Hawkes, T. R.; Howard, J. L.; Poutin, S. E. Herbicides that inhibit the biosynthesis of branched-chain amino acids. In *Herbicides and Plant Metabolism (SEB Seminar Series*; Dodge, A., Ed.; Cambridge University Press: Cambridge, 1989.

LaRossa, R. A.; Schloss, J. V. The herbicide sulfometuron methyl is bacteriostatic due to inhibition of acetolactate synthase. *J. Biol. Chem.* **1984**, *259*, 8753–8757.

Los, M. Synthesis and biology of the imidazolinone herbicides. In *Pesticides and Biotechnology*; Greenhalhg, R., Roberts, T. R., Eds; Blackwell Scientific: Boston, 1987.

Muhitch, J. J.; Shaner, D. L.; Stidham, M. A. Imidazolinones and acetohydroxyacid synthase from higher plants; Properties of the enzyme from maize suspension culture cells and evidence for the binding of imazapyr to acetohydroxyacid synthase *in vivo*. *Plant Physiol.* **1987**, *83*, 451–456.

Newhouse, K. E.; Shaner, D. L.; Wang, T.; Fincher, R. Genetic modification of crop response to imidazolinone herbicides. In *Fundamental and Practical Approaches to Combating Resistance*; Green, M. B., LeBaron, H. M.; Moberg, W. K., Eds.; American Chemical Society: Washington, DC, 1989.

Ray, T B. Site of action of chlorsulfuron: Inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiol.* **1984**, *75*, 827–831.

Saxena, P. K.; King, J. Herbicide resistance in *Datura innoxia*: Cross-resistance of sulfonylurea-resistant cell lines to imidazolinones. *Plant Physiol.* **1986**, *86*, 863–867.

Schloss, J. V.; Ciskanik, L. M.; Drew, E. V. Origin of the herbicide binding site of acetolactate synthase. *Nature* **1988**, *331*, 360–362.

Shaner, D. L.; Anderson, P. C.; Stidham, M. A. Imidazolinones: Potent inhibitors of acetohydroxyacid synthase. *Plant Physiol.* **1984**, *76*, 545–546.

Turner, J. B. Effect of phaseolotoxin on the synthesis of arginine and protein. *Plant Physiol.* **1986**, *80*, 760–765.

Winder, T.; Spalding, M. H. Imazaquin and chlorsulfuron resistance and cross resistance in mutants of *Chlamydomonas reinhardtii*. *MGG, Mol. Gen. Genet.* **1988**, *213*, 394–399.

Received for review July 11, 1989. Accepted January 18, 1990.

Inhibition of Acetyl-Coenzyme A Carboxylase by Two Classes of Grass-Selective Herbicides

Alan R. Rendina,*,‡ Adrienne C. Craig-Kennard, Jacqueline D. Beaudoin, and Mary K. Breen

Chevron Chemical Company, P.O. Box 4010, Richmond, California 94804

The selective grass herbicides diclofop, haloxyfop, and trifop ([(aryloxy)phenoxy]propionic acids) and alloxydim, sethoxydim, and clethodim (cyclohexanediones) are potent, reversible inhibitors of acetyl-coenzyme A carboxylase (ACC) partially purified from barley, corn, and wheat. Although inhibition of the wheat enzyme by clethodim and diclofop is noncompetitive versus each of the substrates adenosine triphosphate (ATP), HCO_3^-, and acetyl-coenzyme A (acetyl-CoA), diclofop and clethodim are nearly competitive versus acetyl-CoA since the level of inhibition is most sensitive to the concentration of acetyl-CoA ($K_{is} < K_{ii}$). To conclusively show whether the herbicides interact at the biotin carboxylation site or the carboxyl transfer site, the inhibition of isotope exchange and partial reactions catalyzed at each site was studied with the wheat enzyme. Only the [^{14}C]acetyl-CoA–malonyl-CoA exchange and decarboxylation of [^{14}C]malonyl-CoA reactions are strongly inhibited by clethodim and diclofop, suggesting that the herbicides interfere with the carboxyl transfer site rather than the biotin carboxylation site of the enzyme. Double-inhibition studies with diclofop and clethodim suggest that the [(aryloxy)phenoxy]propionic acid and cyclohexanedione herbicides may bind to the same region of the enzyme.

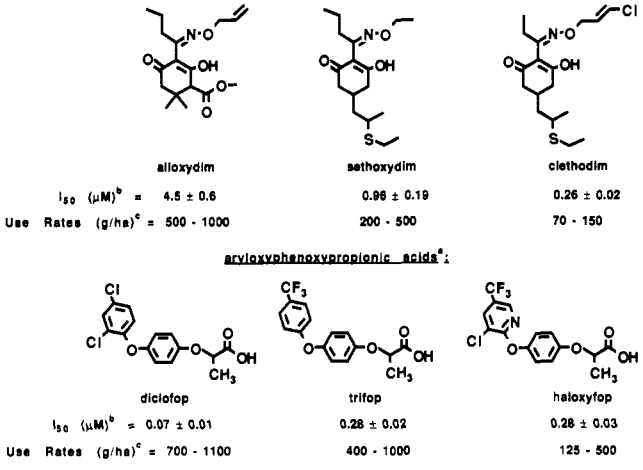
There are two major chemical classes of postemergence herbicides that are used for the control of annual and perennial grasses (monocots) in a large variety of broad-leaved crop plants (dicots): substituted 1,3-cyclohexanediones (alloxydim, sethoxydim, clethodim) (for reviews see Iwataki and Hirono (1979) and Ishikawa et al. (1985)) and various derivatized phenoxypropionic acids (diclofop, haloxyfop, trifop) (for a review see Nestler (1982)). The structures and common names for representatives of each class are shown in Table I.

Studies on the uptake, translocation, and metabolic fate of both classes of herbicides in tolerant and susceptible plants have shown that the susceptibility of monocotyledenous species is probably not due to differential metabolism of the compounds to nonherbicidal forms or to differential uptake or transport (Swisher and Corbin, 1982; Veerasekaran and Catchpole, 1982; Buhler et al., 1985). A number of recent studies have shown that representatives of both classes of herbicides inhibited de novo fatty acid biosynthesis in isolated chloroplasts, cell cultures, or leaves of susceptible grasses such as corn, wheat, and wild oats but not in tolerant broad-leaved plants such as soybean, spinach, and sugar beet (Burgstahler and Lichtenthaler, 1984; Hoppe, 1985; Hoppe and Zacher, 1985;

‡ Present address: Agricultural Products Department, E. I. du Pont de Nemours and Co., Inc., Wilmington, DE 19898.

0021-8561/90/1438-1282$02.50/0

Table I. Inhibition of Wheat Acetyl-CoA Carboxylase



[a] Common names are from the American National Standards Institute. Clethodim (SELECT, Chevron) is the proposed common name for 2-[1-[[(3-chloro-2-propenyl)oxy]imino]propyl]-5-[2-(ethylthio-)propyl]-3-hydroxy-2-cyclohexen-1-one, alloxydim (CLOUT, Nippon Soda and Schering) is the common name for 2-[1-(allyloxy)amino]butylidene]-5,5-dimethyl-4-(methoxycarbonyl)-3-hydroxy-2-cyclohexen-1-one, and sethoxydim (POAST, BASF) is the common name for 2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one. Diclofop, Haloxyfop, and trifop are common names for (*R,S*)-2-[4-(2,4-dichlorophenoxy)phenoxy]propionic acid (the methyl ester of diclofop is HOELON, Hoechst), (*R,S*)-2-[4-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propionic acid (haloxyfop-methyl is VERDICT, Dow), and (*R,S*)-2-[4-[4-(trifluoromethyl)phenoxy]phenoxy]propionic acid (Hoechst), respectively. The free acids diclofop and haloxyfop are the active components of Hoechst's HOELON and Dow's VERDICT, respectively. [b] The concentration of acetyl-CoA was 50 μM for determining the concentration of inhibitor producing 50% inhibition (I_{50} value) from Dixon plots. Data were from Rendina and Felts (1988) and Rendina et al. (1988). [c] Data for alloxydim, sethoxydim, and diclofop-methyl were from Worthing (1987), for trifop-methyl from Nestler (1982), and for clethodim and haloxyfop-methyl from Hopkins (1989).

Cho et al., 1986; Burton et al., 1987; Focke and Lichtenthaler, 1987; Lichtenthaler et al., 1987; Rendina and Felts, 1988; Kobek et al., 1988a,b; Walker et al., 1988a). These results suggest that tolerance of broad-leaved crops and weeds is due to the insensitivity of the target site to these compounds.

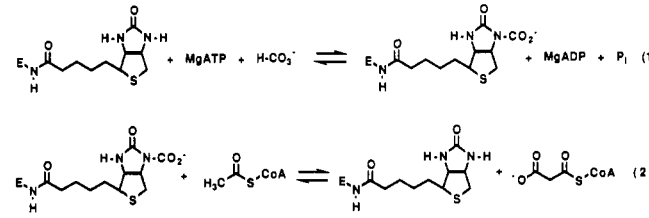
More recently, a key enzyme in the fatty acid biosynthetic pathway, acetyl-CoA carboxylase (E.C. 6.4.1.2), was shown to be the target for the cyclohexanedione (Burton et al., 1987; Focke and Lichtenthaler, 1987; Rendina and Felts, 1988; Secor and Cseke, 1988) and [(aryloxy)phenoxy]propionic acid (Burton et al., 1987; Secor and Cseke, 1988; Rendina et al., 1988; Kobek et al., 1988a; Walker et al., 1988b) herbicides. These studies showed that partially purified ACC from susceptible species such as wheat, corn, and barley was strongly inhibited by sethoxydim, clethodim, diclofop, and haloxyfop (slope inhibition constants or K_{is} values varied from 10^{-6} to 10^{-8} M), while the enzyme from tolerant species such as mung bean and spinach was much less inhibited (K_{is} values varied from 10^{-3} to 10^{-5} M). The relative insensitivity of ACC from broad-leaved plants (inhibition constants were from 300 to 60 000 times higher than the K_{is} values of the enzymes from grasses depending on the species and herbicide) could easily account for the differential selectivity of these herbicides.

With the exception of diclofop, the binding data for the herbicides can be correlated to the efficacy rates for these compounds in the field (Table I). The tighter binding compounds such as clethodim and haloxyfop have lower I_{50} values and use rates than alloxydim and sethoxydim. Diclofop, which is used commercially to control grasses in wheat without damaging the wheat itself, has an extremely high affinity for the wheat enzyme (I_{50} = 70 nM). This apparent inconsistency is explained by studies of the metabolism of diclofop in wheat (Gorecka et al., 1981). Gorecka showed that wheat can detoxify this compound more rapidly than susceptible species (by hydroxylation of the substituted phenoxy ring and subsequent conjugation to sugars). The relatively high use rate for diclofop (700–1100 g/ha), despite its relatively tight binding to the enzyme, may be explained by the poor translocation of this compound in both wheat and wild oats (Brezeanu et al., 1979).

Previous reports also showed that the herbicide binding pocket of the enzyme from grassy species appears to be very specific for the [(aryloxy)phenoxy]propionic acid and cyclohexanedione structures (Rendina and Felts, 1988; Rendina et al., 1988). Studies with corn, barley, or wheat ACC showed that the inhibition by trifop, haloxyfop, and fluazifop was stereoselective for the *R*-(+) enantiomers of the propionic acid moieties of these herbicides (Rendina et al., 1988; Secor and Cseke, 1988; Walker et al., 1988b). These results were consistent with the observed stereoselectivity for (*R*)-(+)-diclofop-methyl and other [(aryloxy)phenoxy]propionic acid herbicides in whole-plant studies (Nestler and Bieringer, 1980).

Malonyl-CoA, the product of acetyl-CoA carboxylase, is a key metabolic intermediate used in the de novo biosynthesis of fatty acids and flavonoids, as well as a number of other metabolic pathways that are important to plants such as the biosynthesis of cuticular waxes, stilbenoids, anthroquinones, napthoquinones, (*N*-malonylamino)cyclopropane-1-carboxylic acid, and malonic acid (Stumpf, 1987). Therefore, in the absence of any other means of synthesizing malonyl-CoA, inhibition of ACC would deprive the plant of an essential intermediate and lead to phytotoxic effects. It is not clear whether blockage of one or more of these pathways is the primary cause of the phytotoxicity.

Acetyl-CoA carboxylases from all species contain covalently bound *d*-biotin, which serves as a carboxyl carrier between a biotin carboxylation site, which catalyzes the ATP and divalent metal ion dependent carboxylation of biotin (eq 1), and a carboxyl transfer site where



the carboxyl group is transferred to acetyl-CoA (eq 2) (Moss and Lane, 1971; Stumpf, 1980). The kinetic mechanism for biotin-dependent carboxylases (Wood and Barden, 1977), including ACC from rat liver (Hashimoto and Numa, 1971), castor oil seeds (Finlayson and Dennis, 1983), and wheat leaves (A. R. Rendina, unpublished observations), has been shown to be two-site ping pong (where the products of the first site may be released before or after the addition of the substrates at the second site). The two catalytic sites are located on separate catalytically active subunits in the *Escherichia coli* enzyme, which interact with a third subunit that contains the covalently bound biotin (Moss and Lane, 1971). In contrast, the

enzyme from mammals and higher plants consists of a single polypeptide chain (molecular weight of 200 000–300 000) containing all three functions (Wood and Barden, 1977; Stumpf, 1987). The catalytic sites can be distinguished by studying isotope exchange reactions and other partial reactions that are functions of the separate half-reactions (Moss and Lane, 1971). Although mammalian acetyl-CoA carboxylases have two types of regulation, by polymerization from the inactive protomer to the active oligomeric form and by a phosphorylation–dephosphorylation cycle, there is no evidence that plant enzymes are under similar regulation (Stumpf, 1987).

In this paper we investigate the kinetic interaction of representatives of each herbicidal class with the normal substrates for wheat ACC. The inhibition of partial reactions catalyzed by the enzyme was studied to determine which catalytic site was affected by the herbicides. A multiple-inhibition study was also conducted to learn whether the two distinct chemical classes of inhibitors shared a common binding site.

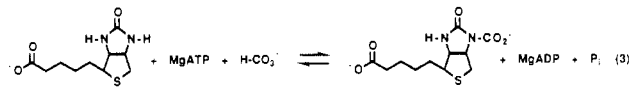
MATERIALS AND METHODS

Chemicals. [^{14}C]$NaHCO_3$ (58 Ci/mol) and [^{32}P]phosphate (P_i, 1000 Ci/mol) was obtained from ICN Radiochemicals, and [1-^{14}C]acetyl-CoA (50.6 Ci/mol; 99.9% radiochemical purity) and [1,3-^{14}C]malonyl-CoA were from NEN–Du Pont. [^{14}C]Malonyl-CoA was further purified to 20.1 Ci/mol and 99% radiochemical purity by anion-exchange chromatography on diethylaminoethyl (DEAE)-Sephadex A25 (Pharmacia) by the method described in Gregolin et al. (1968). The commercial herbicides and other reagents were prepared or obtained as described elsewhere (Rendina and Felts, 1988; Rendina et al., 1988).

Sources of plant materials, radiochemical and spectrophotometric assays for acetyl-CoA carboxylase, and partial purifications of the enzymes from barley (*Hordeum vulgare*), corn (*Zea mays*), wheat (*Triticum aestivum*), spinach (*Spinacia oleracea*), and mung bean (*Phaseolus aureus*) have been described previously (Rendina and Felts, 1988; Rendina et al., 1988).

ATP–[^{32}P]Phosphate Isotope Exchange Assay. ATP–[^{32}P]P_i exchange rates, a measure of the biotin carboxylation site of ACC (eq 1), were determined by a modification of the procedure of Gregolin et al. (1968). Each assay contained in a total volume of 0.25 mL 0.1 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.0), 0.4 mM potassium [^{32}P]phosphate (9 μCi), 2.4 mM $NaHCO_3$, 2.4 mM adenosine diphosphate (ADP), 0.48 mM ATP, 10 mM $MgSO_4$, and varying amounts of inhibitors. Assays were initiated by the addition of 0.01 unit (spectrophotometric assay value; 1 unit = 1 μmol of product formed per min at 25 °C) of wheat ACC. After a 60-min incubation period at 25 °C the reactions were quenched by converting all of the ATP to ADP and glucose 6-phosphate by adding 5 units of hexokinase (Sigma Chemical Co.) and 4 mM glucose in 10 μL. After an additional 10-min incubation period, 0.715 mL of 2% perchloric acid, 1 mL of 1 M sulfuric acid, and 1 mL of 5% ammonium molybdate followed by 2 mL of water-saturated 2-butanol were added. The residual inorganic phosphate was extracted into the organic phase as the phosphomolybdic acid complex. Five additional extractions were made with 2-mL portions of water-saturated 2-butanol, and the organic phases discarded. The incorporation of ^{32}P into ATP was determined by counting a 2-mL aliquot of the aqueous phase in 15 mL of DIMISCINT (National Diagnostics) with use of a Beckman liquid scintillation spectrometer. Control experiments that included 15 μM avidin (Sigma) were carried out at each inhibitor concentration. The control rates were subtracted from each sample to correct for ATP–[^{32}P]P_i exchange catalyzed by contaminating nonbiotin-containing enzymes in the partially purified ACC preparation.

Biotin Carboxylation Assay. The reaction mixture for the carboxylation of free *d*-biotin (eq 3, catalyzed by the biotin carboxylation site of ACC) was essentially that described by Guchhait et al. (1974) with the following changes. Each assay contained in 0.2-mL total volume 0.1 M *N*-tris[(hydroxymethyl)-

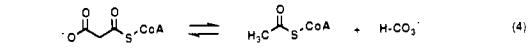


(3)

methyl]-3-aminopropanesulfonic acid (Taps) (pH 8.5), 0.3 M glycerol, 50 mM *d*-biotin (Sigma), 0.006 unit of wheat ACC, 6.5 mM $MgSO_4$, 0.3 mM ATP, 8 mM potassium [^{14}C]bicarbonate (8 μCi), and varying amounts of inhibitors. Control experiments were conducted in the absence of enzyme, and these activities were subtracted from each sample. Reactions were initiated by the addition of enzyme (controls by addition of biotin), incubated for 30 min at 25 °C, and carboxylation was terminated by the addition of 0.8 mL of ice-cold 10 mM ethylenediaminetetraacetic acid at pH 7.0. The solutions were placed on ice, 20 μL of 1-octanol was added to reduce foaming, and unlabeled CO_2 was bubbled through the solution for 1 h to remove excess [^{14}C]HCO_3^- (using metal needles covered with Tygon tubing). Alternately, octanol and then 0.1 mg of carbonic anhydrase (Sigma) were added and the ice-cold solutions were sparged for 20 min with unlabeled CO_2. After gassing, a 0.9-mL aliquot was transferred to a counting vial containing 0.1 mL of 0.1 M KOH, 15 mL of scintillation cocktail (ATOMLIGHT, NEN–Du Pont) was added, and the ^{14}C activity of the residual carboxybiotin was determined on a liquid scintillation spectrometer.

Malonyl-CoA–[^{14}C]Acetyl-CoA Isotope Exchange Assay. The partial reaction catalyzed by the carboxyl transfer site of ACC (eq 2) was monitored by following the isotope exchange between [^{14}C]acetyl-CoA and malonyl-CoA essentially as described by Gregolin et al. (1968). Each assay contained in a total volume of 0.2 mL 0.2 M Taps (pH 8.5), 200 μM malonyl-CoA, 100 μM [^{14}C]acetyl-CoA (0.2 μCi), 0.0026 unit of wheat ACC, and varying amounts of inhibitors. Reactions were initiated by the addition of enzyme and were linear for 10 min at 25 °C. The reactions were terminated by deacylation of acetyl-CoA upon addition of 50 μL of 0.2 M sodium arsenate (pH 8.5) and 9 μL of phosphotransacetylase (2 units, Sigma). After an additional 30-min incubation at 25 °C, the deacylation was stopped by addition of 50 μL of 6 N HCl. The precipitated protein was removed by centrifugation for 10 min, and a 200-μL aliquot was placed in a scintillation vial and dried for 1.5 h in a vacuum oven at 50 °C. Residual acetic acid was removed by addition of 16 μL of water and 200 μL of cyclohexane followed by drying for 45 min at 50 °C. A 1-mL portion of water and 15 mL of scintillation cocktail were added to the dried samples, and the radioactivity of the remaining malonyl-CoA and malonate was determined by scintillation counting.

Malonyl-CoA Decarboxylation Assay. The rate of malonyl-CoA decarboxylation (eq 4, catalyzed by the carboxyl transfer site of ACC) was determined with a standard reaction mix-



(4)

ture (final volume 0.2 mL, pH 8.5) consisting of 0.1 M Taps, 0.3 M glycerol, 6.6 μM [1,3-^{14}C]malonyl-CoA (0.03 μCi), 0.03 unit of wheat ACC, and varying amounts of inhibitors. The reaction was allowed to proceed for 15 min at 25 °C and was terminated by placing the reaction vials on ice, loading the samples onto a 1-mL bed volume of DEAE-Sephadex A25 (Pharmacia) in an 8 × 70 mm disposable polystyrene column previously equilibrated with 50 mM Taps (pH 8.0), and immediately washing with 1 mL of buffer containing 0.04 M NaCl. All of the [^{14}C]bicarbonate formed in the decarboxylation reaction was collected in three 5-mL fractions of the 0.04 M NaCl washes. Under these conditions only the monoanions were eluted. A 1-mL aliquot of each fraction was added to 15 mL of scintillation cocktail, and the activity of the [^{14}C]bicarbonate was determined by liquid scintillation counting. A control experiment containing all components except enzyme was carried out and the rate determined in this experiment was subtracted from each sample rate.

Data Analysis. The determination of I_{50} values and kinetic constants for inhibitors and Michaelis constants for substrates was described previously (Rendina et al., 1988). Equations 5 and 6

$$v = V/(1 + [I]/K_I + [J]/K_J + [[I][J]/\beta]/K_IK_J) \quad (5)$$

$$v = V/(1 + [I]/K_I + [J]/K_J) \quad (6)$$

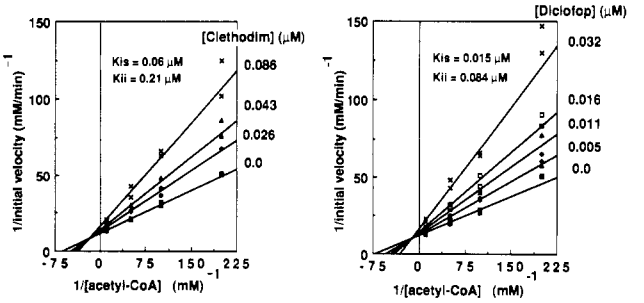


Figure 1. Inhibition of wheat ACC by clethodim (left) and diclofop (right) with acetyl-CoA as the variable substrate at 5 mM $MgSO_4$, 3 mM ATP, and 15 mM bicarbonate. All data were fitted to equations for linear competitive, noncompetitive, and uncompetitive inhibition as previously described (Rendina et al., 1988). The best fit was determined by the lowest σ and the smallest standard errors in the kinetic constants. σ is equal to sum of squares of residuals/degrees of freedom, where degrees of freedom are defined as number of points minus number of parameters. The lines in the figure are the theoretical best fit of the data to noncompetitive inhibition.

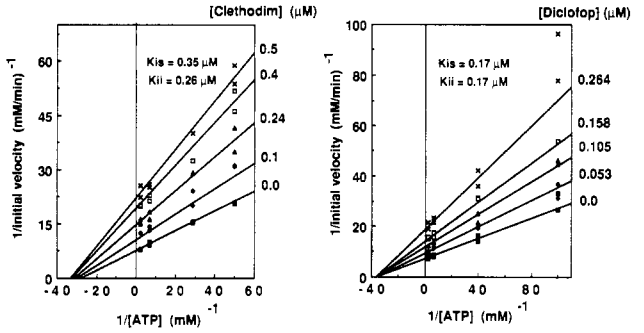


Figure 2. Inhibition of wheat ACC by clethodim (left) and diclofop (right) with ATP as the variable substrate at 150 μM acetyl-CoA, 5 mM $MgSO_4$, and 15 mM bicarbonate. Data analysis was as described in Figure 1.

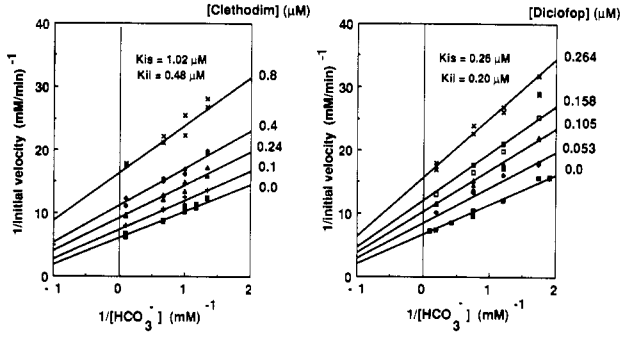


Figure 3. Inhibition of wheat ACC by clethodim (left) and diclofop (right) with bicarbonate as the variable substrate at 150 μM acetyl-CoA, 5 mM $MgSO_4$, and 3 mM ATP. Assays were conducted in stoppered cuvettes and the solutions sparged with argon prior to use. Data analysis was as described in Figure 1.

where v is the initial velocity, V is the maximal velocity, [I] and [J] are the two inhibitor concentrations, K_I and K_J are their apparent inhibition constants, and β is the interaction coefficient (Northrup and Cleland, 1974).

RESULTS AND DISCUSSION

Kinetic Characterization of the Inhibition. Figures 1–3 show that both classes of grass herbicides are linear, noncompetitive inhibitors versus each of the substrates of wheat ACC. Previous work showed that these compounds are reversible inhibitors of the enzymes from grasses (Rendina and Felts, 1988; Rendina et al., 1988). The inhibition is most sensitive to the level of acetyl-CoA ($K_{is} < K_{ii}$) and nearly insensitive to the level of either ATP or bicarbonate ($K_{is} \approx K_{ii}$). The K_{is} value is nearly 10 times lower when acetyl-CoA is varied compared to the apparent K_{is} obtained at 150 μM acetyl-CoA and variable concentrations of ATP and bicarbonate. Although the inhibition data fit best to the equation for noncompetitive inhibition for all three substrates, diclofop and clethodim are nearly competitive versus acetyl-CoA and nearly uncompetitive versus bicarbonate.

When malonyl-CoA is used as a product inhibitor of wheat ACC, very similar patterns of linear, noncompetitive inhibition are observed versus each of the substrates (data not shown). Like the herbicides, malonyl-CoA is also nearly competitive versus acetyl-CoA. Noncompetitive inhibition by malonyl-CoA was also observed for ACC from castor oil seeds (Finlayson and Dennis, 1983) and from rat liver (Hashimoto and Numa, 1971). However, the two-site ping-pong kinetic mechanism of biotin-dependent carboxylases predicts that the product, malonyl-CoA, should be a competitive inhibitor versus acetyl-CoA. This apparent contradiction may be explained if the central complexes of the kinetic mechanism (where the chemical steps take place) are partially rate-determining (Cleland, 1973; Rendina et al., 1988). By analogy to malonyl-CoA, the kinetic data suggest that both classes of herbicides interfere with the carboxylation of acetyl-CoA at the carboxyl transfer site and do not affect the biotin carboxylation site.

Inhibition of Isotope Exchange Reactions and Partial Reactions. Since the kinetic data do not conclusively show that the herbicides are strictly competitive with the substrates of the carboxyl transfer site of the enzyme, the inhibition of the partial reactions that are independently catalyzed at the two separate catalytic sites was studied. For enzymes with ping-pong mechanisms it is possible to demonstrate isotope exchange reactions between the substrates and products of either half-reaction in the absence of any of the substrates or products of the other half-reaction. At the biotin carboxylation site wheat ACC catalyzes the exchange of label between ATP and [^{32}P]phosphate in the presence of ADP, Mg^{2+}, and bicarbonate, the other substrates of this half-reaction (eq 1), and in the absence of either acetyl-CoA or malonyl-CoA. This reaction depends on the integrity of the bound biotin cofactor since carboxybiotin is required to form the putative carboxyphosphate intermediate (Tipton and Cleland, 1988a,b; Ogita and Knowles, 1988). Avidin, which binds free biotin very tightly ($K_D = 10^{-15}$ M; Moss and Lane, 1971), blocks this isotope exchange reaction and allows this biotin-dependent exchange to be distinguished from identical reactions catalyzed by contaminating kinases or ATPases. The biotin carboxylation site can also be distinguished by studying the inhibition of the carboxylation of free *d*-biotin in the presence of Mg-ATP and bicarbonate (eq 3).

At the carboxyl transfer site the enzyme catalyzes the biotin-dependent isotope exchange between [^{14}C]acetyl-CoA and malonyl-CoA (eq 2). We also studied the inhibition of the decarboxylation of [1,3-^{14}C]malonyl-CoA, a reaction that is catalyzed at the carboxyl transfer site of the enzyme (eq 4). Each of the isotope exchange reactions and partial reactions that were studied with the wheat enzyme have been demonstrated previously for other biotin-dependent carboxylases (Moss and Lane, 1971; Wood and Barden, 1977).

The results of the inhibition of the exchange reactions and partial reactions are summarized in Table II. The I_{50} values for inhibition of the reactions catalyzed at the

Table II. Inhibition of Partial Reactions of Wheat Acetyl-CoA Carboxylase

		biotin carboxylation site		carboxyl transfer site	
inhibitor	complete reactn I_{50}, μM	ATP–P_i exchg I_{50}, μM	carboxylation of free biotin I_{50}, μM	acetyl-CoA–malonyl-CoA exchg I_{50}, μM	malonyl-CoA decarboxyln I_{50}, μM
clethodim	0.26 ± 0.02	420 ± 170	13500 ± 1400	4.10 ± 0.4	0.38 ± 0.04
diclofop	0.07 ± 0.01	460 ± 80	7400 ± 1300	1.30 ± 0.05	0.14 ± 0.02

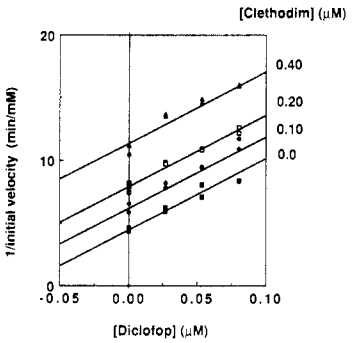


Figure 4. Double-inhibition or Yonetani-Theorell plot of diclofop versus clethodim at 50 μM acetyl-CoA, 5 mM $MgSO_4$, 3 mM ATP, and 15 mM bicarbonate. The best fit of the data was to eq 6, and the lines shown in the figure are to the theoretical fit.

biotin carboxylation site of wheat ACC by clethodim and diclofop were from 200 to 100 000 times larger than the I_{50} values for inhibition of the complete reaction by these compounds. In contrast, clethodim and diclofop were much more potent inhibitors of the reactions catalyzed at the carboxyl transfer site (I_{50} values were only 2–20 times larger than the values for the complete reaction). Taken together, the kinetic inhibition patterns and the inhibition of the isotope exchange and partial reactions strongly suggest that the herbicides interfere with the carboxyl transfer site and not the biotin carboxylation site. It will be interesting to learn whether the herbicides interfere with the movement of biotin between the two catalytic sites, whether the herbicides overlap with both the CoA and biotin binding sites of the carboxyl transfer site, and whether the negatively charged herbicides are acting as mimics of the carbanion intermediate proposed for this half-reaction (O'Keefe and Knowles, 1986), the carboxybiotin anion, or the carboxylate of the product malonyl-CoA.

Double-Inhibition Studies. The extremely similar kinetic behavior of representatives of both classes of herbicidal inhibitors of ACC described above suggested that the two classes might share a common binding site. To determine whether the binding of the two classes of inhibitors was mutually exclusive, the Yonetani–Theorell double-inhibition method (Yonetani and Theorell (1964) as modified by Northrup and Cleland (1974)) was employed. This method, in which two inhibitors are varied in the presence of fixed, subsaturating levels of substrates, yields the factor β (eq 5), which defines the degree of interaction between the inhibitors at the enzyme active site. If the inhibitors can exclude each other from a common binding site, β becomes infinitely large and the pattern becomes a family of parallel lines described by eq 6. The data shown in Figure 4 were fitted to both equations, and the best fit was to eq 6 for a family of parallel lines. These results for clethodim and diclofop demonstrate a high degree of interference in binding between the cyclohexanedione herbicides and the [(aryloxy)phenoxy]propionic acid herbicides. We are currently trying to define those structural features that are important to the overlap.

CONCLUSIONS

The cyclohexanedione and [(aryloxy)phenoxy]propionic acid grass herbicides clethodim and diclofop are potent, reversible, and linear noncompetitive inhibitors versus acetyl-CoA, ATP, and bicarbonate. Since the apparent level of inhibition is most sensitive to the concentration of acetyl-CoA, these herbicides are probably interfering with the carboxyl transfer site and are not affecting the ATP-dependent biotin carboxylation site. This hypothesis was confirmed by studies of the inhibition of the isotope exchange and partial reactions of the separate catalytic sites. Only the reactions catalyzed at the carboxyl transfer site were significantly inhibited by representatives of each class of herbicide. Finally, double-inhibition experiments with clethodim and diclofop showed that the two classes of herbicides are mutually exclusive: that is, the binding sites for these compounds are at least partially overlapping.

ACKNOWLEDGMENT

We thank A. Ho for the preparation and purification of diclofop (free acid), and J. Hagenah, W. Loh, and T. Luo for the preparation of clethodim. The advice and assistance of B. Boettcher, C. Kemal, R. Cherpeck, Y. Nakagawa, W. Loh, A. Omid, and C. Hodges are also gratefully acknowledged.

ABBREVIATIONS USED

ACC, acetyl-coenzyme A carboxylase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoA, coenzyme A; DEAE, (diethylamino)ethyl; P_i, inorganic phosphate; Taps, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

LITERATURE CITED

Brezeanu, A. G.; Davis, D. G.; Shimabukuro, R. H. Ultrastructural Effects and Translocation of Methyl 2-(4-(2,4-Dichlorophenoxy)phenoxy)propionate in Wheat (*Triticum aestivum*) and Wild Oats (*Avena fatua*). *Can. J. Bot.* **1979**, *54*, 2038–2048.

Buhler, D. D.; Swisher, B. A.; Burnside, O. C. Behavior of ^{14}C-Haloxyfop-Methyl in Intact Plants and Cell Cultures. *Weed Sci.* **1985**, *33*, 291–299.

Burgstahler, R. J.; Lichtenthaler, H. K. Inhibition by Sethoxydim of Phospho- and Galactolipid Accumulation in Maize Seedlings. In *Structure, Function and Metabolism of Plant Lipids*; Siegenthaler, P. A., Eichenberger, W., Eds.; Elsevier: New York, 1984.

Burton, J. D.; Gronwald, J. W.; Somers, D. A.; Connelly, J. A.; Gengenbach, B. G.; Wyse, D. L. Inhibition of Plant Acetyl-Coenzyme A Carboxylase by the Herbicides Sethoxydim and Haloxyfop. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 1039–1044.

Cho, H.-Y.; Widholm, J. M.; Slife, F. W. Effects of Haloxyfop on Corn (*Zea mays*) and Soybean (*Glycine max*) Cell Suspension Cultures. *Weed Sci.* **1986**, *34*, 496–501.

Cleland, W. W. Derivation of Rate Equations for Multisite Ping-Pong Mechanisms with Ping-Pong Reactions at One or More Sites *J. Biol. Chem.* **1973**, *248*, 8353–8355.

Corbett, J. R.; Wright, K.; Baille, A. C. *The Biochemical Mode of Action of Pesticides*; Academic Press: Orlando, FL, 1984.

Finlayson, S. A.; Dennis, D. T. Acetyl-Coenzyme A Carboxylase from the Developing Endosperm of *Ricinus communis*. II. A Two-Site Kinetic Mechanism. *Arch. Biochem. Biophys.* **1983**, *225*, 586–595.

Focke, M.; Lichtenthaler, H. K. Inhibition of the Acetyl-CoA Carboxylase of Barley Chloroplasts by Cycloxydim and Sethoxydim. *Z. Naturforsch.* **1987**, *42C*, 1361–1363.

Gorecka, K.; Shimabukuro, R. H.; Walsh, W. C. Aryl Hydroxylation: A Selective Mechanism for the Herbicides, Diclofop-methyl and Clofop-isobutyl, in Gramineous Species. *Physiol. Plant.* **1981**, *53*, 55–63.

Gregolin, C.; Ryder, E.; Lane, M. D. Liver Acetyl-CoA Carboxylase I. Isolation and Catalytic Properties. *J. Biol. Chem.* **1968**, *243*, 4227–4235.

Guchhait, R. B.; Polakis, S. E.; Dimroth, P.; Stoll, E.; Moss, J.; Lane, M. D. Acetyl Coenzyme A Carboxylase System of *Escherichia coli*. Purification and Properties of the Biotin Carboxylase, Carboxyltransferase, and Carboxyl Carrier Protein Components. *J. Biol. Chem.* **1974**, *249*, 6633–6645.

Hashimoto, T.; Numa, S. Kinetic Studies on the Reaction Mechanism and the Citrate Activation of Liver Acetyl-CoA Carboxylase. *Eur. J. Biochem.* **1971**, *18*, 319–331.

Hopkins, W. L., Ed. *Agricultural Chemical New Product Development Review*; Ag Chem Information Service: Indianapolis, IN, 1989; Vol. VII.

Hoppe, H. H. Differential Effect of Diclofop-methyl on Fatty Acid Biosynthesis in Leaves of Sensitive and Tolerant Plant Species *Pestic. Biochem. Physiol.* **1985**, *23*, 297–308.

Hoppe, H. H.; Zacher, H. Inhibition of Fatty Acid Biosynthesis in Isolated Bean and Maize Chloroplasts by Herbicidal Phenoxyphenoxypropionic Acid Derivatives and Structurally Related Compounds. *Pestic. Biochem. Physiol.* **1985**, *24*, 298–305.

Ishikawa, H.; Yamada, S.; Hosaka, H.; Kawana, T.; Okunuki, S.; Kohara, K. Herbicidal Properties of Sethoxydim for the Control of Gramineous Weeds. *J. Pestic. Sci.* **1985**, *10*, 187–193.

Iwataki, I.; Hirono, Y. The Chemical Structure and Herbicidal Activity of Alloxydim-sodium and Related Compounds. In *Advances Pesticide Science*, 4th International Congress on Pesticide Chemistry; Geissbuhler, H., Ed.; Pergamon Press: Oxford, 1979.

Kobek, K.; Focke, M.; Lichtenthaler, H. K. Fatty-Acid Biosynthesis and Acetyl-CoA Carboxylase as a Target of Diclofop, Fenoxaprop and Other Aryloxy-phenoxy-propionic Acid Herbicides. *Z. Naturforsch.* **1988a**, *43C*, 47–54.

Kobek, K.; Focke, M.; Lichtenthaler, H. K.; Retzlaff, G.; Wurzer, B. Inhibition of Fatty Acid Biosynthesis in Isolated Chloroplasts by Cyclooxydim and Other Cyclohexane-1,3-diones. *Physiol. Plant.* **1988b**, *72*, 492–498.

Lichtenthaler, H. K.; Kobek, K.; Ishii, K. Inhibition by Sethoxydim of Pigment Accumulation and Fatty Acid Biosynthesis in Chloroplasts of *Avena* Seelings. *Z. Naturforsch.* **1987**, *42C*, 1275–1279.

Moss, J.; Lane, M. D. The Biotin-Dependent Enzymes. *Adv. Enzymol.* **1971**, *35*, 321–442.

Nestler, H. J. Phenoxy-phenoxypropionic Acid Derivatives and Related Compounds. In *Chemie der Pflanzenschutz und Schladlings-bekampfungsmittel*; Wegler, R., Ed.; Springer-Verlag: New York, 1982; Vol. 8, pp 1–25.

Nestler, H. J.; Bieringer, H. Synthesis and Herbicidal Activity of the D- and L-Methyl 2-[4-(2,4-Dichlorophenoxy)-phenoxy]-propionate Enantiomers. *Z. Naturforsch. B: Anorg. Chem. Org. Chem.* **1980**, *35b*, 366–371.

Northrop, D. B.; Cleland, W. W. The Kinetics of Pig Heart Triphosphopyridine Nucleotide-Isocitrate Dehydrogenase. II. Dead-End and Multiple Inhibition Studies. *J. Biol. Chem.* **1974**, *249*, 5820–5827.

Ogita, T.; Knowles, J. R. On the Intermediacy of Carboxyphosphate in Biotin-Dependent Carboxylations. *Biochemistry* **1988**, *27*, 8028–8033.

O'Keefe, S. J.; Knowles, J. R. Biotin-Dependent Carboxylation Catalyzed by Transcarboxylase Is a Stepwise Process. *Biochemistry* **1986**, *25*, 6077–6084.

Rendina, A. R.; Felts, J. M. Cyclohexanedione Herbicides Are Selective and Potent Inhibitors of Acetyl-CoA Carboxylase from Grasses. *Plant Physiol.* **1988**, *86*, 983–986.

Rendina, A. R.; Felts, J. M.; Beaudoin, J. D.; Craig-Kennard, A. C.; Look, L. L.; Paraskos, S. L.; Hagenah, J. A. Kinetic Characterization, Stereoselectivity and Species Selectivity of the Inhibition of Plant Acetyl-CoA Carboxylase by the Aryloxyphenoxypropionic Acid Grass Herbicides. *Arch. Biochem. Biophys.* **1988**, *265*, 219–225.

Secor, J.; Cseke, C. Inhibition of Acetyl-CoA Carboxylase Activity by Haloxyfop and Tralkoxydim. *Plant Physiol.* **1988**, *86*, 10–12.

Stumpf, P. K. Biosynthesis of Saturated and Unsaturated Fatty Acids. In *The Biochemistry of Plants*; Stumpf, P. K., Conn, E. E.; Eds.; Academic Press: Orlando, FL, 1980; Vol. 4.

Stumpf, P. K. The Biosynthesis of Saturated Fatty Acids. In *The Biochemistry of Plants*; Stumpf, P. K., Ed.; Academic Press: Orlando, FL, 1987; Vol. 9.

Swisher, B. A.; Corbin, F. T. Behavior of BAS-9052 OH in Soybean (*Glycine max*) and Johnsongrass (*Sorghum halepense*) Plant and Cell Cultures. *Weed Sci.* **1982**, *30*, 640–650.

Tipton, P. A.; Cleland, W. W. Catalytic Mechanism of Biotin Carboxylase: Steady-State Kinetic Investigations. *Biochemistry* **1988a**, *27*, 4317–4325.

Tipton, P. A.; Cleland, W. W. Carbon-13 and Deuterium Isotope Effects on the Catalytic Reactions of Biotin Carboxylase. *Biochemistry* **1988b**, *27*, 4325–4331.

Veerasekaran, P.; Catchpole, A. H. Studies on the Selectivity of Alloxydim-sodium in plants. *Pestic. Sci.* **1982**, *13*, 452–462.

Walker, K. A.; Ridley, S. M.; Harwood, J. L. Effects of the Selective Herbicide Fluazifop on Fatty Acid Synthesis in Pea (*Pisum sativum*) and Barley (*Hordeum vulgare*). *Biochem. J.* **1988a**, *254*, 811–817.

Walker, K. A.; Ridley, S. M.; Lewis, T.; Harwood, J. L. Fluazifop, a Grass-Selective Herbicide which Inhibits Acetyl-CoA Carboxylase in Sensitive Plant Species. *Biochem. J.* **1988b**, *254*, 307–310.

Wood, H. G.; Barden, R. E. Biotin Enzymes. *Annu. Rev. Biochem.* **1977**, *46*, 385–413.

Worthing, C. R., Ed. *The Pesticide Manual*, 8th ed.; Lavenham Press: Lavenham, 1987.

Yonetani, T.; Theorell, H. Studies on Liver Alcohol Dehydrogenase Complexes. III. Multiple Inhibition Kinetics in the Presence of Two Competitive Inhibitors. *Arch. Biochem. Biophys.* **1964**, *106*, 243–251.

Received for review July 11, 1989. Accepted December 28, 1989. This paper was presented in a symposium entitled Enzyme Targeted Agrochemicals that was held by the American Chemical Society in Dallas on April 12–13, 1989.

Registry No. ACC, 9023-93-2; diclofop, 40843-25-2; clethodim, 104233-53-6; haloxyfop, 69806-34-4; trifop, 59011-30-2; alloxydim, 55634-91-8; sethoxydim, 74051-80-2.