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Investigations on the Mechanism of the Brassinosteroid Response

I. INDOLE-3-ACETIC ACID METABOLISM AND TRANSPORT

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ABSTRACT

A brassinosteroid treatment of light-grown first internode sections of *Phaseolus vulgaris* results in an increased bending response following unilateral indole-3-acetic acid (IAA) application. Reverse isotope dilution analysis shows that this increased response is not due to an increase in the concentration of applied IAA in the tissue or a change in the amount of IAA conjugated. Treatment with the brassinosteroid also does not affect the rate of IAA transport as measured using the agar block method. These results indicate that even though brassinosteroid potentiates auxin action, it does not have a direct effect on IAA uptake, metabolism, or cell to cell transport.

A steroidal lactone isolated from pollen of *Brassica napus* L. elicits a number of morphological responses when applied to a variety of higher plants at certain developmental stages. In intact plants, many of these effects appear to be unique to this class of compound (13, 15), whereas in more defined systems the effect is one of potentiation of the response of known growth regulating compounds, especially auxins (15). This steroidal lactone has been given the trivial name brassinolide (6) and is 2α,3α,22α,23α-tetrahydroxy-24α-methyl-B-homo-7-oxa-5α-cholestan-6-one (14). Brassinolide and several biologically active analogs were synthesized and the synthetic products were referred to collectively as BS[1] (15).

In most biological systems in which BS have been tested, they appear to act synergistically with auxins (17). However, no effort was made to determine in these test systems the concentration of endogenous hormones. Thus, it is not possible to determine from prior studies whether or not BS have any biological activity in the absence of auxin. In the bean first internode bioassay for auxins of Meudt and Bennett (12), BS lack significant biological activity of their own and demonstrate a potentiation of the bending response obtained by differential application of exogenous auxin.

In this report, we show that the potentiation of the auxin response seen with BS treatment occurs even though the actual concentration of the applied IAA found in the tissue is less in BS treated plants than in control plants treated with IAA alone. In addition, BS have no detectable effect on auxin transport, as measured by using the agar block technique, in either the apical or basipetal direction.

[1] Abbreviations: BS, brassinosteroids (2α,3α,22α,23α-tetrahydroxy-24β-methyl-B-homo-7-oxa-5α-cholestan-6-one and related biologically active isomers).

MATERIALS AND METHODS

IAA and DEAE Sephadex were from Sigma[2], and the 22α,23α-dihydroxy-24β-methyl isomer of brassinolide (Fig. 1) was a gift of Mr. Malcolm Thompson. [1-^{14}C]IAA (59 mCi/mmol) was from Amersham. First internode section (4 cm) of *Phaseolus vulgaris* L. cv Bush Burpee Stringless Bush Bean (Burpee Seed Co.) were grown and prepared as previously described (12). In experiments in which a mean value is reported, that mean is followed by the SE which occurred ($n \geq 3$).

Reverse Isotope Dilution Assay. Twenty internode sections were each treated with 82 nCi (1.4 nmol) of [1-^{14}C]IAA. Filter paper discs (5 mm diameter, Whatman No. 1) were treated with 2 μl of the [^{14}C]IAA solution provided by the supplier in benzene-acetone and the solvent was removed *in vacuo* (160 mm Hg) in a darkened desiccator for 30 min. Some discs were treated with 10 μl of an ethanolic solution of 0.1 ng/μl BS 1 h prior to the [^{14}C]IAA addition. Discs were then placed in contact with the side of the internode section 1.5 cm from its base. A full description of the bioassay system was presented by Meudt and Bennett (12).

After 2 h of treatment, the sections were measured to determine the curvature resulting from the asymmetric auxin application and then the sections were placed in ice-cold water for 1 h to remove [^{14}C]IAA from the tissue surface. After 1 h, the sections were ground using a blender (Hamilton Beach) at high speed for 2 min in a 450-ml glass grinding vessel. The homogenization medium was 100 ml of a 70% acetone:water (v/v) solution containing 1 mg of unlabeled IAA. The homogenate was kept at room temperature for 20 h in the dark to allow for equilibration of labeled and carrier IAA. After gravity filtration through Eaton-Dikeman grade 515 paper, an additional 100-ml 70% acetone was used to wash the filter paper and plant residue. One half of the filtrate was evaporated to dryness *in vacuo* (5 mm Hg) and dissolved in 40 ml of 7 N NaOH. This solution was placed in a 100°C sand bath for 3 h with 30 ml/min of water-saturated N_2 flowing through the hydrolysis vessel. This treatment results in complete hydrolysis of ester and amide conjugates of IAA (2). Following hydrolysis, the sample was neutralized with HCl. The other half of the filtrate was simply reduced to 15 ml and then both the hydrolyzed and nonhydrolyzed fractions were acidified to pH 2.5 with H_3PO_4.

The acidified fractions were partitioned three times against an equal volume of chloroform and the combined chloroform phases were dried for 1 h over an excess of anhydrous granular sodium sulfate. The solution was filtered and the chloroform removed *in vacuo* at 45°C. The residue was dissolved in 5 ml 50% 2-propanol-water and applied to a 2 × 14-cm column of DEAE-Sephadex A-

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

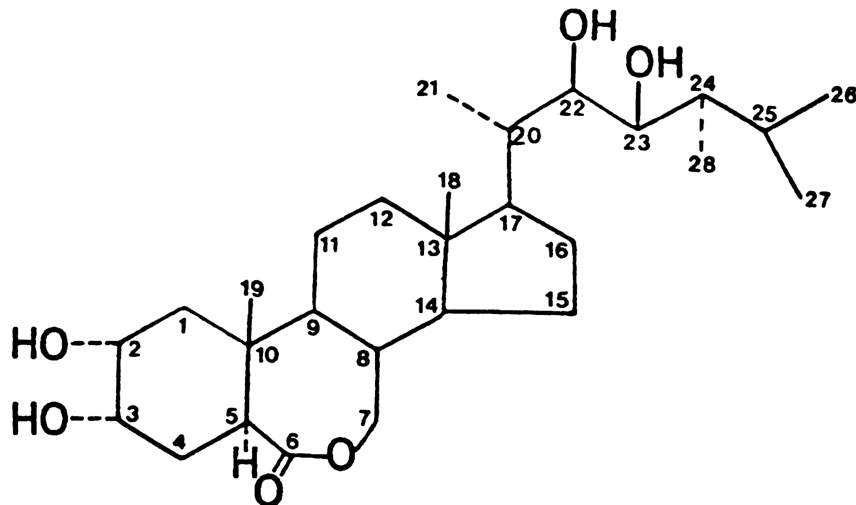

FIG. 1. Structure of the 22α,23α-dihydroxy-24β-methyl isomer of brassinolide.

Table I. *Levels of [^{14}C]IAA in Bean Internodes after 2 Hours of Treatment*

	Free IAA/20 Sections	Total IAA/20 Sections	Growth
	dpm (ng)		*Δmm/2 h*
Experiment 1			
Control ([^{14}C]IAA only)	82,089 (110)	89,520 (120)	6.52 ± 0.43
BS treated	58,190 (78)	62,860 (84)	8.53 ± 0.43
Experiment 2			
Control ([^{14}C]IAA only)	112,905 (151)	123,402 (165)	3.32 ± 0.48
BS treated	78,324 (105)	78,421 (105)	4.58 ± 0.63

25 in the acetate form. The column was washed with 40 ml of 50% 2-propanol:water and eluted with a linear gradient of 100 ml of 0 to 5% acetic acid in 50% 2-propanol:water. IAA eluted between 36 and 42 ml as indicated by TLC (see Ref. 4) of 5-μl aliquots of each 1-ml fraction. These fractions were pooled, reduced to dryness, and redissolved in 100 μl of 50% methanol:water. The 100-μl sample was applied to a 4.6-mm × 25-cm HPLC column of Whatman Partisil PXS 10/25 ODS-3 with a 2.1-mm × 5-cm guard column of Whatman Co:Pell ODS and eluted with 30% methanol:water plus 1% acetic acid. The retention time at a flow rate of 1 ml/min was 26 min. At this stage, the 1-ml fractions from HPLC containing IAA, as indicated by TLC, were pooled, adjusted to 2 ml with 50% methanol:water, and 200 μl removed for counting. The remainder of the fraction was used for the UV spectral analysis using a Beckman model 25 UV-visible spectrophotometer (see Ref. 7 for calculations). The amount of carrier IAA recovered was calculated using the molar extinction coefficient at A_{282} of $\epsilon = 6060$ (1). We found that additional purification of the sample on silica gel TLC (4), followed by methylation and a second HPLC step, resulted in no detectable change in the calculated specific activity.

Polar Transport of [1-^{14}C]IAA. Bean internode segments (4 cm) were preincubated for 2 h in water with or without 40 ng/ml BS. After preincubation, 1-cm sections were cut beginning 1 mm below the apical end. Movement of IAA through tissue segments was measured using agar blocks placed at the ends of a bundle of nine 1-cm-long internode segments (8). Ten ml of 0.75% agar was poured in a 60-mm diameter Petri dish, and labeled IAA (30 μl of benzene-acetone containing 3 μCi; 51 nmol; 8.9 μg of [1-^{14}C]IAA) was added to the hot liquid agar solution. After cooling, the agar was removed, and 3.5-mm-thick 1-cm square blocks were cut. Receiver blocks were prepared similarly except without addition of [^{14}C]IAA.

After 3 h incubation, the donor blocks, tissue segments, and receiver blocks were each transferred to 7-ml scintillation vials containing 5 ml ACS scintillant (Amersham). The samples were counted after equilibration overnight at 4°C in the dark. Correction to dpm was made using the external standard method of the Beckman 9000 liquid scintillation system and then confirmed by addition of [^{14}C]toluene (ICN Chemicals and Radioisotopes Div.) as an internal standard.

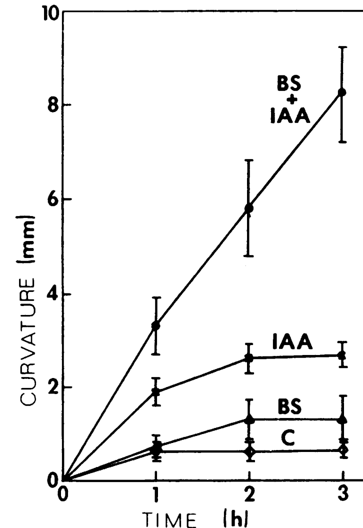

FIG. 2. Bending growth response (as measured in mm displacement from the vertical) of bean internode segments: untreated controls (C) or treated with BS (1 ng/segment), IAA (0.1 nmol/segment), or IAA plus BS. Each data point is the average of 20 determinations with the SE indicated.

CO_2 Evolution. $^{14}CO_2$ evolution was measured by placing a

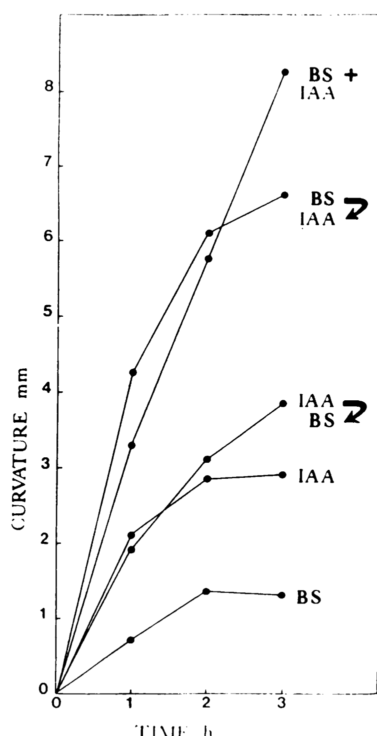

FIG. 3. Effect of order of treatment on the subsequent bending response of bean internode sections. Sections were treated with paper discs containing IAA followed by treatment with discs containing BS (IAA → BS) or the order of application was reversed (BS → IAA). Other conditions were as in Figure 2.

Table II. *Distribution of ^{14}C-Label after 2 Hours Bioassay of 20 Internode Segments*

	Distribution of Radioactivity	
	Exp. 1	Exp. 2
	dpm	
^{14}C extracted into 70% acetone		
Control	121,600	176,640
BS treated	122,700	145,250
^{14}C residual on filter disc[a]		
Control	827,200	873,320
BS treated	690,020	963,835
^{14}C in plant residue after extraction		
Control	428	512
BS treated	493	521
^{14}C in water rinse		
Control	25,400	29,721
BS treated	27,050	29,412
^{14}C Evolved as $^{14}CO_2$[b]		
Control	1,183 ± 91/2 segments	
BS treated	1,325 ± 408/2 segments	

[a] dpm applied to the filter disc not accounted for in the above distribution were absorbed into the sponge used for holding the sections in the bioassay.

[b] Average of three determinations; control and treated not significantly different.

bioassay vial with two internode segments in a 450-ml sealed jar. The jar contained a 7-ml vial with a 5 N NaOH-saturated filter paper strip for trapping the evolved $^{14}CO_2$. After 2 h, the vials were removed and 5 ml ACS scintillant was added to the vial with the filter paper for liquid scintillation counting. Incubation of [^{14}C]IAA with horseradish peroxidase (3) in this trapping system indicated essentially quantitative recovery of radioactivity.

RESULTS

Bean internode segments treated with [^{14}C]IAA and BS contained less [^{14}C]IAA after 2 h than those treated with [^{14}C]IAA alone (Table I). Although the IAA levels were somewhat lower in BS-treated sections, the differential growth response obtained with an equal amount of IAA was greater with BS treatment (Table I; Fig. 2). To affect the IAA response, the BS treatment must be given at the same time as IAA or as a pretreatment. Treatment with BS following IAA application has little effect (Fig. 3). No significant amount of IAA was conjugated during the time course of the experiment (Table I), thus conjugation of IAA would not appear to be involved in the response noted. Although variations are noted in the auxin response of tissue from day to day, this variation also appears unrelated to IAA uptake or tissue level (Tables I and II).

Only small amounts of $^{14}CO_2$ are evolved by the tissue during the bioassay period (Table II) and the total counts found in the treated tissue is comparable to control values (Table II). Thus, it seems that there is no appreciable increase in the rate of IAA turnover in BS-treated tissue. Taken together, these data demonstrate that the potentiation of IAA-induced growth by BS is not based on increasing the effective concentration of IAA.

Similarly, BS does not affect movement of IAA in 1-cm sections of bean internodes (Table III). Bean internodes do not demonstrate the strong polarity of IAA transport noted in other plant tissue systems (5, 8); nevertheless, it is clear that no significant difference between control and BS treatment is seen with these experiments. Variations in the experimental protocol, such as adding BS to the donor block, receiver block, or to both agar blocks, also did not affect the rate of IAA movement (data not shown).

DISCUSSION

Bean internodes treated with BS do not show enhanced tissue levels of [^{14}C]IAA commensurate with the increased bending growth response noted. Thus, the respone to BS is not mediated through changes in tissue levels of IAA as had been previously hypothesized by Mandava *et al.* (11). In addition, we find less than 6% of the [^{14}C]IAA found in the tissue to be in the untreated lateral half of the internode (Dr. K. Bialek, unpublished data). Inhibition of this small amount of lateral movement would not result in the increased bending observed with BS treatment. The response to BS would appear to be different from that noted in various plant systems in response to other steroid treatments. Kopcewicz (10) noted that estrogen treatment increased the extractable auxin activity, as determined by bioassay, in treated plants. Other steroids have shown either auxin-like activity (16) or show a distinct auxin synergism (9); however, BS does not appear to have significant activity in this bioassay in the absence of applied auxin (Fig. 2). At the concentration of BS used in this study, no other steroid has shown growth-promoting or auxin synergism activity in the bean internode bioassay, and over 100 different steroids have been tested (Meudt, unpublished data).

Other indicators of changes in IAA metabolism, such as $^{14}CO_2$ released, amount of ^{14}C fixed in the insoluble residue, amount of IAA taken up by the tissue, and rate of movement through the tissue, remain unchanged by BS treatment. In total, these results lead us to propose, as a working hypothesis, that BS affects some unknown rate-limiting metabolic step involved in IAA-induced growth. If this hypothesis is correct, then BS may prove to be a useful tool for studies of those reactions leading to hormone-mediated cell growth.

Table III. *Effects of a 2-Hour Preincubation with Brassinosteroid on Movement of [^{14}C]IAA from a Donor to a Receiver Agar Block*

Data are the mean of three experiments with SE indicated.

Experiment	Donor	Tissue	Receiver
		dpm	
To	148,705 ± 8857		
Control (basipetal)	108,169 ± 5409	18,564 ± 1848	6,337 ± 1791
+BS (basipetal)	109,000 ± 7200	18,865 ± 2027	6,680 ± 1706
Control (apical)	113,466 ± 11840	11,309 ± 2130	3,703 ± 611
+BS (apical)	109,708 ± 7098	13,405 ± 2872	3,746 ± 447

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