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Inhibition of 2,4-Dichlorophenoxyacetic Acid Conjugation to Amino Acids by Treatment of Cultured Soybean Cells with Cytokinins

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ABSTRACT

Kinetin, and all other cytokinins tested, inhibited the conjugation of [^{14}C]2,4-dichlorophenoxyacetic acid (2, 4-D) to amino acids when supplied simultaneously with the 2,4-D to cultured soybean cells. Upon transfer to hormone-free medium, the cytokinin-treated cells released more of their [^{14}C]2,4-D than did the control cells. Initial exposure to low 2,4-D and high kinetin levels resulted in the greatest release of 2,4-D upon subsequent transfer. The observed alteration in 2,4-D metabolism did not seem to be correlated with growth rate. Appropriate treatment of soybean cells with kinetin resulted in 2,4-D metabolism that resembled the 2,4-D metabolism of embryogenic carrot cells. However, no new morphological structures were observed in these soybean cultures, indicating that other factors are related to the failure of soybean cells to regenerate in culture.

A reduction in the level of exogenously supplied auxin often allows morphogenesis in cultures of higher plant cells (1–3, 9, 10, 16, 17, 19, 21–23, 25). For this reason, the study of auxin metabolism seems important in understanding the biochemical events which lead to regeneration in cell cultures. In previous work (13), substantive differences were demonstrated in the metabolism of 2,4-D between highly embryogenic carrot (*Daucus carota*) and nonregenerable soybean (*Glycine max*) cells (also *cf* 4, 5). Whereas soybean cells conjugated a high percentage of their 2,4-D to amino acids, carrot cells contained primarily free 2,4-D. After long-term exposure to labeled 2,4-D, carrot cells released a much higher percentage of the label upon transfer to 2,4-D-free (embryogenic) medium than did soybean cells. The loss of label to the medium was due to the loss of free 2,4-D and not 2,4-D-amino acid conjugates.

Because the 2,4-D-amino acid conjugates retained by soybean cells might well inhibit morphogenesis (7, 20), it seemed desirable to modify the metabolism of 2,4-D in soybean cells so that it would more closely resemble that in carrot cells. Previous results (13) indicated that, if 2,4-D-amino acid conjugation were inhibited, the relative amount of free 2,4-D would thereby increase and the cells would readily release the 2,4-D upon transfer to 2,4-D-free medium. This paper presents the results of experiments designed to investigate the conjugation of 2,4-D in soybean cells. Several cytokinins are shown to modify the formation of 2,4-D-amino acid conjugates and the subsequent release of 2,4-D.

MATERIALS AND METHODS

Maintenance of Cell Cultures. Carrot and soybean cells were routinely maintained in liquid suspension in B5 medium as described by Gamborg *et al.* (8). The carrot cells were obtained from D. Dougall (W. A. Jones Cell Science Center) and the Wayne soybean cells were obtained from callus initiated from hypocotyl tissue of 7-day-old seedlings grown from seed purchased through Wilkins Seed Grain Co., Pontiac, IL. The carrot cells were subcultured every 3.5 days at 15 mg fresh weight/ml into B5 containing 0.1 μg/ml 2,4-D and the soybean cells were subcultured every 7 days into B5 containing 2 μg/ml 2,4-D. In some experiments, Shenk and Hildebrandt's (SH) medium (18) was used.

Analysis of 2,4-D Metabolites. Cell extraction and metabolite analysis were according to Feung *et al.* (6) and Hamilton *et al.* (11), and the procedures are described in detail in a previous paper (13). Briefly, the cells were harvested and extracted in ethanol; the ethanol extracts were acidified to pH 3 and re-extracted with diethyl ether. Free and conjugated 2,4-D in the ether extracts were separated by TLC on silica gel thin-layer plates developed with isopropanol:ammonia:H_2O (8:1:1, v/v/v). Labeled metabolites were located using a β-camera radiochromatogram scanner (Beta Camera model 6000, Baird Atomic) and quantitated by liquid scintillation using Insta-Gel (Packard Co.) liquid scintillation cocktail. In 2,4-D release studies, the cells were collected by vacuum filtration, washed, and placed directly in scintillation vials. Radiolabeled [2-^{14}C]2,4-D was obtained from Amersham-Searle at a specific radioactivity of about 30 mCi/mmol.

RESULTS

Effect of Kinetin on 2,4-D Metabolism. Table I shows the results of an experiment where soybean cells were given 9 μM [^{14}C]2,4-D in the presence or absence of 5 μM kinetin. The metabolism of the 2,4-D was monitored after 7 days, and the cells then were transferred to hormone-free medium. The amount of label lost to the medium was determined. Control cells showed the usual high percentage of 2,4-D-amino acid conjugate in the ether extract (70%). Treatment with kinetin resulted in a significant reduction in the relative amount of conjugate formed (42%) and an increase in the percentage of free 2,4-D (51%). After 4 h in hormone-free SH medium, the control cells had lost only 14% of their initial label, whereas the kinetin-treated cells had lost 60%. After 4 h, the intracellular metabolites under both treatment conditions showed the same relative percentages of free and conjugated 2,4-D. This is probably due to the fact that free 2,4-D was released from the cells, as shown previously (13).

Dependence on Cytokinin Concentration. Soybean cells were inoculated into SH medium containing 10 μM [^{14}C]2,4-D with various concentrations of either zeatin or kinetin. After 7 days, they were monitored for growth (Fig. 1A) and 2,4-D metabolism (Fig. 1B). Both cytokinins caused a decline in the growth achieved, although, even at the highest concentration tested (5 μM), the cells more than doubled in fresh weight during the 7-day period. Both

Table I. *Effect of Kinetin on 2,4-D Metabolism and Release by Soybean Cells*

Wayne soybean cells were inoculated at 20 mg fresh weight/ml into SH medium containing 9 μM 2,4-D with or without 5 μM kinetin. After 7 days, the cells were again inoculated into the same media except that [^{14}C]2,4-D was used. After 7 days, the cells were harvested and a portion was extracted and later analyzed for 2,4-D metabolites. Another portion was transferred to SH medium without hormones and then the metabolites were analyzed after 4 h.

Medium	Final Fresh Weight	Uptake of Radioactivity after 7 Days		Ether-extractable Metabolites after 7 Days		Radioactivity Released from Cells after 4 h	Ether-extractable Metabolites after 4 h	
		Ether-extractable Fraction	Other Fractions	Free 2,4-D	2,4-D-amino acids		Free 2,4-D	2,4-D-amino acids
	g	*10^6 dpm/g fresh wt*		*%/ether fraction*		%	%	
SH + 9 μM 2,4-D	1.721	0.70	0.75	19	70	14	12	75
SH + 9 μM 2,4-D + 5 μM kinetin	0.377	5.12	1.20	51	42	60	16	73

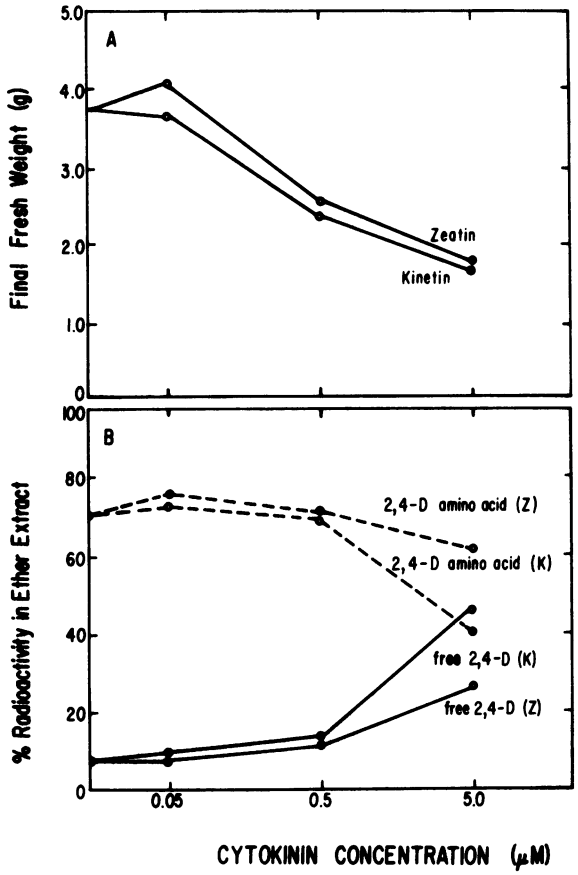

FIG. 1. Effects of kinetin and zeatin on 2,4-D metabolism in soybean cells. Wayne soybean cells previously grown in SH medium containing 10 μM 2,4-D were inoculated at 20 mg fresh weight/ml into SH medium containing 10 μM [^{14}C]2,4-D with various concentrations of zeatin (z) or kinetin (k). After 7 days, the cells were harvested, fresh weights were determined, and the 2,4-D metabolites were analyzed.

cytokinins caused a decline in the amount of 2,4-D-amino acid conjugate formed relative to the amount of free 2,4-D present. This effect was not found until the concentration of cytokinin reached 5.0 μM, even though both cytokinins considerably inhibited growth at concentrations of 0.5 μM. Moreover, although kinetin was more effective than zeatin in reducing conjugate formation, both cytokinins inhibited growth equally. This comparison indicates that the observed alteration in 2,4-D metabolism did not arise because of the inhibition of growth. Other cytokinins were also tested for their effects on 2,4-D release. Both N^6-benzyladenine and N^6-(Δ^2-isopentenyl)adenine enhanced release but neither was superior to kinetin, even at high (160 μM) concentrations (data not shown).

Effect of Varied 2,4-D Concentration. Table II shows the results of three separate experiments in which soybean cells were grown at various concentrations of [^{14}C]2,4-D along with varying kinetin levels. After 7 days, the cells were harvested, fresh weights were determined, and the uptake of radiolabel was measured. The cells then were transferred to hormone-free medium for 4 h, and release of label into the medium was determined. The cells grew well under all hormonal conditions. In contrast to the results of Figure 1, the higher kinetin concentrations were generally more favorable to growth. The interaction between 2,4-D and kinetin levels with regard to growth seems complex, especially when 5 and 10 μM 2,4-D are compared in Experiment 1 of Table II. The cells took up a greater percentage of the 2,4-D when it was supplied at the lower levels. Kinetin did not influence 2,4-D uptake except at the 10 to 20 μM 2,4-D levels where it also stimulated growth. The greatest percentage of release obtained in these experiments was about 60%, even when high levels of kinetin or high kinetin to 2,4-D ratios were employed. In general, the lower 2,4-D to kinetin ratios showed the greatest release of label.

Comparison of Carrot and Soybean Cells. Inasmuch as carrot cells were used as a model system for regeneration, it was important to compare the metabolism of 2,4-D in carrot and soybean cells under identical conditions. Carrot or soybean cells were exposed to 0.5 μM [^{14}C]2,4-D, with or without 5 μM kinetin, for 7 days prior to transfer to SH medium without hormones. Table III shows that all treatments resulted in much the same uptake of label, although kinetin-treated carrot cells took up the least amount. Four h after transfer to hormone-free medium, soybean cells not pretreated with kinetin released only 17% of their label into the medium, whereas comparably treated carrot cells lost 75%. Soybean cells grown with kinetin, however, lost 73%, compared with a value for carrot of 84%. Kinetin clearly made soybean cells much more equivalent to carrot cells with regard to this aspect of 2,4-D metabolism.

DISCUSSION

The metabolism of auxin may play a critical role in the regeneration of plants from cell culture because removal of auxin from the growth medium often induces morphogenesis in callus or suspension cultures (1–3, 9, 10, 16, 17, 19, 21–23). Highly embryogenic carrot cells have served well as a model for the study of the biochemical events which accompany regeneration (12, 14). In a

Table II. *Effect of Various 2,4-D and Kinetin Concentrations on Release of 2,4-D from Soybean Cells*

Wayne soybean cells, growing in SH medium containing 10 μg/ml 2,4-D were inoculated at 20 mg fresh weight/ml into 40 ml SH medium containing various concentrations of 2,4-D and kinetin. The cultures were grown for 7 days, harvested, washed, and transferred to 160 ml SH medium without hormones. After 4 h, loss of intracellular radiolabel was monitored. Three separate experiments were carried out. Initial fresh weights for Experiments 1 and 2 were 0.8 g but, for Experiment 3, where twice the volume of medium was used, it was 1.6 g.

Experiment	Concentration of 2,4-D	Concentration of Kinetin	2,4-D:Kinetin	Final Fresh Weight	2,4-D	
					Taken up by 7 Days	Released by 4 h
	μM	μM	ratio	g	%	
1	5	1.25	4:1	3.11	69.4	50.2
		2.25	2:1	2.67	66.5	50.6
		5.0	1:1	2.19	65.9	56.7
		10.1	1:2	2.24	69.1	55.6
	10	2.5	4:1	1.34	39.2	43.0
		5.0	2:1	1.80	48.1	48.7
		10.0	1:1	1.86	50.9	52.6
		20.0	1:2	2.04	56.9	56.7
	20	5.0	4:1	1.22	27.3	41.8
		10.0	2:1	1.52	34.1	47.4
		20.0	1:1	1.59	36.1	49.1
		40.0	1:2	1.83	40.5	55.8
2	0.5	50	1:100	4.12	89.1	62.1
		100	1:200	4.46	90.2	60.8
		150	1:300	4.71	92.1	58.9
		200	1:400	4.50	91.9	57.5
	1.0	50	1:50	4.21	90.0	57.9
		100	1:100	4.22	92.5	60.8
		150	1:150	4.66	93.3	56.6
		200	1:200	4.91	93.9	57.4
3	0.5	6.25	1:12.5	4.13	83.8	59.9
		12.5	1:25	4.57	84.2	60.3
		25.0	1:50	4.99	85.3	61.7
		50.0	1:100	5.57	85.8	59.9

Table III. *Comparison of 2,4-D Metabolism in Carrot and Soybean Cells*

Wayne soybean (growing in SH medium with 10 μg/ml 2,4-D) or carrot cells (growing in B5 medium with 0.1 μg/ml 2,4-D) were washed and inoculated at 20 mg fresh weight/ml into SH medium containing 0.5 μM [^{14}C]2,4-D with or without 5 μM kinetin. After 7 days, the cells were harvested, washed, and transferred to SH medium without hormones at 2 mg fresh weight/ml.

Cell Culture	Radioactivity	
	Uptake of label during 7 days	Release of label after 4 h
	%	
Soybean		
SH + 0.5 μM 2,4-D	79	17
SH + 0.5 μM 2,4-D + 5 μM kinetin	85	73
Carrot		
SH + 0.5 μM 2,4-D	73	75
SH + 0.5 μM 2,4-D + 5 μM kinetin	63	84

previous paper (13), the metabolism of 2,4-D was compared in nonregenerable soybean cells and in the carrot cell system. Distinct differences were found. Carrot cells conjugated far less 2,4-D to amino acids and released 2,4-D much more rapidly into auxin-free (embryogenic) medium. Because retained 2,4-D-amino acid conjugates might possess or give rise to auxin activity which would inhibit morphogenesis (7, 15), it seemed reasonable that this difference between carrot and soybean cells could be related to the failure of soybean cells to regenerate. For this reason, the regulation of 2,4-D-amino acid conjugation was studied to determine whether an alteration in the pattern of conjugation would lead to enhanced release of auxin from the cells and perhaps lead to morphogenesis.

Our results show that an increase in the intracellular level of free 2,4-D through the inhibition of conjugation by kinetin does indeed lead to enhanced release of the auxin upon transfer to hormone-free medium. All the cytokinins tested caused a similar change in 2,4-D metabolism. Kinetin seemed to be most effective in inhibiting conjugation and enhancing 2,4-D release. The change in 2,4-D metabolism did not appear to result from an effect on growth. In fact, the relationship between 2,4-D concentration, cytokinin concentration, and growth was rather complex in these cells. The effects of cytokinin on growth rate and on 2,4-D metabolism appeared to be independent. Witham (24) already has reported kinetin inhibition of 2,4-D-promoted growth.

The relative concentrations of cytokinin and 2,4-D did influence the rate of 2,4-D release upon transfer to hormone-free medium. Regardless of (*a*) the cytokinin used, (*b*) the relative ratio of 2,4-D to cytokinin, or (*c*) the absolute concentration of 2,4-D and cytokinin, the percentage of 2,4-D released at short times after transfer to 2,4-D-free medium never exceeded 60%. From this result, it appears that the cytokinin effect on 2,4-D release is saturable.

Of course, our experiments do not provide any evidence about the biochemical mechanism that results in the inhibition of conjugate formation. Cytokinin might be inhibiting the activity of the 2,4-D-amino acid conjugating enzyme(s) in some way. This inhi-

bition could be part of the mechanism of action of cytokinins in these cells, in particular if the same effect occurred with regard to the natural intracellular auxins. The resultant higher free auxin levels might in turn lead to many other biochemical events. Skoog and Miller (19) demonstrated a clear interaction between auxin and cytokinin with regard to organogenesis in certain plant tissue cultures. Such an interaction might occur because of an alteration in auxin metabolism caused by the cytokinin.

As shown by the present data, appropriate treatment of soybean cells with cytokinin (kinetin) resulted in auxin (2,4-D) metabolism that closely resembled the 2,4-D metabolism of embryogenic carrot cells. However, no new morphological structures were observed in these soybean cultures, indicating that other factors are related to the failure of soybean cells to regenerate.

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