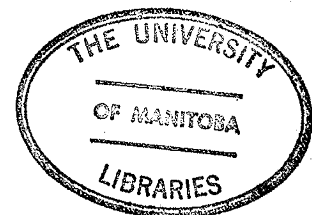
METABOLISM OF THE HERBICIDE
BENZOYLPROP ETHYL

A Thesis
Submitted to the Faculty
of
Graduate Studies
The University of Manitoba
by
Bernard Dale Hill

In Partial Fulfillment of the
Requirements for the Degree

of

Doctor of Philosophy
Department of Plant Science
February 1978



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BERNARD DALE HILL

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ACKNOWLEDGMENTS

Financial support from the Faculty of Graduate Studies, University of Manitoba, and from the National Research Council of Canada is gratefully acknowledged.

The labelled and technical herbicides used in this work were kindly supplied by Shell Research Ltd.

I much appreciate the help and advice received from Dr. Elmer H. Stobbe, Dr. B. L. Jones, Dr. W. Woodbury, Dr. L. J. LaCroix, Dr. R. D. Hill, Dr. F. W. Hougen, Mr. J. A. Menzies, Mr. Barry Todd, and others.

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ABSTRACT

Hill, Bernard Dale. Ph.D. The Department of Plant Science, The University of Manitoba, February, 1978. Metabolism of the Herbicide Benzoylprop Ethyl. Major Professor: Dr. Elmer H. Stobbe.

Since the herbicidal activity of benzoylprop ethyl {ethyl-2-[N-(3,4-dichlorophenyl)benzamido]propionate} is dependent upon its conversion to the biologically active desethyl acid, the metabolism of benzoylprop ethyl was studied to explain variations in wild oat (*Avena fatua* L.) control.

The effect of light and nutrient levels on benzoylprop ethyl metabolism and growth inhibition in wild oat was measured 3, 6, and 12 days after hydroponic dosing with ^{14}C-herbicide. Plants under low light (10.3 Klux) contained more phytotoxic benzoylprop acid due to increased de-esterification at 3 and 6 days, and decreased acid-conjugate formation at 12 days. Under low nutrients (quarter-strength Hoagland's solution), both metabolic reactions were slowed resulting in increased acid at 3 days, but not at 6 or 12 days. Increased growth inhibition after 12 days under high light (17.2 Klux) was attributed to increased ^{14}C-herbicide translocated from plant roots to shoots. After 12 days, there was more growth inhibition at the low nutrient level than at the high nutrient level (half-strength Hoagland's solution). Growth correlated better to the amount (dpm) of acid-conjugate (detoxified acid) than to the amount of ester or acid in plant shoots. Acid concentration, rather than acid

amounts *per se*, had the most influence on subsequent growth. It was concluded that although reduced light and nutrients resulted in higher levels of phytotoxic acid, increased benzoylprop ethyl efficacy is also due to a reduced growth rate of the wild oat.

An investigation into the possible enzymic nature of benzoylprop ethyl de-esterification resulted in the isolation of a carboxyesterase from wild oat which hydrolyzes benzoylprop ethyl to the herbicidally active benzoylprop acid. The esterase was partially purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-100 gel filtration. A 8.3 fold purification was obtained with 46% recovery. The rate of de-esterification of ^{14}C-benzoylprop ethyl was 0.1 ηmoles/16 h at standard assay conditions of 1 mg lyophilized enzyme preparation in 0.1 ml phosphate buffer (0.1M, pH 7.0), substrate 5 μM. The wild oat esterase was stable and activity as a function of reaction time, enzyme and substrate concentration, was linear. Incubation with α-chymotrypsin reduced wild oat esterase activity as did the presence of ethanol in reaction mixtures. Esterase activity for ^{14}C-benzoylprop ethyl was not detected in preparations from wheat (*Triticum aestivum* L.). Evidence was obtained which suggested the presence of an inhibitor in wheat.

The chemical hydrolysis of benzoylprop ethyl, specifically, the carboxyethyl moiety selective of the amide bond, was demonstrated. The ester was treated with ethanolic NaOH, and after a week at room temperature, the reaction product identified as benzoylprop acid was isolated (yield 37%). Thin-layer chromatographic data, infrared spectra, and mass spectra for benzoylprop acid and benzoylprop ethyl are presented and compared.

FOREWORD

This thesis has been prepared in the paper style format outlined in the Department of Plant Science Guide to Thesis Preparation. The main body is composed of three manuscripts prepared in accordance with instructions given to authors by the Weed Research journal. The papers "Effect of light and nutrient levels on ^{14}C-benzoylprop ethyl metabolism and growth inhibition in wild oat (*Avena fatua* L.)" and "Hydrolysis of the herbicide benzoylprop ethyl by wild oat esterase" are to be submitted to Weed Research, while the research note "Chemical hydrolysis of the herbicide benzoylprop ethyl" is not intended for publication.

INTRODUCTION

Benzoylprop ethyl is a post-emergence herbicide used to selectively control wild oats in wheat. Benzoylprop ethyl was first field-tested in Western Canada in 1969, and became commercially available in 1972. Since it is effective on wild oats at the 4-6 leaf stage, benzoylprop ethyl allows later and more flexible timing of application, compared to other wild oat herbicides.

Field experiments have been conducted by the Department of Plant Science, University of Manitoba, to study the herbicidal properties and efficacy of benzoylprop ethyl (Holm, 1972; Lowen, 1975). In these and other experiments, wild oat control was better at later application times and at higher soil fertility levels (Stobbe *et al.*, 1971; Sharma *et al.*, 1977). The variations in wild oat control may have been related to changes in light and nutrient levels caused by crop competition. Crop competition reduces light levels via the development of a crop canopy and decreases nutrient availability via root competition. It has been shown that crop competition enhances the efficacy of benzoylprop ethyl on wild oat (Loubaresse *et al.*, 1971; Jeffcoat and Sampson, 1973). Crop competition would be most severe at later application times and at higher soil fertility levels.

The herbicidal activity of benzoylprop ethyl is dependent upon its metabolic conversion within plants to the biologically active desethyl acid (Jeffcoat and Sampson, 1973; Jeffcoat and Harries, 1973). It has

been suggested that the de-esterification of benzoylprop ethyl in plants is enzymic, and thus depends on levels of esterase (Jeffcoat and Harries, 1973).

Research was conducted in controlled environments and in the laboratory to study the effect of light and nutrient levels on the metabolism and subsequent efficacy of benzoylprop ethyl in wild oats. The enzymic nature of benzoylprop ethyl de-esterification and the chemical hydrolysis of benzoylprop ethyl were investigated.

The objectives of these studies were:

1. To determine whether reduced light and nutrient levels, such as might be expected from crop competition, result in higher levels of phytotoxic benzoylprop acid.

2. To determine whether the increased herbicidal activity of benzoylprop ethyl at reduced light and nutrient levels is due to changes in metabolism, or the reduced growth rate of the wild oat, or both.

3. To demonstrate the enzymic de-esterification of benzoylprop ethyl *in vitro* using wild oat and wheat extracts.

4. To study the properties of isolated wild oat and wheat esterases and thus perhaps gain further insight into the basis of selectivity of benzoylprop ethyl.

5. To chemically hydrolyze benzoylprop ethyl and identify the reaction product(s).

LITERATURE REVIEW

Metabolism of Benzolyprop Ethyl in Plants

Benzoylprop ethyl is a N,N-disubstituted alanine derivative (Fig. 1), whose structure may be abbreviated as DiClAr-NR-CO-R. Although it doesn't belong to a particular herbicide group, benzoylprop ethyl contains structural similarities to certain ureas (DiClAr-NH-CO-NR-R), carbamates (DiClAr-NH-CO-OR), and anilides (DiClAr-NH-CO-R). In general, ureas, carbamates, and anilides containing the dichloroanilino group (DiClAr-N) are dealkylated and reduced to dichloroaniline (Geissbühler, 1969; Herrett, 1969). Dichloroaniline is then conjugated to sugars, lignin, and polysaccharides in plants (Still, 1967; Yih *et al.*, 1968). The nitrogen of the dichloroanilino group of benzoylprop ethyl is trisubstitued and thus, instead of dichloroaniline formation, one might expect the more labile carboxyethyl group to be hydrolyzed.

Beynon *et al.* (1974a) followed the metabolism of benzoylprop ethyl in wheat, oat, and barley seedlings until 15 days after microdroplet application of ^{14}C-herbicide to the foliage. The same major and minor breakdown pathways were proposed for all three plant species (Figs. 1-2). The major pathway (Fig. 1) consisted of hydrolysis of the carboxyethyl group to form the desethyl acid, followed by conjugation of the acid to unidentified sugars. Benzoic acid formation (Fig. 2) following breakage of the amide bond, and an unidentified bound form of original herbicide were detected in minor amounts. There was no evidence of any

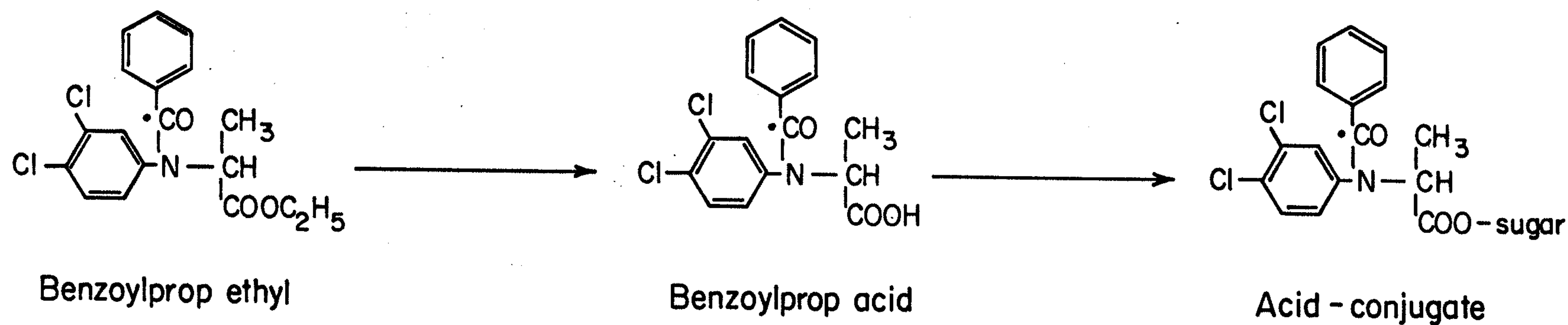


Figure 1. Metabolism of benzoylprop ethyl — Major pathway in plants. Dotted carbon (•C=O) indicates position of ^{14}C-label used in this work.

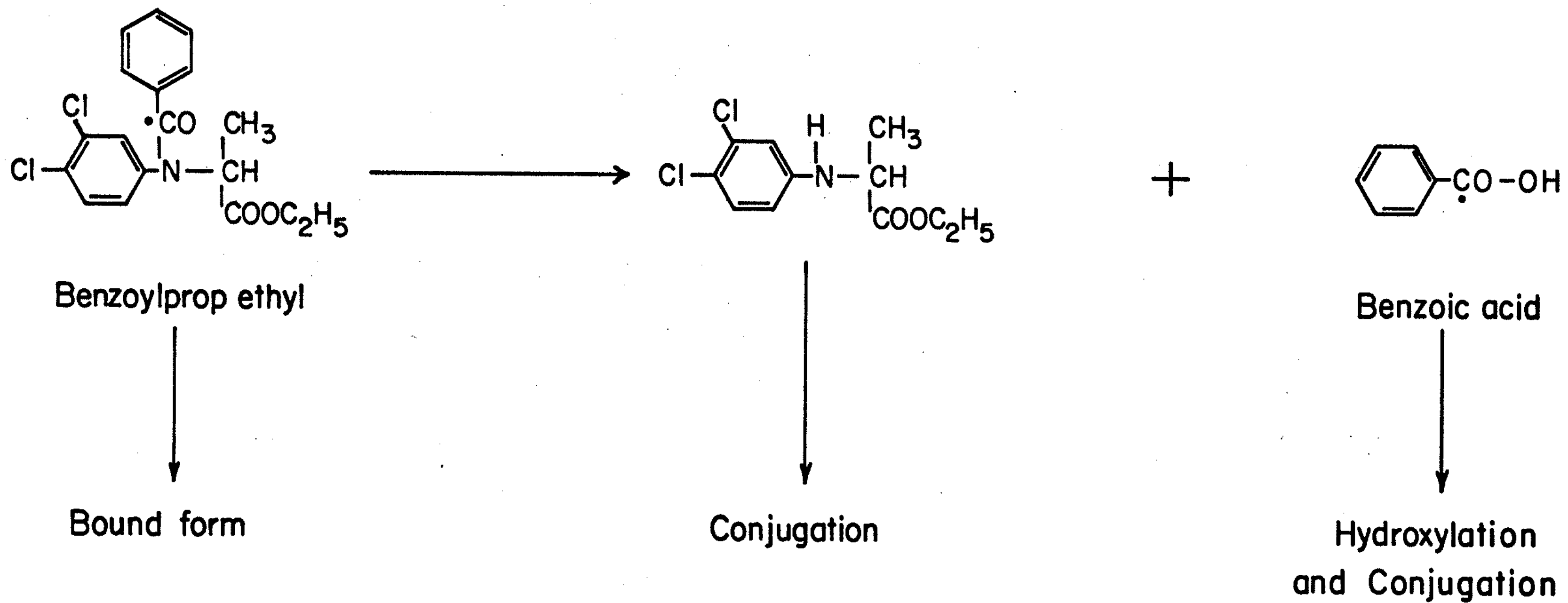


Figure 2. Metabolism of benzoylprop ethyl — Minor pathway in plants. Dotted carbon (•C=O) indicates position of ^{14}C-label used in this work.

dichloroaniline formation. It was noted that penetration of the ^{14}C-herbicide into the foliage was slow, and thus in spite of excessive amounts of ^{14}C-herbicide applied (50 ppm on total F.W. basis), the amount of metabolism over 15 days was limited (5-10% of applied ^{14}C-herbicide).

Beynon *et al.* (1974b) investigated the long-term metabolism of benzoylprop ethyl in spring wheat and winter wheat growing both indoors and outdoors. Plants were examined at harvest 71-98 days after spray application of ^{14}C-herbicide (0.6 - 1.2 kg/ha). The degradation pathways for benzoylprop ethyl were similar to those (Figs. 1-2) reported for short-term indoor studies (Beynon *et al.*, 1974a). The main degradation products were benzoylprop acid and at least two types of benzoylprop acid-sugar conjugates. Benzoic acid (5.5% of total radioactivity) and a previously unreported metabolite, N-benzoyl-3,4-dichloroaniline (< 1% of total radioactivity), were detected in indoor studies only. There was very little bound form of original herbicide and no dichloroaniline detected.

Mode of Action of Benzoylprop Ethyl

Jeffcoat and Sampson (1973), and Jeffcoat and Harries (1973) found that the herbicidal activity of benzoylprop ethyl was dependent upon its conversion to the biologically active desethyl acid. Phytotoxicity appeared to be related to levels of free benzoylprop acid produced by the de-esterification of benzoylprop ethyl. Benzoylprop acid levels were also regulated by detoxification processes, such as conjugation of the benzoylprop acid to sugars. Translocation studies showed that benzoylprop ethyl moved predominately acropetally in treated leaves, however, benzoylprop acid showed greater basipetal movement. It was concluded that benzoylprop

ethyl applied as a foliar spray is first converted in the leaves to benzoylprop acid, which is then translocated via the phloem to the stem, where it inhibits stem elongation through an effect on cell expansion. The resulting stunted wild oat plants were unable to compete successfully with the wheat crop.

Microscopic examination of benzoylprop ethyl-treated tissue has revealed an inhibition of cell elongation in both stem and leaves (Jeffcoat and Harries, 1973); decreased size and organization of vascular bundles in stems (Holm, 1972); and reduced cell length, width, and volume in stem internodes (Lowen, 1975).

Holm (1972) concluded that benzoylprop ethyl does not act as a mitotic poison. Lowen (1975) found some stimulation of cell division in oat shoots treated with benzoylprop ethyl.

Holm (1972), and Jeffcoat and Harries (1973) have described typical injury symptoms for oat plants treated with benzolyprop ethyl. Treated plants are stunted, often tiller more than untreated plants, and have dark green leaves with areas of chlorosis and necrosis extending from the tips. Leaves which develop after benzoylprop ethyl treatment may be deformed and often emerge through the side of leaf sheaths already present.

<u>Basis of Selectivity of Benzoylprop Ethyl</u>

Benzoylprop ethyl applied at the wild oat 4-6 leaf stage, at the rate of 1.12 to 1.40 kg/ha, will selectively control the growth of wild oats in wheat (Shell Canada Ltd., 1977). Bowden *et al.* (1970) found that while 4 kg/ha sometimes produces a 10 - 15% reduction in height, doses up to 8 kg/ha did not affect wheat yields.

Jeffcoat and Sampson (1973), and Jeffcoat and Harries (1973) found that selectivity was dependent upon the relative rates of benzoylprop ethyl de-esterification and benzoylprop acid conjugation in plant species. In oat, de-esterification (activation) was faster than conjugation (detoxification) resulting in accumulations of phytotoxic benzoylprop acid. In wheat, de-esterification was so slow that even a moderate rate of conjugation prevented the occurrence of phytotoxic levels of benzoylprop acid. It was suggested that de-esterification rates were higher in oat than in wheat due to inherently higher levels of esterase activity (Jeffcoat and Harries, 1973).

Effect of Crop Competition and Light and Nutrient Levels on Benzoylprop Ethyl Metabolism and Efficacy

In the field, crop competition enhances the efficacy of benzoylprop ethyl on wild oat (Loubaresse *et al.*, 1971). Growth inhibition of wild oat is of limited duration in the absence of crop competition (Chapman *et al.*, 1969). Improved wild oat control at late application times and at higher soil fertility levels (Stobbe *et al.*, 1971; Holm, 1972; Lowen, 1975; Sharma *et al.*, 1977) may be attributed to increased crop competition. The recommended application time for benzoylprop ethyl is at the wild oat 3-5 leaf stage (Manitoba Department of Agriculture, 1977). This application time represents a compromise between early (3-4 leaf stage) removal of wild oats for maximum yield benefits, and later (5-6 leaf stage) application for maximum wild oat control.

Greenhouse and growth chamber experiments have been conducted to study the effect of light and nutrient levels on benzoylprop ethyl efficacy. Holm (1972) reported increased wild oat injury at a reduced

light intensity. Dry weights of treated plants were 20 - 53% of control at 1050 ft-c, compared to 74 - 116% of control at 2000 ft-c. Similar trends were observed in leaf length measurements. Jeffcoat and Sampson (1973) found that the effectiveness of benzoylprop ethyl increased when oat plants were shaded. Twelve days after spraying, the height of shaded plants was 42% of control compared to 64% of control for unshaded plants. Also, unshaded plants recovered to 94% of control height after 24 days, while shaded plants only recovered to 62% of control after 40 days. Jeffcoat and Sampson (1973) also conducted root competition studies where a single oat plant was surrounded by four wheat plants such that no shading of the oat plant occurred. Although initial height reductions were similar, oat plants with root competition recovered to only 50% of control height after 37 days; compared to 67% of control for plants without root competition. It was concluded that both light and root competition would be important factors in field applications of benzoylprop ethyl. It should be noted that the use of tame oats in this study may preclude any extrapolation of root competition results to a field situation involving wild oats.

There has been limited study of the effects of light and nutrient levels on benzoylprop ethyl metabolism in oats. Jeffcoat and Harries (1973) reported rates of benzoylprop ethyl de-esterification were decreased by 30% in the shoots of oat plants given additional nutrients. This was attributed to a possible reduction of available esterase enzyme in the faster growing plants. In contrast, Jeffcoat and Harries (1975) reporting on flamprop-isopropyl {isopropyl-2-[N-(3-chloro-4-fluorophenyl) benzamido] propionate}, an analogue of benzoylprop ethyl,

suggested that the increased susceptibility of the oat under a reduced nutrient level was probably due to a reduced growth rate. Nutrient availability did not markedly influence ^{14}C-flamprop-isopropyl metabolism.

Plant Esterases

It has been suggested that the de-esterification of benzoylprop ethyl in oats and wheat is enzymic, and thus depends on levels of esterase or more specifically carboxylic ester hydrolase (Jeffcoat and Harries, 1973). While animal esterases have been studied extensively, considerably less work has been done on esterases of higher plants.

Plants such as cucumber, soybean, wheat, and corn contain complex systems of ester hydrolases. Jooste and Moreland (1963), Macko *et al.* (1967) and Sae *et al.* (1971) have found complex esterase isoenzyme patterns in wheat and sorghum grain. Schwartz *et al.* (1964) concluded that carboxylic ester hydrolases may vary in different plant species, in different strains of the same species, and even in different parts of the same plant. Schwartz *et al.* (1964) also speculated that some of the carboxylic ester hydrolase activity of plant tissue may be due to proteases or peptidases which can hydrolyze a number of esters in addition to peptides.

Sae *et al.* (1971) reported the extraction, partial purification, and some properties of sorghum grain esterase. Enzyme assay was based on hydrolysis of indophenyl acetate substrate. Sodium acetate buffer, pH 5.5, 0.01M, was the best extraction solvent. Purification consisted of $(NH_4)_2SO_4$ fractionation, dialysis, and Sephadex G-100 gel-filtration. The purified sorghum grain esterase was relatively stable and had a molecular weight of 60,000. The optimum reaction pH was 7.0. Electro-

phoresis revealed two esterase isoenzymes which reacted differently to organophosphate inhibition.

Mendoza *et al.* (1969) extracted esterases from germinating pea and bean seeds using tris-maleate buffer, pH 6.2, 0.01M. Esterases were partially purified by centrifugation and dialysis. Enzyme assay was based on hydrolysis of p-nitrophenyl propionate or p-nitrophenyl butyrate substrate. It was noted that pH 7.0 was optimum for pea esterase activity. Magnesium ions increased pea esterase activity, while cobalt, manganese, and fluoride ions were inhibitors. The organic pesticides malathion, methyl parathion, carbaryl, and metepa also inhibited esterase activity.

In Vitro Enzymic De-esterification of Herbicides

There are few reports concerning the *in vitro* action of plant esterase on ester-containing herbicides. Morré and Rogers (1960) studied the fate of long chain esters of 2,4-D in plants. They reported that crude protein preparations from cucumber cotyledons and spinach acetone powder catalyzed the hydrolysis of an octyl ester of 2,4-D. Esterase was extracted with 0.1M sodium chloride and was concentrated by centrifugation and $(NH_4)_2SO_4$ precipitation. Enzyme activity was detected by a cucumber root inhibition bioassay for liberated 2,4-D acid. A reaction mixture of 2.26 x 10^{-6}M substrate and 1 mg protein preparation per ml of sodium phosphate buffer, pH 7.0, 0.01M, was incubated for 12 h at 24°C. The resultant rate of hydrolysis, 0.2 x 10^{-6} Moles/1/h, was considered to be extremely low activity, but would account for complete hydrolysis of octyl 2,4-D in intact plants. Esterase activity was linear with respect to 0-12 h reaction times and 0-1.0 mg/ml protein concentrations.

The rate of hydrolysis was proportional to substrate concentration. An apparent Km of 2.8 x 10^{-6}M was reported for the p-nitrophenyl ester of 2,4-D hydrolyzed by cucumber acetone powder preparation. Esterase activity for octyl 2,4-D was also found in preparations from cucumber roots, pumpkin seeds, and corn coleoptile, roots, and embryo. Copper sulfate, at 2 x 10^{-4}M, inhibited esterase activity.

Szabo (1963) reported *in vitro* hydrolysis of butoxyethanol and propylene glycol butylether esters of 2,4-D by bean homogenates. Crude homogenates were prepared in 0.05M phosphate buffer. After 36-h incubation of substrates and homogenate, 2,4-D acid was qualitatively detected.

Jooste and Moreland (1963) cautioned that peptide peptidohydrolases such as trypsin, chymotrypsin, and papain were known to hydrolyze certain carboxyl esters. The possibility that plant proteolytic enzymes may de-esterify benzoylprop ethyl is enhanced by a report that esters of N-substituted aromatic amino acids, similar in structure to benzoylprop ethyl, are good substrates for α-chymotrypsin and trypsin (Schwert *et al.*, 1948).

SECTION 1

EFFECT OF LIGHT AND NUTRIENT LEVELS ON ^{14}C-BENZOYLPROP ETHYL METABOLISM AND GROWTH INHIBITION IN WILD OAT (*Avena fatua* L.)

Summary

Wild oat (*Avena fatua* L.) plants were dosed hydroponically with ^{14}C-benzoylprop ethyl {ethyl-2-[N-(3,4-dichlorophenyl) benzamido] propionate} and were placed under different light and nutrient levels. Herbicide metabolism and growth inhibition in plant shoots were measured after 3, 6, and 12 days. Plants under low light contained more phytotoxic benzoylprop acid due to increased de-esterification at 3 and 6 days, and decreased acid-conjugate formation at 12 days. Under low nutrients, both metabolic reactions were slowed resulting in increased acid at 3 days, but not at 6 or 12 days. Increased growth inhibition after 12 days under high light was attributed to higher shoot ^{14}C-uptake. After 12 days, there was more growth inhibition at the low nutrient level. Growth correlated better to the amount (dpm) of acid-conjugate (detoxified acid) than to the amount of ester or acid in plant shoots. Acid concentration had the most influence on subsequent growth. It was concluded that although reduced light and nutrients result in higher levels of phytotoxic acid, increased benzoylprop ethyl efficacy is also due to a reduced growth rate of the wild oat.

Introduction

Benzoylprop ethyl {ethyl-2-[N-(3,4-dichlorophenyl) benzamido] propionate} is used in Western Canada as a post-emergence herbicide for the selective control of wild oat (*Avena fatua* L.) in wheat (*Triticum aestivum* L.). The basis of selectivity, mode of action, and metabolism of benzoylprop ethyl have been studied (Jeffcoat & Sampson, 1973; Jeffcoat & Harries, 1973; Beynon, Roberts & Wright, 1974). The herbicidal activity of benzoylprop ethyl is dependent upon its conversion to the biologically active desethyl acid. Acid levels are also regulated by detoxification processes, such as conjugation to sugars.

In the field, crop competition enhances the efficacy of benzoylprop ethyl on wild oat (Loubaresse, Moncorgè & Rosher, 1971). The recommended application time is at the wild oat 3 to 5-leaf stage (Manitoba Department of Agriculture, 1977), when the crop is in the "shooting stage" and best able to compete. Oat plants are more susceptible under the reduced light of a crop canopy and the lower nutrient availability with root competition (Jeffcoat & Sampson, 1973). Since activation of benzoylprop ethyl is required, it is of interest to determine whether light and nutrient levels are affecting benzoylprop ethyl metabolism.

Jeffcoat & Harries (1973) found decreased rates of benzoylprop ethyl de-esterification in the shoots of oat plants given additional nutrients. This was attributed to a possible reduction of available esterase enzyme in the faster growing plants. On the contrary, Jeffcoat & Harries (1975) reporting on flamprop-isopropyl {isopropyl-2-[N-(3-chloro-4-fluorophenyl) benzamido]propionate}, an analogue of benzoylprop ethyl, suggested that the increased susceptibility of the oat under a reduced nutrient level

was probably due to a reduced growth rate. Nutrient availability did not markedly influence ^{14}C-flamprop-isopropyl metabolism.

The objectives of this study were to determine: 1) whether reduced light and nutrient levels, such as might be expected from crop competition, result in higher levels of phytotoxic benzoylprop acid; 2) whether the increased herbicidal activity of benzoylprop ethyl at reduced light and nutrient levels is due to changes in metabolism, or the reduced growth rate of the wild oat, or both.

Materials and Methods

Wild oat plants were grown in an Econaire model GR 36 environmental chamber (Econaire Systems Ltd., Winnipeg, Man.) under Sylvania fluorescent lamps (Grolux-VHO-WS). A light intensity of 17.2 Klux, at a plane 30 cm above floor level, was maintained over a 16-h photoperiod. Temperatures were 25°C during the day, and 15°C at night, with a relative humidity of 60%.

Plant culture techniques

Wild oat seeds were germinated at 18.5°C for 3 days. Seeds with a uniform short radicle were chosen for sand culture. The germinated seeds were grown in 20/40-mesh silica sand in 210-ml plastic cups (1 plant/cup). Sand cultures were watered to excess with free drainage. A modified Hoagland's nutrient solution (Hoagland & Arnon, 1950) was used at half strength until the 2-leaf stage, then changed to full strength. When plants were in the late 3-leaf stage, 10 days after emergence, the most uniform were transplanted into 240-ml bottles covered with aluminum foil. They were then grown hydroponically in 180 ml of half-strength nutrient solution until ^{14}C-herbicide dosing.

^{14}C-herbicide dosing

Just prior to dosing, plants were grown for 24 h in a 10^{-4}M $CaSO_4$ solution. At the 4-leaf stage, 14 days after emergence, plants were dosed hydroponically with 0.1 µCi (220,000 dpm) of benzoyl-labelled ^{14}C-benzoylprop ethyl (specific activity 11.4 µCi/mg). A 6-h treatment using a 0.5 x 10^{-5}M ^{14}C-herbicide dose solution resulted in 75% initial uptake, with a final average shoot dose of 38,400 dpm. After dosing, plants were transferred directly to the appropriate nutrient solution without an unlabelled herbicide chase.

Experimental design

After ^{14}C-herbicide dosing, wild oat plants were subjected to varying light intensity and nutrient levels using the same environmental chamber. Light intensity was varied by changing the distance between plants and the light source. Non-herbicide-treated plants were included as growth references. The light intensity levels used were designated as high, 17.2 Klux at 25°C, and low, 10.3 Klux at 23°C. The nutrient levels were designated as high, half-strength, and low, quarter-strength Hoagland's solution. Nutrient solutions were changed every 3 days and solution strengths kept relatively constant with the addition of distilled water when necessary. Plants were sampled 3, 6, and 12 days after ^{14}C-herbicide dosing for metabolism and growth determinations. The experiment was a completely randomized design with treatments composed in a factorial manner. Each treatment was replicated 6 times. Results were analyzed statistically, and unless otherwise noted, the P = 0.10 significance level was used.

Benzoylprop ethyl metabolism

Sampled plants were stored at -20°C until analysis. The frozen shoots were extracted for 1.5 min with 20 ml of acetone using an Omnimixer (Ivan Sorvall Inc., Newton, Conn.). The acetone extract was filtered and the residue re-extracted twice. Extracts were combined and reduced to 1.5 ml using a vacuum rotary evaporator at 40-45°C, then a stream of air. A 250-μl aliquot was taken for total shoot ^{14}C-uptake determination. The remaining extract was reduced to 0.5 ml with air, and filtered prior to determining the relative amounts of ^{14}C-benzoylprop ethyl and metabolites.

The aliquot for total shoot ^{14}C-uptake determination was dark green and required color bleaching before liquid scintillation counting. Exposure to sunlight for 3-4 days turned the extract yellow. The extract was then made virtually colorless with the addition of 1.5 ml of fresh hypochlorous acid (Shneour, Aronoff & Kirk, 1962). The bleached extract was counted in 15 ml of Aquasol (New England Nuclear, Boston, Mass.) using the internal standard method for efficiency determination.

To determine the relative amounts of ^{14}C-benzoylprop ethyl and metabolites, 40 ul of extract was applied to Baker-flex silica gel 1B2-F (J. T. Baker Chemical Co., Phillipsburg, N. J.) thin-layer plates. The plates were developed using 3% acetic acid, 6% acetone in chloroform such that rf-values were: benzoylprop ethyl 0.58, benzoic acid 0.43, benzoylprop acid 0.32, and conjugates (origin). Appropriate portions of the plates were cut directly into scintillation vials and 1 ml of acetone/water (1:1) was added to aid in ^{14}C-desorption. Color bleaching was unnecessary and samples were quantified by liquid scintillation

counting as before.

Growth response

Wild oat growth response to treatments was monitored by measuring the net increase in length (mm) for leaves on the main culm. The last leaf length was not included unless it was judged sizeable in proportion to the second last leaf.

Results and Discussion

Wild oat plants were dosed hydroponically because Jeffcoat & Sampson (1973) and Jeffcoat & Harries (1973) reported that ^{14}C-benzoylprop ethyl uptake and distribution was poor with foliar spot applications. They found that growth response to root and foliar application was similar. The ^{14}C-herbicide dosing rate and procedure used in this work were chosen such that there was some regrowth of plants from benzoylprop ethyl inhibition. Injury symptoms and the amount of growth inhibition were the same as described by Jeffcoat & Harries (1973).

Metabolism of ^{14}C-benzoylprop ethyl

The major breakdown pathway, as described by Beynon, Roberts & Wright (1974), consists of benzoylprop ethyl conversion to the desethyl acid, then conjugation of the acid to sugars. Fig. 3 represents a quantification of this pathway with curves constructed using overall means for 3, 6, and 12 days. Results are expressed as % distribution of ^{14}C-radioactivity between metabolites. Extrapolation of the curves to include 0-3 days was facilitated by assigning functions to ester decline ($y = b/T + a$) and conjugate formation ($y = a - be^{-T}$). When tested statistically, both functions showed a significantly ($P = 0.05$)

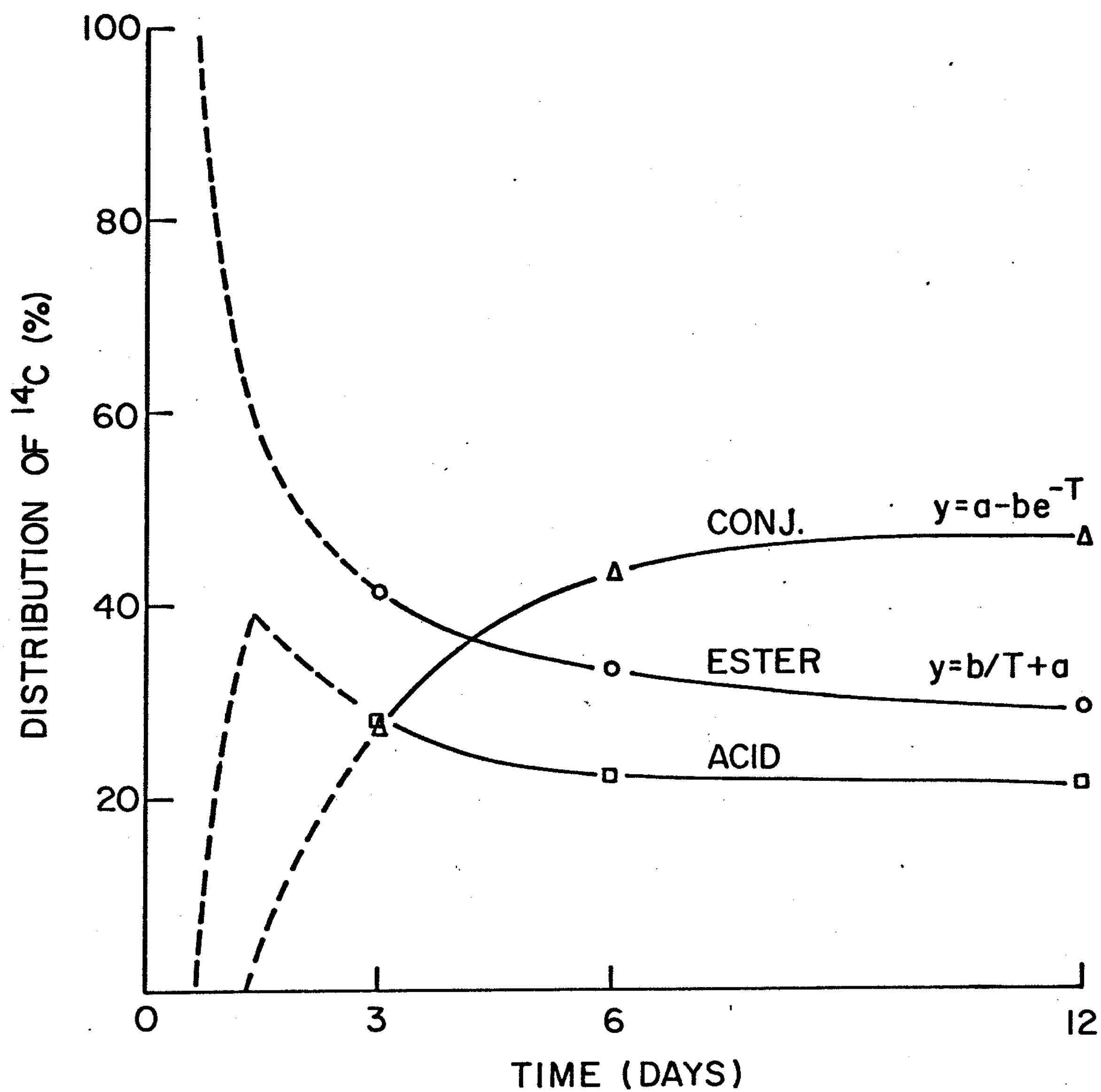


Figure 3. Metabolism of ^{14}C-benzoylprop ethyl in wild oat shoots. Each value is a mean of 24. Curves are extrapolated for 0-3 days.

good fit to the data. The acid curve was extrapolated by determining the remaining % radioactivity. The extrapolations made in this work are supported by curves of similar shape plotted by Jeffcoat & Harries (1973) using 0 to 6-day data. It can be seen that maximum acid levels likely occurred before 3 days. This acid maximum would influence subsequent growth response.

Beynon, Roberts & Wright (1974) proposed a minor pathway in benzoylprop ethyl metabolism where breakage of the amide bond would yield benzoic acid. Plant extracts were examined for benzoic acid, however, levels were variable and low (1-3% of total radioactivity).

Light and nutrient effects on metabolism

Light and nutrients were varied simultaneously, however, results are presented separately to show individual effects. The influence of light intensity on benzoylprop ethyl metabolism is shown in Fig. 4. The rate of de-esterification was faster at low light. Initial conjugate formation was similar at both light levels, then slowed under low light. The net result was higher acid levels in plants under reduced light intensity. The increase in acid was significant at all three sampling dates. Differences in acid levels may have been reduced by the increased shoot ^{14}C-uptake in plants under high light (TABLE 1). Higher initial shoot ^{14}C-uptake tended to increase rates of de-esterification and conjugation. Despite this apparent substrate effect, de-esterification was significantly slower at the high light level.

Nutrient availability did influence benzoylprop ethyl metabolism (Fig. 5). Both de-esterification and conjugate formation were faster at the high nutrient level. At 3 days, there was a significant increase

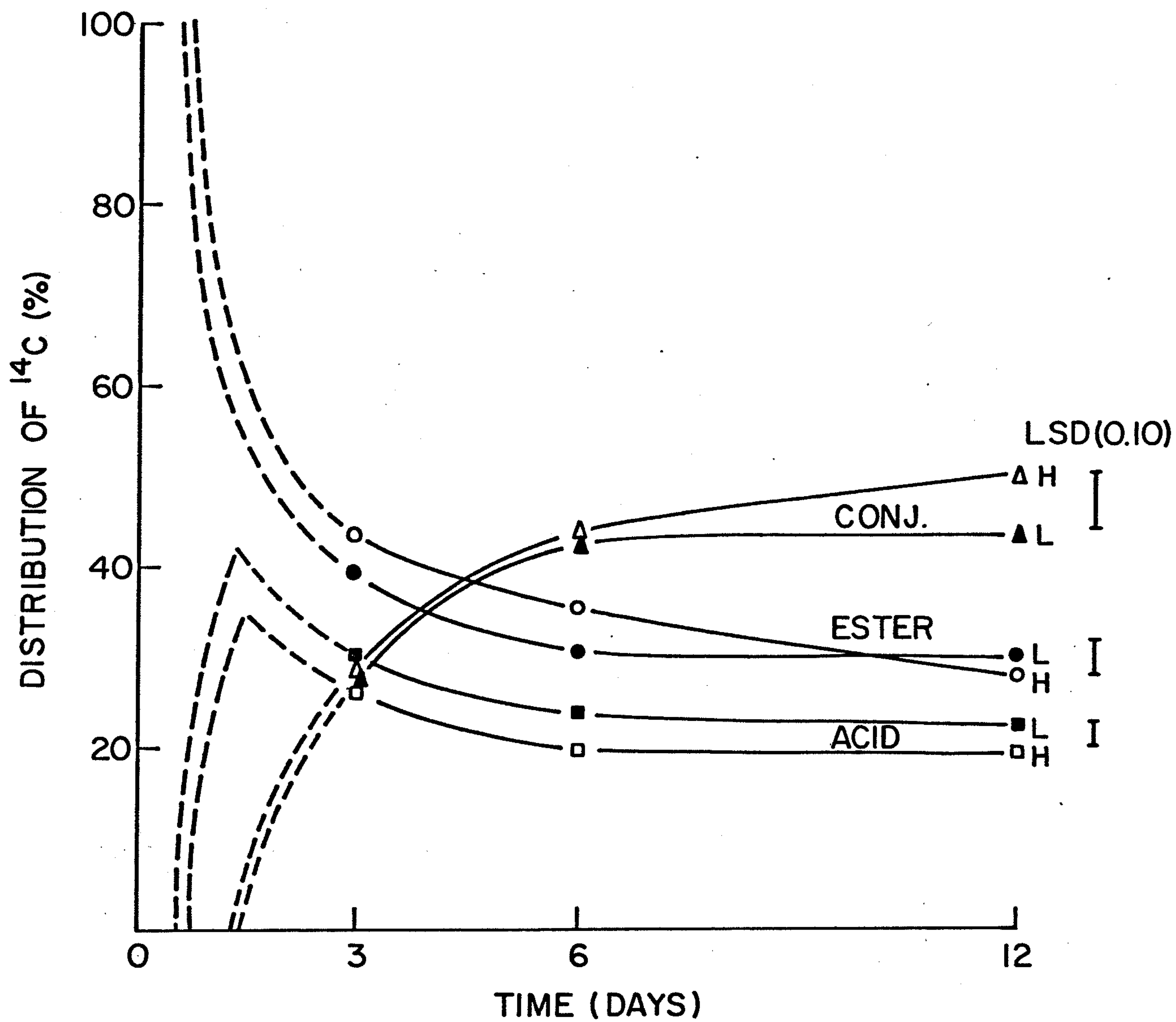


Figure 4. Effect of high (H) and low (L) light levels on the metabolism of ^{14}C-benzoylprop ethyl in wild oat shoots. Each value is a mean of 12. The 0-3 day segments are theoretical.

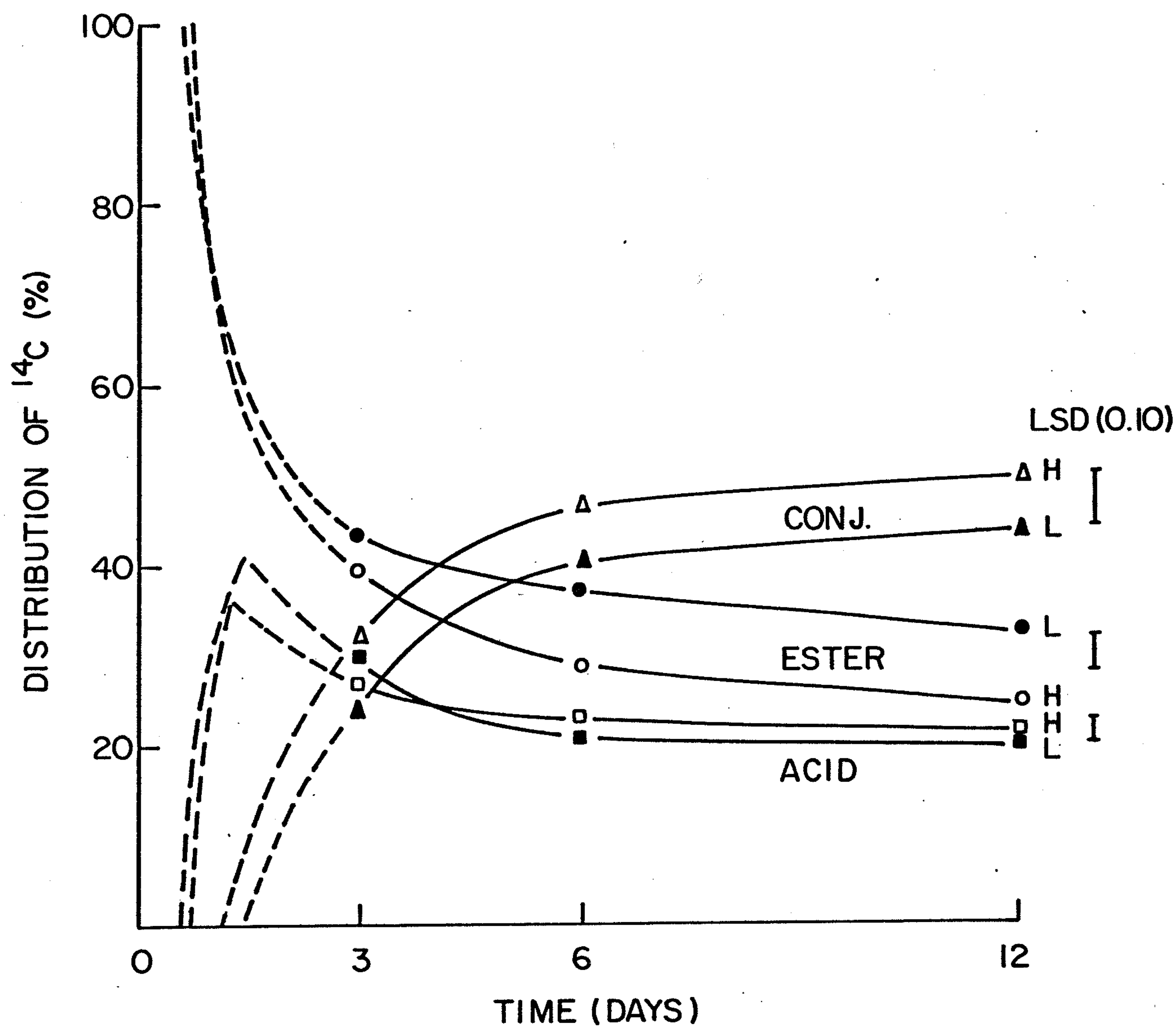


Figure 5. Effect of high (H) and low (L) nutrient levels on the metabolism of ^{14}C-benzoylprop ethyl in wild oat shoots. Each value is a mean of 12. The 0-3 day segments are theoretical.

TABLE 1. Effect of light and nutrient levels on total amount of ^{14}C-radioactivity found in wild oat shoots

	Total shoot ^{14}C-uptake x 10^{-2} (dpm), days after hydroponic dosing[a]		
Treatment	3	6	12
High light	461	393	343
Low light	307	279	305
High nutrients	412	319	301
Low nutrients	356	353	347

[a]Each value is a mean of 12. Least significant difference between light or nutrient treatment means at the same date is 63 at P = 0.10.

in acid at low nutrients. At 6 and 12 days, there was no significant difference between acid levels at high or low nutrients.

There was a significant light x nutrient interaction at 3 days indicating that increased acid levels at low light (Fig. 4) and low nutrients (Fig. 5) were related to the simultaneous application of low nutrients, and low light, respectively.

The increase in phytotoxic acid levels under reduced light and nutrients would help to account for the increase in benzoylprop ethyl herbicidal activity when there is crop competition. Crop competition implies simultaneous reductions in light and nutrients, and at 3 days, the highest acid levels were found under the poorest environment. After 3 days, light intensity appeared to affect acid levels more then nutrient availability. Reduced light intensity sustaining higher

acid levels over 12 days is consistent with the effect of crop competition prolonging herbicide injury.

Growth response

Based on the ^{14}C-herbicide metabolism data (Figs. 3-5), one might expect more benzoylprop ethyl growth inhibition at the low light and nutrient levels. By separating environmental effects, it can be seen that after 12 days herbicide injury was greater at the high light level (TABLE 2).

TABLE 2. Effect of ^{14}C-benzoylprop ethyl on the elongation growth of wild oat at two levels of light and nutrients

Treatment	Net leaf length increase, days after hydroponic dosing[a]		
	3	6	12
High light	57	60	52
Low light	62	62	65
High nutrients	57	63	62
Low nutrients	62	58	54

[a]Expressed as percentage of control plants. Each value is a mean of 12. Least significant difference between light or nutrient treatment means at the same date is 7 at $P = 0.10$.

This may be explained in that initial ^{14}C-herbicide shoot uptake was greater in plants under high light intensity (TABLE 1). Although ^{14}C-herbicide dosing was identical, plants under high light apparently mobilized more ^{14}C-herbicide into the shoots. Growth response to this increased translocation did not become evident until 12 days after ^{14}C-herbicide dosing.

After 12 days, there was slightly more benzoylprop ethyl growth inhibition at the low nutrient level (TABLE 2). Nutrient treatment did not cause a significant difference in the initial ^{14}C-herbicide shoot uptake (TABLE 1).

Multiple linear regression analysis was used to correlate growth response to actual amounts (dpm) of ^{14}C-herbicide and metabolites in the wild oat shoots (TABLE 3). At all sampling dates, growth correlated better to the amount of conjugate formed than to ester or acid levels. This may be explained in that a reduced growth rate due to higher phytotoxic acid levels would require time to become evident, while in that time, the acid originally causing the effect is conjugated. Growth also correlated to total shoot ^{14}C-uptake and initial ^{14}C-herbicide shoot concentrations. These variables were the most important at 3 days. At 6 and 12 days, growth correlated best to the concentration of conjugate in plant shoots. Since conjugate levels represent detoxified acid, it appears that acid concentrations rather than acid levels *per se*, are most important. Acid concentrations will also depend on plant growth rate and plant size.

The relative importance of increased acid levels versus the reduced growth rate of the wild oat can be assessed using the data in TABLE 4. After 3 days under high light, the increased amount of acid, 3050 dpm, did eventually cause a significant growth reduction at 12 days (TABLE 2). Theoretically, had total shoot ^{14}C-uptake been the same, there would have been an increase in acid of 1300 dpm at the low light level. This increase (1300 dpm), in itself, may not cause a significant further growth reduction. A simultaneous reduction in plant size due to low

TABLE 3. Multiple linear regression (MLR) of elongation growth on amounts of ^{14}C-benzoylprop ethyl and metabolites in wild oat shoots[a]

Sampling day	Independent variables[b]	Individual correlations to growth	Partial F-ratio	Significance of variable to MLR	Multiple linear regression
3	Total shoot uptake	-0.36	6.54	0.95	r^2 = 0.66
	Initial shoot conc.[c]	-0.48	14.6	0.99	r = 0.81
	Ester	-0.31	0.56	NS	F-ratio = 5.41
	Acid	-0.09	0.02	NS	Regression is
	Conjugate	-0.44	4.74	0.95	significant at
	Conjugate conc.[d]	-0.54	5.99	0.95	P = 0.99
6	Total shoot uptake	-0.23	3.57	0.90	r^2 = 0.74
	Initial shoot conc.[c]	-0.25	1.32	NS	r = 0.86
	Ester	-0.06	0.08	NS	F-ratio = 8.27
	Acid	0.15	0.04	NS	Regression is
	Conjugate	-0.41	12.6	0.99	significant at
	Conjugate conc.[d]	-0.49	32.0	0.99	P = 0.99
12	Total shoot uptake	-0.43	22.7	0.99	r^2 = 0.86
	Initial shoot conc.[c]	-0.52	24.0	0.99	r = 0.93
	Ester	-0.14	2.00	NS	F-ratio = 17.8
	Acid	0.19	0.01	NS	Regression is
	Conjugate	-0.58	15.0	0.99	Significant at
	Conjugate conc.[d]	-0.72	42.9	0.99	P = 0.99

[a]Elongation growth = net leaf length increase, expressed as percentage of control.

[b]Independent variable units, dpm.

[c]Concentration = dpm/initial 3+4 leaf lengths (mm).

[d]Concentration = dpm/final 3-6 leaf lengths (mm).

light intensity would help increase phytotoxic acid concentrations. At 3 days, there was little difference (304 dpm) in actual acid levels due to nutrient treatment. The significant growth reduction after 12 days at the low nutrient level (TABLE 2) was likely caused by a reduced growth rate effectively increasing acid concentration.

TABLE 4. Calculation of actual and theoretical phytotoxic acid levels in wild oat shoots at 3 days

Treatment	% Acid[a]	Total shoot ^{14}C-uptake (dpm)[b]	Amount of acid (dpm)	Difference in amount of acid (dpm)	
High light	26.4	46,100	12,200	3050	Actual
Low light	29.8	30,700	9,150		
High light	26.4	38,400[c]	10,100	1300	Theoretical
Low light	29.8	38,400[c]	11,400		
High nutrients	26.4	41,200	10,877	304	Actual
Low nutrients	29.7	35,600	10,573		
High nutrients	26.4	38,400[c]	10,138	1267	Theoretical
Low nutrients	29.7	38,400[c]	11,405		

[a]As per Figures 2 and 3.

[b]As per TABLE 1.

[c]Theoretical mean, assuming equal uptake.

In conclusion, reduced light and nutrient levels do result in higher levels of phytotoxic benzoylprop acid in wild oat shoots. Although these increased acid levels will further inhibit growth, it is suggested that increased benzoylprop ethyl efficacy under reduced light and nutrients is also due to a reduced growth rate of the wild oat. In relation to the field, this study supports the observation that crop competition increases benzoylprop ethyl efficacy.

SECTION 2

HYDROLYSIS OF THE HERBICIDE BENZOYLPROP ETHYL BY WILD OAT ESTERASE

Summary

A carboxylesterase from wild oat (*Avena fatua* L.), which hydrolyzes benzoylprop ethyl {ethyl-2-[N-(3,4,-dichlorophenyl)benzamido]propionate} to the herbicidally active benzoylprop acid, was partially purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-100 gel filtration. A 8.3 fold purification was obtained with 46% recovery. The rate of de-esterification of ^{14}C-benzoylprop ethyl was 0.1 ηmoles/16 h at standard assay conditions of 1 mg lyophilized enzyme preparation in 0.1 ml phosphate buffer (0.1M, pH 7.0), substrate 5 μM. The wild oat esterase was stable and activity as a function of reaction time, enzyme and substrate concentration was linear. Incubation with α-chymotrypsin reduced wild oat esterase activity as did the presence of ethanol in reaction mixtures. Esterase activity for ^{14}C-benzoylprop ethyl was not detected in preparations from wheat (*Triticum aestivum* L.). Evidence was obtained which suggested the presence of an inhibitor in wheat.

Introduction

Benzoylprop ethyl {ethyl-2-[N-(3,4-dichlorophenyl)benzamido]propionate} is used in post-emergence foliar applications as a selective herbicide for the control of wild oats (*Avena fatua* L.) in wheat (*Triticum aestivum* L.). The basis of selectivity, mode of action, and metabolism of benzoylprop ethyl have been reported (Jeffcoat & Sampson, 1973;

Jeffcoat & Harries, 1973; Beynon, Roberts & Wright, 1974a; 1974b; Hill & Stobbe, 1977). The herbicidal activity of benzoylprop ethyl is dependent upon its conversion to the biologically active desethyl acid. Rates of de-esterification are high in oat and relatively low in wheat. It has been suggested that the de-esterification of benzoylprop ethyl in these plants is enzymic, and thus depends on levels of esterase or more specifically, carboxylic ester hydrolase (Jeffcoat & Harries, 1973).

Plants such as cucumber, soybean, wheat, and corn contain complex systems of ester hydrolases (Jooste & Moreland, 1963). Studies on wheat and sorghum grain extracts have also revealed complex esterase isoenzyme patterns (Macko, Honold & Stahmann, 1967; Sae, Kadoum & Cunningham, 1971). Carboxylic ester hydrolases vary in different plant species, in different strains of the same species, and even in different parts of the same plant (Schwartz, Biedron, von Holdt & Rehm, 1964). Extraction and partial purification of esterases from pea and bean seeds and sorghum grain have been reported (Mendoza, Grant & McCully, 1969; Sae, Kadoum & Cunningham, 1971).

There is little information concerning the *in vitro* action of plant esterases on ester-containing herbicides. The *in vitro* hydrolysis of 2,4-D esters has been demonstrated using enzyme preparations from cucumber, spinach, and beans (Morré & Rogers, 1960; Szabo, 1963).

This report describes the extraction, partial purification, and some properties of an esterase which hydrolyzes benzoylprop ethyl to the herbicidally active benzoylprop acid. Extracts of susceptible (wild oat) and resistant (wheat) species were studied.

Materials and Methods

Plants were grown in the greenhouse in soil contained in wooden flats. The light intensity was approximately 14 Klux over a 16-h photoperiod. Temperatures ranged from 18-22°C. Plants were harvested at the 2-3 leaf stage.

Enzyme extraction and partial purification

Unless otherwise noted, all steps in the extraction and purification of the enzyme were carried out at 0-4°C. Five g of freshly harvested leaves were cut into small pieces and extracted with 20 ml of 0.1M phosphate buffer at pH 7.0 using a Polytron disintegrator with PT 20ST generator [Brinkmann Instruments (Canada) Ltd., Toronto, Ont.]. Good homogenization with a minimum of foaming was achieved by extracting at moderate speeds for 2 min. The homogenate was centrifuged twice at 10,000 x g for 5 min. The supernatant was then further centrifuged at 30,000 x g for 20 min. The resulting supernatant was called the crude supernatant.

A 15-ml aliquot of crude supernatant was fractioned by: adding 4°C-saturated $(NH_4)_2SO_4$ solution, allowing 1 h for equilibration, and collecting the protein precipitate by centrifugation at 10,000 x g for 10 min. The fraction precipitated between 30 and 60% $(NH_4)_2SO_4$ saturation was redissolved in 0.5 ml of phosphate buffer (0.1M, pH 7.0). This fraction was defined as the 0.3-0.6 $(NH_4)_2SO_4$ fraction and was centrifuged at 30,000 x g for 20 min just prior to gel permeation chromatography.

A 0.35-ml aliquot of the 0.3-0.6 $(NH_4)_2SO_4$ fraction was layered on top of a 1.65 x 26.0-cm Sephadex G-100 (Pharmacia Fine Chemicals,

Uppsala, Sweden) column which had been equilibrated with 0.1M phosphate buffer pH 7.0 at room temperature. The enzyme was eluted using the same buffer at a flow rate of 0.33 ml/min. The absorbance at 280 ηm was measured to estimate the distribution of protein in effluent fractions. Fractions assayed as containing the highest esterase activity were pooled, lyophilized and stored at-20°C.

Standard enzyme assay

Esterase activity was determined by the rate of ^{14}C-benzoylprop ethyl (benzoyl-labelled, specific activity 11.4 μCi/mg) conversion to the desethyl ^{14}C-benzoylprop acid. The reaction mixture consisted of 1 mg of lyophilized enzyme prepration, 0.1 ml of phosphate buffer (0.1M, pH 7.0), and substrate at 5 μM. The substrate, dissolved in ethanol, was introduced into the bottom of 3 x 0.5-cm glass reaction tubes and the ethanol was carefully evaporated using a stream of air. The reaction was started by the addition of enzyme dissolved in buffer, and the reaction mixture incubated at 25°C for 16 h. Controls were heated for 30 min in a boiling water bath and cooled before combining the enzyme solution and substrate. Reactions were terminated by adding 0.1 ml of acetone.

The amount of ^{14}C-benzoylprop acid formed was determined by applying about 75 μl of reaction mix to Baker-flex silica gel 1B2-F (J. T. Baker Chemical Co., Phillipsburg, N.J.) thin-layer plates. The plates were developed using 3% acetic acid, 6% acetone in chloroform such that rf-values were: ^{14}C-benzoylprop ethyl 0.58, ^{14}C-benzoylprop acid 0.32. Appropriate portions of the plates were cut directly into scintillation vials and 1 ml of acetone/water (1:1) was added to aid in ^{14}C-desorption.

Samples were quantified by liquid scintillation counting using 10 ml of Aquasol (New England Nuclear, Boston, Mass.). The relative amounts (dpm) of ester and acid were used to express esterase activity as % ^{14}C-benzoylprop acid formed from original ester substrate.

The protein from wild oat esterase fractions was estimated by the 280-ηm absorbance of 0.1N NaOH solutions compared to crystalline bovine serum albumin standards. Protein in the crude supernatant and 0.3-0.6 $(NH_4)_2SO_4$ fractions was precipitated with 5% TCA and washed twice with 80% acetone before spectrophotometric determination. The lyophilized Sephadex G-100 effluent was dissolved directly in 0.1N NaOH.

Results and Discussion

Purification of benzoylprop ethyl hydrolyzing esterase from wild oat. The results of a typical extraction and purification sequence are shown in TABLE 5. The presence of $(NH_4)_2SO_4$ reduced the activity of wild oat esterase, however, $(NH_4)_2SO_4$ fractionation was useful as a concentration step prior to gel permeation chromatography. No significant esterase activity was found in $(NH_4)_2SO_4$ fractions other than the 0.3-0.6 $(NH_4)_2SO_4$ fraction. An overall purification of 8.3 fold was obtained with 46% recovery. Recovery could have been increased by using larger aliquots. Esterase activity did not change between the 2 and 3-leaf stage of wild oat. Complete maceration of leaves with a minimum of foaming was important for obtaining maximum activity in the crude supernatant. These conditions were better met using the Polytron disintegrator than using a more conventional homogenizer. The use of 1% Triton X-100 as a detergrent, or Antifoam A (Dow Corning Corp., Midland, Mich.) silicone spray to eliminate foaming, reduced esterase activity. Squeezing

TABLE 5. Purification of wild oat esterase

Fraction	Volume (ml)	Protein (mg/ml)	Esterase activity (units[a]/ml)	Total units	Specific activity (units/mg protein)	Recovery[b] (%)	Purification (fold)
Crude supernatant	20	15	189	3780	12.6	100	-
0.3-0.6 $(NH_4)_2SO_4$	0.5	300	206	-	0.7[c]	-	-
Sephadex G-100 effluent (pooled fractions)	6	2.8	292	1752	104	46	8.3

[a]Units = ηmoles x 10^2 ^{14}C-benzoylprop acid formed/16 h, substrate 10 μM.

[b]Recovery shown includes the use of 15/20-ml and 0.35/0.50-ml aliquots between fractions.

[c]$(NH_4)_2SO_4$ inhibited esterase activity; used as a concentration step.

the extract through cheesecloth produced too much foaming and was replaced by short initial centrifugations. Possible metabolites other than ^{14}C-benzoylprop acid, namely ^{14}C-benzoic acid and ^{14}C-herbicide-sugar conjugates (Beynon, Roberts & Wright, 1974a) were not detected after enzymic reactions by the crude supernatant.

The elution of benzoylprop ethyl hydrolyzing esterase from the Sephadex G-100 column is shown in Fig. 6. The activity peak obtained may represent several non-specific esterases. The esterase elution behavior indicates an average molecular weight of 60,000 (Pharmacia Fine Chemicals, Uppsala, Sweden, 1971). A molecular weight of 60,000 has also been reported for sorghum grain esterase (Sae, Kadoum & Cunningham, 1971).

Loss of activity in heated controls and after the addition of acetone both suggest that the observed de-esterification of ^{14}C-benzoylprop ethyl was enzymic. An enzymic reaction was also indicated by the effect of α-chymotrypsin (Worthington Biochemical Corp., Freehold, N.J.) on wild oat esterase preparations. Incubations of wild oat esterase and α-chymotrypsin prior to the addition of substrate greatly reduced activity (TABLE 6). The possibility exists that part of the enzymic nature of wild oat preparations was due to proteolytic enzymes. Some of the carboxylic ester hydrolase activity of plant tissues may be due to proteases or peptidases which can hydrolyze a number of esters in addition to peptides (Schwartz, Biedron, von Holdt & Rehm, 1964). Peptide peptidohydrolases such as trypsin, chymotrypsin, and papain are known to hydrolyze certain carboxyl esters (Jooste & Moreland, 1963). It is significant that α-chymotrypsin itself did not

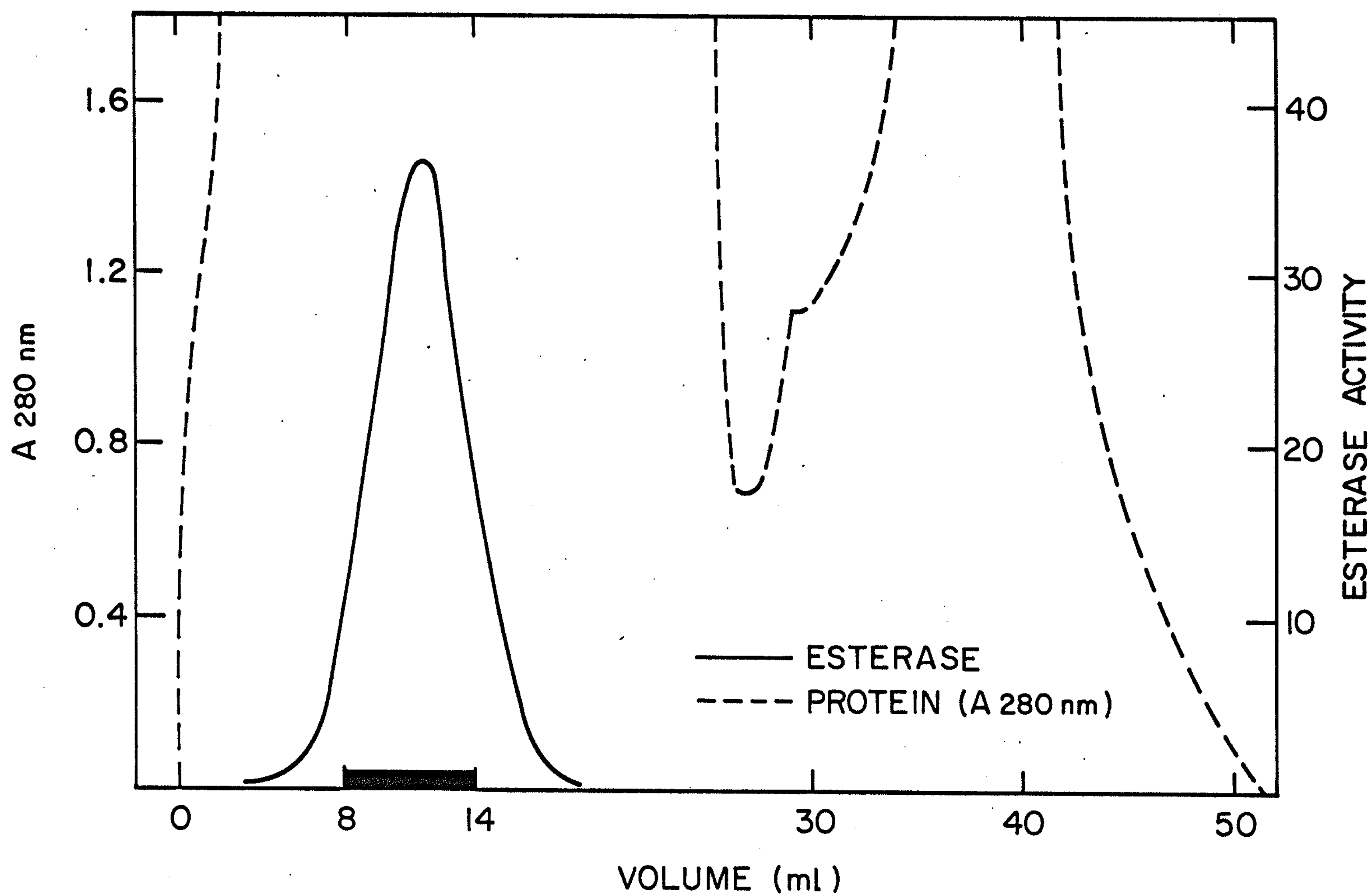


Figure 6. Elution of esterase from Sephadex G-100 column. Elution volume shown as measured from first protein detected. Overall Vo = 10.4, Ve = 22.0, Vt = 61.4 ml. Solid bar indicates pooled 2-ml fractions.

de-esterify ^{14}C-benzoylprop ethyl (TABLE 6), especially since esters of N-substituted aromatic amino acids, similar in structure to benzoylprop ethyl, have been reported to be good substrates for α-chymotrypsin (Schwert, Neurath, Kaufman & Snoke, 1948).

TABLE 6. Effect of α-chymotrypsin on wild oat esterase

Incubation time (h) at 25°C before adding substrate	Activity of wild oat esterase[a]		
	α-chymotrypsin plus esterase[b]	Esterase control	α-chymotrypsin control
0	19.8	30.3	0.1
2	18.2	30.3	
4	18.2	30.7	

[a]% ^{14}C-benzoylprop acid formed in 20 h.

[b]α-Chymotrypsin 0.06 mg, esterase 3 mg/0.1 ml phosphate buffer (0.1M, pH 7.0), substrate 10 μM.

Benzoylprop ethyl hydrolyzing esterase from wheat

Attempts to find esterase activity for benzoylprop ethyl in preparations from wheat (cv. Neepawa and Selkirk) were unsuccessful. Procedures used were similar to those described for wild oat enzyme preparations. The formation of ^{14}C-benzoylprop acid was not detected in reactions using crude supernatants, various $(NH_4)_2SO_4$ fractions, or Sephadex G-100 effluent fractions. Also, esterase activity for ^{14}C-benzoylprop ethyl was not present in preparations from wheat coleoptile. Similar preparations from wild oat coleoptile had shown 20% ^{14}C-benzoylprop acid formed.

The apparent lack of benzoylprop ethyl hydrolyzing esterase in wheat would certainly explain the herbicide's selectivity between wild oats and wheat; however, formation of benzoylprop acid following foliar applications of benzoylprop ethyl to wheat has been reported (Beynon, Roberts & Wright, 1974a; 1974b). It is possible that the substrate levels (5-20 μM) used in reactions of wheat preparations were too low. The solubility of benzoylprop ethyl was limited to approximately 10 μM in aqueous protein solutions. Evidence was obtained (see later) that the use of ethanol to increase substrate solubility would have inhibited the desired reaction.

An experiment was conducted which suggested there may have been an inhibitor of ^{14}C-benzoylprop ethyl de-esterification in wheat preparations. When various fractions of wheat preparations were added to wild oat esterase the expected activity was reduced (TABLE 7). Inhibition was greatest in the wheat 0.25-0.50 $(NH_4)_2SO_4$ fraction. An inhibitor, if present in this wheat fraction, apparently was not removed on Sephadex G-100, since column effluent lacked benzoylprop ethyl hydrolyzing activity. Wheat preparations did have some esterase activity since certain Sephadex G-100 effluent fractions readily hydrolyzed dichlofop methyl {methyl-2-[4-(2,4,-dichlorophenoxy)phenoxy]propanoate}. This suggests that wheat esterase may have either a differential specificity between the two ester-containing herbicides or an inhibitor specific for benzoylprop ethyl.

TABLE 7. Effect of wheat (cv. Neepawa) extracts on wild oat esterase activity

Wheat fraction added to wild oat esterase[a]	Wild oat esterase activity[b]
Control, phosphate buffer (0.1M, pH 7.0)	31.0
Crude supernatant	17.9
0-0.25 $(NH_4)_2SO_4$	17.7
0.25-0.50 $(NH_4)_2SO_4$	0.9
0.50-0.75 $(NH_4)_2SO_4$	3.5

[a]0.1 ml wheat fraction, 1.6 mg wild oat esterase, substrate 10 µM.

[b]% ^{14}C-benzoylprop acid formed in 16 h.

Some properties of wild oat esterase

Stability

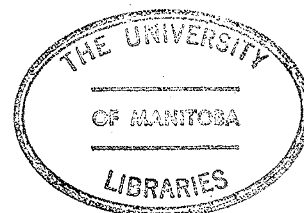
The partially purified esterase was quite stable. The Sephadex G-100 eluate could be lyophilized and stored at -20°C for at least 5 weeks with no loss of activity. The enzyme dissolved in buffer was stable for at least 20 h at 25°C, and for at least 48 h at 4°C. A slight increase in activity was observed when an enzyme solution was frozen and thawed. Activity in the crude supernatant was relatively heat stable in that 30% of the original activity remained after heating at 60°C for 15 min. Heating the enzyme in a boiling water bath for 30 min or adjusting enzyme solutions to 50% acetone ($^{v}/v$) resulted in complete inactivation.

Reaction parameters and kinetics

The effect of concentration of lyophilized enzyme preparation on esterase activity is shown in Fig. 7. The concentration of 1 mg/0.1 ml buffer was chosen for standard assays on the basis of esterase activity and linearity.

The formation of ^{14}C-benzoylprop acid was linear for at least 20 h at 25°C (Fig. 8). The deviation from linearity after 20 h may have been caused by declining substrate levels (initial substrate levels were limited by solubility), rather than by enzyme denaturation or reaction product inhibition. A standard assay reaction time of 16 h was chosen on the basis of esterase activity, linearity, and the convenience of leaving reactions overnight.

A double reciprocal Lineweaver-Burk (1934) plot of reaction velocity versus substrate (^{14}C-benzoylprop ethyl) concentration gave a straight line, $y = 2.36x + 6.15$, $r = 0.998$ (Fig. 9). The maximum reaction velocity, Vmax, was about 1.6 ηmoles/16 h. The "apparent" Km for ^{14}C-benzoylprop ethyl was 3.8 μM. The values for Vmax and Km will be approximate since the reaction itself caused significant reduction in substrate over 16 h. A Km of 2.8 μM has been reported for the enzymic de-esterification of the p-nitrophenyl ester of 2,4-D by cucumber acetone powder (Morré & Rogers, 1960). A substrate concentration for standard assay of 5 μM was chosen on the basis of reaction velocity and substrate solubility. As previously noted, the solubility of benzoylprop ethyl was determined to be approximately 10 μM in aqueous protein solutions. Attempts were made to increase substrate solubility by addition of small amounts of ethanol; however, inhibition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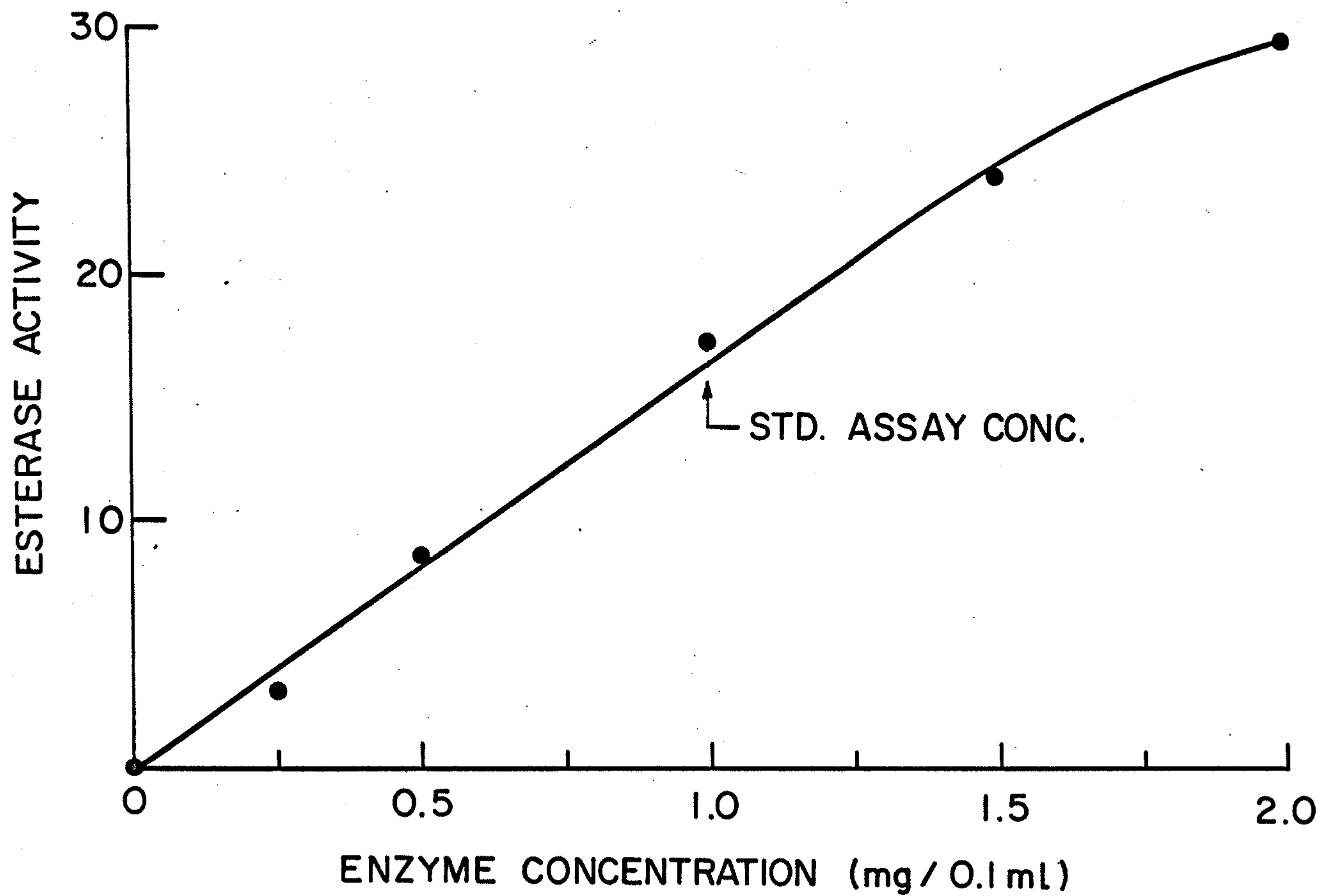

Figure 7. Esterase activity as a function of enzyme concentration (mg lyophilized preparation/0.1 ml buffer). Reaction time 16 h, substrate 5 μM, esterase activity expressed as % ^{14}C-benzoylprop acid formed.

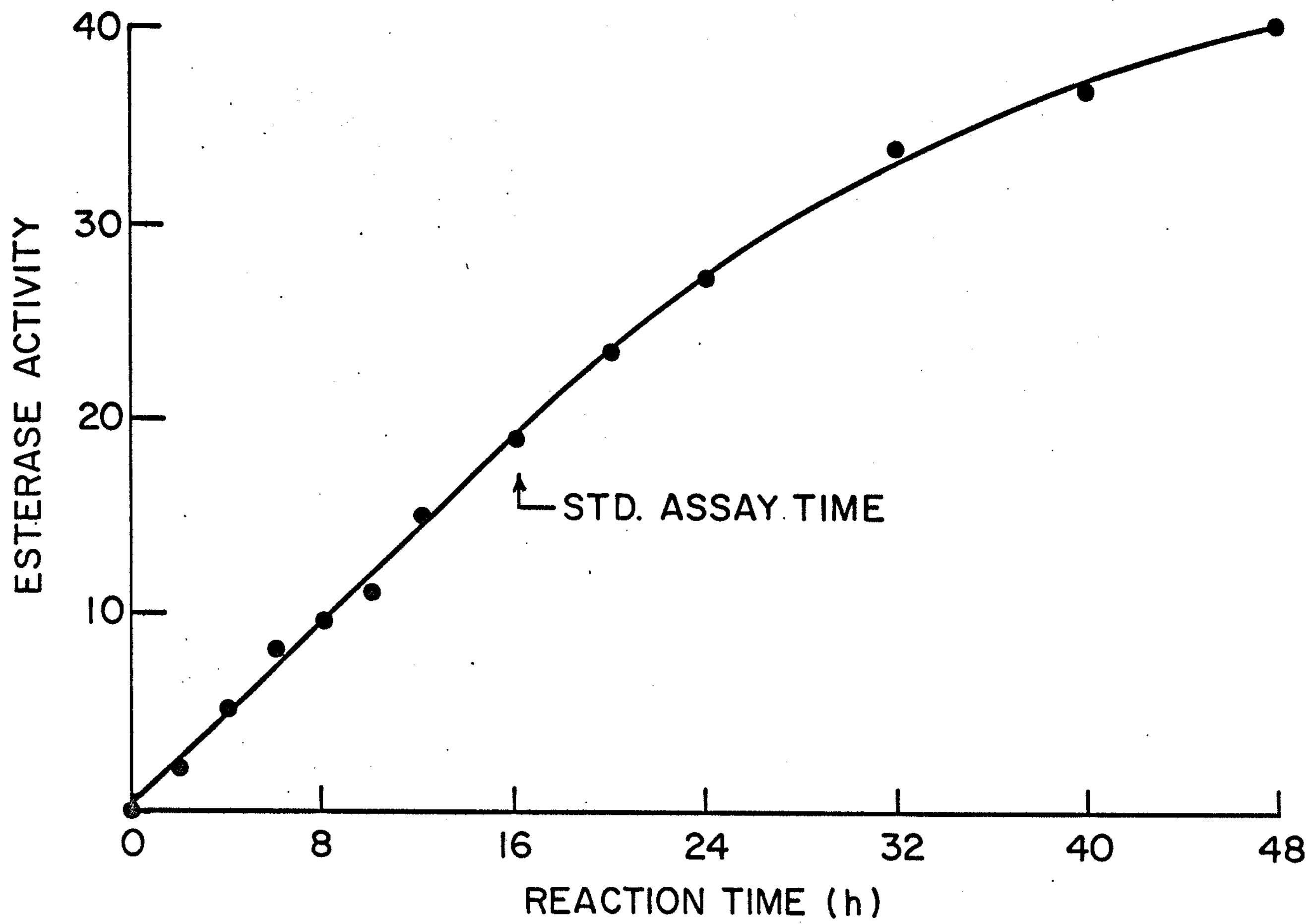

Figure 8. Esterase activity as a function of reaction time. Enzyme concentration 1 mg/0.1 ml buffer, substrate 5 μM, esterase activity expressed as % ^{14}C-benzoylprop acid formed.

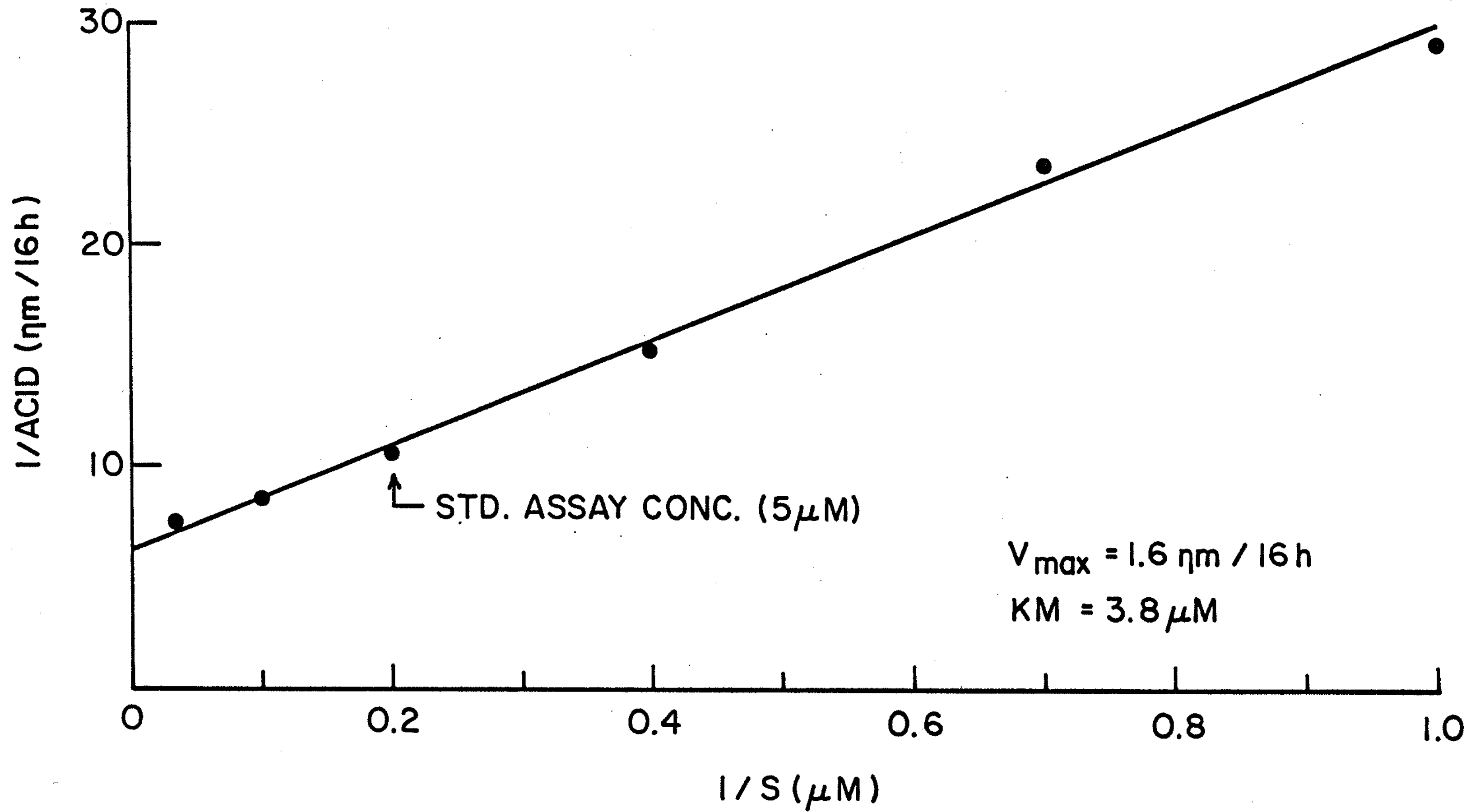

Figure 9. Lineweaver-Burk plot of reaction velocity versus substrate concentration. Enzyme concentration 1 mg/0.1 ml buffer, reaction time 16 h, reaction velocity expressed as ηmoles ^{14}C-benzoylprop acid formed/16 h.

of wild oat esterase became apparent at 0.2% ethanol ($^{V}/v$), and 4% ethanol ($^{V}/v$) virtually stopped all enzyme activity (TABLE 8).

TABLE 8. Inhibition of wild oat esterase by ethanol

% Ethanol in reaction mixture ($^{V}/v$)[a]	Esterase activity[b]
0	16.5
0.2	15.6
1	3.2
4	0.9
10	0.2

[a]Reaction mixture: ^{14}C-benzoylprop ethyl 5 μM, enzyme 1 mg/0.1 ml phosphate buffer (0.1M, pH 7.0).

[b]% ^{14}C-benzoylprop acid formed in 8 h.

The enzymic de-esterification of ^{14}C-benzoylprop ethyl was relatively slow (Vmax ≃ 1.6 ηmoles/16 hr). Standard assay parameters were chosen to maximize esterase activity and thus facilitate ^{14}C-benzoylprop acid (specific activity 11.4 μCi/mg) quantification. Assay parameters were also chosen such that in future inhibition studies, a reduced esterase would still be detectable. The data presented (Figs. 7-9) shows that the reaction of wild oat esterase and ^{14}C-benzoylprop ethyl has the properties of stability and linearity necessary for further study of such a system.

The *in vitro* enzymic de-esterification of ^{14}C-benzoylprop ethyl by a wild oat preparation supports the suggestion (Jeffcoat & Harries, 1973) that the herbicide is enzymically activated *in vivo*. Hydrolysis of "foreign" herbicide molecules requires that plant enzymes be somewhat non-specific. The wild oat esterase prepared in this study is non-specific since it was also observed to readily hydrolyze the herbicide dichlofop methyl. The *in vitro* techniques used in this study should also be applicable to other ester-containing herbicides. Where enzymic de-esterification represents herbicide activation, *in vitro* study of factors affecting de-esterification could help explain changes in *in vivo* herbicide efficacy such as synergism or antagonism.

S E C T I O N   3

RESEARCH NOTE

CHEMICAL HYDROLYSIS OF THE HERBICIDE BENZOYLPROP ETHYL

Benzoylprop ethyl {ethyl-2-[N-(3,4,-dichlorophenyl)benzamido]propionate} is a N,N-disubstituted alanine derivative (Fig. 10) which is used as a post-emergence herbicide to selectively control wild oats (*Avena fatua* L.) in wheat (*Triticum aestivum* L.). The herbicidal activity of benzoylprop ethyl is dependent upon its conversion to the biologically active desethyl acid (Jeffcoat & Sampson, 1973; Jeffcoat & Harries, 1973). The metabolism of benzoylprop ethyl in cereals has been studied and the hydrolysis of the carboxyethyl group to form the desethyl acid (Fig. 10) has been proposed as the major breakdown pathway (Beynon, Roberts & Wright, 1974a; 1974b). The carboxyethyl group is apparently more labile than the amide bond since benzoic acid formation is only detected in minor amounts.

It has been suggested that the de-esterification of benzoylprop ethyl in cereals is enzymic (Jeffcoat & Harries, 1973). *In vitro* enzymic conversion of ^{14}C-benzoylprop ethyl to ^{14}C-benzoylprop acid has been demonstrated using wild oat preparations (Hill, Stobbe & Jones, 1977). It is also conceivable that some non-enzymic de-esterification of benzoylprop ethyl on or in cereals may occur. The report describes the *in vitro* chemical hydrolysis of benzoylprop ethyl to produce benzoylprop acid.

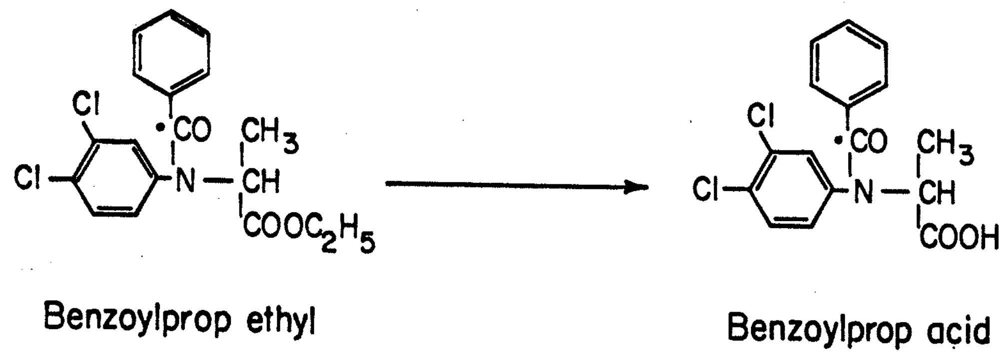

Figure 10. Hydrolysis of benzoylprop ethyl to form benzoylprop acid.

Materials and Methods

The following reaction procedure was adapted from the synthesis of N-Benzoyl-β-anilinopropionic acid reported by Evans, MacKintosh & Szinai (1969). Benzoylprop ethyl (0.25 g) was dissolved in absolute ethanol (2 ml) and treated with 4N NaOH (2 ml). After a week at room temperature, the solution was diluted with water (6 ml) and extracted with petroleum ether (two 15-ml portions). Acidification of the aqueous layer with acetic acid (final pH 5) and dilution with water yielded a precipitate which was collected.

The collected precipitate was analyzed using thin-layer chromatography, infrared spectrophotometry, and mass spectrometry.

Results and Discussion

The reaction product in the form of collected precipitate was identified as benzoylprop acid (yield 37%) by comparing its rf-value, infrared spectrum, and mass spectrum to those obtained from benzoylprop ethyl and benzoylprop acid standards.

The rf-value of benzoylprop acid applied to Baker-flex silica gel 1B2-F (J. T. Baker Chemical Co., Phillipsburg, N.J.) thin-layer plates and developed using 3% acetic acid, 6% acetone in chloroform was 0.32. The rf-value for benzoylprop ethyl on the same thin-layer plate was 0.58.

The infrared spectrum of benzoylprop acid is distinctly different than that of benzoylprop ethyl (Figs. 11-12). When the ester group (CO-OCH_2CH_3) of benzoylprop ethyl is converted to the acid group (CO-OH) of benzoylprop acid, the C=O absorption at 5.75 μ remains relatively unchanged, but the C=O absorption at 8.35 μ becomes broadened and is shifted to 8.22 μ. The amide C=O absorption at 6.09 μ for benzoylprop

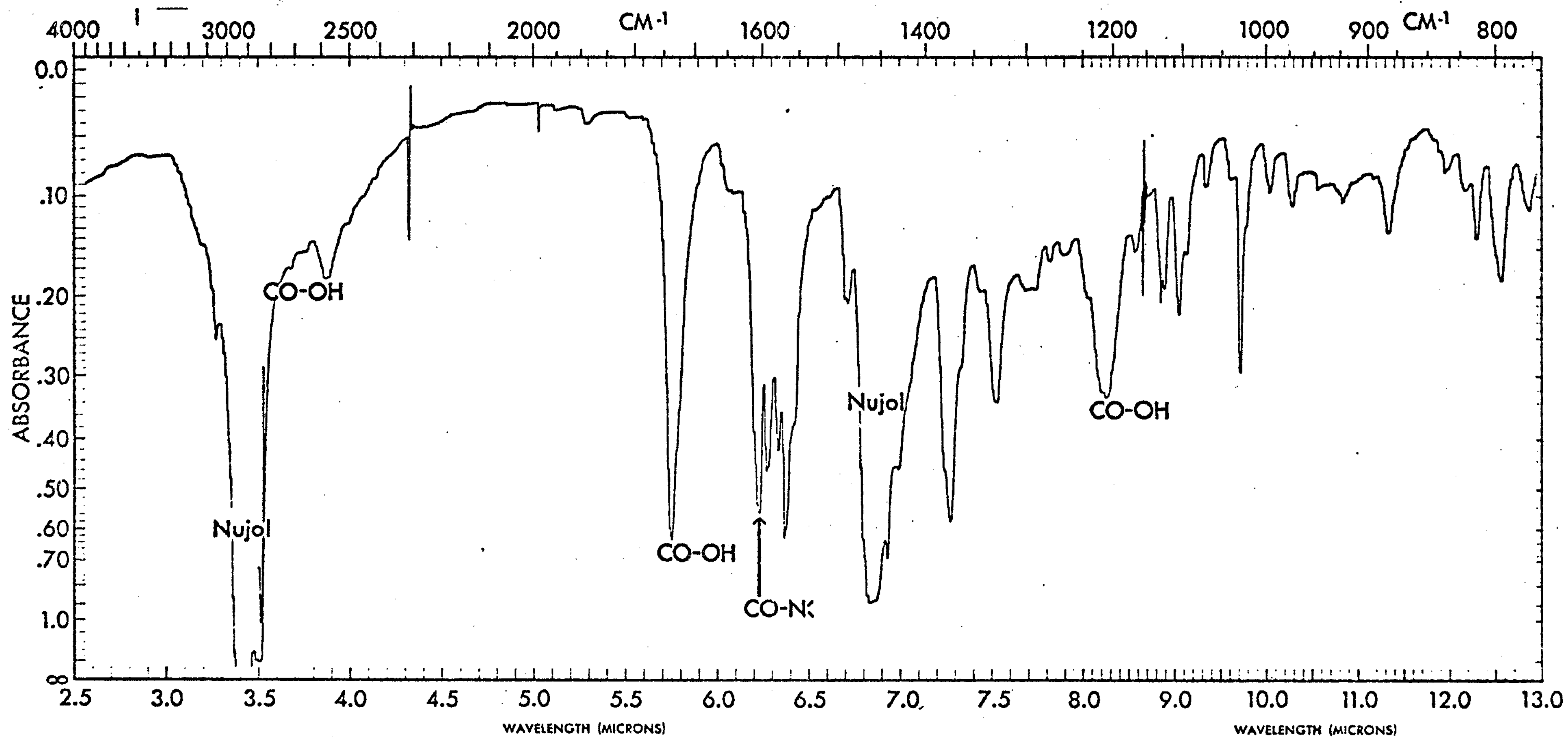

Figure 11. Infrared spectrum of benzoylprop acid. Sample prepared as a Nujol mull. Spectrum recorded using a Perkin-Elmer model 237 spectrophotometer.

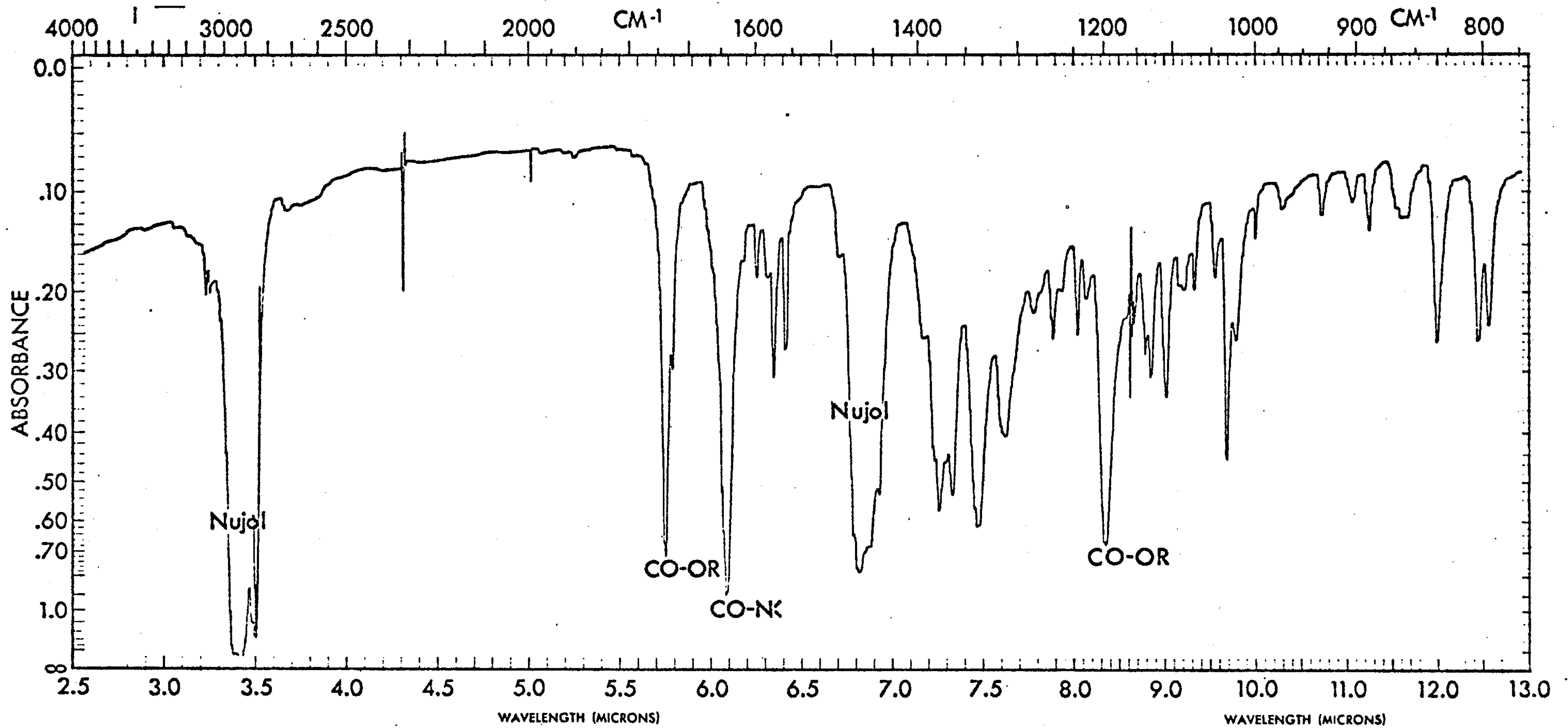

Figure 12. Infrared spectrum of benzoylprop ethyl. Sample prepared as a Nujol mull. Spectrum recorded using a Perkin-Elmer model 237 spectrophotometer.

ethyl is shifted to 6.22 μ for benzoylprop acid. This shift is probably the result of intramolecular hydrogen bonding between the amide C=O and the CO-OH groups of benzoylprop acid. The appearance of a weak absorption at 3.86 μ in the spectrum of benzoylprop acid is likely due to the OH of the acid group (CO-OH).

The mass spectra (Tables 9-10) show slight variations in the fragmentation patterns of benzoylprop acid compared to benzoylprop ethyl. The molecular ion M^+(m/e = 337) of benzoylprop acid is fragmented via the loss of CO_2 (m/e = 293), followed by the loss of the benzoyl moiety, PhCO (m/e = 188). The molecular ion M^+(m/e = 365) of benzoylprop ethyl is fragmented via the loss of OCH_2CH_3 (m/e = 320), followed by the loss of CO (m/e = 292), and then the benzoyl moiety, PhCO, is removed (m/e = 187). Both spectra have relatively abundant ions at m/e = 145 due to the dichlorophenyl ($PhCl_2$) moiety. The base peak of both spectra appears to be m/e = 105 due to the benzoyl (PhCO) moiety.

It was assumed that the mild reaction conditions (5 days at room temperature) reported by Evans, MacKintosh & Szinai (1969) were necessary to prevent simultaneous hydrolysis of the amide bond. However, when benzoylprop ethyl was refluxed (5 min) in ethanolic KOH, the same benzoylprop acid reaction product was obtained. A 20-min reflux in ethanolic Na_2CO_3 also yielded some benzoylprop acid. It appears that the carboxyethyl group of benzoylprop ethyl is more labile than the amide bond. These results are in agreement with the proposal that the major breakdown pathway of benzoylprop ethyl is via benzoylprop acid formation, rather than via benzoic acid formation (Beynon, Roberts & Wright, 1974a).

TABLE 9. Mass spectrum of benzoylprop acid[a]

m/e	Relative abundance[b]	Proposed ion composition
337	1.0	M^+ (Molecular ion)
293	1.6	$(M-CO_2)^+$
188	> 5.6	$(293-PhCO)^+$
187	2.7	$(188-H)^+$
172	2.7	$(187-CH_3)^+$
153	2.3	-
145	3.9	$(PhCl_2)^+$
105	> 5.6	$(PhCO)^+$

[a]Spectrum from Finnigan model 1015 GC-MS, ionization energy 82 ev, solid probe (160°C) sample introduction.

[b]As indicated by peak height, with m/e = 188 and m/e = 105 greater than 67 mm (off-scale).

TABLE 10. Mass spectrum of benzoylprop ethyl[a]

m/e	Relative abundance[b]	Proposed ion composition
365	1.0	M^{+} (Molecular ion)
320	0.3	$(M-OCH_2CH_3)^{+}$
292	2.4	$(320-CO)^{+}$
260	1.2	$(M-PhCO)^{+}$
187	2.2	$(292-PhCO)^{+}$
172	4.1	$(187-CH_3)^{+}$
145	4.5	$(PhCl_2)^{+}$
105	> 6.9	$(PhCO)^{+}$

[a]Spectrum from Finnigan model 1015 GC-MS, ionization energy 70 ev, solid probe (50°C) sample introduction.

[b]As indicated by peak height, with m/e = 105 greater than 125 mm (off-scale).

GENERAL DISCUSSION

In Vivo Study

Outline, Scope, and Methodology

This study was concerned with the interrelationship of the light and nutrient environment, benzoylprop ethyl metabolism, and wild oat growth response (Fig. 13). Light and nutrient levels were varied causing changes in plant biochemistry. In the absence of the herbicide, these changes in plant biochemistry alone, would cause plant growth response to be altered. For example, reduced light intensity causes increased indoleacetic acid activity, which promotes cell elongation. Differences in such "natural" growth response were minimized by selection of appropriate light and nutrient levels, and were monitored by use of non-herbicide-treated control plants. Since benzoylprop ethyl is metabolized by plant biochemistry, changes in plant biochemistry should also result in changes in benzoylprop ethyl metabolism in herbicide-treated plants, and any such changes were monitored. The production of phytotoxic benzoylprop acid would in turn affect some portion of plant biochemistry such that "unnatural" growth response (inhibition of cell elongation) occurs. Changes in plant growth responses to benzoylprop ethyl were measured and through correlations to benzoylprop ethyl metabolism were related back to variations in environment.

It would have been desirable to define a more direct relationship between environment, benzoylprop ethyl metabolism, and wild oat growth

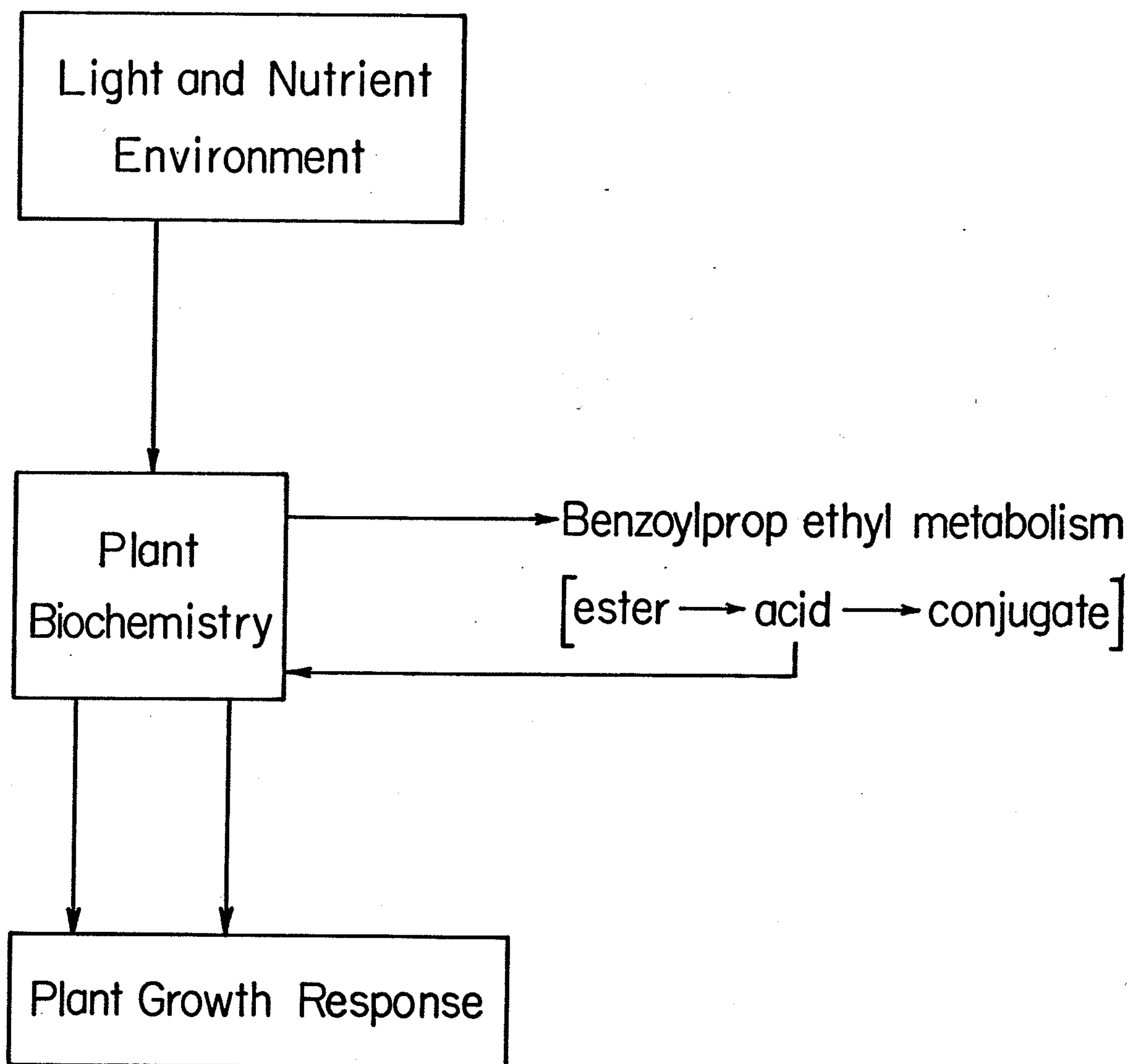

Figure 13. Interrelationship of environment, benzoylprop ethyl metabolism, and plant growth response.

response than is discussed (pgs. 19-29). A more direct relationship may have been defined by using actual amounts (dpm) of herbicide and metabolites in comparisons (Figs. 4-5, pgs. 22-23). Such comparisons were precluded by the variability in wild oat ^{14}C-herbicide uptake, and by the effect of light intensity on subsequent ^{14}C-herbicide translocation from roots to shoots (TABLE 1, pg. 24). Had all plants been dosed with essentially the same shoot concentration of ^{14}C-herbicide, the expected increase in herbicide injury at low light may have been observed (TABLE 2, pg. 25). To overcome the effect of light treatment on ^{14}C-shoot uptake would have required a study in itself, and may have been unachievable.

Due to inherent variability in wild oats, benzoylprop ethyl metabolism and growth response were measured and correlated in the same individual plant. Results were analyzed using a 90% level of significance (rather than a 95% or 99% level) because of wild oat variability. The use of wild oats seemed more appropriate than extrapolating results from less variable tame oats as reported in the literature (Jeffcoat and Sampson, 1973; Jeffcoat and Harries, 1973; Holm, 1972). Since growth response to benzoylprop acid required time to become evident, sampling dates of 3, 6, and 12 days were chosen as a compromise between the relatively fast benzoylprop ethyl metabolism and the relatively slow wild oat growth response. The extrapolation of metabolic curves (Figs. 3-5, pgs. 20-23) gives an important insight into benzoylprop ethyl metabolism 0-3 days after ^{14}C-herbicide dosing. Extrapolated portions are in good agreement with the 0-6 day data plotted by Jeffcoat and Harries (1973), (Fig. 14).

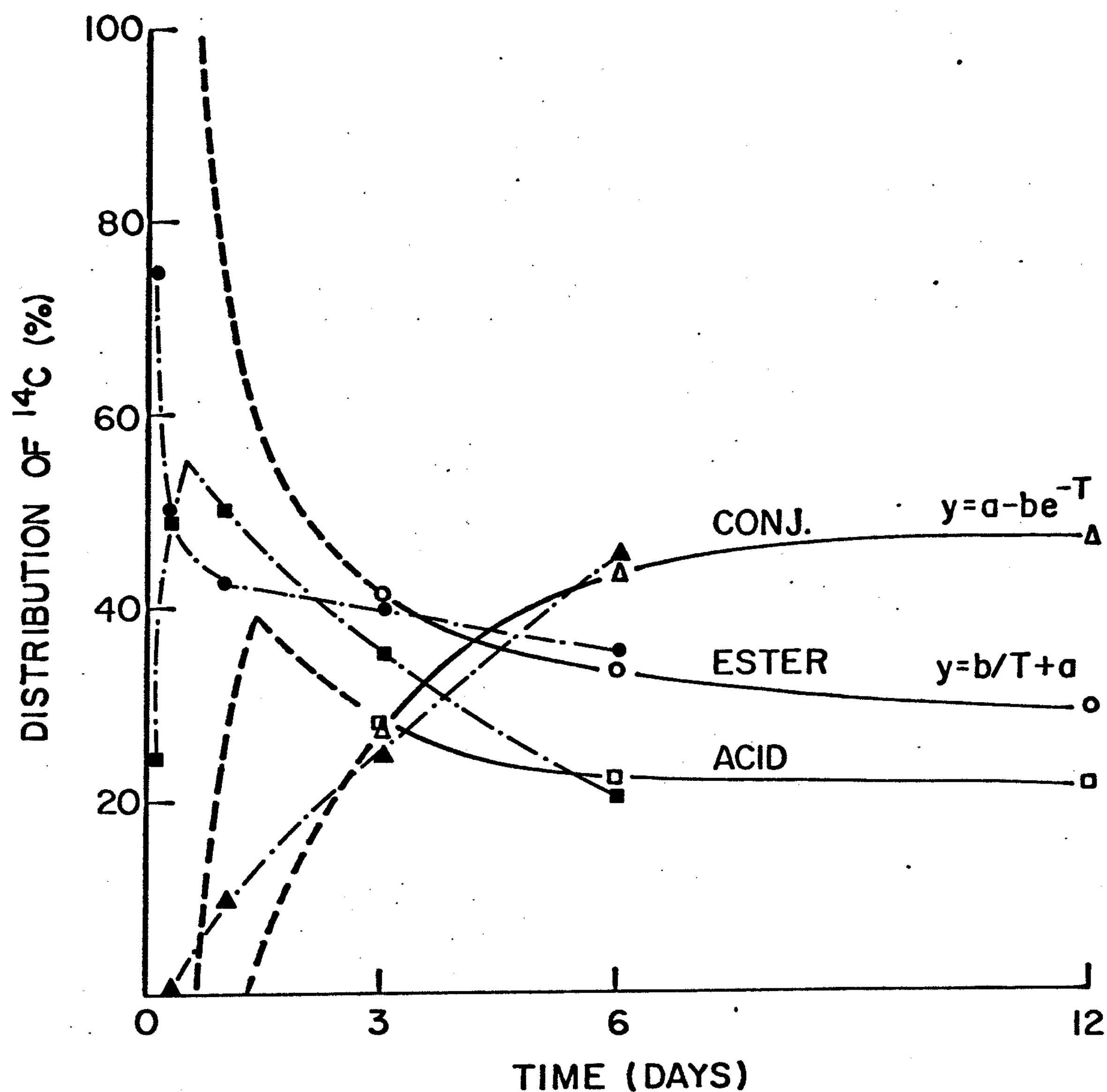

Figure 14. Metabolism of ^{14}C-benzoylprop ethyl in oat shoots. Comparison of data (– – ——) obtained in this study, with 0-6 day data (–·–·–) reported by Jeffcoat and Harries (1973).

Rates of De-esterification and Conjugation Under Different Environments

In general, the initial build-up of phytotoxic benzoylprop acid in wild oats is due to the relative rates of benzoylprop ethyl de-esterification and benzoylprop acid conjugation. The time-lag before the start of acid conjugation may not be as long as shown and predicted by extrapolation (Fig. 3, pg. 20), however, data of Jeffcoat and Sampson (1973), and Jeffcoat and Harries (1973) also indicates acid conjugation is delayed. At the very least, the initial rate of conjugation is much slower than initial de-esterification.

Initial de-esterification was faster at the low light level (Fig. 4, pg. 22). This may have been due to a general surplus of necessary esterase in slower growing plants as suggested by Jeffcoat and Harries (1973). More likely, a specific effect related to decreased photosynthesis or to a phytochrome system is involved in light intensity modifying de-esterification rates. The rate of de-esterification at the high and low light levels tended to equalize after 6 days, due perhaps to decreased ester substrate. Interestingly, the expected increase in acid conjugation at the high light level was not evident until after 6 days. The acid conjugation reaction is not as light sensitive as de-esterification at 0-6 days, perhaps due to a necessary build-up of both benzoylprop acid and plant sugars. It may be concluded that de-esterification is the more important reaction in determining initial benzoylprop acid levels under different light intensities.

Both de-esterification and acid conjugation reactions were faster

at the high nutrient level (Fig. 5, pg. 23). Both reactions appear to be affected to the same extent, resulting in similar acid levels at both nutrient levels. Nutrient availability seems to have a general effect on benzoylprop ethyl metabolism, whereas light intensity had specifically opposite effects on de-esterification and conjugation reactions. The effect of nutrient availability on benzoylprop ethyl metabolism is likely a reflection of the overall metabolic state of the wild oat. Results of this study appear to conflict with the report by Jeffcoat and Harries (1973) that de-esterification was decreased by 30% when plants were given additional nutrients. It should be noted that these workers used tame oats and were really comparing excess and normal nutrient levels, rather than normal and reduced nutrient levels. Jeffcoat and Harries (1975) reported that nutrient availability did not markedly influence the metabolism of ^{14}C-flamprop-isopropyl (an analogue of benzoylprop ethyl), but did not elaborate. It would seem more reasonable to find changes in flamprop-isopropyl metabolism since nutrient availability is likely to influence general plant biochemistry.

It appears that light levels and de-esterification rates are important in determining initial benzoylprop acid concentrations. Nutrient availability and acid conjugation rates are important in relation to sustaining benzoylprop acid levels. Since wild oats can outgrow benzoylprop ethyl inhibition under an environment of high light and nutrients, and since acid levels don't appear to suddenly drop off between 3 and 12 days (Figs. 4-5, pgs. 22-23), a threshold benzoylprop acid level may exist. Herbicide injury to wild oats may depend upon how long acid levels remain above a threshold level.

It can, therefore, be postulated that: the severity of initial wild oat injury depends on initial acid levels produced by the de-esterification of benzoylprop ethyl; but prolonged injury depends upon acid levels being sustained above a threshold level by a slow acid conjugation rate. Since benzoylprop ethyl does not normally kill wild oats, but merely stunts them, it may be more important to prolong herbicide injury.

^{14}C-Benzoic Acid Formation from ^{14}C-Benzoylprop Ethyl Metabolism

As previously noted (pg. 21), ^{14}C-benzoic acid levels detected in this study were low and variable (1-3% of total ^{14}C-radioactivity). This result agrees with reports of only minor amounts of benzoic acid having been detected in other studies (Beynon *et al.*, 1974a; 1974b).

A seemingly unrelated observation is that the total ^{14}C-radioactivity found in wild oat shoots in this study tended to decrease with time (TABLE 1, pg. 24). While ^{14}C-herbicide conjugates would be more difficult to extract with successive sampling dates, it is unlikely this would account for large losses of ^{14}C-radioactivity between sampling dates. One possible mode of loss of ^{14}C-radioactivity would be via ^{14}C-benzoic acid formation, followed by decarboxylation and release of $^{14}CO_2$. If decarboxylation was rapid once ^{14}C-benzoic acid was formed, only low levels of ^{14}C-benzoic acid would be detected. It also appears that the largest losses of ^{14}C-radioactivity occurred under conditions of high light and nutrients, between 3 and 6 days (TABLE 1, pg. 24). One could further postulate that ^{14}C-benzoic acid formation and $^{14}CO_2$ release occurs mostly under conditions of most active wild oat growth, namely high light and nutrient levels. Benzoic

acid formation and $^{14}CO_2$ release could represent a detoxification process which would help account for the transient effect of benzoylprop ethyl in high light and nutrient environments.

Based on results reported by Beynon *et al.* (1974a), no attempt was made to detect possible $^{14}CO_2$ release from ^{14}C-herbicide-treated plants in this study. These workers observed only slight increases in unextractable ^{14}C-residues with time; but also reported larger unaccountable losses of total ^{14}C-radioactivity with successive sampling dates. This was attributed to evaporation from foliar applications of ^{14}C-herbicide. Most important, the total ^{14}C-radioactivity measured after 14 days was similar even when ^{14}C-benzoylprop ethyl was labelled in positions such that ^{14}C-benzoic acid formation would not occur. This evidence seemed to negate the possibility of significant benzoic acid formation and subsequent CO_2 release.

Losses of total ^{14}C-radioactivity in this study likely occurred via root exudates into nutrient solutions which were changed every 3 days.

^{14}C-Benzoylprop Ethyl Metabolism in Wild Oat Roots

Jeffcoat and Harries (1973) reported that roots were less sensitive to benzoylprop ethyl than were oat shoots. They found little metabolism of ^{14}C-benzoylprop ethyl in roots of treated plants. After 6 days, 95% of the radiolabel present in the roots was present as the parent ester. Thus, the roots of wild oat plants in this study were not routinely examined for evidence of ^{14}C-benzoylprop ethyl metabolism. When the roots of two ^{14}C-herbicide-treated plants were analyzed 6 days after ^{14}C-dosing, a total ^{14}C-radioactivity of 8500 dpm was found

distributed as 65% benzoylprop ethyl, 25% benzoylprop acid, and 10% conjugates. While the relative amount of metabolites seems high in view of the report by Jeffcoat and Harries (1973), actual amounts (dpm) are low compared to metabolite levels (dpm) found in the shoots. Metabolites may have been formed in the wild oat shoots and then transported to the roots. No injury was detected in the roots of ^{14}C-herbicide-treated wild oats in this study.

^{14}C-Benzoylprop Ethyl Injury Symptoms

Wild oat plants in this study were dosed hydroponically with ^{14}C-herbicide at the 4-leaf stage. Thus, at the time of ^{14}C-dosing, plants had 4 leaves on the main culm, and usually 2 leaves on a tiller emerging from the first leaf node. In general, wild oat plants had grown to the 5, 5-6, and 6-7 leaf stages by the 3, 6, and 12-day sampling dates, respectively. The injury symptoms of wild oats in this study varied with environmental treatment, but were similar to those described by Holm (1972), and Jeffcoat and Harries (1973). In addition to stunting, plants exhibited the following injury symptoms:

1. Leaves which were the youngest at the time of ^{14}C-dosing later showed necrosis extending from the tips often accompanied by a purplish discoloration.

2. Leaves which emerged after ^{14}C-dosing did so through the side of the previous leaf sheath and exhibited chlorotic blotches.

3. Twelve days after ^{14}C-dosing, normal growth was observed in the tiller from the second leaf node, except that new leaves were slightly chlorotic.

Injury symptoms were less severe in plants under low light due to

less ^{14}C-herbicide translocated from roots to shoots in plants under low light. Plants at the low nutrient level had more severe symptoms and less new growth than plants at the high nutrient level. At the termination of the experiment, non-herbicide-treated plants at the low nutrient level exhibited slight chlorosis and some reddish-purple necrosis on older leaves. Roots of all wild oat plants appeared extremely healthy.

In Vitro Study

Outline, Scope, and Methodology

This study was initiated to detect possible enzymic de-esterification of ^{14}C-benzoylprop ethyl. By extracting, purifying, and comparing esterases from wild oats and wheat, it should have been possible to explain the basis of selectivity of benzoylprop ethyl in specific enzymic terms. This study was limited to forming the basis, in terms of methodology, for possible future investigations of other herbicides and the factors affecting de-esterification.

The methodology of this study was adapted mainly from a report on sorghum grain esterases by Sae *et al.* (1971). Preliminary work using wild oat coleoptiles established that *in vitro* enzymic de-esterification of ^{14}C-benzoylprop ethyl was possible. The use of plants at the 2-3 leaf stage was a compromise between the usual observation that enzymic activity decreases with age and the fact that benzoylprop ethyl is field-applied at the 4-5 leaf stage of wild oats. Good herbicidal activity has been observed in indoor situations where applications of benzoylprop ethyl were made at the 2-3 leaf stages (Holm, 1972). Esterase activity over a range of pH levels was not

examined since several workers have reported pH 7.0 as optimum (Sae *et al.*, 1971; Mendoza *et al.*, 1969).

The enzyme assay used in this *in vitro* study should be particularly relevant since the same substrate (^{14}C-benzoylprop ethyl) was used for *in vivo* work. The substrate levels quoted were based on the amount of ^{14}C-herbicide placed into the bottom of reaction tubes (standard enzyme assay, pg. 34). Actual substrate levels redissolved into protein solution after ethanol evaporation may have been less and somewhat variable, however, the substrate may not have had to be in true solution to be enzymically de-esterified. Regardless, the procedure used to combine substrate and enzyme was dictated by ethanol inhibition (TABLE 8, pg. 46) and excellent substrate linearity was observed (Fig. 9, pg. 45).

<u>^{14}C-Benzoylprop Ethyl Hydrolyzing Esterase from Wild Oat</u>

The observed rate of ^{14}C-benzoylprop ethyl de-esterification in wild oat preparations, 0.1 ηmoles/16 h at standard assay conditions, seems extremely slow. It does, however, compare with the rate of 0.3 ηmoles/16 h reported by Morré and Rogers (1960) for the *in vitro* enzymic de-esterification of 2,4-D octyl ester by spinach acetone powder. The slowness of enzymic de-esterification of benzoylprop ethyl may be an inherent property of its chemical structure. Preliminary work with dichlofop methyl and wild oat esterase revealed that its enzymic de-esterification is about 20 times faster than that of benzoylprop ethyl at similar conditions. The apparent slow enzymic de-esterification of benzoylprop ethyl may be advantageous for *in vivo* herbicide efficacy. A slow steady rate of de-esterification, just faster than acid conjugation, may help prolong herbicide injury. Rapid ester conversion to acid

would produce high initial acid levels, but subsequent detoxification by conjugation would allow stunted (but not killed) plants to regrow.

^{14}C-Benzoylprop Ethyl Hydrolyzing Esterase from Wheat

The lack of ^{14}C-benzoylprop ethyl hydrolyzing esterase in wheat preparations has been previously discussed (pgs. 39-41). Low substrate levels and/or the presence of an inhibitor were used to explain the lack of esterase activity. These explanations were formed on the basis that there should have been *in vitro* esterase activity in wheat preparations, since *in vivo* de-esterification of benzoylprop ethyl in wheat has been reported (Jeffcoat and Harries, 1973; Beynon *et al.*, 1974a; 1974b).

Benzoylprop ethyl is not recommended for application to the wheat variety Selkirk due to low crop tolerance. It is thus especially significant that esterase activity was not detected in preparations from Selkirk wheat. Esterase activity should have been detected in this sensitive wheat variety even at the low substrate levels used (5-20 μM). Wheat coleoptiles were examined in the hope that an inhibitor if present in leaf extracts may not have been present in coleoptile extracts. Lack of esterase activity was again significant since Jeffcoat and Harries (1973) reported inhibition of wheat coleoptile expansion by benzoylprop ethyl. Lack of ^{14}C-benzoylprop ethyl hydrolyzing esterase in wheat preparations is especially puzzling when one considers that the same wheat preparations readily hydrolyzed dichlofop methyl.

The evidence seems to suggest that either wheat preparations contained a specific inhibitor of ^{14}C-benzoylprop ethyl hydrolysis or, wheat esterases are not capable of de-esterifying ^{14}C-benzoylprop ethyl. A specific inhibitor seems unlikely, however, it is possible that

benzoylprop ethyl is not enzymically de-esterified in wheat. One could postulate that limited chemical de-esterification of benzoylprop ethyl occurs in plants. Benzoylprop ethyl can be easily de-esterified *in vitro* by chemical action (pgs. 48-56). There could be only limited chemical de-esterification in wheat compared to chemical and enzymic de-esterification in wild oats. This theory would account for benzoylprop ethyl selectivity and the slow de-esterification of the herbicide in wheat.

Integration of *In Vivo* and *In Vitro* Study

The detection of ^{14}C-benzoylprop ethyl hydrolyzing esterase activity in wild oat preparations supports the suggestion (Jeffcoat and Harries, 1973) that benzoylprop ethyl is enzymically de-esterified *in vivo*. The reaction rate linearity with varying substrate concentration demonstrated for isolated esterase may apply to *in vivo* situations. The double reciprocal Lineweaver-Burk (1934) plot of reaction velocity versus substrate concentration was applied to *in vivo* data. De-esterification reaction velocity was expressed as 1-% ^{14}C-ester of total ^{14}C-radioactivity, while substrate concentration was represented by ^{14}C-shoot uptake (dpm) of herbicide. Since ^{14}C-shoot uptake (dpm) was significantly affected by light intensities (TABLE 1, pg. 24), $^{1}/V$ versus $^{1}/S$ was correlated for both high and low light levels. The correlation coefficients at high and low light levels were 0.47 and 0.45 respectively, compared to a required $r = 0.50$ for significance at the 90% confidence level. Thus, the *in vivo* data for ^{14}C-benzoylprop ethyl de-esterification showed a strong trend towards that reaction being catalytic in nature. Lack of significance (at 90% level) may

have been caused by the inherent variability in wild oats, and the fact that de-esterification is only part of a dynamic equilibrium involving also, conjugation reactions. As previously postulated, part of the de-esterification of benzoylprop ethyl in plants may be non-enzymic.

It is of interest to speculate whether the slow enzymic de-esterification of ^{14}C-benzoylprop ethyl observed *in vitro* could account for rates of ester disappearance in intact plants. Comparison of data shows that the *in vitro* ^{14}C-benzoylprop ethyl de-esterification rate was only 0.4 of that observed *in vivo*, in spite of an *in vitro* substrate level 3.5 times that used *in vivo* (TABLE 11). The presence of simultaneous acid conjugation and non-enzymic de-esterification reactions

TABLE 11. Comparison of *in vivo* and *in vitro* ^{14}C-benzoylprop ethyl dose and subsequent de-esterification by wild oat

Metabolic study	Dose rate (µg ^{14}C-herbicide/g F.W. of wild oat shoot)	De-esterification rate (ng ^{14}C-herbicide converted/h)
In vivo[a]	3.1	17
In vitro[b]	11	6.7

[a]Calculations based on ^{14}C-shoot uptake 38,400 dpm, shoot fresh weight 0.5 g, de-esterification rate 1.125%/h for 0-48 h (Fig. 3, pg. 20).

[b]Calculations based on equivalent of 33.3 mg of original 5.0 g leaves in 0.1 ml reaction mixture, ^{14}C-substrate 10 µM, de-esterification rate 29.2%/16 h (TABLE 5, pg. 36).

in vivo, together with losses during purification and non-optimum conditions *in vitro*, might account for lower de-esterification rates *in vitro*. It should also be noted that *in vivo* de-esterification rates (TABLE 11) are really a measure of ester disappearance part of which could involve direct ester conjugation to plant sugars. On the basis of the data obtained, however, it is concluded that *in vitro* enzymic de-esterification rates would not completely account for *in vivo* de-esterification of benzoylprop ethyl.

Environmental Factors Affecting Benzoylprop Ethyl Metabolism and Efficacy Applied To Field Situations

Although the present study was conducted under controlled environment and laboratory conditions, the ultimate application of results is to field situations. Variations in wild oat control using benzoylprop ethyl have been observed in field experiments (Stobbe *et al.*, 1971); Holm, 1972; Lowen, 1975, Sharma *et al.*, 1977). More specifically, benzoylprop ethyl efficacy increases with application at later 5-6 leaf stages of wild oats, and with increased soil fertility. In order to explain such field observations using information gathered in this study, the following premise is proposed: since benzoylprop ethyl inhibits cell elongation in wild oat, any condition(s) that promote cell elongation will increase benzoylprop ethyl efficacy. Thus, herbicide applications are more effective at the wild oat 5-6 leaf stage because the plant is in the "shooting stage" of most rapid cell enlargement. Concurrently, there may be significant reductions in light intensity upon wild oat plants due to crop competition and wild oat

inter-plant competition. Such reduced light levels would have the compound effect of simultaneously promoting further cell elongation while increasing phytotoxic benzoylprop acid levels. The most significant effect of reduced light levels is likely the corresponding decrease in acid conjugation sustaining acid levels above a threshold and thus prolonging herbicide injury. Good benzoylprop ethyl activity at earlier 2-3 leaf stages of wild oat observed indoors (Holm, 1972), may be attributed to reduced light levels (especially the blue wavelengths) and the resulting promotion of cell elongation. In field situations, any crop management practice which speeds crop development and the resulting crop canopy should increase benzoylprop ethyl efficacy. This may explain the effect of increased soil fertility enhancing wild oat control. Wild oats are extremely good competitors for nutrients and it is questionable whether reduced nutrient availability due to increased crop competition ever becomes a factor in field situations. The opposite effect, increased soil fertility, likely affects benzoylprop ethyl efficacy by speeding development of a crop canopy. Should the condition of nutrient deficient wild oats occur in field situations, the expected increase in herbicidal activity of benzoylprop ethyl would most certainly be due to decreased acid conjugation, sustained acid levels, and thus prolonged injury.

SUMMARY AND CONCLUSIONS

Effect of Light and Nutrient Levels on Benzoylprop Ethyl Metabolism and Growth Inhibition in Wild Oat

Benzoylprop ethyl was metabolized to benzoylprop acid and to polar conjugates in wild oat shoots.

Benzoylprop ethyl was metabolized to benzoic acid in only very small amounts in wild oat shoots.

Changes in the light and nutrient environment of wild oat caused changes in the metabolism (de-esterification and conjugation reactions) of benzoylprop ethyl.

Reduced light and nutrient levels resulted in higher levels of phytotoxic benzoylprop acid in wild oat shoots.

Reduced light intensity was more important than reduced nutrient availability in sustaining higher levels of benzoylprop acid in wild oat shoots.

Since growth response to phytotoxic acid was slower than acid conjugation, at any particular sampling date, growth correlated better to the amount of acid-conjugate (detoxified acid) than to the amount of ester or acid in wild oat shoots.

Benzoylprop acid concentration (dpm/mm leaf length), rather than acid amounts *per se*, had the most influence on subsequent growth.

It is concluded that the increased efficacy of benzoylprop ethyl at reduced light and nutrients is due to higher levels of phytotoxic benzoylprop acid as well as to the reduced growth rate of the wild oat.

It is further concluded that the severity of initial wild oat injury depends on initial benzoylprop acid levels produced by de-esterification of benzoylprop ethyl, but prolonged injury depends upon acid levels being sustained above a threshold level by a slow acid-conjugation rate.

In field situations, it may be most important to prolong herbicide injury, and thus increased efficacy with crop competition is mostly the result of reduced light intensity and slower acid-conjugation rates.

Hydrolysis of Benzoylprop Ethyl by Wild Oat Esterase

In vitro enzymic de-esterification of ^{14}C-benzoylprop ethyl (to form ^{14}C-benzoylprop acid) by a preparation from wild oat leaves was demonstrated.

Wild oat esterase once extracted and partially purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-100 gel filtration was stable and activity as a function of reaction time, enzyme and substrate concentration, was linear.

Incubation with α-chymotrypsin reduced wild oat esterase activity as did the presence of ethanol in reaction mixtures.

^{14}C-Benzoylprop ethyl hydrolyzing esterase activity could not be found in preparations from wheat.

Evidence was obtained which suggested the presence of an esterase inhibitor in wheat preparations.

It is concluded that the *in vitro* enzymic de-esterification rate (6.7 ηg ^{14}C-herbicide/h at standard assay conditions) would not completely account for the observed *in vivo* disappearance rate of ^{14}C-benzoylprop ethyl (17 ηg ^{14}C-herbicide/h).

It is concluded that the *in vivo* de-esterification of benzoylprop ethyl in wild oat shoots is likely enzymic, but the enzymic nature of similar de-esterification in wheat is in doubt.

Chemical Hydrolysis of Benzoylprop Ethyl

Benzoylprop ethyl treated with ethanolic base was readily hydrolyzed to benzoylprop acid.

The apparent lack of benzoic acid formation suggests that the carboxyethyl moiety is more labile than the amide bond, and is consistent with results of enzymic hydrolysis.

It is concluded that chemical hydrolysis of benzoylprop ethyl on or in plants could yield benzoylprop acid and thus may contribute to benzoylprop ethyl de-esterification rates.

SUGGESTIONS FOR FURTHER WORK

Research presented in this thesis has demonstrated that changes in environmental conditions (light and nutrient levels) have a profound effect on benzoylprop ethyl metabolism and growth inhibition in wild oats. In order to better understand this complex subject, research should be conducted to study:

The effect of day length, temperature, humidity, and moisture on benzoylprop ethyl metabolism and growth inhibition.

The effect of environmental conditions, prior to ^{14}C-herbicide dosing, on subsequent metabolism.

The types and amounts of different herbicide-conjugates formed under different environmental conditions.

Whether barban synergism and 2,4-D antagonism of benzoylprop ethyl efficacy is the result of changes in benzoylprop ethyl metabolism.

Since ^{14}C-benzoylprop ethyl hydrolyzing esterase activity was present in preparations from wild oat but not from wheat, the following course of study is recommended:

Literature reports that *in vivo* de-esterification of benzoylprop ethyl occurs in wheat should be verified.

The nature, enzymic or chemical, of benzoylprop ethyl metabolism in

wheat may be determined using substrate versus reaction rate analysis and inhibition studies.

Methods of isolating inhibitor(s) of wheat esterase and of increasing substrate concentration should be applied to wheat preparations.

If ^{14}C-benzoylprop ethyl hydrolyzing esterase activity could be found in wheat preparations, the properties of wild oat and wheat esterases should be compared relative to benzoylprop ethyl selectivity.

Preliminary work has indicated that wild oat and wheat esterases will hydrolyze dichlofop methyl. The *in vitro* enzymic techniques reported in this thesis should be applied to other herbicides and to other weed and crop species. Esterase activation and inhibition studies should be initiated. A study of the effect of environmental conditions on the amount of esterase may explain variations in herbicidal efficacy.

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