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Metabolism of 2-^{14}C-(±)-Abscisic Acid in Excised Bean Axes[1]

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ABSTRACT

Excised embryonic bean axes (*Phaseolus vulgaris*, var. White Marrowfat) rapidly metabolize 2-^{14}C-(±)-abscisic acid to two compounds, M-1 and M-2, which have very low growth-inhibitory activity. Chemical tests indicate the M-1 and M-2 are not previously described abscisic acid metabolites. M-2 accumulates in the axes and evidence is presented for the hypothesis that abscisic acid → M-1 → M-2. Zeatin, which partially reverses the abscisic acid-mediated growth inhibition of axes, neither decreases abscisic acid uptake nor causes any major changes in its metabolism. It was observed that axes transferred from abscisic acid-containing solutions to buffer resume control rates of fresh weight increase while still containing considerable quantities of abscisic acid.

The naturally occurring growth regulator ABA has been shown to be widely distributed in plants and to elicit a variety of physiological responses when applied to plant tissues (1). Although less is known about the metabolism of ABA, Milborrow has recently shown that abscisyl-β-D-glucopyranoside (8) and 6'-hydroxymethyl ABA (9) are the major compounds formed when 2-^{14}C-(±)-ABA is incubated with tomato shoots. These two compounds were apparently also formed when radioactive (±)-ABA was incubated with french bean and *Acer pseudoplatanus* petiole sections (7). The ABA glucoside has been shown to occur naturally in the fruit of *Lupinus luteus* (6) and in the pseudocarp of *Rosa arvensis* (9), and may be a storage and/or transport form of ABA.

We have reported that (±)-ABA is an effective inhibitor of the fresh weight increase of excised embryonic bean axes (14) and have also observed that the inhibitory effect is quickly lost when the axes are removed from solutions containing ABA. With the availability of 2-^{14}C-(±)-ABA (11), we were interested in obtaining information about the metabolic fate of ABA taken up by the axes, the identity of any metabolites formed, and their effects on the growth of the axes.

MATERIALS AND METHODS

Axes were excised from the dry seeds of *Phaseolus vulgaris* L. (var. White Marrowfat) and incubated in Erlenmeyer flasks which were shaken in a Dubnoff metabolic incubator in the dark at 26 to 27 C (13). Under the standard incubation conditions, 100 or 200 mg of axes were incubated in 50-ml flasks with 2 ml of sterile solution containing 10 mM HEPES, pH 6.0, 100 μg of chloramphenicol, and the test compound.

Kinetic Runs. At the end of the incubation period, axes were rinsed with distilled water, ground with Dry Ice, and homogenized with 5 ml of 70% (v/v) ethanol per 100 mg of original axis weight. After centrifugation and washing of the pellet with 70% ethanol, the combined supernatants were evaporated to less than 1 ml *in vacuo* at 30 C. The solution was centrifuged, and aliquots of the supernatant were chromatographed in the dark on Brinkmann TLC[2] plates precoated with silica gel. Several solvent systems were used and solvent composition and R_F values for ABA and its metabolites are given in Table I.

In some instances, prior to TLC, the extract was first chromatographed on a charcoal-celite column (3:2 w/w) with acetone to remove nonradioactive impurities. We have found that both ABA and its metabolites can be extracted into *n*-butanol from an acidified aqueous solution and have used this treatment in some instances prior to TLC. These various methods of partial purification give approximately the same distribution of radioactive compounds.

After chromatography, x-ray film was placed over the plates and left from 3 to 10 days depending on the quantity of radioactivity on the plate. The radioactive material indicated by the developed x-ray film was eluted from the silica gel with 95% ethyl alcohol into vials and counted in a scintillation counter. We have also scanned the TLC plates for radioactivity with a radiochromatogram scanner with the same results as those obtained with x-ray film.

Isolation of Metabolites 1 and 2. A total of approximately 4.5 gm of axes on a dry wt basis was incubated for 13 hr in the standard incubation medium which also contained 22 μg of 2-^{14}C-ABA per ml (5 μc/μmole). After incubation the axes were rinsed, ground with Dry Ice, and then homogenized with 50 ml of 80% ethanol. After centrifugation the pellet was resuspended in 40 ml of 80% ethanol and recentrifuged. The combined supernatants were evaporated *in vacuo* at 30 C to about 15 ml. After acidification to pH 2.5 with HCl, the solution was extracted three times with 25 ml of ether. The acidic ether fraction was chromatographed on Brinkmann precoated Silica Gel G TLC plates and developed three times in benzene-ethyl acetate-acetic acid (50:5:2, v/v). Radioactive compounds were located by autoradiography and eluted from the plates with 95% ethanol. Besides ABA and *trans*-ABA there were two additional bands of radioactivity on the plate with R_{ABA} values of 0.66 and 0.16. They contained approximately 5% and 23% of the total radioactivity on the plate and will be referred to as metabolites 1 and 2 (M-1, M-2) respectively. M-1 and M-2 were further purified by TLC in chloroform-

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[2] Abbreviation: TLC: thin layer chromatography.

Table I. *Solubility and Chromatographic Properties of M-1 and M-2 Compared with ABA*

Compound	Solubility Properties	Retardation Factor in Various Solvent Systems		
		Benzene-ethyl acetate-acetic acid (50:5:2)	Benzene-acetic acid (50:20)	Chloroform-methanol-water (75:22:3)
			R_F	
ABA	Soluble in water at pH 8, extracted into ether at pH 3, extracted into *n*-butanol at pH 3	0.22	0.65	0.70
M-1	Soluble in water at pH 8, extracted into ether at pH 3, extracted into *n*-butanol at pH 3	0.11	0.45	0.60
M-2	Soluble in water at pH 8, partially extracted into ether at pH 3, extracted into *n*-butanol at pH 3	0.02	0.29	0.46

methanol-water (75:22:3, v/v) in which they have R_F values of approximately 0.60 and 0.46, respectively.

The radioactivity remaining in the aqueous phase after ether extraction was adsorbed on a charcoal-celite column (3:2, w/w). The column was washed with water, and the radioactivity was eluted with acetone. The eluted material was streaked on TLC plates and developed in chloroform-methanol-water (75:22:3, v/v). Although autoradiography showed that there were several bands of radioactivity, more than 95% of the radioactivity was contained in a single band. The major band was eluted and rechromatographed in the same solvent and a single radioactive band was observed. After elution this material was further chromatographed on paper in *n*-butanol-propanol-ammonia-water (6:2:1:2, v/v) and autoradiography indicated a single band with R_F 0.63. This material cochromatographed with M-2 in several solvent systems, and treatment of both with diazomethane produced new compounds which also cochromatographed. We have found that when extracts from axes are chromatographed without a prior acidic ether extraction there is no evidence for two metabolites in the M-2 region of the plate. On the basis of these results we have concluded that M-2 and the major metabolite remaining in the aqueous phase after acidic ether extraction are the same.

Chemical Tests. For the determinations of the functional groups of the ABA metabolites, a convenient amount of material was deposited in 0.3-ml conical tubes (Microflex tubes, Kontes Glass Company, Vineland, N. J.) from alcoholic solutions by means of a nitrogen stream. For acetylation, 25 μl of acetic anhydride-pyridine (1:1) was added at room temperature. After 24 hr in the dark at room temperature, the reagents were removed with nitrogen, and the residue was taken up in ethanol and spotted on TLC plates. Tests for carbonyl groups were conducted by exposing the compounds to 10 to 20 μl of 0.15% 2,4-dinitrophenylhydrazine in acetic acid for 24 hr at room temperature in the dark. The mixture was spotted directly onto TLC plates. Methyl esters were prepared by adding ethereal diazomethane to the samples. The reagent was removed after 1 min at room temperature by a nitrogen stream, and the residue was taken up in ethanol and spotted on TLC plates.

Synthesis of Compounds. 2-^{14}C-ABA was prepared at a specific radioactivity of 10 mc/mmole by the procedure of Sondheimer and Tinelli (11) and zeatin by the procedure of Shaw *et al.* (10). The *cis*- and *trans*-1',4' diols of ABA were prepared by sodium borohydride reduction of ABA (2).

RESULTS

Both the metabolism of ABA and its inhibitory effects on fresh weight increase occur rapidly in excised bean axes which had been previously incubated in buffer for 6 hr at 26 C. A slight reduction in the rate of fresh weight increase can be detected in axes 30 min after exposure to 17 μM ABA, and this inhibition reaches 60% during the 2nd hr after transfer. Conversely, axes which are then transferred back to buffer after 2 hr in 17 μM ABA regain the control rate of fresh weight increase after 1 hr (Fig. 1).

Two radioactive metabolites of ABA can be detected 15 min after transfer to solutions containing 17 μM 2-^{14}C-ABA (Fig. 2). Both of these metabolites, designated M-1 and M-2, appear to be more polar than ABA (Table I). These metabolites are not formed from 2-^{14}C-ABA in the standard incuba-

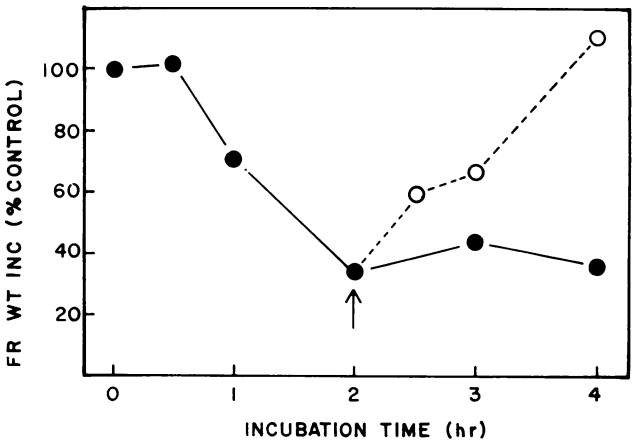

FIG. 1. Inhibition of fresh weight increase by ABA and loss of inhibition after transfer to buffer. Axes initially incubated in buffer for 6 hr. ●: 17 μM ABA; ○: transferred to buffer at time indicated by ↑.

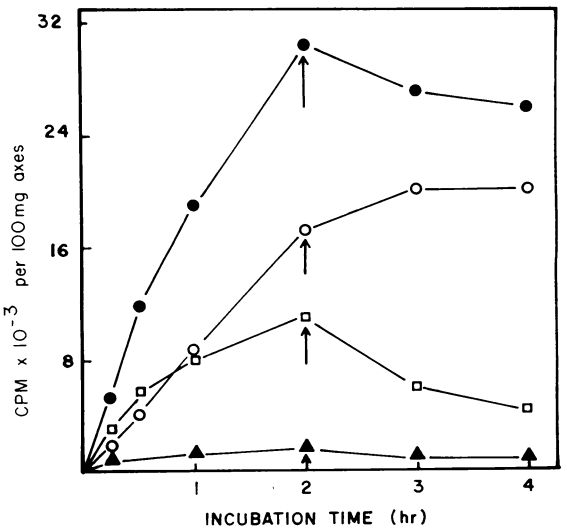

FIG. 2. Uptake of 2-^{14}C-ABA and conversion to M-1 and M-2. Axes incubated in buffer for 6 hr and then transferred to 17 μM 2-^{14}C-ABA. ●: Total 70% ethanol-soluble radioactivity; ○: M-2; □: ABA; ▲: M-1; transferred to buffer at time indicated by ↑.

tion buffer without axes, with axes which had been heated at 100 C for 5 min, or in incubation medium in which axes had previously been incubated for 6 hr.

Figure 3 shows the results of an experiment in which axes were incubated in 17 μM 2-^{14}C-ABA for periods of up to 12 hr without prior incubation in buffer. The rate of ^{14}C uptake was essentially constant except between the 2nd and 6th hr when both ^{14}C and water uptake by the axes were minimal (13). At the end of 2 hr, the ^{14}C-ABA level in the axes reached a plateau, stayed approximately the same to the 6th hr, and then rose slowly at a constant rate. At the end of the 12th hr, the internal and external ^{14}C-ABA concentrations were approximately equal. Only 10% of the radioactivity taken up by the axes at the end of the initial 2 hr of incubation had been converted to M-2. However, M-2 accumulated and accounted for 70% of the radioactivity in the axes at the end of 12 hr. This low initial conversion to M-2 contrasts with the more than 50% conversion of ABA to M-2 in axes during 2 hr if they had been previously incubated in buffer for 6 hr (Fig. 2). This difference in metabolic rate suggests that the capacity to metabolize ABA to M-2 develops during incubation. M-1, on the other hand, does not appear to accumulate and accounts for only 3 to 6% of the total radioactivity in the axes during the entire incubation period.

Washout Experiments. When axes were transferred to ABA-free solutions after 5 hr in ^{14}C-ABA, approximately 33% of the radioactivity was lost to the medium in the initial 3 hr. There was relatively little further loss during the succeeding 4 hr (Fig. 4). The radioactivity in the leachate consisted of 80 to 90% ABA, and the radioactive ABA in the axes dropped by 70% during the 7 hr. M-2, which made up the remainder of the radioactivity detected in the leachate, remained at an almost constant level in the axes. Since more than 50% of the radioactivity in the axes at the time of transfer was M-2, it is apparent that the rate of loss of ABA from the axes is much greater than that of M-2. We have calculated that almost all of the decrease in the ^{14}C-ABA level within the axes during the 7 hr is attributable to loss to the medium rather than continued metabolism.

When axes which had been incubated in 17 μM ABA for 5 hr are transferred to buffer, most of the inhibition of fresh weight increase is relieved within 2 hr after transfer (Fig. 5), although more than 60% of the radioactive ABA remains within the axes. Even during the 4th hr after transfer at which time there are no significant differences between the control rates of fresh weight increase and those found for the treated axes, approximately a third of the radioactive ABA is still present in the axes.

Feeding of M-1 and M-2. The observation that M-2 builds up with time while M-1 levels remain low and relatively constant is consistent with the hypothesis that ABA → M-1 → M-2, with the conversion of ABA → M-1 being the rate-limiting step. The fact that the M-2 content in the axes rose slightly while that of M-1 decreased after axes were transferred to solutions containing no ABA is also consistent with this hypothesis (Fig. 2). Feeding of partially purified M-1 and M-2 provided further experimental verification. After a 12-hr incubation with M-1, 80 to 90% of the radioactivity was recovered from the axes as M-2, and no products that cochromatographed with ABA were detected. Feeding of M-2 under these conditions led to the reisolation of material with unchanged chromatographic characteristics. These experiments also showed that neither M-1 nor M-2 has demonstrable growth-inhibiting activity at concentrations exceeding those attainable within the axes when ABA is fed (Table II). When M-2 was fed to axes that also contained growth-inhibitory

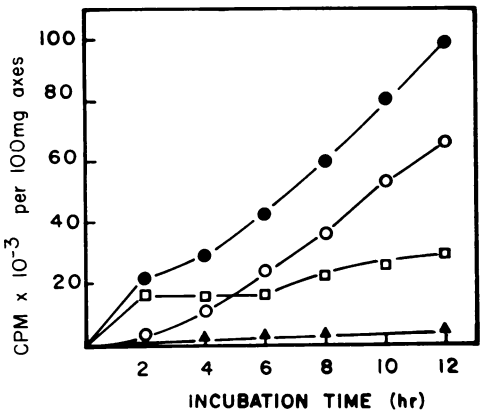

FIG. 3. Uptake of 2-^{14}C-ABA and conversion to M-1 and M-2 over a 12-hr period. ●: Total 70% ethanol-soluble radioactivity; ○: M-2; □: ABA; ▲: M-1.

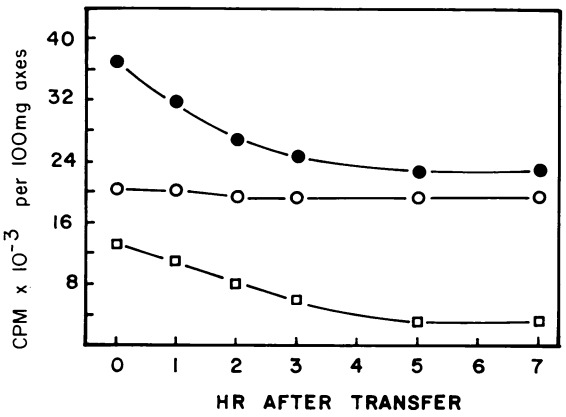

FIG. 4. Change in distribution of radioactivity in axes after transfer from 17 μM 2-^{14}C-ABA to buffer. Axes were incubated in ABA for 5 hr before transfer. ●: Total 70% ethanol-soluble radioactivity; ○: M-2; □: ABA.

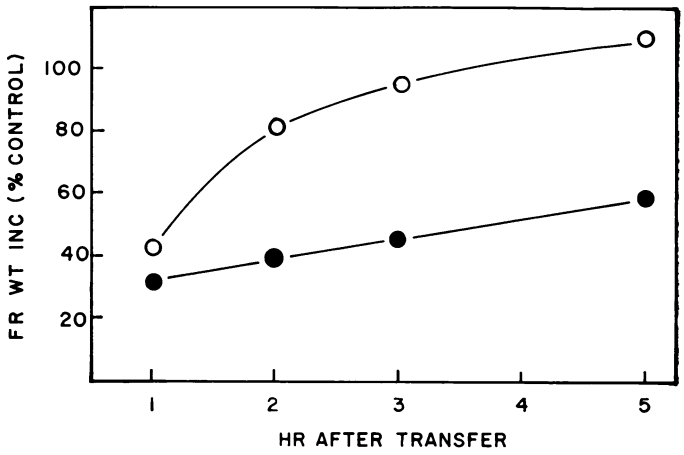

FIG. 5. Recovery from inhibition of fresh weight increase caused by ABA. Axes were incubated in 17 μM ABA for 5 hr, then transferred to fresh ABA solution (●) or buffer (○).

amounts of ABA, no synergistic effects were observed (Table III). It is therefore concluded that M-1 and M-2 are deactivation products of ABA and that M-2 is devoid of anti-ABA activity at the concentrations tested.

Effects of Zeatin. We have previously reported that exogenous zeatin, which alone has no effect on the fresh weight in-

Table II. *Growth Inhibitory Effects of ABA Compared with M-1 and M-2*

Axes (50 mg) were incubated for 12 hr in 1 ml of 10 mM HEPES, pH 6.0, 50 μg chloramphenicol, and test compound.

Compound	Quantity Fed[1]	Uptake	Fresh Weight Increase
	dpm	*dpm/50 mg axes*	*% of control*[2]
ABA	35,700	7,100	71
	71,500	13,700	55
M-1	71,500	5,700	89
M-2	71,500	5,150	94

[1] 71,500 dpm of ABA is 1.63 μg.

[2] After imbition and prior to growth initiation the original 100 mg of axes weigh 260 ± 5 mg. At the end of 12 hr incubation, the axes fresh weight of controls is 360 ± 10 mg.

Table III. *Effects of M-2 on Axis Fresh Weight Increase in the Presence and Absence of ABA*

Axes were incubated under standard conditions for 12 hr.

Treatment	Fresh Weight Increase
	% of control
12 μg/ml[1] M-2	94
2 μg/ml ABA	28
12 μg/ml M-2 + 2 μg/ml ABA	23

[1] Concentration was calculated from the radioactivity assuming a molecular weight and specific radioactivity identical to ABA.

Table IV. *Effect of μM Zeatin on Uptake and Metabolism of 2-^{14}C-ABA*

Axes were incubated for 11 hr under standard conditions in 0.26 μc of ABA (25 μM).

Treatment	Total Uptake	ABA Metabolism	Fresh Weight Increase
	dpm/100 mg axes	*% conversion to M-1 + M-2*	*% of control*
With zeatin	69,000	73	54
Without zeatin	95,000	68	30

Table V. *Reactivity and Chromatographic Behavior of Derivatives of M-1, M-2 and Related Compounds*

Treatment	Compound				
	M-1	M-2	ABA	1′,4′-*trans* diol of ABA	1′,4′-*cis* diol of ABA
Acetic anhydride[1]	No reaction	R_F 0.09	No reaction	R_F 0.35	R_F 0.38
2,4-Dinitrophenylhydrazine[1]	R_F 0.38	No reaction	R_F 0.50	—	—
Diazomethane[2]	—	R_F 0.08	R_F 0.65	R_F 0.46	R_F 0.30

[1] TLC on silica gel in benzene-ethyl acetate-acetic acid (50:5:2).

[2] TLC on silica gel in ethyl acetate-isooctane (1:1).

crease of axes, gives partial reversal of the ABA-induced growth inhibition (14). Because of this observation 2-^{14}C-ABA was fed to axes that were also exposed to zeatin (Table IV). Under conditions where zeatin reversed the growth inhibition by about 40%, total ABA uptake was stimulated slightly, possibly due to higher water uptake. However, little or no effect on the metabolism of ABA could be detected, indicating that the observed growth effects cannot be attributed either to reduced uptake of ABA or to increased inactivation. These data do not rule out the possibility that zeatin affects the conversion of ABA to an "active species" which we failed to detect.

Partial Chemical Characterization. In tomato shoots ABA is converted to 6′-hydroxymethyl ABA [3-methyl-5-(2′,6′-dimethyl-1′-hydroxy-6′-hydroxymethyl-4′-oxo-cyclohex-2′-enyl)-*cis,trans*-2,4-pentadieneoic acid] and abscisyl-β-D-glucopyranoside (8, 9). The major conversion products observed in bean axes exhibit chromatographic behavior similar to that reported for the above two metabolites but were shown to be different by means of chemical tests. M-1 reacts with 2,4-dinitrophenylhydrazine, but contrary to 6′-hydroxymethyl ABA, it is not acetylated (Table V). M-2 reacts with diazomethane and acetic anhydride but not with 2,4-dinitrophenylhydrazine (Table V). Basic hydrolytic conditions used for the cleavage of abscisyl-β-D-glucopyranoside to ABA did not affect the chromatographic behavior of M-2 or give any radioactive spot with the R_F value of ABA. These properties show that M-2 is not abscisyl-β-D-glucopyranoside but do not eliminate the *cis*- or *trans*-1′,4′-diols of ABA as possible structures. However, Table V shows that both M-2 and its ester have different R_F values from the diol acids and esters. In addition MnO_2 does not oxidize the methyl ester of M-2 under conditions in which the diol esters are converted to ABA. We conclude from these data that M-2 is not *cis*- or *trans*-1′,4′-diol of ABA.

DISCUSSION

Excised bean axes are able to metabolize 2-^{14}C-ABA very rapidly to two compounds, one of which appears to be the precursor of the second, which accumulates. These compounds are neither abscisyl-β-D-glucoside nor 6′-hydroxymethyl abscisic acid which are formed when 2-^{14}C-ABA is incubated with several other plant tissues (8, 9). We have obtained evidence, however, that the glucoside may be a major metabolite when the shoots from 7-day light-grown bean seedlings are incubated with ABA (unpublished data).

Neither of the two metabolites formed in the axes has significant growth-inhibitory activity, so their formation appears to be a deactivation mechanism. The rapidity with which ABA is metabolized to these less active compounds explains, partially at least, the speed with which the axes resume control growth rates after they are transferred from ABA-containing solutions to buffer.

There are several apparent anomalies which occur when axes are transferred from solutions containing ABA to buffer. When the changes in distribution of radioactivity within the axes and the leachate with time are analyzed, it is found that the reduction in radioactive ABA within the axes after the transfer to buffer is due almost entirely to loss to the medium rather than to continued metabolism. This seems surprising in view of the rapidity with which ABA is converted to M-2 during incubation. However, at least two explanations can be made for this observation. Since the radioactive ABA fed is a racemic mixture, it seems quite possible that one of the isomers is preferentially or exclusively metabolized so that only a small fraction of the radioactive ABA observed within the axes at any given time is actually metabolizable at a significant rate. Milborrow has reported that (+)-ABA is preferentially, if not exclusively, metabolized to 6′-hydroxymethyl-ABA in tomato (9). Another explanation is that a portion of the entering ABA, regardless of differences in optical isomerism,

becomes unavailable for metabolism. This may be the result of physical separation of substrates and enzymes within the tissue.

The fact that in wash-out experiments growth rates comparable to those of untreated controls are observed at a time when the axes still contain relatively high concentrations of 2-^{14}C-ABA also requires comment. Since (+)-ABA is the naturally occurring enantiomorph it is possible that it has higher hormonal activity and is also more rapidly degraded than the (−)-isomer. Since we are feeding 2-^{14}C-(±)-ABA, the ABA remaining in the axes during the wash-out experiment may become enriched with the (−)-isomer. Information on these points is still very limited, although it has been reported that (+)- and (−)-ABA are equally effective in inhibiting coleoptile growth of excised wheat embryos (9). It is also possible that a portion of the incoming ABA becomes unavailable for growth regulation. This has been suggested for IAA where a close correlation has been observed between the amount of transportable hormone and growth stimulation (4, 5). Finally, the metabolism of 2-^{14}C-ABA may lead to the formation of substances with anti-ABA properties. While this possibility cannot be discounted, M-2, the major ABA metabolite, was found to be devoid of anti-ABA activity.

The partial reversal of the ABA-mediated growth inhibition in axes by zeatin cannot be attributed to a reduction of ABA uptake nor to any major changes in the metabolism of ABA. The latter observation differs from that made for lentil root, in which kinetin appears to affect the metabolism of indole acrylic acid (3). ABA does have an effect on the metabolism of 8-^{14}C-zeatin in bean axes, but no evidence has been obtained that this alteration is related to the partial reversal of growth inhibition by zeatin (12).

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