Plant Metabolism of Xenobiotics. Comparison of the Metabolism of 3,4-Dichloroaniline in Soybean Excised Leaves and Soybean Cell Suspension Cultures

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Excised leaves and cell suspension cultures of soybean (*Glycine max* L.) were incubated with [UL-^{14}C]-3,4-dichloroaniline. The compound was almost completely metabolized after 48 h in both systems; it was apparent that the major detoxification pathways present in the excised leaves were also present in the cultured cells. Besides considerable amounts of insoluble residues, the N-glucosyl and the N-malonyl conjugates of 3,4-dichloroaniline, and a yet unknown metabolite was formed in the excised leaves; tentatively the latter was identified with the 6′-O-malonylester of N-glucosyl-3,4-dichloroaniline. The cell suspension cultures produced predominantly the N-malonyl conjugate, besides negligible amounts of the N-glucosyl conjugate, and insoluble residues; the majority of the N-malonyl compound was excreted into the medium. It was shown, that the metabolism of 3,4-dichloroaniline in excised leaves and cell suspension cultures of soybean was directed towards different end products: the excised leaves were able to make extensive use of cell wall structures as a deposit for xenobiotic bound residues, resembling plants in this respect; lack of these structures in the cultured soybean cells resulted in a soluble end product, namely the N-malonyl conjugate.

Introduction

Several methods have been elaborated in order to study the metabolic fate of xenobiotics (*e.g.* pesticides) in plants. These include studies using whole, intact plants grown in nutrient solution, excised leaves, cell cultures, and enzyme extracts [1, 2]. Though each of these methods tends to separate metabolic effects caused by the plant material itself from transformations caused by microorganisms, and abiotic factors, they present different degrees of simplification with regard to plants found in the environment. It seems reasonable to examine the metabolism of xenobiotics at some of these levels, first to get a more detailed insight into the processes involved in xenobiotic metabolism, and second to detect the differences among individual procedures, and to reveal their facilities and limitations. Cell suspension cultures have been used to some extent for metabolism investigations [3, 4]; due to the use of different conditions, results are often not comparable to each other. These difficulties could be overcome by using a standard screening procedure [5–9], which was tested for its practicability and reliability [10]. Excised leaves used by several laboratories are a "step closer" to the plant; in case the applied xenobiotic is taken up *via* the petioles, they seem to be a suitable system in order to study the fate of a given compound once the chemical has entered the xylem, and is transported under the influence of the transpiration stream from roots to shoots. The chlorophyll-deficient cell suspension cultures used in the present investigation were cultured in the dark, thus minimizing undesirable photochemical transformations of the applied parent xenobiotic and its metabolites. By contrast excised leaves are endowed with a functioning photosynthetic apparatus; results provided by this system should complete data obtained from experiments using cell suspension cultures.

Abbreviations: DCA, 3,4-dichloroaniline; DCA-Glu, N-β-D-glucopyranosyl-3,4-dichloroaniline; DCA-Mal, N-malonyl-3,4-dichloroaniline; DCA-Mal-methylester, N-malonyl-3,4-dichloroaniline methylester; 2,4-D, 2,4-dichlorophenoxyacetic acid; parathion, O,O-diethyl-O-4-nitrophenyl-phosphorothionate; PCP, pentachlorophenol; AcOH, acetic acid; BuOH, butanol; Et_2O, diethylether; EtOAc, ethylacetate; HFBA, heptafluorobutyric anhydride; MeOH, methanol; GC, gaschromatography; MS, mass spectrometry; TLC, thin-layer chromatography.

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3,4-Dichloroaniline is the aromatic part of certain pesticides, and is released into the environment due to metabolism of the parent chemicals by plants, animals and microorganisms. In rice plants the metabolic fate of one of these compounds (N-propionyl 3,4-dichloroaniline) was studied [11]; subsequent investigations were directed towards the metabolism of the 3,4-dichloroaniline moiety [12, 13], especially with regard to bound residues. More recently, DCA has become one of those reference chemicals examined in different ecochemical, and ecotoxicological testing procedures. Results concerning the metabolism of DCA in wheat and soybean cell suspension cultures were published [9, 14]; the screening procedure mentioned above was used in these investigations. Enzyme systems, responsible for the conjugation of the compound with glucose and malonic acid, were characterized and isolated from different plant tissues [15–17]. The objective of the present investigation was, first to study the metabolic fate of 3,4-dichloroaniline in excised leaves and plant cell suspension cultures of soybean, and secondly to compare and value the data resulting from the two systems, in view of the processes observed or to be expected in intact plants.

Materials and Methods

Chemicals

[UL-^{14}C]-3,4-Dichloroaniline (12.9 mCi/mmol) was provided by Sigma Chemical Company; the radiochemical purity was greater than 98% by thin-layer chromatography (solvent systems: CH_2Cl_2, toluene, hexane/Et_2O 25/75). 3,4-Dichloroaniline (DCA), pestanal-quality, was supplied by Riedel-de Haen.

Reference compounds

N-β-D-Glucopyranosyl-3,4-dichloroaniline (DCA-Glu) was prepared by the reaction of 3,4-dichloroaniline with D-glucose [18]. The pure β-isomer was isolated by preparative TLC (system B, see below) and confirmed by ^{1}H NMR (500 MHz, CD_3OD, ppm relative to TMS): 3.33–3.40 (m, 2H); 3.45 (t, J = 8.7 Hz, 1H); 3.64–3.68 (m, 2H); 3.86 (dd, J = 12.1, 2.3 Hz, 1H); 4.48 (d, J = 8.5 Hz, 1H, H–C(1′), anomeric proton); 6.70 (dd, J = 8.9, 2.7 Hz, 1H, H–C(6)); 6.91 (d, J = 2.5 Hz, 1H, H–C(2)); 7.22 (d, J = 8.8 Hz, 1H, H–C(5)). Acidic hydrolysis of the product (see below) yielded 3,4-dichloroaniline and glucose. N-Malonyl-3,4-dichloroaniline (DCA-Mal) was synthesized from malonic acid and 3,4-dichloroaniline in the presence of dicyclohexylcarbodiimide [16]; the crude product was purified by column chromatography on silica gel (CH_2Cl_2/cyclohexane 1/1, CH_2Cl_2/cyclohexane/AcOH 50/45/5, CH_2Cl_2/AcOH 95/5), and confirmed by ^{1}H NMR (500 MHz, pyridine-d_5, ppm relative to TMS): 3.87 (s, 2H, H–C(2′)); 7.39 (d, J = 8.9 Hz, 1H, H–C(5)); 7.65 (dd, J = 8.7, 2.6 Hz, 1H, H–C(6)); 8.37 (d, J = 2.4 Hz, 1H, H–C(2)); 11.50 (s, 1H, COOH); 13.6 (br. s, 1H, NH).

Derivatization reagents and procedures

The methyl ester of DCA-Mal (DCA-Mal-methylester) was prepared by reaction with BF_3-MeOH (20%, Merck) at 20 °C overnight [19]. Heptafluorobutyric acid derivatization of the aniline was achieved at 20 °C for 15 min using a 1/1 mixture of EtOAc/heptafluorobutyric anhydride (HFBA; Fluka), solvent and reagent were carefully evaporated with a stream of N_2, and the remaining product redissolved in EtOAc.

Hydrolysis

The N-glucoside was hydrolyzed with 1 M HCl at 20 °C for 45 min [18]; the reaction mixture was adjusted to pH = 9 (NH_3) and extracted with CH_2Cl_2. Alkaline hydrolysis of DCA-Mal was accomplished by 2 M NaOH at 40 °C for 2 h [20]; the hydrolysate was extracted with CH_2Cl_2.

Chromatography

Silica gel plates (SIL G-25 UV_{254}, 0.25 mm, Macherey & Nagel; silica gel 60 F_{254}, 1.00 mm, Merck) were used for all thin-layer chromatography (TLC). TLC solvent systems mainly used were: A: CH_2Cl_2, B: system A, and $CHCl_3$/MeOH/H_2O 65/25/4 consecutively, C: EtOAc/AcOH:H_2O (65/10/6), D: CH_2Cl_2/EtOAc/AcOH 1/1/0.1, E: toluene/acetone 75/25. Separated ^{14}C-zones were located with a Berthold Tracemaster-40 radiochromatogram scanner, non-labeled compounds by fluorescence quenching under UV light (254 nm). A Hewlett-Packard 5890 Series II gas chromatograph equipped with ECD or MSD

(Hewlett-Packard 5971 A) and a capillary column (SE-54, 50 m, i.D. 0.25 mm, film thickness 0.58 µm; CS Chromatography Service GmbH) was used for GC and GC/EI-MS, respectively.

Radioanalysis

Quantitative ^{14}C measurements were made with Hydroluma (Canberra-Packard) counting cocktail and a Beckmann LS 5000 TD liquid scintillation spectrometer. Insoluble ^{14}C residues were determined by combustion analysis with a Biological Oxidizer OX 500 (Zinsser/Harvey Instrument Corporation) and with Oxysolve C400 (Zinsser) counting cocktail.

Biological materials and treatments

Excised leaves

Greenhouse soybean plants (*Glycine max* L. *cv.* Harasoy 63) were germinated and grown in soil. Fully expanded leaves from 5- to 12-week old plants including petioles were excised under distilled H_2O. Each petiole was placed into a 1.9 cm × 3.5 cm small flask (application vial) containing 10 µg [^{14}C]DCA (270000 dpm) in 1 ml of distilled H_2O. After an uptake phase of approximately 3 h the petioles were transferred to 1.7 cm × 10.0 cm test tubes (incubation vial) filled with distilled H_2O and maintained at 20 °C under white neon light (Osram-L 65W/25 Universal-White; 5500 lx) with a photoperiod of 16 h. The H_2O phases remaining in the application vials were analyzed for ^{14}C; on an average 70% of the applied radioactivity was absorbed by the excised leaves under these conditions. Metabolism was stopped by MeOH extraction of the treated leaves after 24 h, and 48 h respectively; the experiments were carried out in 5/5 (24 h/48 h) replicates.

Cell suspension cultures

A standard screening procedure was used to study the metabolic fate of xenobiotics in cell cultures (for details refer to references cited above). Soybean (*Glycine max* L. *cv.* Merrill var. Mandarin) cell suspension cultures were grown in the dark at 27 °C in 20 ml Gamborg B5 media supplemented with 4.5 µmol/l of 2,4-D (Aldrich); the nutrient solution was adjusted to pH = 5.5. 1 g of cells was transferred every 7th day into fresh sterile medium by passing them through a spoon-shaped sieve of 0.8 mm mesh width. 20 µg of [^{14}C]DCA (500000 dpm) in 20 µl of MeOH was added to the cell cultures 5 days after the transfer of cell inoculum into fresh media. Incubation was ended 48 h after treatment; the experiments were performed in 7 replicates.

Extraction procedures and isolation of metabolites

Excised leaves

DCA treated leaves were ground and extracted separately by means of an Ultraturrax mixer (Jahnke & Kunkel) with 50 ml of MeOH. Extracts were vacuum filtered (Schwarzband; Schleicher & Schuell) and analyzed for ^{14}C, and by TLC without further concentration (systems A and B) for unreacted DCA and metabolites. Insoluble plant materials were air-dried, and analyzed for bound ^{14}C by means of a sample oxidizer. H_2O phases in the incubation vials were analyzed for ^{14}C, combined, adjusted to pH = 9, and extracted with CH_2Cl_2; the organic phase (78% recovery) was analyzed by TLC (system A).

Combined MeOH extracts were concentrated on a rotary evaporator, the residue was dissolved in H_2O and extracted with *n*-BuOH. The organic phase was concentrated, and the residue redissolved in MeOH/H_2O 9/1; the solution was extracted with cyclohexane. The aqueous MeOH, which contained about 87% of the soluble absorbed radioactivity, was concentrated to an appropriate volume *in vacuo*. An analytical TLC (system B) of this concentrate mainly showed 4 radioactive zones: a) R_f = 0.15, 10%; b) R_f = 0.35, 35%; c) R_f = 0.58, 25%; d) R_f = 0.82, 30%; zone b cochromatographed with DCA-Mal and zone c with the N-glucoside DCA-Glu. – The entire concentrate was subjected to preparative TLC using solvent system B. Individual areas were scraped off and eluted with MeOH. Recoveries were: a) 40%, b) 46%, c) 84%, and d) 20% respectively. – Zone c was subjected to acidic hydrolysis; the ^{14}C-labeled aglycone (recovery 76%) was analyzed by TLC (system A), derivatized with HFBA, and analyzed by GC and GC/MS; EI mass spectrum of N-heptafluorobutyryl-3,4-dichloroaniline, *m/z* (relative abundance %): 359/357 (61/100), 340/338 (5/6), 190/188 (39/64), 162/160 (66/86), 147/145 (11/18), 135/133 (11/18). – Zone b was further purified by preparative TLC (system C, 61% re-

covery). Part of the purified metabolite was analyzed by TLC (system B), and a second part hydrolyzed by 2 M NaOH; the ^{14}C-labeled exocon (68% recovery) was analyzed by TLC (system A), and by GC, and GC/MS (HFBA derivative). A third part of purified b was derivatized in 67% yield to the corresponding methyl-ester using BF_3-MeOH, and analyzed by TLC (system A). – Zone a was further purified by TLC (system B, recovery 34%), hydrolyzed in 1 M HCl (recovery 71%), and analyzed by TLC (system A).

Cell suspension cultures

Media were separated from treated cells by vacuum filtration, and analyzed separately for ^{14}C by liquid scintillation counting, and for unreacted DCA and metabolites by TLC (systems A and B). – 30 ml of $CHCl_3$/MeOH 1/2, so-called Bligh-Dyer system [21], was added to the separated cells. The cells were stored overnight at −20 °C, ground and extracted as described for the excised leaves; the extracts were separated from cell debris by vacuum filtration, washed with $CHCl_3$/MeOH/H_2O 1/2/0.8, and analyzed for ^{14}C by liquid scintillation counting, and by TLC (systems A and B) without further concentration. – Insoluble residues were air-dried, and analyzed for bound ^{14}C as described.

The organic portions of the combined Bligh-Dyer extracts were removed *in vacuo,* the residue diluted with H_2O to a final volume of 250 ml, and extracted as described below for the media (recovery 78%). – The combined media were adjusted to pH = 3 (HCl) extracted with EtOAc, dried over Na_2SO_4, and concentrated *in vacuo* (recovery 73%). The extracts were analyzed by TLC (systems A, B, C, D, and E) and showed 3 radioactive zones: a) R_f = 0.34 (89%), b) R_f = 0.56 (2%), c) R_f = 0.95 (9%) for the media and a) R_f = 0.39 (49%), b) R_f = 0.56 (7%), c) R_f = 0.96 (44%) for the Bligh-Dyer fraction respectively (values listed refer to system B). Zone a and b cochromatographed with DCA-Mal and DCA-Glu, respectively. – The extract of the combined media was subjected to preparative TLC (system C); zone a was scraped off and eluted with MeOH (99% recovery). One part of the purified metabolite was analyzed by TLC (system A and B), a second part was derivatized by BF_3-MeOH (yield 100%) and analyzed by TLC (solvents: A, Et_2O); the third part was hydrolyzed by 2 M NaOH (recovery 90%), and analyzed by TLC (solvent A), GC, and GC/MS (HFBA derivative). – The Bligh-Dyer fraction was subjected to preparative TLC (system C); two fractions were collected. Zone a (recovery 79%) was analyzed as described for the corresponding fraction obtained from the media (yield for BF_3-MeOH derivatization 100%; recovery for alkaline hydrolysis 83%).

Results and Discussion

Excised leaves

[^{14}C]-3,4-Dichloroaniline was metabolized rapidly by the excised soybean leaves; already within 24 h, almost 100% of the absorbed ^{14}C-compound was metabolized yielding soluble and insoluble products. In detail, the distributions of radioactivity after incubation periods of 24 h and 48 h, respectively are summarized in Table I. Because of considerable differences in uptake of the labeled compound among parallel experiments (20% to 99%) only average values are shown. The radioactivity left in the application vials after treatment was analyzed for ^{14}C only; the greater part of the activity found in the incubation vials consisted of unchanged DCA (65%), possibly released into the aqueous phase by desorption from the petioles.

41.9% (24 h) and 35% (48 h) of the radioactivity were found in the MeOH extracts. These findings should be contrasted with the results obtained for the non-extractable residues, which amounted to 21.3% (24 h) and 29.0% (48 h). Apparently the bound residues fraction, resulting from the metabolism of DCA, increased with residence time. Investigations on the metabolism of N-propionyl-3,4-dichloroaniline (propanil) and DCA [11–13] using rice plants (monocotyledon), showed a high

Table I. Incubation of excised leaves of soybean *(Glycine max)* – distribution of radioactivity (in % of applied radioactivity) after application of 10 µg (= 270000 dpm) of [^{14}C]-3,4-dichloroaniline (average values from 5/5 parallel experiments are shown).

Incubation time	24 h	48 h
Application vial:	28.8	25.3
Incubation vial:	2.7	2.1
MeOH extract:	41.9	35.6
Non-extractable residues:	21.3	29.0
Total recovered radioactivity:	94.7	92.0
Recovered unchanged 3,4-DCA: (excepting application and incubation vials)	0.0	0.0

turnover of the parent compound and complexation of the DCA moiety to insoluble, polymeric cell constituents, predominantly lignin. Using excised leaves it was possible to demonstrate (unpublished results), that the dicotyledonous tobacco incorporated about 20% (48 h incubation period) of the applied [^{14}C]DCA into insoluble macromolecular structures.

TLC and radioanalysis of the MeOH extracts showed no significant differences neither among individual leaves nor between the 24 h and the 48 h incubation period, and in addition, that the soluble fraction derived from [^{14}C]DCA consisted entirely of polar products. The combined MeOH extracts were subjected to clean-up; radioanalysis of a TLC of this fraction showed four distinct zones (a–d), which were separated by preparative TLC (Fig. 1). Zone a (10%) was markedly instable and released DCA-Glu (zone c), and traces of DCA. With regard to chemical and chromatographical behaviour, compound d was tentatively identified with the 6′-O-malonylester of DCA-Glu. An analogous compound formed from PCP was recently detected, and suggested as the product to be deposited in the cellular vacuole [22]. – Zone b (35%) was identified with the N-malonyl conjugate of DCA (Fig. 2). GC/MS of the aglycone showed only the presence of the HFBA derivative of DCA. – Zone c (25%) was identified unequivocally with the N-glucoside of 3,4-dichloroaniline by cochromatography with reference compound DCA-Glu (Fig. 2), hydrolysis, and analysis of the ^{14}C-labeled aglycone. DCA-Glu exhibited a certain degree of decomposition to DCA when using acidic (AcOH) TLC solvent systems; this acid sensitivity was studied thoroughly [14]. – Zone d (30%) demonstrated an unusual behaviour; though extensively analyzed, the identity of this radioactive fraction remained obscure. It is reasonable to assume, that fraction d resulted from non-enzymatic interactions of DCA with cell constituents or artefacts during work-up procedures. – The results concerning the soluble metabolite fraction discussed above are in agreement with published data concerning the formation of the N-glucoside [11, 12]. A N-glucosyltransferase for arylamines was detected in soybean plants [15]; recently, published results [16, 17] demonstrated the existence of N-malonyl-, and N-glucosyltransferases in peanut and soybean with specificity for xenobiotics. The data of the study in hand indicate, that in excised soybean leaves these enzyme activities are present and able to metabolize 3,4-DCA to its glucose and malonic acid conjugates.

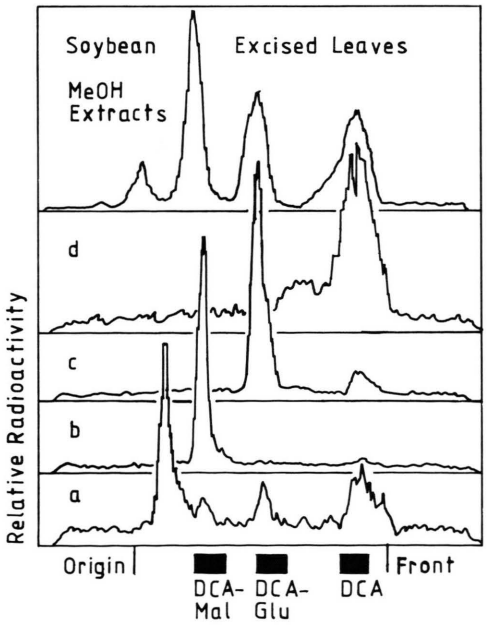

Fig. 1. Radioactivity profiles: Combined MeOH extracts of the excised soybean leaves, separated zones a–d, and positions of the unlabeled reference compounds DCA-Mal, DCA-Glu, and DCA (solvent system B).

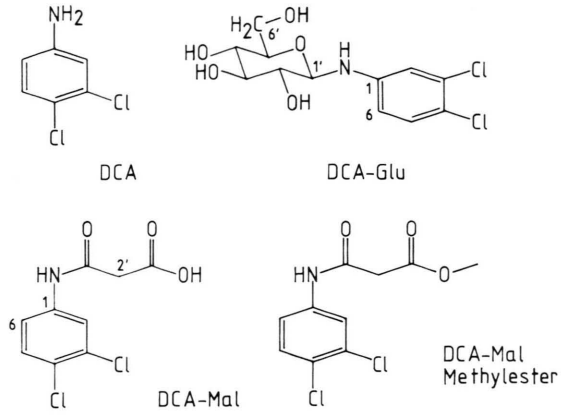

Fig. 2. Chemical structures of 3,4-dichloroaniline (DCA), N-β-D-glucopyranosyl-3,4-dichloroaniline (DCA-Glu), N-malonyl-3,4-dichloroaniline (DCA-Mal), and N-malonyl-3,4-dichloroaniline methylester (DCA-Mal-methylester).

Cell suspension cultures

The cell suspension cultures of soybean were capable to metabolize [^{14}C]DCA rapidly and almost completely; within 48 h after application the labeled compound was transformed to polar soluble products, and to minor amounts of insoluble bound residues. The details of the present investigation discussed below are in agreement with published data [9, 14], and show the reliability of the method. Table II summarizes the distribution of radioactivity among different fractions. Only 3.6% of the applied radioactivity were found as non-extractable residues; this rather low percentage is commonly attributed to the low lignin content of the cultured soybean cells. Not expected, unusually low levels of bound residues in soybean cell suspension cultures were also found in metabolism studies with other pesticides, *e.g.* parathion, 2,4-D, and PCP [10, 23]. Unlike cell suspension cultures, intact soybean plants are expected to produce large amounts of aniline derived bound residues [24]. The present results concerning cell cultures, and excised leaves as a model "closer" to intact plants (3.6% and 21.3/29.0%, respectively) are in agreement with these data.

78% of the applied radioactivity were found in the media after removal of the cells. Individual replicates were analyzed by TLC and showed, first no significant differences, and second a prominent metabolite peak; this metabolite was also present in the cell extracts, which amounted to 13% of the applied radioactivity (discussed below). The EtOAc extract of the media showed three radioactive zones (a–c; Fig. 3). Peak a (89%) was identified with DCA-Mal; this is in agreement with published data [14]. – On account of low amounts, zones b and c were not further characterized; zone b (2%) cochromatographed with synthesized reference compound DCA-Glu. An EtOAc extract of the combined Bligh-Dyer fractions was analyzed by TLC; three radioactive zones were detected (a–c; Fig. 3). Peak a (49%) was identified with DCA-Mal; this result agrees with published data [14] – Zone b (7%) cochromatographed with DCA-Glu. – The nature of peak c (44%) recovery) remained obscure; it is reasonable to assume, that the radioactive material was formed by analogous processes as discussed for fraction d derived from the excised leaves.

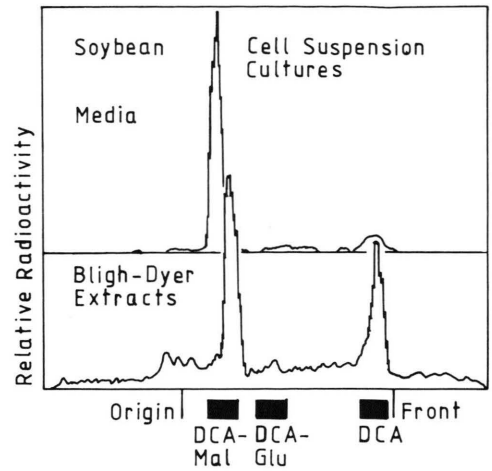

Fig. 3. Radioactivity profiles: EtOAc extract of combined media, EtOAc extract of combined Bligh-Dyer extracts of the soybean cell suspension cultures, and positions of the unlabeled reference compounds DCA-Mal, DCA-Glu, and DCA (solvent system B).

Table II. Incubation of cell suspension cultures of soybean *(Glycine max)* – distribution of radioactivity (in % of applied radioactivity) after application of 20 µg (= 500000 dpm) of [^{14}C]-3,4-dichloroaniline (average values from 7 parallel experiments and relative standard deviations at the 95% confidence interval are shown).

Culture medium:	78.1 ± 3.1
Bligh-Dyer extract:	13.2 ± 2.7
Non-extractable residues:	3.6 ± 0.4
Total recovered radioactivity:	94.9 ± 3.8
Total recovered unchanged 3,4-DCA:	0.8 ± 1.2
Turnover rate:	94.1 ± 3.8

Acknowledgements

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