AN ABSTRACT OF THE THESIS OF

MARIDA JANE NORGAARD for the MASTER OF SCIENCE
(Name of student) (Degree)

in FOOD SCIENCE presented on August 11, 1967
(Major) (Date)

Title: SOME CARBOXYLIC ESTERASES OF THE PEA (PISUM SATIVUM L.)

Abstract approved: _____
Dr. Morris W. Montgomery

Although esterases have been reported in peas, their properties have not been studied. The purpose of this investigation was to determine the substrate and inhibitor specificity of the esterases present in a water extract of lyophilized peas, and to determine if pea esterases could be classified according to the criteria established for animal esterases.

Esterase activity was determined manometrically using the Gilson differential respirometer. The effect of pH on esterase activity was determined using phenyl acetate, phenyl propionate, tripropionin, and tributyrin as substrates. The pH optima appeared to lie between 6.9 and 7.2, and pH 7 was selected for use in this study. The acetyl, propionyl, and n-butyryl esters of phenol, 2-naphthol-6-SO_3Na and glycerol were hydrolyzed by the enzyme extract. Long chain esters of 2-naphthol-6-SO_3Na, however, were

not hydrolyzed. Cholinesterases and lipases did not appear to be present in the extract since only a very small amount of activity was observed when the choline esters and triolein were used as substrates.

Using phenyl propionate and phenyl butyrate as substrates, esterase activity, based on the original extract, decreased with dilution. Later work revealed that the esterase(s) which hydrolyzed phenyl propionate were inhibited by heavy metal ions and activated by metal complexing agents. Hence, a possible explanation for the decrease in activity was inhibition by metal ions in the distilled water.

The effects of the inhibitors parathion, tetraethyl pyrophosphate, and diisopropyl phosphorofluoridate at concentrations ranging from 10^{-1} M to 10^{-10} M on esterase activity were determined. The data suggested the presence of as many as six esterases in the aqueous extract of peas, three for which the evidence was quite conclusive. On the basis of their inhibition by organophosphorus compounds, all but one of the esterases appeared to be of the B type.

Physostigmine sulfate (10^{-5} M) had no effect on esterase activity with the nine substrates used indicating that the activity was not due to cholinesterases.

At least one of the esterases which hydrolyzed each of the substrates was sensitive to 10^{-3} M p-chloromercuribenzoic acid

sodium salt suggesting the importance of sulfhydryl groups for enzyme activity.

Some Carboxylic Esterases of the Pea (*Pisum sativum* L.)

by

Marida Jane Norgaard

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1968

APPROVED:

Assistant Professor of Food Science and Technology
In Charge of Major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented August 11, 1967

Typed by Donna Olson for Marida Jane Norgaard

ACKNOWLEDGEMENTS

The author wishes to express her sincere thanks to Dr. Morris W. Montgomery, major advisor, for his guidance and help during this investigation and in the preparation of this thesis, to Dr. Robert R. Becker for his advice and encouragement and to Dr. William D. Davidson for his helpful suggestions in the preparation of this manuscript.

This research was supported by the Public Health Service under grant AM 09306-01.

Special thanks is extended to my husband, Dick, for his patience and encouragement during the preparation of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SOME CARBOXYLIC ESTERASES OF THE PEA (PISUM SATIVUM L.)

INTRODUCTION

Esterases of animal tissues have been studied rather extensively, and many have been purified. Animal esterase classifications have been developed, the most common of which is based on substrate and inhibitor specificity. With the exception of acetylcholinesterase which functions in the transmission of nerve impulses, the physiological roles of esterases have not been determined.

Relatively little work has been done on plant esterases. Recent studies by Jooste and Moreland (1963) and Schwartz et al. (1964) have indicated that plant tissues may contain esterase systems as complex as those of animals. One study has been reported on pea esterases other than those on pea lipase activity. Frankel and Garber (1965) reported a maximum of six bands with esterase activity which were separated by starch gel electrophoresis from extracts of twelve varieties of germinating peas. The substrate used was α-naphthyl acetate.

Plant esterases are involved in food spoilage and are, therefore, of particular interest to food scientists. Fat hydrolysis by lipases causes spoilage of grains, flours, oil seed etc. (Reed, 1966).

Lipases of improperly blanched frozen peas have been shown to contribute to the deterioration of lipids and development of off-flavors and color changes during storage (Lee and Wagenknecht, 1958).

Esterases can also be of use to the food industry. Pectin methyl esterase is used in the preparation of low sugar, low-calorie jellies, gelled milk desserts, puddings and pudding powders, gelled soups, fruit juices, vegetable juices, tomato cocktail, sauces, purees, frozen desserts, canned fruits, and coatings for certain meat products and candied fruits (Cruess, 1958). More knowledge of plant esterases could lead to new uses of esterases in the food industry.

The purpose of this study was to determine the substrate and inhibitor specificity of the esterases present in a water extract of lyophilized peas and to determine if pea esterases could be classified according to the criteria established for animal esterases.

REVIEW OF LITERATURE

Esterases are generally distinguished from other hydrolytic enzymes by their ability to hydrolyze esters. There is, however, some overlap in substrate specificity between esterases and proteases. A few esterases have been shown to hydrolyze amide derivatives (Myers, Tol, and de Jonge, 1957) and acid anhydrides (Wilson, 1954), while some proteolytic enzymes such as trypsin, chymotrypsin and thrombin have been reported to hydrolyze carboxyl esters. These proteolytic enzymes hydrolyzed esters by the same general mechanism as esterases and were inhibited by many of the esterase inhibitors (Myers, 1960). Proteolytic enzymes are, therefore, usually considered esterases as well as proteases.

Some non-enzymatic proteins may also hydrolyze esters. Esterase activity has been reported for serum albumin (Rongone and Bocklage, 1957; Casida and Augustinsson, 1959; Harris, Hopkinson, and Robson, 1962; Tove, 1962; Wilde and Kebwick, 1964; Downey and Andrews, 1965) and for the γ-globulins (Downey and Andrews, 1965). However, the question of whether or not the esterase activity is actually a property of these proteins or of a contaminating enzyme still remains to be answered.

Esterase Classification

Since most of the esterase studies and all of those from which the classifications were developed have been with animal esterases, pertinent animal esterase literature will be included in this review.

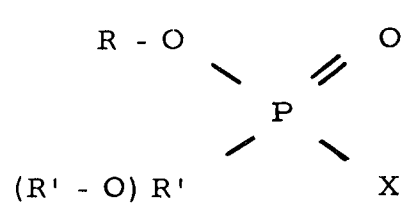
Some confusion in the literature exists in regard to the classification of esterases and no completely satisfactory system has been developed. For example, in early definitions of the general classes of esterases, most fatty acid esterases were included in the ill-defined terms aliesterases, simple esterases and lipases. For most authors, lipase was an esterase which hydrolyzed long chain esters, especially fats, and aliesterase or simple esterase was an esterase which hydrolyzed short-chain aliphatic esters (Hofstee, 1960). Later, liver esterase, which does not hydrolyze true fats (Sarda and Desnuelle, 1958), was shown to hydrolyze both long and short chain aliphatic and aromatic esters. Longer chain esters were hydrolyzed at a higher rate (Hofstee, 1954). The original definitions were no longer meaningful, and it was suggested later that the term lipase be reserved for the fat-splitting enzymes (Hofstee, 1960). The term aliesterase has taken on the new meaning of a B-esterase which will be discussed later.

The finding that the activity of purified pancreatic lipase became pronounced only when saturation of substrate was reached

led to another classification. Fatty acid esterases were grouped into 1) esterases proper acting on soluble substrates or 2) lipase-type esterases acting on undissolved substrates (Desnuelle, 1951; Sarda and Desnuelle, 1958). Most esterases fall into the former group. Those in the latter group are assumed to be active when adsorbed at an ester-water interface because only then is the active center of the enzyme available to the substrate (Dixon and Webb, 1964).

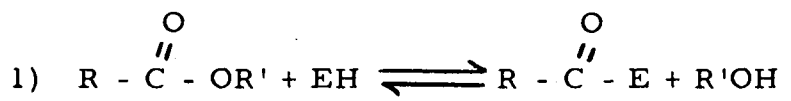
The most common approach to esterase classification and the one used in this study is to group esterases according to their substrate and inhibitor specificity. Esterases usually are not classified on substrate specificity alone since they do not show absolute substrate specificity.

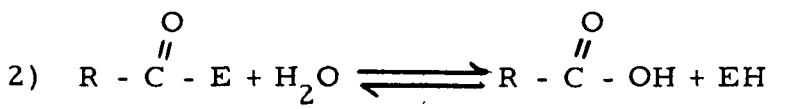
Inhibitors commonly used in esterase classification are organophosphorus (OP) compounds, physostigmine, and p-chloromercuribenzoate (PCMB). The OP inhibitors have the general formula:



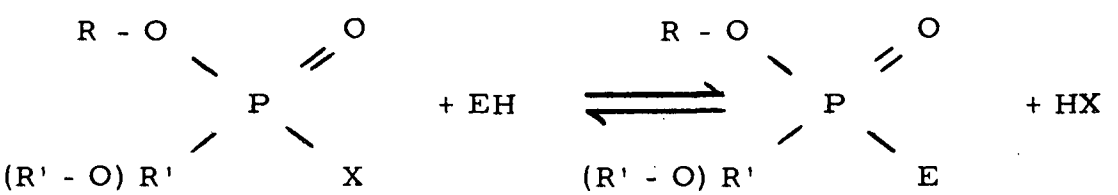
where R and R' are alkyl groups and X is -F, -CN, or $-0C_6H_4NO_2$. OP inhibitors are highly specific for esterases since

the enzyme and the OP compound form the inhibitory group by a process analogous to the first stage of hydrolysis of the substrate (Dixon and Webb, 1964). Hydrolysis of the substrate occurs in two stages. First, the acyl group is transferred to the enzyme with the formation of an acylated enzyme and liberation of alcohol. Second, the acylated enzyme is hydrolyzed yielding the acid and free enzyme.





Similarly, with OP inhibitors the substituted phosphoryl group is transferred to the enzyme with the formation of a phosphorylated enzyme and liberation of HX.



In the case of more effective inhibitors, the phosphorylated enzyme is, however, a comparatively stable compound which hydrolyzes only very slowly. OP inhibitors have been shown to combine with the esterase through the hydroxyl group of a serine at the active

site (Osterbaan and Cohen, 1964)

The action of physostigmine has been shown to be similar to the OP inhibitors. Wilson (1961) reported that the carbamoyl group was transferred to the active center of the enzyme, resulting in a carbamoyl-enzyme which was relatively slowly hydrolyzed. PCMB inhibition has been shown to occur by the reversible formation of mercaptides from the mercury of PCMB and the enzyme thiol groups (Dixon and Webb, 1964).

Aldridge (1953a) was one of the first to use inhibitors in esterase classification. Working with a variety of mammalian serums he observed that there were two distinct types of esterases which hydrolyzed p-nitrophenyl acetate, propionate, and butyrate. One type, which he called A-esterase, was not inhibited by paraoxone (E600) at concentrations as high as 10^{-3} M, while the other, which he called B-esterase, was inhibited by concentrations as low as 10^{-8} M. Neither A- nor B-esterase was inhibited by physostigmine which indicated that these esterases were not cholinesterases. Aldridge (1953b) also showed that the A-esterase of rabbit, rat and horse serum could hydrolyze E600. Later he (Aldridge, 1954) extended his classification by using aromatic esters and triglycerides as substrates and a variety of OP inhibitors to characterize some esterases of rat intestinal mucosa, pancreas, liver and serum. He reported a variety of esterases, all of which were either A or B

type. Aldridge (1954) pointed out the importance of using more than one inhibitor in classification. Although the A-esterase of pancreas was not inhibited by tetraethyl pyrophosphate (TEPP), DFP or mipafox, it was inhibited by 10^{-5} M E600. This inhibition, however, was shown to be reversible.

Aldridge's work was performed with plasma or crude enzyme preparations. Using partially purified esterases, Augustinsson (1958, 1959) separated blood plasma esterases of 13 different mammalian species by electrophoresis on cellulose columns. He then classified them using a variety of inhibitors and a series of aliphatic, aromatic, heterocyclic and choline esters as substrates. The results showed vetebrate plasma to contain as many as three types of esterases with the following characteristics: Arylesterases (A): aromatic esterases which hydrolyzed phenyl acetate at a higher rate than phenyl butyrate; aliphatic esters were normally not attacked. They were resistant to certain OP compounds and to physostigmine, but highly sensitive to p-hydroxymercuribenzoate. Aliesterases (B): both aliphatic and aromatic esters were hydrolysed, but not choline esters. They were sensitive to 10^{-5} M of a great number of OP inhibitors, but resistant to 10^{-5} M physostigmine. Some were sensitive to higher concentrations of the latter inhibitor. Cholinesterases (Ch): choline esters were split at a higher rate than both aliphatic or aromatic esters. These esterases were

sensitive to many OP inhibitors 10^{-6} M or less and to physostigmine, the latter giving complete inhibition in 10^{-5} M or lower concentrations.

The classification developed by Aldridge and Augustinsson has been applied to esterases of numerous animal tissues. This classification was not exclusive, however. In 1957 Bergman discovered a new type of esterase in hog-kidney which he called C-esterase. Like A-esterase, C-esterase was inhibited by DFP, but unlike A-esterase which was inhibited by PCMB, C-esterase was activated by PCMB. An eight fold increase in activity was observed using 10^{-4} M PCMB. This effect was shown to be pH dependent (Bergman and Rimon, 1958). The stimulating effect was thought to be due to the liberation of the esterase from an inhibitor by PCMB (Bergman and Rimon, 1960). Other C-esterases have been further reported by Barron, Bernsohn and Hess (1963) in human brain and by Bernsohn et al. (1966) in rat brain.

Another approach to classification is that of the Commission on Enzymes of the International Union of Biochemistry (1961). In 1961, in an attempt to clarify the confusion in enzyme classification, the commission proposed a new system in which all enzymes were divided into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Each of these classes was further divided into a number of subclasses and sub-subclasses.

Each enzyme was given a number and two names, a systematic name and a trivial name. The trivial name was usually shorter and often the one in current use. This system works well for enzymes which are specific for certain substrates. Many esterases, however, have wide substrate specificity and cannot be grouped by substrate specificity alone.

Plant Esterases

Whereas animal esterases have been studied quite extensively, comparatively little work has been done on plant esterases.

Much of the plant esterase literature merely reports the presence of plant esterases, but does not involve detailed studies and classification. Lipases have been found in cotton (Olcott and Fontaine, 1941), castor beans (Longenecker, 1946; Ory, Angelo and Altschul, 1960), oats (Martin and Peers, 1953), peas (Lee and Wagenknecht, 1958), rice embryo (Obara and Ogasawara, 1959) and malt and soybeans (Hofstee, 1960). Other esterases have been reported in tomato and alfalfa (Mac Donnel et al., 1950), maize (Schwartz, 1960), root tip of the broad bean Vicia faba (Benes, 1962), wheat germ (Mounter and Mounter, 1962), shoot tips of white spruce (Born, 1963), spherosomes of onion (Walek-Czanecka, 1963), peas (Frankel and Garber, 1965), spherosomes of corn and tobacco (Matile et al., 1965), germinating seeds, flowers and leaves of 12

species of Collinsia (Wennstrom and Garber, 1965), and bean leaves (Rudolph and Stahmann, 1966). Pectinesterase has been found in a variety of plants (Mac Donnel et al., 1950) and Krossing (as cited by Hofstee, 1960) reported the presence of chlorophyllase in the chloroplasts of green plants.

More extensive studies have been done on plant esterases. An acetylesterase from citrus fruits was purified and shown to hydrolyze esters of acetic acid and to be inhibited by diisopropyl phosphorofluoridate (Jansen, Jang and Mac Donnell, 1947; Jansen et al., 1948). Jooste and Moreland (1963) attempted to characterize and identify plant esterases using the criteria established for animal esterases. Evidence obtained from electrophoretic mobilities and substrate and inhibitor specificities indicated that cucumber, soybean, wheat, and corn contain complex esterase systems. Using as substrates the 2-naphthol esters of acetic, butyric, caprylic and phenoxyacetic acids and as inhibitors diisopropyl phosphorofluoridate (DFP), PCMB, and physostigmine, both A-esterases and B-esterases were indicated. Their data suggested the presence of A-esterases in soybean, wheat, and corn, and B-esterases in cucumber and soybean. Jooste and Moreland (1962) also reported an esterase in soybean which did not fit into the classification used for animal esterases. The activity of this esterase was increased 17 percent by 10^{-3} M DFP, similar to some A-esterases that

hydrolyze OP compounds, but was unaffected by PCMB unlike A- or C-esterases. The enzyme had considerable specificity for 2-naphthyl-phenoxyacetate and was named phenoxyesterase.

Schwartz et al. (1964) found a variety of esterases in green beans, cabbage, potato tuber, citrus albedo and flavedo, and fruits of many cucurbits. Using starch gel electrophoresis they showed that all extracts contained a multiplicity of esterases active toward α-naphthyl acetate which differed in different species, in different strains of the same species, and in different parts of the same plant.

These studies suggest that plants, like animals, contain a multiplicity of esterases and the criteria used for classification of animal esterases may be very useful with some modifications for plant esterase classification.

MATERIALS AND METHODS

Preparation of Enzyme

Freshly harvested peas (Pisum sativum L., variety Dark Skin Perfection) were obtained from Lamb-Weston Inc., Weston, Oregon. The peas were lyophilized, flushed with nitrogen, sealed in cans in an atmosphere of nitrogen, and stored at -18°C. During lyophilization the temperature of the heating plates was not allowed to exceed 38°C. Enzyme extracts were prepared by grinding the dried peas in a Waring Blendor for two minutes, diluting one part powder with ten parts distilled water, and mixing. Slurries were centrifuged in the cold (3°C) at 31,000 x G for 25 minutes. Appropriate dilutions of the supernatants were used as the enzyme preparations. Enzyme for the controls was prepared by heating portions of the preparations in a boiling water bath for ten minutes and removing the precipitate by filtration (Whatman No. 12).

Preparation of Substrates

Substrates used in this study were phenyl acetate[1] (PA), phenyl propionate[1] (PP), phenyl n-butyrate[2] (PB), triacetin[1] (TA),

[1] Eastman Organic Chemicals

[2] K & K Laboratories, Inc.

tripropionin[1] (TP), tri-n-butyrin[1] (TB), triolein[2], acetylcholine iodide[1], propionylcholine iodide[2], n-butyryl choline iodide[2], and the acetyl (NA), propionyl (NP), n-butyryl (NB), hexyl, octyl, decyl, and hexadecyl esters of 2-naphthol-6-SO_3Na[3]. The concentrations of phenyl esters and triglycerides were 1M with the exception of 0.23M triacetin and 0.5M triolein. Choline esters and naphthyl esters were 0.1M and 0.06M, respectively. All substrates were prepared in three percent (w/v) Triton X-155 (Rohm and Haas) and 0.1 percent gum arabic (Matheson Coleman & Bell) and homogenized for two minutes in a water-cooled microblender. Homogenization was omitted in the preparation of the water soluble naphthyl esters.

Preparation of Inhibitors

OP inhibitors used in this study were diethyl p-nitrophenyl thiophosphate[2] (parathion), diisopropyl phosphorofluoridate[2] (DFP), and tetraethyl pyrophosphate[2] (TEPP). Triton X-100 (Rohm and Haas) was required to promote solubility of the higher concentrations of parathion. Studies have indicated that Triton X-100 activated

---

[1] Eastman Organic Chemicals

[2] K & K Laboratories, Inc.

[3] Donated by Dr. T. L. Forster, Washington State University, Pullman, Washington.

certain esterases (Allen, Allen and Licht, 1965). Since activation was also observed in this study, all OP inhibitors were prepared in one percent (w/v) Triton X-100. The activating effect was then constant and comparisons could be made between the effects of the different OP inhibitors. The 0.1M parathion solution required 30 seconds homogenization in a microblender to form a semi-stable emulsion. DFP and TEPP were soluble at the concentrations used. Prescribed concentrations were prepared by serially diluting the more concentrated solutions or emulsions. p-Chloromercuribenzoic acid sodium salt[4] (PCMB) solutions were also prepared in one percent Triton X-100, while physostigmine sulfate[4] solutions were prepared in water.

Assay Procedure

Esterase activity was determined manometrically using the Gilson differential respirometer. The H^+ from the acid, produced as the substrate was hydrolyzed, shifts the following equilibrium to the right, evolving CO_2:

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2(\text{dissolved}) \rightleftharpoons CO_2(\text{gas})$$

[4] K & K Laboratories, Inc.

The volume of CO_2 evolved from the bicarbonate buffer was used as a measure of esterase activity.

The assay was performed at a constant pressure of 770 mm Hg and water bath temperature of 37°C. The procedure used was similar to that of Forster, Bendixen and Montgomery (1959). One and a half ml $NaHCO_3$ buffer, one ml enzyme preparation, and one ml of either distilled water, one percent Triton X-100, or inhibitor solution depending on the experiment were pipetted into the main compartments of the respirometer flasks. One tenth ml $NaHCO_3$ buffer and 0.4 ml substrate were pipetted into the side arms. Bicarbonate concentrations required to give the desired pH were determined according to Umbreit, Burris and Stauffer (1964). Flasks were attached to the respirometer and gased for ten minutes with a mixture of five percent CO_2 and 95 percent N_2 with the side-arm vents open. After gassing, side-arm vents were closed, the pressure was adjusted to 770 mm Hg, and the flasks were equilibrated for ten minutes. Side arms contents were tipped into the main compartment at zero-time. Readings were taken at ten minute intervals for 30 minutes.

Calculation of Results

The microliters of gas produced in 30 minutes was determined from the difference in the amount of gas produced by the sample and the heated control--thus accounting for nonenzymatic hydrolysis.

This value was then multiplied by a correction factor described by equation D of Gregory and Winter (1965). The corrected value (A_{30}) was the actual change in amount of assayed gas, expressed in microliters at standard conditions. The correction factor accounts for the fact that the cylinders holding the variable gas volume and the tygon tubing were not immersed in the water bath and, therefore, were subject to daily temperature changes. The correction factors calculated for the experimental conditions of this study are given in Table I.

Table I. Correction factors.

Room Temperature (^{o}C)	Factors
20	1.0618
21	1.0567
22	1.0514
23	1.0460
24	1.0407
25	1.0354

Results from the substrate specificity studies are expressed in units of esterase activity. One unit was defined as the amount of esterase which hydrolyzes one microequivalent of substrate per minute per milliliter of the original pea extract.

The results of the inhibitor studies were expressed as percentage inhibition, calculated from the following formula:

$$\text{Percentage inhibition} = \left[\frac{A_{30}\ (\text{not inhibited}) - A_{30}\ (\text{inhibited})}{A_{30}\ (\text{not inhibited})} \right] 100$$

RESULTS AND DISCUSSION

Determination of pH Optima

The effect of pH on pea esterase activity was investigated to determine a suitable pH to be used throughout this work. Since a mixture of esterases were presumed to be present, a detailed pH study was not deemed necessary. Esterase activity was determined at various pH values between 6.0 and 8.0, the limits allowed by the assay procedure, using PA, PP, TP, and TB as substrates. The results are presented in Figures 1A and 1B. Each point represents the results of a single or the average of as many as five determinations. The pH optima appear to lie between 6.9 and 7.2. A pH of 7 was, therefore, chosen to be used throughout this work since it was desirable for comparative purposes to maintain constant conditions.

Substrate Specificity

Esterase activity of the enzyme extract with the various substrates is presented in Table II. Activity is expressed in terms of the original extract, although various dilutions of the enzyme extract were used with the different substrates to obtain reaction rates which were constant with time. If too low a dilution was used the reaction rates decreased with time. When PP and PB were used as substrates, dilutions high enough to give a constant reaction rate had

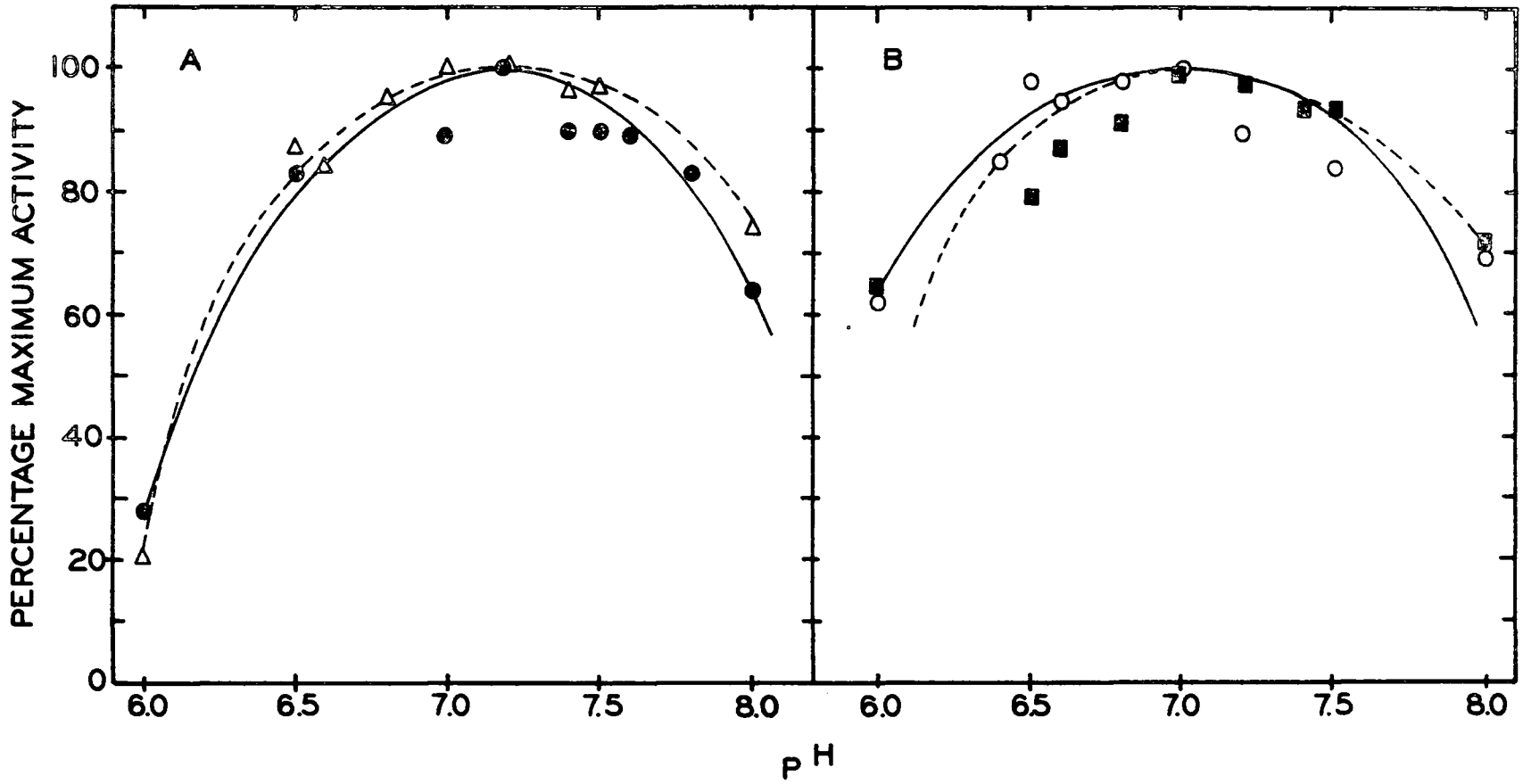


Figure 1. Effect of pH on esterase activity of pea extracts. LEGEND: △ , phenyl acetate; ⊕ , phenyl propionate; ○ , tripropionin; ▣ , tributyrin.

Table II. Hydrolysis of various esters by the pea extract.

Substrate	Number of Replications	Activity Units*	Standard Deviation
Phenyl Acetate	11	3.07	0.71
Phenyl Propionate	7	6.92	1.13
Phenyl n-Butyrate	3	6.81	1.01
Triacetin	5	0.73	0.17
Tripropionin	6	1.17	0.07
Tri-n-butyrin	8	0.66	0.14
Triolein	3	0.03	--
Acetyl ester of 2-Naphthol-6-SO_3Na	8	0.82	0.09
Propionyl ester of 2-Naphthol-6-SO_3Na	8	1.83	0.16
n-Butyryl ester of 2-Naphthol-6-SO_3Na	6	1.06	0.08
Hexyl, Octyl, Decyl, Hexadecyl esters of 2-Naphthol-6-SO_3Na	1	0.00	--
Acetyl Choline Iodide	1	0.03	--
Propionyl Choline Iodide	1	0.02	--
n-Butyryl Choline Iodide	1	0.02	--

*One activity unit represents one microequivalent of substrate hydrolyzed/min/ml of pea extract.

reduced activities. With these two substrates a 1:10 dilution of the extract was used for the assay, and activity was estimated from the reaction rate for the first ten minutes.

The phenyl esters were hydrolyzed the most rapidly, while the triglycerides and the naphthol esters were hydrolyzed to a lesser extent but at approximately the same rate. It is interesting to note that approximately twice as much activity was obtained with the propionyl esters than with the acetyl or n-butyryl esters of the triglycerides and naphthols. Since a mixture of esterases was present, however, it is not possible to determine if this was due to a higher specificity for the propionyl ester or the presence of more esterases which hydrolyze this ester. The esterases which hydrolyze the 2-naphthol-6-SO_3Na esters appear to be specific for the shorter chain esters since no activity was observed with the hexyl, octyl, decyl and hexadecyl esters.

The low activity observed toward the three choline esters suggests the absence of cholinesterases, since cholinesterases split choline esters at a higher rate than either aliphatic or aromatic esters (Augustinsson, 1959). This does not rule out the possibility, however, of the presence of a cholinesterase specific for substrates not tried.

As previously discussed, the term lipase has never been very well defined. Triolein, however, has been used as a substrate to

determine if lipase is present (Aldridge, 1954). Since triolein is water insoluble, it was suggested that only the lipase-type esterases will cleave it. Using this criteria, the very small amount of activity observed using triolein as substrate suggests the absence of lipase in the extract. The presence of lipase in peas has been reported, however (Lee and Wagenknecht, 1958). It appears, therefore, that either pea lipase was not extracted, was not stable under the experimental conditions employed, or that the pea lipases reported are actually esterases proper rather than lipase-type esterases.

As mentioned earlier, esterase activity decreased with dilution of enzyme when PP and PB were used as substrates. The effect was quite pronounced using PB as substrate (Table III).

Table III. Effect of enzyme dilution on activity using phenyl butyrate as substrate.

Extract Dilution	Number of Replications	Activity Units	Standard Deviation
1:10	3	6.81	1.01
1:20	4	3.11	0.56

The activity, calculated on the basis of the original extract, was about twice as much at a 1:10 dilution as it was at a 1:20 dilution. Two possible explanations for this effect might be dilution of an activator or the presence of an inhibiting substance in the distilled

water. Dixon and Webb (1964) state that ordinary distilled water is apt to contain sufficient heavy metal ions to produce an appreciable inhibition of sensitive enzymes. Diluting a sensitive enzyme with distilled water could very likely cause an increase in inhibition. After most of the work on this study was completed, evidence presented in the following section was obtained which supports this interpretation.

Effect of Heavy Metal Ions and Metal Complexing Agents

The effect of heavy metal ions on esterase activity was determined using PP as substrate and the results are presented in Table IV. Each value represents one determination. The esterase(s) which hydrolyzed PP were sensitive to all of the heavy metal ions tried. The degree of sensitivity varied with Zn^{++} and Cd^{++} being most inhibitory, followed by Ni^{++} and Co^{++}, while Mn^{++}, Ca^{++} and Mg^{++} were effective only at the higher concentrations.

The esterase(s) which hydrolyzed PP were also activated by metal complexing agents (Table V). EDTA and cysteine showed maximum activation at 1 mM. The large variation in percentage activation obtained with replication could be due to variation in concentration of metal ions in the distilled water, although this variation was not determined.

Table IV. Effect of heavy metal ions on esterase activity using phenyl propionate as substrate.

Compound	Concentration (mM) in Final Reaction Mixture	Percentage Inhibition
$CdCl_2 \cdot 4H_2O$	100	84
	10	62
	1	44
$CoCl_2 \cdot 6H_2O$	100	56
	10	37
	1	32
$NiCl_2 \cdot 6H_2O$	100	63
	10	40
	1	34
$ZnCl_2$	100	97
	10	60
	1	37
$MnCl_2 \cdot 4H_2O$	100	28
	10	14
	1	9
$CaCl_2$	100	20
	10	7
$MgCl_2$	100	10
	10	0
	1	0

Table V. Effect of metal complexing agents.

Compound	Number of Replications	Concentration (mM)	Percentage Activation	
			Mean	Range
EDTA	1	10	14	--
	2	1	56	30-82
	9	0.1	43	16-86
	3	0.0025	23	8-33
Cysteine Hydrochloride	1	10	20	--
	1	1	44	--
	1	0.1	0	--
Ethyl Mercaptan	1	0.05	14	--

The inhibition of esterase activity by metal ions and the activation by metal complexing agents suggests that the presence of metal ions in the distilled water may have caused the decrease in activity with dilution. This suggests that the activity determined with a metal complexing agent was probably the most accurate estimate of the true activity. Results (Table VI) show that the activity was considerably higher and less variable when EDTA was included in the assay mixture.

Table VI. Effect of EDTA on activity and standard deviation using phenyl propionate as substrate.

Treatment	Number of Replications	Activity Units	Standard Deviation
Without EDTA	8	5.63	1.11
With 0.1mM EDTA	8	8.30	0.52

With PP as substrate, 8.3 is probably the best estimate of esterase activity of the extract. This indicates that the results in Table II are low for PP and probably also for PB which also had the decrease in activity with dilution. Other values in the table may also be low if the substrates were hydrolyzed by metal sensitive enzymes. Had this effect been realized earlier, EDTA should have been incorporated into the standard assay procedure.

As seen by the standard deviation (Table II), the amount of variation between determinations was fairly low for the naphthyl esters and the triglycerides, but quite high for the phenyl esters, especially PP and PB. This variation might also be explained by the variation in the concentration of metal ions in the distilled water.

Effect of OP Inhibitors

Plots of inhibitor concentration versus percentage inhibition

or percentage activity have been used to distinguish between esterases. Aldridge (1953) was able to distinguish between two esterases which hydrolyzed the same substrates in this manner. One esterase was not inhibited by the OP compounds and 100 percent activity was observed at all the inhibitor concentrations used. The other esterase was inhibited by the OP compounds and a sigmoid curve was observed when activity was plotted against inhibitor concentration. Hence, if a sigmoid curve is obtained, at least one enzyme is being inhibited. Since different esterases could have the same inhibitor sensitivities, a single sigmoid curve does not necessarily imply the inhibition of only a single esterase. It is only when different sigmoid curves are obtained that sensitive esterases can be distinguished. For example, two esterases could be distinguished from one another if one was inhibited between pI (-log of M inhibitor concentration) 2 and 4 and the other between pI 6 and 8.

If both a sensitive and a resistant enzyme are present in the same preparation, hydrolyzing the same substrate, the sigmoid curve would be expected to level off or reach a plateau before 100 percent inhibition is reached. It is at this plateau that the percentage activity due to each of the esterases can be estimated.

Esterases, which have different inhibitor sensitivities and which are in the same preparation, hydrolyzing the same substrates can also be distinguished from each other. In this case the curve

obtained would be expected to approximate a double sigmoid curve. Myers, Tol, and de Jonge (1957) reported such a curve, a plateau was reached where an increasing concentration had little effect on activity until the inhibitor concentration was sufficient to start inhibiting the second enzyme. Again, it is at the plateau that the percentage activity of the two enzymes can be estimated.

The effects of the inhibitors parathion, TEPP, and DFP, at concentrations ranging from pI 1 to 10 on esterase activity of the pea extract were studied. The results are plotted in Figures 2, 3 and 4. In general, parathion and TEPP showed more selective inhibition toward the pea esterases than DFP.

The results indicate the presence of a mixture of esterases. The double sigmoid curves in Figures 2A, 3A, and 4A demonstrate that at least two esterases were hydrolyzing the acetyl and propionyl esters of phenol, 2-naphthol-6-SO_3Na and glycerol. The leveling off of the butyryl ester curves in the region pI 2 to 5 or 6 suggests that two esterases were also hydrolyzing the butyryl esters.

The leveling off of the NA curve in Figure 4C strongly suggests the presence of a third esterase. This esterase was not inhibited by DFP and represented 11 percent of the activity toward NA. It did not hydrolyze any of the other substrates tried, since 100 percent inhibition was observed using DFP with all other substrates.

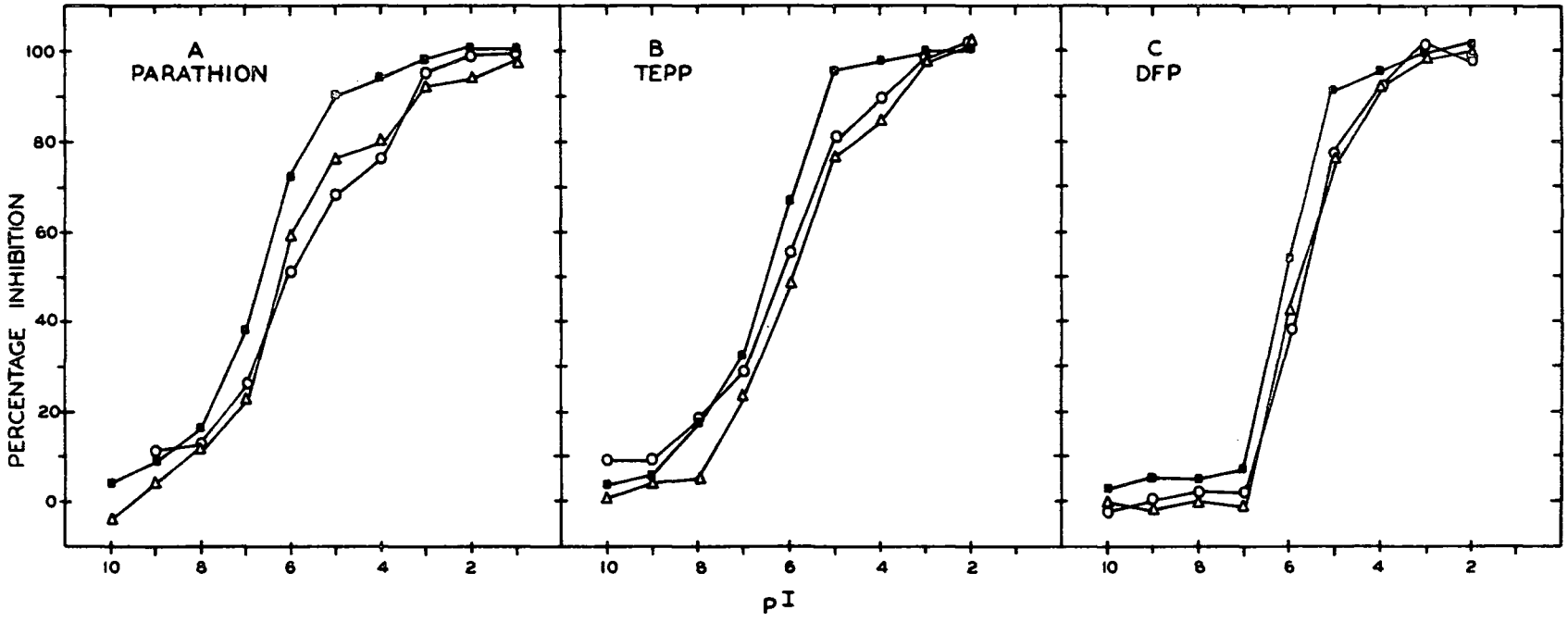


Figure 2. Inhibition curves for the hydrolysis of phenyl esters by extracts of peas. Number of replications for Figures A, B and C, respectively, are given in parenthesis. LEGEND: △, phenyl acetate (3, 7, 2); o, phenyl propionate (2, 6, 3); ■, phenyl butyrate (4, 3, 2).

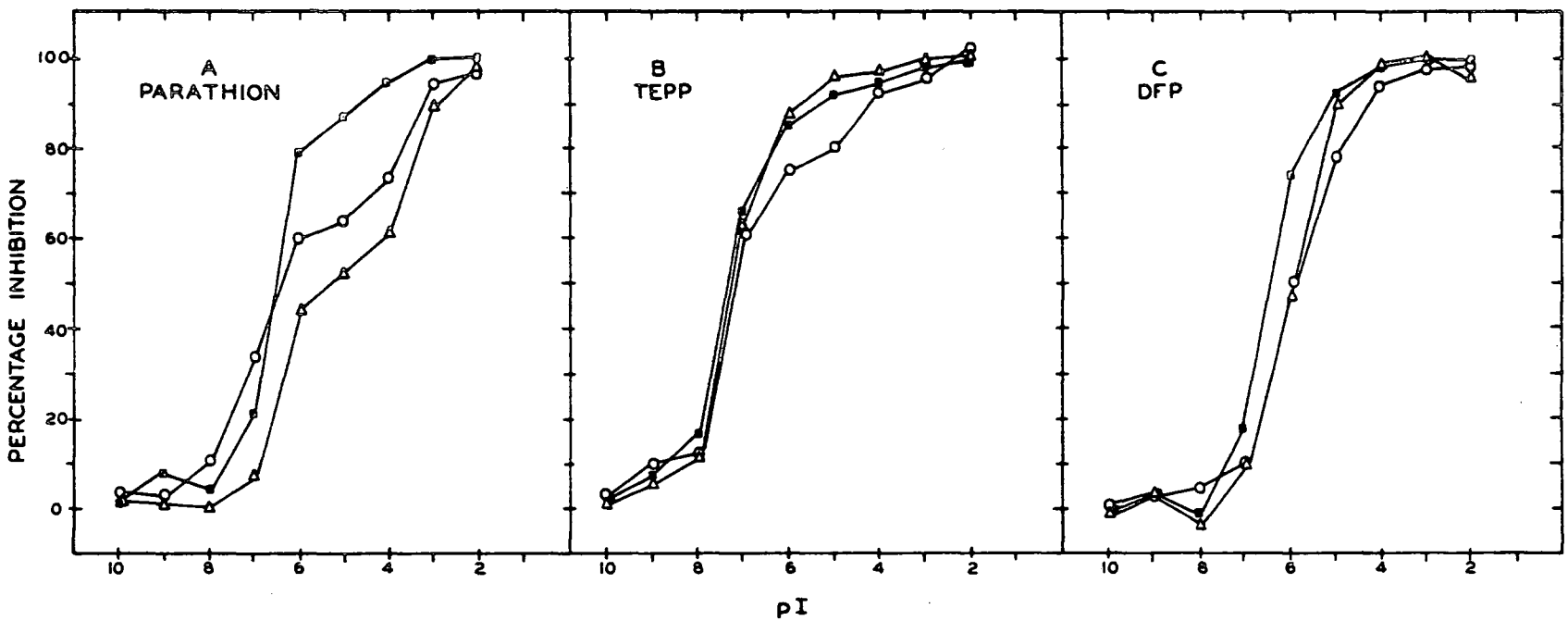


Figure 3. Inhibition curves for the hydrolysis of triglycerides by extracts of peas. Number of replications for Figures A, B and C, respectively, are given in parenthesis. LEGEND: △, triacetin (3,2,2); ○, tripropionin (3,2,2); ■, tributyrin (3,4,2).

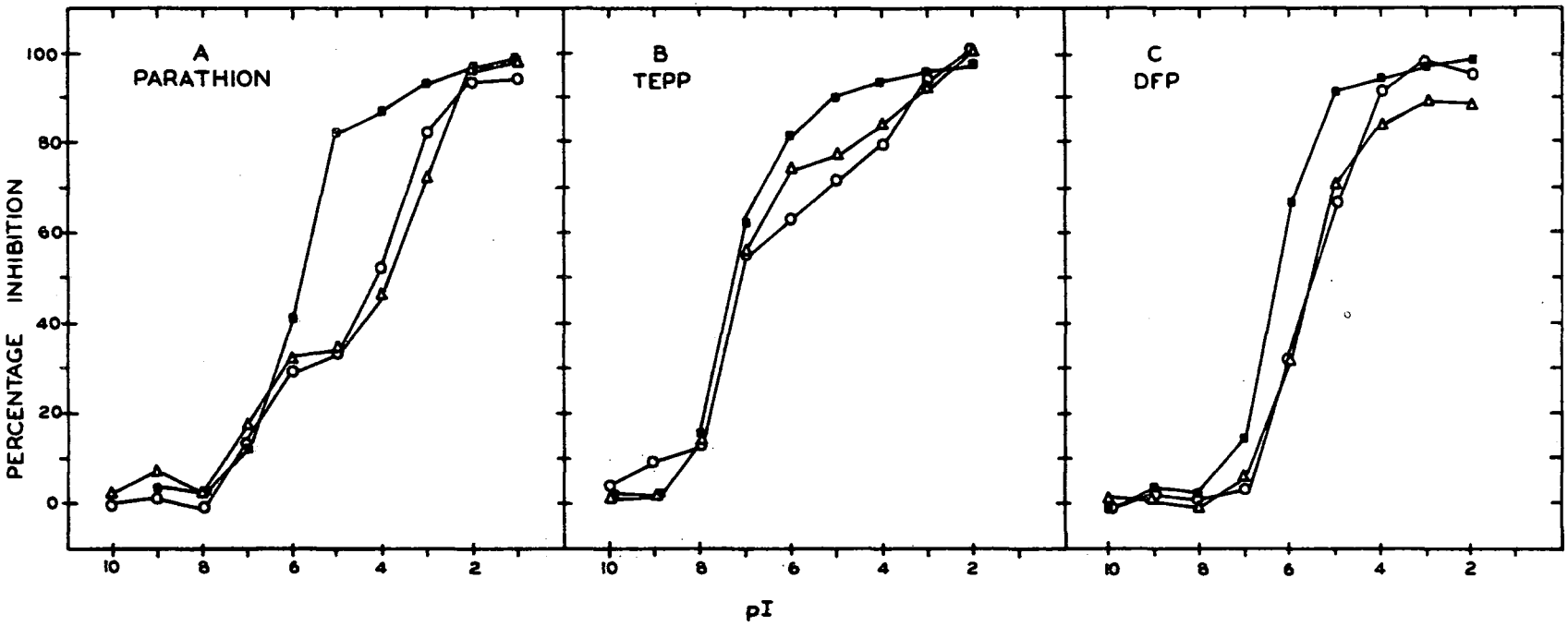


Figure 4. Inhibition curves for the hydrolysis of esters of 2-naphthol-6-SO_3Na by extracts of peas. Number of replications for Figures A, B and C, respectively, are given in parenthesis. LEGEND: Δ, acetyl ester (4,2,2); O, propionyl ester (4,2,2); ■, butyryl ester (3,2,2).

The curve for PA (Figure 2A) is suggestive of a triple sigmoid curve which would indicate the presence of three esterases which hydrolyzed PA. Eight percent of the activity toward PA could, therefore, represent a fourth esterase which was inhibited between pI 1 and 3 paration. However, this small difference occurring only at pI 2, may not be significant.

There is some evidence that the most sensitive esterase that hydrolyzed the phenyl esters (Figure 2A) and NB (Figure 4A) was not the same as the most sensitive esterase that hydrolyzed the triglycerides (Figure 3A), NA, and NP (Figure 4A), and that a fifth esterase may be present. The curves for the latter group reached a leveling off point at pI 6, but the former group did not reach this point until pI 5, and a substantial amount of inhibition occurred between pI 5 and 6.

There is also some evidence that the inhibition observed between pI 6 and 8 parathion and pI 6 and 8 TEPP was not due to the same enzyme. A larger portion of the activity toward NA, NP, and TA was inhibited at pI 6 by TEPP than parathion. Since pI 6 was the point where each curve reached a plateau, the percentage activity due to the esterases can be estimated. However, the estimates are vastly different with the two inhibitors. For example, using PA and parathion (Figure 4A), the percentage activity due to the most sensitive esterase is 30 percent; using TEPP, (Figure 4B), it is approximately 70 percent. This

would suggest the presence of a sixth esterase unless the esterase most sensitive to parathion was the same as the one least sensitive to TEPP.

The data, therefore, suggests the presence of as many as six esterases, three for which the evidence is quite conclusive. More may be present, if they have the same inhibitor sensitivities as those observed.

On the basis of their inhibition by the OP compounds all but one of the esterases appeared to be of the B type. The esterase which represented 11 percent of the activity toward NA, however, was not inhibited by DFP. This suggests that it was an A-esterase. However, the fact that it was inhibited by parathion and TEPP makes such a classification questionable. Similar problems have occurred in the classification of animal esterases. As pointed out earlier, Aldridge (1954) observed an A-esterase of pancreas which was not inhibited by three OP compounds, but which was inhibited by paraoxone. He found this inhibition to be reversible, however, and classified the esterase as an A type. Further study is needed to determine the status of this pea esterase. The fact that esterases have been found which cannot be placed in the established classification shows a weakness in the classification. The current classification probably will become less and less satisfactory as more esterases are studied, as exemplified by the soybean phenoxyesterase

(Jooste, 1962) discussed earlier.

Effect of Physostigmine and PCMB

The effect of 10^{-5} M physostigmine sulfate on esterase activity was determined with each of the nine substrates used in the previous section. No inhibition was observed. Since this is the concentration which completely inhibits cholinesterases (Augustinsson, 1959), none of the activity towards these substrates was due to a cholinesterase.

The effect on esterase activity of 10^{-3} M PCMB was determined with the same nine substrates. The results are presented in Table VII. Each value listed represents one determination. Varying amounts of inhibition were observed with all of the substrates, indicating that at least one of the esterases which hydrolyzed each of the substrates was sensitive to PCMB. Sensitivity to PCMB suggests that sulfhydryl groups are required for enzyme activity, although there is some question since a relatively high concentration of PCMB was used. According to Sohler (1952), inhibition caused by relatively high concentrations of the mercurials may not be specific for sulfhydryl groups on the enzyme. This high concentration of PCMB was used to check for PCMB activation as described by Bergman (1957). However, since at least one of the esterases hydrolyzing each of the substrates was inhibited by this

concentration of PCMB, it was not possible to determine if any activation occurred.

Table VII. Effect of 10^{-3} M PCMB on esterase activity.

Substrate	Percentage Inhibition
Phenyl acetate	17
Phenyl propionate	24
Phenyl-n-butyrate	46
Triacetin	56
Tripropionin	59
Tri-n-butyrin	62
Acetyl ester of 2-naphthol-6-SO_3Na	23
Propionyl ester of 2-naphthol-6-SO_3Na	13
n-Butyryl ester of 2-naphthol-6-SO_3Na	33

SUMMARY

The effect of pH on esterase activity of the pea extract was determined using PA, PP, TP, and TB as substrates. The pH optima appeared to lie between 6.9 and 7.2, and pH 7 was selected for use in this study.

Esterase activity of the enzyme extract was determined using a variety of substrates. The acetyl, propionyl and n-butyryl esters of phenol, 2-naphthol-6-SO_3Na and glycerol were hydrolyzed. The phenyl esters were hydrolyzed the most rapidly, while the triglycerides and the naphthyl esters were hydrolyzed to a lesser extent and at approximately the same rate. The long chain naphthyl esters were not hydrolyzed. The small amount of activity observed with the choline esters and triolein suggested the absence of cholinesterases and lipases in the extract.

Using PP and PB as substrates, the activity based on the original extract decreased with dilution of the enzyme. Since the esterase(s) which hydrolyzed PP were inhibited by heavy metal ions and activated by metal complexing agents, a possible explanation was inhibition of the esterase(s) by metal ions in the distilled water.

The effects of the inhibitors parathion, TEPP and DFP at concentrations ranging from pI 1 to 10 on esterase activity were determined. The data suggested the presence of as many as six

esterases in the aqueous extract of peas, three for which the evidence was quite conclusive. On the basis of their inhibition by OP compounds, all but one of the esterases appeared to be of the B type.

Physostigmine sulfate (10^{-5} M) had no effect on esterase activity with the nine substrates used indicating that the activity was not due to cholinesterases.

At least one of the esterases which hydrolyzed each of the substrates was sensitive to 10^{-3} M PCMB suggesting the importance of sulfhydryl groups for enzyme activity.

The water extract of lyophilized peas thus appeared to contain a mixture of mostly B-esterases which hydrolyzed a variety of esters.

BIBLIOGRAPHY

Aldridge, W. N. 1953a. Serum esterases. 1. Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate, propionate and butyrate, and a method for their determination. Biochemical Journal 53:110-117.

_____ 1953b. Serum esterases. 2. An enzyme hydrolysing diethyl p-nitrophenyl phosphate (E600) and its identity with the A-esterase of mammalian sera. Biochemical Journal 53:117-124.

_____ 1954. Some esterases of the rat. Biochemical Journal 57:692-702.

Allen, Sally Lyman, John M. Allen and Barbara Morrison Licht. 1965. Effects of triton X-100 upon the activity of some electrophoretically separated acid phosphatases and esterases. Journal of Histochemistry and Cytochemistry 13:434-440.

Augustinsson, Klas-Bertil. 1958. Electrophoretic separation and classification of blood plasma esterases. Nature 181:1786-1789.

_____ 1959. Electrophoresis studies on blood plasma esterases. I. Mammalian plasmata. Acta Chemica Scandinavica 13:571-592.

_____ 1964. Arylesterases. Journal of Histochemistry and Cytochemistry 12:744-747.

Barron, Kelvin D., Joseph Bernsohn and Adeline R. Hess. 1963. Separation and properties of human brain esterases. Journal of Histochemistry and Cytochemistry 11:139-156.

Benes, Karel. 1962. Histochemical demonstration of esterase in the root tip of Vicia faba with the azo coupling method. Biologia Plantarum Academia Scientiarum Bohemoslovenica 4:211-219. (Abstracted in Chemical Abstracts 56:14567g. 1962)

Bergman, F. and Sara Rimon. 1958. The effect of pH Variations on the activities of C-esterase. Biochemical Journal 70:339-344.

_____ 1960. Fractionation of C-esterase from the hog's kidney extract. Biochemical Journal 77:209-214.

Bergman, F., Ruth Segal and Sara Rimon. 1957. A new type of esterase in hog-kidney extract. Biochemical Journal 67:481-486.

Bernsohn, Joseph, Kevin D. Barron, Paul F. Doolin, Adeline R. Hess and Marjorie J. Hedrick. 1966. Subcellular localization of rat brain esterases. Journal of Histochemistry and Cytochemistry 14:455-72.

Born, W. H. Vanden. 1963. Histochemical studies of enzyme distribution in shoot tips of white spruce (Picea glauca (Moench) Voss). Canadian Journal of Botany 41:1509-1527.

Casida, J. E. and Klas-Bertil Augustinsson. 1959. Reaction of plasma albumin with 1-napthyl n-methylcarbamate and certain other esters. Biochimica et Biophysica Acta 36:411-426.

Cruess, W. V. 1958. Commercial fruit and vegetable products. 4th ed. New York, McGraw-Hill. 884p.

Desnuelle, P. 1951. Conférences hydrolyse enzymatique des triglycerides. Societié de Chimie Biologique Bulletin 33:909-923. Cited In: Hofstee, B. H. J. Fatty acid esterases of low eserine sensitivity and related enzymes. In: The enzymes, ed. by P. D. Boyer, J. Lardy and K. Myrback. Vol. 4. New York, Academic, 1960. p. 485-500.

Dixon, Malcolm and Edwin C. Webb. 1964. Enzymes. 2nd ed. New York, Academic. 950 p.

Downey, W. K. and P. Andrews. 1965. Esterase activity of the non-enzymic proteins of milk and serum. Biochemical Journal 96:21c-22c.

Forster, T. L., H. A. Bendixen and M. W. Montgomery. 1959. Some esterases of cow's milk. Journal of Dairy Science 42: 1903-1912.

Frankel, Trina A. and E. D. Garber. 1965. Esterases in extracts from germinating seeds of twelve pea varieties. Botanical Gazette 126:221-222.

Gregory, Kenneth F. and Harry C. Winter. 1965. Data reduction with constant-pressure respirometers. Analytical Biochemistry 11:519-531.

Harris, H., D. A. Hopkinson and E. B. Robson. 1962. Two-dimensional electrophoresis of pseudocholinesterase components in normal human serum. Nature 196:1296-1298.

Hofstee, B. H. J. 1954. Specificity of esterases. IV. Behavior of horse liver esterase towards a homologous series of n-fatty acid esters. Journal of Biological Chemistry 207:219-224.

_____ 1960. Fatty acid esterases of low eserine sensitivity and related enzymes. In: The enzymes, ed. by P. D. Boyer, J. Lardy and K. Myrback. Vol. 4. New York, Academic. p. 485-500.

International Union of Biochemistry. 1961. Report of the Commission on Enzymes. New York, Pergamon. 159 p. (I. U. B. Symposium series, vol. 20)

Jansen, Eugene F., Rosie Jang and L. R. Mac Donnell. 1947. Citrus acetylesterase. Archives of Biochemistry 15:415-431.

Jansen, Eugene F., M. D. Fellows Nutting and A. K. Balls. 1948. The reversible inhibition of acetylesterase by diisopropyl fluorophosphate and tetraethyl pyrophosphate. Journal of Biological Chemistry 175:975-987.

Jooste, Jasper van der W. and Donald E. Moreland. 1962. Soybean phenoxyesterase. Nature 195:907-908.

_____ 1963. Preliminary characterization of some plant carboxylic ester hydrolases. Phytochemistry 2:263-271.

Lee, F. A. and A. C. Wagenknecht. 1958. Enzyme action and off-flavor in frozen peas. II. The use of enzymes prepared from garden peas. Food Research 23:584-590.

Longenecker, H. E. 1946. Esterases. In: Enzymes and their role in wheat technology, ed. by J. A. Anderson. New York, Interscience. p. 127-152.

Mac Donnell, L. R., R. Jang, E. F. Jansen and H. Lineweaver. 1950. The specificity of pectinesterases from several sources with some notes on purification of orange pectinesterase. Archives of Biochemistry 28:260-273.

Martin, H. F. and F. G. Peers. 1953. Oat Lipase. Biochemical Journal 55:523-529.

Matile, P. H., J. P. Balz, E. Semadeni and M. Jost. 1965. Isolation of spherosomes with lysosome characteristics from seedlings. Zeitschrift für Naturforschung 20:693-698. (Abstracted in Chemical Abstracts 63:16780a. 1965)

Mounter, L. A. and Mary-Elizabeth Mounter. 1962. Specificity and properties of wheat-germ esterase. Biochemical Journal 85:576-580.

Myers, D. K. 1960. Carboxyl ester cleavage. In: The enzymes, ed. by P. D. Boyer, H. Lardy, and K. Myrback. Vol. 4. New York, Academic. p. 475-483.

Myers, D. K., A. Kemp, Justina W. Tol and Maria H. T. de Jonge. 1957. Studies on ali-esterases. 6. Selective inhibitors of the esterases of brain and saprophytic mycobacteria. Biochemistry 65:232-241.

Obara, Tetsujiro and Yasokichi Ogasawara. 1959. Esterases from seeds. IV. Activation of esterase from rice embryo. Nippon Nogei Kagaku Kaishi 33:876-880. (Abstracted in Chemical Abstracts 57:3778c. 1962)

Olcott, H. S. and T. D. Fontaine. 1941. Composition of cotton seeds. IV. Lipase of germinated seed. Journal of the American Chemical Society 63:825-827.

Osterbaan, R. A. and J. A. Cohen. 1964. The active site of esterases. In: Structure and activity of enzymes: Symposium No. 1 of the Federation of European Biochemical Societies, London, March 24, 1964. New York, Academic. p. 87-95.

Ory, R. L., A. J. St. Angelo and A. M. Altschul. 1960. Castor bean lipase: action on its endogenous substrate. Journal of Lipid Research 1:208-213.

Reed, Gerald. 1966. Enzymes in food processing. New York, Academic. 482 p.

Rongone, E. L., B. C. Bocklage, D. R. Strength and E. A. Doisy. 1957. Hydrolysis of steroid acetates by bovine albumin. Journal of Biological Chemistry 225:969-975.

Rudolph, K. and M. A. Stahmann. 1966. Multiple hydrolases in bean leaves (Phaseolus vulgaris L.) and the effect of the halo blight disease caused by Pseudomonas phaseolicola (Burkh.) Dowson. Plant Physiology 41:389-394.

Sarda, L. and P. Desnuelle. 1958. Action de la lipase pancreatique sur les esters en emulsion. Biochimica et Biophysica Acta 30:513-521. Cited in: Hofstee, B. H. J. Fatty acid esterases of low eserine sensitivity and related enzymes. In: The enzymes, ed. by P. D. Boyer, J. Lardy and H. Myrback. Vol. 4. New York, Academic, 1960. p. 485-500.

Schwartz, Drew. 1960. Genetic studies on mutant enzymes in maize: synthesis of hybrid enzymes by heterozygotes. National Academy of Sciences Proceedings 46:1210-1215.

Schwartz, Helen M., Sieglinde I. Biedron, Mavis M. von Holdt and S. Rehm. 1964. A study of some plant esterases. Phytochemistry 3:189-200.

Sohler, M. Rogatia, M. Angelice Seibert, Cornelius W. Kreke and Elton S. Cook. 1952. Depression of catalase activity by organic mercurial compounds. Journal of Biological Chemistry 198:281-291.

Tove, S. B. 1962. The esterolytic activity of serum albumin. Biochimica et Biophysica Acta 57:230-235.

Umbreit, W. W., R. H. Burris and J. F. Stauffer. 1964. Manometric techniques. 4th ed. Minneapolis, Burgess. 305 p.

Walek-Czanecka, A. 1963. Nonspecific esterase in the spherosomes of onion. Acta Societatis Botanicorum Poloniae 32(2): 405-408. (Abstracted in Chemical Abstracts 60:3271c. 1964)

Wennstrom, Joan I. and E. D. Garber. 1965. The genus Collinsia. XXVII. Separation of esterases and acid phosphatases in extracts from twelve species and one interspecific hybrid by

starch-gel electrophoresis. Botanical Gazette 126:223-225.

Wilde, C. E. and R. G. O. Kekwick. 1964. The arylesterases of human serum. Biochemical Journal 91:297-307.

Wilson, Irvin B. 1954. The mechanism of enzyme action. Baltimore, Johns Hopkins. 642 p.

Wilson, Irwin B. and M. A. Harrison. 1961. Turnover number of acetylcholinesterase. Journal of Biological Chemistry 236: 2292-2295.