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Fluazifop, a grass-selective herbicide which inhibits acetyl-CoA carboxylase in sensitive plant species

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Fluazifop is a grass-selective herbicide that appears to act by inhibiting fatty acid synthesis *de novo* in sensitive species. Results from four different types of experiment show that this inhibition is due to an action of fluazifop on acetyl-CoA carboxylase and not on fatty acid synthetase. The acetyl-CoA carboxylase from sensitive barley (*Hordeum vulgare*), but not from resistant pea (*Pisum sativum*), is inhibited by the *R* stereoisomer, a finding that agrees with the herbicidal specificity of fluazifop.

INTRODUCTION

Fluazifop {2-[4-(5-trifluoromethyl-2-pyridyloxy)-phenoxy]propionate} is a grass-specific herbicide marketed under the trade formulation 'Fusilade'. Fluazifop and other aryloxyphenoxypropionates act by causing a rapid necrosis of the meristematic tissue of sensitive plant species (Shimabukuro *et al.*, 1979). The mode of action of these herbicides appears to be through an inhibition of fatty acid and, hence, acetyl lipid synthesis (see Harwood *et al.*, 1988). Furthermore, this action on lipid formation occurs in the absence of any detectable effects on CO_2 fixation or carbohydrate, amino acid or nucleic acid metabolism (Hoppe, 1981; Walker *et al.*, 1988). Cyclohexanediones, such as sethoxydim, also appear to act in a similar manner (see Hoppe, 1987; Harwood *et al.*, 1988).

Since the absorption, translocation and metabolism of aryloxyphenoxypropionates or cyclohexanediones appear to be the same in monocotyledons and in dicotyledons, it seems probable that selectivity is based on differences in the target enzyme. For that reason we have studied the inhibition of lipid synthesis by fluazifop in more detail. Reports of our initial observations have been published (Walker *et al.*, 1988; Harwood *et al.*, 1987, 1988). These studies led us to conclude that fatty acid synthesis *de novo*, but not elongation, was the target site and, because of fluazifop-induced changes in labelling patterns, a possible target was suggested to be acetyl-CoA carboxylase. We now report several experiments, findings from all of which indicate that this carboxylase is, indeed, the target enzyme for fluazifop action.

MATERIALS AND METHODS

Growth of tissue and leaf-segment labelling

These were carried out as detailed by Walker *et al.* (1988). Sample sizes were 16.5 ± 0.6 mg fresh wt. (100 mm^2; $n = 10$) for pea (*Pisum sativum*) discs and 35.2 ± 2.3 mg fresh wt. (200 mm^2; $n = 10$) for barley (*Hordeum vulgare*) leaf pieces.

Isolation of intact chloroplasts

This was carried out as detailed by Walker *et al.* (1988). Incubation mixtures contained approx. 210 μg of chlorophyll per sample.

Acetyl-CoA carboxylase assay

This enzyme was assayed in a desalted Sephadex G-25 fraction of barley and pea leaf tissue prepared as described by Nikolau *et al.*, (1984), but at 4 °C. Activity was measured in the acid-stable fraction by liquid-scintillation counting after subtracting blanks for non-acetyl-CoA-dependent labelling. In barley the enzyme was assayed by the method of Thomson & Zalik (1981) at pH 8.3. The enzyme in pea extracts was assayed by the methods of Mohan & Kekwick (1980) and Thomson & Zalik (1981) at pH 7.5. Assays were started by the addition of acetyl-CoA (250 μM final concn.).

Fatty acid synthesis

Stromal fractions prepared from the disrupted chloroplasts of barley (Hoj & Mikkelsen, 1982) were assayed for fatty-acid-synthesis activity. When [2-^{14}C]-malonyl-CoA was used as precursor, incubation conditions were: 670 μM-NADH, 670 μM-NADPH, 4.5 mM-ATP, 50 μg of *Escherichia coli* acyl-carrier protein (ACP), 50 nCi of [2-^{14}C]malonyl-CoA (sp. radioactivity 55.8 mCi/mmol) in 6.25 mM-potassium phosphate, final pH 8.5. For synthesis from [1-^{14}C]acetate, the above-mentioned reaction conditions were used, but 1 μCi of [1-^{14}C]acetate (sp. radioactivity 53 mCi/mmol) was used instead of [^{14}C]malonyl-CoA, and 25 μM-CoA, 1.2 mM-$MgCl_2$, 1.2 mM-$MnCl_2$, in 6.25 mM-potassium phosphate, final pH 8.5, were included. Incubation mixtures contained 710 μg of stromal protein per sample.

Additions of malonyl-CoA, acetyl-CoA, avidin (Sigma Chemical Co., Poole, Dorset, U.K.) and acetyl-ACP were made as detailed in the Table legends. Acetyl-ACP was synthesized by the method of Alberts *et al.* (1969), with *E. coli* ACP purified by a method similar to that of Majerus *et al.* (1969), but including a final step using fast protein liquid chromatography on a Mono-Q column (Pharmacia, Uppsala, Sweden).

Abbreviation used: ACP, acyl-carrier protein.

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Table 1. Changes in the chain length of fatty acids labelled from [1-^{14}C]acetate caused by inhibitory concentrations of fluazifop in barley

For incubation conditions with leaf pieces or with isolated chloroplasts see the Materials and methods section. Results are expressed as means ± S.D. for experiments performed with three replicates. Abbreviation: nd, none detected.

Organ/ suborganelle	Stereo-isomer	*n*	[Fluazifop] (μM)	[^{14}C]Fatty acids (% of total)			10^{-3} × Total radioactivity (d.p.m.)
				< C_{18}	C_{18}	Other	
Leaf pieces	*R*	2	0	11±1	89±1	nd	537±68
			5	29±4	58±7	13±2	197±9
			25	24±4	55±3	21±5	129±10
Isolated chloroplasts	*R,S* mixture	3	0	50±4	50±4	nd	32.6±1.8
			5	62±7	39±7	nd	14.10±2.9
			15	69±6	31±7	nd	8.8±0.7
			25	100	nd	nd	7.2±2.2

Table 2. Effect of fluazifop on fatty acid synthesis from [2-^{14}C]malonyl-CoA in stromal preparations from barley chloroplasts

Incubation conditions are described in the Materials and methods section. Results are expressed as means ± S.D. (n = 3). Abbreviation: tr, trace (0.5). The control activity was 6.3 pmol/min per mg of protein.

Addition to incubation mixture	Final concn. (μM)	Activity (% of control)	Distribution of radioactivity (% of total [^{14}C]fatty acids)			
			$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$
None	—	100	2±1	36±2	7±1	55±2
Acetyl-CoA	20	38±14	4±1	72±3	6±2	18±5
(*R,S*)-Fluazifop	35	127±15	3±1	36±5	10±tr	51±5
Acetyl-CoA +fluazifop	20+35	772±12	4±2	60±6	8±1	28±7

RESULTS AND DISCUSSION

We had noted previously that concentrations of fluazifop which caused only partial inhibition of fatty acid synthesis in leaf segments or isolated chloroplasts caused a change in the pattern of fatty acids labelled. Results from typical experiments are shown in Table 1, where it can be seen that an increase in the relative labelling of shorter chains (C_{14} and C_{16}) relative to C_{18} acids occurs. Such a result could be produced either by a relative sensitivity of condensing (β-oxoacyl-ACP synthetase) enzyme II rather than condensing enzyme I, or because acetyl-CoA carboxylase is being inhibited (Walker *et al.*, 1988). In the latter case, a relative increase of acetyl over malonyl units for condensation is known to decrease the chain length of fatty acyl products in several plant systems (see Harwood, 1988). Even from [2-^{14}C]malonate, which is used preferentially by the endoplasmic-reticulum elongation systems (which synthesize stearate at appreciable rates), a shift in the ratio of C_{16} to C_{18} products was seen also at intermediate herbicide concentrations (Walker *et al.*, 1988) (see Table 2 for the effect of increasing primer acetyl-CoA concentration on labelling patterns).

Unfortunately, because of decarboxylation reactions in barley and pea tissues, the use of [2-^{14}C]malonate does not necessarily by-pass acetyl-CoA carboxylase. Thus the conclusion by Hoppe & Zacher (1982, 1985) that another aryloxyphenoxypropionate derivative, diclofop, inhibits fatty acid synthetase may not be correct, since labelling of fatty acids from both [1-^{14}C]acetate and [1-^{14}C]malonate in their experiments may be decreased because of an inhibition of acetyl-CoA carboxylase.

We failed to demonstrate any inhibition of fatty acid synthetase (measured with [2-^{14}C]malonyl-CoA) by fluazifop (Table 2). However, we did note that, when unlabelled acetyl-CoA was added to ensure that primer substrate was not limiting, the decrease in radioactivity observed was partly reversed by fluazifop addition (Table 2). Acetyl-CoA carboxylase is present in the stromal preparations used, so that addition of unlabelled acetyl-CoA not only provides primer substrate, but would also be expected to dilute the [2-^{14}C]malonyl-CoA specific radioactivity, owing to carboxylation. This would result in less incorporation of radioactivity into fatty acid products. The diminution in this decrease in labelling by fluazifop is consistent with its action in inhibiting acetyl-CoA carboxylase.

Although fluazifop actually slightly stimulated labelling of fatty acids from [2-^{14}C]malonyl-CoA in the usual stromal incubation system (Table 2), (a phenomenon even more pronounced at half the malonyl-CoA concentration; results not shown), it did inhibit their labelling from [1-^{14}C]acetate. Incorporation of radioactivity from the latter substrate is, of course, due to both acetyl-CoA carboxylase and fatty acid synthetase. In order to try to eliminate fatty acid synthetase as a site of action for fluazifop, we carried out incubations where either the acetyl-CoA:acetyl-ACP transacylase (catalysing the first partial reaction of fatty acid

Table 3. Experiment to distinguish fluazifop inhibition of fatty acid synthesis from [1-^{14}C]acetate via fatty acid synthetase from that via acetyl-CoA carboxylase

Incubations were conducted with formal fractions from barley chloroplasts using [1-^{14}C]acetate as described in the Materials and methods section. Means ± S.D. are shown. Addition of avidin and unlabelled malonyl (Mal)-CoA was designed to prevent labelling via acetyl-CoA carboxylase, whereas addition of an excess of unlabelled acetyl-ACP prevented labelling of fatty acids via the priming reaction of fatty acid synthetase.

Unlabelled addition (final concn.)	Fatty acid labelling (d.p.m.)	Fatty acid labelling (% of control)
Avidin (100 μg/ml) + Mal-CoA (64 μM)	393 ± 166	100
Avidin (100 μg/ml) + Mal-CoA (64 μM) + fluazifop (35 μM)	363 ± 24	92.1
Acetyl-ACP (50 μg/ml)	25238 ± 1798	100
Acetyl-ACP (50 μg/ml) + fluazifop (35 μM)	3363 ± 71	13.3

synthetase) or acetyl-CoA carboxylase were essentially unable to account for fatty acid labelling. These incubations included a large excess of unlabelled acetyl-ACP or avidin plus a large excess of unlabelled malonyl-CoA respectively (Table 3). In experiments where only acetyl-CoA:acetyl-ACP transacylase activity could result in radiolabelling of fatty acids from [1-^{14}C]acetate, fluazifop had no significant effect on incorporation. In contrast, for those experiments where labelling was dependent on the activity of acetyl-CoA carboxylase, fluazifop severely inhibited fatty acid labelling (Table 3).

The above three experiments had all indicated that acetyl-CoA carboxylase, rather than fatty acid synthetase, was the site of fluazifop action. More importantly, the systems used ranged from tissue preparations to subcellular fractions, so that we were confident that we were measuring a consistent physiological phenomenon.

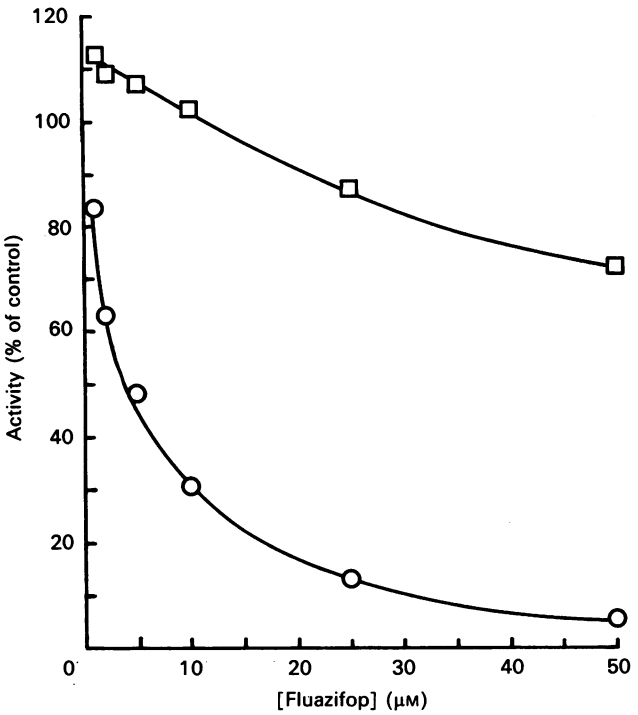

Fig. 1. Dose response of barley leaf-tissue acetyl-CoA carboxylase to stereoisomers of fluazifop

Incubations were carried out as described in the Materials and methods section with either the *R* isomer (○) or the *S* isomer (□) of fluazifop. The latter contained 7.5% of the *R* isomer as a contaminant. Control activity was 2.89 nmol/min per mg of protein.

A direct action of the herbicide on acetyl-CoA carboxylase assays was found for barley (Table 4), but not pea, preparations (results not shown). Burton *et al.* (1987) also noted that haloxyfop (2-{4-[3-chloro-5-(trifluoromethyl)-2-pyridinyloxy]phenoxy}propionic acid) and sethoxydim {2-[1-(ethoxyimino)butyl]-5-(2-ethylthiopropyl)-3-hydroxy-2-cyclohexen-1-one} failed to inhibit the acetyl-CoA carboxylase from pea leaves, but were active on that from maize (*Zea mays*). Since fluazifop shows stereospecificity in its herbicidal action, we tested purified preparations of the *R* and *S*

Table 4. Inhibition of barley acetyl-CoA carboxylase by fluazifop

Acetyl-CoA carboxylase was assayed by measuring the acetyl-CoA-dependent fixation of $^{14}CO_2$ into acid-stable product using a desalted preparation (see the Materials and methods section). The product co-chromatographed with malonyl-CoA on t.l.c. Results are expressed as means ± S.D. for triplicate determinations with three separate preparations. Fluazifop was present at 25 μM.

Expt.	Incorporation (d.p.m.)	Incorporation (% of control)	Acetyl-CoA carboxylase activity (nmol/min per mg of protein)
1 Control	10869 ± 720	100	2.60
+(*R*,*S*)-Fluazifop	3422 ± 761	31.5	
2 Control	9821 ± 178	100	2.67
+(*R*,*S*)-Fluazifop	2702 ± 17	27.5	
3 Control	9208 ± 214	100	2.77
+(*R*,*S*)-Fluazifop	3029 ± 625	32.9	

stereoisomers. Inhibition studies showed that a half-maximal concentration for the *R* isomer was about 4 μM (see Fig. 1). When the contamination of the *S* isomer by 7.5% of the *R* isomer was taken into consideration, no activity could be detected for the *S* isomer (Fig. 1). This agreed with the herbicidal action of the *R* isomer (Walker *et al.*, 1988).

Previous data (see Harwood *et al.*, 1988; Hoppe, 1987) have shown that oxyphenoxypropionate herbicides act by inhibiting lipid synthesis in sensitive plants. The experiments described above indicate that the inhibition of lipid synthesis by fluazifop is at the level of acetyl-CoA carboxylase. Taken together with the data of Burton *et al.* (1987) for haloxyfop and sethoxydim, they raise the intriguing question as to why the acetyl-CoA carboxylases from those monocotyledons and dicotyledons so far tested should differ in their sensitivities to such herbicides. In addition, the relative insensitivity of the labelling of very-long-chain fatty acids from [1-^{14}C]-acetate by elongation reactions (Harwood *et al.*, 1988; Walker *et al.*, 1988) raises the question as to whether there is an extra-plastidic acetyl-CoA carboxylase in monocotyledons which has a structure different from that of the chloroplast enzyme.

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