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Metabolism of 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Soybean Root Callus[1]

EVIDENCE FOR THE CONVERSION OF 2,4-D AMINO ACID CONJUGATES TO FREE 2,4-D

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ABSTRACT

An auxin-requiring soybean root callus metabolized [1-^{14}C]-2,4-dichlorophenoxyacetic acid (2,4-D) to diethyl ether-soluble amino acid conjugates and water-soluble metabolites. The uptake in tissue varied with incubation time, concentration, and amount of tissue. Uptake was essentially complete (80%) after a 24-hour incubation and the percentage of free 2,4-D in the tissue fell to its lowest point at this time. At later times, the percentage of free 2,4-D increased and the percentage of amino acid conjugates decreased, whereas the percentage of water-soluble metabolites increased only slightly. Similar trends were seen if the tissue was incubated for 24 hours in radioactive 2,4-D, followed by incubation in media without 2,4-D for 24 hours. Inclusion of nonlabeled 2,4-D during the 24-hour chase period did not reduce amino acid conjugate disappearance but did reduce the percentage of free [1-^{14}C]2,4-D. Thus, an external supply of 2,4-D does not directly prevent amino acid conjugate metabolism in this tissue. It is concluded that 2,4-D amino acid conjugates were actively metabolized by this tissue to free 2,4-D and water-soluble metabolites.

The auxin-type herbicide 2,4-D is readily metabolized by several kinds of callus tissue (4–6). In Acme soybean cotyledon callus, we have identified (4, 7) primarily ether-soluble aspartic and glutamic acid conjugates of 2,4-D, as well as water-soluble glycosides of various 4-hydroxy 2,4-D metabolites with lesser amounts of other metabolites (including the presumed 2,4-D glucose ester). Although we have not characterized the metabolites in Amsoy root callus as extensively, the metabolites found are qualitatively similar to the Acme cotyledon callus. The synthesis and degradation of 2,4-D or IAA conjugates may contribute to the regulation of auxin levels in tissue (9).

Bandurski *et al.* (2) found that a light flash, causing photoinhibition of growth in corn seedlings, decreased the level of free IAA but increased the level of conjugated IAA. Thus, growth regulation by light and other factors can perhaps be correlated with changes in auxin conjugation.

Excised Amsoy roots and 9-week-old soybean root callus appeared to regulate the level of free 2,4-D by converting excess 2,4-D into amino acid conjugates (3) but younger soybean callus (3-week-old tissue) did not regulate 2,4-D or accumulate amino acid conjugates. We have utilized this auxin-requiring Amsoy root callus tissue to extend our observations on the relationship between amino acid conjugates and free 2,4-D levels in this tissue.

The objectives of this study were to determine if: (*a*) the accumulated glutamic and aspartic acid conjugates of 2,4-D could supply free 2,4-D in this tissue as the medium becomes depleted in 2,4-D or if 2,4-D was removed, and (*b*) the presence of unlabeled 2,4-D in the medium would alter the metabolism of 2,4-D conjugates in the tissue.

MATERIALS AND METHODS

Soybean root callus cultures were initiated from 3-day-old roots of soybean (*Glycine max* [L.] Merrill var. Amsoy) and cultured on agar-solidified medium with 10 μM α-naphthaleneacetic acid and 2.32 μM kinetin (10). Cultures were maintained at 25 C under continuous low intensity fluorescent light (0.5 μE/m^2·s).

Eight-week-old callus tissue (2.5–6.5 g) was aseptically transferred to 125-ml flasks containing 50 ml liquid medium (without added auxin) to which 1 to 5 μM [1-^{14}C]2,4-D (55 or 57 mCi/mmol, Amersham/Searle) was added. Tissues were incubated for 8 to 72 h (as indicated) and the incubation mixture was decanted. The tissue was washed three times with sterile H_2O, weighed, and extracted with hot ethanol. In some experiments, after a 14- to 24-h incubation with [1-^{14}C]2,4-D, the washed tissues were returned to liquid medium with or without unlabeled 9 μM 2,4-D for 24 or 48 h. All tissues were incubated on a reciprocal shaker (120 oscillations/min) at room temperature (21 C) under ambient fluorescent light (17 μE/m^2·s, 9 h light, 15 h dark).

In all cases the tissue was harvested on filter paper, surface-rinsed with cold distilled H_2O, and weighed. The tissue was ground in a VirTis "45" tissue homogenizer with 10 times its weight of hot 95% ethanol. The homogenate was filtered and the residue was washed with 80% ethanol. The filtrate was concentrated at 50 C by rotary evaporation (aspirator), adjusted to pH 3 (H_3PO_4), and extracted four times with equal volumes of diethyl ether. The aqueous phase was extracted three times with equal volumes of 1-butanol saturated with H_2O (water-soluble or aqueous fraction), which removed 90% of the water-soluble radioactive metabolites.

The butanol fraction was concentrated in a rotating evaporator (aspirator) at 50 C to 0.5 ml. An aliquot (0.2 ml) was diluted to 20 ml with distilled H_2O and adjusted to pH 6 prior to incubation with β-glucosidase (8 mg of Emulsin, United States Biochemical Corp.) for 24 h at 27 C. The solution then was acidified to pH 3 (H_3PO_4) and extracted with diethyl ether.

The ether fractions were chromatographed on Supelcosil 12A thin-layer plates containing a fluorescent indicator (zinc silicate). Metabolites were located by autoradiography and co-chromatography with nonlabeled standards. The plates were developed in diethyl ether/petroleum ether (38–40 C)/formic acid (70:30:2, v/

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v) or other solvents as previously reported (4, 7).

The radioactivity of each fraction was measured by liquid scintillation counting in Aquasol (New England Nuclear). The ethanol-insoluble residue was combusted by the O_2 flask method (8) prior to counting. All counts were corrected for quenching using the channels ratio method.

RESULTS AND DISCUSSION

The uptake of 2,4-D by Amsoy root callus tissue increased with incubation time, with increasing concentration of 2,4-D, and with the amount of tissue in the flask. Uptake after 24 h was 70 to 80% if 3 to 4 g tissue were incubated with about 1.2 to 2.5 μM 2,4-D (Fig. 1), but with 4 to 5 g tissue/flask uptake could approach 90% in 24 h. The remaining radioactivity in the culture solution consists mainly of 2,4-D but did contain some metabolites. Approximately 97% of the applied radioactivity can be recovered after 72 h incubation, implying minimum loss of radioactivity via the chain degradation pathway.

The ethanol-soluble fraction contained amino acid conjugates, glucosides of free and hydroxylated 2,4-D, and unmetabolized 2,4-D. The ethanol fraction was partitioned into an ether-soluble and an aqueous fraction. The ether fraction contained primarily unmetabolized 2,4-D and amino acid conjugates (mostly the glutamic and aspartic conjugates), whereas the aqueous fraction contained primarily hydroxylated 2,4-D glycosides (3, 7). The 2,4-D metabolites remaining in the residue fraction have not been identified.

A summary of 30 experiments is shown in Table I in which the results are expressed in nmol/g fresh weight (calculated as [1-^{14}C]2,4-D). Comparisons are difficult where the weight of tissue and, hence, the nmol/g 2,4-D used varied greatly. Therefore, the data were converted to percentage of the total in the tissue for each fraction (Fig. 2). The percentage of ether-soluble amino acid conjugates increased from 43% (8 h) to almost 63% (24 h) of the label in the tissue, whereas the free 2,4-D dropped from about 32% at 8 h to 11% at 24 h (Fig. 2). After 2,4-D in the medium was largely depleted at 24 h (Fig. 1), the percentage of amino acid conjugates of 2,4-D fell from 63% to about 21% (72 h), which mainly correlated with a rise in free 2,4-D (11–27%) and water-soluble metabolites (22–43%. The percentage water-soluble metabolites tended to increase with increasing incubation time.

In subsequent experiments, the tissues which had been incubated with labeled 2,4-D were rinsed with sterile H_2O and returned to medium with or without 2,4-D (Fig. 3). When tissue was

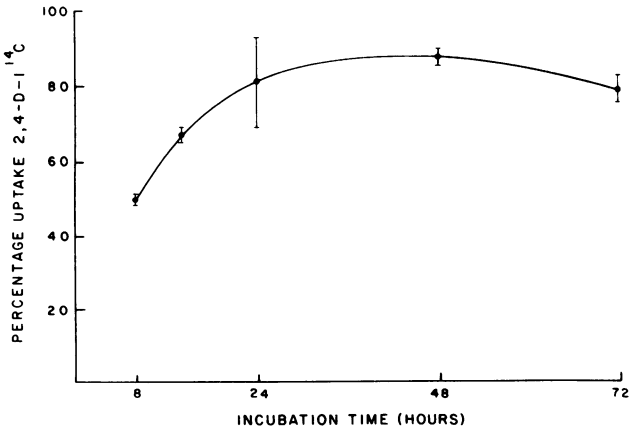

FIG. 1. The percentage of applied [1-^{14}C]2,4-D taken up by 8-week-old soybean root callus over a 72-h incubation time. The average fresh weight of the tissue was from 3.3 to 4.1 g and the amount of 2,4-D in the medium was 1.2 to 2.6 μM. The number of experiments and average nmol/g are indicated in Table I.

incubated for 14 or 24 h with radiolabeled 2,4-D and then incubated (34-24 h) without 2,4-D, the percentages of free 2,4-D, ether-soluble metabolites and aqueous-soluble metabolites were similar to the percentages found in tissue incubated for 48 h in [1-^{14}C]2,4-D (Fig. 2). This was so because uptake of most of the 2,4-D occurred during the first 24 h (Fig. 1). When tissue was incubated with [1-^{14}C]2,4-D for 24 h followed by 48 h without 2,4-D (Fig. 3), the percentages of metabolites were similar to those in tissue incubated for 72 h in [1-^{14}C]2,4-D (Fig. 2).

Tissues were incubated also with radiolabeled 2,4-D for 24 h, rinsed with sterile H_2O, and returned to an incubation mixture containing unlabeled 9 μM 2,4-D for an additional 24 h (Fig. 3). In tissue incubated in this manner, the percentage of label in the free 2,4-D component remained at 11%, whereas, without 2,4-D, the percentage of [1-^{14}C]2,4-D in tissue increased to 22% (Fig. 3). When the tissue was continuously incubated with [1-^{14}C]2,4-D, the conjugate fraction decreased from 63% at 24 h to 40% at 48 h (Fig. 2), which was similar to the 40% found after 48 h in the chase experiment (24 h with [1-^{14}C]2,4-D, 24 h with 2,4-D; Fig. 3) or the 37% found after 48 h incubation with and without 2,4-D (24 h with [1-^{14}C]2,4-D, 24 h without 2,4-D; Fig. 3). The increases in the percentage of radioactivity in the aqueous metabolite components were similar with or without the unlabeled 2,4-D chase. Thus, the metabolism of amino acid conjugates seemed to be independent of the presence or absence of free 2,4-D in the medium. The percentage label in free 2,4-D was lower and that in the residue fraction was somewhat higher with the 2,4-D chase. The total pool sizes in various fractions after incubation with unlabeled 2,4-D are unknown.

Previous long-term studies with 2,4-D have shown that an increase in incubation time alters the distribution of radioactivity found in Acme cotyledon callus tissue (7). The water-soluble hydroxylated glycosides of 2,4-D increased to 51% after 24 days with a concomitant decrease in radioactivity in the ether-soluble glutamic and aspartic acid conjugates, whereas the radioactivity in the residue fraction remained at about 5%. The glutamic acid conjugate of 2,4-D reached 78% of the ether-soluble fraction after 1 day and then decreased to 15% after 8 days. During this same period, 2,4-D increased from 21 to 65% of the ether-soluble fraction. It was also previously shown (4) that exogenous 2,4-D glutamic acid can be metabolized by soybean cotyledon callus to other amino acid conjugates, free 2,4-D, and mainly aqueous-soluble metabolites after 12 days incubation. These results (7) are in general similar to the short term changes reported here.

It appears that the increase in free 2,4-D in the tissue after 48 to 72 h incubation was due to the conversion of ether-soluble amino acid conjugates to free 2,4-D. Uptake of label was almost complete after 24 h incubation (Fig. 1), so that the rise in free 2,4-D must be accomplished at the expense of conjugates. This was confirmed in experiments where tissue was removed from labeled 2,4-D at 24 h. With increasing incubation times, the water-soluble fraction also increased in radioactivity at the expense of the amino acid conjugate fraction.

Free 2,4-D was at its lowest level after 24 h incubation, followed by a steady increase in concentration. One explanation for the minimum 2,4-D level at 24 h would be that there is over induction of "conjugation enzyme" along with medium depletion of external 2,4-D at this time. Perhaps this phenomenon can also be correlated with the resumption of cell division in the tissue. In a partially synchronized suspension of tobacco cells, cell division is correlated with an increase in tissue 2,4-D levels when grown in media containing 2,4-D (11). Since the callus used in our experiments was not synchronously dividing, the gradual increase in free 2,4-D after 24 h may be related to the conversion of tissue to a younger physiological state with an increasing number of cell divisions. In our previous 48-h 2,4-D-labeling studies with this tissue, we found young tissue was not controlling internal 2,4-D

Table 1. *The Concentration of [^{14}C]2,4-D Metabolites in Various Fractions after Incubation of Soybean Root Callus with [^{14}C]2,4-D*

All values are averages ± SD.

Time of Incubation		No of Exp.	Nanomol of ^{14}C Label/g fresh wt Calculated as [^{14}C]2,4-D					
With [^{14}C]2,4-D	Without 2,4-D		Applied [^{14}C]2,4-D (average)	Total in Tissue[b]	Residue	Free 2,4-D	Aqueous Soluble Metabolites	Ether Soluble Metabolites
h								
8	0	2	25.3 ± 0.3	11.9 ± 0.6	0.7 ± 0.1	3.8 ± 0.4	2.2 ± 0.1	5.1 ± 0.9
14	0	3	17.3 ± 2.8	11.6 ± 2.3	0.7 ± 0.1	2.2 ± 0.3	2.8 ± 0.8	6.0 ± 1.5
14	34	5	15.2 ± 2.8	9.8 ± 0.7	1.0 ± 0.4	2.1 ± 0.4	3.1 ± 0.4	3.6 ± 1.0
24	0	4	21.3 ± 11.0	16.1 ± 5.6	0.7 ± 0.4	1.8 ± 0.7	3.6 ± 1.6	10.0 ± 3.1
24	24	5	17.7 ± 6.0	13.5 ± 3.7	1.5 ± 0.8	3.2 ± 1.7	4.2 ± 1.7	4.7 ± 0.4
24	(24)[a]	4	25.0 ± 8.5	14.5 ± 3.5	2.2 ± 0.6	1.8 ± 1.2	4.8 ± 1.0	5.6 ± 1.0
24	48	2	26.6 ± 1.3	17.6 ± 0.6	1.6 ± 0.1	4.7 ± 0.5	7.0 ± 0.1	4.3 ± 1.2
48	0	3	14.9 ± 3.5	13.1 ± 2.5	1.0 ± 0.2	2.7 ± 0.8	4.0 ± 0.3	5.3 ± 2.5
72	0	2	25.1 ± 2.6	19.7 ± 1.3	1.6 ± 0.1	5.4 ± 0.8	8.4 ± 0.9	4.3 ± 1.4

[a] Incubated with 9 μM unlabeled 2,4-D.

[b] The average fresh wt were, respectively, 4.03, 4.05, 4.37, 3.51, 4.21, 4.12, 3.04, 4.10 and 3.27 g.

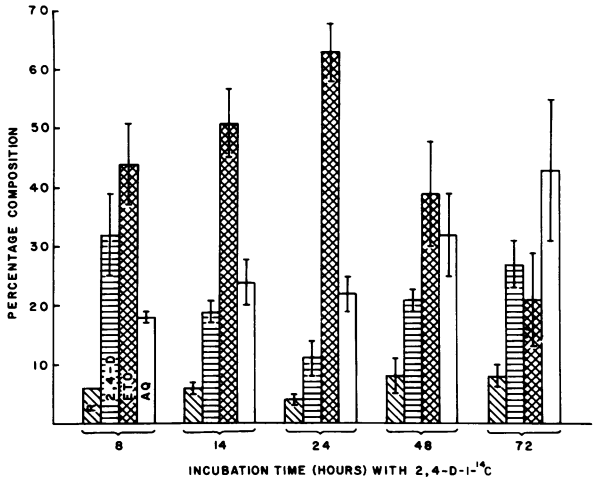

FIG. 2. The distribution of radioactivity in 8-week-old soybean root callus tissue incubated with [1-^{14}C]2,4-D for 8-72 h; R: residue; 2,4-D: free 2,4-D; ETO: ether-soluble metabolites; AQ: aqueous-soluble metabolites.

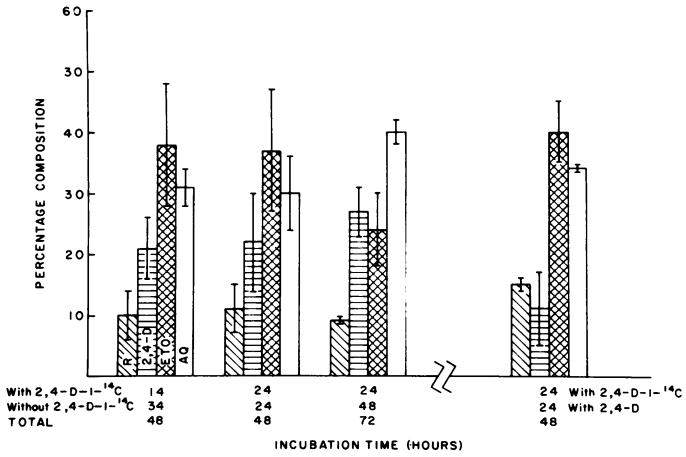

FIG. 3. The distribution of radioactivity in 8-week-old soybean root callus tissue incubated with [1-^{14}C]2,4-D for 14 or 24 h, followed by incubation with or without unlabeled 2,4-D. The bar graphs are represented by the same symbols used in Figure 2.

levels through amino acid conjugate formation (3).

It is apparent that free 2,4-D can be supplied at the expense of ether-soluble conjugates. The induction of the auxin conjugation system by exogenous auxin in several tissues is well known (1, 9, 13), and the presence of natural aspartate conjugates has been reported (12). It also seems that hydrolysis, as well as formation, of such conjugates would be closely regulated. Removal or depletion of 2,4-D in this callus tissue does cause a decrease in the conjugate pool and an increase in free 2,4-D and other typical 2,4-D metabolites. We thought that chasing with unlabeled 2,4-D might prevent hydrolysis of the conjugate pool but the labeled pool of 2,4-D conjugates decreased just as rapidly with or without a 24-h chase with 2,4-D. However, the labeled 2,4-D level was lower following the unlabeled 2,4-D chase. Either the 2,4-D conjugates can be directly hydroxylated (with subsequent glycoside formation) rather than hydrolyzed if 2,4-D levels are high or perhaps compartmentation allows selective conversion of 2,4-D released from the conjugate to be metabolized if internal 2,4-D levels are high. Experiments are in progress to evaluate the total pool size changes during the chase.

This auxin-dependent, rapidly growing callus tissue may have some advantages for studies on possible regulation of auxin levels by conjugate metabolism. There appears to be little auxin synthesis, and the metabolic pathways for degradation of 2,4-D are better understood (and more limited) than those for IAA. Some auxin-type herbicides are even more stable than 2,4-D, and study of regulation of their levels in this sort of callus tissue would be informative.

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