To the Graduate Council:

I am submitting herewith a thesis written by George Howard Gregg entitled "Preliminary Characterizations of Esterase and Cholinesterase Activities in Etiolated Barley, Rice, and Maize Seedlings." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

Otto J. Schwarz, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

PRELIMINARY CHARACTERIZATIONS OF ESTERASE AND CHOLINESTERASE ACTIVITIES IN ETIOLATED BARLEY, RICE, AND MAIZE SEEDLINGS

A Thesis

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Master of Science

Degree

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ABSTRACT

This study represents preliminary characterizations of esterase and cholinesterase activities in barley (Hordeum vulgare cv Himalaya), in rice (Oryza sativa japonica cv Calrose 76), and in maize (Zea mays cv Pioneer 3145). The activities were partially purified by 30% to 90% ammonium sulfate fractionation and Sephadex G-200 column chromatography. Characterizations of activities were attempted by observing the effects of pH, temperature, dicationic metals, and cholinesterase inhibitors.

Barley and maize were found to exhibit relatively strong esterase and cholinesterase activities and rice was found to have a strong esterase activity but lacks cholinesterase activity. Activities from all three sources had pH optima between 7.0 and 8.0 and were temperature stable to at least 30 C. Inhibition by metal ions varied greatly depending on source and activity while only maize activities exhibited sensitivity to inhibitors. Based on this research, the activities from these sources do not exhibit cholinesterase or acetylcholinesterase activities by the criteria used to describe similar activities in animal systems.

Little information is available concerning the metabolic roles of the esterase activities and their substrates in vivo. Conjectures related to possible functions of the esterase system in the whole plant system are considered.

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CHAPTER I

INTRODUCTION

Esterases (carboxylic ester hydrolases, EC 3.1.1) are characterized by their ability to cleave ester linkages. The function of the esterase system in plants is unknown, but several workers have proposed an involvement with the phytochrome system (e.g., Jaffe, 1972; Ernst and Hartmann, 1980). This view has been discounted by others (Kasemir and Mohr, 1972; Saunders and McClure, 1973). Other possibilities, still more conjecture than fact at this time, are that acetylcholine may be involved in ion uptake by membranes (Bennet-Clark, 1956) or in the biosynthesis of gibberellic acid or other metabolic processes involved in growth (Riov and Jaffe, 1973c; Hartmann, 1979; Ernst and Hartmann, 1980) while acetylcholinesterase presumably regulates the activity or effect of acetylcholine.

The esterase enzymes are known to be inhibited by various cholinergic drugs, including organophosphates (e.g., Paraoxon,[1] Parathion,[2] Dimethoate[3]) and quaternary ammonium compounds (e.g., AMO-1618,[4] eserine,[5] and neostigmine[6]) (Austin and Berry, 1953; Holmes

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[1]Diethyl p-nitrophenyl phosphate.

[2]O,O-diethyl O-p-nitrophenyl phosphorodithioate.

[3]O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate.

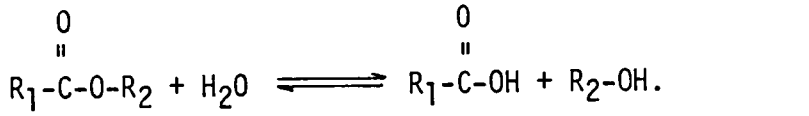
[4](2'-isopropyl-4'-(trimethylammonium chloride)-5-methylphenyl piperidine-1-carboxylate.

[5]Physostigmine.

[6](m-hydroxyphenyl)-trimethylammonium bromide dimethylcarbamate.

and Masters, 1967; Schwarz, 1967; Riov and Jaffe, 1973c; Kasturi and Vasantharajan, 1976; Hovanec et al., 1977; Lanks et al., 1977). Newhall (1969, 1971) was able to correlate the inhibition of blood serum esterase by quaternary ammonium compounds with plant growth retardation by the same inhibitors, suggesting that perhaps an activity which is biochemically similar to serum esterase is involved in plant growth and metabolism. Since these inhibitors are often used as pesticides, research concerning the esterase system of higher plants may yield information concerning the effects of insecticides and herbicides on crop plants during growth and grain storage. Such studies may also provide a basis for the evaluation of herbicide effectiveness and quality.

Carboxylic ester hydrolases catalyze the reaction:



Schwarz (1967) lists a classification scheme based on Dixon and Webb (1964) for the carboxylic ester hydrolase activities which are of interest in plant systems. These enzymes as described in animal systems, the reaction which they catalyze, and their responses to certain inhibitors are (Froede and Wilson, 1971; Holmes and Masters, 1967; Krisch, 1971; Schwarz, 1967):

3.1.1.1 Carboxylesterases (ali-esterase, B-esterase) = carboxylic ester hydrolase

A carboxylic ester + H_2O $\rightleftharpoons$ an alcohol + carboxylate

Inhibited by and do not hydrolyze organophosphates; not inhibited by eserine.

3.1.1.2 Arylesterase (A-esterase) = aryl-ester hydrolase

A phenyl acetate + $H_2O \rightleftharpoons$ a phenol + acetate

Not inhibited by and hydrolyze organophosphates; not inhibited by eserine.

3.1.1.6 Acetylesterase (C-esterase) = acetic ester hydrolase

An acetic ester + $H_2O \rightleftharpoons$ an alcohol + acetate

Not inhibited by and does not hydrolyze organophosphates; not inhibited by eserine.

3.1.1.7 Acetylcholinesterase (true cholinesterase, cholinesterase) = acetylcholine hydrolase

Acetylcholine + $H_2O \rightleftharpoons$ choline + acetate

Inhibited by both organophosphates and eserine; shows strong substrate inhibition.

3.1.1.8 Cholinesterase (pseudocholinesterase, butyrylcholinesterase) = acylcholine acyl-hydrolase

An acylcholine + $H_2O \rightleftharpoons$ choline + carboxylate

Inhibited by both organophosphates and eserine; does not exhibit substrate inhibition; hydrolyzes butyrylcholine much faster than acetylcholine.

Due to certain differences between plant and animal esterases (e.g., lack of inhibition of enzyme by high substrate levels in plants, priority of inhibition by certain metabolic and metallic ion inhibitors) (Ernst and Hartmann, 1980), the propriety of employing a similar classification scheme in the description of plant esterases is in question and such classifications should no doubt be modified for applications to plant systems.

The research in this report deals with the preliminary characterizations of esterase activities in three agriculturally significant monocots, Hordeum vulgare, Oryza sativa, and Zea mays. Since available

useful information concerning characterization of esterase activities in maize and barley (Fluck and Jaffe, 1974c, 1975) is limited, these two sources were investigated. Esterase activities in rice were studied because this plant has been studied only from the viewpoint of electrophoretic mobility of esterases as related to the genetics of the plant (Gupta and Singh, 1977).

CHAPTER II

HISTORICAL REVIEW

The first definite observaticn of a plant esterase other than pectin-esterase was reported by Jansen et al. (1947, 1948; see Appendix A: Historical Summary) in citrus fruits. Esterases had been observed by MacDonnell et al. (1945) as "contaminants" in pectinesterase assays (Jansen et al., 1947). Subsequent identifications were reported by Mounter and Mounter (1962) in wheat germ and by Tzagoloff (1963a, b) in mustard. Jooste and Moreland (1963) extracted esterases from several plants (wheat, maize, soybean, and cucumber) and performed substrate (2-naphthyl acetate) and inhibitor studies and electrophoretic mobility experiments. A thorough survey of the esterases of the Cucurbitaceae was presented by Schwartz et al. (1964). Following the early reports, subsequent electrophoretic, substrate, and inhibitor studies were performed with a variety of plants from several families including the Leguminoseae, Malvaceae, Graminae, Solanaceae, and Pinaceae (Schwarz, 1967; Norgaard and Montgomery, 1968; Cherry et al., 1972; Williamson et al., 1968; Bhatia and Nilson, 1969; Shahi et al., 1969; Prentice et al., 1971; Sae et al., 1971; Orgell et al., 1958; Desborough and Peloquin, 1967; Bartels, 1971; Rudin and Rasmusson, 1973).

Most of the research concerning the esterases has been performed using animal systems. In these systems, ion fluxes and bioelectrical potential changes are induced by the neurohumor hormones such as acetylcholine and γ-aminobutyric acid. In the central nervous system,

for example, acetylcholine is involved directly with the elicitation and propagation of neural response and action potential. When the potential reaches the dendron, acetylcholine is released from the motor end plate, presumably from the synaptic vesicles. Receptor sites in the opposing axonal membrane are activated due to diffusion of acetylcholine across the synapse with the result that the permeability of the axonal membrane to ions is increased.

The enhanced ionic flow generates a potential in the end plate which results in waves of depolarization when a threshold is attained, eventually resulting in muscular contraction. The response is limited in duration due to the hydrolysis of acetylcholine by acetylcholinesterase at the axon (Bloom and Fawcett, 1968).

Dettbarn (1962) compared acetylcholinesterase activity in animal systems and in Nitella, a fresh-water alga, and discussed the similarity of bioelectrical potential generation in plants and animals. Jaffe (1970) described the induction of a bioelectrical potential in mung bean roots that he attributed to increased H^+ efflux caused by exogenously applied acetylcholine. In this case, Jaffe found that red light elicits a similar response. Such a phenomenon suggests that red light irradiation effects the release of bound acetylcholine. Jaffe believes this response to be mediated by phytochrome, with acetylcholine acting as a local hormone which enhances H^+ efflux. The primary observation is that ion flux alterations and membrane permeability regulation due to acetylcholine correspond to responses observed in animal systems.

Yunghans and Jaffe (1972) reported that exogenously applied acetylcholine exhibits a photomimetic effect in the regulation of ATP

concentration and increased rate of oxygen uptake, implying that acetylcholine may serve as the intermediary between phytochrome and morphogenic responses. While no specific mechanism has been observed, these workers propose that acetylcholine causes changes in membrane permeability by acting on an unidentified receptor which may induce conformational changes resulting in the efflux and influx of ions or hormones.

The model of regulation of phytochrome-mediated responses by acetylcholine as proposed by Jaffe (1972) involves the "activation" of acetylcholine by red light and the "activation" of acetylcholinesterase by far-red light. When acetylcholine is synthesized or activated under the influence of red light, the compound moves to the so-called target sites by random diffusion. These target sites consist of the nucleus where Ca^{2+} is released, the plasmalemma where a proton pump is initiated, and the mitochondria where monovalent cations are transferred across the mitochondrial membrane. Calcium accumulation and minimal calcium levels for movement have been demonstrated in the slime mold Physarum. Analogies have been drawn between the slime mold system and animal muscle requirements for Ca^{2+} and the Ca^{2+} sequestering capability of the muscular sarcoplasmic reticulum (Britz, 1979). It should be pointed out, however, that such phenomena have been neither reported nor alluded to in higher plant systems.

Opponents to these views point out that the potential changes observed by Jaffe (1970) are due to interference of K^{+} flow by acetylcholine (Tanada, 1972). Further, Kasemir and Mohr (1972) and Saunders and McClure (1973) found that acetylcholine does not exhibit photomimetic effects and Satter et al. (1972) and Satter (1979) report

that acetylcholine titer remains constant during the rhythmic movement cycle and following phytochrome conversion in Albizzia.

Hartmann (1971) found that endogenous levels of acetylcholine (as determined by frog heart ventricular contraction) are higher in moss callus that has been grown under red light as opposed to callus grown under red and far-red light. More recently, Hartmann (1979) and Ernst and Hartmann (1980) continue to insist that levels of acetylcholine are controlled secondarily by light and primarily by phytochrome. They concede, however, that there is no information as yet concerning the significance of any interactions between phytochrome and acetylcholine.

Hartmann (1979) has demonstrated the in vivo synthesis under red light of labelled acetylcholine from labelled acetate with 97% efficiency in Phaseolus vulgaris seedlings. Based on this observation and on the proven presence of choline acetyltransferase[1] in several plant systems (Hartmann, 1979), including bean hypocotyl hooks (noted by Hartmann but no citation given), Hartmann proposes that the machinery exists in vivo for the synthesis of the naturally occurring substrate of acetylcholinesterase.

A model involving a link between choline acetyltransferase and acetylcholinesterase was advanced by Bennet-Clark (1956). He proposed that the esterase system of higher plants is involved with the lecithinase-D and choline acetyltransferase system in ion uptake by the formation of a lipid-soluble lecithin-salt complex at the membrane level.

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[1] Choline + acetyl CoA $\xrightarrow{\text{choline acetyltransferase}}$ acetylcholine.

This complex is hydrolyzed and the salt is released inside the membrane boundary, along with choline and phosphatidic acid. Acetylcholine is then resynthesized by choline acetyltransferase. At this point, cholinesterase acts as a transferase, replacing the acetyl ion with a phosphatidyl radical, so that lecithin is resynthesized at the membrane and the cycle is repeated.

Other effects of exogenously applied acetylcholine include enhanced slime mold amoeboid movement (Britz, 1979) and the inhibition of mycelial sporulation (Gressel et al., 1971). In addition to the presence of acetylcholine in slime mold, Nakajima and Hatano (1962) report the presence of a cholinesterase activity in Physarum. The activities have been localized near invaginations in the plasma membrane of Physarum and near possible sites of actin filament attachment. Britz (1979) leaves open the possibility that the activities in Physarum may be involved in differences in potential related to cytoplasmic streaming.

Some workers have suggested interactions between acetylcholine and certain growth regulators. In those species in which ethylene inhibits the opening of hypocotyl hooks, acetylcholine has been observed to inhibit the biosynthesis of ethylene (Kang, 1979; Parups, 1976). Riov and Jaffe (1973c) have proposed that acetylcholine functions as a growth inhibitor by inhibition of gibberellic acid biosynthesis. This suggestion is based on the observation that AMO-1618, a potent cholinesterase inhibitor, is also inhibitory to the synthesis of gibberellic acid (Dennis et al., 1965). This inhibition would presumably function as follows: (1) AMO-1618 inhibits the hydrolytic activity of cholinesterase, leading to increased levels of

acetylcholine; (2) increased levels of acetylcholine inhibit the synthesis of gibberellic acid; (3) decreased levels of gibberellic acid result in growth inhibition. This suggestion is based in part on the observation of Jaffe (1970) who found that exogenous acetylcholine inhibits the development of secondary roots in mung bean seedlings.

Dekhuijzen (1973), however, reports that acetylcholine reverses the inhibition of growth of wheat seedlings by CCC (2-chloroethyl trimethyl ammonium chloride). CCC decreases endogenous levels of gibberellic acid in higher plants and is known to inhibit the cyclization of gibberellic acid precursors in the fungus Gibberella (Lang, 1970). Jaffe (1972) proposed that CCC merely enhances the effectiveness of acetylcholinesterase, resulting in lower endogenous levels of acetylcholine, so that, in effect, CCC should work as a growth enhancer, not inhibitor. According to Dekhuijzen, this is not the case. The effect of CCC on cholinesterase activity has never been reported in the literature.

Jaffe (1972) believes that the active principle in his mung bean root adhesion studies is acetylcholine, based on the specificity of the clam heart ventricle assay for acetylcholine and its responses to eserine, atropine, and benzoquinonium chloride[2] (a skeletal muscle relaxant). Nonetheless, the metabolic role of acetylcholine is still unclear, and indeed, acetylcholine may not be the natural substrate of acetylcholinesterase-like activity found in higher plants. Riov and

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[2]Mytolon; WIN 2747; (2,5-p-benzoquinonylenebis(iminotrimethylene)) bis(benzyldiethylammonium chloride).

Jaffe (1973c) suggest two possibilities: (1) acetylcholine may be a native growth retardant, as mentioned earlier, so that a buildup in acetylcholine concentration occurs when acetylcholinesterase is inhibited, or (2) cholinesterase is biochemically similar to those enzymes which are truly responsible for cholinesterase activity *in vivo*, its inhibition is merely coincidental, and there is no relation between acetylcholine and growth.

Recent attempts at characterization of plant esterases have been mainly limited to dicots, primarily within the genus *Phaseolus*. Further, most of the extraction processes have involved the investigation of the cell wall bound fraction of the activity (see Table 1). Based on cytochemical localization, Fluck and Jaffe (1975) found that 90% to 95% of the extractable acetylcholine hydrolyzing activity is associated with the cell wall in the roots of *Phaseolus aureus*. Lees and Thompson (1975), however, found the primary source of this activity in the cotyledons of *Phaseolus vulgaris* to be soluble and the extractable portion from the cell wall to be highly variable. Schwarz (1967) found strong activity in the soluble root extracts of *Pisum*, but Kasturi and Vasantharajan (1976) found no soluble activity in the same source. The material here deals only with soluble activities.

Reports dealing with the relationship between certain inhibitors and esterase activities are summarized in Tables 2, 3, and 4. Inhibitors which have been studied have been limited mainly to eserine, neostigmine, and AMO-1618. The I_{50} effects of eserine (Table 2) appear to be in the approximate range of 0.1 mM to 1.0 mM in the sources which have been studied. The inhibitory effects of neostigmine (Table 3) appear to be

Table 1. Reported K_m values for cholinesterase and acetylcholinesterase activities from several sources.

Source	Cell Wall/ Soluble Fraction	Substrate	K_m	Reference
Pisum sativum	Soluble	IPA*	--	Schwarz, 1967
Sorghum	Cell wall	IPA	--	Sae et al., 1971
Phaseolus aureus	Cell wall	ATCh**	84 µM	Riov and Jaffe, 1973b
		ACh***	72 µM	
Solanum melongena	Soluble	ATCh	--	Fluck and Jaffe, 1975
Zea mays	Soluble	ATCh	--	Fluck and Jaffe, 1975
Pisum sativum	Cell wall	ATCh	200 µM	Kasturi and Vasantharajan, 1976
		ACh	--	
Phaseolus vulgaris	Cell wall	ATCh	56 µM	Mansfield et al., 1978
Phaseolus vulgaris	Cell wall	ATCh	460 µM	Ernst and Hartmann, 1980
Phaseolus vulgaris	Soluble	ATCh	--	Lees and Thompson, 1975

*Indophenyl acetate.

**Acetylthiocholine.

***Acetylcholine.

Table 2. Reported percent inhibition of esterase activities by eserine in several sources.

Source	Substrate	Inhibitor Conc.	% Inhibition	Reference
Solanum melongena	ATCh	0.1 mM	50	Fluck and Jaffe, 1975
	ATCh	0.7 mM	100	
Zea mays	ATCh	0.1 mM	10.6	
	ATCh	0.7 mM	50	
Phaseolus aureus	ATCh	0.1 mM	26	Riov and Jaffe, 1973b
	ATCh	0.7 mM	45	
Phaseolus vulgaris	ATCh	0.1 µM	10	Ernst and Hartmann, 1980
	ATCh	10.0 mM	65	
	ATCh	0.9 mM	50	Mansfield et al., 1978
Pisum sativum	ATCh	0.9 mM	50	Kasturi and Vasantharajan, 1976
	ATCh	6.0 mM	100	
	ACh	12.0 µM	27	Schwarz, 1967
	ACh	12.0 mM	91	
	IPA	5.1 µM	13	
	IPA	5.1 mM	33	

Table 3. Reported percent inhibition of esterase activities by neostigmine in several sources.

Source	Substrate	Inhibitor Conc.	% Inhibition	Reference
Solanum melongena	ATCh	16.0 nM	50	Fluck and Jaffe, 1975
	ATCh	8.0 µM	100	
Zea mays	ATCh	16.0 nM	0	
	ATCh	8.0 µM	50	
Phaseolus aureus	ATCh	16.0 nM	0	
	ATCh	8.0 µM	50	
Phaseolus vulgaris	ATCh	0.1 µM	5	Ernst and Hartmann, 1980
	ATCh	1.0 mM	100	
Pisum sativum	ATCh	0.6 µM	50	Kasturi and Vasantharajan, 1976
	ATCh	4.0 µM	100	

Table 4. Reported percent inhibition of esterase activities by AMO-1618 in several sources.

Source	Substrate	Inhibitor Conc.	% Inhibition	Reference
Eel	ATCh	1.0 mM	100	Riov and Jaffe, 1973c
Serum	ATCh	1.0 mM	100	
Phaseolus aureus	ATCh	1.0 mM	83.3	
Citrus	IPA	1.0 mM	0	
Wheat germ	IPA	1.0 mM	0	
Phaseolus aureus	IPA	1.0 mM	0	
Phaseolus aureus	ATCh	0.1 mM	32	Fluck and Jaffe, 1975
	ATCh	10.0 mM	94	
Solanum melongena	ATCh	0.1 mM	50	
	ATCh	10.0 mM	74	
Zea mays	ATCh	0.1 mM	0	
	ATCh	10.0 mM	39.2	

of a somewhat wider range, 19 mM to 8 μM. From the available data, it is difficult to determine the I_{50} for AMO-1618 (Table 4), but it is obvious that this value in most cases is less than 1.0 mM.

In summary, the esterase enzymes are believed to be responsible for the regulation of ester metabolism *in vivo*, including, perhaps, acetylcholine. While the substrate(s) of the system is (are) still in question, the activities undoubtedly occur in plants. Riov and Jaffe (1973a) purified and characterized a cell wall bound esterase from mung bean roots that was described as a cholinesterase which is unique (Table 5) from those described in animal systems on the basis of the response of the enzyme to eserine. Schwartz et al. (1964) described esterase activity in the Cucurbitaceae. Schwarz (1967) observed a soluble esterase activity in etiolated pea seedlings and also performed various inhibitor studies. Sae et al. (1971) studied soluble esterase activity inhibition by organophosphorus compounds in sorghum grain. Recently, Chandra and Toole (1977) investigated the possibility of esterase activity on the cellulose membrane which surrounds lettuce seed embryos. A number of workers have employed esterase zymograms for genetic and taxonomic studies, specifically Gupta and Singh (1977) in rice and Prentice et al. (1971) in barley.

Within the family Graminae, esterase activity has undergone little study. Fluck and Jaffe (1974c) list only three graminaceous species in which the esterases have been studied from the viewpoint of isolation, *Avena sativa*, *Hordeum vulgare*, and *Zea mays*. In all three studies, however, these authors report no activity in extracts of *Avena* or

Table 5. Comparison of esterase characteristics from P. aureus and animal sources (adapted from Riov and Jaffe, 1973b).

Characteristics*	P. aureus cholinesterase	Eel acetyl-cholinesterase	Serum cholinesterase
Localization	Membrane	Membrane	Soluble
Rate of hydrolysis of choline esters	Acetyl > propionyl > butyryl	Acetyl > propionyl > butyryl	Butyryl > propionyl > acetyl
pH optimum	8.5 to 8.7	8.0 to 8.3	7.6 to 8.0
Activity curve	Bell-shaped (inhibition by excess substrate)	Bell-shaped (inhibition by excess substrate)	S-shaped (no inhibition by excess substrate)
K_m	72 to 84 μM	100 to 300 μM	Does not follow Michaelis-Menten kinetics
Inhibition by eserine	+	+++	+++
Inhibition by neostigmine	+++	+++	+++

*All characteristics with the exception of rate of hydrolysis are considered solely with acetylcholine or acetylthiocholine as substrate.

Hordeum. The activity reported for Zea (Fluck and Jaffe, 1975) was found to be different from that found in Zea by this worker, so it was felt necessary to pursue characterization of the esterase activities not only from maize, but also from barley. The total lack of biochemical information concerning esterase activities in rice prompted investigation of this source.

CHAPTER III

MATERIALS AND METHODS

A. PREPARATION OF EXTRACTS

Seedlings employed in this study were of 7-day-old maize (Zea mays cv Pioneer 3145), 5-day-old barley (Hordeum vulgare cv Himalaya), and 5-day-old rice (Oryza sativa japonica cv Calrose 76). Maize seedlings were grown in the dark at 25 C in cylinders of moistened paper toweling. The cylinders were placed upright in beakers containing approximately 3 cm water and covered with plastic wrap to conserve moisture. The water level was replenished throughout the growth period as necessary. Barley and rice seedlings were grown in sterilized covered culture dishes containing 2 circles of sterilized Whatman #1 filter paper (sterilized Reeve-Angel glass fiber filter paper was used for rice germination) and 10 ml of sterilized double-distilled water. The seeds were germinated in the dark at 25 C.

The harvesting procedure consisted of removing the embryonic axes from the cotyledons and placing the axes immediately into ice-cold water. When a sufficient number of axes had been collected (100 to 200, depending on organism), the material was blotted dry and weighed. For purposes of surface sterilization, the axes were placed in a beaker containing ice-cold 5% Clorox bleach and swirled manually for 30 sec. This was followed by two or three 15 sec rinses with cold 0.1 N HCl and several rinses with cold double-distilled water until the scent of

bleach was no longer detectable. Subsequent preparations proceeded as follows.

Maize seedlings were ground three times at 5 sec each time at top speed in cold 50 mM potassium phosphate buffer, pH 7.6 containing 10% non-acid-washed polyvinyl pyrrolidone (Polyclar AT) and 1% ascorbic acid in a cold Sorvall Omni-Mixer. The slurry was passed through buffer saturated cheesecloth and the liquid fraction centrifuged. This and all subsequent centrifugations were at 27000 x g for 15 min. Following centrifugation, the supernatant was brought to 30% $(NH_4)_2SO_4$ with stirring over an hour and centrifuged. The supernatant of the 30% fraction was brought to 90% $(NH_4)_2SO_4$ and centrifuged.

The pellet of the 90% fraction was then dissolved in cold 50 mM potassium phosphate buffer, pH 7.6 and applied to a Sephadex G-25 column (commercially available PD-10 disposable column; 1.5 cm x 5 cm). This step is necessary with maize extracts due to the presence of constituents which tend to obstruct flow in the subsequent Sephadex G-200 column application and which cause interference in UV monitor readings. The PD-10 column removes an appreciable amount of this material. The eluant of the PD-10 column was then applied to a Sephadex G-200 column (approximately 2.5 cm x 20 cm). The PD-10 column was run in the cold and the G-200 column was run with a flowing ice water-alcohol cooling jacket. The G-200 column was eluted with 50 mM potassium phosphate buffer at pH 7.6. A flow rate of 1 ml per min was used and fractions of 2 ml each were collected. Protein was monitored by the change in absorbance at 280 nm.

Seedlings of barley and rice were extracted in a similar manner except that polyvinyl pyrrolidone and ascorbic acid were not added to the buffer and the Sephadex G-25 (PD-10) column was not employed. With these sources, the pellet from the 90% $(NH_4)_2SO_4$ precipitation was dissolved in 3 to 5 ml potassium phosphate buffer (50 mM, pH 7.6) and dialyzed overnight at 4 C against the same buffer. The dialysate was applied to a Sephadex G-200 column as was done with maize.

The fractions following the void volume were assayed spectrophotometrically for esterase activity using indophenyl acetate as substrate (IPA; see Appendix B for structure) (Kramer and Gamson, 1958; Schwarz, 1967; Riov and Jaffe, 1973b), for cholinesterase activity using acetylthiocholine as substrate (ATCh; see Appendix B for structure) (Ellman et al., 1961; Riov and Jaffe, 1973b), and for protein as described later in this chapter. Fractions which showed an absorbance change of at least 0.001 per min with acetylthiocholine as substrate were pooled and assayed for enzymatic activity with IPA and ATCh both alone and in the presence of metal ions, inhibitors, at various pH points, and at various temperatures.

B. PREPARATION OF BUFFERS, SUBSTRATES, AND REAGENTS

The buffer used throughout this study, except where noted, was 50 mM potassium phosphate, pH 7.6. Substrates employed were indophenyl acetate (IPA) and acetylthiocholine (ATCh). IPA stocks were prepared by dissolving the solid in 70% ethanol to a final concentration of 25 mM. ATCh stocks were prepared by dissolving the solid in buffer to a final concentration of 20 mM.

DTNB (5,5' dithiobis-(2-nitrobenzoic acid)), the reagent which reacts with the enzymatic product of thiocholine esters, is prepared by dissolving the solid in a minimal amount of alcohol followed by slow addition of buffer containing 9 mg $NaHCO_3$ per 100 ml to a final concentration of 7.4 mM (Riov and Jaffe, 1973b).

All inhibitors and metal stocks were dissolved in buffer and the pH adjusted to 7.6. Stock concentration of all additives was 20 mM.

C. ASSAY PROCEDURES

Esterase assay mixtures contained 150 µl of extract added to 2.8 ml of buffer. The reaction is initiated by the addition of 50 µl of 25 mM IPA (IPA reaction concentration 0.42 mM). The mixture was allowed to equilibrate for 30 sec and was followed at 625 nm at 25 C (except for temperature studies). The extinction coefficient $e^{1\ cm}_{625\ nm}$ of the product (indophenol) is 13.6 mM^{-1} (Riov and Jaffe, 1973b). The unit of activity for this reaction is defined as the formation of 1 µM of indophenol per min. The use of IPA as a substrate in the spectrophotometric determination of esterase activity was originally proposed by Kramer and Gamson (1958, 1960). The reaction involves the hydrolysis of IPA to indophenol and is presented in Appendix C.

Cholinesterase assay mixtures contained 300 µl of extract added to 2.25 ml of buffer. To this was added 300 µl of 20 mM ATCh (final assay concentration of 2 mM) and 150 µl DTNB (final assay concentration 0.37 mM) which initiates the colorimetric reaction. The mixture was allowed to equilibrate for 3 to 5 min and was followed at 412 nm at 25 C (except for temperature studies), with the unit of activity being

defined as 1 μM of product formed per min. The reaction employed in this assay was first described by Ellman et al. (1961) and involves the hydrolysis of the thiocholine ester to a thiocholine group and an acetate ion. DTNB undergoes reduction due to cleavage of the disulfide bond to form two molecules of 5-thionitrobenzoate. One thiocholine molecule oxidizes one 5-thionitrobenzoate molecule, resulting in the formation of 2-nitro, 5-thiobenzoate. This chromophore absorbs maximally at 412 nm and has an extinction coefficient $e^{1\ cm}_{412\ nm}$ of 13.8 mM^{-1} (Riov and Jaffe, 1973b). The reaction is presented in Appendix C.

Assay methodologies for the determination of the effects of temperature, pH, metal ions, and inhibitors are outlined as follows:

Temperature:

1. Prepare buffer and extract in test tube with respective volumes adjusted to allow for final assay volume of 3 ml.
2. Allow to equilibrate to desired temperature and allow to incubate for 10 min. Temperatures studied were at approximate 10 degree increments from 2 C to 70 C.
3. Chill in ice bath for 30 to 60 sec.
4. Add substrate to desired concentration.
5. Transfer to cuvette.
6. Record reaction at appropriate wavelength following appropriate equilibration period.

pH:

In addition to phosphate buffer (KH_2PO_4), borate buffer (H_3BO_3) at 50 mM was used from pH 8.0 to 9.0. Also, problems

were encountered due to the sensitivity of indophenol to pH below pH 7.0 and above pH 8.0 (Kramer and Gamson, 1958) and the sensitivity of the thiocholine ester-DTNB assay below pH 6.5 and above pH 8.5 (Gorun et al., 1978).

Between pH 7.0 and pH 8.0, the following procedure was followed:

1. Adjust buffer to desired pH.
2. Prepare buffer and extract in cuvette to give a final assay volume (including substrate) of 3 ml.
3. Add substrate to desired concentration.
4. Record as before following equilibration.

Below pH 7.0 and above pH 8.0, the following procedure was followed:

1. Adjust buffer to desired pH.
2. Prepare buffer and extract in 10 ml graduated cylinder to allow to a final assay volume (including substrate) of 5 ml.
3. Add substrate to desired concentration.
4. Allow reaction to proceed for time period desired.
5. Adjust to pH 7.6 with HCl or KOH.
6. Bring volume to 5 ml with phosphate buffer (50 mM at pH 7.6).
7. Transfer to cuvette.
8. Record absorbance for that time point.
9. Repeat procedure for each different time point desired.

Metal ions and inhibitors:

1. Prepare buffer and extract in test tube allowing for the addition of additive and substrate and for pH adjustment with final assay volume of 5 ml.

2. Add metal solution or inhibitor to appropriate concentration.
3. Adjust pH if necessary (this can be determined beforehand).
4. Bring to volume allowing for addition of substrate.
5. Allow to incubate for 10 min in ice bath.
6. Add substrate to desired concentration.
7. Record as before.

All blanks were without extract. All inhibitor, metal, and substrate studies were assayed in cuvette volumes of 3 ml. All experiments were performed at least twice and all points represent a minimum of two replicates per experiment.

D. PROTEIN ASSAYS

Samples reserved for protein determination were treated with an equal volume of 10% trichloroacetic acid and kept in the cold for 30 min. Following centrifugation and two washes with 10% trichloroacetic acid, each followed by centrifugation, the pellets were dissolved in 0.1 N NaOH. Replicate protein assays were performed by the method of Lowry et al. (1951) and protein concentrations were determined from a standard curve of bovine serum albumin.

CHAPTER IV

RESULTS

A. PURIFICATION

Methods of purification of barley and rice esterase activities are shown in Figure 1 and the purification protocol for the activities from maize are shown in Figure 2. Purification and percent recovery data for barley, rice, and maize are presented in Tables 6, 7, and 8, respectively. The cause of the decrease of protein concentration between ammonium sulfate fractions was not determined. The elution profiles from Sephadex G-200 gel filtration are shown in Figures 3, 4, and 5. Greatest activity was eluted just after the void volume in all three sources.

B. TIME COURSE OBSERVATIONS

Following appropriate equilibration periods as outlined in the Materials and Methods Chapter, all time course observations were found to be linear with time (Figures 6 and 7). The rate of reaction was found to be proportional to enzyme concentration in all cases.

C. REPRODUCIBILITY OF RESULTS

All data which are presented represent the results of at least two experiments with at least two data points for each value per experiment. Replicates within experiments varied by less than 10% and

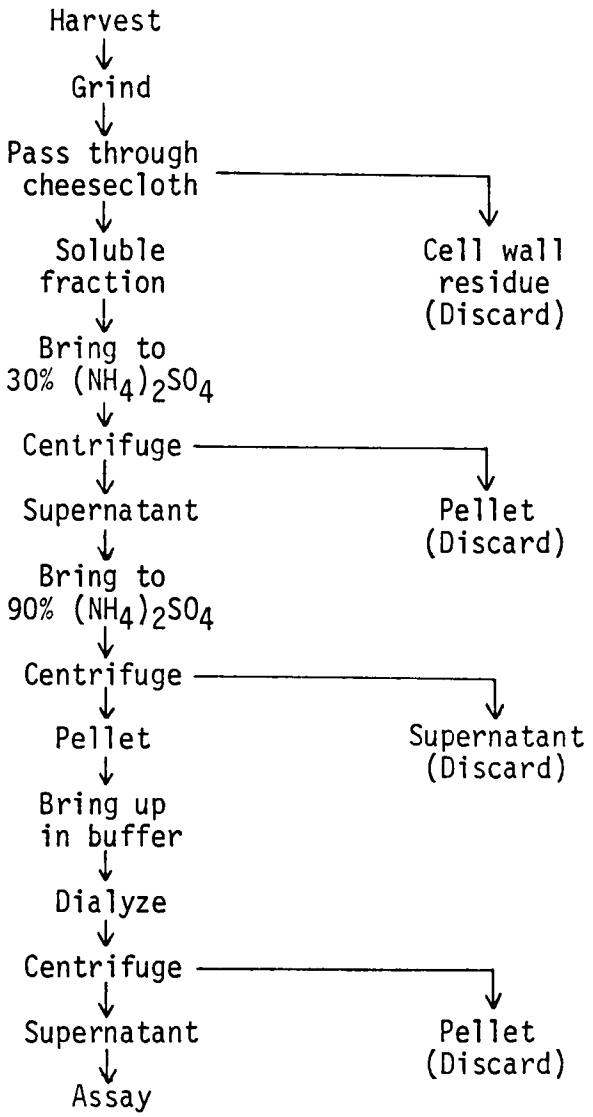

Figure 1. Purification protocol for barley and rice.

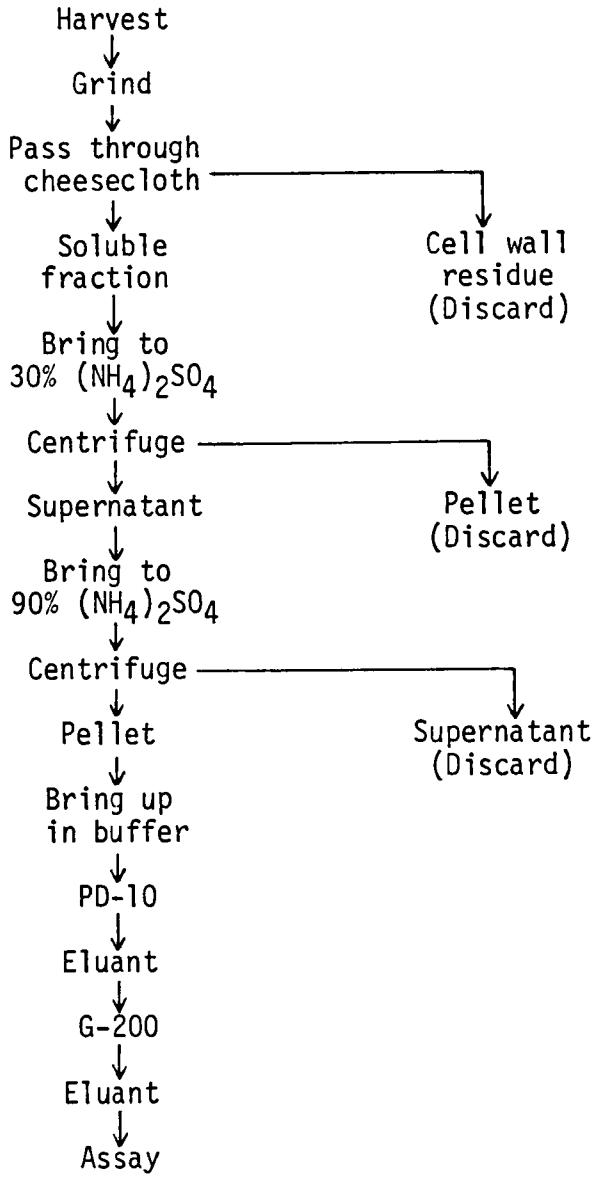

Figure 2. Purification protocol for maize.

Table 6. Purification data for ATCh and IPA hydrolytic activity in barley seedlings.

Fraction	Specific Activity (Units/mg Protein)	Total Protein (mg)	Total Activity (Units)	Recovery
	ATCh Hydrolytic Activity			
Crude	0.18	260	46.8	100%
30% supernatant	0.18	212	38.2	82%
30% to 90% pellet	0.31	85	26.0	56%
G-200 eluant	1.68	10	16.8	36%
	IPA Hydrolytic Activity			
Crude	1.18	260	306.8	100%
30% supernatant	1.31	212	277.7	91%
30% to 90% pellet	1.45	85	123.0	40%
G-200 eluant	8.32	10	83.2	27%

Table 7. Purification data for IPA hydrolytic activity in rice seedlings.

Fraction	Specific Activity (Units/mg Protein)	Total Protein (mg)	Total Activity (Units)	Recovery
Crude	0.92	354	325.7	100%
30% supernatant	0.96	283	271.7	83%
30% to 90% pellet	1.66	117	194.6	60%
G-200 eluant	14.64	9	131.8	40%

Table 8. Purification data for ATCh and IPA hydrolytic activity in maize seedlings.

Fraction	Specific Activity (Units/mg Protein)	Total Protein (mg)	Total Activity (Units)	Recovery
	ATCh Hydrolytic Activity			
Crude	0.24	390	93.6	100%
30% supernatant	0.28	280	78.4	84%
30% to 90% pellet	0.43	105	45.4	49%
PD-10 eluant	0.43	102	43.9	47%
G-200 eluant	2.28	13	29.6	32%
	IPA Hydrolytic Activity			
Crude	1.77	390	689.0	100%
30% supernatant	2.15	280	602.0	87%
30% to 90% pellet	3.02	105	316.8	46%
PD-10 eluant	3.04	102	309.6	45%
G-200 eluant	19.11	13	248.4	36%

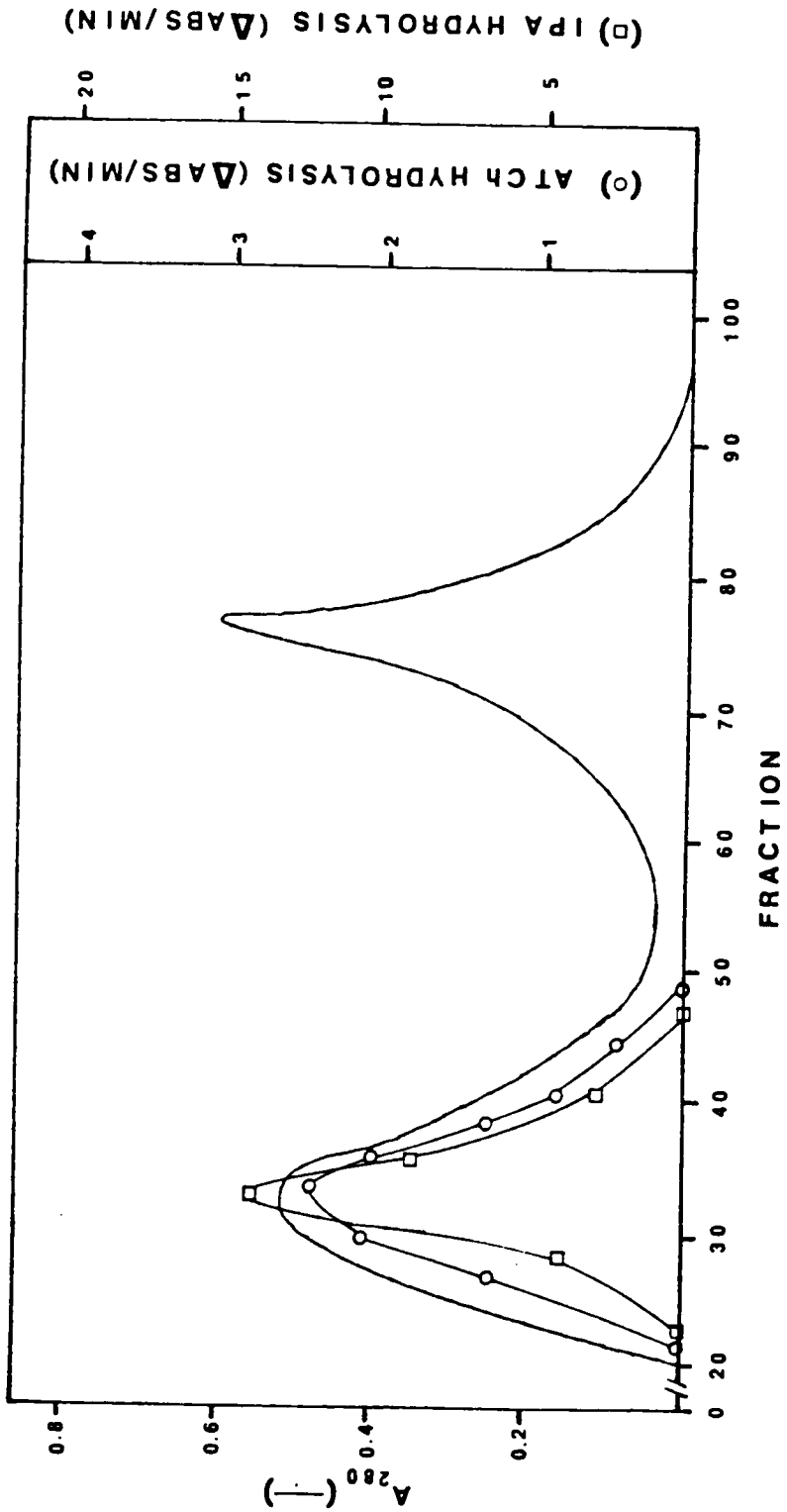

Figure 3. Elution profile of barley extract from Sephadex G-200 gel filtration. Protein concentration was monitored at 280 nm while ATCh and IPA hydrolytic activities were assayed at 412 nm and 625 nm, respectively. The solubilized pellet of the 30% to 90% $(NH_4)_2SO_4$ fraction was applied to the column and fractions were collected as described in Materials and Methods: Preparation of Extracts.

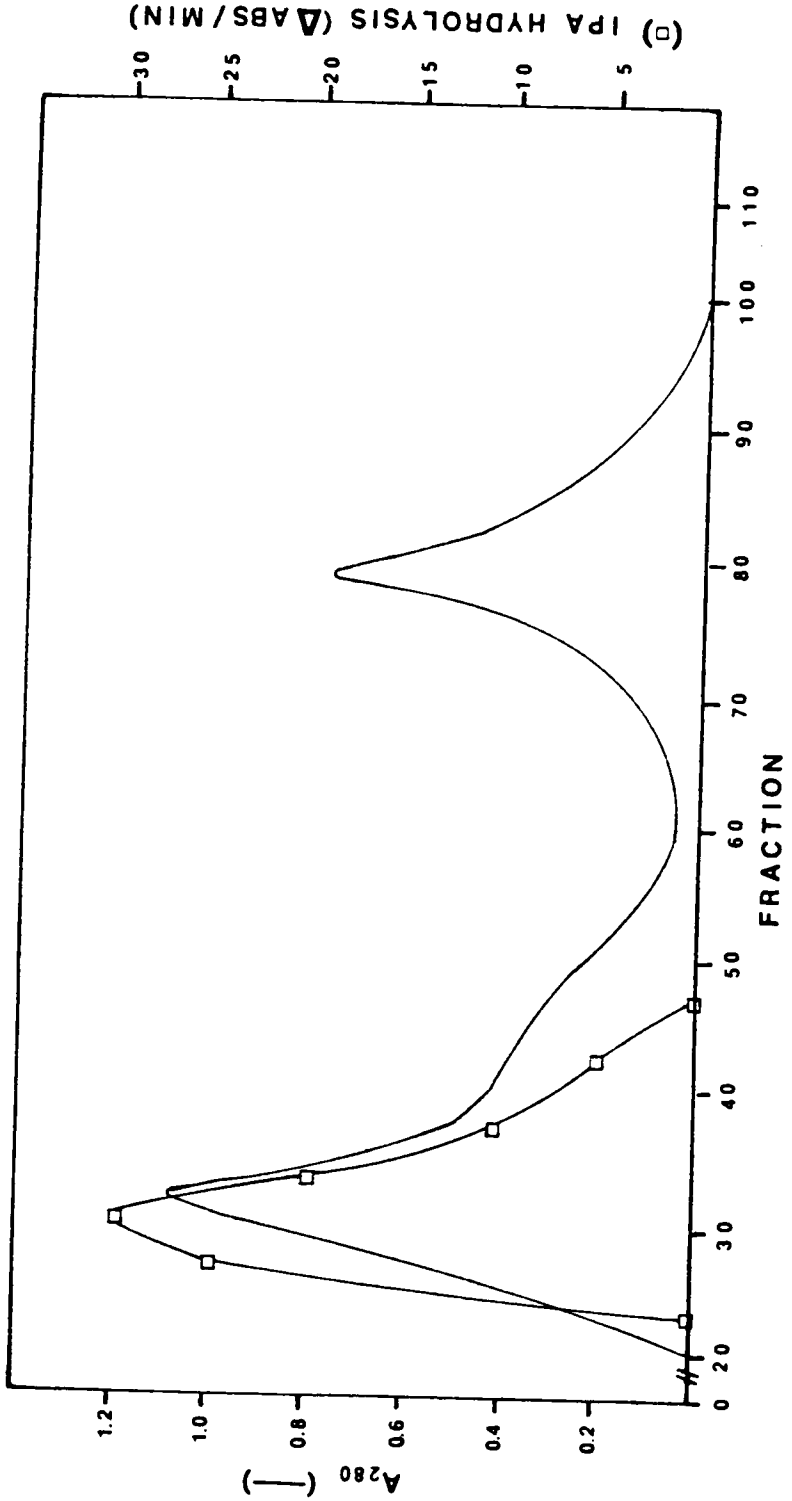

Figure 4. Elution profile of rice extract from Sephadex G-200 gel filtration. Protein concentration was monitored at 280 nm and IPA hydrolytic activity was assayed at 625 nm. The solubilized pellet of the 30% to 90% $(NH_4)_2SO_4$ fraction was applied to the column as described in Materials and Methods: Preparation of Extracts.

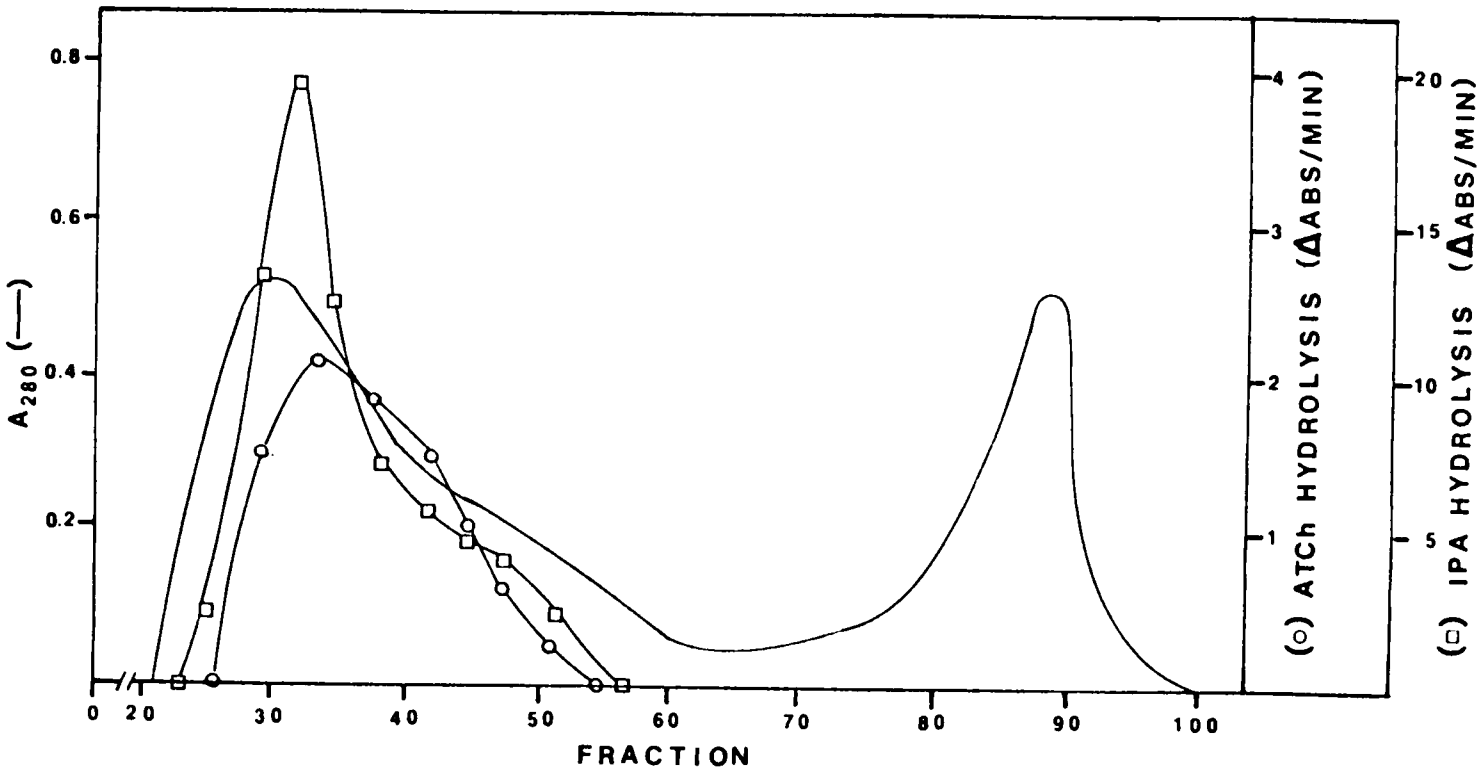

Figure 5. Elution profile of maize extract from Sephadex G-200 gel filtration. Protein concentration was monitored at 280 nm while ATCh and IPA hydrolytic activities were assayed at 412 nm and 625 nm, respectively. The G-25 eluant of the solubilized 30% to 90% $(NH_4)_2SO_4$ fraction was applied to the column and fractions were collected as described in Materials and Methods: Preparation of Extracts.

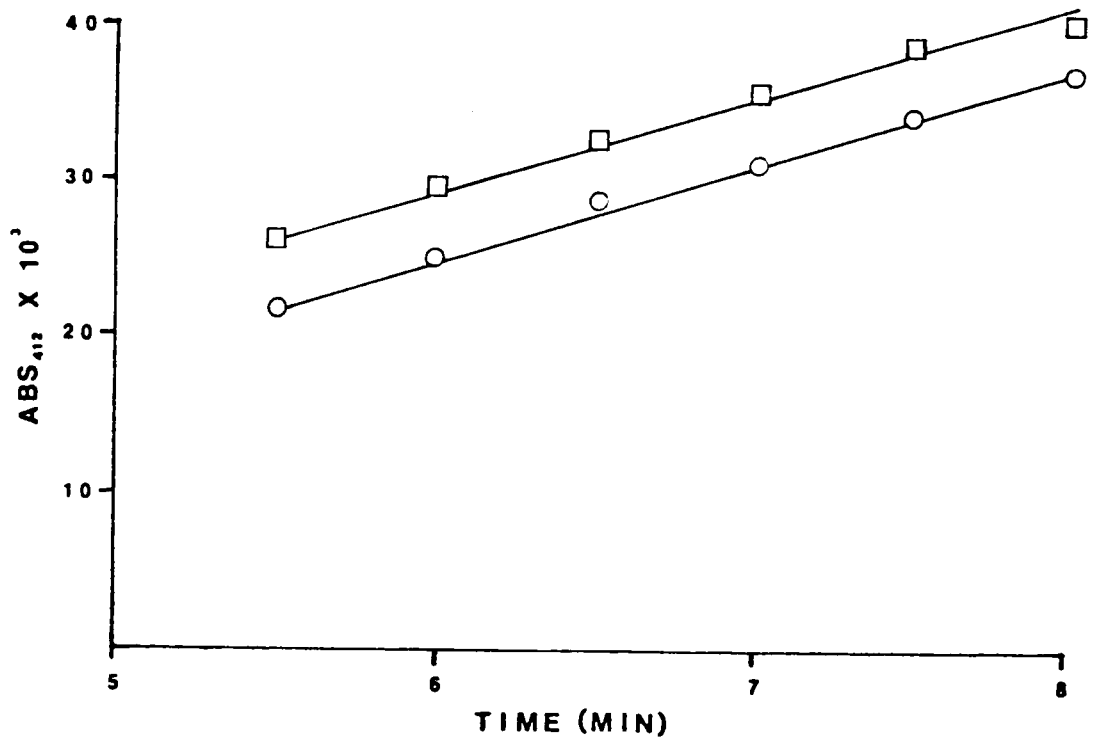

Figure 6. Linearity of ATCh hydrolysis over time. The assay mixture was allowed to equilibrate for 5 minutes and assayed as described in Materials and Methods: Assay Procedures. ATCh concentration was 2 mM with DTNB concentration of 0.37 mM. (□) Maize extract; (○) Barley extract.

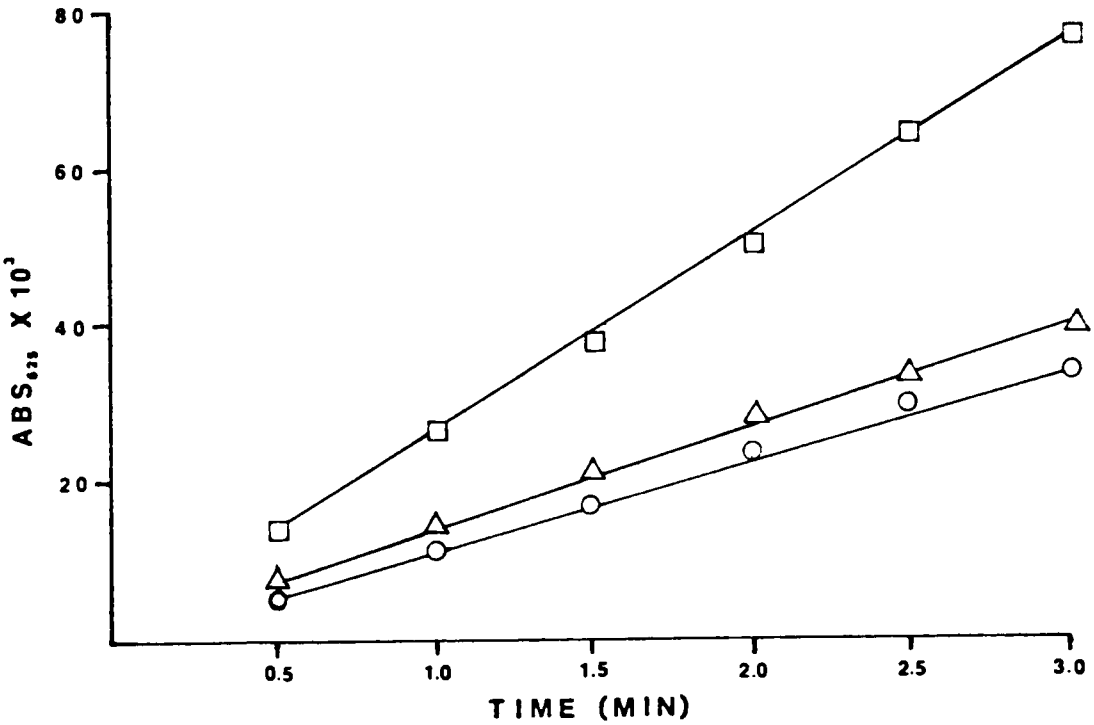

Figure 7. Linearity of IPA hydrolysis over time. The assay mixture was allowed to equilibrate for 30 seconds and assayed as described in Materials and Methods: Assay Procedures. IPA concentration was 0.42 mM. (□) Maize extract; (△) Rice extract; (○) Barley extract.

variation among experiments, after correction for protein concentration, was less than 20%.

D. pH OPTIMA

Figures 8, 9, and 10 show the effects of pH on ATCh and IPA hydrolytic activities in the G-200 fractions of barley, rice, and maize, respectively. The optimum pH for ATCh hydrolysis in both maize and barley occurs in the range of pH 7.8 to 8.2, while that of IPA hydrolysis is between pH 7.0 to 7.6 in barley and maize and between pH 7.0 to 7.4 in rice.

E. TEMPERATURE EFFECTS

The enzyme extracts were incubated at various temperatures for 10 min, cooled in an ice bath and assayed as previously described. In all cases, sensitivity to temperatures above 30 C is observed. IPA hydrolytic activity is apparently less sensitive to increasing temperature than is ATCh hydrolytic activity, but definite decreases in enzyme activity occur in both assays at extreme temperatures regardless of enzyme source. Results from these experiments are shown in Figures 11, 12, and 13.

F. METAL EFFECTS

The effects on esterase activities by various divalent metal cations and EDTA were investigated. The results are summarized in Table 9. The concentration of all ions and EDTA was 1 mM and all assays

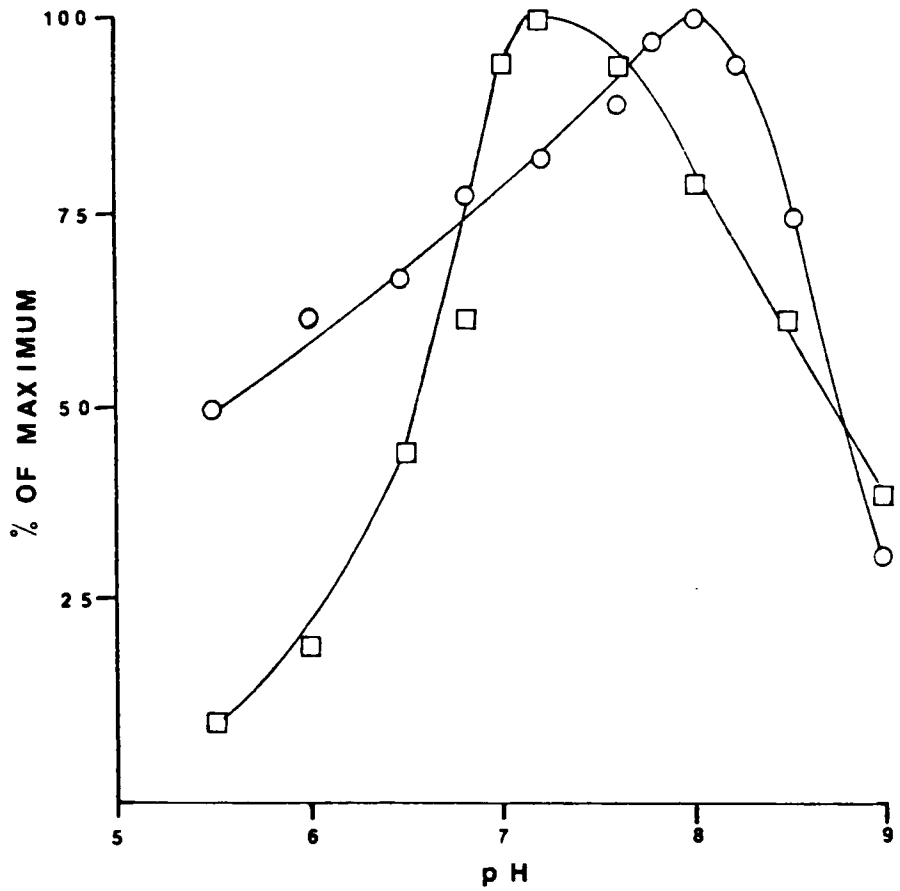

Figure 8. Effect of pH on ATCh and IPA hydrolytic activities in barley extract. Phosphate buffer was used from pH 5.5 to pH 8.0 while borate buffer was used above pH 8.0. Assays were performed as described in Materials and Methods: Assay Procedures. (□) IPA; (○) ATCh.

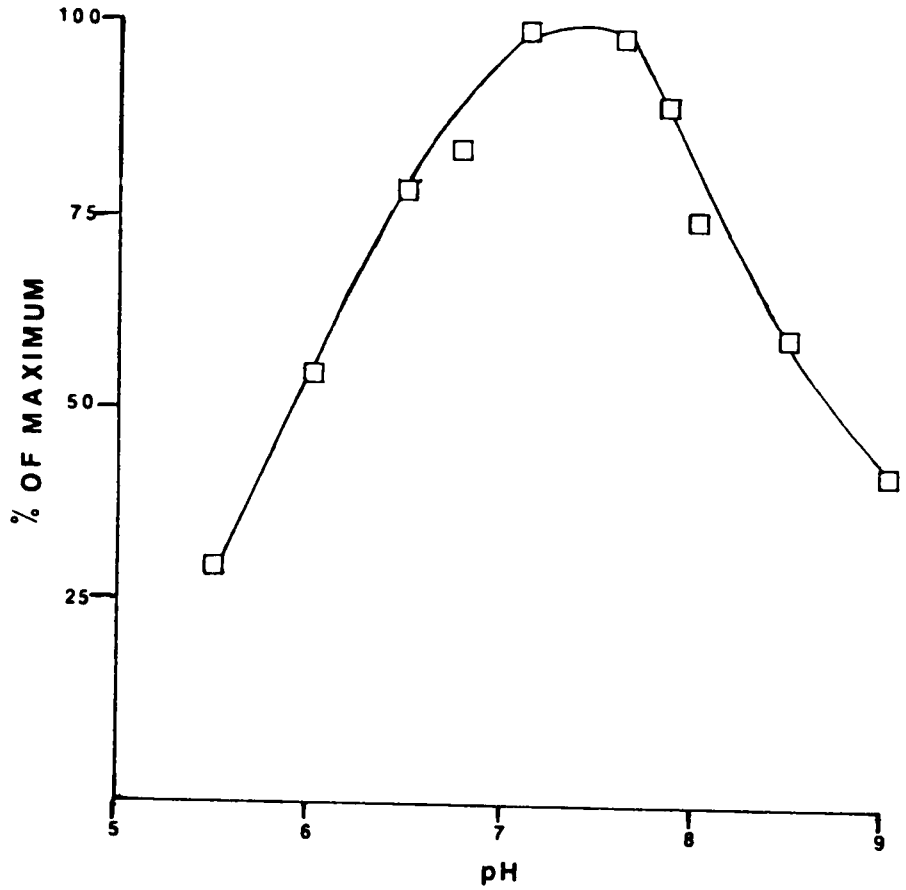

Figure 9. Effect of pH on IPA hydrolytic activity in rice extract. Phosphate buffer was used from pH 5.5 to pH 8.0 while borate buffer was used above pH 8.0. Assays were performed as described in Materials and Methods: Assay Procedures. (□) IPA.

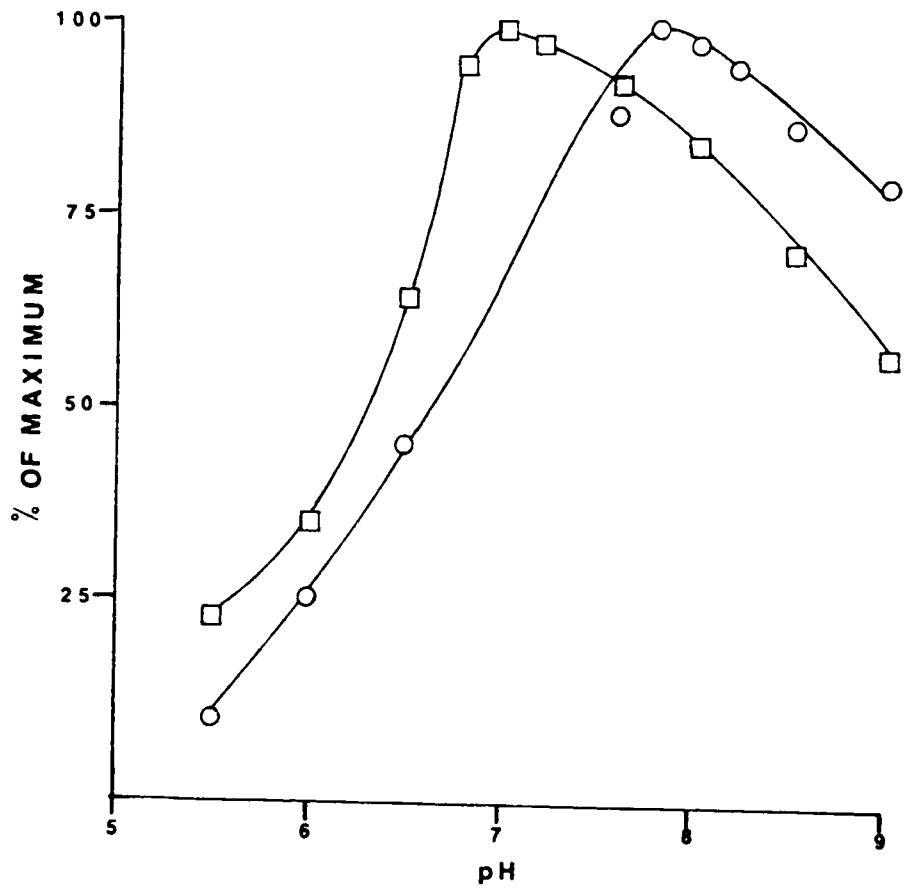

Figure 10. Effect of pH on ATCh and IPA hydrolytic activities in maize extract. Phosphate buffer was used from pH 5.5 to pH 8.0 while borate buffer was used above pH 8.0. Assays were performed as described in Materials and Methods: Assay Procedures. (□) IPA; (○) ATCh.

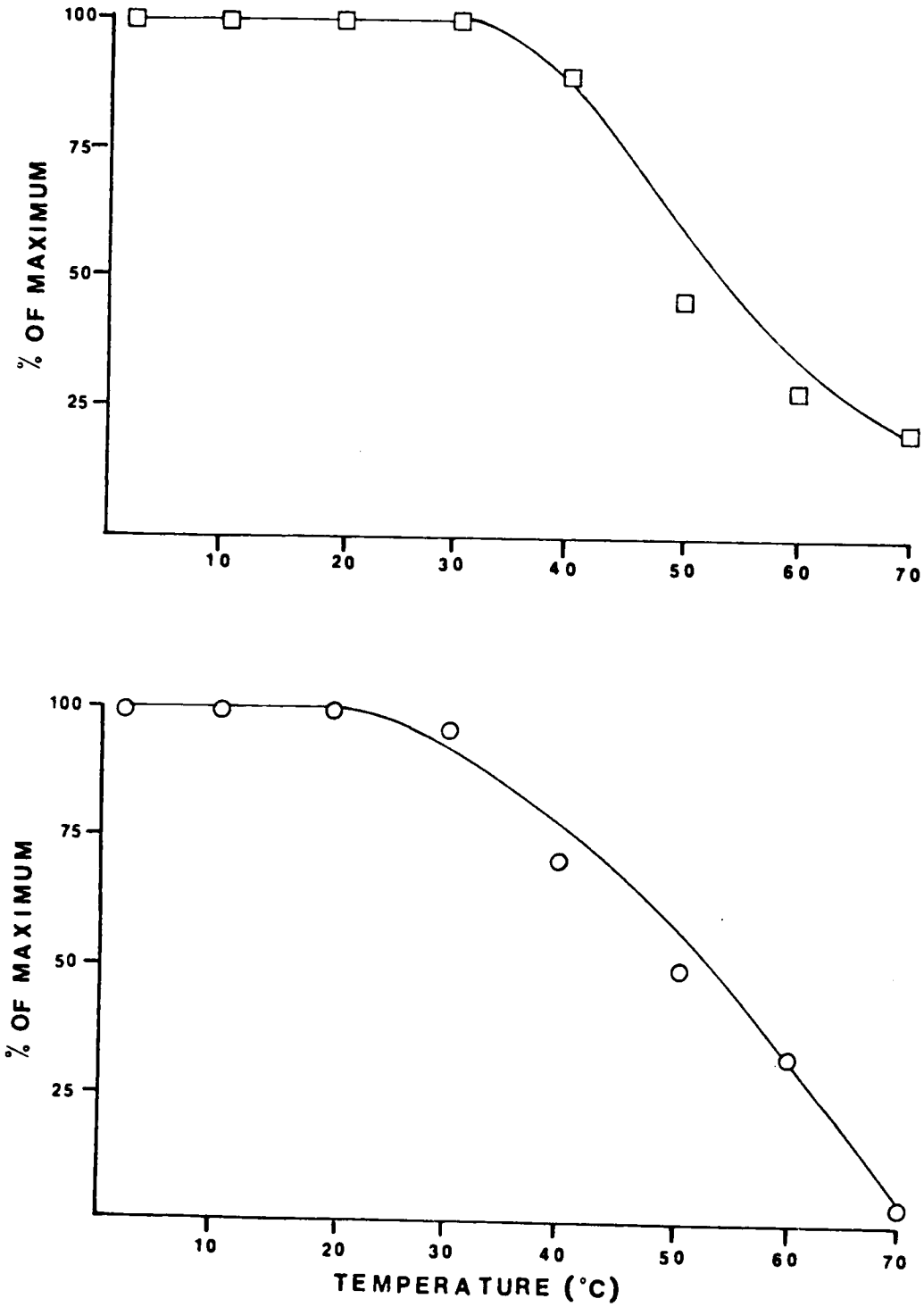

Figure 11. Effect of temperature on ATCh and IPA hydrolytic activities in barley extract. Following incubation at the designated temperature, the assay mixtures were chilled in an ice bath and assayed as described in Materials and Methods: Assay Procedures. (□) IPA; (○) ATCh.

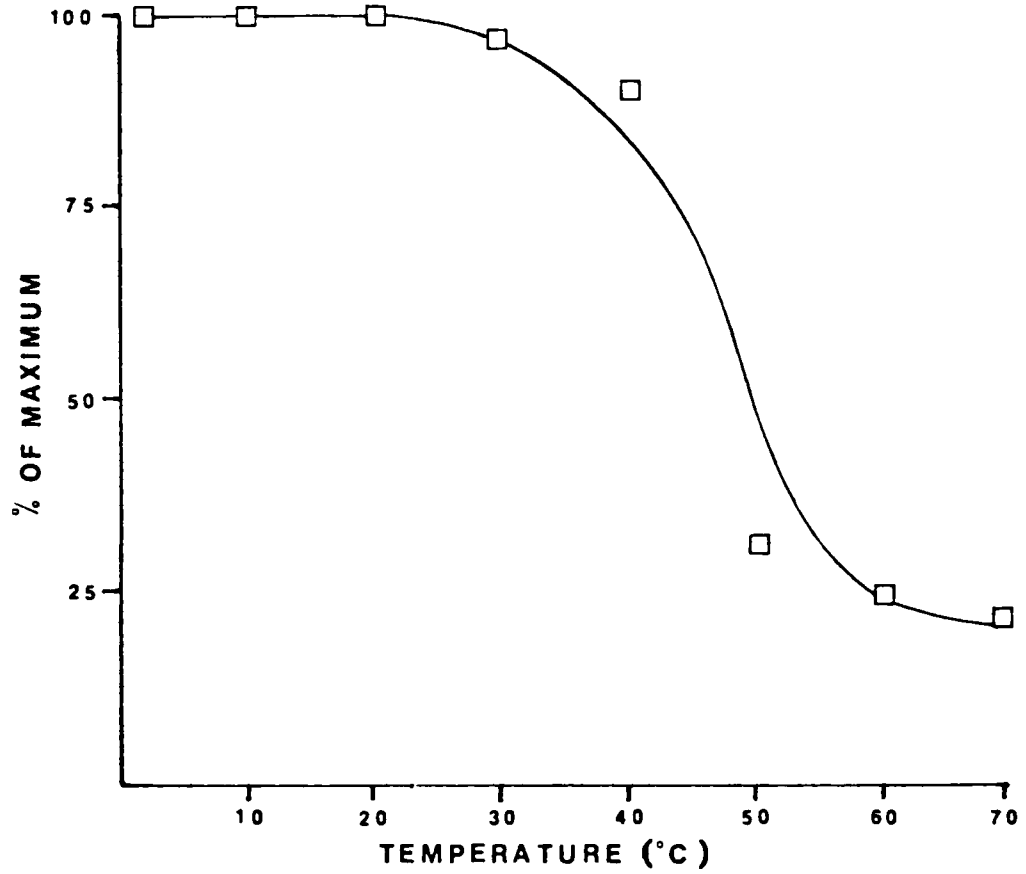

Figure 12. Effect of temperature on IPA hydrolytic activity in rice extract. Following incubation at the designated temperature, the assay mixtures were chilled in an ice bath and assayed as described in Materials and Methods: Assay Procedures. (□) IPA.

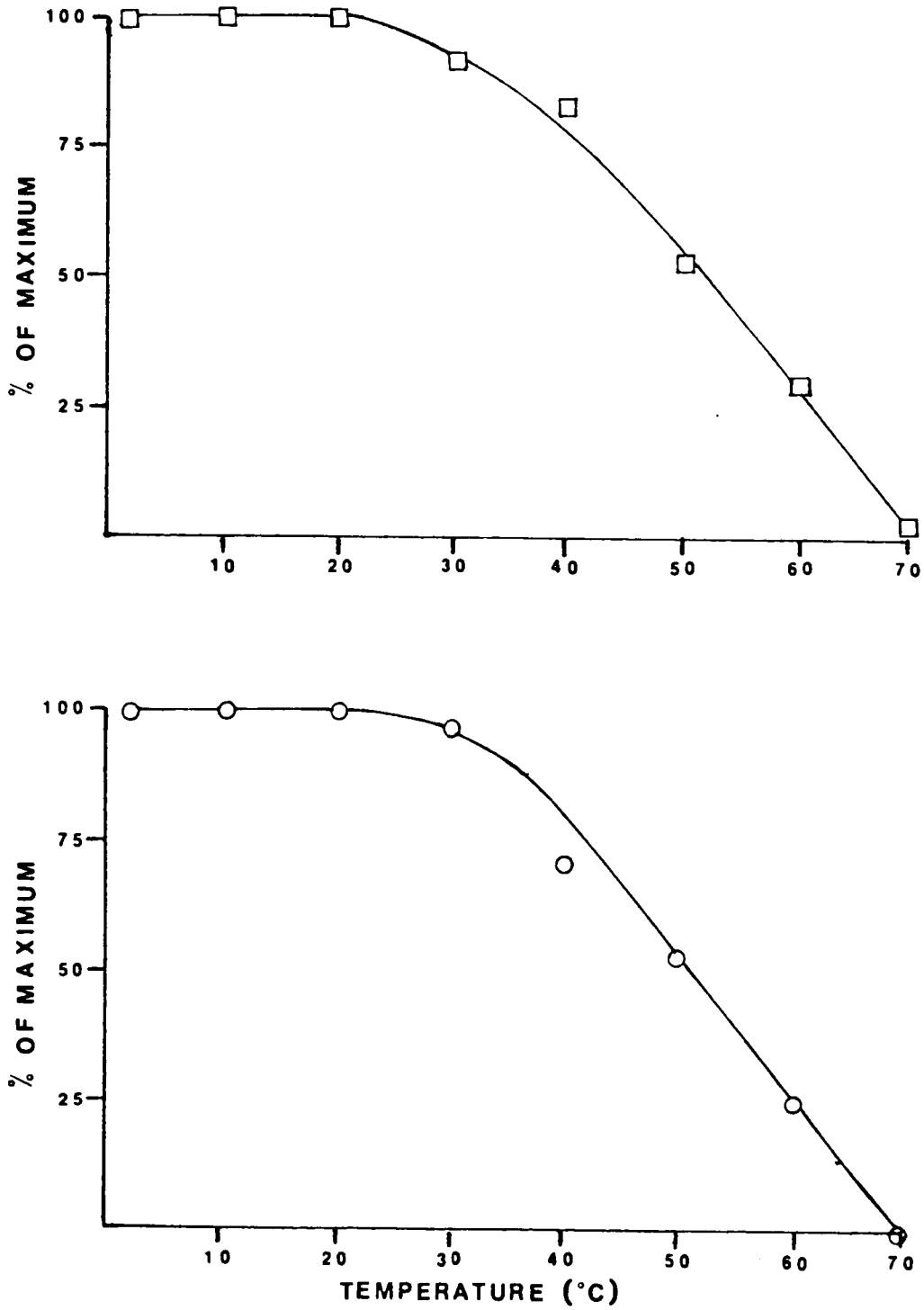

Figure 13. Effect of temperature on ATCh and IPA hydrolytic activities in maize extract. Following incubation at the designated temperature, the assay mixtures were chilled in an ice bath and assayed as described in Materials and Methods: Assay Procedures. (□) IPA; (○) ATCh.

Table 9. Effects of metal cations on enzyme activity.

Metal	Concentration (mM)	% of Control				
		ATCh		IPA		
		Barley	Maize	Barley	Maize	Rice
Cu^{++}	1.0	118	17	23	46	0
Mg^{++}	1.0	77	92	105	55	71
Mn^{++}	1.0	115	0	29	56	100
Ca^{++}	1.0	86	94	25	61	134
Fe^{++}	1.0	110	75	41	63	90
Zn^{++}	1.0	112	86	120	57	0
EDTA	1.0	69	85	46	65	47

were performed at pH 7.6. ATCh hydrolytic activity showed extreme differences between the two sources, barley and maize, especially with the addition of copper or manganese to the reaction solution. In these two sources, enzymatic activity in barley was somewhat enhanced while the activity in maize was strongly inhibited by these cations. Iron and zinc elicited similar responses but in maize the inhibition was less severe. On the other hand, magnesium and calcium resulted in slight inhibition of activity in both sources. EDTA gave responses similar to magnesium and calcium.

Extreme differences in IPA hydrolytic activities were again observed among the three sources in their responses to metal ions. Between barley and maize, the responses did not follow the pattern set by ATCh hydrolytic activity responses. Greatest extremes occurred in responses to manganese, calcium, and zinc. The differences among the three sources in their responses to copper, magnesium, and iron were obvious but not as great. EDTA elicited similar responses in barley, rice, and maize hydrolytic activities.

Generally speaking, comparable responses of IPA and ATCh hydrolytic activities within a source were not observed except in the case of zinc in which both activities were enhanced in barley extracts. However, also in barley extracts, fairly large differences in responses between the two activities were observed with the addition of copper, manganese, calcium, or iron. In maize, copper, magnesium, and manganese showed differences between the activities.

G. INHIBITORS

Inhibition responses of the hydrolytic activities from the three sources were investigated by observing the effects of choline, acetylcholine, AMO-1618, eserine, and neostigmine (structures are presented in Appendix B). Concentrations were 0.1 mM, 1 mM, or 10 mM. Responses were recorded as percent inhibition (control = 0%) and are summarized in Table 10.

IPA hydrolysis was inhibited in maize and rice at all concentrations of acetylcholine and choline, while barley was not affected by 0.1 mM acetylcholine or 0.1 to 1.0 mM choline. Maize IPA hydrolytic activity was most strongly affected by AMO-1618 while barley and rice extract responses were somewhat similar to one another. Eserine and neostigmine show definite inhibitory (and comparable) effects on maize extract IPA hydrolytic activity. Rice extract responses to eserine and neostigmine are comparable, as are barley extract responses. However, barley shows the least sensitivity to these two inhibitors as compared to the other two sources.

Choline had no effect on ATCh hydrolytic activity in either barley or maize. Acetylcholine had no effect on barley extract at concentrations of less than 10 mM, while maize extract showed definite sensitivity at 1 mM. AMO-1618 had no effect on barley at concentrations up to 10 mM and only 23% inhibition of maize extract activity at 10 mM. Barley extract was insensitive to eserine up to a concentration of 10 mM but was strongly inhibited at 10 mM. Maize extract activity showed

Table 10. Effects of inhibitors on enzyme activity.

Inhibitor	Concentration (mM)	% Inhibition				
		ATCh		IPA		
		Barley	Maize	Barley	Maize	Rice
Acetylcholine	0.1	0	4	0	30	29
	1.0	0	25	10	35	29
	10.0	35	40	51	35	36
Choline	0.1	0	0	0	24	8
	1.0	0	0	0	46	14
	10.0	0	0	33	65	14
AMO-1618	0.1	0	0	0	31	10
	1.0	0	0	10	35	18
	10.0	0	23	14	48	21
Eserine	0.1	0	0	0	56	29
	1.0	0	30	14	71	36
	10.0	70	54	14	100	43
Neostigmine	0.1	0	70	3	54	21
	1.0	9	70	5	73	50
	10.0	39	100	12	100	50

inhibition at eserine concentration of 0.1 mM. Conversely, maize extract showed greatest sensitivity to neostigmine compared to the response of barley extract.

CHAPTER V

DISCUSSION

This report deals with a preliminary characterization of esterase activities from three agriculturally important genera, Hordeum vulgare, Oryza sativa, and Zea mays. While barley and maize have been studied from the viewpoint of determination of the presence of esterase activity (Fluck and Jaffe, 1974c, 1975; Prentice et al., 1971), there have been no attempts at characterization. The activity in rice has never been investigated from the standpoint of isolation. The several other papers related to the subject of plant esterases deal primarily with the effects of exogenously applied acetylcholine or with chemotaxonomic observations involving electrophoretic studies, including barley (Prentice et al., 1971) and rice (Gupta and Singh, 1977).

Limited information is available concerning the isolation and characterization of esterase activity in graminaceous genera. Fluck and Jaffe (1974c) reported an attempt to identify an acetylcholine hydrolytic activity from the roots and leaves of oat, barley, and maize. No activity was found, however. Prentice et al. (1971) isolated an α-naphthyl acetate hydrolytic activity from barley endosperm. Sae et al. (1971), using IPA as a substrate, characterized an esterase from sorghum grain. Fluck and Jaffe (1975) performed inhibitor studies on an acetylthiocholine hydrolyzing activity from maize but found that the activity from this source is not inhibited by high substrate concentrations. Inhibition by high substrate concentration is considered to

be a basic criterion for the classification of an activity as acetylcholinesterase or cholinesterase (Riov and Jaffe, 1973b).

The assays involved in this study include the use of substrates which are specific for cholinesterases (thiocholine esters; Ellman et al., 1961) and a substrate for general esterase activity (indophenyl acetate; Kramer and Gamson, 1958). The thiocholine ester substrate employed here is acetylthiocholine (ATCh). Both ATCh and IPA are highly amenable to an uncomplicated and extremely adaptable spectrophotometric assay method for the determination of cholinesterase and esterase activities. All substrates and reagents are quite stable and the chromophores are stable within the physiological ranges of temperature and pH. The ranges of instability of indophenol are out of practical assay range since the chromophore undergoes spontaneous self-hydrolysis at pH 8.5 and 40 C (Schwarz, 1967) while thiocholine assays involving DTNB are limited to the range of pH 6.5 to pH 8.5 (Gorun et al., 1978).

Although all three experimental sources involved in this study are members of the Graminae, a definite dichotomy of response exists as is seen in the differences in experimental results between maize and barley on the one hand and rice on the other, specifically in the lack of acetylthiocholine hydrolytic activity in rice. Since the minimum activity which can be assayed with reasonable accuracy under the condition employed (observation time of approximately 15 min) is 0.072 μM per min (based on an absorbance change of 0.001 per min) and the specific activity is 1.7 and 2.3 units/mg protein for barley and maize, respectively (Table 6, page 29; Table 8, page 38), it is possible that

the absence of detectable activity in rice is due simply to lack of sensitivity of the assay.

Since rice apparently lacks a detectable ATCh hydrolytic activity, the sensitivity of the rice IPA hydrolytic activity to acetylcholine and choline is somewhat surprising. The inhibition by acetylcholine of this activity compares to that found in maize, which has a strong ATCh hydrolytic activity. The extent of the inhibition of the ATCh activity by acetylcholine and choline in both cases is fairly constant over the range from 0.1 mM to 10 mM. Inhibition of the IPA hydrolytic activity by choline, however, varies among the three sources, but, again in rice, even though relatively low, is constant over the range of interest.

The pH optimum for the IPA hydrolytic activity in all three sources occurs at approximately pH 7.2 while the optimum pH for the hydrolysis of ATCh by enzyme extracts of barley and maize is observed to be approximately pH 8.0 (Figures 8 and 10, pages 38 and 40, respectively). Sae et al. (1971) found a broad IPA hydrolytic response from pH 6.5 to pH 8.0 in sorghum grain. Riov and Jaffe (1973b) reported a pH optimum of pH 8.5 to pH 8.7 for the hydrolysis of ATCh in Phaseolus aureus. Lees and Thompson (1975) reported the pH optimum for ATCh hydrolysis in Phaseolus vulgaris to be pH 8.0. Reports for other sources are pH 7.2 for IPA and pH 8.5 for labelled acetylcholine in Pisum (Schwarz, 1967), a broad optimum of pH 7.5 to 8.5 in Solanum melongena, and pH 8.0 in Zea mays (Fluck and Jaffe, 1975).

No reports concerning temperature stability or inactivation of esterase activities are available. All IPA hydrolytic activities under

study here apparently have similar responses to incubation at elevated temperatures, as do ATCh hydrolytic activities. In all three cases, greater than 80% of the IPA hydrolytic activity remains up to 40 C while 80% of ATCh hydrolytic activity in barley and maize can be found up to approximately 35 C. This analysis sheds no light on the question of separate activities.

Influence of metal ions and EDTA on activities varied greatly depending on enzyme source. ATCh hydrolytic activity inhibition in barley and maize showed some parallels in responses to magnesium and calcium in that both cations have relatively little effect on enzyme activity (Table 9, page 44). In maize, all metal ions and EDTA showed inhibition to approximately half-maximal rates. In rice, activity was enhanced by calcium, while manganese and iron had little or no effect. Activity was totally inhibited by copper and zinc. EDTA reduced activity in rice to half that observed in controls. In barley extracts, zinc enhanced activity but in all other cases except calcium, an opposite effect to that found with ATCh hydrolytic activity was observed, i.e., where enhancement was observed with ATCh as substrate, inhibition occurred with IPA as substrate and vice versa. The strongly divergent responses in barley and maize extracts suggest that there is little if any overlap of IPA hydrolytic and ATCh hydrolytic activities within each source relative to pH, metals, and inhibitors. There are no comparative studies available in the literature.

The majority of data concerning plant cholinesterase responses to metabolic inhibitors has been limited to dicotyledonous species. Eserine has been reported to not greatly inhibit either IPA or ATCh hydrolytic activities while neostigmine, even at low concentrations, is inhibitory (Schwarz, 1967; Sae et al., 1971; Riov and Jaffe, 1973b; Fluck and Jaffe, 1975; Kasturi and Vasantharajan, 1976; Mansfield et al., 1978; Ernst and Hartmann, 1980). In the case of eserine, IPA and ATCh hydrolytic activities from maize and IPA hydrolytic activity from rice were either partially or totally inhibited, while both activities from barley were not inhibited except at high inhibitor concentration. It should be noted that Augustinsson (1963) employs the criterion of total inhibition by 10 μM eserine as part of the definition of cholinesterase. By this criterion, none of the three sources has true cholinesterase activity. As for the response to neostigmine, it was found that only maize extract (ATCh and, to a lesser extent, IPA hydrolytic activities) exhibited similarities to true cholinesterase activity. Both activities in barley were only slightly inhibited in response to neostigmine while rice extract was limited to half-maximal rates. It is noteworthy that even though AMO-1618 and neostigmine are structurally related (i.e., both are quaternary ammonium carbamate esters), different responses are elicited in maize and rice. In both sources, inhibition in response to AMO-1618 is only half that which occurs in response to neostigmine.

The nature of the differences among the inhibition responses exhibited by these three sources may be reflected in the response of the

whole plant to growth retardants and growth regulators. Riov and Jaffe (1973c) have suggested that endogenous acetylcholine may affect the biosynthesis of the growth regulator gibberellic acid (GA; structure of GA_3 presented in Appendix B), thereby functioning as a growth retardant. Dekhuijzen (1973), however, found that acetylcholine can replace GA in the reversal of wheat seedling growth inhibition by CCC (2-chloroethyltrimethyl ammonium chloride). Lang (1970) reported varied results from experiments dealing with the counteractivity of GA to growth retardant effects and also suggested that CCC inhibits GA biosynthesis. One report of relevant interest deals with the observation that CCC enhances choline utilization by stimulating both choline kinase activity and choline incorporation into lipids (Tanaka and Tolbert, 1966). While no data are available concerning the effects of CCC on acetylcholinesterase activity, it should be noted that CCC, neostigmine, and AMO-1618 bear structural similarities to acetylcholine (see Appendix B).

Therefore, since neostigmine and AMO-1618 are inhibitory to acetylcholinesterase activity, it might be reasonably assumed that CCC could be inhibitory to acetylcholinesterase activity. Tanaka and Tolbert (1966) and Lang (1970) report an antagonistic relationship between GA and CCC in the incorporation of choline into lipids. Thus, if there is a relationship between GA and CCC (suggested by Tanaka and Tolbert) and a relationship between CCC and acetylcholinesterase (there is no experimental support for this), then a relationship could reasonably exist among GA, acetylcholinesterase, and CCC specifically, or GA, acetylcholinesterase, and other quaternary ammonium carbamate esters in

general. Dennis et al. (1965) have suggested that one of the primary sites of inhibition by AMO-1618 or CCC is GA biosynthesis. They found that the addition of AMO-1618 to cultures of Fusarium moniliforme results in the accumulation of trans-geranylgeranyl pyrophosphate, an intermediate in the biosynthesis of GA. If acetylcholine is native to the plant and produces GA-like results, then an alternate source of growth regulator may present itself since acetylcholine would not be hydrolyzed due to inhibition of acetylcholinesterase along with the enzyme which converts trans-geranylgeranyl pyrophosphate to (-)-kaurene in the pathway for the synthesis of GA.

There may also be a functional interaction between acetylcholine, cholinesterase, and another plant growth regulator, indole acetic acid (IAA; structure presented in Appendix B). Percival and Bandursky (1976) have reported that 95% of the total extractable IAA from oat flour and from maize is esterified to several compounds including glucose, inositol, inositol glycosides, and cellulosic glucans. Further, at high concentrations (10^{-3} to 10^{-4} M), IAA inhibits cholinesterase activity (Bennet-Clark, 1956).

A structural similarity exists between α-naphthyl acetate, an esterase substrate commonly used in electrophoretograms (Prentice et al., 1971; Veerabhadrappa and Montgomery, 1971) and 1-naphthalene acetic acid (NAA; structure presented in Appendix B), which is used as a rooting stimulating compound (Noggle and Fritz, 1976). Also of interest is the fact that eserine is structurally similar to IAA (see Appendix B). Further, acetylcholine has been found to mimic the IAA cell elongation response in Avena coleoptiles. The inhibition of the cell elongation response by calcium is also reversed by acetylcholine (Evans, 1972),

perhaps due to membrane permeability effects which displace the interfering calcium ions from cell walls and membranes.

The well-known involvement of auxins with phototropism may also be linked to acetylcholine based on the findings of Ernst and Hartmann (1980), who reported that an acetylthiocholine hydrolyzing activity is at its highest levels in the hypocotyl hooks of Phaseolus vulgaris as compared to the remainder of the plant. The alleged and unsubstantiated involvement of phytochrome with this system notwithstanding (Hartmann, 1972; Jaffe, 1972), the possibility of an interaction among IAA, acetylcholine, and cholinesterase may exist. Obviously, no corollary can be extended at this time based on the status of current research, but the possibility of a metabolic relationship among esterases, cholinesterases, growth regulators, and growth modifiers should not be discounted.

An hypothesis set forth by Bennet-Clark (1956) concerning the possible role of acetylcholine and cholinesterase in salt accumulation and ion pumps at the membrane level was expanded to higher plant esterase systems by Schwarz (1967). The model involves the enzymatic hydrolysis of acetylcholine to choline and acetate, whereupon the choline is transferred, perhaps by cholinesterase, to form phosphatidylcholine. This polar compound forms a complex with salts at the outside of the membrane. The phosphatidylcholine-salt complex is reduced by lecinthinase-D to phosphatidic acid and choline, and the salt is released intramembranously. Choline acetylase resynthesizes acetylcholine from the choline radical and acetate, and the cycle is

repeated. The implications of inhibition of this system by metabolic inhibitors are obvious.

CHAPTER VI

CONCLUSIONS AND SUMMARY

This research represents an attempt to characterize the ester hydrolytic activities from three agriculturally significant monocots, Zea mays, Hordeum vulgare, and Oryza sativa. Characterization of the esterase activities of these plants is presented from the viewpoint of pH and heat stability (Figures 8 through 13, pages 38 to 43) along with the effects of several divalent metal ions (Table 9, page 44) and metabolic inhibitors (Table 10, page 47). Summaries of hydrolytic characteristics described in this study are presented in Tables 11, 12, and 13.

As has been pointed out, the actual roles of the esterase system in plants may be many but the point of major importance at this time is a description of the nature of the system itself. Isolation and characterization work has been performed (Ernst and Hartmann, 1980; Fluck and Jaffe, 1975; Riov and Jaffe, 1973b; Sae et al., 1971; Schwarz, 1967; Veerabhadrappa and Montgomery, 1971) but, in general, the content of the majority of these reports has been limited or the work has been restricted to a search for an explanation of a theory.

As is apparent, many conjectures and few facts are as yet available concerning not only the function of the esterase systems in higher plants but also the presence of acetylcholine as a naturally occurring substrate. Conjecturally, at least four hypotheses have been proposed:

Table 11. Characteristics of acetylthiocholine and indophenyl acetate hydrolytic activities from barley.

Characteristic	ATCh	IPA
pH optimum	8.0	7.4
Heat stability	At least 90% of both activities stable to 30 C.	
Metal ion effect (metal ion at 1.0 mM)	Inhibition by Mg, Ca, and EDTA; Enhancement by Cu, Mn, Fe, and Zn.	Strong inhibition by Cu, Mn, and Ca; Enhancement by Mg and Zn.
Metabolic inhibitor effect (inhibitor at 0.1 mM)	No inhibition by inhibitors studied.	
$(NH_4)_2SO_4$ fraction	30% to 90% fraction for both activities.	

Table 12. Characteristics of indophenyl acetate hydrolytic activity from rice.

Characteristic	IPA
pH optimum	7.4
Heat stability	At least 90% of activity stable to 40 C.
Metal ion effect (metal ion at 1.0 mM)	No inhibition by Mn; Enhancement by Ca; Strong inhibition by Cu and Zn.
Metabolic inhibitor effect	Slight or no inhibition by all inhibitors studied.
$(NH_4)_2SO_4$ fraction	30% to 90% fraction.

Table 13. Characteristics of acetylthiocholine and indophenyl acetate hydrolytic activities from maize.

Characteristic	ATCh	IPA
pH optimum	7.6	7.0
Heat stability	At least 90% of both activities stable to 30 C.	
Metal ion effect (metal ion at 1.0 mM)	Strong inhibition by Cu and Mn.	Inhibition by all ions studied.
Metabolic inhibitor effect (inhibitor at 0.1 mM)	Strong inhibition by neostigmine. No inhibition by other inhibitors studied.	25% to 30% inhibition by acetylcholine, choline, and AMO-1618. 50% inhibition by eserine and neostigmine.
$(NH_4)_2SO_4$ fraction	30% to 90% fraction for both activities.	

(1) involvement with the phytochrome system,

(2) involvement with gibberellic acid,

(3) involvement with idole acetic acid, and

(4) involvement in ion accumulation.

It may not yet be safe to assume that there are no interactions among or within the four systems. Based on current research, however, in vivo involvement of the esterase system with phytochrome is still unsubstantiated for the most part.

Acetylcholine has been found to both mimic and antagonize the effects of both gibberellic acid and indole acetic acid. Thus an experimental link between acetylcholine and growth regulators, although somewhat tenuous, has been established. Whether this relationship is a relevant facet in the development of the plant awaits further study.

The ion accumulation hypothesis has received support only indirectly and in association with the other three effects. It seems most likely that this last effect may be the primary role of the esterase system while the other effects are under secondary or indirect influence.

It would be highly premature to make a definite decision as to characterizations of the activities observed in this study as cholinesterase or acetylcholinesterase. A tentative conclusion, however, is that the activities from barley, rice, and maize are not cholinesterase or acetylcholinesterase as currently defined from animal sources. This determination is based on comparative inhibitory responses to eserine, neostigmine, and choline. These comparisons are presented in Table 14.

Table 14. Comparative summary of inhibition responses to eserine, neostigmine, and choline.

Source	Substrate	Inhibition by[1]		
		Eserine	Neostigmine	Choline
Barley seedlings	ATCh	-	-	-
	IPA	-	-	+
Rice seedlings	IPA	+	+	+
Maize seedlings	ATCh	-	+++	-
	IPA	++	++	++
Mung bean roots[2]	ATCh	++	+++	-
Eel electroplax cells[2]	ACh	+++	+++	+
Horse serum[2]	ACh	+++	+++	+

[1]Eserine and neostigmine at 100 μM except in eel electroplax and horse serum in which the inhibitors are at 10 μM. Choline is at 10 mm in all cases.

[2]Data for mung bean, eel, and horse are from Riov and Jaffe (1973b). See also Table 5 in this report.

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APPENDICES

APPENDIX A

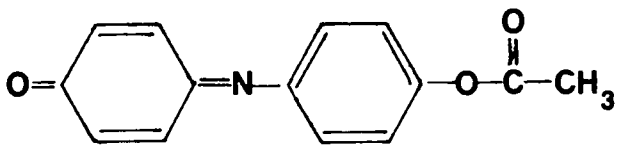
HISTORICAL SUMMARY

1947	Jansen, Jang, and MacDonnell	Acetylesterase observed in citrus fruits.
1956	Bennet-Clark	Proposed the involvement of acetylcholine and acetylcholinesterase in a membrane transport system for salt accumulation.
1958	Kramer and Gamson	Colorimetric assay of esterases employing indophenyl acetate.
1962	Mounter and Mounter	Comparison between wheat germ esterase and acetylcholinesterase.
1963	Jooste and Moreland	Studies on electrophoretic mobilities, substrate specificities, and inhibitor effects on extracts of various plants.
1964	Schwartz, Biedron, von Holdt, and Rehm	Survey of esterases in the Cucurbitaceae.
1967	Schwarz	Substrate and inhibitor studies in Pisum.
1968	Norgaard and Montgomery	Described various esterases in Pisum.
1970	Jaffe	Suggested that acetylcholine is involved in ion flux and is regulated by phytochrome.
1971	Sae, Kadoum, and Cunningham	Purification and study of Sorghum esterases.
1971	Veerabhadrappa and Montgomery	Purification and study of Phaseolus esterases.
1972	Jaffe	Review of relation between acetylcholine and phytochrome.
1972	Kasemir and Mohr	Disputed Jaffe's (1970) suggestion that acetylcholine is mediator between phytochrome and morphogenic response.

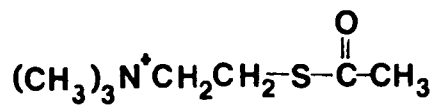
1972	Tanada	Suggested that Jaffe's (1970) root tip adhesion study conclusions are in error due to ionic interference of K^+ by acetylcholine.
1973(a)	Riov and Jaffe	Observed a correlation between the effectiveness of a compound in retarding root production and the extent of enzyme inhibition.
1973(b)	Riov and Jaffe	Described the presence of esterases in Phaseolus roots.
1973(c)	Riov and Jaffe	Described the inhibition of Phaseolus esterase by AMO-1618.
1974(a)	Fluck and Jaffe	Survey of the plant acetylcholine system.
1974(b)	Fluck and Jaffe	Described the subcellular localization of cholinesterase in Phaseolus.
1974(c)	Fluck and Jaffe	Survey of cholinesterases in several plant systems.
1974(d)	Fluck and Jaffe	Demonstrated that the esterase which had been studied in Phaseolus is not pectin esterase.
1975	Fluck and Jaffe	Study of cholinesterases from Solanum melongena and Zea mays.
1975	Lees and Thompson	Subcellular distribution of cholinesterase during germination of Phaseolus.
1976	Kasturi and Vasantharajan	Electrophoresis of esterases and pesticide effects on the esterases of Pisum.
1978	Mansfield, Webb, Clark, and Taylor	Partial purification of cholinesterase from Phaseolus roots.
1979	Hartmann	Demonstrated the in vivo incorporation of labelled acetate into acetylcholine in Phaseolus.
1980	Ernst and Hartmann	Characterized an acetylcholinesterase from Phaseolus.

APPENDIX B

CHEMICAL STRUCTURES OF REFERENCED COMPOUNDS

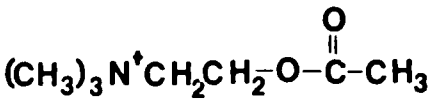


Indophenyl acetate

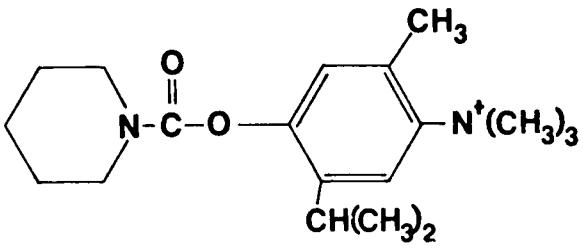


Acetylthiocholine

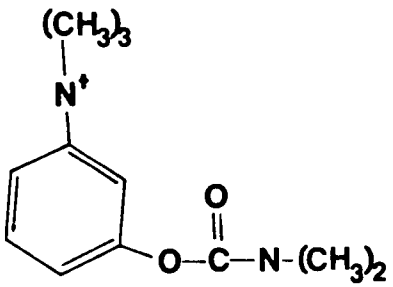
Choline



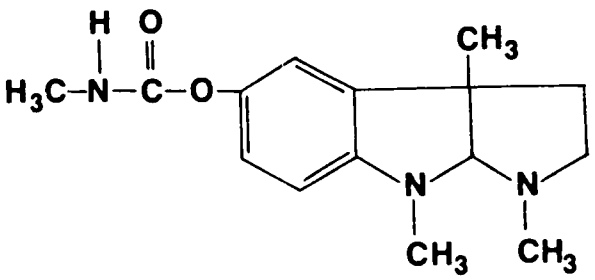
Acetylcholine



AMO-1618

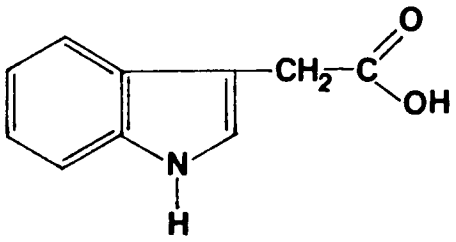


Neostigmine

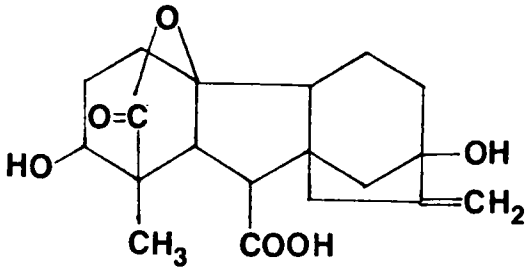


Eserine

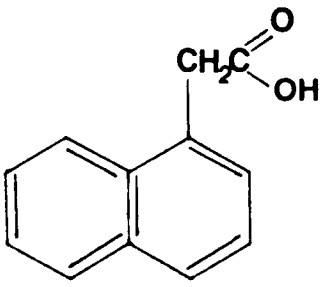
CCC



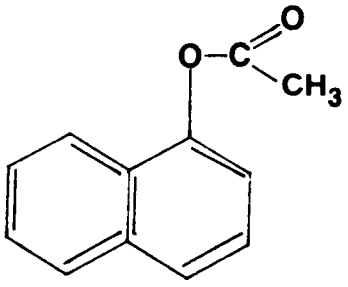
IAA



GA_3



1-Naphthalene
acetic acid
(NAA)



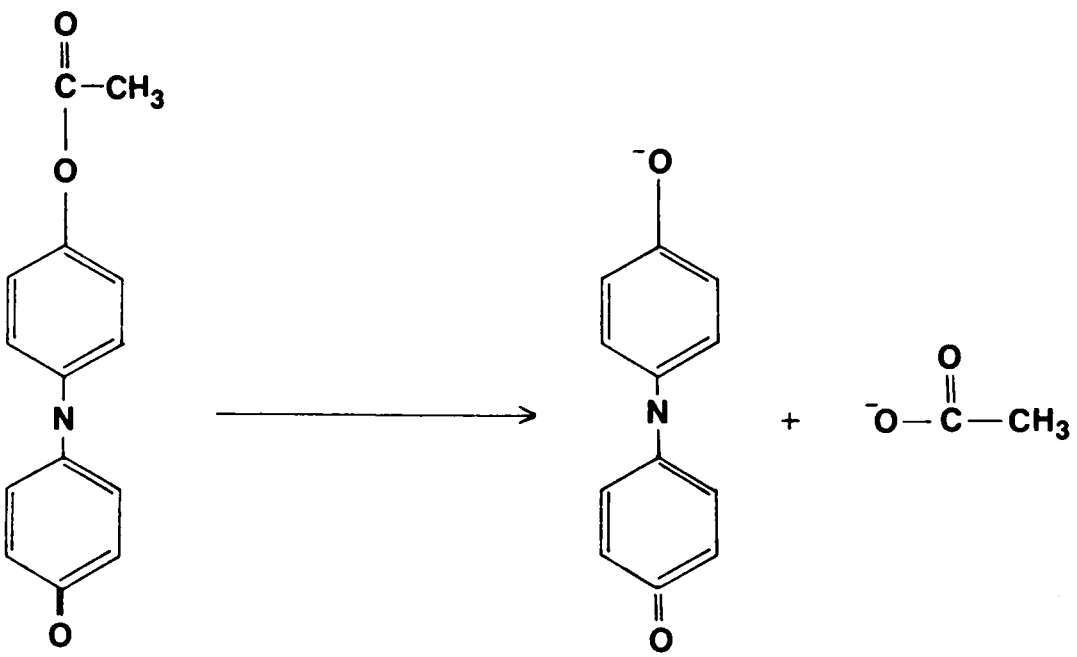
α-Naphthyl
acetate

APPENDIX C

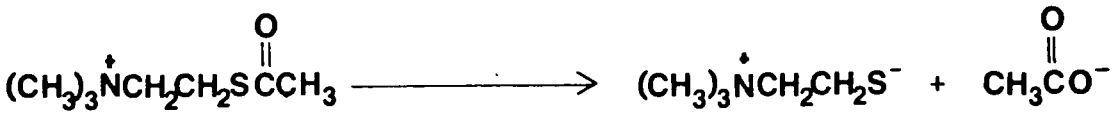
HYDROLYTIC REACTIONS OF INDOPHENYL ACETATE

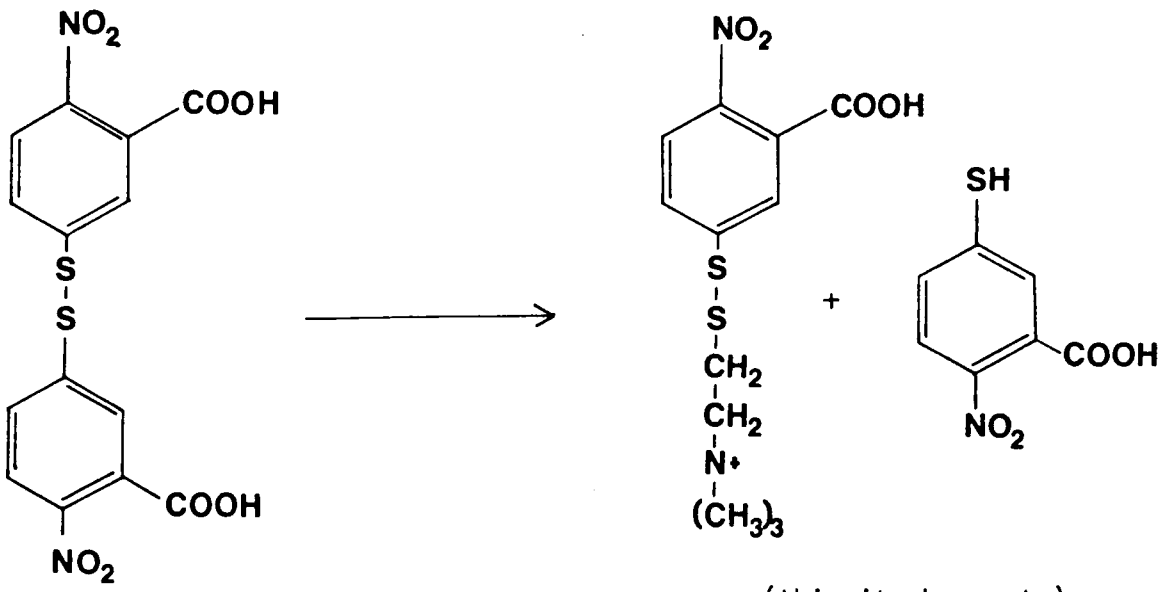
AND ACETYLTHIOCHOLINE

Enzymatic hydrolysis of indophenyl acetate to indophenol:



Enzymatic hydrolysis of acetylthiocholine to 2-nitro, 5-thiobenzoate with DTNB as oxidizing agent:





(thionitrobenzoate).

VITA

George Howard Gregg was born in Knoxville, Tennessee on August 13, 1949. He attended elementary schools in that city and graduated from Rule High School in June 1967. He entered The University of Tennessee in September 1967 and received the Bachelor of Science degree in March 1972 with a major in Biology and a minor in Anthropology. He entered the United States Army in April of 1972 and served with the United States Army Security Agency as a Russian Translator until April 1975.

He entered The Graduate School of The University of Tennessee, Knoxville in June 1975 and was a lab assistant in General Biology before accepting a graduate assistantship from the Department of Botany in January 1976. He accepted a full-time position as laboratory assistant at the Comparative Animal Research Laboratory at Oak Ridge in September 1978. He is currently employed by the Department of Botany at The University of Tennessee as Research Associate.

He is married to the former Zoe Kathleen Rush of Corryton, Tennessee and they have two children, Matthew Duncan and Kerry Lauren.