



RESEARCH PAPER

Fatty acid elongation is important in the activity of thiocarbamate herbicides and in safening by dichlormid

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Abstract

The thiocarbamates, such as pebulate (S-propyl butyl (ethyl) thiocarbamate) are a well-established class of herbicides. They inhibit fatty acid elongation, which is necessary for the biosynthesis of constituents of surface waxes and suberin and this has been proposed to be important for their toxicity. In this study lipid metabolism was investigated in herbicide-treated barley (*Hordeum vulgare*) and a pernicious weed, wild oats (*Avena ludoviciana*), to test the hypothesis that inhibitory effects on fatty acid elongation could be counteracted by the safer, dichlormid. Pebulate and its sulphoxide derivative (thought to be the active metabolite *in vivo*) were tested against lipid metabolism in barley or wild oat shoots. In both plants there was a significant inhibition of very long chain fatty acid (VLCFA) synthesis at herbicide concentrations $\geq 25 \mu\text{M}$. The extent to which safer dichlormid could prevent the inhibition of VLCFA synthesis was different in the two species. Previous treatment of barley with dichlormid (*N,N*-diallyl-2,2-dichloroacetamide) enabled fatty acid elongation in the presence of pebulate or pebulate sulphoxide, but had no effect on wild oats. The effects on fatty acid elongation mimicked the differential safening action of dichlormid observed on shoot elongation and growth in the two species. These data provide further evidence that inhibition of VLCFA formation is important for the mechanism of action of thiocarbamates.

Key words: *Avena ludoviciana*, barley, dichlormid, fatty acid elongation, *Hordeum vulgare*, thiocarbamate herbicide, wild oats.

Introduction

Thiocarbamates are a well-established group of herbicides which were noted some 30 years ago to alter the production of surface waxes in plants (Still *et al.*, 1970). This effect was postulated to provide a basis for the toxicity of such compounds (Harwood, 1991). Waxes and other components of the surface layer of plants are derived from very long chain (>18C) fatty acids (Kolattukudy, 1980; von Wettstein-Knowles, 1995) and elongation reactions used for their synthesis were inhibited by several thiocarbamates (Harwood and Stumpf, 1971). These early studies have been extended to a variety of plants (Bolton and Harwood, 1976; Abulnaja and Harwood, 1991a) and have included *in vitro* experiments on the elongation enzyme reactions themselves (Walker and Harwood, 1986; Barrett and Harwood 1998a). The data show that thiocarbamates do not affect *de novo* synthesis of fatty acids, but only inhibit the fatty acid elongases responsible for extra-chloroplast VLCFA formation (Cassagne *et al.*, 1987).

Generally, thiocarbamates exert their major herbicidal effects after metabolic conversion to their sulphoxide derivatives (Owen, 1991; Katagi and Mikami, 2000). Although the mechanism of this activation is unknown it may be through the activity of a cytochrome P450 monooxygenase or, more likely, a peroxygenase (Blée, 1998). The increased effectiveness of sulphoxide derivatives compared to the parent thiocarbamate has been reported previously (Barrett and Harwood, 1998b). Such results on VLCFA synthesis are consistent with the postulate that sulphoxides are the herbicidally-active compounds formed from thiocarbamates (Owen, 1991; Katagi and Mikami, 2000).

Safening compounds are agrochemicals which can be used to treat crop plants so that they are resistant to the subsequent application of a herbicide (Pallos and Casida, 1978). In effect, safening of a crop turns a broad-spectrum

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Abbreviations: FAME, fatty acid methyl-ester; GLC, gas-liquid chromatography; GST, glutathione S-transferase; VLCFA, very long chain (>18C) fatty acid.

herbicide into a selective one in that it no longer kills the crop. For thiocarbamates, a number of different safeners have been used successfully in different crops (Devine *et al.*, 1993; Gronwald, 1989). In these cases, the production and/or detoxification of their sulphoxide derivatives may be altered by the use of safeners (Hatzios, 1991; Wiegand *et al.*, 1986). Therefore, if fatty acid elongation is central to the mechanism of action of thiocarbamates, through their active sulphoxide derivatives, then safeners which modify the metabolism of sulphoxides should also change elongation. In studies using 1-aminobenzotriazole (Abulnaja and Harwood, 1991b) or naphthalic anhydride (Barrett and Harwood, 1998c) it was found that they did, indeed, have a protective effect on the inhibition of fatty acid elongation by thiocarbamates.

To test this proposal further, a well established safening compound, dichlormid, was used (Fig. 1) (Hatzios, 1991). Its use has been restricted to the Gramineae and, most commonly, for the protection of maize crops against the herbicidal effects of thiocarbamates (Devine *et al.*, 1993; Gronwald, 1989).

Various reasons have been proposed for the safening capacity of dichlormid, including competitive antagonism,

increased detoxifying-enzyme activity, down-regulation of herbicide activation, up-regulation of defence gene transcription, and induction of novel enzymes (Ezra and Stephenson, 1989). In addition, dichlormid is known to induce glutathione *S*-transferases (GSTs), a diverse group of enzymes involved in herbicide detoxification via conjugation with glutathione (Jepson *et al.*, 1994) and this is thought to be a major mechanism of its action (Hatzios, 2000).

In the study reported here, the effectiveness of dichlormid has been examined in antagonizing the herbicidal activity of pebulate (or its sulphoxide) in barley and the common weed, wild oat. The spread of wild oats (*Avena ludoviciana*), a pernicious weed, is a major factor competing with, and reducing yields of, barley (Gressel, 1991). Both species are known to be susceptible to thiocarbamates, grow rapidly and show good labelling of VLCFAs and, thus, were appropriate experimental systems. The herbicidal effects and safening were then compared to changes in the elongation of fatty acids. The data show that the specific inhibition of VLCFA synthesis by thiocarbamates can be reversed by dichlormid in barley but not in wild oats and that these effects are paralleled by changes in growth. These results, therefore, add to the evidence that the elongation of VLCFAs by thiocarbamates is physiologically relevant and central to their mechanism of action.

Materials and methods

Plant treatments

Seeds of barley (*Hordeum vulgare* cv. Maris Otter) and wild oats (*Avena ludoviciana*) were obtained from Plant Breeding International, Cambridge. Seeds were sown in vermiculite and grown in an incubator under a 16/8 h light/dark cycle at 20 °C, provided by fluorescent tubes (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Shoots were sampled from 7-d-old barley and 10-d-old wild oat plants.

Pebulate (*S*-propyl butyl (ethyl) thiocarbamate) and its sulphoxide (Fig. 1) were a kind gift from CJ Spiller (ICI Americas). They were dissolved in absolute ethanol and then diluted before application to the whole plant or for incubations of primary shoots. For primary shoot incubations the final concentration of ethanol was 2.5% (v/v), while this was lowered to 0.1% (v/v) when applied to the soil in which the plants were growing. Primary shoots were detached and incubated with their cut ends in herbicide for 4 h prior to lipid labelling (4 h) unless otherwise stated. A draught (5 m s^{-1}) was applied to ensure good transpiration. Control experiments showed that ethanol at the above concentrations affected neither shoot elongation nor lipid metabolism.

The herbicide safener, dichlormid (*N,N*-diallyl-2,2-dichloroacetamide), was obtained from ChemService, Birkenhead, UK. Dichlormid was dissolved in absolute ethanol and diluted to a concentration of 100 μM in 0.1% (v/v) ethanol and seeds were incubated in this for 3–24 h before planting. Lipid labelling involved the incubation of primary shoots with 2 μCi [1- ^{14}C]acetate for 4 h under conditions previously detailed (Barrett and Harwood, 1998b).

Extraction and analysis of lipids

Lipid extracts were obtained using a two-phase partition method (Garbus *et al.*, 1963). Fatty acid methyl esters (FAMES) were produced by acid-catalysed methanolysis and radiolabelled FAMES

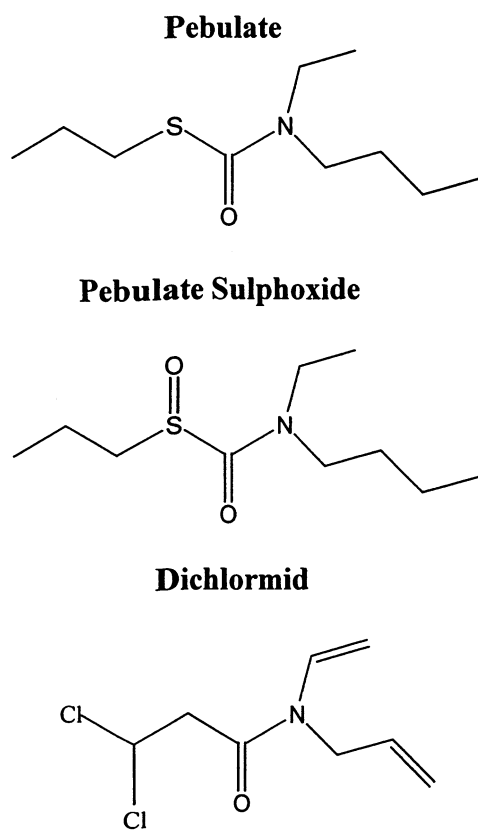


Fig. 1. The structures of the thiocarbamate herbicide pebulate, its sulphoxide derivative and the herbicide safener dichlormid.

analysed by radio-GC using a Pye-Unicam GCD gas chromatograph connected to a LabLogic RAGA (LabLogic Systems Limited, Sheffield, UK) gas flow proportional counter. A glass column (1.5 m×4.5 mm) packed with 5% SP-2100 (equivalent to SE-30) on Supelcoport 100/120 (Supelco, Sigma-Aldrich, Poole, UK) was routinely used. A temperature programme of 10 min at 210 °C followed by a 4 °C min⁻¹ rise to 250 °C, followed by holding at 250 °C for the remainder of the run, was used. Routine identification was by co-chromatography with authentic standards, although the barley FAME products were further identified by chemical degradation and chromatographic methods (Abulnaja, 1989).

Results and discussion

Fatty acid labelling patterns

Both barley and wild oats incorporated radioactivity from [¹⁴C]acetate effectively into fatty acids, including very long chain products (Table 1). In previous studies (Abulnaja, 1989) it was demonstrated that these VLCFAs were all produced by elongation, as expected for such compounds (Cassagne *et al.*, 1994). A notable advantage of monocotyledons when studying elongation is that they seem particularly active for such reactions (Abulnaja and Harwood, 1991a) and, in the present experiments, up to half of the total products were saturated very long chain fatty acids. Chain lengths of up to 26C (cerotic acid) were easily detected.

Effect of pebulate and its sulphoxide

Pervious work with pebulate and its sulphoxide had indicated that these were effective in the 5–100 µM range (Barrett and Harwood, 1998a, b); effects on the fatty acid labelling of barley shoots could be seen at 25 µM (data not shown). 100 µM was routinely used since this produced a strong inhibitory effect on the labelling of VLCFAs. Notably, 100 µM pebulate caused an almost total inhibition of the labelling of 22–26C fatty acids (i.e. it inhibited the elongation reactions that led to these

products) in both barley and wild oats (Table 1). By contrast, the absolute labelling of the 16C acid palmitate and the 18C products stearate and oleate, which are produced by *de novo* synthesis, was not significantly affected. Thus, total labelling of 16C and 18C fatty acids for barley was 1.68, 1.20 and 1.47×10⁴ dpm in controls, pebulate and pebulate sulphoxide-treated, respectively. For wild oats the equivalent figures were 1.64, 1.64 and 1.71×10⁴ dpm, respectively (Table 1). This selective effect of pebulate fits well with previous results for this (Barrett and Harwood, 1998a, b) and other thiocarbamates (Abulnaja and Harwood, 1991a; Bolton and Harwood, 1976) on fatty acid biosynthesis. Pebulate did not affect labelling of arachidic acid. However, this acid was a minor product, so that any statistically significant effect would be difficult to detect. Nevertheless, because separate elongases (condensing enzyme component) are used to form different very long chain products (Walker and Harwood, 1986; Cassagne *et al.*, 1994), the lack of inhibition of arachidate (20:0) labelling may be due to an insensitivity of that particular elongase. Such an effect was noted when thiocarbamates were used to inhibit fatty acid elongation in different experiments (Abulnaja and Harwood, 1991c).

As the sulphoxide derivations of thiocarbamates are believed to be their herbicidally effective metabolites (Hatzios, 1991), pebulate sulphoxide was also tested against barley and wild oats (Table 1). The results were very similar to those with pebulate showing that labelling of the fatty acids behenate, lignocerate and cerotate was effectively inhibited, while the total *de novo* synthesis of palmitate and oleate was unaltered. Although labelling of arachidate appeared to be reduced in both plants, the effect was not statistically significant.

Safening by dichlormid

If the inhibition of fatty acid elongation by pebulate or its sulphoxide was central to the herbicidal mechanism of

Table 1. Mean (±SE) percentage radiolabelling from [¹⁴C]acetate of fatty acids from 7-d-old barley and 10-d-old wild oat shoots with and without exposure to pebulate or pebulate sulphoxide

Total labelling in barley was 2.9±0.2, 1.3±0.4 and 1.6±0.2 dpm ×10⁴ for the control, 100 µM pebulate and 100 µM pebulate sulphoxide-treated incubations, respectively. Total labelling in wild oats was 3.1±0.4, 1.8±0.2 and 1.9±0.1 dpm ×10⁴ for the control, 100 µM pebulate and 100 µM pebulate sulphoxide-treated incubations, respectively. * Significantly different from control (*P* <0.05) using Mann–Whitney test (*n*=3). n.d., None detected. The non-polar (SP-2100) column used for GC did not separate saturated from unsaturated fatty acids enough for their individual radioactivity to be determined. However, independent experiments showed that, with the exception of 18C fatty acids (mainly oleate) virtually all the radioactivity was associated with saturated components.

| Radiolabelled fatty acids | Barley | | | Wild oats | | |
|---------------------------|----------|-----------------|----------------------------|-----------|-----------------|----------------------------|
| | Control | 100 µM pebulate | 100 µM pebulate sulphoxide | Control | 100 µM pebulate | 100 µM pebulate sulphoxide |
| 16C | 23.7±3.0 | 35.6±3.6* | 25.4±6.8 | 21.9±3.9 | 31.3±6.2* | 19.3±1.1 |
| 18C | 34.7±4.2 | 56.0±3.1* | 66.6±5.3* | 30.9±4.2 | 59.8±6.4* | 70.7±3.5* |
| 20C | 3.8±1.1 | 3.8±0.3 | 2.4±0.9 | 4.5±2.8 | 2.6±0.6 | 2.4±1.3 |
| 22C | 14.8±4.7 | 3.8±0.7* | 4.3±2.0* | 19.1±4.5 | 4.2±0.2* | 3.7±0.1* |
| 24C | 12.6±1.3 | 0.8±0.6* | 0.8±1.5* | 9.7±2.6 | 2.0±0.9* | 1.7±1.3* |
| 26C | 10.3±1.8 | n.d.* | n.d.* | 13.9±5.9 | n.d.* | n.d.* |

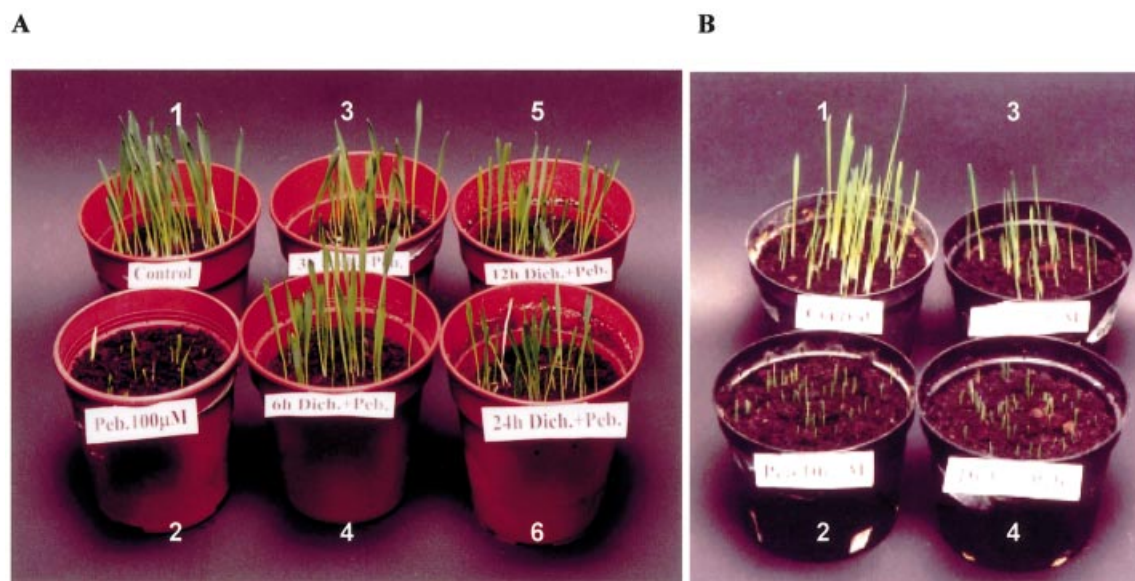


Fig. 2. Effect of pebulate and safening on growth. (A) 7-d-old barley after various pretreatments (see Materials and methods) with 100 μM dichlormid and subsequent exposure to 100 μM pebulate. Control plants were exposed to 0.1% (v/v) ethanol (1); or pebulate dissolved in ethanol (2); or pebulate but with a dichlormid pretreatment for 3 h (3), 6 h (4), 12 h (5) or 24 h (6). (B) 10-d-old wild oats following similar pretreatments/treatments. Controls as above (1); pebulate in ethanol (2); 100 μM dichlormid for 24 h (3); or pebulate with a 24 h dichlormid pretreatment (4).

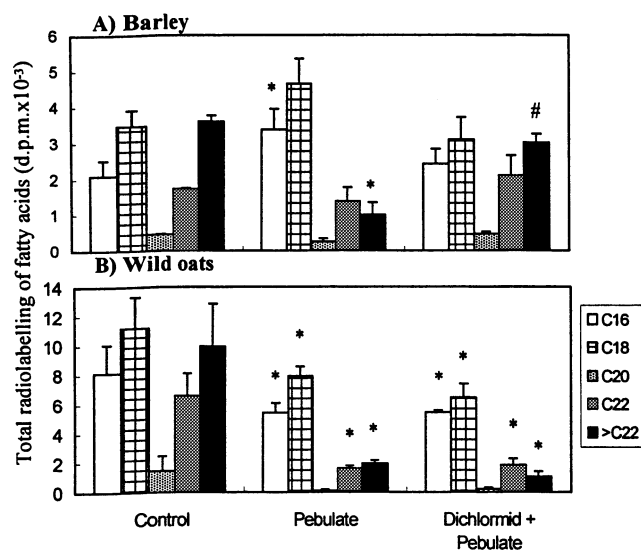


Fig. 3. Fatty acid labelling patterns from shoot incubations of (A) 7-d-old barley or (B) 10-d-old wild oats with [1-¹⁴C]acetate after various treatments. Safened seeds were exposed to 100 μM dichlormid for 24 h before planting. Shoots were exposed to 100 μM pebulate applied directly to the plants growing in vermiculite for 24 h before detaching the shoots for a 4 h incubation with radiolabel. Results are means ± SE (*n*=3). * Significantly different from the control (*P* < 0.05). # Significantly different from pebulate-treated (*P* < 0.05), by Mann–Whitney test.

action, then compounds that can reverse thiocarbamate toxicity *in vivo* should also protect fatty acid elongation (Abulnaja and Harwood, 1991*b*). For Gramineous crops, dichlormid is an effective safener against thiocarbamates

(Farago *et al.*, 1994; Walton and Casida, 1995) and this compound was tested with barley and wild oats. Preliminary experiments showed that dichlormid alone had no significant effect on plant height or lipid labelling in barley, although some inhibition was detected for wild oats (data not shown). However, when used as a seed pretreatment, it effectively reversed the inhibitory action of pebulate on shoot elongation in barley (Fig. 2A), but not in wild oats (Fig. 2B). Pretreatment for as little as 3 h was effective in barley. The effect of pebulate was mainly on plant height rather than germination (Fig. 2). The postulated main activity of thiocarbamates in preventing surface wax formation (Still *et al.*, 1970) would be in agreement with an inhibition of growth rather than germination.

Dichlormid was partially effective at protecting fatty acid elongation in barley (Fig. 3A). As discussed above, pebulate did not affect labelling of fatty acids formed *de novo*, but produced a statistically significant inhibition of elongation. Likewise, the restoration of total fatty acid labelling by dichlormid, restored elongation to either control or near control levels.

In marked contrast, in wild oats, dichlormid was neither able to protect against loss of total fatty acid labelling nor specifically, to reverse the reduced elongation that was induced by pebulate (Fig. 3B). Thus, differential effects of the safener, dichlormid, on fatty acid elongation in the two plant species paralleled the differential ability of the chemical to safen shoot growth. The ability of dichlormid to safen barley against pebulate could be because it prevents activation of the herbicide to its sulphoxide

derivative or, more likely, because of the known effect of this safener in inducing GST activity (Jepson *et al.*, 1994). Increased levels of GST activity should allow more rapid detoxification of pebulate sulphoxide which, as described above, is thought to be the herbicidally-active derivative of pebulate herbicide (Katagi and Mikami, 2000). Certainly, in separate experiments, dichlormid was able to induce increased mRNA levels of a cDNA identified by sequence homology as a GST (Baldwin, 2001).

An attempt was made to eliminate safener effects on herbicide activation as a mode of action by repeating the experiments shown in Fig. 3 with pebulate sulphoxide. Although sulphoxide derivatives of thiocarbamates are accepted generally as the herbicidally-active derivatives (Owen, 1991), they are not used to treat intact plants because of poorer uptake compared to the parent compounds. Indeed, although pebulate sulphoxide did reduce the growth of barley (data not shown), the inhibition was much less than for pebulate itself. Pretreatment of barley with dichlormid allowed increased labelling of VLCFAs compared to treatment with pebulate sulphoxide alone (Fig. 4). As expected, absolute labelling of 16C and 18C acids (produced by *de novo* synthesis) was unaffected.

These data are consistent with the safener, dichlormid, acting by increasing GST activity and, hence, detoxification of the thiocarbamate sulphoxide (herbicidally-active) metabolite. They are also consistent with the conclusion that thiocarbamate sulphoxides are selective against the elongation reactions involved in the production of VLCFAs. However, the results are not clear-cut because of the small inhibition that pebulate sulphoxide had on growth and VLCFA labelling *in vivo*, in comparison to pebulate.

Taken together, these results support previous studies (Abulnaja and Harwood, 1991*b*; Barrett and Harwood, 1998*c*) in suggesting that the inhibition of fatty acid elongation caused by thiocarbamates is central to their

herbicidal action. The differential ability of safening compounds to protect against the reduction in VLCFA labelling in safened species, but not in a non-safened species, is also consistent with this conclusion. It also appears likely that the safening compound dichlormid acts by enhancing detoxification of the metabolically-active sulphoxide derivatives (probably via GST activity) although further experiments are needed to prove this unequivocally.

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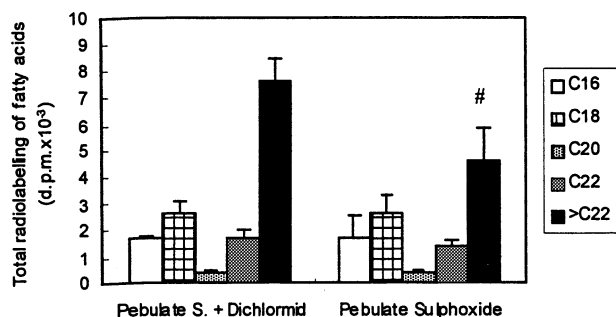


Fig. 4. Fatty acid labelling from shoot incubations of 7-d-old barley. Safened seeds were imbibed in 100 μ M dichlormid for 24 h prior to planting. Shoots were exposed to 100 μ M pebulate sulphoxide applied directly to the plants growing in vermiculite for 24 h before detaching the shoots for a 4 h incubation with radiolabel. Results shown are means \pm SE ($n=3$). # Significantly different by Mann–Whitney test ($P < 0.05$).

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