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Cytokinin-controlled Indoleacetic Acid Oxidase Isoenzymes in Tobacco Callus Cultures[1]

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ABSTRACT

Indoleacetic acid oxidase in tobacco callus tissues (*Nicotiana tabacum* L., cultivar White Gold) was resolved into seven anionic isoenzymes by polyacrylamide gel disc electrophoresis. Different concentrations of kinetin and zeatin in the presence of indoleacetic acid affected the level of this enzyme, particularly two fast-moving isoenzymes, A_5 and A_6. The optimal concentration of kinetin was 0.2 μM; increasing concentrations above this level progressively lowered the total activity of indoleacetic acid oxidase and repressed the development of isoenzymes A_5 and A_6. Actinomycin D and cycloheximide inhibited the development of these two isoenzymes under the influence of 0.2 μM kinetin, suggesting a requirement for RNA and protein synthesis. The cytokinin-promoted indoleacetic acid oxidase isoenzymes A_5 and A_6 increased with time and paralleled the dry weight increase of tobacco callus tissues, but the total activity of indoleacetic acid oxidase per unit dry weight of tobacco callus varied with time depending on the stage of plant growth.

Little is known of the effect of cytokinin on indoleacetic acid oxidase in plants. Kinetin (6-furfurylaminopurine) was reported to lower the IAA oxidase activity in dwarf beans (11) but to increase the activity of this enzyme in lentil roots (8) and barley seedlings (7). Many factors might contribute to the inconsistency in the apparent increase or decrease of IAA oxidase activity in response to kinetin. For an effective approach, it is necessary to have a plant system which is responsive to cytokinin and permits less restricted growth both in cell enlargement and cell division, and to have an enzyme preparation free of natural cofactors and inhibitors. Consequently, I chose the tobacco callus cultures as the plant material and the disc electrophoresis technique for the separation of the enzyme. With this system I found that the level of IAA oxidase, certain specific isoenzymes in particular, was affected by cytokinin and other growth regulators and that the relationship between this enzyme and plant growth was complex, depending not only on the type, concentration, and interaction of growth regulators but also on the stage of plant growth.

[1] Contribution 455.
[2] Abbreviation: DCP: 2,4-dichlorophenol.

MATERIALS AND METHODS

Plant Material. Callus cultures of *Nicotiana tabacum* L., cultivar White Gold, were used throughout. The cultures were originally derived from the pith of the stem and maintained on a modified Murashige and Skoog medium (15, 20). The concentration of kinetin for the stock culture was 2 μM but varied in the test medium. The concentration of IAA was 6 μM for the stock culture and 10 μM for the experiments. The cultures were grown at 28 C under weak light (20 ft-c) supplied by fluorescent and incandescent lamps on a 16-hr day and 8-hr night cycle. Five pieces of tissue were placed in each 125-ml Erlenmeyer flask containing 50 ml of the medium, and five replicate flasks were used for each treatment. The cultures were grown for 3 to 27 days as required for individual experiments. Plant growth was expressed as dry weight per callus.

Preparation of Extracts. Five grams of the fresh tissue were extracted in a Potter-Elvehjem tissue homogenizer with 10 ml of phosphate buffer (0.05 M, pH 7.5) containing 0.8 M KCl and 0.05 M sodium ascorbate. The slurry was centrifuged at 20,000*g* for 10 min, and the residue was reextracted twice with fresh medium and centrifuged. The total volume of the combined extract was 25 ml. A portion of the extract was dialyzed for 22 hr against 4 liters of deionized water, which was changed twice during this period. All operations were done at 2 to 4 C. The dialyzed extract was centrifuged, and the clear supernatant was used as the enzyme source.

Electrophoresis. The disc electrophoresis procedure used was basically that of Davis (3). The Buchler analytical polyacrylamide disc electrophoresis apparatus was used. Each tube was 75 mm long and 5 mm in inner diameter. For an effective separation, the gel column was composed of two sections, the upper 10 mm of 4.5% (w/v) polyacrylamide gel and the lower 55 mm of 5.5% polyacrylamide gel. The extract, after mixing with a 60% (w/v) sucrose solution (2:1), was applied (0.2 ml) to the top of the gel just before electrophoresis. The amount of proteins per gel was from 15 to 75 μg. The temperature of the buffer in the lower reservoir was kept at 4 C during electrophoresis by circulating cold water through the cooling jacket from a refrigerated water bath. The current applied was 2 ma per gel and the electrophoresis was completed in 1.5 hr. Immediately after the completion of electrophoresis, the gel column was removed from the tube, frozen on Dry Ice, and sliced into 1-mm sections. Each section was extracted with 2 ml of 0.03 M phosphate buffer, pH 6.0, with constant shaking for 20 hr at 4 C. The anionic isoenzymes thus separated were designated as A_1 to A_7.

IAA Oxidase Assay. The tubes containing 1 mm gel slices and buffer after 20 hr extraction were brought to 37 C immediately before assay for IAA oxidase. To each tube was added 0.5 ml of a freshly prepared solution of IAA, 2,4-dichlorophenol, and $MnCl_2$. The final concentrations of IAA, DCP,[2] and $MnCl_2$ were

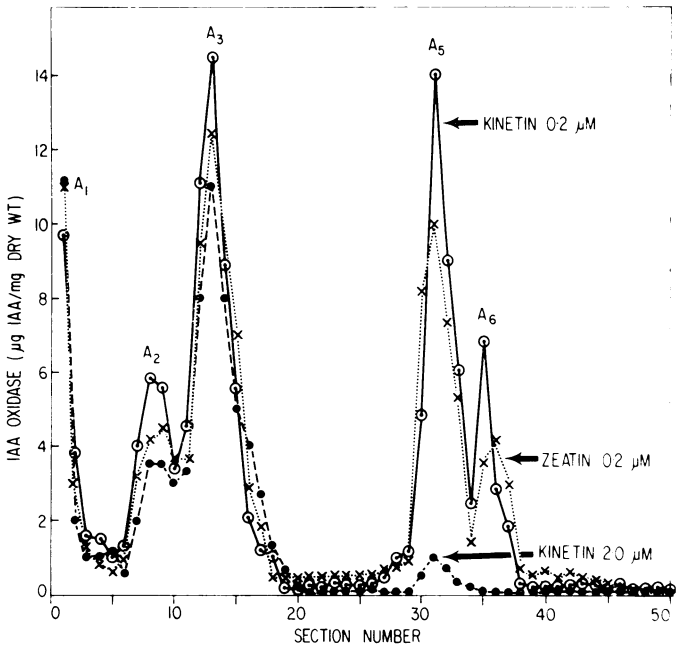

FIG. 1. Effect of concentration of kinetin and zeatin on IAA oxidase isoenzymes in tobacco callus tissues. The growth period was 24 days. IAA oxidase was expressed as μg IAA destroyed per mg dry wt in 30 min at 37 C.

Table I. *Effect of Kinetin and Zeatin on IAA Oxidase and Growth of Tobacco Callus Cultures*

The medium contained 10 μM IAA. The average initial dry weight per stock callus was 7.8 mg. The growth period was 24 days. IAA oxidase was expressed as μg or mg IAA destroyed per mg dry wt or callus in 10 min at 37 C.

Treatment	IAA Oxidase	Total IAA Oxidase	Tissue Dry Weight
	μg IAA/mg dry wt	*mg IAA/callus*	*mg/callus*
Kinetin			
0.2 μM	320	59	214
2.0 μM	168	20	118
Zeatin			
0.2 μM	356	70	240
2.0 μM	211	33	159

Table II. *Effect of Concentration of Kinetin on IAA Oxidase and Growth of Tobacco Callus Cultures*

The medium contained 10 μM IAA. The average initial dry weight per stock callus was 4.9 mg. The growth period was 24 days.

Kinetin Concn	IAA Oxidase	IAA Oxidase	Total IAA Oxidase per Callus	IAA Oxidase A_5	Proteins	Tissue Dry Weight
μM	*μg IAA/mg dry wt*	*μg IAA/μg protein*	*—fold increase*	*μg IAA/mg dry wt*	*μg/mg dry wt*	*mg/callus*
0	243	7.8	19	9.6	31	69
0.2	386	28.8	78	51.1	13	181
0.5	289	20.9	55	27.4	14	171
1.0	203	10.8	28	16.8	19	123
2.0	192	5.0	20	6.9	38	92
5.0	175	3.6	10	0	49	49

0.15, 0.1, and 0.1 mM, respectively. The tubes were incubated in a shaking water bath at 37 C. After 30 min, 5 ml of modified Salkowski reagent (10) were added. The absorbance was read at 530 nm with a Hitachi-Perkin Elmer spectrophotometer 30 min later. For the total IAA oxidase activity the same procedure was used except that 0.05 ml of the dialyzed crude extract, after being properly diluted, was added to 2.5 ml of the reaction mixture and the reaction was stopped after 10 min. The final concentrations of IAA, DCP, and $MnCl_2$ were the same as mentioned above. The enzyme activity was expressed as μg of IAA destroyed per mg dry weight of tissue at 37 C in 10 or 30 min.

The proteins in the extract were precipitated with 10% (w/v) trichloroacetic acid and quantitatively estimated by the procedure of Lowry *et al.* (16). Bovine serum albumin was used as the standard.

Kinetin, zeatin (6-(4-hydroxy-3-methylbut-*trans*-2-enyl)aminopurine) and IAA were purchased from Calbiochem. Actinomycin D was a generous gift from the Research Laboratories of Merck, Sharp and Dohme Co. Cycloheximide was obtained through the Sigma Chemical Co.

RESULTS

IAA Oxidase Isoenzymes. IAA oxidase in tobacco callus tissues was fractionated into seven anionic isoenzymes by polyacrylamide gel electrophoresis. Under the various growth conditions shown in Figure 1, however, the isoenzymes A_7 and A_4 were absent or unresolved. Isoenzyme A_4 was clearly detectable under a growth condition described in Figure 4, and isoenzyme A_7 was a fast-moving IAA oxidase induced by gibberellic acid in a later stage of growth (Lee, unpublished). Among the major IAA oxidase isoenzymes, A_1, A_2, and A_3 were consistently present, but the presence and magnitude of A_5 and A_6 varied to a great extent with the concentration of cytokinins and other types of growth regulators supplied.

Concentration Effect of Cytokinin. The tobacco callus tissues grown on a medium supplemented with 2 μM kinetin and 10 μM IAA contained none or little of IAA oxidase isoenzyme A_5 and none of A_6. When the concentration of kinetin was lowered to 0.2 μM, isoenzymes A_5 and A_6 developed and the activity of A_5 was 14 times that with 2 μM kinetin, although there was little change in the activity of other IAA oxidase isoenzymes (Fig. 1). Tobacco callus tissues grown on a medium with 0.2 μM zeatin produced a profile of anionic IAA oxidases similar to that with 0.2 μM kinetin (Fig. 1). Zeatin at 2 μM repressed A_5 and A_6 also to a similar extent as did 2 μM kinetin.

Both the activity of total IAA oxidase per unit dry weight of tissue and plant growth in terms of dry weight per callus also increased when the concentration of kinetin and zeatin was lowered from 2 to 0.2 μM (Table I). Consequently, the total activity of IAA oxidase per callus was two to three times higher with 0.2 μM than that with 2 μM cytokinin. Evidently, a higher concentration of cytokinin decreased IAA oxidase, particularly the anionic isoenzymes A_5 and A_6.

Further experiments with a series of concentrations of kinetin revealed that a low level of kinetin was definitely required for the apparent increase of IAA oxidase. The optimal concentration of kinetin was 0.2 μM (Table II). At this concentration, the activity of IAA oxidase per μg of protein or per mg dry weight of tissue and the activity of isoenzyme A_5 were the highest. So were the total IAA oxidase activity per callus and the dry weight yield of tobacco tissues. On a medium without kinetin, the tissues had a moderate rate of growth in the 24-day period and produced a low level of IAA oxidase isoenzyme A_5 (Table II). Presumably the limited growth was due to a small amount of kinetin carried over by the stock callus, which was originally grown on a kinetin-rich medium (2 μM).

Increasing the concentration of kinetin from 0.2 to 0.5, 1.0, 2.0, and 5.0 μM progressively decreased the activity of IAA oxidase and the growth of tobacco tissues in terms of dry weight increase although the total amount of extractable protein per callus was less affected (Table II). At 5.0 μM, kinetin completely repressed IAA oxidase isoenzymes A_5 and A_6 and lowered the total IAA oxidase activity per unit dry weight by 50% as compared with that at 0.2 μM. A significant decrease was also noted in the specific activity of IAA oxidase per μg of protein, and this was partially due to the increase of extractable protein per unit dry weight with increasing concentrations of cytokinin.

Thus the development of two specific isoenzymes of IAA oxidase, which in turn affected the over-all concentration of this enzyme, was dependent on the concentration of cytokinin.

Time Course Study. In the fast-growing tobacco callus cultures, the level of IAA oxidase isoenzymes A_5 and A_6 per unit dry weight of tissue increased with time, while in the control culture with 2 μM kinetin and 10 μM IAA the activity of A_5 remained at a low level throughout the experimental period. With kinetin at 0.5 μM the level of A_5 increased by 11-fold in 15 days and by 43-fold in 27 days, whereas the dry weight increase was 10- and 20-fold, respectively (Fig. 2). Isoenzyme A_6 developed later than A_5 but also increased with time. Because of the rapid growth of the callus tissues and because of the steady increase of IAA oxidase A_5 and A_6 per unit weight of tissue, the total activity of these isoenzymes per callus increased sharply during the growth period. The increase in A_5 was 3-fold in 6 days, 114-fold in 15 days, and 700-fold in 27 days.

The total IAA oxidase activity per unit dry weight of tissue showed a different trend. It increased in the first 5 days, then decreased with time for about 2 weeks and then started to increase thereafter (Fig. 3). This trend remained regardless of whether the kinetin concentration was high or low, although the activity of IAA oxidase was higher with a low concentration of kinetin. It appears that the concentration of IAA oxidase in tobacco callus tissues was determined not only by the rate of synthesis and degradation of this enzyme but also by the rate of plant growth. When the tissue just began to grow, the rate of enzyme synthesis exceeded the rate of enzyme degradation and the rate of plant growth; under this condition accumulation of IAA oxidase occurred. When the tissue reached a stage of rapid growth, the rate of growth exceeded the rate of increase of this enzyme; thus IAA oxidase per unit weight of tissue decreased. The level of IAA oxidase rose again when the rate of plant growth passed the peak.

Effect of Inhibitors. Cycloheximide (1 mg/liter), which was added to a medium containing 0.2 μM kinetin and 10 μM IAA, inhibited the development of IAA oxidase isoenzymes A_5 and A_6 (Fig. 4). This suggests that synthesis of new proteins was involved in the increase of these isoenzymes under the influence of kinetin at a low concentration. During the 24-day growth period, cycloheximide also inhibited the growth of tobacco callus by 95%.

Despite the inhibition of isoenzymes A_5 and A_6 by cycloheximide, the total activity of IAA oxidase per unit dry weight of the cycloheximide-treated tissues was higher than that of the control (Table III) and increased steadily with time for 20 days (Fig. 3). This increase probably was due to the increased activities of isoenzymes A_1, A_3, and A_4 (Fig. 4). This particular aspect of IAA oxidase has not been fully investigated; perhaps the increase was due in part to slower degradation of these isoenzymes because of inhibition of synthesis of proteolytic enzymes and in part to continued synthesis of these isoenzymes in the presence of cycloheximide at the 1 mg/liter level.

Similar experiments with actinomycin D (5 mg/liter) show that the development of IAA oxidases A_5 and A_6 were also repressed (Fig. 5). This may be regarded as an indication of a requirement for DNA-dependent RNA synthesis for the development of these

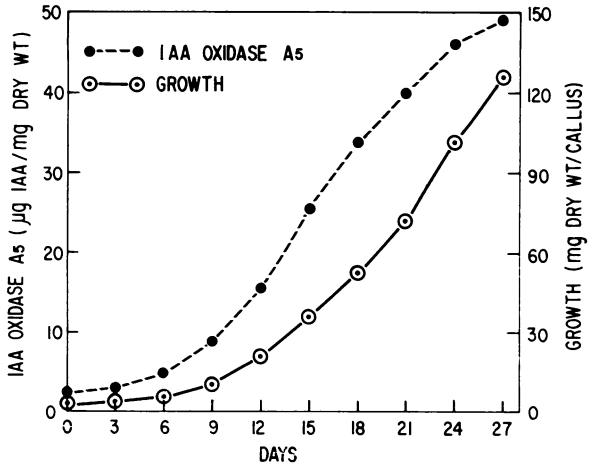

FIG. 2. Parallel increase of IAA oxidase isoenzyme A_5 and dry weight of tobacco callus tissues. The medium contained 0.5 μM kinetin and 5 μM IAA. The average initial dry weight per callus was 7.4 mg. IAA oxidase was expressed as μg IAA destroyed per mg dry wt of tissue in 30 min at 37 C.

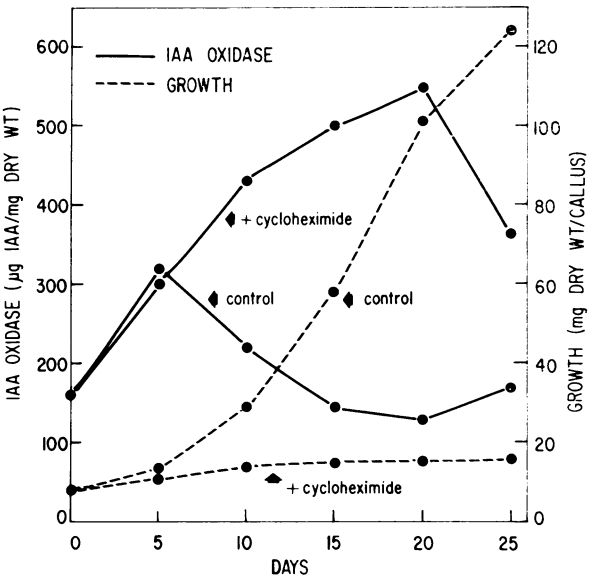

FIG. 3. Changes in total IAA oxidase activity at different stages of growth of tobacco callus tissues with and without cycloheximide. The medium contained 1.5 μM kinetin and 10 μM IAA. The concentration of cycloheximide was 1 mg/liter. The average initial dry weight per callus was 7.8 mg. IAA oxidase was expressed as μg IAA destroyed per mg dry wt of tissue in 10 min at 37 C.

isoenzymes affected by a low concentration of cytokinin. Actinomycin D also lowered the activity of isoenzymes A_2, A_3, and possibly A_4 (Fig. 5), although the inhibition was not as severe as that of A_5 and A_6. Furthermore, actinomycin D inhibited growth of tobacco callus cultures and synthesis of proteins (Table III).

DISCUSSION

A remarkable feature of the biological activity of cytokinin is the diverse effect of different concentrations. This was first shown by Skoog and Miller (23) in the regulation of growth and differentiation in tobacco tissue cultures. Results from the present study show evidence for the control of IAA oxidase by different concentrations of cytokinin. The quantitative and negative association of the activity of anionic IAA oxidase isoenzymes A_5 and

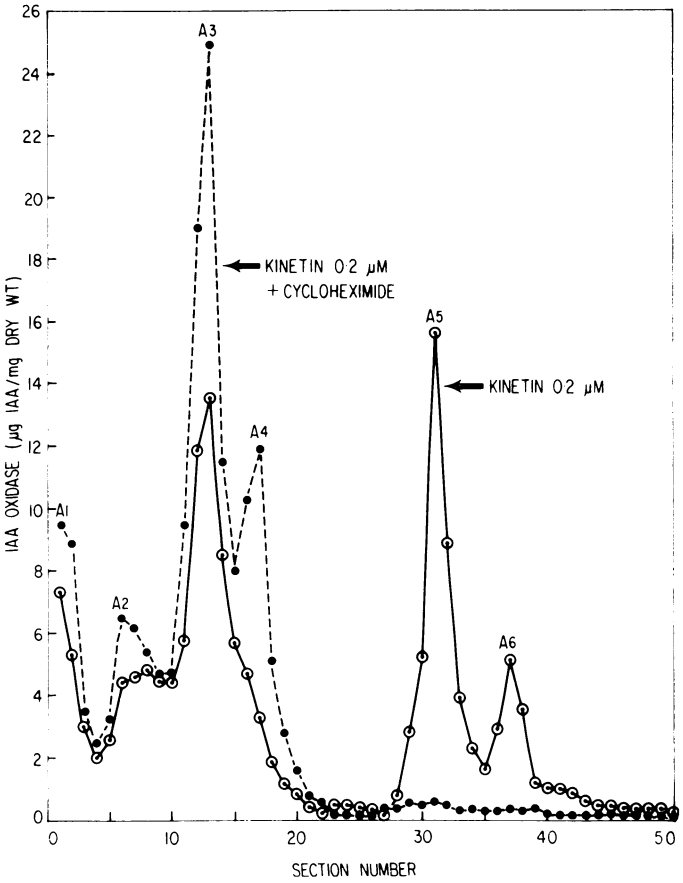

FIG. 4. Effect of cycloheximide on kinetin-promoted IAA oxidase isoenzymes in tobacco callus tissues. The medium contained 10 μM IAA. The concentration of cycloheximide was 1 mg/liter. The growth period was 24 days. IAA oxidase was expressed as μg IAA destroyed per mg dry wt of tissue in 30 min at 37 C.

Table III. *Effects of Cycloheximide and Actinomycin D on IAA Oxidase and Growth of Tobacco Callus Cultures*

Cycloheximide (1 mg/liter) and actinomycin D (5 mg/liter) were filter-sterilized and added to the medium containing 0.2 μM kinetin and 10 μM IAA. The average initial dry weight per stock callus was 7.7 mg. The growth period was 22 days.

Treatment	IAA Oxidase	Total IAA Oxidase		Proteins	Tissue Dry Weight
	μg IAA/mg dry wt	*mg IAA/callus*	*—fold increase*		
Control	385	75	74	10.2	30
Cycloheximide	532	9	8	0.7	1
Control	342	73	72	11.6	30
Actinomycin D	209	4	3	0.5	1.5

A_6 with the concentration of kinetin above the 0.2 μM level is of particular interest. The inhibition of the cytokinin-controlled isoenzymes A_5 and A_6 by actinomycin D and cycloheximide is suggestive of a requirement for RNA and protein synthesis for the increase of these isoenzymes. Cytokinin has been known to increase RNA and protein synthesis in plants (1, 2, 21), while higher concentrations of cytokinin inhibited RNA synthesis (2, 26), particularly the ribosomal RNA (26). Presumably the increase or decrease of IAA oxidase isoenzymes A_5 and A_6 was a result of action of different concentrations of cytokinin at the site of RNA synthesis. A derepressor function of cytokinin was suggested by Osborne (22). On the other hand, kinetin was reported to suppress the activity of nucleases (17, 24). Thus it is also possible that the development of IAA oxidase isoenzymes A_5 and A_6 was due to a multiple effect of cytokinin on synthesis and degradation of RNA.

Peroxidase has long been considered a component of IAA oxidase (5, 9, 13, 25). The enzyme preparations from different treatments reported in this paper also possessed peroxidase activity, and the peaks of IAA oxidase activity of isoenzymes A_5 and A_6 coincided with those of peroxidase activity determined by densitometry with benzidine as the substrate. Evidently, under the same growth conditions as described earlier, kinetin at low concentrations enhanced the development of two fast-migrating anionic peroxidases but at higher concentrations inhibited them. With tobacco pith tissues cultured *in vitro* Galston *et al.* (6) reported an increase in total peroxidase activity by low concentrations (0.02–2.0 mg/liter) of kinetin but observed no change in peroxidase isoenzyme patterns separated by starch gel electrophoresis. In *Pelargonium* tissues, kinetin was found to inhibit the development of an isoperoxidase (14). The quantitative data of IAA oxidase isoenzymes obtained in this work and others (Lee, unpublished) with different concentrations and types of growth substances permit a close comparison of this enzyme with peroxidase, and the detailed results will be reported later.

Galston *et al.* (6) reported an interaction of kinetin and IAA on peroxidase in tobacco pith tissues in which the enhanced peroxidase activity by kinetin was dependent on the level of IAA. In the present work, the increase of IAA oxidase isoenzymes A_5 and A_6 by low concentrations of kinetin also required IAA. Furthermore, addition of GA_3 in the presence of appropriate concentrations of kinetin and IAA further increased the level of IAA oxidase, particularly isoenzymes A_5 and A_6 (Lee,unpublished). Evidently the development of isoenzymes A_5 and A_6, which in turn affected the total concentration of IAA oxidase, was controlled not only by cytokinin but also by auxin and gibberellin. This is a good

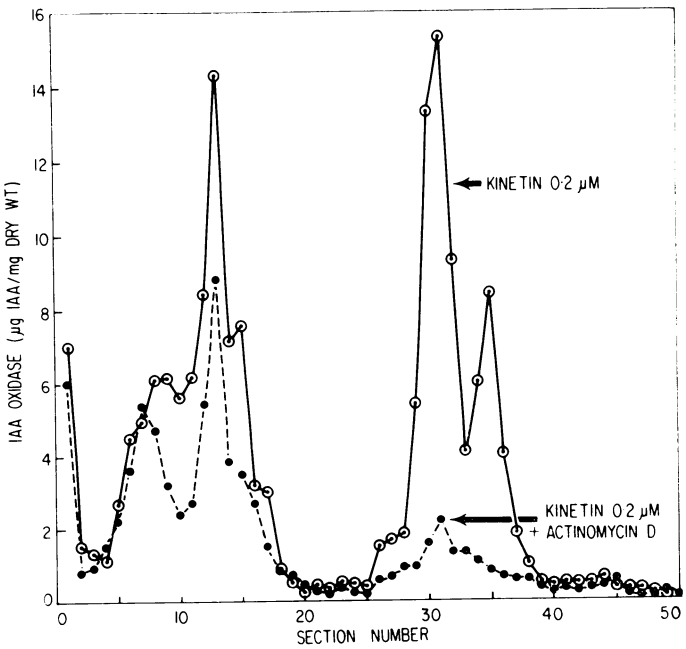

FIG. 5. Effect of actinomycin D on kinetin-promoted IAA oxidase isoenzymes in tobacco callus tissues. The medium contained 10 μM IAA. The concentration of actinomycin D was 5 mg/liter. The growth period was 20 days. IAA oxidase was expressed as μg IAA destroyed per mg dry wt of tissue in 30 min at 37 C.

example for multiple control of an enzyme by plant growth hormones.

The multiple control of IAA oxidase isoenzymes by plant growth hormones may have far-reaching implications because all of these substances profoundly affected the growth of tobacco tissues in this defined system. Under different conditions these isoenzymes may function in different capacities. For example, different isoperoxidases from other plant tissues were found to have different catalytic properties such as pH optima, specific activities, and affinity toward different substrates and inhibitors (4, 12, 18, 19). In the present work, the isoenzyme A_5, which was a major IAA oxidase most responsive to cytokinin, auxin, and gibberellin, was distinctly different from another major isoenzyme A_3, not only in electrophoretic mobility and response to different growth hormones but also in precipitation with different concentrations of $(NH_4)_2SO_4$. Further purification and characterization of these isoenzymes may reveal any differences in biochemical properties which relate to the metabolism of IAA. This work is being undertaken.

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