Phytochemistry, Vol. 27, No. 6, pp. 1709–1711, 1988.
Printed in Great Britain.

0031-9422/88 $3.00+0.00

METABOLISM OF CHLORTOLURON IN CELL SUSPENSIONS OF *LACTUCA SATIVA*: A QUALITATIVE CHANGE WITH AGE OF CULTURE

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(*Received* 13 *November* 1987)

Key Word Index—*Lactuca sativa*; Compositae; lettuce; suspension culture; chlortoluron; pesticide metabolism; demethylation; hydroxylation.

Abstract—A cell suspension culture originating from newly isolated hypocotyl callus of *Lactuca sativa* cv 'Suzan' metabolized the dimethylphenylurea herbicide chlortoluron by sequential *N*-demethylation and by hydroxylation of the 4-methylphenyl group. These two modes of attack occurred in equal proportion, giving rise to four oxidised products. In a cell suspension established from five-year-old hypocotyl callus the ability to carry out *N*-demethylation predominated over hydroxylation of the 4-methylphenyl group by a ratio of 3:1. It is suggested that a selective decline in the ability to perform ring methyl hydroxylation of chlortoluron had occurred in the five-year-old tissue culture.

INTRODUCTION

Plant cell suspensions are able to degrade a wide variety of pesticides using enzymic mechanisms similar to those displayed by intact plants. Because of this, cell cultures have found increasing favour for pesticide metabolism studies and the advantages of tissue culture over whole plants have been discussed by Mumma and Davidonis [1]. However, if tissue culture methods are to be used routinely in assessing the metabolic fate of pesticides, it will be necessary to be aware of the factors involved in the initiation and maintenance of cultures which may limit their application.

The selective herbicide chlortoluron (*N'*-(3-chloro-4-methylphenyl)-*N*,*N*-dimethylurea) is susceptible to combined metabolic attack by progressive oxidative *N*-demethylation and by hydroxylation of the 4-methylphenyl group in intact plants [2–4] and in cell suspensions [5, 6]. This gives rise to several derivatives of modified phytotoxicity. The benzyl alcohol derivatives may oxidise slowly to the corresponding benzoic acids although these are minor, long term products in whole plants [2]. All oxidation products of chlortoluron may form glycosyl conjugates. Since the biotransformation of chlortoluron occurs by competing mechanisms, this herbicide is a useful tool with which to detect factors which may influence the qualitative metabolism of pesticides by cell suspensions.

In view of the genetic variation which occurs over time in cultured plant cells [7, 8], age is potentially the most important factor which will affect the fidelity of metabolic pathways operating in tissue culture to those in intact plants. This requires investigation since tissue cultures used in pesticide metabolism laboratories have often been in existence for many years. We have already examined the influence of nutrient medium composition on the metabolism of chlortoluron in cotton (*Gossypium hirsutum* L) and maize (*Zea mays* L.) cell suspensions [6].

RESULTS AND DISCUSSION

In a suspension culture established from newly isolated lettuce hypocotyl callus two major products of chlortoluron (**1**) were found after 24 hr (Fig. 1). These were *N'*-(3-chloro-4-methylphenyl)-*N*-methylurea (**2**) and *N'*-(3-chloro-4-hydroxymethylphenyl)-*N*,*N*-dimethylurea (**4**). Minor compounds resulted from the further *N*-demethylation of **2** (forming **3**) and the mono-*N*-demethylation of **4** (forming **5**). At this time, by far the greater proportion of chlortoluron and its oxidized products were detected in the culture fluid rather than the cells, although it was determined previously that culture fluid which had supported cell growth possessed no enzymic activity towards chlortoluron [6]. During this early stage of metabolism the polar conjugate fraction was minor and therefore it was unnecessary to hydrolyse this to ascertain the major products of primary metabolism. These results demonstrate that in common with intact plants or cell suspensions of several species including cotton, maize and wheat (*Triticum aestivum* L.) [2–6], lettuce cells converted chlortoluron by both successive *N*-demethylation and ring-methyl hydroxylation. We did not detect *N'*-(3-chloro-4-hydroxymethylphenyl) urea as in other species but it is likely that this would have appeared in longer term incubations.

By contrast, a cell suspension arising from a lettuce hypocotyl callus culture initiated five years previously displayed a more limited ability to carry out ring methyl hydroxylation (forming **4** and **5**). As a result, *N*-demethylation predominated over hydroxylation (Fig. 1), giving compound **2** as the major product and giving compound **3** as a more significant product than in the new culture. The ratio of total demethylated products was 3:1 whereas in the new culture this ratio was 1 : 1. Although rates of overall metabolism were comparable for the two cultures the fresh weights of cells at harvest were 1.52 $\pm$0.08 g and 3.08 $\pm$0.18 g for the new and five year cul-

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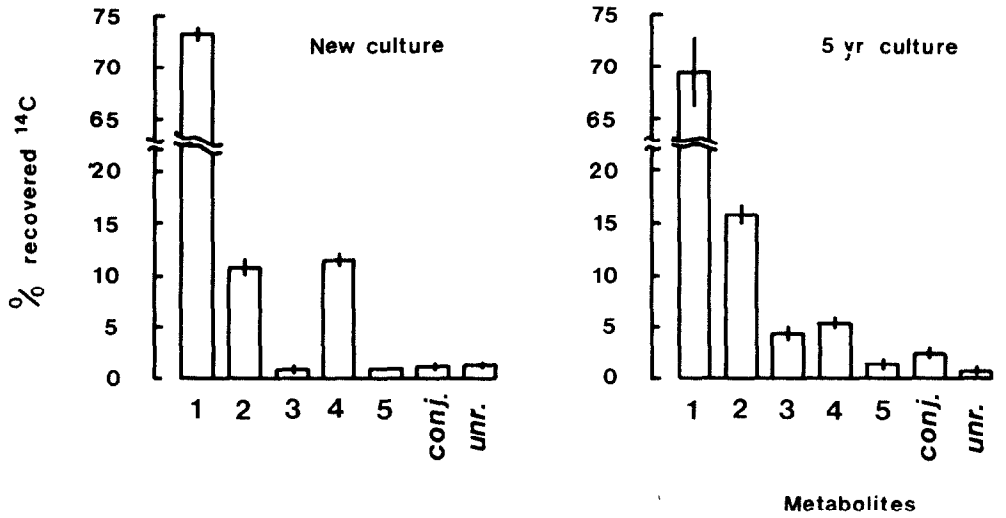

Fig. 1. Metabolism of ^{14}C-chortoluron after 24 hr in suspension cultures established from newly isolated or five year-old hypocotyl callus tissue of lettuce. Data are the sums of residues found in the cell extract and in the culture fluid. See Fig. 2 for key to compounds 1–5. Conj. = polar conjugates, unr. -unresolved radioactivity on TLC plates.

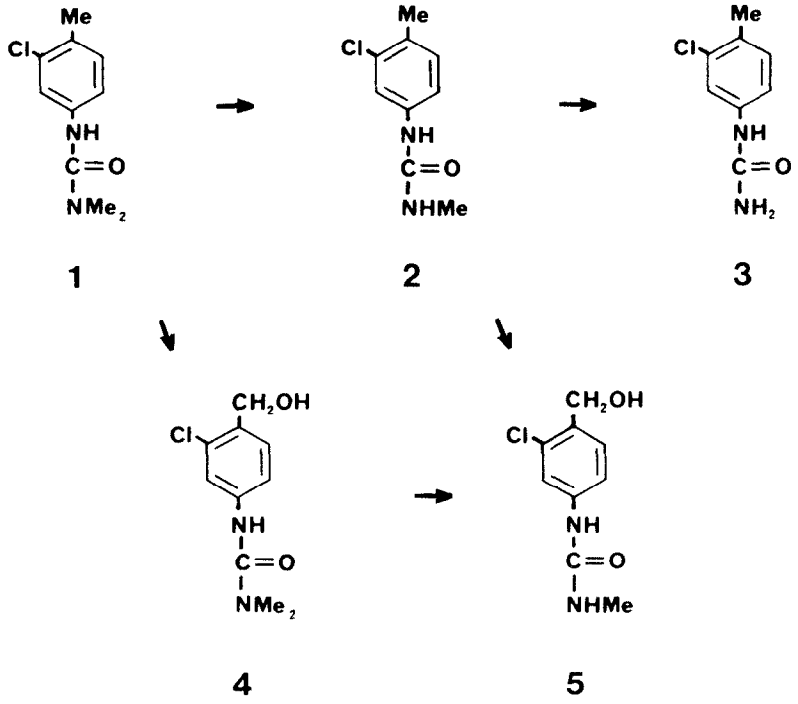

Fig. 2. Biotransformation of chlortoluron in lettuce suspensions.

tures respectively. Therefore the five year culture appeared to be less metabolically competent overall with respect to chlortoluron but particularly in the case of methylphenyl hydroxylation. The metabolic pathways for chlortoluron metabolism in lettuce cells are given in Fig. 2.

It has been stated that mechanisms of pesticide metabolism possessed by whole plants are generally retained in the derivative cell cultures [1]. However, in this laboratory several exceptions have been found. In a parallel study of the breakdown of the acylalanine fungicide metalaxyl in these two lettuce cell suspension lines the contributions of ring methyl hydroxylation and ester hydrolysis were lower compared with other forms of metabolism in the older culture than the new culture [9]. It is asserted that in the case of both these pesticides suspensions produced from callus which had been in existence for several years displayed an impaired ability

to carry out hydroxylation of the ring methyl group, resulting in a change in the profile of expected metabolites. Suspensions produced from maize callus which had been maintained for several years failed to conjugate the herbicide atrazine with glutathione [10] even though this reaction occurred readily in maize plants. It is unclear whether conjugation ability was lost upon dedifferentiation of tissue or whether decline was gradual.

It is concluded that age-dependent changes in metabolic capabilities may seriously limit the application of cell cultures to pesticide metabolism studies and that age may be a more important limiting factor than nutrient medium composition or explant source.

EXPERIMENTAL

Suspension cultures. For the purpose of this study, a cell suspension was established from a newly isolated seedling hypocotyl callus of *Lactuca sativa* L. cv 'Suzan'. Full details of culture initiation, maintenance conditions and subculture are given elsewhere [9]. The metabolism of chlortoluron in this line was compared with that in a suspension culture established from hypocotyl callus of the same cultivar which had been initiated 5 years previous to conducting these experiments [5]. This latter line had been maintained throughout as callus tissue on solid medium. Both lines were grown on a nutrient medium containing 10% (v/v) coconut water [11].

Metabolic experiments. At 5 days after inoculation, when cells were in log growth phase, [U-^{14}C-*phenyl*]-chlortoluron, sp. act. 415 MBq/mmol was added aseptically to cells in 20 μl MeOH, giving 1.5×10^6 DPM or 60 nmol per flask. Radiolabelled chlortoluron was found to be 98% radiochemically pure by TLC.

Harvest and extraction of cells and culture fluid After incubation with ^{14}C-chlortoluron for 24 hr, cells and culture filtrates were collected and cells were extracted as previously, the 80% MeOH extracts of cells being reduced *in vacuo* for analysis. Radioactivity in culture filtrates was concentrated by passing 15 ml through C_{18} reversed phase cartridges. After washing with water, bound material was eluted in 3 ml 100% MeOH. Cell extracts and culture filtrates were both radioassayed prior to analysis. For all experiments, recovery of radioactivity was 95–97% of the applied dose.

Analytical methods. Chlortoluron and its products in cell extract concentrates and C_{18} eluates of culture media were initially analysed by 2D TLC on plastic-backed silica gel 60 F_{254} plates (Merck) in $CHCl_3$–EtOH–Me_2CO (9:2:1), followed by $CHCl_3$–EtOH–HOAc (9:1:1) and thereafter routinely by 1D TLC in the latter solvent system. Parent herbicide and products were identified by co-chromatography with a series of unlabelled reference compounds. Polar conjugates were retained at the origin of TLC plates. Radioactive areas were visualized with X-ray film, cut out and quantified by liquid scintillation spectrometry. Areas of lanes not containing discrete bands were also assayed to determine residual radioactivity.

Experimental replication. Results are the means from two independent experiments conducted on the 4th and 5th growth cycles after initiation of suspensions from both new and 5 year old callus. Experiments were internally triplicated and data means are given with standard errors.

Acknowledgement—We thank Ciba-Geigy AG (Basle) for financial support and for generous provision of radiolabelled material and reference compounds.

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