Metabolism of 2,4-Dichlorophenoxyacetic Acid. VI. Biological Properties of Amino Acid Conjugates

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Twenty amino acid conjugates of 2,4-dichlorophenoxyacetic acid (2,4-D) were synthesized and evaluated for their ability to stimulate cell division and elongation. All the conjugates stimulated *Avena coleoptile* elongation at an optimum concentration of 10^{-5}–10^{-6} *M* and were nearly equivalent to 2,4-D at its optimum of 10^{-6} *M*. These conjugates also stimulated growth of soybean callus tissue at an optimum concentration of 10^{-6}–10^{-7} *M*. The growth resulting from the addition of many of these conjugates exceeded the growth stimulation produced by 2,4-D at its optimum of 10^{-7} *M*. Elongation and growth were inhibited when higher concentrations of the conjugates were used. All of these properties are typical of auxins and the reported metabolism of 2,4-D to conjugates Glu, Asp, Ala, Val, Leu, Phe, and Trp cannot be considered as a detoxification mechanism.

The effect of auxin on the growth of tissues of various organs has been extensively investigated in the last three decades. But few studies have been performed concerning its regulation of amino acid conjugate formation and its role in the growth of tissues. Tissues of soybean cotyledon callus, when exposed to a specific and defined chemical medium, require kinetin as well as auxins such as indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), or 2,4-dichlorophenoxyacetic acid (2,4-D) for continuous growth and maintenance in culture (Miller, 1963). However, it has been found that a high level of 2,4-D does induce some growth alone (Witham, 1968). Also 2,4-D alone stimulates the growth of soybean root cell cultures (Gamborg and Ojima, 1967), different somatic organs of rice plant (Wu and Li, 1971), and rice root callus tissues (Lieb *et al.*, 1972, 1973; Yatazawa *et al.*, 1967). 2,4-D has also been used in the culture of other tissues without addition of cytokinins (Filner, 1965; Harvey, 1967).

Recent studies with cultured callus tissues of soybean cotyledon, for the purpose of the detection, isolation, and chemical characterization of 2,4-D metabolites, have demonstrated that 2,4-D is rapidly conjugated by means of an amide bond with a number of amino acids (Feung *et al.*, 1971, 1972, 1973a, b). Such conjugates were characterized by paper and thin-layer chromatography and by mass spectrometry. Seven amino acid conjugates have been isolated and identified (2,4-D–glutamic acid, 2,4-D–aspartic acid, 2,4-D–alanine, 2,4-D–valine, 2,4-D–leucine, 2,4-D–phenylalanine, and 2,4-D–tryptophan). To better understand the action of plant growth regulators and to evaluate the biological significance of the recently identified conjugates of 2,4-D, 20 L-form amino acid conjugates of 2,4-D were synthesized and characterized (Feung *et al.*, 1973a). These conjugates have now been evaluated for their comparative ability to effect growth of *Avena coleoptile* sections and soybean cotyledon callus cultures. Previous studies (Krewson *et al.*, 1956; Wood and Fontaine, 1952) had shown that several of these derivatives had 2,4-D like herbicidal activity.

EXPERIMENTAL SECTION

Oat seeds were surface sterilized in hydrogen peroxide for 10 min and rinsed three times with sterilized distilled water. The sterilized seeds were spread over moist filter paper in glass trays and were kept in the dark at room temperature. After 24 hr in the dark, these seeds were exposed to red light for 4 hr (red-filtered incandescent lamp) and then allowed to germinate in the dark for 48 hr. Seedlings with coleoptiles 2.5–3.0 cm in length were selected, from which 6.5-mm coleoptile sections were obtained by excision 2 mm from the apical end (Wang *et al.*, 1968) and then placed in basal medium (2% sucrose in 0.01 *M* KH_2PO_4). Eight randomly selected coleoptile sections were transferred to each petri dish, which contained 25 ml of the basal medium with or without 2,4-D or the conjugate at selected concentrations. The *Avena coleoptile* sections were then allowed to incubate in a dark room at 25° for 24 hr, at which time the length of the sections was measured.

The callus tissue used in these studies was originally derived from soybean cotyledon (*Glycine max.* [L] Merr. var. Acme). Stock callus cultures were grown on an agar medium of Miller (1963) with 3% sucrose, kinetin (0.5 mg/l.), and naphthaleneacetic acid (NAA, 2.0 mg/l.) under continuous low intensity fluorescent light at 25° for about 5 weeks.

For bioassays, three small clumps (about 5–10 mg each) of callus tissue were aseptically inoculated in each 125-ml Erlenmeyer flask containing 50 ml of the standard solidified medium (0.8% agar) without NAA. The medium (Miller, 1963) also contained 3% sucrose and 0.5 mg/l. of kinetin with varying concentrations of 2,4-D or the amino acid conjugates of 2,4-D at pH 5.8. The tissues were allowed to grow under fluorescent light (*ca.* 40 ft-candles) for a period of 6 weeks at room temperature. At the completion of each experiment the tissues were removed from the nutrient agar medium, blotted briefly, and fresh weight was determined.

RESULTS AND DISCUSSION

All the amino acid conjugates of 2,4-D stimulated the elongation of *Avena coleoptile* sections (Table I). The optimum elongation response appeared to be at the concentration of 10^{-6} *M* for 2,4-D, 2,4-D-glycine, 2,4-D-alanine, 2,4-D-valine, 2,4-D-leucine, 2,4-D-isoleucine, 2,4-D-cysteine, 2,4-D-methionine, and 2,4-D-phenylalanine, although none of the amino acid conjugates quite equal the stimulation of 2,4-D itself at 10^{-6} *M*. The concentration of 10^{-5} *M* was optimum for 2,4-D-serine, 2,4-D-proline, 2,4-D-threonine, 2,4-D-hydroxyproline, 2,4-D-aspartic acid, 2,4-D-lysine, 2,4-D-glutamic acid, 2,4-D-histidine, 2,4-D-arginine, 2,4-D-tryosine, 2,4-D-tryptophan, and 2,4-D-cystine. All of these conjugates exceeded the response of 2,4-D at 10^{-5} *M* and most nearly equalled

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Table I. The Elongation of *Avena coleoptile* Sections Induced by Amino Acid Conjugates of 2,4-D

2,4-D or conjugate	% elongation			
	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Control (no additive, 8.0 mm)				
2,4-D	38.8	73.8	45.0	38.8
Glycine	16.3	42.5	27.5	25.0
Alanine	35.0	51.3	27.5	25.0
Proline	62.5	51.3	35.0	22.5
Serine	70.0	42.5	25.0	23.8
Valine	33.8	60.0	32.8	30.0
Threonine	62.5	47.5	35.0	25.0
Leucine	47.5	56.3	36.3	28.8
Isoleucine	45.0	55.0	47.5	23.8
Cysteine	30.0	46.3	33.8	23.8
Hydroxyproline	53.8	33.8	28.8	23.8
Aspartic acid	57.5	35.0	26.3	22.5
Lysine	51.3	32.5	26.3	25.0
Glutamic acid	63.8	37.5	25.0	23.8
Methionine	30.0	48.8	31.3	20.0
Histidine	68.8	30.0	26.3	25.0
Phenylalanine	48.8	58.8	32.5	26.3
Arginine	71.3	48.8	33.8	30.0
Tyrosine	58.8	37.5	28.8	23.8
Tryptophan	66.3	41.3	23.8	22.5
Cystine	58.8	38.8	27.5	25.0

the response of 2,4-D at 10^{-6} *M*. The variability as measured by the standard deviation was less than 7% at the optimal concentrations. A concentration of 5×10^{-5} *M* conjugates caused a decrease in the length of coleoptile sections when compared to the length observed at 10^{-5} *M*.

All the amino acid conjugates of 2,4-D stimulated the growth of soybean cotyledon callus tissues, showing an optimum response at a concentration of 10^{-6} to 10^{-7} *M* (Table II) and a standard deviation of less than 10%. At the optimum concentration for 2,4-D (10^{-7} *M*), many conjugates equalled the stimulation of 2,4-D and several conjugates, namely 2,4-D-leucine, 2,4-D-isoleucine, 2,4-D-glutamic acid, 2,4-D-methionine, 2,4-D-tryptophan, and 2,4-D-cystine, exceeded (>15-55%) 2,4-D induced growth. At a higher concentration (10^{-6} *M*) 2,4-D-hydroxyproline, 2,4-D-lysine, 2,4-D-histidine, and 2,4-D-phenylalanine also greatly exceeded (>35%) the optimum response of 2,4-D. Seven amino acid conjugates, 2,4-D-glycine, 2,4-D-alanine, 2,4-D-proline, 2,4-D-serine, 2,4-D-valine, 2,4-D-cysteine, and 2,4-D-arginine, did not equal the response of 2,4-D at any concentration. All the amino conjugates, as well as 2,4-D, show less stimulation of growth (inhibition) at the highest tested concentration of 10^{-5} when compared to 10^{-6} *M*. A small portion of the tissue in the center of the callus clumps was found to be dead when the tissue was grown on media possessing the conjugates at a concentration of 10^{-7} *M*. These small portions of dead tissue may be due to depletion and/or metabolism of the amino acid conjugate since at 10^{-8} *M* some of the entire callus tissue clumps were found to be dead. Generally the tissues resulting from the bioassay of the amino acid conjugates were yellow to pale green in color and were composed of loose masses of cells. In contrast, the tissues grown in stock cultures (plus NAA) were yellowish green to green in color and formed a dense compact callus.

All the 20 amino acid conjugates of 2,4-D stimulated cell division and/or cell elongation in the two bioassays performed. A number of the 2,4-D conjugates stimulated growth in the soybean callus tissue to a greater extent than 2,4-D at its optimum concentration (10^{-7} *M*). In fact, 2,4-D-methionine and 2,4-D-glutamic acid at 10^{-7} *M* gave 50% more growth than 2,4-D at this concentration. When higher concentrations were used the conjugates inhibited growth of soybean tissue and *Avena* section elongation. All of these properties are typical of auxins. The reported conversion of 2,4-D to seven amino acid conjugates (glutamic acid, aspartic acid, alanine, valine, leucine, phenylalanine, and tryptophan) (Feung *et al.*, 1973b) thus cannot be considered as a detoxification mechanism but perhaps is an activation step.

There are at least two possibilities with regard to the conjugates activity. The first is that only 2,4-D is the active molecule, since it can be formed as a product of the metabolism of the amino acid conjugates of 2,4-D as we have previously shown for the glutamic conjugate (Feung *et al.*, 1971). In this view the higher activity of the 2,4-D conjugates compared to 2,4-D in soybean callus growth would be related to secondary factors such as altered uptake or metabolism.

The second possibility is that these conjugates are required active intermediates. Soybean callus tissue rapidly

Table II. The Growth of Soybean Cotyledon Callus Tissue Induced by Amino Acid Conjugates of 2,4-D

2,4-D or conjugate	Weight (mg)[a] soybean cotyledon callus tissue			
	10^{-5} *M*	10^{-6} *M*	10^{-7} *M*	10^{-8} *M*
Control (no additive)			All died	
Control (2,4-D)	65.0 ± 4.2	224.2 ± 15.4	285.8 ± 25.3	71.7 ± 21.7
Glycine	73.3 ± 6.6	215.0 ± 15.9	245.0 ± 27.5	46.7 ± 8.3
Alanine	81.7 ± 15.0	168.0 ± 33.3	275.8 ± 32.5	54.8 ± 9.6
Proline	113.4 ± 6.8	247.5 ± 41.3	255.9 ± 20.8	40.2 ± 3.7
Serine	84.2 ± 12.5	188.3 ± 17.4	*b*	No growth
Valine	60.0 ± 9.9	188.3 ± 26.7	280.8 ± 35.8	61.4 ± 11.5
Threonine	97.5 ± 19.2	200.0 ± 20.0	258.3 ± 31.7	53.7 ± 6.2
Leucine	81.7 ± 9.2	287.5 ± 27.5	385.9 ± 27.1	183.3 ± 11.7
Isoleucine	72.5 ± 5.8	274.4 ± 25.8	332.5 ± 4.2	91.2 ± 8.2
Cysteine	109.2 ± 9.4	222.5 ± 19.1	257.5 ± 21.3	48.7 ± 5.8
Hydroxyproline	265.9 ± 27.5	388.4 ± 31.9	143.3 ± 23.3	No growth
Aspartic acid	88.4 ± 10.0	272.5 ± 27.5	302.2 ± 16.3	92.1 ± 13.3
Lysine	330.0 ± 11.1	480.9 ± 30.9	144.2 ± 10.8	No growth
Glutamic acid	101.7 ± 7.5	288.4 ± 25.0	437.5 ± 17.9	132.6 ± 9.8
Methionine	110.0 ± 6.5	291.7 ± 24.2	442.5 ± 26.7	113.6 ± 10.9
Histidine	282.5 ± 31.3	433.3 ± 30.9	275.0 ± 19.3	No growth
Phenylalanine	125.0 ± 20.0	433.3 ± 28.3	312.5 ± 37.5	150.8 ± 4.2
Arginine	188.3 ± 12.5	274.2 ± 30.9	278.3 ± 14.6	No growth
Tyrosine	132.5 ± 7.5	301.7 ± 30.8	255.0 ± 23.4	81.4 ± 7.3
Tryptophan	129.2 ± 14.2	304.2 ± 30.5	348.3 ± 20.9	No growth
Cystine	109.2 ± 2.5	293.4 ± 25.8	348.4 ± 16.7	94.6 ± 8.5

[a] Wet weight. [b] Died after 3 weeks.

forms the active glutamic conjugate, making it difficult to understand why the glutamic conjugate would be more active than 2,4-D in this tissue. Studies (Feung *et al.*, 1973b) with 2,4-D-[1-^{14}C]-glutamic acid indicate a more rapid conversion of it to the inactive 4-OH-2,3-D or 4-OH-2,5-D than conversion to 2,4-D itself. This is again inconsistent with the higher activity of the 2,4-D-glutamic conjugate. It thus seems possible that the conjugated form of 2,4-D could be exclusively the physiologically active form. In any event some of these active conjugates may be more suitable auxins for plant tissue cultures than either NAA or 2,4-D.

Different responses were observed for the various conjugates; however, it is possible that at the subcellular level no real differences exist. The observed differences in physiological responses may reflect variations in permeability and/or metabolism which thus affect micro-pool environments.

Amino acid conjugates of auxins can effect responses at very low concentrations and it has been postulated one site of action is at the nucleic acid level. Cellular localization of 2,4-D in the nucleus (Liao and Hamilton, 1966) as well as in nuclioli (Zwar and Brown, 1968) has been shown. Key and Shannon (1964) reported that IAA and 2,4-D, at the concentration which promoted cell elongation, enhanced ^{14}C-nculeolide incorporation into ribonucleic acid (RNA) of excised soybean hypocotyl tissue, whereas inhibitory levels decreased incorporation. It has in general been concluded (Key, 1969) that RNA and protein synthesis are essential for the process of cell elongation to proceed at the normal rate. The observed differences in physiological response may also reflect a difference in binding to a protein and/or a selectivity in an enzyme reaction leading to biological function. These amino acid conjugates could compete with amino acid or specifically react with transfer ribonucleic acid to affect selective protein synthesis. Labeled 2,4-D has been reported bound to macro-molecules (Galston and Davies, 1969). Additional studies are needed to elicit the function of these amino acid conjugates of auxins in plants.

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Extraction of Nitrogenous Constituents from the Jack Bean (*Canavalia ensiformis*)

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Studies were carried out on the nitrogen (protein) solubility of the Jack bean (*Canavalia ensiformis*). Using a solvent-to-meal ratio of 100:6, the optimum conditions for a one-step protein extraction, using an aqueous system, were pH 13, 70° for 1 hr. The point of minimum solubility was found at pH 4.9, and nitrogen solubility increased toward the acidic and basic sides. Jack bean meal proteins displayed a different pH-nitrogen solubility profile in 0.5 *N* NaCl solution. At pH 4.9, 70% of the extracted nitrogen was recovered. The recovered proteins showed a higher essential amino acid content than the original meal. Methionine was the most limiting amino acid in both cases. When using methionine, the chemical score for the protein concentrate was lower than for the original meal. The reverse was true when the second most limiting essential amino acid was used, indicating a better quality protein for the concentrate once the methionine deficiency is overcome.

Jack bean (*Canavalia ensiformis*) can be grown relatively easily, producing high yields in regions of low altitude, high temperature, and relative humidity, inadequate for the growth of other edible legume foods such as *Phaseolus vulgaris* (Rachie, 1973). Thus, the Jack bean has a high potential in regions of varying climates and altitudes like the Central American area where it would not compete with the black bean (*Phaseolus vulgaris*) and could yield an additional protein source.

Although the Jack bean has a relatively high protein,

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