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Auxin-induced changes in the patterns of protein synthesis in soybean hypocotyl

(wounding/2,4-dichlorophenoxyacetic acid/cytokinin/two-dimensional gel electrophoresis)

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ABSTRACT **The patterns of protein synthesis in elongating and mature (basal) sections of soybean hypocotyl were examined after incubation in a medium containing auxin (auxin-treated) or a medium lacking auxin (untreated). The hypocotyl sections (1.2 cm) were labeled with [^{35}S]methionine, and polypeptide patterns were analyzed by one- and two-dimensional polyacrylamide gel electrophoresis. Auxin treatment altered the pattern of protein synthesis in both elongating and basal soybean hypocotyl sections. Excision of terminal segments from incubated sections was required to clearly observe auxin-induced changes in the synthesis of polypeptides. Polypeptides synthesized in terminal segments, possibly in response to wounding, can mask subtle changes in the spectrum of polypeptides synthesized in response to auxin. Cytokinin treatment caused a decrease in [^{35}S]methionine incorporation into polypeptides and altered the pattern of protein synthesis in untreated and auxin-treated elongating hypocotyl sections.**

There is substantial evidence that continued RNA (1–4) and protein (1, 3–5) syntheses are required for auxin to induce cell elongation in excised tissues. Controversy has existed, however, over whether auxin-induced cell elongation involves the synthesis of any specific "growth-limiting" or "growth-essential" proteins (6, 7). To determine whether auxin alters the types of proteins synthesized, we have employed high-resolution one-dimensional and two-dimensional gel electrophoresis systems to examine the spectrum of polypeptides synthesized in both elongating and mature (basal) soybean hypocotyl sections that were incubated in a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) (auxin-treated) or a medium lacking 2,4-D (untreated). We have also examined the effect cytokinin (isopentenyladenosine) has on the patterns of polypeptides synthesized under conditions in which this growth substance inhibits auxin-induced cell elongation (8–10). Our results indicate that auxin treatment alters the pattern of protein synthesis, causes a charge modification of proteins, or both in both elongating and basal sections of soybean hypocotyl. In addition, our results indicate that cytokinin treatment causes a decrease in [^{35}S]methionine incorporation into polypeptides and changes the types of polypeptides synthesized in untreated and auxin-treated elongating hypocotyl sections.

MATERIALS AND METHODS

Chemicals and Radioisotopes. [^{35}S]Methionine (600 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear. 2,4-D was obtained from Sigma and recrystallized twice from benzene. Ampholines were purchased from LKB. Nonidet P-40 was obtained from Gallard-Schlesinger (New York). Isopentenyladenosine was purchased from Sigma.

Plant Material. Soybean seeds (*Glycine max* var. Wayne) were germinated in a 1:1 mixture of moistened vermiculite and perlite in the dark at 30°C. After 72 hr of germination, the hypocotyls were harvested; 1.2-cm sections were excised from the elongating region (0.5–1.7 cm below the cotyledons) and from the mature (basal) region (the 1.2-cm region just above the root–shoot transition zone). Hypocotyl sections were rinsed with deionized distilled water and placed in the appropriate incubation solution.

Radioactive Labeling of Proteins in Hypocotyl Sections. Twenty-five elongating hypocotyl sections were incubated at 30°C with continuous shaking in 25-ml erlenmeyer flasks in 5 ml (or 12 sections in 10-ml erlenmeyer flasks in 2.4 ml) of a solution containing 2% (wt/vol) sucrose, chloramphenicol at 50 μg/ml, and 10 mM sodium phosphate or potassium phosphate (pH 6) buffer containing 50 μM 2,4-D (auxin-treated) or lacking 2,4-D (untreated). After the sections had been incubated for 2 hr, [^{35}S]methionine (100 μCi/ml) was added to the medium and incubation was continued for 3 hr unless indicated otherwise. Basal sections were incubated and labeled in the same manner with the exception that the medium for auxin-treated sections contained 500 μM 2,4-D. Labeling experiments were conducted at least three times for elongating and basal sections, and representative examples were utilized in all results presented.

Preparation of Labeled Proteins for Polyacrylamide Gel Electrophoresis. After the 5-hr incubation period, the terminal 1-mm segments of each hypocotyl section were excised and the sections were divided into terminal segments and internal segments. Twenty-five (or 12) unlabeled 0.8-cm sections were combined with the 1-mm terminal segments to facilitate preparation of the labeled proteins in a manner similar to that for internal segments. The hypocotyl segments were homogenized in 15 ml (or 7 ml) of 50 mM Tris·HCl (pH 7.9 at 4°C)/1 mM EDTA/0.1% (vol/vol) 2-mercaptoethanol/500 mM ammonium sulfate. A high ionic strength homogenization buffer was chosen to solubilize nuclear proteins (11) and other proteins that are insoluble in dilute buffers. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at $8000 \times g$ for 15 min. Proteins from the supernatant were concentrated by ammonium sulfate precipitation (approximately 90% saturation with ammonium sulfate), and the precipitate was collected by centrifugation at $5000 \times g$ for 5 min. The precipitate was solubilized in 10 mM Tris·HCl (pH 7.9 at 4°C)/1 mM EDTA/0.1% (vol/vol) 2-mercaptoethanol at 2°C and dialyzed against several changes of the same buffer at 2°C; the sample was then made 1.7% (wt/vol) $NaDodSO_4$ and 8% (vol/vol) 2-mercaptoethanol and boiled for 3 min; after the sample was cooled to 20°C, urea and Ampholines (pH 3.5–10) were added to 9.5 M and 0.2% (wt/vol), respectively (except for Fig. 3 *A* and *D*, for which the precipitate was solubilized directly in sample buffer). Dialysis of the sample was preferred because greater resolution and less polypeptide aggregation and streaking were observed on two-dimensional gels.

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; IEF, isoelectric focusing.

We found it advantageous to precipitate the labeled proteins with ammonium sulfate prior to electrophoresis because this procedure allowed us to concentrate the protein into a small volume and eliminated a large amount of background radioactivity on fluorographs. No significant differences in polypeptide patterns between ammonium sulfate-precipitated proteins and proteins solubilized directly in a homogenization buffer containing $NaDodSO_4$ were observed by analysis on one-dimensional concave exponential gradient [10–16% (wt/vol)] polyacrylamide gels (data not shown).

Polyacrylamide Gel Electrophoresis. One-dimensional gel electrophoresis in the presence of $NaDodSO_4$ was performed on 0.75-mm thick slab gels by the method of Laemmli (12). Two-dimensional gel electrophoresis with isoelectric focusing (IEF) in the first dimension and $NaDodSO_4$ in the second dimension was performed as described by O'Farrell fr(13, 14). Sample treatment and loading on first-dimension gels were similar to the $NaDodSO_4$ protocol described by O'Farrell *et al.* (15) with a ratio of $NaDodSO_4$ to Nonidet P-40 of 1:4 in the final protein sample loaded. Isoelectric focusing was conducted for a total of 6800 V·hr (15 hr at 400 V plus 1 hr at 800 V). Concave exponential gradient [10–16% (wt/vol)] polyacrylamide gels were prepared as described by O'Farrell (14). Slab gels were stained with 0.2% (wt/vol) Coomassie brilliant blue R-250 in 50% (vol/vol) ethanol and 10% (vol/vol) acetic acid for 15 hr and destained with 7.5% (vol/vol) acetic acid.

To facilitate comparison of labeled polypeptides extracted from untreated and hormone-treated hypocotyl sections, equal amounts of protein were loaded on first-dimension gels in order to avoid differences in resolution of the polypeptides on the second-dimension gels. Protein concentration was determined by the method of Bradford (16).

Fluorography. Fluorography was performed according to the method of Bonner and Laskey (17). Slab gels were dried onto Whatman filter paper under reduced pressure and exposed to Kodak X-Omat R x-ray film at −70°C. For one-dimensional gels, fluorographs were exposed 1 day per 84,000 cpm of ^{35}S applied. For two-dimensional gels, fluorographs were exposed 2 days per 1,000,000 cpm of ^{35}S applied to the first-dimension gel.

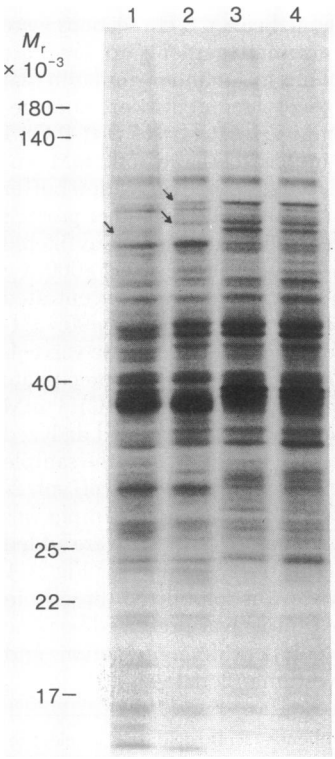

FIG. 1. Fluorograph of ^{35}S-labeled polypeptides from elongating sections resolved by electrophoresis on a concave exponential gradient (10–16%) polyacrylamide gel in the presence of $NaDodSO_4$. To each lane, 28,000 cpm was applied. Lane 1, untreated internal segments; lane 2, auxin-treated internal segments; lane 3, untreated terminal segments; lane 4, auxin-treated terminal segments. Molecular weights indicated on the left were determined with cauliflower RNA polymerase IIB subunits and bovine serum albumin as molecular weight standards. Arrows indicate examples of differences in polypeptides synthesized in untreated and auxin-treated sections.

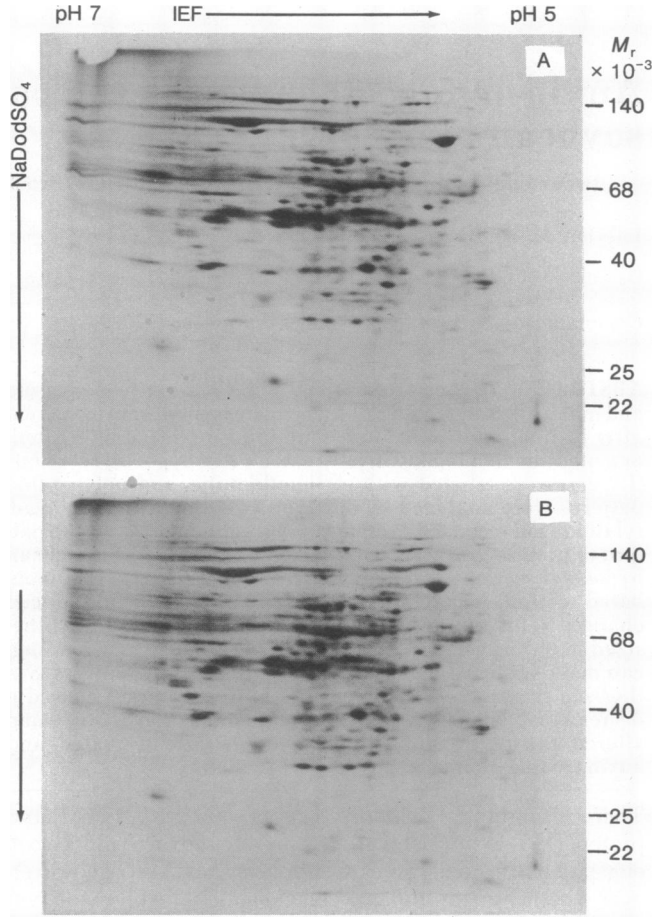

FIG. 2. Fluorographs of ^{35}S-labeled polypeptides from elongating terminal segments resolved by electrophoresis on two-dimensional polyacrylamide gels (13). Samples loaded on first-dimension gels were 54 μg of protein and 272,000 cpm for both untreated and auxin-treated tissue. Second-dimension gels were 10% polyacrylamide. (*A*) Untreated segments; (*B*) auxin-treated segments.

RESULTS

Elongating Sections. During the 5-hr period of incubation, the increases in hypocotyl length for untreated and auxin-treated sections were 8% and 28%, respectively. Because of possible wounding effects on the patterns of polypeptide synthesis in excised sections of hypocotyl tissue, we removed the terminal 1-mm segments of each section after incubation in [^{35}S]methionine. The terminal 1-mm segments of both untreated and auxin-treated sections contained approximately 70% of the [^{35}S]methionine incorporated into protein, and the ^{35}S-labeled polypeptides observed in terminal segments were significantly different from those observed in internal segments (Fig. 1). One-dimensional polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ revealed no obvious differences in the polypeptides synthesized in untreated and auxin-treated terminal segments (Fig. 1) or in whole sections without their termini removed (data not shown); however, several differences (especially evident in the range of 80,000–100,000 daltons) were observed in the polypeptide patterns of internal segments from untreated and auxin-treated elongating hypocotyl sections (Fig. 1). These results indicate that polypeptides synthesized in the terminal segments can mask subtle changes in the spectrum of polypeptides synthesized in response to auxin. The cells at the

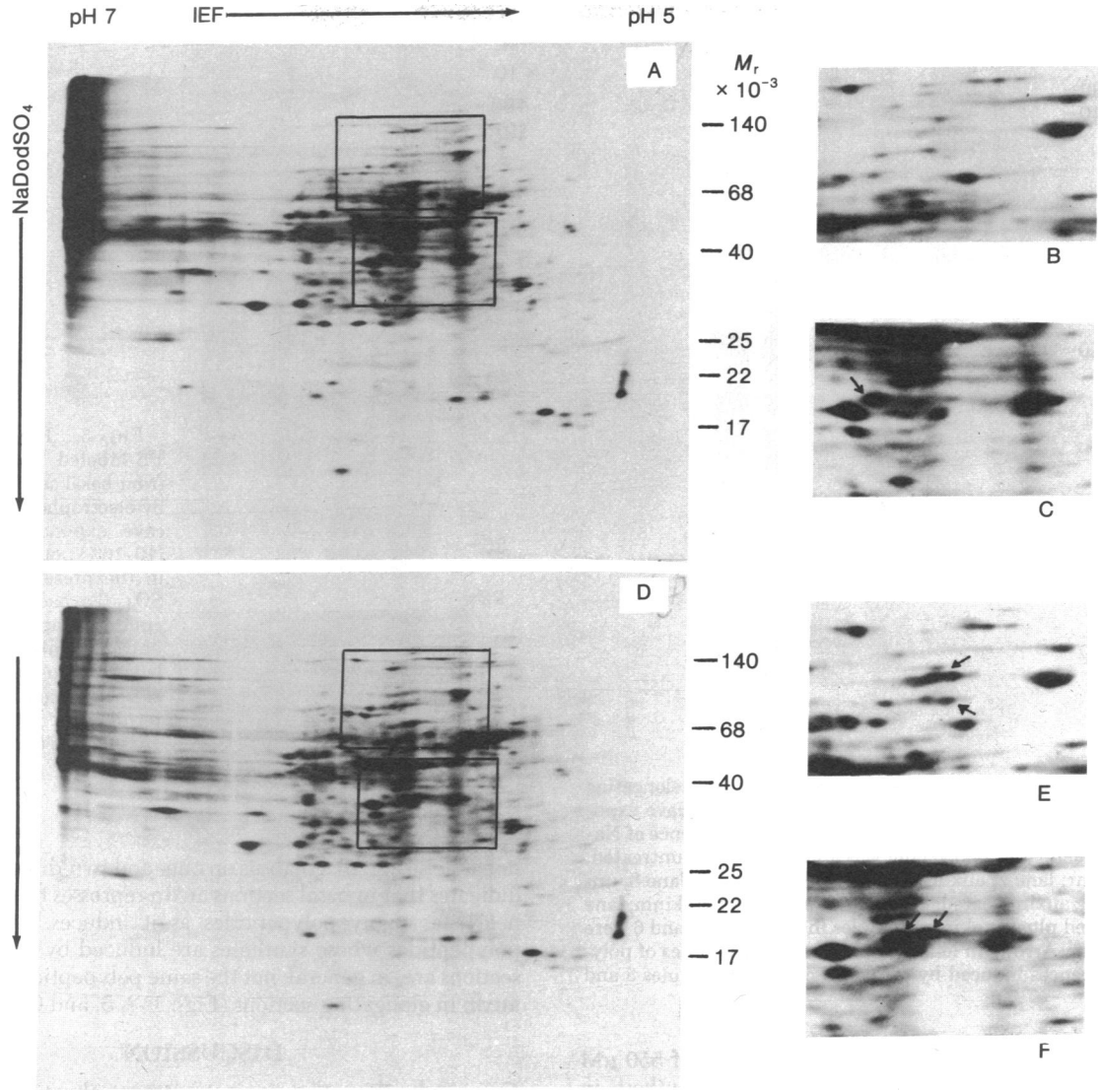

FIG. 3. Fluorographs of ^{35}S-labeled polypeptides from elongating internal segments resolved by electrophoresis on two-dimensional polyacrylamide gels. Samples loaded on first-dimension gels were 52 μg of protein in each case; 78,000 cpm was applied in the case of untreated and 118,000 cpm was applied in the case of auxin-treated tissue. Second-dimension gels were concave exponential gradient (10–16%) polyacrylamide gels. (*A*) Untreated segments; (*B*) enlargement of upper region outlined in *A*; this enlargement is taken from a similar gel in which high molecular weight polypeptides were better resolved and more intense than in *A*; (*C*) enlargement of lower region outlined in *A*; (*D*) auxin-treated segments; (*E*) enlargement of upper region outlined in *D*; this enlargement is taken from a similar gel in which high molecular weight polypeptides were better resolved and more intense than in *D*; (*F*) enlargement of lower region outlined in *D*.

ends of excised tissue sections may be more active in protein synthesis in response to wounding or may possess a higher specific activity of [^{35}S]methionine. This higher specific activity may result from [^{35}S]methionine being primarily absorbed at the cut ends, where a cuticle does not restrict amino acid uptake.

Most of the [^{35}S]methionine was incorporated into polypeptides in the molecular weight range of 30,000–60,000, and we utilized two-dimensional gel electrophoresis (13) to enhance the resolution of these polypeptides. Comparison of Fig. 2 *A* and *B* reveals that the patterns of polypeptides synthesized in untreated and auxin-treated elongating terminal segments are similar although minor differences in intensity of some polypeptides are evident. The overall patterns of polypeptides synthesized in untreated and auxin-treated elongating internal segments are similar, but differences in the intensity of some polypeptides are more striking than those observed with terminal segments, and the synthesis of several polypeptides appears to be strongly activated or repressed by auxin treatment (Fig. 3).

The most significant differences between untreated and auxin-treated elongating internal segments observed on two-dimensional gels are the appearance of a polypeptide of approximately 100,000 daltons with an isoelectric point of about 6.1, the appearance of two polypeptides of approximately 40,000 daltons with isoelectric points of about 6.1 and 5.9, and the disappearance of a polypeptide of approximately 40,000 daltons with an isoelectric point of about 6.3 in auxin-treated tissue (Fig. 3).

Because auxin-induced proteins that are required for cell elongation may possess short half-lives (18, 19), we attempted to analyze the polypeptides synthesized in untreated and auxin-treated elongating internal segments labeled for 1 hr rather than 3 hr. The polypeptide patterns of the elongating internal segments labeled for 1 hr were similar but not identical to those observed after labeling for 3 hr (Fig. 4); however, a polypeptide of approximately 100,000 daltons synthesized in response to auxin was observed in both 1- and 3-hr labeling periods.

Because cytokinins have been shown to inhibit auxin-induced

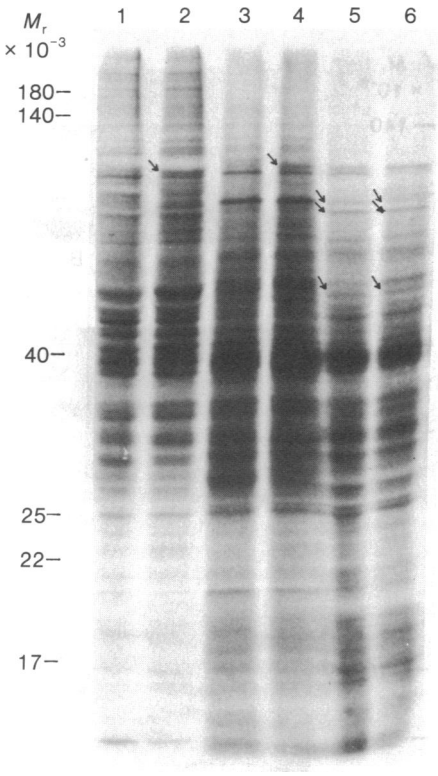

FIG. 4. Fluorograph of ^{35}S-labeled polypeptides from elongating internal segments resolved by electrophoresis on a concave exponential gradient (10–16%) polyacrylamide gel in the presence of NaDodSO$_4$. To each lane, 14,000 cpm was applied. Lane 1, untreated, labeled for 1 hr; lane 2, auxin-treated, labeled for 1 hr; lane 3, untreated; lane 4, auxin-treated; lane 5, untreated plus cytokinin; lane 6, auxin-treated plus cytokinin. Samples in lanes 3, 4, 5, and 6 were labeled for 3 hr. Arrows in lanes 5 and 6 indicate examples of polypeptide differences induced by cytokinin compared to lanes 3 and 4.

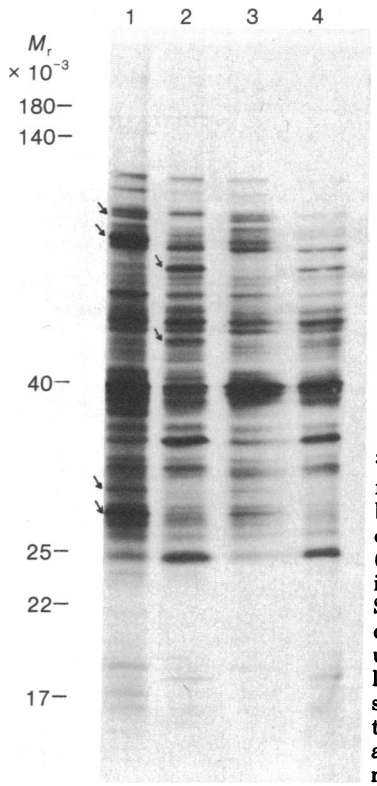

FIG. 5. Fluorograph of ^{35}S-labeled polypeptides from basal sections resolved by electrophoresis on a concave exponential gradient (10–16%) polyacrylamide gel in the presence of NaDodSO$_4$. To each lane, 28,000 cpm was applied. Lane 1, untreated internal segments; lane 2, auxin-treated internal segments; lane 3, untreated terminal segments; lane 4, auxin-treated terminal segments.

cell elongation (8–10), we investigated the effect of 550 μM isopentenyladenosine on the patterns of protein synthesis in untreated and auxin-treated elongating hypocotyl sections. This concentration of cytokinin inhibited auxin-induced cell elongation by 70% during the 5-hr period of incubation and inhibited [^{35}S]methionine incorporation into protein by 60% and 50% for untreated and auxin-treated sections, respectively. One-dimensional polyacrylamide gel analysis of polypeptides synthesized in response to cytokinin in the presence or absence of auxin is shown in Fig. 4. Isopentenyladenosine treatment results in some change in the patterns of protein synthesis in internal segments of both untreated and auxin-treated hypocotyl sections.

Mature Sections. In contrast to elongating hypocotyl sections, mature (basal) sections of soybean hypocotyl fail to elongate when exposed to auxin; instead, this tissue responds to auxin treatment by increases in the levels of RNA (20) and protein synthesis (21) relative to untreated sections. We have found that basal sections do not display the preferential labeling of polypeptides in severed ends that we observed with elongating sections; 75% of the [^{35}S]methionine was incorporated into internal segments in both untreated and auxin-treated basal sections. Terminal 1-mm and internal segments displayed similar patterns of protein synthesis, and auxin-induced differences were striking as revealed by both one-dimensional (Fig. 5) and two-dimensional (Fig. 6) polyacrylamide gel electrophoresis. Synthesis of a large spectrum of polypeptides was promoted and repressed in response to auxin treatment as evidenced by the polypeptide patterns observed on two-dimensional gels (Fig. 6). Analysis on one- and two-dimensional gels indicates that in basal sections auxin represses the synthesis of nearly as many polypeptides as it induces. Furthermore, polypeptides whose syntheses are induced by auxin in basal sections are, in general, not the same polypeptides induced by auxin in elongating sections (Figs. 1–3, 5, and 6).

DISCUSSION

Our results show that auxin treatment alters the pattern of protein synthesis in both elongating and basal sections of soybean hypocotyl by promoting the synthesis of specific polypeptides, by repressing the synthesis of certain polypeptides, or by causing a charge modification of polypeptides synthesized in the absence of auxin. Changes in the patterns of protein synthesis induced by auxin are also shown to be tissue specific, because elongating and basal sections synthesize largely different polypeptides in response to auxin. In addition, we have shown that cytokinin alters the pattern of protein synthesis in both untreated and auxin-treated elongating hypocotyl sections.

In order to clearly observe auxin-induced changes in the spectrum of polypeptides synthesized in soybean hypocotyl sections, it is necessary to employ high-resolution techniques, such as electrophoresis on one-dimensional exponential gradient gels and two-dimensional polyacrylamide gels. With elongating sections, it is also important to remove the terminal segments because polypeptides synthesized in the cut ends may be induced by wounding, and these polypeptides can mask the changes in the spectrum of polypeptides induced by auxin. Failure to detect differences in polypeptides synthesized in untreated and auxin-treated *Avena* coleoptile sections reported by Bates and Cleland (22) could have resulted from insufficient resolution of polypeptides on low percentage one-dimensional polyacrylamide gels as well as masking of auxin-induced polypeptide changes by polypeptides synthesized in response to wounding, because wounded termini were not removed and

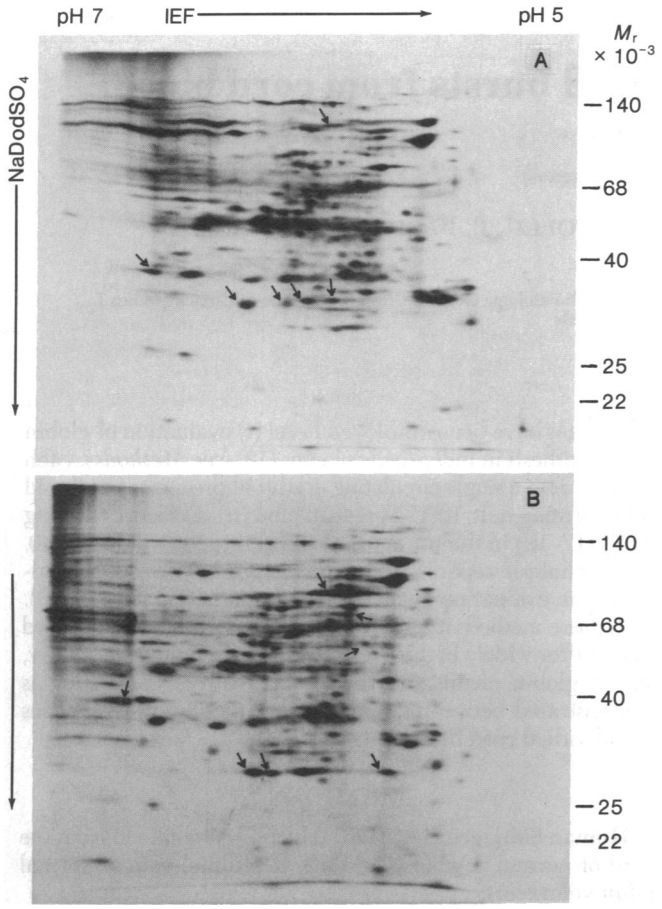

FIG. 6. Fluorographs of ^{35}S-labeled polypeptides from basal internal segments resolved by electrophoresis on two-dimensional polyacrylamide gels. Samples loaded on first-dimension gels were 18 μg of protein in each case; 424,000 cpm was applied in the case of untreated and 409,000 cpm was applied in the case of auxin-treated tissue. Second-dimension gels were 10% polyacrylamide. (*A*) Untreated segments; (*B*) auxin-treated segments.

the sections could have been wounded extensively by peeling off the cuticle (23). However, it is also possible that coleoptile tissue, unlike hypocotyl tissue, synthesizes an identical spectrum of polypeptides in the presence or absence of auxin. It is likely that auxin-induced changes in the patterns of proteins synthesized in elongating tissue sections are quantitative (i.e., differences in the levels of synthesis of specific polypeptides) rather than qualitative (i.e., differences in the types of polypeptides), because untreated sections exhibit a low rate of cell elongation in the absence of auxin, and application of auxin simply enhances this rate. In the basal sections of soybean hypocotyl, auxin causes a striking difference in the pattern of protein synthesis observed on both one- and two-dimensional gels.

That auxin causes alterations in the pattern of protein synthesis in tissue sections was suggested earlier by studies using subapical sections of pea epicotyl (24). Patterson and Trewavas were able to demonstrate auxin-induced differences in the overall pattern of protein synthesis by utilizing a technique of limited resolution, analysis of $^{14}C/^{3}H$ ratios of labeled polypeptides fractionated on columns of Sephadex G-100 or G-150. Our results indicate that auxin-induced changes in the patterns of protein synthesis would not have been detected by utilizing techniques of limited resolution (e.g., gel filtration or uniform-percentage, one-dimensional polyacrylamide gel electrophoresis) with elongating sections of soybean hypocotyl; however, with basal sections such changes might be observed even with techniques of low resolving power.

Further research is required to determine: (*i*) whether auxin-induced changes in the patterns of polypeptide synthesis result from changes in the population of specific messenger RNAs, (*ii*) what specific enzymatic activities or structural roles these polypeptides represent, and (*iii*) whether these changes in polypeptide patterns represent changes in "growth-limiting" or "growth-essential" proteins (18, 19) required for auxin-induced growth. Changes in the population of specific messenger RNAs can be tested by *in vitro* translation experiments, and polypeptides synthesized *in vitro* can be compared to those observed in our *in vivo* labeling experiments. *In vitro* translation experiments will permit analysis of short-term auxin treatment because problems such as uptake of label required for *in vivo* experiments will be alleviated.

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