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The Hormone Content of Ripening Grape Berries and the Effects of Growth Substance Treatments

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ABSTRACT

Berries on field-grown *Vitis vinifera* cv. Doradillo were treated at different times during stage II with benzothiazole-2-oxyacetic acid or 2-chloroethylphosphonic acid, and measurements were made of their growth and hormone content. The concentration of ethylene was low during stage II and declined as berries ripened. Both 2-chloroethylphosphonic acid and benzothiazole-2-oxyacetic acid caused increases in ethylene concentration, yet they had varying effects on ripening: the former applied at the start of stage II and the latter applied 1 week before the end of stage II delayed ripening, while 2-chloroethylphosphonic acid applied at the end of stage II hastened ripening.

The abscisic acid content of berries increased as they ripened, and the effects of 2-chloroethylphosphonic acid and benzothiazole-2-oxyacetic acid on abscisic acid levels were correlated with the effect of these compounds on ripening. The roles of abscisic acid and ethylene in the regulation of the ripening of grapes are discussed.

The growth curve of the grape berry, like those of many berry and drupaceous fruits, is double sigmoid (Fig. 1). The second phase of rapid growth (stage III), unlike the first (stage I), is nonmeristematic and is entirely due to enlargement of pericarp cells. As well as an increased growth rate, it is characterized by changes in deformability (softening) and chemical composition, changes which are associated with fruit ripening. The inception of this growth phase (the boundary between stages II and III in Fig. 1) is called "veraison" by viticulturists and is marked by a change in color and inflexions in curves for size, sugar concentration, acidity, and deformability.

The several stages of growth are probably regulated by hormones. Endogenous ethylene may be involved during stages II and III: this is suggested because of its generally accepted role in the ripening of fruits and also because of the effects of exogenous ethylene and 2-chloroethylphosphonic acid. Explanations of the regulation of these growth stages must take account of the dual nature of the effect of CEPA[1] (9), of the delaying effect of exogenous auxin (8, 23), and of the suggestion that auxin-like, biologically active compounds are involved (3, 15).

We are studying the changes in the amounts of growth substances in the berry before and after veraison to try to elucidate their role in controlling the onset of stage III. This paper emphasizes the changes in concentration of ethylene and ABA.

MATERIALS AND METHODS

Mature grapevines, *Vitis vinifera* L. cv. Doradillo, growing in the Waite Agricultural Research Institute orchard, were used for two experiments in consecutive seasons. To improve uniformity, inflorescences with synchronous flowering were selected and late-developing berries were removed 5 weeks after anthesis. Plots of four clusters were selected for randomized block experiments with three replicates of 10 (experiment 1) and 4 (experiment 2) treatments.

Treatments. Clusters were treated with CEPA at 400 mg/liter, benzothiazole-2-oxyacetic acid at 20 mg/liter, or triiodobenzoic acid at 30 mg/liter, at the times indicated below, by immersing them in the appropriate solution containing Tween 20 (0.05%, v/v).

Experiment 1. The effect of time of application of CEPA and BTOA during stage II was investigated. Treatments were applied at four times estimated to be 3, 2, 1, and 0 weeks before veraison. As stage II of Doradillo grapes lasts for about 3 weeks, the first treatment was applied at the start of stage II. In addition, one group of plots was treated with TIBA at 2 weeks before veraison and another group was left untreated.

Experiment 2. The results of experiment 1 were used to select treatments to give maximal promotion of veraison by CEPA and inhibition by CEPA and BTOA. The treatments were (*a*) untreated; (*b*) CEPA 3 weeks before veraison (start of stage II); (*c*) CEPA at end of stage II; (*d*) BTOA 1 week before veraison. These times turned out to be −18, +3, and −6 days after veraison for (*b*), (*c*), and (*d*), respectively.

Berry Size and Deformability Measurements. Two berries were labeled on each cluster, and their equatorial diameters were measured between 8 and 10 AM once or twice weekly with a constant tension caliper. The diameters were measured with two spring tensions, one giving a force of 60*g* and the other 240*g*. The diameter with the weak spring gives a measure of berry size while the difference between the two diameters provides a measure of deformability or "softening" (Coombe, unpublished results), these being two indicators of veraison.

Sampling. Samples of 60 berries (15 berries from each cluster) were taken from each plot at weekly intervals, the first being at the start of stage II and the last 3 weeks after veraison. The berries were cut carefully from the cluster with scissors and were handled quickly but gently since mechanical shock

[1] Abbreviations: CEPA: 2-chloroethylphosphonic acid; BTOA: benzothiazole-2-oxyacetic acid; TIBA: tri-iodobenzoic acid; GLC: gas-liquid chromatogram; t-ABA: the *trans*-isomer of ABA; Me-ABA, and Me-t-ABA: their methyl esters.

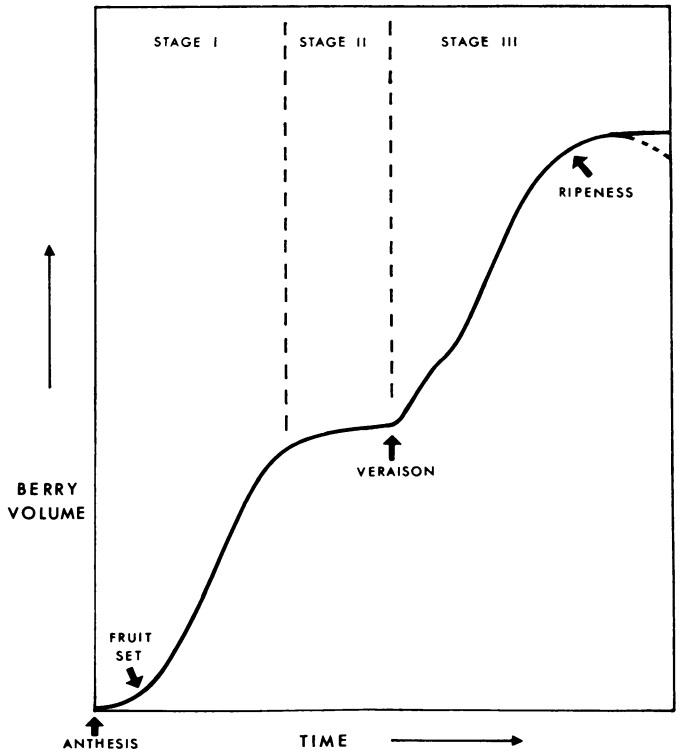

FIG. 1. Diagram of the growth curve of a grape berry showing the extent of the three stages and the location of veraison.

results in a large though transient production of ethylene in grape berries (Hale, unpublished results).

The samples were weighed and divided into two subsamples, one being used immediately for ethylene measurement and the other placed in a refrigerator at −20 C and held there until used for measurement of acidic hormones.

Ethylene Measurement. Intercellular gases were extracted from samples by the method of Maxie *et al.* (12) except that the berries were not punctured and a vacuum of 1 cm Hg was applied for 1 min. The stem of the funnel was calibrated to give a direct measure of the volume of extracted gas. The berries were washed with water before extraction, and for each extraction a fresh lot of saturated NaCl was used. The ethylene concentration in the gases was measured by gas-solid chromatography; the minimal detectable level was 0.03 μl of C_2H_4 per liter. Measurements were made within 20 min of removal of the first berry of the sample from the vine.

Extraction and Assay of Acidic Hormones, Experiment 1. Acidic plant hormones in the frozen berries were extracted and measured within 8 months of sampling. The extraction and purification methods were developed by a comparison of the recovery of added GA_3, GA_{4+7}, IAA, and ABA, assessed by TLC (Faull and Coombe, unpublished data). In preliminary tests, 10-berry samples were dissected into flesh and seed, weighed, extracted with ethanol, partitioned from aqueous acid into ether and chromatogrammed on DEAE-Sephadex columns and paper; segments of the paper at the R_F levels of IAA, ABA, and GA_3 were bioassayed using the barley endosperm (4) and wheat coleoptile (14) bioassays. The same extracts were methylated with diazomethane and applied to a GLC with a 3% OV17 column and a flame ionization detector (5).

Extraction and Purification of ABA, Experiment 2. A subsample of four berries was dissected into flesh and seed and weighed. Each part was ground separately under N_2 in a mixture of H_2O, 4 ml; acetone, 4 ml; and Na diethyldithiocarbamate, 2 mg, using an UltraTurrax tissue grinder at room temperature. The mixture was titrated to pH 9 with 3 N NH_4OH and centrifuged, and the supernatant was removed. The pellet was washed twice with 4 ml of 0.25 M NH_4HCO_3. The supernatants were combined, partitioned once with chloroform, 6 ml, and the aqueous layer was removed. The chloroform layer was washed with 2 ml of 0.25 M NH_4HCO_3. The combined alkaline-aqueous fractions were acidified to pH 3 with 1 M H_3PO_4 and extracted successively with 15, 4, and 2 ml of ethyl acetate with centrifuging to assist partitioning. The combined ethyl acetate fractions were partitioned with 4, 2, and 2 ml of 0.25 M NH_4HCO_3, and the alkaline aqueous fractions were combined and held at 4 C.

In the second stage the fractions were acidified to pH 3 with Amberlite IR 120 H (with warming) then partitioned with 10, 3, and 3 ml of ethyl acetate. The combined supernatants were concentrated to about 0.5 ml with a stream of warm N_2, then applied as a 7-cm streak to acid-washed Whatman 3MM paper. IAA, ABA, Methyl Red, and 5-AMP (later 2′,3′-AMP) were used as side markers. To gauge the effects of extracts on mobility, Methyl Red and AMP were also spotted at three points on each extract streak. The paper was developed in a flat-bed electrophoresis at 2000 v for 1 hr in 0.1 M NH_4HCO_3 at pH 8.5. The position of markers was determined with UV light using Alfoil to shield the body of the chromatogram. The ABA-containing pieces of paper were cut out and eluted with 3 to 5 ml of methanol-water (1:4), and the eluate was dried with a stream of warm N_2. After washing with dry petroleum spirit, the dried extract was dissolved in ethyl acetate and methylated with diazomethane (17).

GLC-Electron Capture Detection of ABA, Experiment 2. This sensitive method was adapted from Seely and Powell (22). ABA fractions from four berries were methylated, and aliquots of from 1/100th to 1/3000th were applied to a Varian 2700 GLC fitted with a tritium foil electron capture detector. Typical GLC traces are shown in Figure 2. The minimal detectable amount of ABA was 3 pg, and the standard curve was linear between 0 and 1 ng.

Proof of the identification of ABA was provided by: (*a*) coincidence of the retention times of Me-ABA and Me-t-ABA (C and D, Fig. 2) in authentic samples and in extracts; (*b*) the parallel effects on both of UV irradiation, *i.e.*, a decrease in C and an increase in B and D (Fig. 2); (*c*) matched retention times and evidence of isomerisation when a 3% OV17 column was used; (*d*) greatly reduced discrimination of C and D compared with other compounds when a flame ionization detector was used instead of an electron capture detector; (*e*) the inhibitory activity of the unmethylated extracts in bioassays.

Lenton *et al.* (10) have described the use of UV irradiation to help in the identification of ABA. Isomerization under our conditions was 24 times faster, probably owing to differences in the method and type of irradiation (Table I). The data suggest isomerization of Me-ABA (C) to Me-t-ABA (D) and compound B. Irradiation for longer than 5 min led to considerable decreases in compounds B, C, and D but not A. Chromatograms of our grape extracts contained more Me-t-ABA than described by Lenton *et al.* (10). These facts deterred us from using Me-t-ABA as an internal standard. To quantify ABA, the areas of peaks coincident with Me-ABA and Me-t-ABA were compared with those of Me-ABA standards and summed. Recoveries of between 70 and 95% were achieved when grape samples were spiked with 100 ng of ABA.

RESULTS

Experiment 1. Data on deformability and concentration of ethylene in the extracted gas are shown in Figures 3 and 4.

CEPA applied during the first 2 weeks of stage II delayed veraison, the earliest treatment causing the greatest delay. When applied at the end of stage II, CEPA accelerated ripening. While none of the CEPA treatments had large effects on the rate of ripening (as displayed by the rate of increase in deformability) BTOA affected both the onset and the rate. Application of BTOA 6 days before normal veraison, *i.e.*, at the end of the 2nd week of stage II, led to a delay of 14 days; earlier and later treatments had lesser effects. TIBA caused a slight delay.

The concentration of ethylene in untreated berries decreased 5-fold from the start of stage II to a low and steady level of 0.05 μl/liter during and after veraison (Fig. 4). Treatment with CEPA during stage II caused large increases within 1 day to about 6 μl/liter, *i.e.*, 10-to 100-fold; the levels then decreased slowly, but they remained at least 10 times greater than untreated at all times. BTOA applied during stage II caused small but rapid increases (to about 0.6 μl/liter, *i.e.*, 3- to 10-fold) in ethylene concentration, but, in contrast to CEPA treatment, the levels returned to those of untreated by the time of normal veraison. Some small increases were obvious again 15 days later. Parallel but smaller changes occurred following the application of TIBA. When treatments were delayed until near to veraison, the effects on ethylene levels were quite different: CEPA caused an increase one-tenth of that following earlier application while BTOA had no immediate effect.

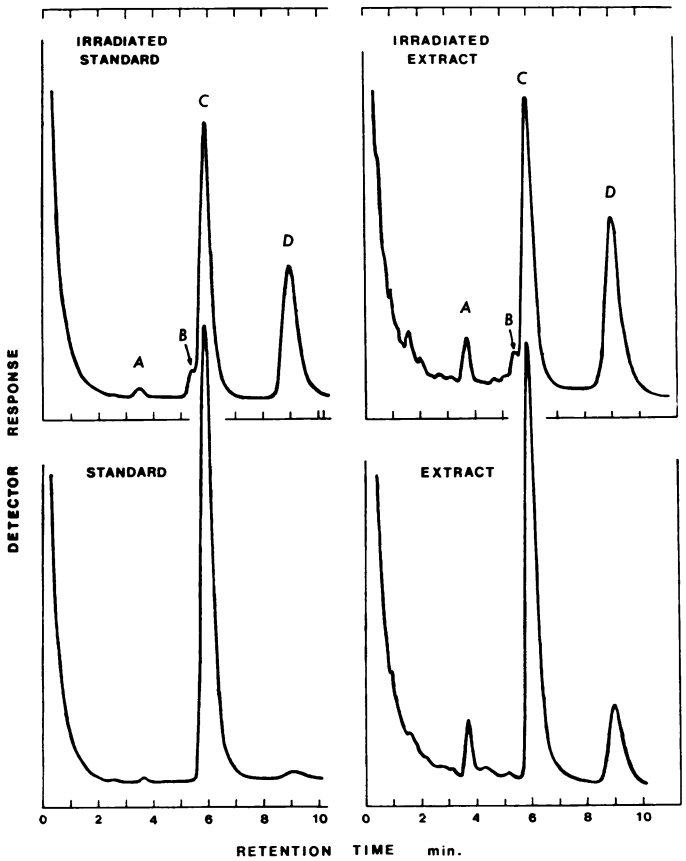

FIG. 2. GLC-electron capture traces of 1 ng of methyl-abscisate and a methylated grape extract before and after irradiation of the ethyl acetate solutions in unstoppered vials for 5 min under an unfiltered Oliphant UV lamp emitting predominantly at 2537A. A and B are unknowns, C is Me-ABA, and D is Me-t-ABA. Columns 150 × 0.2 cm glass; packing, 0.8% QFI plus 0.2% DC200 12500 CS on Varaport 30 100/120 mesh; temperature, 187 C; carrier gas, N_2 at 40 ml/min.

Table I. *Effect of Time of UV Irradiation of Ethyl Acetate Solutions of Me-ABA on the Area of GLC Peaks*

Conditions as in Figure 2.

Peak	Area of Peak after Exposure for:				
	0 min	3 min	5 min	8 min	240 min
	mm^2				
A. unknown	8	13	21	18	27
B. unknown	0	18	71	54	6
C. Me-ABA	1300	1030	781	610	110
D. Me-t-ABA	28	260	452	421	85
Total (A + B + C + D)	1336	1321	1325	1103	228

While extracting gas for the measurement of ethylene, differences were noticed in the volume obtained: the amounts extracted 15 and 22 days after veraison of untreated fruit were inversely correlated with the degree of deformability at that time (results not presented). This parameter was measured in experiment 2.

No promotive activity was found in the barley endosperm bioassays of any of the paper segments. Slight promotion of wheat coleoptile growth was found at the R_F of IAA in flesh extracts, but in the GLC-flame ionization measurements only 11 out of 61 chromatograms showed peaks corresponding to methyl-IAA, and these were small (equivalent to 1–5 ng of IAA per berry). Pronounced inhibition of coleoptiles was shown by extracts at the R_F of ABA and all GLC-flame ionization chromatograms showed peaks at the position of Me-ABA and Me-t-ABA.

Experiment 2. The measurements made on untreated berries are shown in Figure 5. The time of veraison was established from the fresh weight curve. It marked a decrease in gas volume and an increase in ABA. No abrupt change occurred in the ethylene concentration. The three treatments had the expected effects on berry weight (Fig. 6A): BTOA slowed the rate of increase, while CEPA, applied early and late, decreased and increased the initial rate of weight increment but subsequently had little effect. The increase in berry weight was correlated with a decrease in gas volume from untreated and treated berries (Figs. 5, 6A, and 6B). The changes in concentration of ethylene generally resembled those in experiment 1, *i.e.*, large sustained increases following CEPA and a smaller transient increase following BTOA treatments (Fig. 6C).

The weight of ABA in the flesh of untreated berries increased from 33 ng 7 days before to a level of 100 ng per berry at veraison (Figs. 5 and 6D); this increase was significant on logged data. By 13 days after, ABA had increased to 490 ng per berry; the amount then declined. The two treatments which delayed veraison also delayed the increase in ABA, especially BTOA treatment (Fig. 6D). The late CEPA treatment, which accelerated initial ripening, increased the weight of ABA at 13 and 20 days. The same differences are shown when the data are calculated as ng ABA/g fresh weight (Table II).

The amounts of ABA in the seeds are shown on three bases in Table II. Although the concentration is relatively high on a fresh weight basis, neither the concentrations nor the weights per seed fluctuated more than 2- to 3-fold, while the amount in the flesh varied 15-fold.

DISCUSSION

The concentration of ethylene increases considerably in many fruits in association with the onset of ripening (13). Reid and Pratt (18) have suggested that the increased ethylene causes

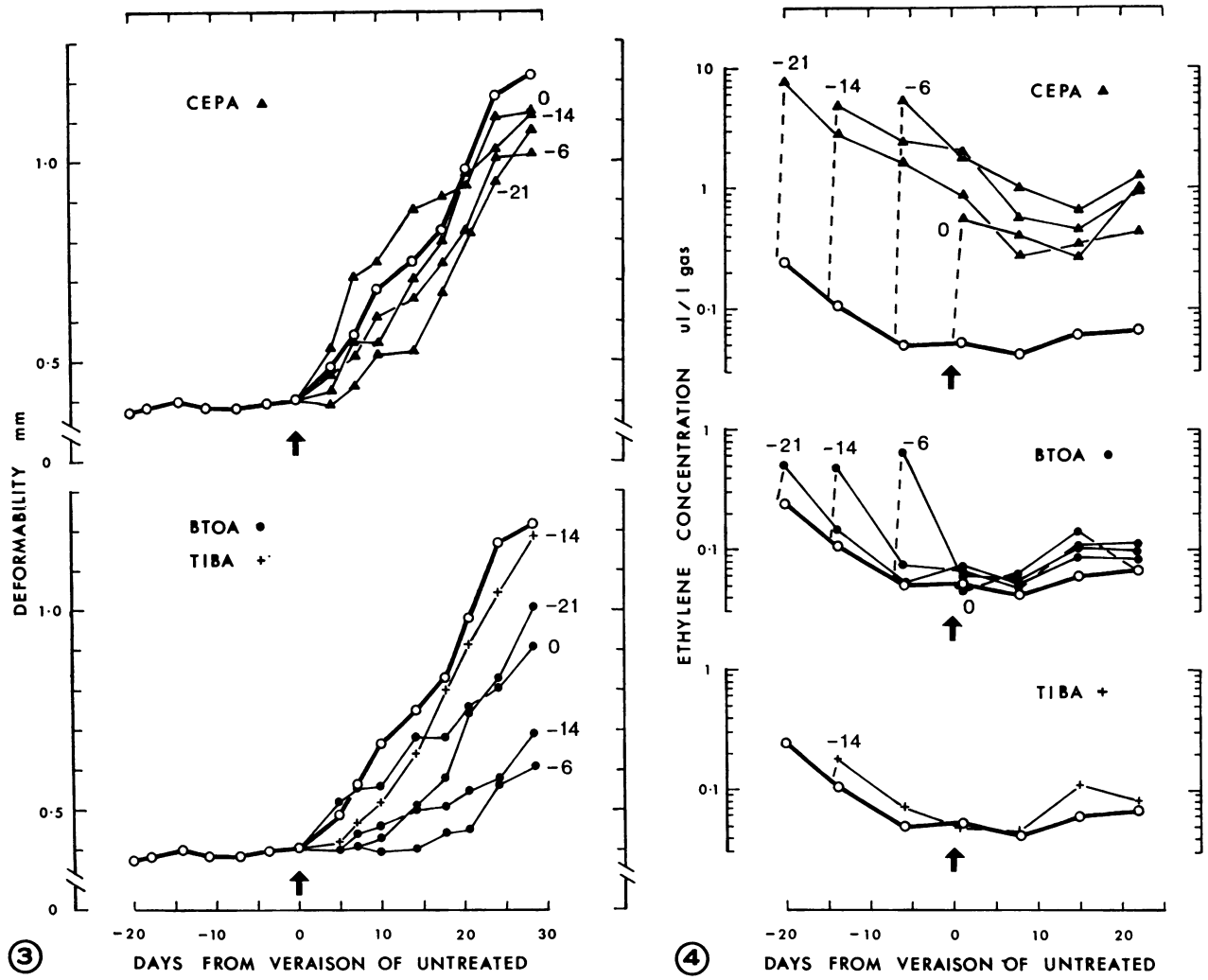

FIG. 3. Deformability of marked berries at intervals before and after veraison of untreated grapes (○) and of grapes treated at the times indicated with CEPA 400 mg/l (▲), BTOA 20 mg/l (●), and TIBA 30 mg/l (+).

FIG. 4. Concentration of ethylene (μl/l) in the gas extracted from 30-berry samples. Data identified as in the legend of Figure 3.

the rise in respiration (the climacteric rise) which is a feature of the ripening process in these fruits. In other fruits ethylene levels are low and there is no respiratory climacteric. Our results show that, in the grape, the concentration of ethylene is low before and during ripening. There is controversy over whether a grape displays a respiratory climacteric (16), but, on the basis of the foregoing, it would not be expected.

The ethylene curve for Doradillo berries resembles that of cherry, a nonclimacteric fruit (2). The concentrations in the grape are exceedingly low when calculated on the same basis used for cherry: 2.6 pl/g fresh weight at −15 days declining to 0.1 at +19 days, compared with a minimal value of about 60 pl/g fresh weight in cherry. The concentrations of ethylene on a gas volume basis (0.14–0.02 μl/liter) are more in line with quoted values but nevertheless would be regarded as low (19). Similarly low values have been found in the grape cultivar Shiraz; the results calculated on an ethylene production basis were 0.05 μl/kg/hr at −7 days declining to 0.005 at +35 days (C. R. Hale, unpublished data).

Ethylene is unlikely to have the dominant role in the ripening of grapes that it appears to have in the ripening of other fruits. This conclusion is reinforced by the discrepancies between ethylene concentration and veraison following treatment with growth substances: CEPA and BTOA increased the levels of endogenous ethylene even though ripening was either hastened or delayed by these treatments.

The differences between the CEPA- and BTOA-induced rises in ethylene levels are noteworthy (Fig. 4). The rises following CEPA applied during stage II were large and prolonged, while those following treatment with the auxin BTOA were small and transient. At the end of stage II, however, CEPA's effect was reduced and BTOA's eliminated. This signifies a change in an ethylene-synthesizing mechanism at this time. It is interesting that this is correlated with an increasing ABA content since it has been shown that ABA suppresses IAA-induced ethylene synthesis (11).

In contrast to ethylene, the changes in endogenous ABA (Figs. 5 and 6D) suggest the hypothesis that the onset and rate of ripening in the grape berry are a function of the accumulation of ABA. The amount of ABA increases, gradually before veraison and rapidly as ripening begins. A comparison of the fresh weight and ABA curves (Figs. 6A and 6D) suggests that the onset and rate of increase of growth and ABA are correlated. For example, (*a*) the slow growth following BTOA is correlated with a smaller and slower increase in ABA per berry. (*b*) The early CEPA treatment delays the inception, but not the rate, of the increase in both growth and ABA. (*c*) ABA rises to a high level following late CEPA, as does growth.

The datum for ABA at +5 days following late CEPA is the only one between −8 and +13 days in these results which is not consistent with the hypothesis. The decline in ABA after 13 days, evident in all treatments, is not associated with any obvious change in growth rate. On the basis of these results we have applied ABA to grapes during stage II and have found that ripening was hastened (unpublished data).

If ethylene has a role in the initiation of ripening, it may derive from the berry's changing sensitivity to ethylene. Perhaps once ABA has accumulated to a certain level, exogenous ethylene or CEPA is synergistic with ABA in promoting ripening. As a corollary, the berry will not ripen when ABA is below this level, and applied or induced increases in ethylene have the effect of maintaining ABA at a low level.

There are other reports of the accumulation of ABA during the maturation of fleshy fruits, *e.g.*, strawberry (21), pear (20), and tomato (6), but this is the first time ABA has been assigned a definite role in the maturation process rather than roles connected with stress effects or with abscission or inhibition of seed germination. The increase in ABA levels following CEPA treatment is also novel though Goldschmidt *et al.* suggested this (7) and the possibility was mooted by Addicott and Lyon (1). It is possible that this is a common phenomenon.

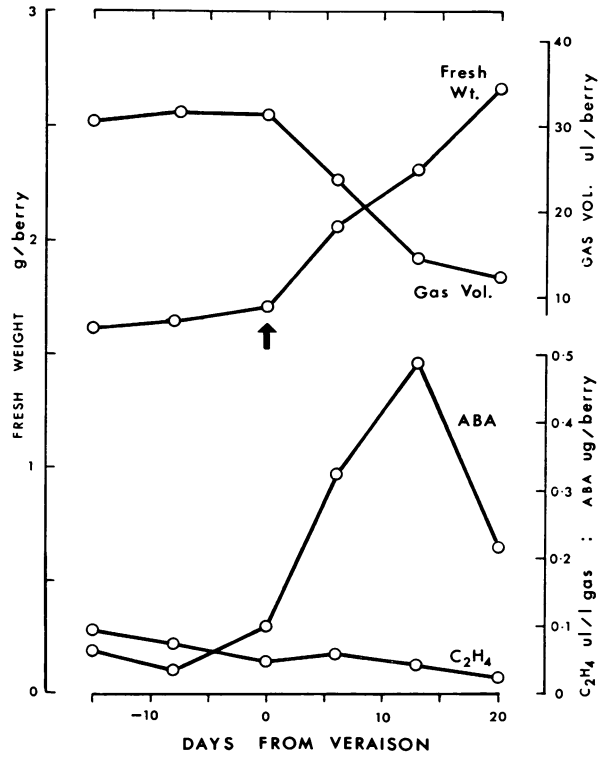

FIG. 5. Fresh weight and volume of gas per berry, ethylene concentration in the extracted gas, and weight of ABA in the flesh, all of untreated grapes.

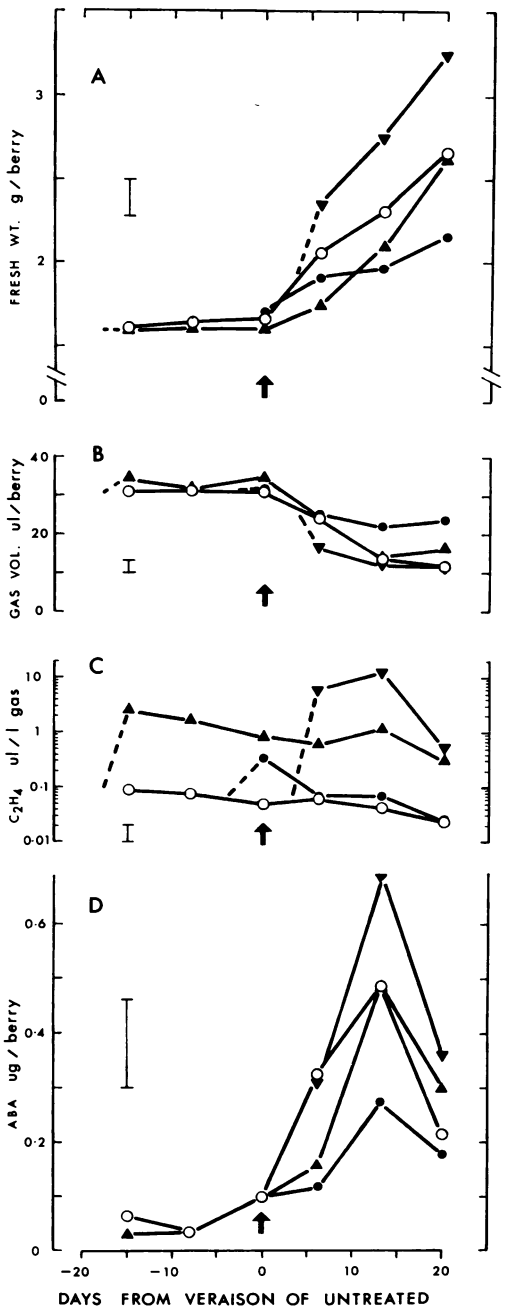

FIG. 6. Fresh weight per berry (A), volume of gas per berry (B), ethylene concentration in the extracted gas (C), and weight of ABA in the flesh (D) of untreated grapes (○) and of grapes treated with CEPA 400 mg/l on −18 days (▲) and on +3 days (▼) and with BTOA 20 mg/l on −5 days (●). Vertical bars represent LSD at $p < 0.05$.

Table II. *Content of ABA (Total of Both Isomers) in the Flesh and Seed of Grape Berries, Vitis vinifera cv. Doradillo, Expressed on Different Bases*

ABA	Days after Veraison					
	−15	−8	0	6	13	20
ng in flesh of 1 berry	63	33	100	326	489	216
ng in seeds of 1 berry	29	30	46	47	59	41
ng per seed	12	17	18	17	37	27
ng/g fresh wt flesh	41	21	62	158	212	81
ng/g fresh wt seed	203	250	336	314	535	495

The accumulation of ABA in the flesh was not accompanied by an accumulation in the seed. The data in Table II show that, although the concentration in the seed was high, the total amount of ABA in the seeds of one berry was small and relatively constant.

In our previous paper (9) we suggested that auxin maintained homeostasis of the berry during stage II. The delaying

effects of CEPA and BTOA on the start of ripening were explained by their effects on auxin level and the opposite effect of late CEPA or ethylene by the changed sensitivity to ethylene consequent to a declining auxin level. The low levels of IAA found during stage II cause us to question one of the bases of this hypothesis. However, there are other compounds which could account for the rise and fall of biological activity during stage II, and this question needs further investigation.

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