Fatty-Acid Biosynthesis and Acetyl-CoA Carboxylase as a Target of Diclofop, Fenoxaprop and other Aryloxy-phenoxy-propionic Acid Herbicides

Klaus Kobek, Manfred Focke, and Hartmut K. Lichtenthaler

Botanisches Institut der Universität, Kaiserstraße 12,
D-7500 Karlsruhe 1, Bundesrepublik Deutschland

Z. Naturforsch. **43c**, 47–54 (1988); received November 2, 1987

Acetyl-CoA Carboxylase, Chloroplast Metabolism, *de novo* Fatty-Acid Biosynthesis, Fluazifop, Haloxyfop, Herbicide Sensitivity

The effect of the herbicides and aryloxy-phenoxy-propionic acid derivatives diclofop, fenoxaprop, fluazifop and haloxyfop and their ethyl, methyl or butyl esters on the *de novo* fatty-acid biosynthesis of isolated chloroplasts was investigated with intact chloroplasts isolated from sensitive grasses (Poaceae) and tolerant dicotyledonous plants *(Pisum, Spinacia)*. The 4 herbicides (free-acid form) block the *de novo* fatty-acid biosynthesis ([2-^{14}C]acetate incorporation into the total fatty-acid fraction) of the sensitive *Avena* chloroplasts in a dose-dependent manner. The I_{50}-values (a 50% inhibition of the [^{14}C]acetate incorporation) lie in the range of 10^{-7} to 2×10^{-6} M. The ethyl or methyl esters (diclofop, fenoxaprop, haloxyfop) and butyl ester (fluazifop) do not affect the *de novo* fatty-acid biosynthesis of isolated chloroplasts or only at a very high concentration of *ca.* 10^{-4} M. In contrast, the *de novo* fatty-acid biosynthesis of the tolerant dicotyledonous species (pea, spinach) is not affected by the 4 aryloxy-phenoxy-propionic acid herbicides.

In an enzyme preparation isolated from chloroplasts of the herbicide-sensitive barley plants the *de novo* fatty-acid biosynthesis from [^{14}C]acetate and [^{14}C]acetyl-CoA is blocked by all 4 herbicides (free acids), whereas that of [^{14}C]malonate and [^{14}C]malonyl-CoA is not affected. This strongly suggests that the target of all 4 herbicides (free-acid form) is the acetyl-CoA carboxylase within the chloroplasts. The applied ester derivatives, in turn, which are ineffective in the isolated chloroplast test system, have equally little or no effect on the activity of the acetyl-CoA carboxylase. It is assumed that the acetyl-CoA carboxylase of the tolerant dicot plants investigated is modified in such a way that the 4 herbicides cannot bind to and affect the target.

Introduction

Several aryloxy-phenoxy-propionic acid derivatives such as diclofop, fenoxaprop, fluazifop and haloxyfop (for structure see Fig. 1) are very effective herbicides for postemergence control of annual and perennial grasses (mainly Poaceae) in a large variety of dicotyledonous crop plants [1–6]. All 4 herbicides induce in growing seedlings white chlorotic leaf zones and inhibit growth and further development. The exact mode of action of these herbicides is not known hitherto. In the case of diclofop and fenoxaprop it was shown by Hoppe [7, 8] that in intact chloroplasts isolated from herbicide-sensitive maize plants both herbicides block the *de novo* fatty-acid biosynthesis from [^{14}C]acetate, whereas their esters were not effective [7, 8]. The inhibition of fatty-acid biosynthesis in isolated chloroplasts by the free-acid form of the two herbicides was confirmed by Burgstahler [9], who found a similar action also for the cyclohexane-1,3-dione herbicide sethoxydim [9, 10].

In higher plants the *de novo* fatty-acid biosynthesis is exclusively bound to the chloroplasts (plastids) [11–13]. All enzymatic activities of the *de novo* fatty-acid synthetase are present in the plastid (chloroplast) compartment [11, 12] including the acyl-carrier-protein (ACP) as well as the preceding enzymes, such as acetyl-CoA synthetase and acetyl-CoA carboxylase [14, 15]. Since in intact plants the [^{14}C]-malonate incorporation was blocked by diclofop to an equal degree as that of [^{14}C]acetate, it was concluded that the site of inhibition must be a step in the *de novo* fatty-acid biosynthesis after the malonate and malonyl-CoA formation [7]. Whether in isolated chloroplasts [^{14}C]malonate could be incorporated

Abbreviations: D,L-diclofop, 2-(4-(2,4-dichlorophenoxy-)phenoxy-)propionic acid; D,L ring hydroxylated diclofop, 2-(4-(2,4-dichloro-5-hydroxyphenoxy-)phenoxy-)propionic acid; D,L-fenoxaprop, 2-(4-(6-chloro-2-benzoxazolyloxy-)phenoxy-)propionic acid; D,L-fluazifop, (4-(5-trifluoromethyl-2-pyridoxyloxy-)phenoxy-)propionic acid; D,L-haloxyfop, ((4-(3-chloro-5-trifluoromethyl-2-pyridinyl-)oxy-)phenoxy-)propionic acid.

Reprint requests to Prof. Dr. Hartmut K. Lichtenthaler.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0100–0047 $ 01.30/0

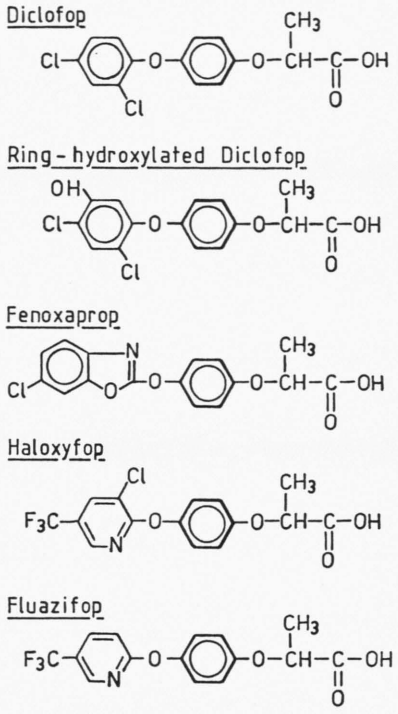

Fig. 1. Chemical structure of several aryloxy-phenoxy-propionic acid derivatives with herbicide activity.

into the fatty-acid fraction and be blocked by diclofop or fenoxaprop had, however, not been tested.

In our attempt to localize the site of interaction of the aryloxy-phenoxy-propionic acids with the *de novo* fatty-acid biosynthesis we repeated the investigations with diclofop and fenoxaprop in a newly developed test system of intact isolated *Avena* chloroplasts, which exhibits a much higher control rate of [^{14}C]acetate incorporation [18] than in the older experiments [8–10]. The herbicides were also tested in a crude enzyme preparation isolated from barley chloroplasts which possesses *de novo* fatty-acid biosynthesis capacity starting from [2-^{14}C]acetate [19, 20]. We also wanted to see whether the newer aryloxy-phenoxy-propionic acid derivatives fluazifop and haloxyfop have similar effects to diclofop and fenoxaprop in the isolated chloroplast test system and in the chloroplast enzyme preparation with *de novo* fatty-acid biosynthesis capacity.

Material and Methods

Oat (*Avena sativa* L. var. "Flämingnova"), *Spinacia oleraceae* L. var. "Matador" and pea seedlings (*Pisum sativum* L. var. "Kleine Rheinländerin") were cultivated on peat (TKS II) in a 14/10 h day/night cycle at 25 °C at a light intensity of 1500 μE $m^{-2}s^{-1}$ (light source: Osram HQI-E 400 W, 5 cm water filter). The chloroplasts were isolated from 7-day-old oat seedlings (2 leaf stage from 2-week-old pea and 5-week-old spinach plants using a medium containing sorbitol 500 mM, phosphate buffer 67 mM (pH 8.0), $MgCl_2$ 2 mM and BSA 0.2% [21]. The chlorophyll content was determined using the new equations of Lichtenthaler [22]. The incubation with [2-^{14}C]acetate or [2-^{14}C]malonate was carried out at room temperature in 1 ml chloroplast suspensions in light (1400 μE $m^{-2}s^{-1}$) applied from below. The reaction mixture contained 300 mM sorbitol, 50 mM tricin, 50 mM posphate buffer (pH 7.9), 30 mM $NaHCO_3$, 2.5 mM DTT, 2 mM ATP, 0.5 mM CoA, 0.5 mM $MgCl_2$, 0.2 mM NADH, 35 μM [2-^{14}C]acetate (1 μCi per ml) or 50 μM [2-^{14}C]malonate (1 μCi per ml) and chloroplasts with a chlorophyll content of *ca.* 100 μg per ml [18]. The herbicides diclofop, fenoxaprop, haloxyfop and fluazifop were added in methanolic solution (final methanol concentration 0.2%). After an incubation time of 20 min the reaction was stopped by 30% KOH, the lipids were saponified at 70–80 °C for 90 min. After acidification with *ca.* 500 μl 12 M sulfuric acid per sample and subsequent addition of 300 μl 30% trichloroacetic acid, the fatty acids and remaining non-acyllipids were extracted 3 times with 2 ml of light petrol (b.p. 50–70 °C) [18]. The fatty acids were separated by TLC (silica gel Merck Nr. 5626, solvent: light petrol/diethyl ether/acetic acid: 90/20/1, by volume) and the radioactivity was measured with a liquid scintillation counter. At least 98% of the total radioactivity incorporated in organic material was found in the total fatty-acid fraction.

Isolation of an enzyme fraction active in fatty-acid biosynthesis

Barley seedlings (*Hordeum vulgare* var. "Alexis") were cultivated on peat (TKS 2) for 6 days in the dark. The etiolated plants were illuminated for 6 h. Chloroplasts were isolated from the primary and secondary leaves following the procedures of Høj and Mikkelsen [19] with some modifications. Leaves were homogenized in 0.1 M tricin, 0.6 M glycerol, pH 9, with a warring blendor and centrifuged for 5 min at 8000 × *g*. The pellet was resuspended in a

buffer containing 0.1 M tricin, 0.3 M glycerol, 1 mM $MgCl_2$, 2 mM DTE at pH 9 and passed through a French press at 8000 psi. After an ultracentrifugation step (1 h, 105,000 × *g*) the resulting supernatant was fractionated by adding saturated $(NH_4)_2SO_4$ solution. The 40% to 70% $(NH_4)_2SO_4$-saturation fraction was dialyzed against 0.1 M tricin, 0.3 M glycerol, 1 mM $MgCl_2$ and 2 mM DTE of pH 8.8 overnight and then concentrated against polyethylenglycol 20,000. Protein was determined as described by Bach *et al.* [23].

Enzyme assay

100 µl of the enzyme fraction was assayed for fatty-acid synthesis capacity with following cofactors in a total volume of 0.15 ml:

1) assay with [2-^{14}C]malonyl-CoA: acetyl-CoA 25 µM, NADPH 1.25 mM, NADH 0.625 mM, ATP 2.5 mM, ACP 7 µM, $MgCl_2$ 1.25 mM, Na_2HPO_4 6.25 mM, malonyl-CoA 82.4 µM (= 0.02 µCi),

2) assay with [2-^{14}C]malonic acid: cofactors etc. in the same concentrations as above, but without [^{14}C]-malonyl-CoA but 100 µM malonic acid (0.3 µCi) plus 25 µM CoA,

3) assay with [1-^{14}C]acetyl-CoA: cofactors etc. in same concentrations as above, but without [^{14}C]-malonyl-CoA but 12.5 mM $NaHCO_3$ and 1.25 mM $MnCl_2$ and 53 µM [^{14}C]acetyl-CoA = 0.02 µCi),

4) assay with [2-^{14}C]acetic acid: cofactors as above without [^{14}C]acetyl-CoA, but with additional 25 µM CoA and 100 µM acetic acid (= 0.86 µCi) in place of [^{14}C]acetyl-CoA.

The herbicides and active ingredients were added by addition of 2 µl 16% methanolic solution (final methanol concentration in the incubation medium 0.2% by volume in all samples including the controls).

The assays were performed at 30 °C for 15 min (in the case of [^{14}C]acetyl- and malonyl-CoA substrates) or 30 min (in the case of labelled malonic or acetic acid) and stopped by the addition of 200 µl 40% KOH. 10 µl 1% oleic acid in methanol was added as carrier. After 45 min in a 70–80 °C hot-water bath, the assay mixtures were acidified by addition of 200 µl 12 M H_2SO_4 and 10 µl 30% trichloroacetic acid. The labelled fatty acids together with cold oleic acid were extracted three times by the addition of 600 µl light petrol. The petrol was concentrated by evaporation. After separation by TLC (see above), the radioactivity of the fatty acids was determined in a LSC (Tri-carb 2000 CA) using a toluene-scintillator (Packard Instruments). Since in several experiments more than 98% of the radioactivity was found in the fatty-acid fraction, the TLC separation step for fatty acids can be omitted for routine analysis. The chemicals were obtained from Sigma, Deisenhofen, and Merck, Darmstadt. The ACP was purchased from Calbiochem and the radiochemicals from Amersham.

Results

Inhibition effects in isolated chloroplasts

The aryloxy-phenoxy-propionic acid herbicides such as diclofop, fenoxaprop, haloxyfop and fluazifop as well as the ring-hydroxylated metabolization product of diclofop (s. Fig. 1) inhibit the *de novo* fatty-acid biosynthesis from [^{14}C]acetate of isolated intact *Avena* chloroplasts in a dose-dependent manner (Fig. 2). The I_{50}-values lie in the range of 1×10^{-7} M to 3×10^{-6} M (Table I). The most efficient substance in the *Avena* chloroplast-test system is diclofop.

The methyl-, ethyl- or butyl-ester derivatives of the free-acid forms of these herbicides, which are the

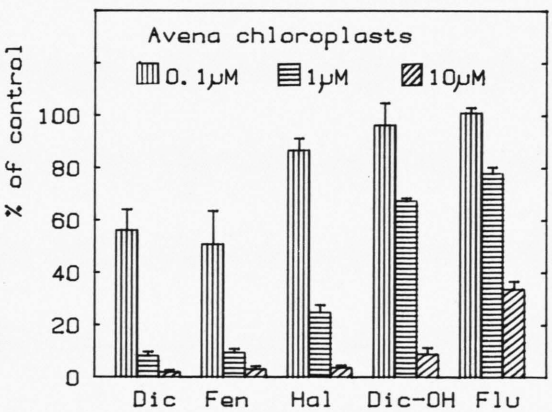

Fig. 2. Inhibition of *de novo* fatty-acid biosynthesis from [2-^{14}C]acetate in intact chloroplasts isolated from *Avena* seedlings by the herbicides diclofop (Dic), fenoxaprop (Fen), haloxyfop (Hal), fluazifop (Flu) and the metabolization product (of diclofop) ring-hydroxylated diclofop (Dic-OH). Radioactive labelling of fatty acids in the methanol-treated (0.2%) controls: 9–11 kBq per mg chlorophyll in 20 min, which corresponds to *ca.* 3.5 to 4% of the applied label and to *ca.* 26–32 nmol acetate incorporation per mg chlorophyll × h. Mean values of at least 6 determinations from 2 chloroplast isolations with standard deviation.

Table I. I_{50}-Values of the aryloxy-phenoxy-propionic acid derivatives and their methyl or butyl ester for the 50% inhibition of [2-^{14}C]acetate incorporation into the total fatty-acid fraction of isolated *Avena* chloroplasts.

Substance	I_{50}-value [M]
Free acids:	
Diclofop	10^{-7}
Fenoxaprop	10^{-7}
Haloxyfop	3×10^{-7}
Ring-hydroxylated diclofop	2×10^{-6}
Fluazifop	*ca.* 3×10^{-6}
Esters:	
Diclofop-methyl	10^{-5}
Fenoxaprop-ethyl	10^{-5}
Haloxyfop-methyl	5×10^{-5}
Fluazifop-butyl	10^{-4}

active ingredients applied to whole plants, do not affect the *de novo* fatty-acid biosynthesis of intact *Avena* chloroplasts or only in much higher concentrations than the corresponding free-acid forms (Fig. 3). The I_{50}-values for the inhibition of the [^{14}C]acetate incorporation into fatty acids are therefore higher by a factor of 10 to 100 than those of the free acids (Table I). The inhibition activity of the ester forms is higher for diclofop-methyl and fenoxaprop-ethyl and decreases *via* to haloxyfop-methyl and fluazifop-butyl. This activity sequence of the ester forms corresponds to that of the free diaryloxy-propionic acids.

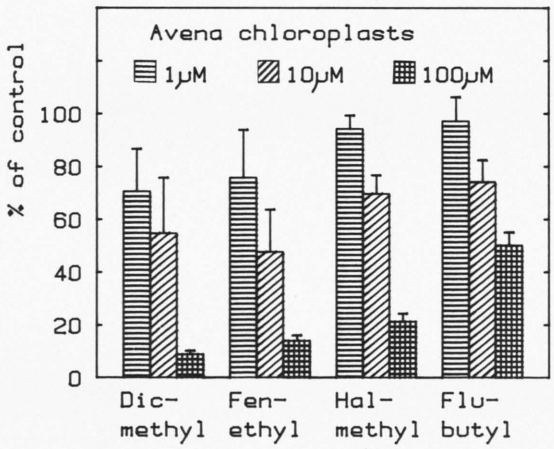

Fig. 3. Inhibition of the *de novo* fatty-acid biosynthesis from [2-^{14}C]acetate in isolated oat chloroplasts by the herbicide esters diclofop-methyl (Dic-methyl), fenoxaprop-ethyl (Fen-ethyl), haloxyfop-methyl (Hal-methyl) and fluazifop-butyl (Flu-butyl). Radioactive labelling of controls: 11 kBq per mg chlorophyll and 20 min (4% incorporation of applied label; = *ca.* 32 nmol acetate per mg chlorophyll × h). Mean values of 3 determinations with standard deviation.

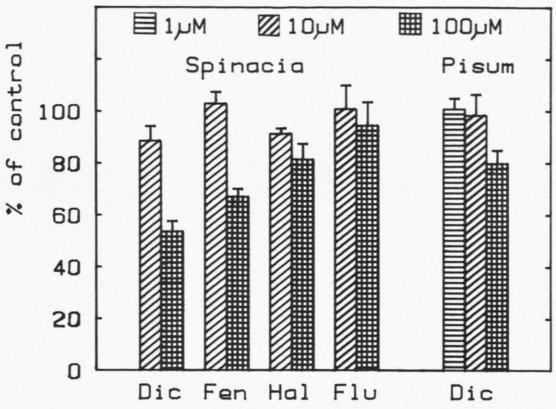

Fig. 4. Effects on the *de novo* fatty-acid biosynthesis from [2-^{14}C]acetate in isolated spinach and pea chloroplasts by the herbicides diclofop (Dic), fenoxaprop (Fen), haloxyfop (Hal) and fluazifop (Flu). Radioactive labelling of controls: *Pisum:* 17 kBq per mg chlorophyll and 20 min (= 5.8% of applied label; = *ca.* 49 nmol acetate per mg chlorophyll × h). *Spinacia:* 22 kBq per mg chlorophyll (7.6% of applied label; = *ca.* 64 nmol acetate per mg chlorophyll × h). Mean values of 4 determinations from 2 chloroplast isolations with standard deviation.

The dicotyledonous plants such as spinach or pea are known to be tolerant as intact plants towards these diaryloxy-propionic acid type herbicides [1–6]. Isolated spinach chloroplasts, however, are not fully tolerant towards diclofop or fenoxaprop treatment. At a 10^{-4} M concentration they significantly affect the *de novo* fatty-acid biosynthesis (Fig. 4). This is not found for haloxyfop and fluazifop. Pea chloroplasts are less sensitive than spinach chloroplasts towards treatment with diclofop. The ester forms of the four active ingredients did not significantly effect the *de novo* fatty-acid biosynthesis from [^{14}C]acetate in spinach chloroplasts (Fig. 5).

The incorporation of label from [2-^{14}C]malonate into the fatty-acid fraction of intact *Avena* chloroplasts proceeds at a much lower rate (ca. 10–15%) than that of [2-^{14}C]acetate (Table II) indicating that, when applied to intact chloroplasts, malonate is not a suitable substrate for fatty-acid biosynthesis. The chloroplast envelope seems to be a barrier for the ready uptake of malonate. The low incorporation rate of label from [^{14}C]malonate was blocked by

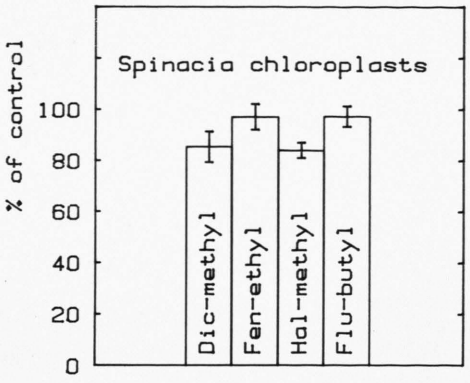

Fig. 5. Effects on the *de novo* fatty-acid biosynthesis from [2-^{14}C]acetate in isolated spinach chloroplasts by the herbicide esters diclofop-methyl (Dic-methyl), fenoxyprop-ethyl (Fen-ethyl), haloxyfop-methyl (Hal-methyl) and fluazifop-butyl (Flu-butyl). Radioactive labelling of controls: 24 kBq per mg chlorophyll and 20 min (= 8.3% of applied label; = *ca.* 69 nmol acetate per mg chlorophyll × h). Mean values of 3 determinations with standard deviation.

Table II. Incorporation of [2-^{14}C]malonate into the total fatty-acid fraction of isolated *Avena* chloroplasts with and without application of diclofop. In the controls only about 0.5% of the applied radioactivity (= *ca.* 5.7 nmol malonate per mg chlorophyll × h) was incorporated. Mean values of 3 determinations (maximum deviation ± 7% or less).

Condition	kBq per mg chlorophyll × h	Inhibition [%]
Controls	4.2	0
Diclofop 1 μM	*ca.* 1.8	57
Diclofop 100 μM	*ca.* 0.6	86

diclofop in a dose-dependent manner (Table II). We have also applied [^{14}C]acetyl-CoA and [^{14}C]malonyl-CoA to intact chloroplasts of oat and spinach, but no label was taken up into the fatty-acid fraction.

Assay using an enzyme preparation from barley chloroplasts

From the chloroplasts of barley seedlings, which belong to the group of Poaceae which are sensitive towards diphenoxy-propionic acid-type herbicides, we isolated an enzyme preparation capable of synthesizing fatty acids from [^{14}C]acetate when provided with cofactors and the acyl carrier protein ACP. The incorporation of radioactive label from [^{14}C]acetate was similarly affected by these active ingredients (Fig. 6). In both cases diclofop and fenoxaprop were more efficient inhibitors in the enzyme assay than haloxyfop and fluazifop. The sequence of the inhibition activity in the enzyme assay was the same as in the test system of intact *Avena* chloroplasts.

In contrast to [^{14}C]acetate and [^{14}C]acetyl-CoA the incorporation of the label from [^{14}C]malonyl-CoA

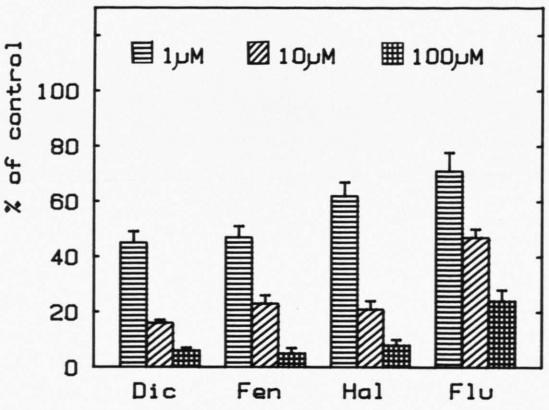

Fig. 6. Inhibition of the [1-^{14}C]acetyl-CoA incorporation into the fatty-acid fraction of an enzyme preparation from barley chloroplasts. The incorporation rate of the control amounted to 300 kBq per mg protein × h. About 6.7% of the applied radioactivity (0.02 μCi = 8.4 nmol) were incorporated. Mean of 3 determinations (diclofop, fenoxaprop) or 2 determinations (haloxyfop, fluazifop).

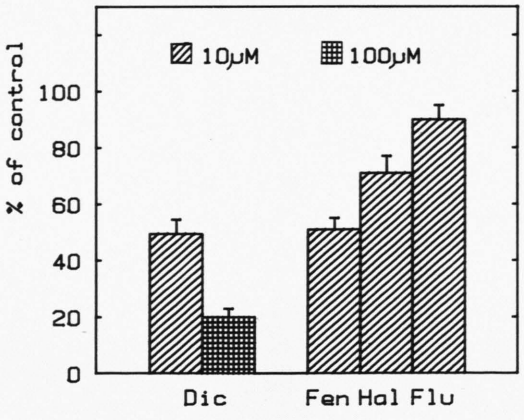

Fig. 7. Inhibition by diphenoxy-propionic acid type herbicides of [2-^{14}C]acetate incorporation into the total fatty-acid fraction of an enzyme preparation isolated from chloroplasts of barley seedlings. Applied were 0.86 μCi (*ca.* 15 nmol) [2-^{14}C]acetate per condition. The incorporation rate of the control amounted to 7–8 kBq per mg protein × h and to an incorporation of *ca.* 7% of the applied radioactivity. Mean of 2 determinations from two separate chloroplast isolations with standard deviation. Abbreviations of the herbicides are the same as in Fig. 2.

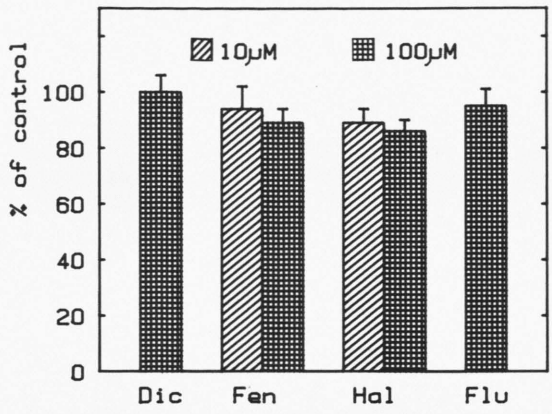

Fig. 8. Effect of the phenoxy-propionic acid type herbicides on the incorporation of [2-^{14}C]malonyl-CoA into the total fatty-acid fraction synthesized in an enzyme preparation from barley chloroplasts. The control incorporated *ca.* 12% of the applied radioactivity (0.02 µCi = 16 nmol). Mean of two determinations.

into the fatty-acid fraction of the enzyme preparation was, however, not inhibited by the four active ingredients (Fig. 8). Even at a 100 µM concentration we could not observe a significant inhibition. There was also no inhibition when [^{14}C]malonate was applied as substrate. From the 15 nmol malonic acid (0.3 µCi) applied to the enzyme preparation 8.3% was incorporated into fatty acids which could not be affected by 10^{-5} or 10^{-4} M diclofop or fenoxaprop.

Discussion

The results of this investigation show that the *de novo* fatty-acid biosynthesis of isolated intact *Avena* chloroplasts is sensitive to the different aryloxy-phenoxy-propionic acids and can be inhibited by these in a dose-dependent manner. There are differences in the inhibition activity of these active ingredients, which are due to their differing chemical structure. Diclofop is the most active substance. Its ring-hydroxylated derivative, a metabolization product formed from diclofop in intact wheat seedlings [2] is much less effective in the isolated oat-chloroplast test system. The relative tolerance of wheat towards diclofop as compared to other members of Poaceae, therefore seems to lie in the ring-hydroxylation of diclofop [2] yielding a product which is less active at the target site *i.e.* the *de novo* fatty-acid biosynthesis in the chloroplasts. The rather low I_{50}-values of these herbicides, *e.g.* diclofop 1×10^{-7} M up to 3×10^{-6} M (fluazifop), as well as the rate of [^{14}C]acetate incorporation of 27 to 33 kBq per mg chlorophyll and h indicates that the isolated intact oat chloroplasts still reflect the approximate *in vivo* functional organization of the *de novo* fatty-acid biosynthesis from acetate and represent a very suitable screening system for herbicide activity.

The relative ineffectiveness of the ester forms diclofop-methyl, fenoxaprop-ethyl, haloxyfop-methyl and fluazifop-butyl in the isolated chloroplast test system demonstrates that the ester forms of the herbicides cannot be the real inhibitors at the target site. Though the ester forms are applied in the treatment of whole plants for various reasons (*e.g.* uptake and distribution within the plant) [1–6], their free-acid forms seem to represent the actual active ingredient. In fact, for some diphenoxy-propionic acid type herbicides it was shown that the applied esters are fast hydrolyzed within the plant cell to the free acids [33]. Similar results as described here for *Avena* chloroplasts, were also found for diclofop and fenoxaprop using maize chloroplasts [10]. In maize it was also shown that the relative ineffectiveness of the ester form (diclofop-methyl) was not due to a lower rate of uptake of the ester form as compared to the free acid. In fact, the uptake rate of diclofop-methyl into maize chloroplasts was ca. 7 times higher than for diclofop [10].

The tolerance of intact dicotyledonous plants such as spinach, pea or bean towards the diphenoxy-propionic acid-type herbicides seems to lie in the properties and tolerance of their chloroplasts. This is demonstrated by the relative ineffectiveness of these herbicides (free-acid form) in isolated chloroplasts of spinach and pea (Fig. 4). In the case of diclofop and fenoxaprop this was also found for bean chloroplasts [10]. Our results with spinach and pea chloroplasts, however, indicate, that the target of this group of herbicides within the chloroplasts is not fully tolerant towards the more effective compounds diclofop and fenoxaprop (Fig. 4).

Though the test system of isolated intact chloroplasts of the sensitive monocot *Avena* or the tolerant dicot spinach plants is a very efficient screening system for the activity of diphenoxy-propionic acid-type herbicides, the results with intact chloroplasts do not indicate the exact site of interaction. They only show that these herbicides block somewhere in the full sequence of *de novo* fatty-acid biosynthesis starting from [^{14}C]acetate. Isolated intact chloroplasts from

oat and spinach, which are capable of good rates of *de novo* fatty-acid biosynthesis from acetate, do not take up label from [^{14}C]acetyl-CoA or [^{14}C]malonyl-CoA. The incorporation of label from applied [2-^{14}C]malonate into the fatty acids of intact oat chloroplasts amounted to only a maximum of 10% of the rate of the [2-^{14}C]acetate incorporation. This indicates that malonate is not a suitable substrate for *de novo* fatty-acid synthesis when applied to intact isolated chloroplasts.

A better test system for applying different precursor substances of fatty-acid biosynthesis is the enzyme preparation obtained by $(NH_4)_2SO_4$ fractionation from isolated barley chloroplasts. When supplied with an active acyl-carrier protein from *Escherichia coli* and the proper cofactors, this enzyme fraction is capable of *de novo* synthesis of fatty acids from acetate including the whole sequence from acetate to C16 and C18 fatty acids. The chloroplast envelope, which in intact chloroplasts represents a barrier for acetyl- and malonyl-CoA and to a large extent also for malonate, does not do so under the incorporation of the diverse labelled precursors into the fatty acids in this enzyme preparation.

The dose-dependent block by diclofop, fenoxaprop, haloxyfop and fluazifop of the incorporation of ^{14}C-label from [^{14}C]acetyl-CoA into the fatty-acid fraction (Fig. 6), and the total lack of effect on that of [2-^{14}C]malonyl-CoA (Fig. 7) clearly demonstrates that the major target of these diphenoxy-propionic acid-type herbicides is the acetyl-CoA carboxylase (EC 4.6.1.2.). The concentrations of the herbicides needed in the isolated enzyme preparation is higher than in the isolated intact chloroplasts and the inhibition is not yet complete at a 100 μM concentration. This is, however, to be expected and is well known in research with isolated enzymes. One should note in this respect that the intact chloroplasts still represent a predominantly intact *in vivo* test system, whereas the enzyme preparation is a crude preparation which contains many other proteins and also some inactive or denaturated acetyl-CoA carboxylase, which may bind herbicides but is not enzymatically active. One could also assume that the herbicides may block allosterically or in such a way that they do not fully inhibit the carboxylation of [^{14}C]acetyl-CoA to malonyl-CoA.

Our results further show that the activation of acetic acid to acetyl-CoA as catalyzed by the acetyl-CoA synthase (acetate: CoA ligase AMP forming) is not the target of the applied herbicides. The sequence of activity of those herbicides at the barley enzyme preparation level from diclofop *via* fenoxaprop and haloxyfop to fluazifop (Fig. 6) corresponds to the activity found in intact *Avena* chloroplasts (Fig. 2). The relative ineffectiveness of the herbicide esters in intact chloroplasts is confirmed for the enzyme preparation. This is a further proof that the free-acid forms are the active ingredients of diphenoxy-propionic acid-type herbicides.

Fluazifop is significantly less active in the inhibition of the fatty-acid biosynthesis in isolated oat chloroplasts as well as in the isolated enzyme preparation than the three other herbicides. Whether fluazifop may have a further target in addition to the blocking of the acetyl-CoA carboxylase, or whether oat and barley are less sensitive towards fluazifop than towards the other three herbicides, must be a matter of further research.

Another group of plant-lipid-biosynthesis inhibitors, the cyclohexane-1,3-dione derivatives such as sethoxydim and cycloxydim, which block *de novo* fatty-acid biosynthesis in isolated intact chloroplasts [18, 20, 24], also exert their activity by inhibition of the acetyl-CoA carboxylase. The latter is apparently a key enzyme in plant fatty-acid biosynthesis [25, 26] as it is known for the animal fatty-acid synthesis [27].

A group of active ingredients, which are structurally somewhat related to the diphenoxy-propionic acid type herbicides, have been described as hypolipidemic agents [28]. These drugs, *e.g.* CPIB, 2-ethyl-2-(*p*-chlorophenyl)-2-methyl-propionate and TPIA, 2-methyl-2-(*p*-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy-propionic acid, are inhibitors of the avian and mammalian acetyl-CoA carboxylase [28]. These active compounds are thought to interfere with the activator citrate [29], which in mammalian tissue promotes the formation of active polymeric forms of acetyl-CoA carboxylase from inactive protomers. Whether citrate may play a role in the activation of the plant acetyl-CoA carboxylase is not known hitherto. The plant acetyl-CoA carboxylase, which is thought to be of the eucaryotic type, consists of one major polypeptide of 220 kDa containing all 3 catalytic subunits [30]. An inhibition of the soybean acetyl-CoA carboxylase by citrate could be relieved by magnesium [31], whereas the enzyme from avocado and spinach was stimulated by citrate [32].

From the earlier observations in intact plants or cut shoots, where the inhibition of the [2-^{14}C]malo-

nate incorporation into fatty acids by diclofop and sethoxydim could be demonstrated [8, 11], it was concluded that the target site of the diclofop and the cyclohexane-1,3-dione derivative sethoxydim in the biosynthetic sequence from acetate to fatty acids is after the malonyl-CoA formation. This is, however, not the case with the isolated enzyme as shown here for the diphenoxy-propionic acid type compounds. This has also been demonstrated for cyclohexane-1,3-dione herbicides [20]. Our present assumption is that in intact plants and in isolated chloroplasts, the malonate is partially decarboxylated to acetate, the incorporation of which into fatty acids can then be blocked by diclofop and sethoxydim. This explanation needs experimental proof and is part of our present research.

Acknowledgements

We wish to thank Dr. H. Köcher, Hoechst AG, D-6000 Frankfurt for providing the herbicides diclofop, fenoxaprop and their derivatives. The compounds, haloxyfop and haloxyfop-methyl were a gift of Dr. W. A. Kleschick, Dow Chemical Research Center, Walnut-Creek, California and fluazifop (butyl) of Dr. S. Ridley, ICI Jealott's Hill Research Station, Bracknell, Berkshire, which we gratefully acknowledge. Thanks are also due to Dr. T. J. Bach for the use of his scintillation counter.

[1] H. Köcher and K. Lötzsch, Z. Pflkrankh. Pflschutz **9,** 171–179 (1981).
[2] A. Jacobson, R. H. Shimabukuro, and C. McMichael, Pestic. Biochem. Physiol. **24,** 61–67 (1985).
[3] P. E. Plowman, W. C. Stonebridge, and N. Hawtree, Proceed. 1980 British Crop Protect. Conf.-Weeds **1,** 29–37 (1980).
[4] J. W. Dicks, J. W. Slater, and D. W. Bewick, Proceed. 1985 British Crop Protect. Conf.-Weeds **3c-2,** 271–280 (1985).
[5] P. Hendley, J. W. Dicks, T. J. Monaco, S. M. Slyfield, O. J. Tummon, and J. C. Barrett, Weed Science **33,** 11–24 (1985).
[6] Technical Data on new Verdict* Herbicide, Dow Chemical Company, Agricultural Products Department, Midland, Michigan 48640.
[7] H. H. Hoppe, Weed Res. **20,** 371–376 (1980).
[8] H. H. Hoppe, Habilitationsschrift der Universität Göttingen (1978).
[9] H. H. Hoppe, Pestic. Biochem. Physiol. **23,** 297–308 (1985).
[10] H. H. Hoppe and H. Zacher, Pestic. Biochem. Physiol. **24,** 298–305 (1985).
[11] R. Burgstahler, Karlsruhe Contribut. Plant Physiol. **13,** 1–111 (1985) (ISSN 0173-3133).
[12] H. K. Lichtenthaler, in: The Metabolism, Structure and Function of Plant Lipids (P. K. Stumpf, J. B. Mudd, and W. D. Nes, eds.), pp. 63–73, Plenum Press, New York 1987.
[13] R. Lessire and P. K. Stumpf, Plant Physiol. **73,** 614–618 (1982).
[14] J. B. Ohlrogge, D. N. Kuhn, and P. K. Stumpf, Proc. Natl. Acad. Sci. USA **76,** 1194–1198 (1979).
[15] J. W. M. Hemskerk and J. F. G. M. Wintermans, Physiol. Plant. **70,** 558–568 (1987).
[16] D. N. Kuhn, M. Knauf, and P. K. Stumpf, Arch. Biochem. Biophys. **209,** 441–450 (1981).
[17] K. C. Eastwell and P. K. Stumpf, Plant Physiol. **72,** 50–55 (1983).
[18] H. K. Lichtenthaler, K. Kobek, and K. Ishii, Z. Naturforsch. **42c,** 1275–1279 (1987).
[19] P. B. Høj and J. D. Mikkelson, Carlsberg Res. Communications **47,** 119–141 (1982).
[20] M. Focke and H. K. Lichtenthaler, Z. Naturforsch. **42c,** 1361–1363 (1987).
[21] J. Hawke, M. Rumsby, and R. Leech, Phytochemistry **13,** 403–413 (1974).
[22] H. K. Lichtenthaler, in: Methods in Enzymology (R. Douce and L. Packer, eds.), **Vol. 148,** pp. 350–382, Academic Press Inc., New York 1987.
[23] T. J. Bach, D. H. Rogers, and H. Rudney, Eur. J. Biochem. **154,** 103–111 (1986).
[24] K. Kobek, M. Focke, H. K. Lichtenthaler, G. Retzlaff, and B. Würzer, Physiol. Plant. **72,** in press (1988).
[25] Y. Nakamura and M. Yamada, Plant Sci. Lett. **14,** 291–295 (1979).
[26] A. Sauer and K. P. Heise, Z. Naturforsch. **39c,** 268–275 (1984).
[27] J. J. Volpe and P. R. Vagelos, Annu. Rev. Biochem. **42,** 21–60 (1973).
[28] M. E. Maragoudakis, J. Biol. Chem. **244,** 5005–5013 (1969).
[29] M. E. Maragoudakis and H. Hankin, J. Biol. Chem. **246,** 348–358 (1971).
[30] A. R. Slabas and A. Hellyer, Plant Sci. **33,** 177–182 (1985).
[31] D. J. Charles and J. H. Cherry, Phytochemistry **25,** 1067–1071 (1986).
[32] S. B. Mohan and R. G. O. Kekwick, Biochem. J. **187,** 667–676 (1980).
[33] F. B. Holl, S. A. Tritter, and B. G. Todd, Weed Res. **26,** 421–425 (1986).