Plant Physiol. (1989) 91, 1329-1338
0032-0889/89/91/1329/06/$01.00/0

Received for publication May 2, 1989
and in revised form August 15, 1989

Gravitropism in Higher Plant Shoots[1]

VI. Changing Sensitivity to Auxin in Gravistimulated Soybean Hypocotyls

Patricia A. Rorabaugh and Frank B. Salisbury*
Plant Science Department, Utah State University, Logan, UT 84322-4820

ABSTRACT

Although the Cholodny-Went model of auxin redistribution has been used to explain the transduction phase of gravitropism for over 60 years, problems are apparent, especially with dicot stems. An alternative to an auxin gradient is a physiological gradient in which lower tissues of a horizontal stem become more sensitive than upper tissues to auxin already present. Changes in tissue sensitivity to auxin were tested by immersing marked *Glycine max* Merrill (soybean) hypocotyl sections in buffered auxin solutions (0, 10^{-8} to 10^{-2} molar indoleacetic acid) and observing bending and growth of upper and lower surfaces. The two surfaces of horizontal hypocotyl sections responded differently to the same applied auxin stimulus; hypocotyls bent up (lower half grew more) in buffer alone or in low auxin levels, but bent down (upper half grew more) in high auxin. Dose-response curves were evaluated with Michaelis-Menten kinetics, with auxin-receptor binding analogous to enzyme-substrate binding. V_{max} for the lower half was usually greater than that for the upper half, which could indicate more binding sites in the lower half. K_m of the upper half was always greater than that of the lower half (unmeasurably low), which could indicate that upper-half binding sites had a much lower affinity for auxin than lower-half sites. Dose-response curves were also obtained for sections 'scrubbed' (cuticle abraded) on top or bottom before immersion in auxin, and 'gravitropic memory' experiments of L. Brauner and A. Hagar (1958 Planta 51: 115-147) were duplicated. [1-^{14}C]Indoleacetic acid penetration was equal into the two halves, and endogenous plus exogenously supplied (not radiolabeled) free auxin in the two halves (by gas chromatography-selected ion monitoring-mass spectrometry) was also equal. Thus, differential growth occurred without free auxin redistribution, contrary to Cholodny-Went but in agreement with a sensitivity model.

Gravitropism has been explained for more than 60 years by the C-W[2] theory, which suggests that auxin is preferentially transported into lower tissues where the higher concentrations cause increased growth, and thus, upward bending. Discrepancies between the C-W theory and experimental results, especially with dicot stems, have been noted. Enough evidence has now accumulated to suggest that auxin concentration might not be the only factor controlling asymmetric growth in gravitropism (see reviews in refs. 16 and 19). An alternative model involves changing tissue sensitivities to auxin. As we note in our previous paper (19), until recently few investigators except for Brauner (*e.g.*, refs. 2 and 3) and Trewavas (26) have mentioned a sensitivity model for gravitropism.

Went and Thimann (27, pp. 75 to 86) observed that, in overall growth of *Avena* coleoptiles and various stems, auxin is supplied from the apex or the cotyledons and flows downward with more auxin in apical tissues and decreasing levels with distance from the apex. But, contrary to the assumption that auxin concentration controls growth, maximum elongation occurred at some distance from the apex in tissues where auxin was reduced. They invoked a 'food factor' transported from the base, which acted in concert with auxin, with apical growth limited by the food factor and basal growth limited by auxin. This hypothetical food factor was never found. We note, however, that Went and Thimann (27) also mentioned changes in sensitivity to auxin in regard to aging of coleoptile tissue, and Erickson and Silk (9, 21, 22) have studied the developmental displacement of cells and their aging downward through the hypocotyls. As cells mature and become displaced from the apex, structural and metabolic changes take place. Might one of these changes be the synthesis and/or activation of enzymes, including the auxin receptor? These maturing receptors would then bind auxin, perhaps reducing the amount of free auxin in that region of the shoot, with elongation in those cells commencing.MacDonald and Hart (13) have suggested a modified and elegant C-W model that includes differentially auxin-sensitive tissue across the dicot stem with the epidermis being most auxin responsive and the subepidermal tissue less responsive. They still envision auxin transport during gravitropism but only between these two tissues rather than across the whole stem: auxin in the upper half would move down into the nonresponsive subepidermis, but auxin in the lower half would move down into the responsive epidermis.

Clearly, growth of vertical as well as gravistimulated tissue is a complex chain or even web of events that involves auxin and other growth regulators and tissue responses to them. But in order to study this intricate phenomenon, we often measure only the end result: stem or root elongation or bending. There is much room for error, but we have little other choice. To simplify in this paper, we assume that growth is directly related

[1] This work was supported in part by Utah State Experiment Station Project 283 and by National Aeronautics and Space Adiministration grant NSG-NAG10-0014. It was submitted by the senior author in partial fulfillment of the requirements for the Doctor of Philosophy degree. This is Experiment Station technical paper 3814.

[2] Abbreviations: C-W, Cholodny-Went model (roughly, auxin-gradients and/or auxin transport); CS, cotyledonary side; ACS, anticotyledonary side; DSIDA, double-standard isotope dilution assay.

to tissue auxin concentration combined with tissue sensitivity to the auxin. From this simplification, we then define sensitivity as the capacity of an organism to respond to a given stimulus. It can be measured by varying the stimulus and observing the response (16, 19). The greater the stimulus required for a given response, the less sensitive that organism is to the stimulus. In our study, the stimulus causing growth of soybean hypocotyls was exogenously supplied auxin. The concentration was varied over a wide range (0 and 10^{-8} to 10^{-2} M IAA), and stem bending and lengths of the upper and lower sides were measured. Dose-response curves for the two halves were then compared.

As discussed in our earlier paper (19), dose-response curves can be analyzed using Michaelis-Menten kinetics (Fig. 1) for enzyme reactions. Two important values, which define two types of sensitivity changes, are derived from the kinetic curves. First, V_{max} is the theoretical rate limit approached as the substrate concentration is increased to the point where all the enzyme present is bound to the substrate. Its value depends on the maintenance of a finite number of enzyme binding sites in the reaction mixture. The analogous value in an auxin dose-response curve is the maximum growth rate induced by auxin. According to the kinetics analogy, V_{max} could only change if the number of enzyme (or in this case auxin) binding sites changed. Thus, one type of sensitivity, V_{max} sensitivity, is likely to be a function of the number of auxin binding sites; it could involve the synthesis, destruction, activation or inactivation of those sites. It could also involve changes in penetration and other factors (19). Second, the Michaelis constant (K_m) is the substrate (auxin) concentration that yields a reaction (growth) rate one-half of V_{max}. It indicates affinity of the enzyme (binding site) for its substrate. The lower the K_m, the higher the apparent affinity. Thus, the

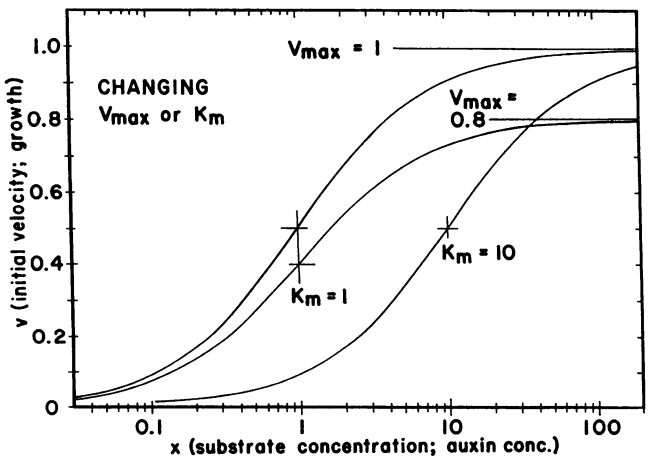

Figure 1. The Michaelis-Menten curves, where initial velocity of an enzymatic reaction is plotted as a function of the log of the substrate concentration. In the analogy with stem elongation, growth is substituted for velocity, and auxin concentration is substituted for substrate concentration. If V_{max} is indicative of the growth when a specific number of auxin binding sites are active, and K_m is representative of the affinity of those binding sites for auxin, then this shows how the growth curves might change when the number or affinity of the auxin binding sites changes (see text for details).

K_m sensitivity in an auxin dose-response curve could involve changes in the affinity of the binding sites for auxin. In support of this we note the report (23) that increasing auxin concentration enhanced both the synthesis (*i.e.*, number) and affinity of auxin binding sites.

Hypothetical dose-response curves can be predicted based on the C-W and sensitivity models. For the C-W theory, we started with a typical bell-shaped dose-response curve for the *Avena* coleoptile (Fig. 2A, redrawn from ref. 10). Two assumptions were then made. First, in accordance with the C-W theory, more endogenous auxin should accumulate initially in the lower halves, say 2×10^{-8} M IAA, with 10^{-8} M IAA in the upper half (consistent with the literature). Second, we assume that 10% of the exogenous IAA penetrates equally into all tissues, without internal transport (consistent with

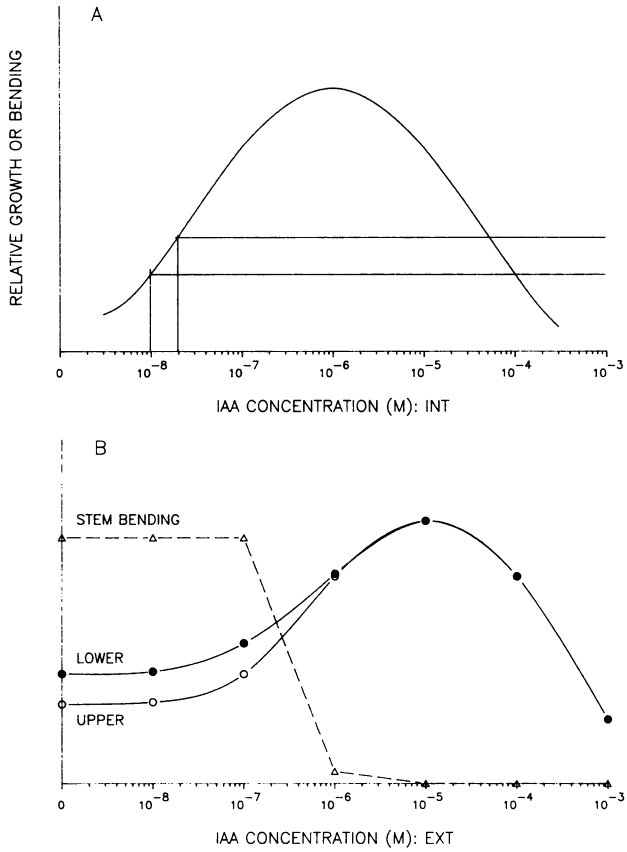

Figure 2. Hypothetical growth curves suggested by the C-W theory. A, Typical bell-shaped dose-response curve of the *Avena* coleoptile (redrawn from ref. 10). Note that the inhibition of growth at high auxin levels has no counterpart in the curves of Figure 1, but we can think of maximum growth at the optimum auxin concentration as representative of V_{max}. B, Predicted growth curves for the upper and lower halves of immersed, horizontal stems. To obtain predicted from *Avena* curves, internal auxin concentrations for each external concentration are determined from Table I. Internal concentrations are then located on the abscissa of the curve in part A, and growth at each concentration is then transferred (horizontally, in the figure) to the appropriate external concentration in part B. As is clear from Table I, the endogenous auxin makes an insignificant contribution to total auxin when the external concentration equals about 10^{-5} M IAA or higher.

actual [^{14}C]IAA uptake studies reported later in this paper). Table I then shows the total endogenous plus applied auxin concentrations that would be predicted in the two halves. Using these predicted values, and the procedures outlined in the caption of Figure 2, a set of hypothetical growth curves for the upper and lower halves can be derived, with a third curve representing the curvature that should result (Fig. 2B). One important point: as the exogenous auxin increases it should soon overwhelm the endogenous auxin, so this interpretation of the C-W never allows the upper half to grow more than the lower half; negative or downward bending is not predicted. If, however, changes in sensitivity to auxin do occur, with the lower side becoming more sensitive relative to the upper side, then exposure of the two halves to equal concentrations of auxin (as by immersion) should elicit differential growth, producing displaced dose-response curves for the two halves, as predicted in Figure 3. Either V_{max} or K_m or both might be displaced. Unlike the C-W model, the hypothetical curves for the sensitivity hypothesis do predict downward bending.

Three approaches with dose-response curves were used to study sensitivity changes during gravitropic bending. First, we obtained basic dose-response (as described above) by immersing hypocotyls in a wide range of auxin concentrations and then comparing them via kinetic analysis (as in refs. 16 and 19). Second, hypocotyls were scrubbed to promote differential IAA uptake from the solution. If auxin concentration is most important (as suggested by the C-W model), then abrading the cuticle on one side (upper or lower) to increase auxin uptake on that side should produce an auxin gradient in the tissue with a consequent predicted bending, up or down. If a change in sensitivity has taken place, however, then the top, when abraded, might still be less responsive at a given auxin concentration than the abraded bottom with a consequent effect on bending. The results from three such experiments were combined and are presented here. Third, the phenomenon of gravitropic memory, noted early in this century (see refs. 16 and 19 for review), was pursued by Brauner and Hagar (2) who performed experiments on sunflower by decapitating and 'depleting' the hypocotyls of auxin, gravistimulating them (no bending occurred), then returning them to the vertical and applying 10^{-4} M IAA to their cut surfaces. Bending occurred as predicted: what had been the lower side was now the convex side. Thus, auxin did not appear to be the signal in gravitropism but was required for growth. In light of these and other results, they suggested three alternatives to the C-W theory, one of which was that sensitivities to auxin might change. Brauner and Böck (3) later reported that decapitation caused more loss in sensitivity to auxin than actual depletion of auxin itself; hypocotyls were mostly desensitized, the term we will use. In any case, this technique appeared to be an excellent way to study the possible involvement of changes in sensitivity to auxin in gravitropism, but rather than adding back only the one auxin level, we tested several concentrations to obtain dose-response curves. If auxin enters the desensitized, gravistimulated (though unbending), and now vertical hypocotyls equally on both sides and is not transported, then any bending observed is most likely caused by differential sensitivity of the tissue achieved during gravistimulation.

A key difference between the C-W and sensitivity theories is the requirement or lack thereof for an auxin gradient; it was clear that auxin should be measured in upper and lower halves. Two questions were addressed: First, although the hypocotyls were immersed, was the auxin distributed homogeneously in the hypocotyls; that is, did it penetrate evenly, and once inside, was it pumped to the lower side? This was tested by addition of radiolabeled IAA to the solutions. Second, although exogenous auxin affects the gravitropic response, endogenous auxin is obviously also important, espe-

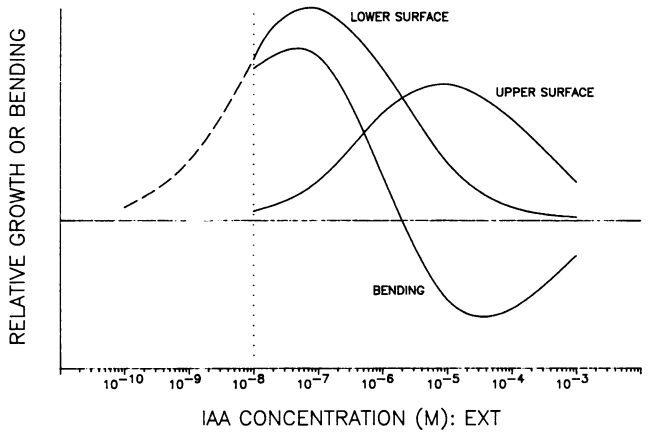

Figure 3. Predicted growth curves for the upper and lower halves of a horizontal stem, with associated stem bending, suggested by the sensitivity model. Because the bottom surface is predicted to be more sensitive, the curve of Figure 2A has been displaced so V_{max} is larger and K_m smaller for the bottom surface; the curve for the top surface has been displaced to a relatively smaller V_{max} and a larger K_m. The dashed part of the curves indicates concentrations even lower than those normally found in stem tissues. The bending curve is the algebraic difference between the curves for top and bottom surfaces. Note negative bending when upper surface grows more than lower surface.

Table I. *Predicted Amounts of Auxin Entering from the Solution, and in the Upper and Lower Halves of a Hypothetical Gravistimulated Stem*

Assumptions necessary for this simple hypothetical determination are as follows: first, in accordance with the C-W theory, more endogenous auxin accumulates initially in the lower halves; second, 10% of the exogenous IAA penetrates equally into all the tissue and is not transported.

Solution IAA	Amount Enters IAA	Total IAA	
		Lower	Upper
		M	
0	0	2×10^{-8}	1×10^{-8}
1×10^{-8}	1×10^{-9}	2.1×10^{-8}	1.1×10^{-8}
1×10^{-7}	1×10^{-8}	3×10^{-8}	2×10^{-8}
1×10^{-6}	1×10^{-7}	1.2×10^{-7}	1.1×10^{-7}
1×10^{-5}	1×10^{-6}	1.02×10^{-6}	1.01×10^{-6}
1×10^{-4}	1×10^{-5}	1.002×10^{-5}	1.001×10^{-5}
1×10^{-3}	1×10^{-4}	1.0002×10^{-4}	1.001×10^{-4}
1×10^{-2}	1×10^{-3}	1.00002×10^{-3}	1.00001×10^{-3}

cially when exogenous levels are low. Therefore, what is the total auxin (endogenous plus nonradiolabeled, exogenously applied) in the two halves? This was determined by GC-MS.

MATERIALS AND METHODS

Plant Material

Seeds of *Glycine max* (L.) Merrill (soybean: A-3127; Asgrow Seed Co.[3], Ames, IA) were soaked in deionized water for 3 h, sown in moist vermiculite, and grown 4 d in a growth room under fluorescent and incandescent lights with an average photosynthetic photon flux at the level of the seedlings of 120.8 ± 1.64 μmol m^{-2} s^{-1} and a photoperiod of 16 h day/8 h night. The temperature was 30.5°C ± 2°C day/27°C ± 2°C night, with a relative humidity between 30 and 40%. The young seedling is initially asymmetric with the cotyledons folded to one side, which is identified by a pubescence and designated as the cotyledonary side (CS). The opposite side was designated the anti-cotyledonary side (ACS). Seedlings used for the scrubbing, gravitropic memory, and radioactive uptake experiments were grown 5 d in a dark room at 32°C with a relative humidity between 35 and 45%. Bending response was similar whether seedlings were grown in light or dark.

Basic Experimental Apparatus and Procedures

Hypocotyls were decapitated with a diagonal cut, immediately cut to 35 mm, and marked by applying a ring of white oil paint (titanium oxide) near the apex and a second ring 20 mm below the first by rotating each stem against two brushes (16, 19). Segments were secured with a drop of 2% Bacto agar into holes drilled in one of two types of Plexiglas holders, each of which held up to 10 hypocotyls either vertically or horizontally. Because orientation was found to be important, hypocotyls were oriented so cotyledons of horizontal hypocotyls would have hung downward if they had been attached, and those of vertical hypocotyls would have pointed in toward the holder. Hypocotyls tested horizontally were kept vertical during the 30 min required for cutting and marking. Hypocotyls were immersed in one of two large Plexiglas tanks (illustrated in refs. 16 and 19), one for vertical controls, the other for horizontal test plants. Each tank was divided into eight vertical tanklets, with each tanklet accommodating one Plexiglas holder. Hypocotyls were tested in control buffer and in auxin at seven concentrations (10^{-8} to 10^{-2} M IAA). A 1-mM (pH 6.5), K-phosphate was made by mixing 100 mM K_2HPO_4 and 100 mM KH_2PO_4, then diluting to 1 mM. IAA (Sigma Chemical Co.) was dissolved in 100 mL of deionized water plus four to five pellets of KOH and mixed with 1 mM K-phosphate to a final volume of 2 L and final auxin concentration of 10^{-2} M IAA. Tenfold dilutions with buffer were made to obtain the seven IAA levels. Solutions were adjusted to pH 6.5 with 1 M HCl or KOH, added to the tanks, and placed in the dark room for 1 h at 32°C before the experiment to allow for temperature equilibration.

Data were recorded at 0.5-h intervals during the first 2 h and at hourly intervals for 5 to 7 h thereafter using two view cameras. Negatives (4 × 5 inches, same format as tanks) were projected through an *f* 1.2, 50-mm, Pentax camera lens via two first-surface mirrors from below (enlargement to 4.5 times life size) onto a 304.8-mm square Scriptel glass digitizing tablet (Scriptel Corp., Columbus, OH). Bending was initially measured as grid coordinates that were then translated into angles by a BASIC program. Lengths of upper and lower surfaces between edges of the marks (indicating the growth mechanism by which bending was achieved), and of 10-mm-long Plexiglas rectangles (cemented near each hole and used for calibration), were measured directly with the Sigma-Scan 3.0 Measurement System software package (Jandel Scientific). Data were processed through a SuperCalc4 spread sheet (Computer Associates International Inc.) to provide actual lengths, percent length increases, and average angles, and plotted using Sigma-Plot (Jandel Scientific). Bending of horizontal hypocotyls in degrees away from the horizontal zero line, and of vertical controls in degrees away from the vertical zero line, were measured so each could be plotted on the same graph for quick comparison.

Scrubbing Experiments

Early in the research (July 1984), before the methods just described had been developed, effects of 'scrubbing' (abrading) the cuticles were tested. Scrubbing was not used in other experiments reported here. Hypocotyls (cotyledons left intact) were cut to 40 mm, rubbed gently four times between thumb and forefinger with a slurry of carborundum and deionized water (8), rinsed, and secured in Plexiglas holders (16). These were placed in small glass tanks with hypocotyls either horizontal or vertical. At intervals the holders were removed from the solutions, laid flat on an overhead projector, the hypocotyl images projected onto a screen, and angles measured with a protractor.

Gravitropic Memory Experiments

In order to initiate the auxin desensitization process, seedlings were decapitated but left in the vermiculite for 2 h. They were then uprooted, cut to 40 mm, marked with carbon black in immersion oil, and secured in holders. The holders were placed in glass tanks filled with deionized water so hypocotyls (five per treatment) were vertical, and the desensitization process was continued for another 3 h. The now auxin-desensitized hypocotyls were turned to the horizontal in water and gravistimulated for 3 h, after which little or no bending occurred. The hypocotyls were finally turned to the vertical and immersed in buffered auxin solutions, with photographs taken every half-hour with a Pentax ME Super 35-mm camera and Plus-X Pan ISO 125 film. Negatives were mounted in standard slide mounts, and angles and lengths were analyzed with the digitizer/computer system.

[3] Mention of trade names is to provide detailed information only and does not imply endorsement to the exclusion of other products that might also be suitable.

Measurement of Radioactive IAA Uptake

Special Plexiglas holders (each for 10 horizontal plants or 5 vertical controls) and glass tanks bonded with silicone sealant were constructed for these experiments (16). K-phosphate, 1 mM (pH 6.5) was prepared as a control, and unlabeled IAA was added to produce final concentrations of 10^{-1} M, 10^{-2} M, 10^{-4} M, 10^{-6} M, and 10^{-7} M. Fifty μL of [1-^{14}C]IAA (61 Ci/mol, 50 mCi/L solution in toluene/acetone, Amersham Corporation) were added per 300 mL as a tracer to each tank but the control. (Experiments were performed in November 1985.)

For labeling analysis of the basic dose-response experiments, cotyledons were not removed, and hypocotyls, cut to 35 mm, were marked (carbon black in immersion oil), secured in the vertical and horizontal holders, and allowed to incubate 3 h in the labeled auxin solutions. For labeling analysis during the gravitropic memory studies, decapitated seedlings were desensitized as described above, gravistimulated in water for 3 h (after which little or no bending occurred) and then returned to the vertical, transferred to labeled auxin solutions, and incubated for 1.5 h (long enough for bending but prior to autotropic straightening). Data were recorded as for the gravitropic memory studies except that images were projected onto a screen (enlargement 5.5 times life size), with angles and lengths (calibrated with a ruler in each photograph) measured with a protractor and flexible ruler.

Hypocotyls were removed from the holders and rinsed, with cotyledons being removed from intact hypocotyls, and all hypocotyls were split into upper and lower halves. Half-hypocotyls were rinsed three times in cold, nonlabeled buffer, blotted between Kimwipes, weighed, and ground with mortar and pestle in an appropriate volume of 100% ethyl alcohol to yield a final concentration of 70%, accounting for tissue water. Extracts were poured into beakers wrapped in foil, incubated at 4°C overnight, filtered through tared Whatman No. 1 Qualitative (11 μm pore size) filter paper, and the auxin-containing filtrate taken to dryness in a hood under a constant stream of air. The residue was resuspended in 2 mL of 50% ethyl alcohol and sampled (1 mL in 10 mL Aquasol-2 Universal LSC Cocktail, Dupont: NEF:952) for counting. The resulting cpm were corrected for background and divided by the g fresh weight. Since a maximum of about 10^{-7} M radiolabeled IAA was added to each solution, the results are expressed as an adjusted cpm/g fresh weight calculated by multiplying the actual cpm/g fresh weight by a factor to correct for dilution.

Double-Standard Isotope Dilution Assay (DSIDA) for Auxin Using GC/SIM/MS

Cotyledons were removed, and 35-mm hypocotyl segments were marked with two rings of white oil paint 15-mm apart, secured vertically or horizontally in the large Plexiglas holders that were then lowered into the large tanks containing one of three buffered auxin solutions, pH 6.5: 0, 10^{-7} M, or 10^{-3} M IAA, the latter being repeated once. A total of 80 hypocotyls per treatment were used. Photographs were taken every 0.5 h with bending and lengths analyzed with the digitizer/computer system. After 2 h the holders were removed and each set of 10 segments was rinsed in deionized water. Each segment was removed, rinsed, and manually bisected (upper and lower for horizontal; ACS and CS for verticals), with tissue outside the marks removed so only the tissue between the marks (in the zone of bending) was saved for auxin analysis. The half-hypocotyls were placed in preweighed plastic bags, weighed, frozen, and subsequently transported on Dry Ice to the laboratory of Robert Bandurski at Michigan State University, East Lansing, MI, for auxin determination.

The frozen half-hypocotyls were ground in 70% acetone with a cold mortar and pestle, after which two auxin isotopes were added: a radioisotope [^{3}H]IAA (10 μL per sample of an 18,000-cpm μL^{-1} stock solution; Amersham) as a tracer; and the stable isotope [^{13}C-6]IAA (custom preparation by and gift from J. D. Cohen, USDA Plant Hormone Laboratory, Beltsville, MD) used for the quantitative assay of auxin, with 530 ng added to each of the 0 and 10^{-7} M IAA treated hypocotyl extracts and either 4825 ng (first run) or 5300 ng (second run) added to the extracts of hypocotyls treated with 10^{-3} M auxin. Each ground extract was incubated overnight at 34°C then gravity-filtered through a funnel-held, preweighed, No. 1 Whatman filter into a round-bottom vacuum flask, rinsed with 70% acetone, and taken to near-dryness in a flash evaporator. The ethanol residues, with two ethanol rinses of the flask, were applied to a DEAE-Sephadex column using 16 to 20 mL of 50% ethanol, and the auxin was removed with about 20 mL of a linear gradient of increasing acetic acid from 0 to 2.5% in 50% ethanol using a custom-made double-beaker gradient maker. Fractions were sampled (20 μL in 5 mL scintillation fluid Safety Solve, Research Products International Corp. Mount Prospect, IL) and counted, and auxin-containing fractions were combined, dried completely on the flash evaporator (to remove all traces of acetic acid from the column), redissolved in 200 μL of 50% ethanol, and stored frozen overnight. Further purification was by reverse-phase HPLC using a Varian 5000 Liquid Chromatograph (Houston Instruments). The precolumn was a Whatman Pellicular ODS, 4.6 by 250 mm, followed by a Whatman Partisil 10 ODS, 4.6 by 250-mm main column, with a flow rate of 1 mL min^{-1}. Isocratic elution for the first run was with 30% ethanol and for the second run was with 30% ethanol plus 0.07% acetic acid. A UV-5 Selectable Wavelength detector with a 280-nm filter was used, which gives sub-nanogram sensitivity for strong UV-absorbers, including IAA. After the first HPLC run, fractions were sampled (10 μL in 5 mL scintillation fluid) and counted; those containing auxin were combined, dried, redissolved in 200 μL 50% ethanol, and methylated for 5 min with 300 μL dried diazomethane (see ref. 5 for details). Methylated samples were dried under a gentle flow of N_2 gas, redissolved in 200 μL 50% ethanol, and reapplied to the HPLC. Fractions were sampled for counting and those with auxin combined and dried. Purified auxin extracts were redissolved in 1:1 methanol:acetonitrile (200 μL for the 10^{-3} M auxin treated samples; 40 μL for the 0 and 10^{-7} M samples), and analyzed with a Hewlett-Pachard 5970 mass selective detector (MS) coupled to a Hewlett-Packard 5890 GC using SIM, analyzing the ions at 130, 136, 189, and 195 amu with the following specifications: GC column was a 12.5-m by

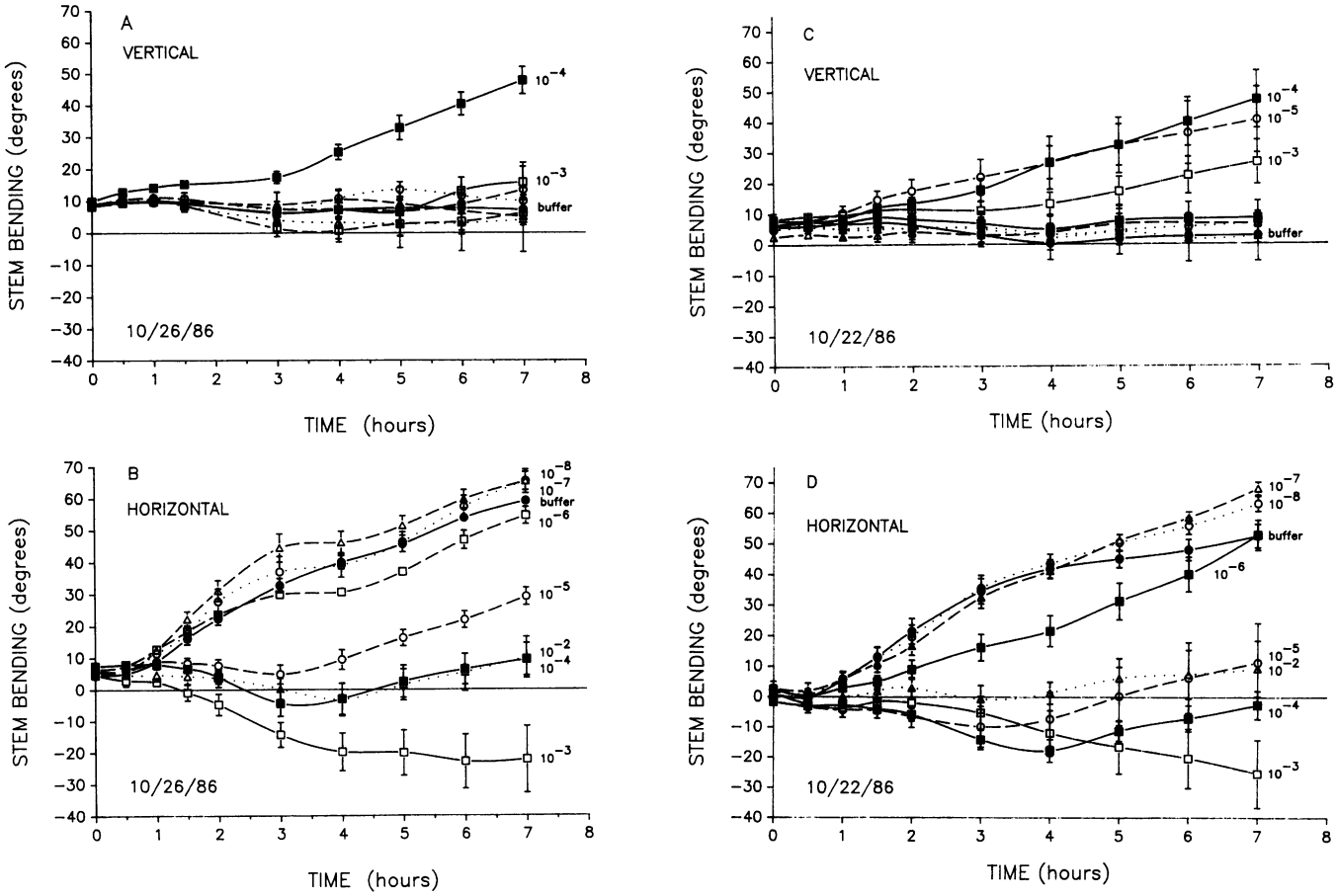

Figure 4. Average stem bending as a function of time for light-grown, decapitated, soybean hypocotyls immersed in buffered auxin solutions. Bars = 1 SE. Results from two separate experiments are given: A and C show stem bending for vertical controls, and B and D show stem bending for the corresponding horizontal hypocotyls. Note that there is at least a 30-min lag period in horizontal hypocotyls prior to bending, and that increasing the auxin concentration inhibits upward bending. Hypocotyls consistently bent down in 10^{-3} M IAA.

0.25-mm Chrompack Sil-19CB column with a film thickness of 0.19 μm; the carrier gas was helium with a flow rate of about 1 mL min^{-1}; electron impact ionization at 70 eV; the detector and ion source were at 280°C. After an initial 1 min, the oven temperature was 100°C, after which the oven was ramped at 30°C min^{-1} to a final temperature of 230°C and held for 1 min until the end of the run.

The DSIDA depends on the ability to distinguish between the masses of the endogenous compound (in this case [^{12}C] IAA methyl ester: 189 amu—native IAA) and the chemically related internal standard added in a known amount ([$^{13}C_6$]-[benzene ring]-IAA methyl ester: 195 amu—heavy IAA). The mass spectrometer ionizes the auxin molecules (native and heavy) into their respective indole and acetic acid components. The native indole ring has a molecular mass of 130 amu, whereas the heavy indole ring has a molecular mass of 136 amu. The masses of native and heavy fractions were measured, and the ratio (mass of heavy/mass of heavy + mass of native) was used as the C_f value below. The amount of total free auxin in the tissue extracts was calculated with the standard isotope dilution equation (6, 15): $Y = ([C_i/C_f] - 1)(X/R)$, where Y = amount of auxin in pmol/half stem (this is converted to pmol g fresh weight $^{-1}$ and plotted on a log scale); C_i = initial specific activity of the internal standard (in this case 100%); C_f = the final specific activity of the internal standard (as given above); one (1) is subtracted so that, if there is no dilution, $C_i/C_f = 0$; X = the amount (ng) of internal standard added; R = a correction factor (found to be 1.13 as per J. Cohen, personal communication to R. Bandurski).

RESULTS AND DISCUSSION

The Basic Dose Response Curve

Figure 4 shows bending versus time in two experiments for vertical and horizontal hypocotyls decapitated and immediately immersed in seven auxin solutions plus buffer. More than 20 experiments were performed with essentially the same results. Vertical controls remained fairly straight in buffer or low auxin concentrations, with some outward bending in higher auxin levels after 3 h, but not for the highest level (10^{-2} M). Horizontal hypocotyls immersed in buffer and in low auxin solutions consistently showed a 30-min lag period (no bending), followed by rapid upward bending, lasting about 2.5 h. By 4 h the rate of upward bending decreased. Higher auxin levels inhibited bending, and downward bending consistently occurred in 10^{-3} M IAA, with little or no bending in 10^{-2} M IAA.

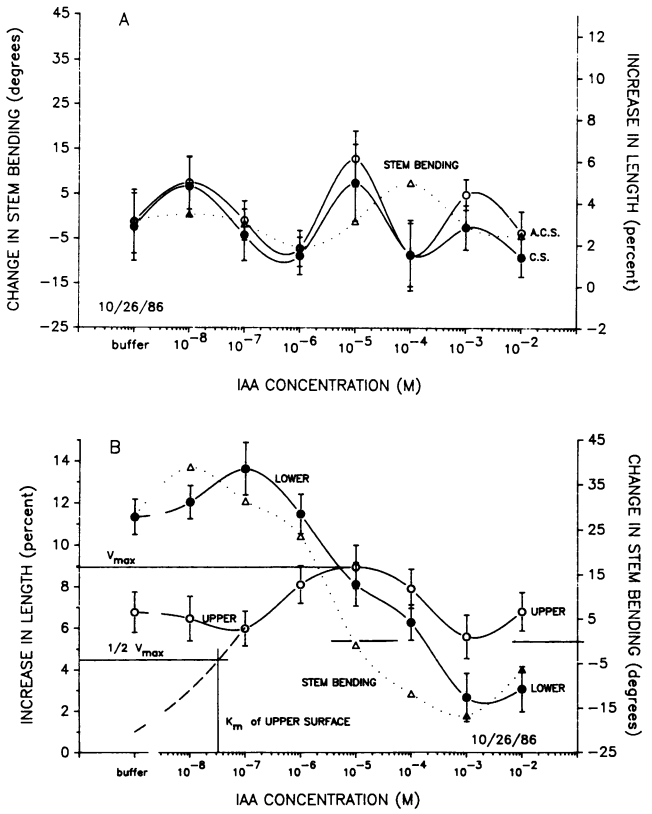

Figure 5. Stem bending and increase in length of light-grown, decapitated soybean hypocotyls after 3 h as a function of the exogenous auxin concentration. Bars = 1 SE. This is the same experiment shown in Figure 4, A and B. Note that auxin concentration did not differentially affect the ACS or CS of vertical controls (A), but auxin did affect the upper and lower halves of the horizontal hypocotyls differently (B). The dashed line indicates a possible shape of the curve for the upper surface if there were no endogenous auxin and allows an estimate of K_m for the upper surface; K_m for the lower surface is to the left of the scale. V_{max} is larger for the lower surface as usual. (We have seen V_{max} larger or about as large for the upper surface, as in Fig. 8A, illustrating the variability that is common to these experiments.)

Figure 5 shows lengths of the two surfaces and bending after 3 h. Bending in vertical controls was not significant, with no significant differences in growth between the ACS and CS. Upward bending of horizontal hypocotyls immersed in buffer or low IAA levels was caused by increased growth in the lower half relative to both the vertical controls and the upper half; downward bending in higher auxin levels was caused by growth inhibition in the bottom and growth stimulation in the top. Horizontal hypocotyls generally grew more in response to auxin than vertical controls, indicating that the gravity stimulus increased sensitivity to auxin of the hypocotyl as a whole.

Figure 5 also shows that V_{max} for the lower half of horizontal hypocotyls was greater than V_{max} for the upper half although this generalization does not always hold with soybeans (for example, see Fig. 8 below). Since an increase in V_{max} may be explained by an increase in the number of effective auxin binding sites, this number most likely increases in the lower half with respect to the upper half. A K_m value was also

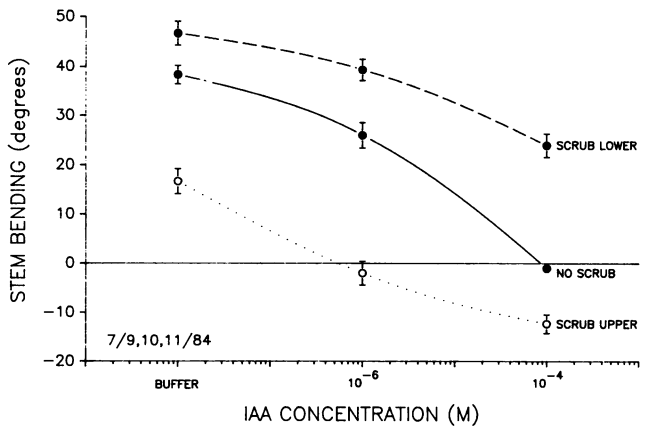

Figure 6. Effect of scrubbing on stem bending of dark-grown, horizontal soybean hypocotyls (with cotyledons) as a function of exogenous auxin concentration after 3 h. Each point represents the average stem bending of 15 hypocotyls (5 plants each from three separate experiments). Bars = 1 SE. Note specifically that the response at 10^{-4} M IAA should be completely controlled by the high exogenous auxin levels, which should swamp out any endogenous levels, and that bottom-scrubbed hypocotyls bent up more than top-scrubbed hypocotyls bent down. The two tissues are responding differently to the same exogenous auxin stimulus.

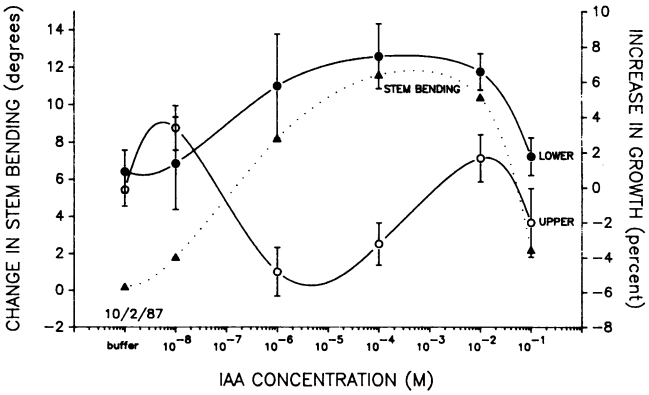

Figure 7. Gravitropic memory studies: stem bending and increase in lengths as a function of exogenous auxin concentration for vertical hypocotyls after 1.5 h in auxin solutions. Hypocotyls were previously decapitated and desensitized for a total of 5 h and gravistimulated in water for 3 h. Bars = 1 SE. In comparing these data with those for nondesensitized horizontal stems (Fig. 5B), note that a completely opposite pattern emerges with no bending and little growth of either side in buffer, and increased bending (but negative growth of the upper surface) with added auxin.

estimated for the upper half: about 3×10^{-8} M IAA, with the lower-half K_m off scale on the low side and thus not calculable with these curves. If K_m indicates binding site affinity, then auxin binding sites in the lower half (with a much lower K_m and a much higher K_m sensitivity) have a greater affinity for auxin than those in the upper half.

The Effect of Scrubbing on the Gravitropic Response

After 3 h, hypocotyls in all scrubbing treatments showed the typical inhibition of bending with increased auxin (Fig. 6). If auxin concentration is the only factor influencing the

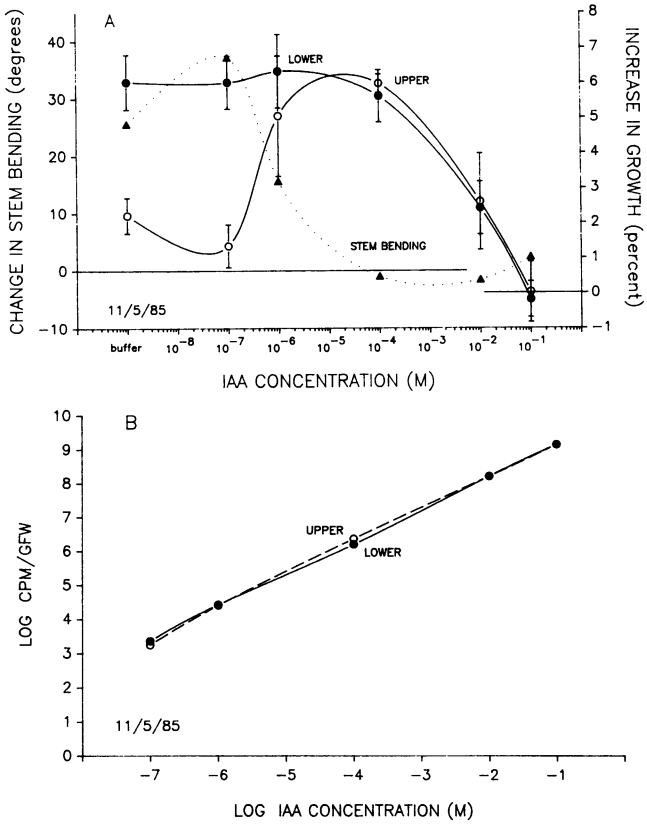

Figure 8. Radioactive IAA uptake: basic experiment. A, Stem bending and increase in lengths as a function of exogenous auxin concentration for dark-grown, horizontal soybean hypocotyls (with cotyledons) after 3 h in radiolabeled IAA solutions. Bars = 1 SE. Compare with Figure 5B. B, Radioactive IAA uptake into the upper and lower halves of the hypocotyls shown in A, presented as the adjusted log cpm/g fresh weight as a function of the log of the exogenous IAA concentration. Note that there was virtually no difference in the radiolabeled uptake even when the two halves were growing at different rates.

response, and if high levels of applied IAA swamp out endogenous concentration differences, then in the highest level of auxin tested (10^{-4} M IAA) the downward bending observed when the top is scrubbed should equal the upward bending observed when the bottom is scrubbed. Bending of unscrubbed hypocotyls in 10^{-4} M IAA was conveniently zero, making the comparison at this concentration more obvious. Downward bending of hypocotyls scrubbed on top (about −10°) was less than the upward bending of hypocotyls scrubbed on the bottom (about +25°), suggesting reduced responsiveness in the upper half.

Gravitropic Memory Studies

Bending and length after a 1.5-h immersion in auxin solutions of previously desensitized and gravistimulated soybean hypocotyls (Fig. 7) show that low levels of auxin produced little or no bending and no length differences of the two halves. This agrees with the overall decrease in sensitivity to auxin caused by decapitation, as suggested by Brauner and Böck (3). Increased auxin levels produced increased bending in the appropriate (what had been the upward) direction, however, with a slight increase in growth of the lower surface, and a marked decrease in growth of the top. Further increases in auxin reduced bending mainly by causing increased growth of the upper side. Bending and growth were inhibited at the highest auxin level. We were suspicious of the negative growth of the upper side at some auxin concentrations, but repeated calibrations and measurements of the photographic negatives validated the results, and shrinkage of upper surfaces has been reported by several other workers (*e.g.* refs. 7 and 14).

Radioactive Uptake Experiments

Bending and growth of horizontal segments responding in labeled IAA solutions during the basic experiment (Fig. 8A) followed the expected pattern. Uptake of labeled IAA into the two halves after 3 h (Fig. 8B) was the same. Stem bending and lengths after 1.5 h for a labeled gravitropic memory

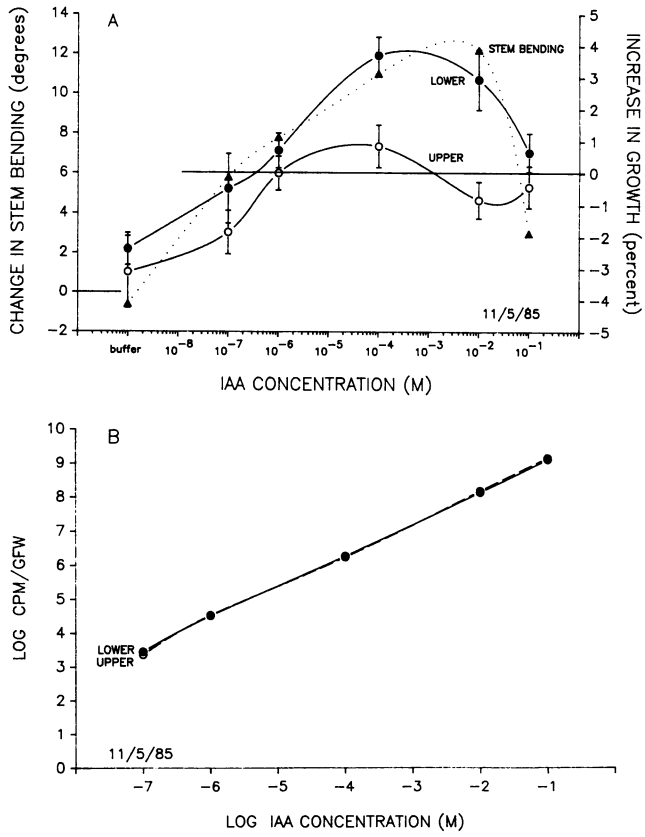

Figure 9. Radioactive IAA uptake: gravitropic memory experiment. A, Stem bending and increase in lengths as a function of the exogenous auxin concentration for dark-grown, vertical hypocotyls after 1.5 h in radiolabeled IAA solutions. Hypocotyls were decapitated and auxin desensitized for a total of 5 h and gravistimulated for 3 h in water before being turned to the vertical and put in the auxin solutions. Bars = 1 SE. Compare with Figure 8. B, Radioactive IAA uptake into what had been the upper and lower halves of the hypocotyls shown in A presented as the adjusted log cpm/gfw as a function of the log of the exogenous IAA concentration. Note that there was virtually no difference in the radioactive uptake even when the two halves were growing at different rates.

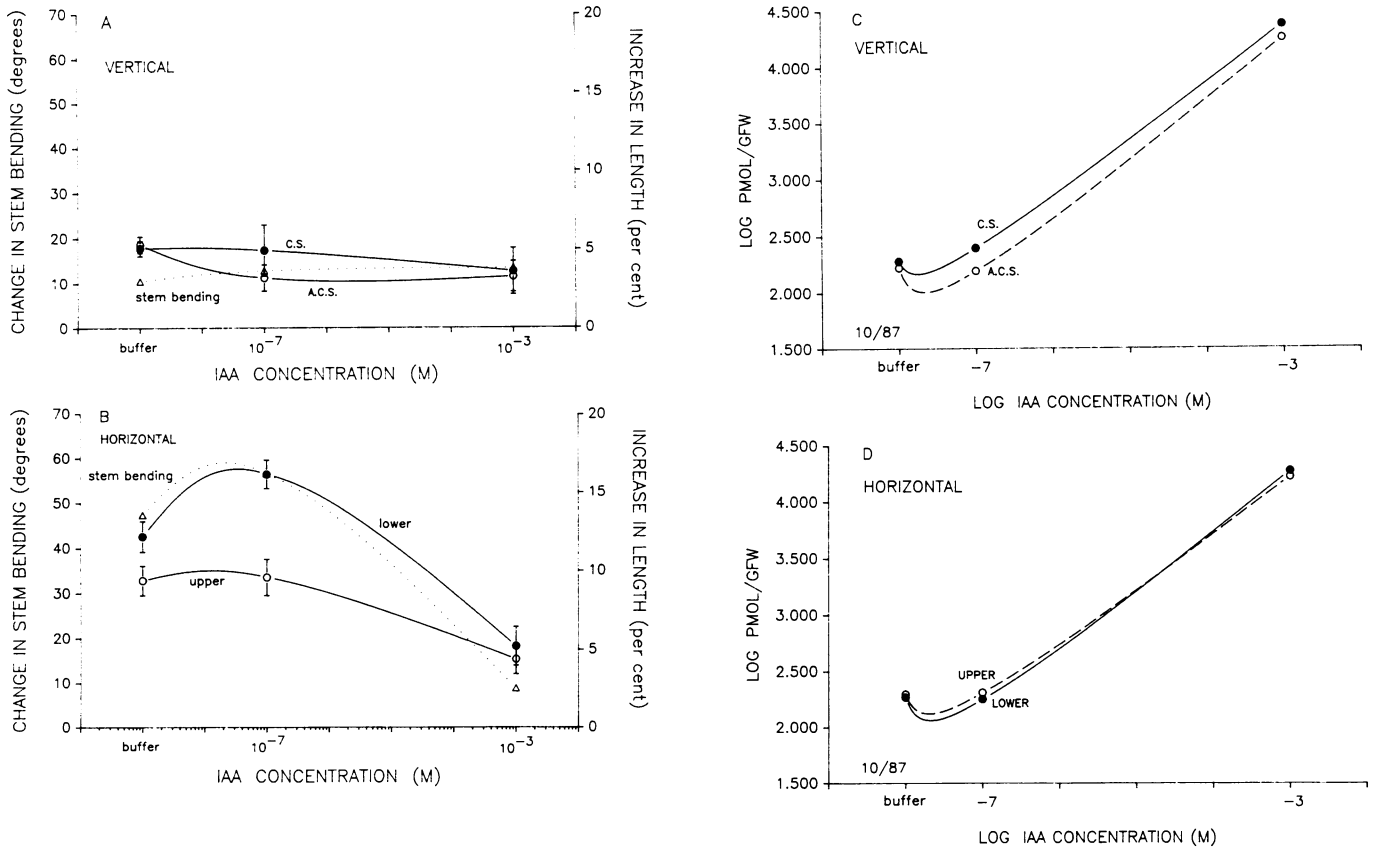

Figure 10. Total auxin as measured by GC/SIM/MS. A and B, Stem bending and length increase after 2 h as a function of exogenous auxin concentration for vertical and horizontal light-grown, decapitated soybean hypocotyls. Bars = 1 SE. C and D, Total free auxin (measured by GC/SIM/MS, presented as the log pmol/g fresh weight) in the two halves of hypocotyls shown in A and B. Note that at low auxin levels, when internal concentration may be equal to or greater than external levels, there is at least as much, if not slightly more, auxin in the upper, slower growing half as in the lower half.

experiment (Fig. 9A) followed the same general pattern as previously described for desensitized stems, including shrinkage of some surfaces. Radiolabeled IAA uptake after 1.5 h into what had been the upper and lower halves (Fig. 9B) was essentially the same. Since growth and bending were entirely dependent on the exogenously supplied auxin, and since concentrations of that auxin were equivalent throughout all the tissue, it is difficult to escape the conclusion that tissue sensitivity to auxin must differ in the two halves of the hypocotyl.

Total Auxin Content of Upper and Lower Halves of Soybean Hypocotyls Using GC-SIM-MS

Bending and growth, for vertical and horizontal hypocotyls (decapitated though not desensitized) exposed to three levels of auxin (Fig. 10, A and B), followed the general patterns discussed previously. In vertical controls there was more total free IAA in the CS (corresponding to the lower half of horizontal hypocotyls) than in the ACS at all three IAA concentrations (Fig. 10C). But in horizontal hypocotyls (Fig. 10D) total free IAA was at least as high in the upper (or slower growing) half exposed to low auxin concentrations, with no difference in free auxin content between the two halves when immersed in the highest auxin concentration (undoubtedly due to a swamping of the endogenous auxin by high exogenous auxin levels).

CONCLUSIONS

We used dose-response curves in three ways to compare the role of an auxin concentration gradient with that of a physiological gradient in sensitivity to auxin: simple dose-response curves for vertical, upper, and lower surfaces of hypocotyl segments; effects of scrubbing top or bottom surfaces on response to added auxin; and the gravitropic memory experiments.

As with sunflower hypocotyls (19), the two halves of horizontal soybean hypocotyls reacted differently to the same exogenous auxin stimulus over a range of concentrations. The upper half was consistently less responsive to added auxin than the lower half (both V_{max} and K_m sensitivity), not only for intact (data not shown here) and decapitated hypocotyls but also for scrubbed and for previously desensitized hypocotyls. Downward bending, not easily predicted by C-W, occurred in horizontal hypocotyls immersed in high auxin concentrations. The two sides also reacted differently to auxin in the gravitropic memory experiments. Of the three kinds of

experiments, results of the scrubbing tests are least impressive. These and the memory experiments were done before development of our most recent methods; they should be repeated (now impossible for us).

The dose-response experiments taken alone cannot be conclusive evidence that changes in auxin binding are occurring because auxin could be redistributed within the tissues in ways that might explain the results (*e.g.* ref. 13). (Sensitivity, response as a function of stimulus, would be different for the two surfaces, but differences might involve auxin penetration or internal distribution rather than auxin binding sites.) Radioactive IAA uptake studies examined these possibilities. Although there is a possibility that initial uptake into the tissues may have been unequal owing to differential penetratability (24), bending of both normal and desensitized hypocotyls after 3 h was not accompanied by a redistribution of applied auxin. Redistribution between epidermis and subepidermal tissues (13), which we did not measure, might explain our results, but this seems unlikely because auxin concentrations in all the epidermal cells would surely be very high in the high auxin solutions.

Endogenous auxin, not measured in the ^{14}C-IAA studies, could also influence the results although this seems quite unlikely at the high exogenous auxin levels. In any case, total free auxin after 2 h, as measured by GC/SIM/MS, was at least as great in the upper half of horizontal hypocotyls as in the lower half, contrary to C-W. These measurements also need to be repeated with more auxin concentrations and at more time points; perhaps one of us will sometime be able to do so. Meanwhile, one wonders if the slightly higher levels of free auxin in upper halves of control and low-auxin segments after 2 h (Fig. 10D) are real and an indication that a larger number of more active auxin binding sites in the lower tissues have sequestered more of the free auxin there.

How might sensitivity to auxin, say V_{max} and K_m of auxin binding sites, change in response to gravistimulation? Although this is unknown at present, we note that it is probably as easy to imagine testable mechanisms for sensitivity change as it is to propose mechanisms that account for auxin transport or synthesis/destruction. For example, amyloplasts in the root cap or the starch sheath of stems are massive enough to sediment quickly (18), and they bind Ca^{2+} (4, 12) and carry a negative charge (17). Settling could thus result in a change in electrical fields, and such changes have been reported (1, 11, 20, 25, 27, 28). Changing electrical fields could influence auxin receptors, perhaps those located in membranes. (For a more in-depth discussion, see ref. 16.) In any case it now seems appropriate to wonder about mechanisms of sensitivity change as well as those of auxin transport or synthesis/destruction.

ACKNOWLEDGMENTS

We wish to thank Rosemary White, who took part in our early work on changing sensitivity; Schuyler Seeley, who read the manuscript and made many valuable suggestions; Sharon Goalen for help with the manuscript; and Linda Gillespie for technical assistance. We are especially indebted to Robert Bandurski who allowed us to make the GC/SIM/MS measurements of auxin in his laboratory, where Aga Schultz and Dennis Reinecke were especially helpful.

LITERATURE CITED

1. **Bjorkman T, Leopold AC** (1987) An electric current associated with gravity sensing in maize roots. Plant Physiol **84:** 841–846
2. **Brauner L, Hager A** (1958) Versuche zur Analyse der geotropischen perzeption. I. Planta **51:** 115–147
3. **Brauner L, Böck F** (1963) Versuche zur Analyse der geotropischen Perzeption. IV. Untersuchungen über die Auswirkung der Dekapitierung auf den Wuchsstoffgehalt, das Längenwachstum und die geotropische Krümmungsfähigkeit von Helianthus-Hypokotylen. Planta **60:** 109–130
4. **Chandra S, Chabot JF, Morrison GH, Leopold AC** (1982) Localization of calcium in amyloplasts of root-cap cells using ion microscopy. Science **216:** 1221–1223
5. **Cohen JD** (1984) Convenient apparatus for the generation of small amounts of diazo-methane. J Chromatogr **303:** 193–196
6. **Cohen JD, Schulze A** (1981) Double-standard isotope dilution assay. I. Quantative assay of indole-3-acetic acid. Anal Biochem **112:** 249–257
7. **Cosgrove D** (1985) Direct turgor pressure measurements during plant gravitropism. Physiologist **28:** 297
8. **Dowler MJ, Rayle DL, Cande WZ, Ray PM, Durand H, Zenk MH** (1974) Auxin does not alter the permeability of pea segments to tritium-labeled water. Plant Physiol **53:** 229–232
9. **Erickson RO, Silk WK** (1980) The kinematics of plant growth. Sci Am **242:** 134–151
10. **Foster RJ, McRae DH, Bonner J** (1952) Auxin-induced growth inhibition, a natural consequence of two-point attachment. Proc Natl Acad Sci USA **38:** 1014–1022
11. **Jaffe LF, Nuccitelli R** (1977) Electrical controls of development. Annu Rev Biophys Bioeng **6:** 445–476
12. **Krauss H** (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu Rev Plant Physiol **38:** 47–72
13. **MacDonald IR, Hart JW** (1987) New light on the Cholodny-Went theory. Plant Physiol **84:** 568–570
14. **Mueller WF, Salisbury FB, Blotter PT** (1984) Gravitropism in higher plant shoots. II. Dimensional and pressure changes during stem bending. Plant Physiol **76:** 993–999
15. **Rittenberg D, Foster GL** (1940) A new procedure for quantitative analysis by isotope dilution, with application to the determination of amino acids and fatty acids. J Biol Chem **133:** 737–744
16. **Rorabaugh PA** (1988) A possible role for changes in cell sensitivity to auxin in gravitropic bending of soybean hypocotyls. PhD dissertation. Utah State University, Logan UT
17. **Sack F, Priestley DA, Leopold AC** (1983) Surface charge on isolated maize-coleoptile amyloplasts. Planta **157:** 511–517
18. **Sack F, Suyemoto MM, Leopold AC** (1986) Amyloplast sedimentation and organelle saltation in living corn columella cells. Am J Bot **73:** 1692–1698
19. **Salisbury FB, Gillespie L, Rorabaugh P** (1988) Gravitropism in higher plant shoots. V. Changing sensitivity to auxin. Plant Physiol **88:** 1186–1194
20. **Salisbury FB, Ross CW** (1985) Plant Physiology. Wadsworth Publishing, Belmont, CA
21. **Silk WK** (1984) Quantitative descriptions of development. Annu Rev Plant Physiol **35:** 479–518
22. **Silk WK, Erickson RO** (1978) Kinematics of hypocotyl curvature. Am J Bot **65:** 310–319
23. **Starling RJ** (1984) The question of plant hormone binding sites. Trends Biochem Sci **9:** 48–49
24. **Tanada T** (1978) A rapid gravitational effect on the translocation of fluorescein in mung bean hypocotyl sections. Planta **142:** 221–223
25. **Tanada T, Vinten-Johansen C** (1980) Gravity induces fast electrical field change in soybean hypocotyls. Plant Cell Environ **3:** 127–130
26. **Trewavas AJ** (1981) How do plant growth substances work? Plant Cell Environ **4:** 203–228
27. **Went FW, Thimann KV** (1937) Phytohormones. Macmillan, New York.
28. **Woodcock AEK, Hertz CH** (1972) The geoelectric effect in plant shoots. V. A discussion. J Exp Bot **23:** 953–957