Comparative Investigations on the Metabolism of the Herbicide 2-(2,4-Dichlorophenoxy)-propionic Acid in Plants and Cultured Cells of Tomato*

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The metabolism of [1-^{14}C]2-(2,4-dichlorophenoxy)-propionic acid was studied in excised plants and cell suspension cultures of tomato. It was rapidly taken up and metabolized by both the plants and the cultured cells. The metabolites, isolated by extraction with aqueous acetone, separated and purified by TLC and HPLC, were identified by chemical and spectrometric methods. Conjugates with carbohydrates were detected. Glucose, diglucose as the main conjugating moiety, and triglucose were found as carbohydrate components within the conjugates. Almost the same conjugates occurred in plants and cultured cells.

Introduction

The metabolism of the auxin herbicide dichlorprop has been studied recently in a monocotyledon species (barley) [1]. Hydroxylation in position 4 of the aromatic ring involving the shift of the chlorine to positions 5 and 3 takes place which was also known for the closely related herbicide 2,4-D [2]. The parent compound and also the 4-hydroxy derivative undergo conjugation with mono- und diglucoses in the carboxyl and the hydroxyl group.

The investigations reported in this paper on the one hand were carried out to compare the metabolism of dichlorprop in excised plants and cultured cells of one species, *i.e.* tomato, and, on the other hand, to compare the above metabolism with that of barley as a monocotyledon species. Furthermore, the results of this study are also the basis for regarding the main metabolites of dichlorprop in residual analysis [3].

Abbreviations: dichlorprop, 2-(2,4-dichlorophenoxy)-propionic acid; GOD-POD, glucoseoxidase-peroxidase; ss, solvent system; glc, glucosyl; ac, acetyl; LSC, liquid scintillation counting.

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Materials and Methods

Radiochemicals and synthetic standards

[1-^{14}C]Dichlorprop was synthesized from [1-^{14}C]2-bromopropionic acid (Isocommerz GmbH, Dresden-Rossendorf) and 2,4-dichlorophenol in 5 N aqueous NaOH, and converted to the potassium salt by using methanolic K_2CO_3 [4]. Specific radioactivity was 55 MBq × mmol^{-1}. Dichlorprop-glcac$_4$ was synthesized according to [5]. The structure was confirmed by ^{1}H NMR, electron impact and electron attachment MS [1].

Plant material and application

The tomato plants (*Lycopersicon esculentum* Mill., *cv.* "Lukullus") were cultivated in soil in a greenhouse at 23 to 25 °C. The 4-week-old excised plants were immersed in an aqueous solution of [1-^{14}C]dichlorprop (K-salt). To each tomato plant 0.5 mg dichlorprop in 1 ml water (1.8×10^{-3} mol × l^{-1}) was offered for 72 h. The origin and cultivation of the tomato cell cultures were published by Tewes *et al.* [6]. The medium did not contain any 2,4-D or other auxins. At the end of the logarithmic growth phase, which was determined by measuring the optical density at 570 nm ($E = 1.9$, fresh weights 37 mg × ml^{-1}, 3 days after inoculation), each subculture (100 ml medium each) was provided with 4 mg [1-^{14}C]dichlorprop (7.3×10^{-5} mol × l^{-1}, altogether 40 mg for 10 times 100 ml medium), and incubated for 3 days.

Isolation of metabolites

After incubation, the excised plants were rinsed several times with water, cut into small pieces, homogenized with an Ultra-Turrax (Janke u. Kunkel KG, Staufen i. Br., F.R.G.) in 50% aqueous acetone, and filtered. The solid material was shaken for 15 h with another portion of 50% aqueous acetone. The combined filtrates were evaporated to remove the acetone, and the remaining aqueous phase was extracted successively using *n*-hexane and ethyl acetate.

The cells in suspension cultures were filtered, washed with 100 ml water, and sucked for 1 min to a relative dryness; then the fresh weights were determined. The culture medium was extracted using ethyl acetate. The cell material was lyophilized, pulverized, and extracted with 50% acetone. The 50% acetone extract was treated as the extract of the plants. The radioactivity of aliquots of all fractions was determined by LSC employing a Tricarb 2660 (Packard Instruments, Chicago, U.S.A.). Before LSC the solid residue was combusted using a Micro-Mat BF 5010 sample oxidizer (Berthold-Frieseke GmbH, Karlsruhe, F.R.G.). The metabolites of the different extracts were separated and purified by TLC and HPLC. For quantifying the metabolites the ratios of aglycones, determined after hydrolysis of an aliquot, were used, and the integrals of peak areas of radioscans were evaluated.

Chromatography

Preparative TLC was carried out on self-coated plates (silica gel 60 PF_{254}, Merck, 0.9 mm thick) having a concentration zone of 3 cm (Kieselgur, Merck). For comparative TLC precoated silica gel plates ("Silufol UV_{254}", Kavalier, C.S.S.R.) were used, employing the following solvent systems:

1. toluene : dioxane : acetic acid 90 : 25 : 2 (v : v : v)
2. benzene : acetone : acetic acid 20 : 80 : 3 (v : v : v)
3. ethyl acetate : 2-propanol : water 50 : 30 : 12 (v : v : v)

The TLC plates were analyzed for radioactive zones using the Radioscanner II (Berthold, Wildbad, F.R.G.).

HPLC was carried out using a Serva SI 100 polyol RP 18 column (0.005 mm, 4.6 × 250 mm), which was fitted with an RCT HPLC eluent supply and a PYE Unicam PU 4020 detector set at 254 nm. Isocratic elution was carried out using 57% aqueous acetonitrile (1 ml × min^{-1}).

Hydrolyses and derivatization

Samples of conjugates (0.002 to 0.008 mg supposed glucose, calculated from their radioactivity) were dissolved in 1 ml McIlvain buffer, pH 3.0, and incubated with 0.25 mg dialyzed cellulase for 20 h at 37 °C. Glucose was quantified by applying the GOD-POD method [7]. For alkaline hydrolysis, an aliquot was treated with 1 ml 2.5% aqueous NH_4OH (24 h, room temperature). Acid hydrolysis was carried out by treating an aliquot with 1 ml 1 N HCl for 1 h at 100 °C. Acetylation was performed using pyridine/acetic anhydride (ratio 2 : 1).

Spectrometric methods

^1H NMR spectra were recorded on a Bruker WP 200 spectrometer (Karlsruhe, F.R.G.) at 200.13 MHz in $CDCl_3$. Chemical shift values are related to TMS.

Electron impact (2 to 4 eV) and electron attachment (10 to 16 eV) mass spectra were obtained using a "Manfred von Ardenne" mass spectrograph (Dresden, G.D.R.).

Results

Metabolism in tomato plants

The excised tomato plants absorbed the offered dichlorprop very rapidly. After the incubation time of 72 h, 93% of the radioactivity applied were present in the plants (92% were extracted by using aqueous acetone, and 1% remained in the insoluble residue). The medium still contained 3% (Table I).

Table I. Distribution of radioactivity in the fractions obtained from excised tomato plants and tomato cell suspension cultures treated with [^{14}C]dichlorprop.

Fractions	% of applied radioactivity	
	Excised plants	Cell cultures
Plant/cell material	93	81
Solid residue	1	4
50% aqueous acetone extract	92	77
n-Hexane extract	17	9
Ethyl acetate extract	6	7
Aqueous phase	69	61
Medium	3	13
Ethyl acetate extract	n.d.*	4
Aqueous phase	n.d.	9

* Not determined.

Extraction, separation and purification of the radioactive metabolites of the aqueous acetone extract were carried out corresponding to the experiments with barley seedlings [1]. For additional purification HPLC was used and some HPLC fractions were investigated by MS. Besides 5% dichlorprop, isolated from *n*-hexane and ethyl acetate extracts, 3 conjugates were isolated mainly from the aqueous phase. In Table II the metabolites are listed according to their decreasing TLC mobility. It also shows TLC data and quantities of the metabolites extracted from the plants after 72 h of incubation time. Peak 2 cochromatographed with dichlorprop (R_F 0.42 in ss 1). Peak 3 is identical with dichlorprop-glc from barley [1] as demonstrated by TLC (R_F 0.6 in ss 2). Furthermore, the peracetyl derivative cochromatographed with synthetic dichlorprop-glcac$_4$ ([1], R_F 0.51 in ss 1). Alkaline and enzymic hydrolysis yielded dichlorprop. Following the enzymic hydrolysis, a molar ratio was observed of dichlorprop to glucose of about 1:1 (GOD-POD test). Peak 5, the main metabolite of dichlorprop in tomato plants, was identical with the corresponding conjugate, dichlorprop-glc$_2$, from barley (R_F 0.5 in ss 3). The peracetyl derivatives, isolated from both plant species and acetylated, also cochromatographed. Hydrolyses yielded dichlorprop. The GOD-POD test after enzymic hydrolysis revealed two glucose units per mol dichlorprop. HPLC of the peracetylated derivative indicated that it consists of two conjugates having very similar retention times. The mass spectra of these two fractions showed equal fragmentation patterns (parent peak *m/z* 852, carbohydrate fragments, *e.g.* *m/z* 635 and *m/z* 347) but different relative intensities. Therefore, isomerism was suggested in the sugar moiety.

Table II. TLC data and quantities of dichlorprop and its metabolites isolated from excised plants and cultured cells of tomato.

Peak No.	Metabolite[1]	TLC[2]		% of applied radioactivity	
		R_F	ss	Excised plants	Cultured cells
1	dichlorprop-me[3]	0.63	1	–	35
2	dichlorprop	0.42	1	5	12
3	dichlorprop-glc	0.6	2	16	11
4	unknown-monosaccharide conjugate of dichlorprop	0.5	2	–	4
5	dichlorprop-glc$_2$[4]	0.5	3	57	43
6	dichlorprop-glc$_3$	0.3	3	14	7

[1] Metabolites are listed according to the decreasing TLC mobility.
[2] For TLC conditions, see "Materials and Methods".
[3] This artifact was present only after extracting the cells by using methanol instead of acetone.
[4] Two conjugates with different isomerism in the carbohydrate moiety.

Alkaline and enzymic hydrolysis of peak 6 yielded dichlorprop. The mass spectrum (parent peak *m/z* 1140, carbohydrate fragments, *e.g.* *m/z* 923, *m/z* 635 and *m/z* 347, and some aglycon fragments) indicated a trisaccharide conjugate of dichlorprop. Since the molar ratio of dichlorprop to glucose, determined after enzymic hydrolysis and GOD-POD test, was about 1:3, the carbohydrate chain seems to be a triglucose.

Metabolism in cultured tomato cells

For the comparison with tomato plants, the metabolism of dichlorprop was studied in cultured cells of the same species. After the incubation time (3 d) the fresh weights (135 mg × ml^{-1}), extinction, and the number of plasmolytic cells of all subcultures coincided with the control.

Only 13% of the applied radioactivity were found in the medium, 4% of which were extracted using ethyl acetate, and 9% remained in the aqueous phase. Applying TLC revealed that the medium contained about equal quantities of dichlorprop and dichlorprop-glc.

The greater part (81%) of the applied radioactivity was present in the cells, 77% of which were extracted using aqueous acetone, and 4% remained in the insoluble residue (Table I). The methods used before to determine the metabolites of dichlorprop in barley seedlings [1] and tomato plants were also applied to cultured cells. The carbohydrate conjugates (dichlorprop-glc, dichlorprop-glc$_2$, and dichlorprop-glc$_3$), which before had been found in tomato plants, were detected in the cultured cells, too. TLC data and quantities are listed in Table II. By applying HPLC and MS the main metabolite dichlorprop-glc$_2$, like the corresponding conjugate of tomato plants, was shown to consist of two compounds with different isomerism of the sugar moiety. In addition to these conjugates, another conjugate of dichlorprop was found, which was very similar to dichlorprop-glc and not completely separated from it. TLC properties of

this conjugate (R_F 0.5 in ss 2), of its peracetyl derivative (R_F 0.5 in ss 1), and of the hydrolytically released dichlorprop (R_F 0.42 in ss 1) and carbohydrate moiety indicated a monosaccharide conjugate.

Extracting the cells by using aqueous methanol instead of aqueous acetone resulted in the formation of considerable amounts of dichlorprop methyl ester as an artifact (Table II). It was identified by cochromatography using synthetic methyl ester.

Discussion

The results of this study demonstrate that the metabolism of dichlorprop in excised plants essentially leads to the same conjugates as in cultured cells of tomato. Similar to an earlier study on 2-(2,4-dichlorophenoxy)-isobutyric acid [8] applied to the same plant systems, conjugates with mono-, di-, and triglucoses were formed. The diglucoside fraction of dichlorprop was shown to consist of two different conjugates. Probably the diglucose moieties of both isomeric conjugates are gentiobiose and cellobiose. Gentiobiosyl esters and cellobiosyl esters are known for other xenobiotics to occur in tomato [9]. Also in barley, beside gentiobiose, another diglucose was found as a conjugating moiety of a xenobiotic [10]. Unfortunately in our experiments the metabolites were not obtained in amounts sufficient to give evidence for structural details by means of NMR. Thus it also remained uncertain if the glucose moieties have α or β configuration. However, because the most xenobiotic conjugates have β configuration [11], it is very probable that this is also the case with carbohydrate esters of dichlorprop. Furthermore it cannot be excluded that labile conjugates, *e.g.* malonyl glucosides [12], were hydrolyzed during the extraction procedure. But no attempts were performed to isolate them.

Unlike barley [1] tomato plants did not hydroxylate dichlorprop. This difference in the metabolism of both species may arise from their belonging to mono- and dicot plants. The different capacity of tomato and barley to hydroxylate dichlorprop is possibly the reason for the different susceptibility and tolerance to the herbicide. However, 2,4-D was hydroxylated by many plants and cell cultures irrespective of mono- and dicot [2].

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