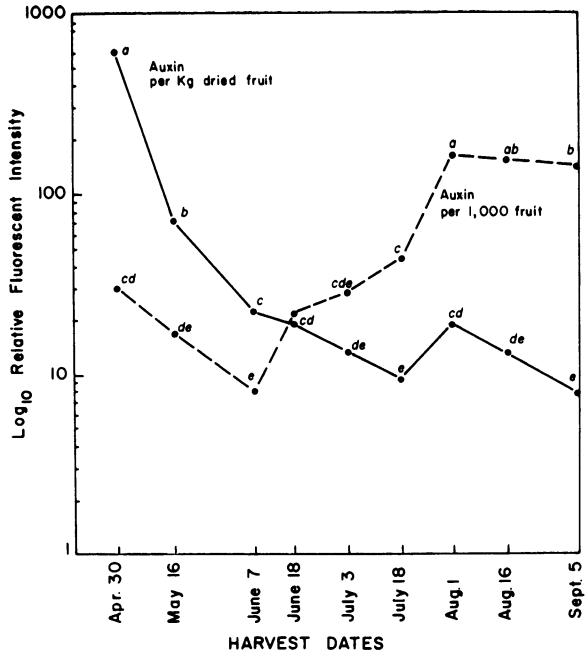

FIG. 2. Seasonal changes in relative fluorescent intensity, assumed to be correlatable with *Citrus* auxin based on relative amount per g and total amount per fruit. The activation and fluorescent maxima were 350 mμ and 450 mμ, respectively. On a given line, points not having a common letter are significantly different at the 5 % level.

Higher RFI values than 35 or lower than 1.5 gave no activity.

Variations in the concentration per kg and relative amount of *Citrus* auxin per fruit are shown in figure 2. The concentration of auxin decreased rapidly from a high on April 30 to 4 % of this level on June 7. After this date, the decline in concentration continued at a slower rate; on August 1 there was a slight increase, followed by a return to the July 18 level by September 5.

The disposition of auxin within the fruit can best be visualized by looking at changes in the total amount of the compound per fruit, as related to concentration changes. The total amount per fruit decreased from April 30 to June 7 and increased from June 7 to August 1. The rate of increase during this latter period was not sufficient to maintain the concentration as indicated by the drop in RFI per kg. Apparently the growth rate had decreased sufficiently by August 1 to allow a build-up in concentration of auxin on August 1. Net accumulation per fruit began decreasing after August 1.

Auxin Antagonists. There were 2 auxin antagonists or inhibitors present in the developing fruit. Both of these compounds inhibited IAA-induced curvature of the *Avena* coleoptile. Neither of them was active alone in the *Avena* curvature, dwarf pea, dwarf corn, or cucumber hypocotyl tests in the concentrations tested. The chromatographic and spectrofluorometric characteristics for both compounds are given in table I.

A 10-ml solution of inhibitor I with RFI of 4.0 completely inhibited the curvature typically induced by 250 μg of IAA per liter of 1.5 % agar (table II). A solution with a RFI of 0.40 partially inhibited this curvature, but that with a RFI of 0.04 did not

Table II. *Correlation of Relative Fluorescent Intensity and Bioassay Values*

IAA			Citrus auxin		Inhibitor I		Inhibitor II	
	Conc	Curvature		Curvature		Conc of IAA inhibited		Conc of IAA inhibited
RFI	μg/liter	Degrees/50 μl	RFI	Degrees/10 ml	RFI	by 10 ml	RFI	by 10 ml
			>35.0	No activity				
10.10	250	24–28	15.0	17	4	250 μg/liter	4.0	250 μg/liter
4.50	100	11–13	5.0	7–11	0.40	100 μg/liter	0.4	250 μg/liter
1.10	25	3–5	1.5	2–5	0.04	No inhibition	0.1	100 μg/liter
0.45	10	None	< 1.5	None			0.04	No inhibition

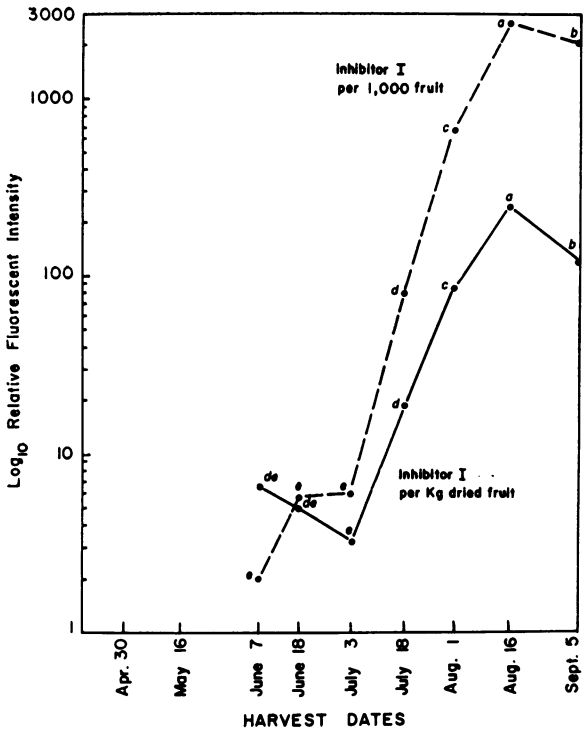

FIG. 3. Seasonal changes in relative fluorescent intensity, assumed to be correlatable with inhibitor I based on relative amount per g and total amount per fruit. The activation and fluorescent maxima were 330 and 410 mμ, respectively. On a given line, points not having a common letter are significantly different at the 5 % level.

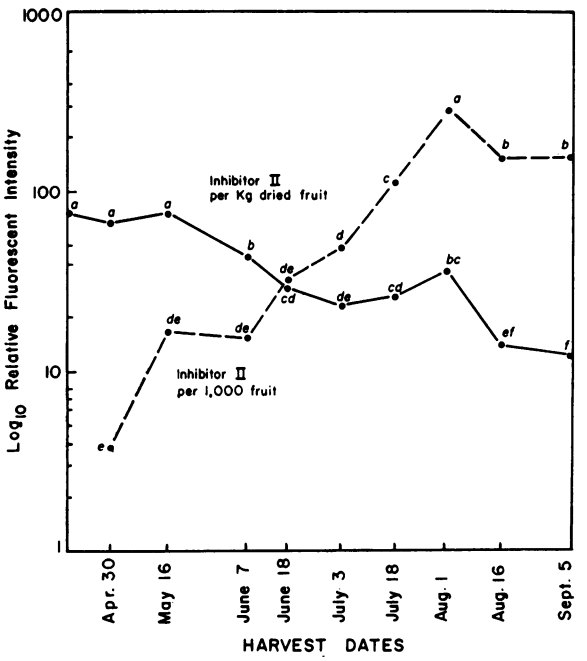

FIG. 4. Seasonal changes in relative fluorescent intensity, assumed to be correlatable with inhibitor II based on relative amount per g and total amount per fruit. The activation and fluorescent maxima were 280 and 330 mμ, respectively. On a given line, points not having a common letter are significantly different at the 5 % level.

inhibit curvature at all. This inhibitor also prevented the curvature normally induced by *Citrus* auxin.

Inhibitor I was not detected prior to June 7. The amount of inhibitor I per fruit increased rapidly from June 7 to August 16 (fig 3). The early increase was not sufficient to keep up with the rate of fruit growth so there was an initial decrease in concentration followed by a continual rise in concentration until August 16.

Inhibitor II with a RFI of 0.40 in 10 ml of water inhibited the curvature typically induced by 250 μg/l of IAA (table II). No inhibition was obtained with the solution of RFI 0.04 in 10 ml. Apparently, the 2 compounds differ in their relative antagonistic activity. Inhibitor I was active over at least a 10-fold range but inhibitor II was not.

Inhibitor II was present throughout the period studied (fig 4). The rate of accumulation per fruit was fairly constant until August 1 and then the amount per fruit began decreasing. This rate of accumulation did not keep up with the growth rate so that the concentration of the compound gradually decreased to a low at the end of the experiment. There was a temporary increase in concentration on August 1 corresponding with the end of exponential growth.

Discussion

Seasonal Changes in Auxin. The principal peak in auxin concentration was seen after anthesis and probably accompanied fruit set. It was followed by a rapid decrease in auxin concentration and total amount per fruit until June 7. This approximates the most rapid period of fruit growth; and, according to the work done by Bain (1) in Australia, is probably the period of cell division.

From June 7 to August 1, during the period of exponential growth, there was a net accumulation of auxin per fruit as evidenced by the increasing RFI per fruit. This is the period of growth associated with cell enlargement on the basis of Bain's work. The increase per fruit means there had to be synthesis of the compound and it would have to be in some portion of the fruit other than the seed since all the unfertilized ovules have aborted by this time.

After August 1, there is a final drop in the concentration of auxin and in the total amount per fruit. This may mean that auxin synthesis has essentially stopped. Perhaps at this point, cell differentiation begins with an accompanied slowing of cell enlargement. The morphological and histological details of this correlation will need further study.

The seasonal changes in *Citrus* auxin levels seem

typical of auxin changes observed in other parthenocarpic fruit, but differ from those observed in seeded fruit. In seeded fruits, the highest concentration of auxin usually comes about halfway between anthesis and maturation according to the reports on apple (15), black currant (25), grape (4, 18), peach (14,21) and strawberry (17). One or 2 smaller peaks have been observed at other stages including 1 shortly after full bloom. In parthenocarpic grapes, Coombe (4) observed the peak in auxin concentration early in fruit development in the Black Corinth and Sultania but past mid-season in the Seedless Emperor. This latter variety is seedless because of embryo abortion and so may be atypical of parthenocarpic fruit. It does amplify the importance of distinguishing the type of parthenocarpy in this work. Nitsch (18) observed seasonal changes in the auxin content of the Seedless Concord grape. Here the pattern of change was very similar to that of the navel orange. The principal peak in auxin concentration was shortly after full bloom, followed by a rapid decline in concentration with a secondary peak or shoulder later in the season.

The stimuli responsible for these rapid changes in auxin level are even more obscure in parthenocarpic fruit than seeded ones. Wittwer (24) reported a stimulation in auxin production accompanying synapsis in *Zea mays*. Perhaps that is the cause of the initial auxin peak in parthenocarpic fruit. The cause of the rapid disappearance of auxin following a period of high concentration is also a mystery. Utilization of the auxin in metabolism and cell growth may be part of the picture, but the hypothesis of Wittwer (24) that auxin is translocated out of the fruit to stimulate vegetative growth could also be important. Nitsch (18) also suggests that auxin may flow out of a berry in discussing the relation of auxin and fruit drop.

Fruit Set and Auxin. First Gustafson and subsequently many other workers have shown the dependence of fruit set on an adequate supply of auxin.

Gustafson (7) also reported on the high levels of auxin activity in crude extracts of parthenocarpic citrus and grape at the time of fruit set. The peak concentration of *Citrus* auxin soon after full bloom suggests that this compound could satisfy the requirement of an auxin for navel orange fruit set.

Fruit Drop and Auxin. The change in the pattern of auxin accumulation around June 7 is particularly interesting since this is also the period of heavy fruit drop or "June drop" for the navel orange. In this study, June drop coincided approximately with the change from decreasing to increasing auxin per fruit, the end of the period of rapid auxin concentration drop, the change from cell division to cell enlargement, and the initial detection of an auxin antagonist inhibitor I.

There was also a variable deviation of the growth rate from the linear relationship on June 7; although this difference was assumed to be insignificant, further work may show it to be correlated with fruit drop.

This association between heavy fruit drop and low auxin level has also been shown for apples (15), Concord grapes (18) and peaches (14,21). Such a simple correlation between low auxin level and fruit drop is probably an oversimplification since so many factors are known to be in transition at this stage and the state of others such as gibberellin and kinin are unknown.

Auxin Antagonists. It is significant to note that inhibitor I reached its highest level per fruit and highest concentration at the harvest date following the August 1 change in growth rate and decrease in auxin level. This compound was only evident during the phase of growth associated with cell enlargement and seems to be correlated with the cessation of rapid cell enlargement.

Inhibitor II does not seem to be closely correlated with a growth phenomenon in that its concentration change throughout the season was small. Its accumulation per fruit dropped off at the cessation of exponential growth, but this may be due to a general slowing of all metabolic functions.

Conclusions. The correlations between *Citrus* auxin levels and fruit set, growth, and abscission support the hypothesis that *Citrus* auxin is not only an auxin in the sense of the classical *Avena* curvature bioassay but also as it relates to the Navel orange. At least one auxin antagonist may also be an important regulator in the development of the fruit.

Before this data can be more than correlations, details will be needed on the histological and morphological changes associated with these auxin changes as well as the changes in other growth regulators such as kinins and gibberellins.

Summary

Quantitative changes in *Citrus* auxin and 2 auxin antagonists have been correlated with the growth and development of the parthenocarpic Navel orange, *Citrus sinensis* (1) Osbeck, to estimate the importance of these compounds to the source fruit.

Fruit set in the parthenocarpic orange is correlated with a high level of *Citrus* auxin typical of the pattern shown for grapes and suggested for citrus.

The period of maximum fruit drop seems to coincide with the change from cell division to cell enlargement, a change in the pattern of auxin accumulation, and the initial detection of an auxin antagonist, inhibitor I.

The highest concentration of inhibitor I was associated with a change in the growth rate of the fruit and a decrease in auxin level.

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