AN ABSTRACT OF THE THESIS OF

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Title: MULTIPLE FORMS OF CARBOXYLESTERASES IN THE

GREEN BEAN (PHASEOLUS VULGARIS L.) AND PEA

(PISUM SATIVUM L.)

Abstract approved: _____
                              Morris W. Montgomery

Esterase activity of an aqueous extract of the green bean was separated into fourteen bands, while aqueous extracted pea esterases revealed seven bands, by polyacrylamide-gel electrophoresis. The fourteen bands of bean esterase activity formed three groups; slow, intermediate and fast moving. α-Naphthyl acetate, propionate, and n-butyrate and AS naphthol acetate were hydrolyzed at various rates by the bean and pea esterases. No hydrolysis of β-naphthyl laurate was observed, indicating the absence of a lipase in the aqueous extracts of these vegetables. Since all the esterase bands active toward α-naphthyl acetate were inhibited by organophosphorus compounds (diisopropylphosphorofluoridate, diethyl p-nitrophenyl thiophosphate and diethyl p-nitrophenyl phosphate), these esterases were classified as carboxylesterases (carboxylic ester hydrolase, EC 3.1.1.1).

To study the carboxylesterases of the green bean in greater detail, a protamine sulfate treated aqueous extract was separated into three fractions (S_I, S_{II} and S_{III}) by chromatography on Sephadex G-100. Subsequent analysis of each fraction by polyacrylamide-gel electrophoresis demonstrated the presence of the slow moving group in fraction S_I, slow and intermediate moving groups in fraction S_{II} and the fast moving group in fraction S_{III}. Hence, these studies suggest that the three groups of esterase activity in beans were dissimilar in molecular size and the relative molecular size was slow > intermediate > fast moving group.

Chromatography of fraction S_I on carboxymethyl (CM) cellulose with sodium chloride linear-gradient elution resulted in three fractions (CM_I, CM_{II} and CM_{III}). Similarly, fraction S_{II} yielded three fractions (DE_I, DE_{II} and DE_{III}), while fraction S_{III} produced two fractions (DE_{IV} and DE_V), by chromatography on microgranular diethylaminoethyl (DEAE) cellulose. Polyacrylamide-gel electrophoresis revealed the presence of the first three bands of the slow moving group in CM_I and only the first two bands in CM_{II}. DE_I possessed mainly the first two bands and DE_{II} the last two bands of the five bands in the slow moving group. The five bands of the intermediate moving group of esterase activity was found only in fraction DE_{III}. The first two bands of the four bands of the fast moving group were separated into fraction DE_{IV}, while the last two bands were in DE_V.

Nine substrates and various concentrations of three inhibitors were used to characterize some of the fractions obtained from ion-exchange chromatography. Although most of the fractions hydrolyzed the substrates used in this study, each fraction differed to some extent in substrate specificity. Inhibitor studies indicated the presence of a sensitive and a resistant component of esterase activity in each fraction studied. These results suggest that the esterase fractions were composed of two enzymes. To account for the fourteen bands of esterase activity a hypothetical model of polymers consisting of two monomers was proposed. This hypothesized model suggests that the slow moving group contained six pentamers, the intermediate group five tetramers and the fast moving group four trimers. Most characteristics of the carboxylesterases of beans observed in these studies could be explained on the basis of the hypothetical model.

Multiple Forms of Carboxylesterases in the Green Bean
(Phaseolus vulgaris L.) and Pea (Pisum sativum L.)

by

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INTRODUCTION

Esterases, unlike most enzymes, possess a low order of substrate specificity and have been studied with a wide variety of biochemical techniques. An important contribution to the study of this group of enzymes has been made by the use of electrophoretic methods followed by characterization of isolated enzymes based on their reactions with various substrates and inhibitors (Augustinsson, 1958 and 1959).

Introduction of starch gel and polyacrylamide gel as a medium in which to carry out electrophoretic separations has increased our capacity to resolve proteins. Using these techniques, evidence has been provided to show the presence of multiple forms of esterases in various plant species (Jooste and Moreland, 1963; Schwartz et al., 1964; Schwartz, 1962; Rudolph and Stahmann, 1966; Macko, Honold and Stahmann, 1967; Desborough and Peloquin, 1967; Fottrell, 1968). Plant esterases have not been investigated as thoroughly as animal esterases (Hunter and Markert, 1957; Markert and Hunter, 1959; Allen and Hunter, 1960; Paul and Fottrell, 1961; Ecobichon and Kalow, 1964; Holmes and Masters, 1967a, 1967b, 1968a, 1968b; Ecobichon, 1968). Recent reports from this laboratory (Norgaard and

Montgomery, 1968; Putnam and Montgomery, 1968; Carino and Montgomery, 1968) show that plant tissues contain complex systems of esterases. Considerable variation in regard to the number of esterases present and substrate and inhibitor specificities was found to exist among the vegetables, peas, beans and carrots.

Biological function of these vegetable esterases is not known. The possibility that esterases are involved in various metabolic systems which affect flavor, texture and color of food substances are of interest to the food scientist. Certain esterases are known to hydrolyze certain organophosphorus insecticides while others are inhibited by these compounds (Main and Braid, 1962; Rowlands, 1965). Therefore, esterases could detoxify insecticides, which otherwise would be harmful to humans. By inhibiting esterases, insecticides might affect the metabolism of the plant and cause undesirable changes in the flavor, texture and color of those plant parts used for food.

To understand the biological significance of plant esterases, detailed biochemical studies of each enzyme are essential. These investigations would require purified preparations of esterases. Therefore, the objectives of these studies were: (1) to determine the esterases present in aqueous extracts of peas and beans; (2) to classify these esterases according to their sensitivity to certain organophosphorus inhibitors; (3) to fractionate and purify the esterases of

the bean; and (4) to characterize these purified fractions of bean esterases.

REVIEW OF LITERATURE

Esterases are distinguished from other hydrolytic enzymes by their ability to catalyze the hydrolysis of ester linkages. Esterases have been classified into five distinct groups by Dixon and Webb (1958). This classification was based on the ability of the enzymes to preferentially hydrolyze one of the following types of esters--carboxylic, phosphoric, sulfuric, thiol or phenolic. Moreover, a few esterases have been found to hydrolyze amide derivatives (Myers et al., 1957) and acid anhydrides (Wilson, 1954). Proteolytic enzymes such as trypsin, chymotrypsin and thrombin have been reported to hydrolyze carboxyl esters (Myers, 1960). Erythrocyte carbonic anhydrase (Pocker and Stone, 1967) and 3-phosphoglyceraldehyde dehydrogenase (Park et al., 1961) have been reported to hydrolyze p-nitrophenyl acetate. This literature review deals only with those esterases which catalyze the hydrolysis of carboxylic esters.

Research on esterases has been centered mainly in the area of animal biochemistry, while plant esterases have been investigated to a limited extent. Therefore, the literature dealing with animal esterases will be discussed briefly as a background material.

Animal Esterases and Their Classification

In the literature, terminology associated with the designation of

various esterases is vague and at certain times confusing. The terms aliesterases, simple esterases and lipases have been used to designate the various esterases. Aliesterases or simple esterases were considered to be esterases catalyzing the hydrolysis of short-chain aliphatic esters (Hofstee, 1960); whereas, lipases were esterases which hydrolyzed the long-chain fatty acid esters, especially fats. These definitions changed however, when the following observations were made. Working with a liver esterase preparation, Sarda and Desnuelle (1958) found that solutions of long-chain aliphatic and aromatic esters were attacked at a much higher rate than short-chain esters while fats were not hydrolyzed. These authors also found activity of purified pancreatic lipase to be significantly pronounced only when the saturation point of the substrate was reached. These results led to the conclusion that fatty acid esterases could be divided into those active on substrates in solution (esterases proper) and those active on insoluable substrates (lipases). Considering the characteristics of liver esterase, Hofstee (1960) suggested that "lipase" refer to fat-splitting enzymes only.

For the purpose of this review, the term esterase will apply only to enzymes which exhibit their maximal activity on short-chain fatty acid esters.

Esterases from blood plasma have been divided into A- and B-esterases (Aldridge, 1953a; Augustinsson, 1958) on the basis of their

response to organophosphorus inhibitors. B-esterase was inhibited ·

by organophosphorus compounds, whereas A-esterase was not. Some

A-esterases hydrolyze certain organophosphorus inhibitors such as

diethyl-p-nitrophenylphosphate (E600, paraoxon) (Aldridge, 1953b)

and diisopropylphosphofluoridate (DFP) (Mounter, 1954). Esterases

were also grouped according to their substrate specificity

(Augustinsson, 1958, 1959). A-esterase, which hydrolyzed phenyl

acetate at a higher rate than phenyl butyrate, did not normally attack

aliphatic esters. B-esterase hydrolyzed both aliphatic and aromatic

esters but not choline esters.

Bergmann, Segal and Rimon (1957) also referred to A- and B-

esterases and suggested the presence of an imidazole ring at the active

site of the two enzymes. This was demonstrated by the reversal of

the inhibition of both enzymes by cupric ions with histidine. A-

esterase was also inhibited by p-chloromercuribenzoate (PCMB).

Hence, the presence of a sulfhydryl group at or near the active

center, in addition to the imidazole ring, was suggested. Because of

inhibition by DFP, B-esterase was considered to have a seryl hydroxyl

group in addition to the imidazole ring at the active center.

Unlike A-esterase, an esterase present in hog-kidney was

shown to be activated by PCMB at concentrations which inhibited A-

esterase (Bergmann et al., 1957). At higher concentrations, PCMB

inhibited this esterase. This enzyme, C-esterase, was not inhibited

by DFP. Other C-esterases have been reported by Barron, Bernsohn and Hess (1963) in human brain and by Bernsohn et al. (1966) in rat brain.

Cholinesterases, called C-esterase by Augustinsson (1958), hydrolyzed choline esters at higher rates than aliphatic and aromatic esters. These esterases were completely inhibited by 10^{-5} $\underline{M}$ or less of many organophosphorus compounds (Augustinsson, 1958 and 1959).

In 1961, in an attempt to clarify the confusion in enzyme classification, the Commission of Enzymes of The International Union of Biochemistry (1961), 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 systematic name identified the enzyme and indicated the substrate on which it acted as precisely as possible. The trivial name, was usually shorter and often the one in current use. The trivial name carboxylesterase (EC 3.1.1.1) was designated for aliesterase or B-esterase, while carboxylic ester hydrolase was the designated systematic name. Similarly, A-esterase was given the trivial name arylesterase (EC 3.1.1.2) and the systematic name of arylester hydrolase. This system is well suited for enzymes which are specific for certain substrates. However, many esterases have

wide substrate specificities and can not be grouped by this character-
istic alone.

Esterases from Plants

Putnam and Montgomery (1968), working with bean esterases,
suggested the absence of esterases or lipases capable of hydrolyzing
long-chain esters. Therefore, the literature dealing with plant lipases
will not be reviewed. Esterases from wheat germ, active toward
esters of acetic acid, were first reported by Sullivan and Howe (1933).
This enzyme was studied extensively by Singer and Hofstee (1948a and
1948b) and was classified as lipase. However, under present nomen-
clature it must be classified as an esterase (Hofstee, 1960). Jansen,
Nutting and Balls (1948) suggested that wheat germ esterase may be
classified as an acetylesterase (EC 3.1.1.6). An enzyme, similar to
wheat germ esterase, was isolated and purified from citrus (Jansen,
Jang and MacDonnell, 1947; Jansen et al., 1948). This enzyme,
classified as an acetylesterase, was inhibited by DFP and activated
by sodium chloride.

Jooste and Moreland (1962) reported an esterase from soybean
seedlings which differed from enzymes previously described. This
enzyme was highly specific for 2-naphthyl phenoxyacetate. It was not
inhibited by PCMB and only slightly activated by DFP. Electro-
phoresis, inhibitor and substrate specificities were used to

characterize esterases from wheat seeds, corn, cucumber and soy-
bean seedlings by Jooste and Moreland (1963). Results of starch-gel
electrophoresis revealed the presence of seven to nine bands of
esterase activity. Substrate and inhibitor studies indicated the
presence of A-esterases in soybean, wheat and corn, and B-esterases
in cucumber and soybean. Results of the electrophoretic studies sug-
gested that plant esterases probably exist in multimolecular forms
(isozymes).

Genetic control of the nature of the esterases present in maize
kernel has been reported by Schwartz (1960). Three forms of esterase
activity, which showed differential migration rates in starch-gel
electrophoresis, were found to be under the control of three allelic
genes.

Esterase activity towards indophenylacetate was found in green
beans, cabbage, potato tuber, citrus albedo and flavedo and fruits of
many cucurbits (Schwartz and Coworkers, 1964). Starch-gel electro-
phoresis, using α-naphthyl acetate as substrate, showed that all
extracts contained multiplicity of esterases which varied in different
species, in different strains of the same species, and even in different
parts of the same plant. Six bands of esterolytic activity were shown
to be present in green bean extract.

Frankel and Garber (1965) detected a maximum of six esterases
from germinating seeds of twelve varieties of the pea by starch-gel

electrophoresis. Polyacrylamide-gel electrophoresis of an aqueous

extract of bean leaves revealed nine esterase bands active toward α-

naphthyl acetate and seven active towards α-naphthyl butyrate

(Rudolph and Stahmann, 1967).

Norgaard and Montgomery (1968) made a study of esterases

present in peas. The activities of esterases of the pea were deter-

mined toward nine different substrates in the presence of three dif-

ferent organophosphorus inhibitors. At least six esterases appeared

to be present in the pea that differed in their substrate and inhibitor

specificity, five of which were classified as carboxylesterases.

Hexyl, octyl, decyl, and hexadecyl esters of sodium-2-naphthol-6-

sulfonate, triolein and acetyl, propionyl and n-butyryl choline esters

were not hydrolyzed or hydrolyzed slowly, which suggested the absence

of lipases and choline esterases. Based on inhibitor and substrate

specificity studies, Putnam and Montgomery (1968) identified three

esterases from aqueous extracts of beans. One esterase was resist-

ant to certain organophosphorus compounds, suggesting similarity to

A-esterase. Various concentrations of organophosphorus compounds

inhibited the activity of the other two esterases. These were classified

as carboxylesterases. In similar studies, Carino and Montgomery

(1968) reported six esterases from carrots.

Multiple Forms of Esterases

Until recently, the methods of selective inhibition offered the only effective approach to the determination of various esterases. Development of zone-electrophoresis techniques, utilizing charge and size differences to separate proteins in a semi-solid matrix, such as starch gel (Smithies, 1959) or polyacrylamide gel (Raymond and Weintraub, 1959), provided a method for separating enzymes in a crude homogenate. Application of histochemical reagents to such gels has revealed the presence of numerous molecular forms of enzymes which possess the ability to catalyze the same reaction. Hunter and Markert (1957) used the term "zymogram" to describe an electrophoretic matrix, which had been stained for specific enzyme activity.

Enzymes hydrolyzing simple esters (Richter and Croft, 1962) were shown quite early to exist in more than one form within the same species. The same phenomenon was demonstrated for lysozyme (Tallan and Stein, 1951), ribonuclease (Martin and Porter, 1951), and cytochrome c (Paleus and Neilands, 1950). After the observation by Neilands (1952) that crystalline lactic acid dehydrogenase (LDH) of beef heart contained two electrophoretically separable components, Markert and Moller (1959) coined the term "isozyme" to describe these components. An isozyme was defined as different molecular

forms in which proteins may exist with the same enzymatic activity.

The concept of isozymes has in recent years aroused great interest among biologists and biochemists working in many different areas. Through the use of such techniques as described above, an ever-increasing number of enzymes have been reported as existing in more than one molecular form. In fact, it appears that the enzyme existing in only one form is an exception. For the majority of such enzyme systems, the genetic and functional significance of the multiple forms has not yet been made clear. Groups of isozymes for which the structure, genetic origin and significance of tissue distribution have been partly elucidated are the lactic acid dehydrogenase (LDH), and malate dehydrogenase (MDH) (Kaplan, 1968). For most other enzyme systems, such detailed studies have not been undertaken.

In a recent review, Kaplan (1968) proposed various mechanisms to account for multiple molecular forms of enzymes. Polymerization of two or more types of subunits, which have different amino acid composition, illustrated by the LDH isozymes (Markert, 1963), is one of the simpler mechanisms for the production of multiple forms. Creatin kinases (Dawson, Eppenberger and Eppenberger, 1968) and adolases (Penhoet, Rajkumar and Rutler, 1966) represent a group of enzymes with isozymes similar to the LDH's. In these cases the number of subunits per molecule is the same for all the molecular forms. Differences in electrophoretic migration are brought about

by different charges on the various types of subunits.

Kaplan (1968) designated another group of multiple forms of enzymes as 'conformers' which are not caused by genetic phenomenon. Conformers have the same amino acid sequence, but exist in varying conformations. These differences in conformations may be such that differences in exposed charges are involved, and that variations in electrophoretic migration arise, as well as, in behavior on cation or anion exchange resins. Mitochondrial malate dehydrogenases of chicken heart represent this group of isozymes (Kitto, Wassarman and Kaplan, 1966).

In addition to isozymes due to non-genetic phenomena, Kaplan (1968) discussed other causes of multiple forms of enzymes. Artifacts may arise as a result of cleavage of peptide bonds by proteases. Proteolysis appears to be responsible for some of the multiple forms that have been found for yeast hexokinase (Kaplan, 1968). Impurities in substrates and other reagents might also lead to a misleading interpretation concerning the number of isozymes present in a given tissue. For example, electrophoretic investigation of a dehydrogenase in liver may lead to erroneous conclusions because there are a number of isozymes of alcohol dehydrogenase present in liver. Ethanol is present in many solutions, in substrates, and even in nicotinamide-adenine dinucleotide phosphate preparations. Hence, in order to establish with accuracy the number of multiple forms of a given

dehydrogenase in liver, one must rule out the artifacts that can be induced by ethanol and alcohol dehydrogenase. It is also possible, according to Kaplan (1968) that isozymes may result from the same protein having varying degrees of amidation.

Binding of varying amounts of substrate, coenzyme, carbo-hydrate, lipid and salts might be responsible for the existence of some multiple forms. Different degrees of acetylation also may be a cause for the appearance of multiple forms (Kaplan, 1968).

Multiple molecular forms may also arise from the existence of different aggregates of a given enzyme. The structure of the aggre-gate might be of such a nature that their charge exposure may be dif-ferent, resulting in a difference in mobility. Some of the serum choline esterases appear to exist as aggregates (Lamotta, McComb and Wetstone, 1965).

Since esterases have broad substrate specificity and share esterolytic activity with other proteins, evidence for molecular mechanism of isozyme formation is not easily obtainable. However, the work of Holmes and Masters (1967a), in which they discussed possible molecular mechanisms to account for the isozymic nature of cavian (guinea pig) esterases, indicates that esterases can be suitable material for the study of molecular mechanisms of isozymes. With starch-gel electrophoresis as many as 24 bands of esterase activity were separated and the occurrence of these individual forms

in the different tissues of cavian were inter-related. From the inhibitor and substrate studies, the presence of carboxylesterases, arylesterases, acetylesterases, and choline esterases were proposed. The arylesterase activity of cavian tissues were resolved in four separate bands. From detailed observations of these bands, Holmes and Masters (1967a) suggest that different arylesterase forms possessed similar activity sites but differed primarily in the electrical charge of the carrier protein. Confirmation of this concept will require extensive information on the primary structure of these arylesterases.

Esterases also have been used conveniently for the study of genetics of isozymes. Allen (1961), working with the protozoan, Tetrahymena pyriformis, found that four distinct esterases were under the control of a single gene. He reasoned that esterases, which were under the control of a single gene, showed similar substrate and inhibitor specificities, while other esterases present in the same species were of a different type and were not affected by changes in the gene. It seems likely that such similarity can be taken as an indication of increased probability of finding common structural subunits in two or more different enzymes.

On the other hand, two forms (slow and fast) of insect esterases, which were under the control of two allelic genes, were shown to be quite different with regard to their sensitivity to an organophosphorus

inhibitor (Wright, 1963). At 10^{-4} $\underline{M}$ NN'-diisopropylphosphorodiamidic

fluoride (Mipafox), the activities of both the fast and slow forms of

esterase were only reduced. However, at 10^{-3} $\underline{M}$, the slow form

showed no activity, whereas the fast form was still active, although

the activity was reduced to about half that of the control. Another

difference between these two forms was that the fast form was more

heat labile than the slow form. Thus the two genetically controlled

forms of insect esterase were differentially sensitive to organo-

phosphorus inhibitor and to heat (Wright, 1963).

A hypothesis formulated by Desborough and Peloquin (1967)

to explain the occurrence of 15 isozymes in $\underline{Solanum}$ tubers is worth

mentioning. The biochemical hypothesis was based on an active

isozyme of a tetramer composed of one, two, or three types of

monomers. Three monomers may be arranged in fifteen isozymes,

each representing an individual band. It was also hypothesised that

all three monomers were under the control of three allelic genes.

Recently, Montgomery, Norggard, and Veerabhadrappa (1968),

in an attempt to establish the nature of seven multiple forms of pea

carboxylesterases, obtained three fractions. Two of these fractions

showed identical inhibition and substrate specificities and were sug-

gested to be isozymes. The third fraction exhibited differences in

inhibitor and substrate specificities and was shown to contain one

major esterase band on polyacrylamide-gel electrophoresis.

SECTION I

Electrophoretic Studies of Pea and Bean
Carboxylesterases

MATERIALS AND METHODS

Preparation of Pea Extract

Freshly harvested peas (variety Dark Skin Perfection) were obtained from Lamb-Weston Inc., Weston, Oregon. The peas were freeze-dried, with the temperature of the heating plates remaining below 38°C, packed in No. 2 1/2 cans under N_2, and stored at -18°C until needed. Peas were ground in a Waring Blendor for two minutes and the resulting dry powder was extracted with nine volumes of cold water (5°C). The slurry was centrifuged in the cold (5°C) at 27,000 x G for 30 to 40 minutes in a Super-Speed Servall centrifuge. The resulting supernatant was treated with 0.1 N hydrochloric acid to pH 5.5 and centrifuged to remove the precipitate at 12,000 x G for 10 minutes. The clear supernatant solution constituted the crude enzyme.

Preparation of Bean Extract

Freshly harvested green beans (variety Tender-Crop) were obtained from Blue Lake Packers, Inc., Corvallis, Oregon. The

beans were cut into 1 inch pieces, treated, and stored under the same conditions as for the peas.

The aqueous extract of the bean was prepared by grinding the freeze-dried beans in Waring Blendor for two minutes, suspending with four volumes of cold water (5°C) and mixing. The slurry was centrifuged in the cold (5°C) at 27,000 x G for 40 minutes in a Super-Speed Servall centrifuge. Ten ml of the resulting supernatant was treated with one ml of two percent protamine sulfate (pH 6.5). This solution was allowed to stand for 30 minutes at 4°C and centrifuged at 12,000 x G for ten minutes. The resulting supernatant was lyophilized and stored in the dry form. This solid material was dissolved in water and used routinely for electrophoresis.

Substrates

The following esters were used as substrates: α-naphthyl acetate,[1] α-naphthyl propionate,[1] α-naphthyl butyrate,[1] AS naphthol acetate[2] and β-naphthyl laurate.[3]

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[1] Sigma Chemical Company

[2] K & K Laboratories, Inc.

[3] Mann Res. Laboratories, Inc.

Inhibitors

Inhibitors used in this study included the following: diisopropyl phosphorofluoridate,[4] (DFP), diethyl-p-nitrophenylphosphate[4] (E600, paraoxon), diethyl-p-nitrophenylthiophosphate[4] (parathion) and physostigmine sulfate[4] (eserine). Inhibitors were prepared in one percent (w/v) Triton X-100[5]. The 0.1 $\underline{M}$ parathion and paraoxon solutions required 60 seconds homogenization in a microblender to form a semi-stable emulsion. DFP and eserine were soluable at the concentrations used.

Electrophoretic Procedure

Zone electrophoresis was performed vertically in a plexiglass cell (Model EC-470, E-C Apparatus Corporation) with a companion power supply (Model EC-454). Cyanogum-41[6], a mixture of acrylamide and $\underline{N},\underline{N}$-methylenebisacrylamide, was used as the supporting medium. Approximately 150 ml of seven percent cyanogum-41 was prepared in 0.017 $\underline{M}$ Tris-0.2 N HCl buffer (pH 7.5) and filtered through Whatman No. 1 paper. After adding a small drop (approximately 0.02 ml) of Tween 80, gel polymerization was catalyzed by

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[4]K & K Laboratories, Inc.

[5]Rohm and Haas Company

[6]E-C Apparatus Corporation

adding 0.15 ml N,N,N',N'-tetramethylethylenediamine[7] (TMED) and

0.15 gm ammonium persulfate.[8] The gel solution was immediately

poured into the space between the cooling plates of the electrophoretic

cell maintained in a horizontal position. The slot former was inserted

and the polymerization occurred within 60 minutes to form a colorless

gel (17 cm x 12 cm x 0.4 cm). By insertion of a slot former prior to

the polymerization reaction it was possible to vary the number and

volume of the samples. In most cases a former containing 4 slots

and permitting a maximum sample volume of approximately 200 μl

per slot was used.

After polymerization, the exposed block of gel above the slot

former was excised. The remaining electrophoretic operation was

carried out in the cold room at 5°C. The cell was turned to a verti-

cal position, and the electrode buffer (0.03 $\underline{M}$ Boric acid-0.1 N NaOH,

pH 8.7) was poured into upper compartment until overflow covered

the platinum wire electrode in the lower compartment by two to three

cm. The closed buffer system holds two liters. Water circulation

through the cooling channels of the cell was maintained continuously

during the run. The slot former was removed by gently sliding

directly upward and the buffer was circulated before sample application

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[7]Sigma Chemical Company

[8]E-C Apparatus Corporation

for 20 minutes to allow equilibration of the gel-buffer system. At the end of 20 minute period, buffer circulation was stopped.

Samples in ten percent sucrose, containing a small quantity of bromophenol blue (as marker), were applied directly onto the slots at the top of the gel to form a layer under the buffer. Electrophoresis was performed at a constant potential of 300 volts for 2.5 hours. Buffer circulation was started after the samples were completely stacked in the gel.

Treatment of the Gel

On completion of electrophoresis, the gel was removed from the cell and cut into four strips. Techniques were essentially those of Markert and Hunter (1959) for detection of hydrolysis of the naphthol esters. Fast blue RR[9] was the diazonium salt used for simultaneous coupling with the naphthol moiety released upon hydrolysis. The gel strips were placed in 100 ml of 0.1 $\underline{M}$ sodium acetate (pH 5.6) containing 70 mg of fast blue RR salt and 40 mg of ester dissolved in 2 ml of acetone. The time of incubation at 37°C was 30 minutes and 1.5 hours for peas and beans, respectively.

To study the effect of inhibitors, the gel strips were incubated in a solution of the inhibitor for one hour at 37°C. Longer incubations

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[9]K & K Laboratories, Inc.

did not cause further inhibition. A control gel strip was incubated in one percent Triton X-100 without inhibitor. At the end of the incubation period, inhibitor solution was poured out and the gel was placed in the substrate solution.

RESULTS

Substrate Specificity of Esterases

The reactivity of the bean and pea esterases with four different substrates is shown in Figures 1 and 2. A number of enzymes were shown to be capable of hydrolyzing α-naphthyl acetate. In bean extract (Figure 1) at least 14 bands of esterolytic activity were separated. All bands of esterolytic activity could be divided into three groups depending upon their electrophoretic mobility. The slow-moving group, containing the first five bands constitutes the first group. The second group, intermediate in mobility, appeared to contain a wide zone of activity which was later shown to contain five bands (6-10). The fast-moving group, containing bands 11 through 14, belong to the third group.

Bands 1 and 2 in the first group were active towards α-naphthyl acetate and α-naphthyl propionate, but not with α-naphthyl butyrate. Bands marked 3, 4, and 5 were moderately active and reacted equally well with α-naphthyl acetate, propionate, and butyrate. The second group, containing bands 6 through 10, is shown to be active on both α-naphthyl acetate and α-naphthyl propionate, but did not appear to hydrolyze α-naphthyl butyrate. The third group was active with all three substrates, however, the activity followed descending order
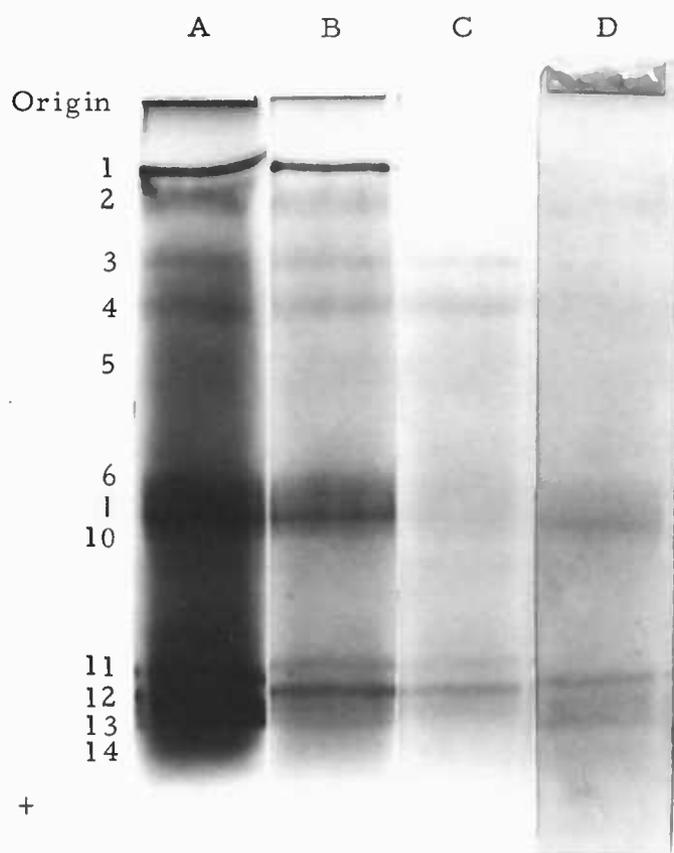
Figure 1. Esterase zymogram of bean (Phaseolus vulgaris L.).
Stained with: A, α-naphthyl acetate; B, α-naphthyl
propionate; C, α-naphthyl butyrate; D, AS naphthol
acetate.
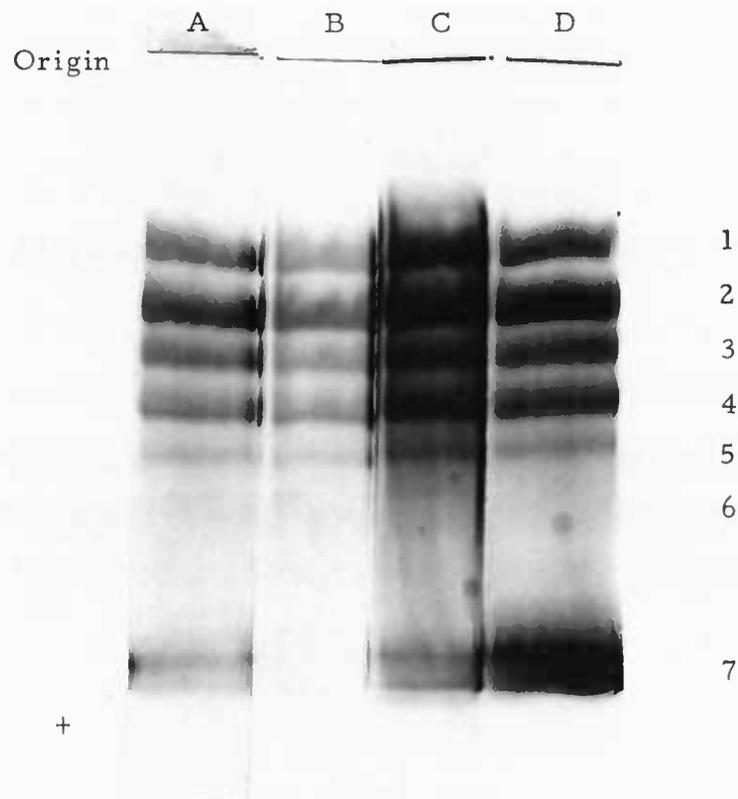
Figure 2. Esterase zymogram of pea (<u>Pisum sativum</u> L.).
Stained with: A, AS naphthol acetate; B, α-
naphthyl butyrate; C, α-naphthyl propionate; and
D, α-naphthyl acetate.

from α-naphthyl acetate to α-naphthyl butyrate. This group developed within two to three minutes after immersing the gel in the substrate mixtures and seemed to be the major group of esterase enzymes in beans.

Zymogram produced with AS naphthol acetate showed only slight activity with first and second groups and the largest activity with the third group. These bands appeared purple in color and developed after 12 hours.

Esterase zymogram prepared from pea extract is shown in the Figure 2. At least seven esterolytic activity bands have been demonstrated. These bands were divided into two groups; first group being slow in mobility, contained 1 through 6 bands, and the second, faster moving group, band 7, had a wide zone of esterase activity.

All bands, except band 6 in Figure 2, appeared within two to three minutes after the gel was immersed in the substrate mixture. After five minutes of incubation with α-naphthyl acetate and α-naphthyl propionate, bands 2 and 7 were very intense in color, 3 and 4 moderate, 5 the least intense, and 6 was not visible. However, after five minutes of incubation with α-naphthyl butyrate, bands 2 and 7 were moderate in color, 3 and 4 the least intense, and 5 and 6 were not developed. It appears that α-naphthyl propionate was the preferred substrate for the slow-moving bands followed by α-naphthyl acetate and butyrate, respectively. The preferred substrates for the

fast-moving bands were α-naphthyl acetate and propionate while α-naphthyl butyrate showed only slight activity. This observation indicates, perhaps, a degree of chain length steric specificity for this enzyme. All seven bands indicated activity with AS naphthol acetate and required 7 hours to develop.

The absence of activity both in the case of peas and beans with β-naphthyl laurate indicates that these esterases preferentially hydrolyze esters of short-chain fatty acids.

<div align="center">Inhibitor Studies</div>

Organophosphorus inhibitors have been used by various workers (Augustinsson, 1958 and 1959; Bergmann, Segal and Rimon, 1957; Jansen, Nutting and Balls, 1948; Aldridge, 1953a; and Menzel, Craig and Hoskins, 1963) in classification and identification of esterases. In the present studies, α-naphthyl acetate was employed as the substrate. Figure 3 shows the effect of various concentrations of paraoxon on the esterase activity of beans. The streaking effect was noticed only in the gels incubated with solutions containing Triton X-100. Paraoxon at 10^{-2} $\underline{M}$ inhibited most of the activity of the bean extract. The first group was the most sensitive to paraoxon, while the second and third groups were more resistant and appeared to be approximately of equal resistance. Due to the streaking, the effect of paraoxon on the individual bands could not be determined;
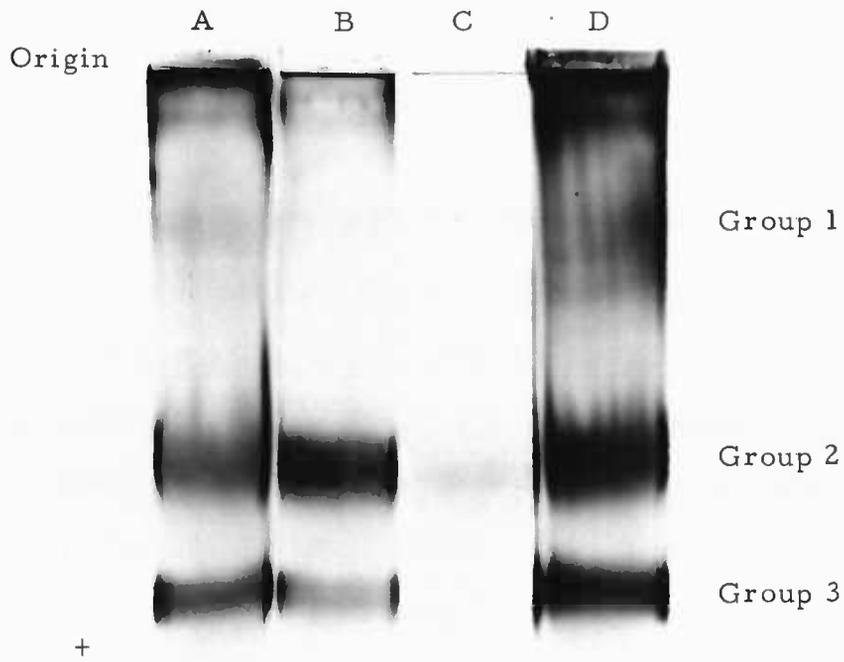
Figure 3. Esterase zymogram of bean after treatment with different concentrations of paraoxon: A, 10^{-4} $\underline{M}$; B, 10^{-3} $\underline{M}$; C, 10^{-2} $\underline{M}$; and D, α-naphthyl acetate control.

however, all bands within a given group appeared to behave
similarly.

Figure 4 shows that even at 10^{-2} $\underline{M}$ parathion considerable
activity remained, particularly with the second and third groups.
This observation is in agreement with the work of Putnam and
Montgomery (1968), who showed that 60 percent of the bean esterase
activity was inhibited at 10^{-2} $\underline{M}$ parathion. The first group was
quite sensitive to parathion with most of the activity inhibited at
10^{-4} $\underline{M}$.

At all concentrations used, the first group was sensitive to
DFP (Figure 5). At 10^{-4} $\underline{M}$ DFP, the activities of both the second
and third group of esterases were only slightly reduced. However,
at 10^{-3} $\underline{M}$ and 10^{-2} $\underline{M}$, the third group showed nearly complete inhibi-
tion, whereas the second group was still active, although the activity
was reduced to about half that of the control.

The effect of inhibitors on pea esterases is shown in the
Figures 6, 7 and 8. At the highest concentrations of all the inhibitors
used in this study (10^{-2} $\underline{M}$), complete inhibition was obtained, except
with parathion where band 7 still exhibited a small amount of
activity. At 10^{-3} $\underline{M}$, a different response between fast and slow
moving bands was quite apparent. Band 7 seemed to be quite
resistant to both parathion and paraoxon at 10^{-3} $\underline{M}$, whereas with
DFP, it showed only slight activity. At 10^{-4} $\underline{M}$, most of the activity
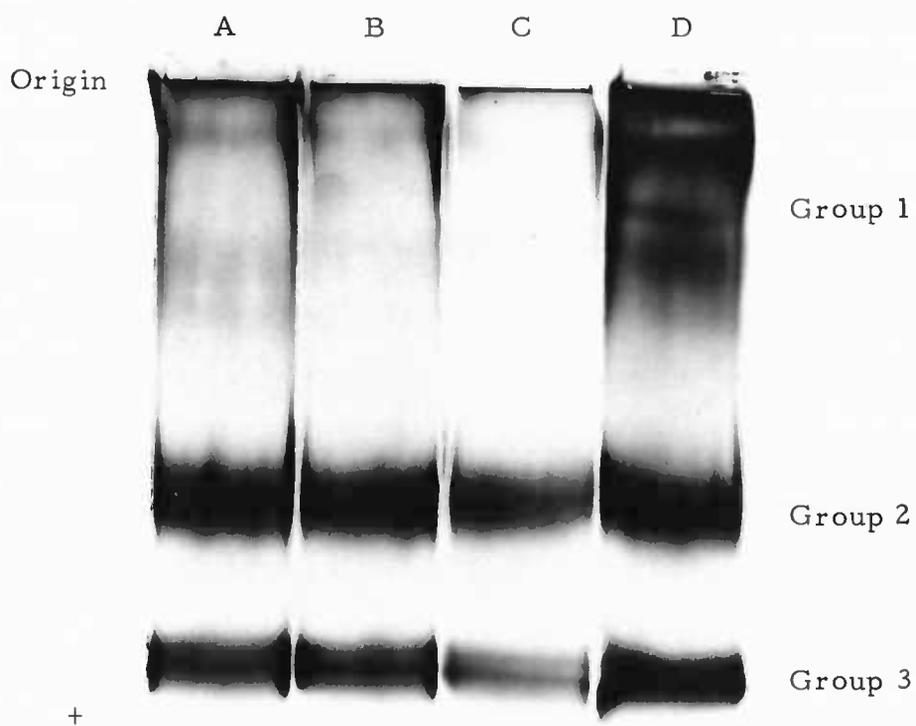
Figure 4. Esterase zymogram of bean after treatment with
different concentrations of parathion: A, 10^{-4} $\underline{M}$;
B, 10^{-3} $\underline{M}$; C, 10^{-2} $\underline{M}$; and D, α-naphthyl acetate
control.
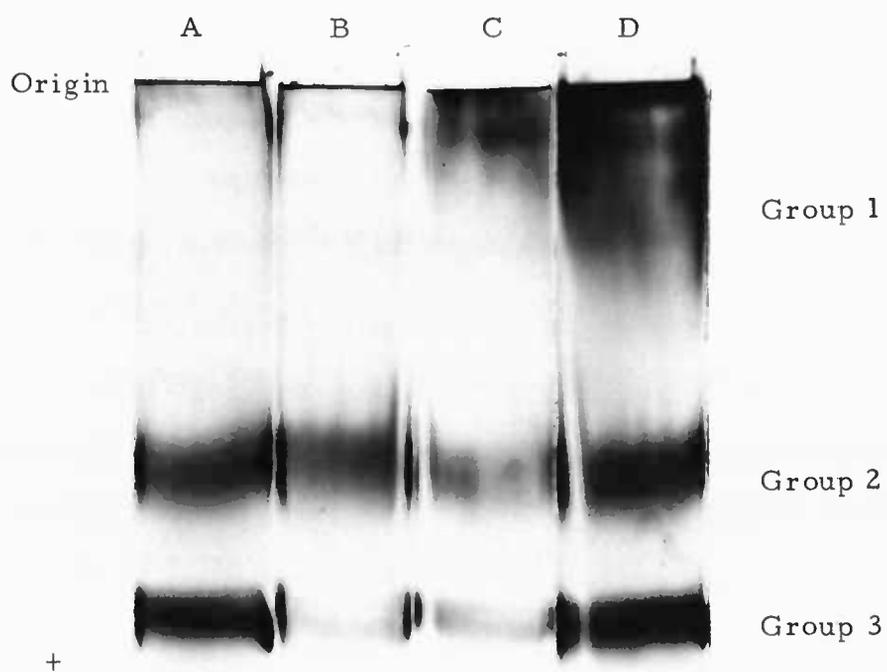
Figure 5. Esterase zymogram of bean after treatment with different concentrations of DFP: A, 10^{-4} $\underline{M}$; B, 10^{-3} $\underline{M}$; C, 10^{-2} $\underline{M}$; and D, α-naphthyl acetate control.
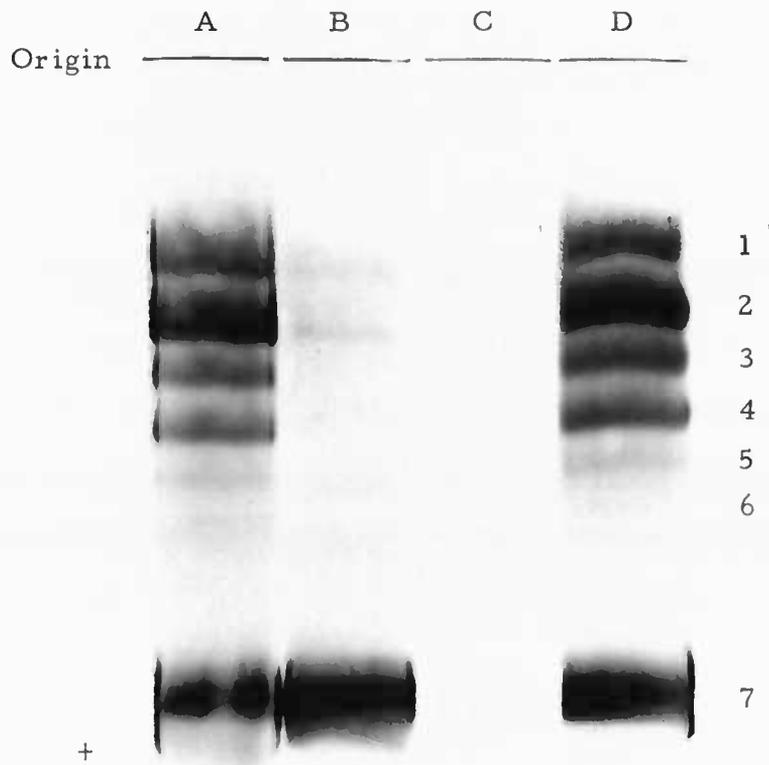
Figure 6. Esterase zymogram of pea after treatment with different concentrations of paraoxon: A, 10^{-4} $\underline{M}$; B, 10^{-3} $\underline{M}$; C, 10^{-2} $\underline{M}$; and D, α-naphthyl acetate control.
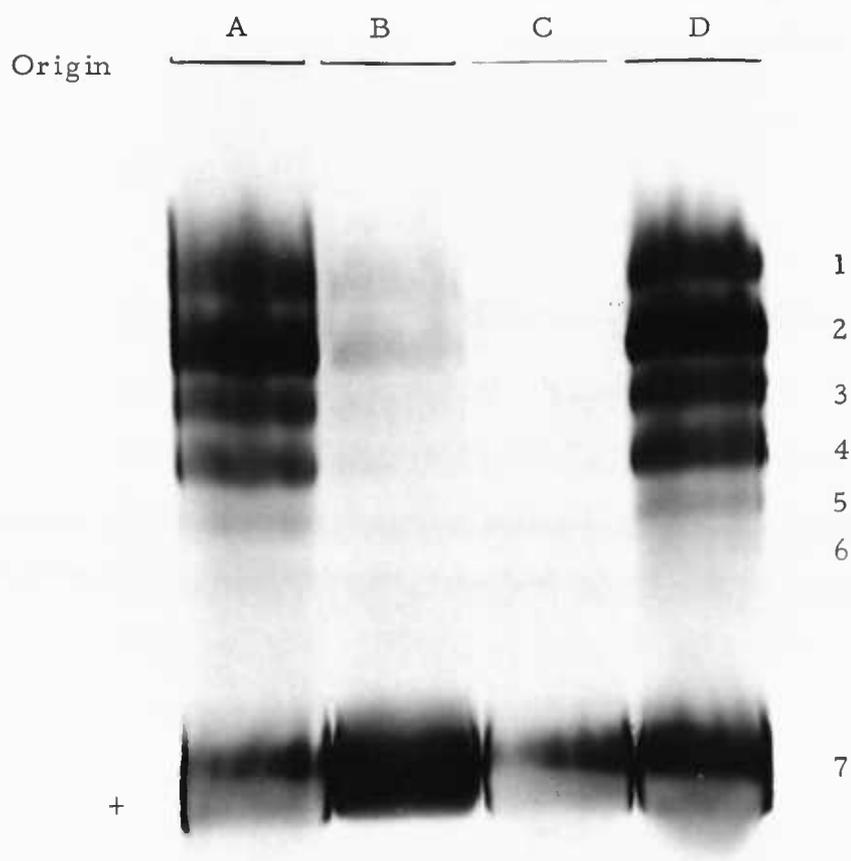
Figure 7. Esterase zymogram of pea after treatment with different concentrations of parathion: A, 10^{-4} $\underline{M}$; B, 10^{-3} $\underline{M}$; C, 10^{-2} $\underline{M}$; and D, α-naphthyl acetate control.
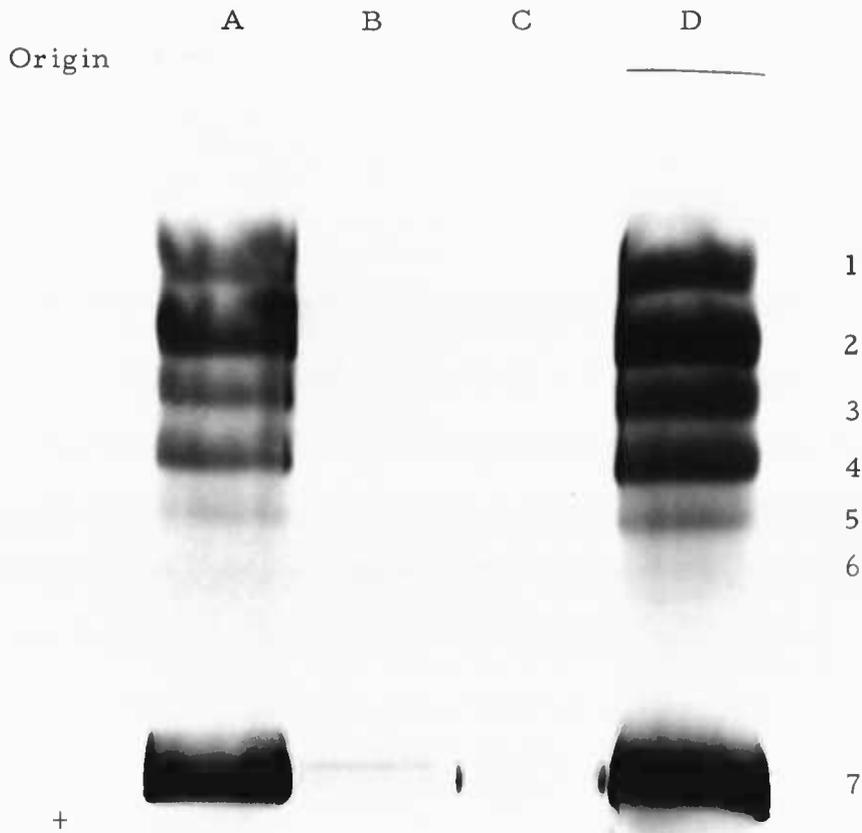
Figure 8. Esterase zymogram of pea after treatment with different concentrations of DFP: A, 10^{-4} $\underline{M}$; B, 10^{-3} $\underline{M}$; C, 10^{-2} $\underline{M}$; and D, a-naphthyl acetate control.

of pea esterases was observed with all three inhibitors. This observation does not correlate well with the work of Norgaard and Montgomery (1968), where 10^{-4} DFP and parathion, the pea extract exhibited 80 and 50 percent inhibition, respectively. This phenomenon might be explained on the basis that the concentration of inhibitor inside the gel was less than 10^{-4} M. Moreover, the results (Figures 6, 7 and 8) are only an approximation without quantitative data.

DISCUSSION

Esterases consist of a heterogenous family of enzymes. However, only with the use of electrophoretic techniques has the complexity of this group of hydrolases been fully realized. Seven and 14 bands were noted in the extracts of peas and beans, respectively. The difference between these two vegetables appeared to be the presence of the second group of esterases in the beans. Reasons for this difference are not readily apparent at this time. Both plants belong to the same family (Leguminosae) and similar tissue (seeds) was used from both plants. The bean did include the pod while the pea did not, which could account for the difference. However, the esterase bands of the two vegetables migrated at different rates under identical conditions. Therefore, the individual esterase components of the two vegetables must be dissimilar. Various numbers of esterase bands have been reported in extracts of cucumber, soybean, wheat seeds, and corn using starch-gel electrophoresis (Jooste and Moreland, 1963).

Although the esterases of the bean and pea did appear electrophoretically different, their substrate and inhibitor specificities were somewhat similar. Most esterase bands from both vegetables hydrolyzed α-naphthyl acetate and propionate in preference to α-naphthyl butyrate and AS naphthol acetate, although the substrate specificity differences appeared more pronounced in the beans than

the peas (Figures 1 and 2). These results are in close agreement with the substrate specificity of the root nodule esterases (Fottrell, 1968). Interestingly, all the bands in group one of the beans did not have the same substrate specificity. Bands 1 and 2 did not hydrolyze α-naphthyl butyrate, while bands 3, 4 and 5 did. With the pea, α-naphthyl butyrate was not hydrolyzed by band 7. It is remarkable that esterase bands showing similar substrate specificities possess striking differences in electrophoretic mobility and inhibitor specificities (Figures 3-8).

All organophosphorus inhibitors used in this study inhibited the esterases of both peas and beans. However, the bean esterases were more resistant than the pea esterases, which confirms previous results from this laboratory (Norgaard and Montgomery, 1968; Putnam and Montgomery, 1968). With purified fractions, band 1 through 6 of the pea zymogram have been shown to be isozymes (Montgomery, Norgaard and Veerabhadrappa, 1968). The similar substrate and inhibitor specificities (Figures 2, 6, 7 and 8) of these bands would confirm the isozymic nature. Band 7 was originally thought to be homogeneous, however, the zymogram in Figure 8-B shows that this band contained at least three and possibly four components. The similar substrate and inhibitor specificities of these components, as well as, those in groups 2 and 3 of the bean zymograms (Figures 3-5) suggests that the components of these groups

are isozymes. On the other hand, group 1 of the beans, due to the difference in substrate specificity of bands 1 and 2 from 3, 4 and 5, do not appear to be isozymes.

Esterase isozymic forms have also been demonstrated in extracts from maize kernels (Schwartz, 1962), plants belonging to cucurbiraceae (Schwartz et al., 1964), wheat seeds, cucumber, corn, soybean seedlings (Jooste and Moreland, 1963) and Solanum tubers (Desborough and Peloquin, 1967).

Esterases, unlike lactate dehydrogenases, represent a complex group of enzymes whose substrate specificity and biological function have not been fully defined. Therefore, the isozymic nature of these esterases can only be established conclusively after the enzymes have been isolated individually in pure state and structural or genetic studies have been carried out.

The apparent absence of choline esterase in our preparation was established in previous work (Norgaard and Montgomery, 1968; Putnam and Montgomery, 1968). This information, with the almost complete inhibition of esterase activity with 10^{-2} $\underline{M}$ paraoxon, indicates that the majority of esterases present in both peas and beans were carboxylesterases (EC 3.1.1.1, carboxylic ester hydrolases).

Finally, it should be mentioned that the bands observed did not represent esterases from an individual bean or pea, or from beans and peas from the same plant, but represented esterases from

composite samples of each plant material. With the above observations in mind, a study dealing with the separation and characterization of multiple forms of carboxylesterases of beans was carried out and constitutes the following section of this investigation.

SECTION II

Fractionation, Substrate and Inhibitor Studies of Bean
Carboxylesterases

MATERIALS AND METHODS

Preparation of Enzyme Extract

The procedure used to prepare the aqueous extract of the bean

was essentially the same as that given in the previous section. Glass

distilled water was used throughout this work.

Preparation of Substrates

Substrates used in this study were phenyl acetate,[10] phenyl

propionate,[10] phenyl-n-butyrate,[11] triacetin,[10] tripropionin,[10]

tri-n-butyrin,[10] and acetyl,[12] propionyl,[13] and n-butyryl[12] esters

of sodium-2-naphthol-6-sulfonate. The concentrations of the phenyl

esters and triglycerides were 1 $\underline{M}$ with the exception of 0.23 $\underline{M}$

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[10]Eastman Organic Chemicals

[11]K & K Laboratories, Inc.

[12]Donated by Dr. T. L. Foster, Washington State University,
Pullman, Washington.

[13]Prepared in this laboratory

triacetin; the naphthyl esters were 0.06 $\underline{M}$. All substrates were pre-
pared in three percent (w/v) Triton X-155[14] and 0.1 percent gum
arabic.[15] The phenyls and triglycerides were homogenized for two
minutes in a water cooled microblender. Homogenization was
omitted in the preparation of the water soluble naphthyl esters.

Preparation of Inhibitors

Inhibitors used in this study were parathion, DFP, and PCMB.
Inhibitors were prepared in one percent (w/v) Triton X-100. The
0.1 $\underline{M}$ parathion solution required 30 seconds homogenization in a
microblender to form a semistable emulsion. DFP was soluble at
the concentrations used. Prescribed concentrations were prepared
by serially diluting the more concentrated solutions or emulsions.
PCMB solutions were prepared in water and serially diluted.

Assay Procedure

Esterase activity was determined by manometric techniques
similar to those described by Forster, Bendixen, and Montgomery
(1959), and Norgaard (1968), using Gilson differential respirometer.
The acid produced after substrate hydrolysis shifts the following

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[14] Rohm and Haas Company

[15] Matheson, Coleman and Bell

equilibrium to the right evolving CO_2:

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 \text{ (dissolved)} \rightleftharpoons CO_2 \text{ (gas)}$$
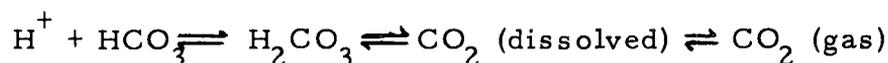
The volume of CO_2 evolved from the bicarbonate buffer was used as the measurement of activity. Main compartment of the respirometer flasks contained 1.5 ml of 2.8×10^{-2} $\underline{M}$ $NaHCO_3$ buffer, one ml of enzyme, and one ml of either distilled water, one percent (w/v) Triton X-100, or inhibitor preparation depending upon the experiment. Side arm contained 0.1 ml of 4.07×10^{-2} $\underline{M}$ $NaHCO_3$ and 0.4 ml substrate. A mixture of 95 percent N_2 and five percent CO_2 was passed through the flasks for ten minutes. After gassing, the side arms were closed and the system equilibrated for ten minutes at a pressure of 770 mm of Hg. The contents of the side arms were tipped into the main compartments at zero time and readings were taken at ten minute intervals for 30 minutes. All observations were made in triplicate unless otherwise stated.

Esterase activity was measured in units. One unit of esterase activity was defined as the amount of esterase which hydrolyzed one microequivalent of the substrate per minute per ml of the enzyme preparation. Corrections for room temperature differences were calculated according to formula D of Gregory and Winter (1965). Protein was determined by absorption at 280 mμ (Dixon and Webb, 1964).

Protamine Sulfate Treatment

The strong cationic nature and apparent specificity (Felix, 1960)
makes protamine sulfate (from salmon sperm) a very potent agent for
precipitating nucleic acids and their anionic biopolymers. A two per-
cent solution of protamine sulfate was prepared and titrated with
1 N NaOH to pH 6.5. It was necessary to prepare and warm this
solution to 50°C before use to dissolve an oil which separated upon
storage in the cold. One ml of this solution was used per ten ml of
extract and allowed to stand in the cold for 30 minutes. The pre-
cipitate was removed by centrifugation at 15,000 x G for ten minutes.

Sephadex Chromatography

Sephadex (Pharmacia) was prepared according to the recom-
mendations from the manufacturer (Technical Data Sheet No. 6).
Complete swelling of Sephadex (G-100) gel before column packing
was carried out by suspending the gel in excess buffer (1 m$\underline{M}$
potassium phosphate, pH 7.0) at 24°C for three days. The gel was
stirred, allowed to settle, and the fines were decanted intermittently
during swelling. A Sephadex column (Pharmacia, 2.5 x 95 cm) was
packed by pouring a portion of the gel slurry into the column with
the outlet closed. The remainder of the gel slurry was placed in a
reservoir connected to the top of the column by means of a siphon.

The column outlet was opened and was packed by gravity flow. The head pressure during packing and running the column did not exceed 25 cm. After equilibration with two to three column volumes of buffer, typical flow rates were 10 to 20 ml per hour. Upper surfaces of the Sephadex bed was protected by a sample applicator.

Sample Application

Sixty ml of enzyme extract (1:5 w/v), after treatment with two percent protamine sulfate, were lyophilized. The gummy material obtained from lyophilization was dissolved in 15.0 ml of water and placed on the Sephadex column. The sample was rinsed into the gel twice with 10 ml portions of buffer (1 m$\underline{M}$ potassium phosphate, pH 7.0). Approximately 30 ml of the buffer was placed on the bed before the column was attached to a reservoir. Effluent from the column was continuously monitored at 280 mμ by a Gilson absorption meter and collected in 10 ml fractions.

Ion-Exchange Chromatography

General procedures for handling and working with cellulose ion-exchangers have been derived from the work of Peterson and Sober (1962). Fibrous and Microgranular cellulose ion-exchangers were used in this investigation.

Fibrous Diethylaminoethyl (DEAE) Cellulose

DEAE-cellulose (Schliecher and Schuell, Selectacel type 20, 0.83 m eq./g), after removal of fines, was conditioned by successive treatment with 1 N NaOH, 1 N HCl, and finally washed with distilled water as described by Ting, Montgomery and Anglemier (1968). A suspension of the cellulose in 0.1 N NaOH was used to pack a column (2.0 x 35 cm) under gravity pressure at room temperature. After packing, the column was placed in a cold room at 5°C, and washed free of NaOH with starting buffer (1 m$\underline{M}$ Tris-phosphate, pH 7.4).

Microgranular Diethylaminoethyl (DEAE) Cellulose

Microgranular DEAE-cellulose columns were prepared in accordance to instructions from the manufacturer (Whatman Technical Bulletin IE 2). Preswollen, microgranular (DE-52), anionic exchanger (Whatman) was obtained from Reeve Angel Inc. This exchanger was supplied in wet form and had the capacity of 1.0 ± 0.1 meq/g of material. DEAE-cellulose (140 g) was suspended in 1 m$\underline{M}$ Tris-phosphate buffer, pH 7.4 (25 ml/dry g) and was titrated with the acid component of the buffer (1.0 $\underline{M}$ KH$_2$PO$_4$) to the pH 7.4. After filtering, the cellulose was resuspended in the buffer (25 ml/dry g) for ten minutes and filtered. This treatment was repeated six to eight times until the pH of the filtrate was the same as the buffer.

To remove the fines, equilibrated exchanger was dispersed in buffer (30 ml/dry g) and poured into a two liter measuring cylinder. After standing for 54 minutes, the fines were removed by suction and the exchanger was degassed by stirring the slurry in a stoppered Buchner flask connected to a water pump.

A column (Pharmacia, 2.7 x 40 cm) was packed by the same procedure as the Sephadex column. The packed column was placed in the cold room and eluted with the starting buffer (1 m$\underline{M}$ Tris-phosphate buffer, pH 7.4) until the pH of the eluent was 7.4. After the chromatographic run, the exchanger was reequilibrated in the column by eluting first with 100 ml of 0.1 $\underline{M}$ KH_2PO_4 and then overnight with 1 m$\underline{M}$ starting buffer.

Microgranular Carboxymethyl (CM) Cellulose

Preswollen, microgranular (Whatman) cationic exchanger was obtained in the wet form from Reeve Angel Inc. Equilibration of cationic exchanger and packing of the column were similar to the procedure used for microgranular DEAE-cellulose column. The packed column was placed in the cold room and eluted with starting buffer (1 m$\underline{M}$ phosphate, pH 6.6) overnight. A constant flow micro-pump adjusted to 45 ml per hour was used. For re-use the CM-cellulose column was eluted with 100 ml of 0.1 $\underline{M}$ phosphate (pH 6.6) followed with 1 m$\underline{M}$ phosphate (pH 6.6) until the pH of the effluent was

6. 6

Chromatography of Fractions

Each enzyme fraction obtained from the Sephadex column was concentrated by lyophilization. The concentrated samples were dissolved in five mililiter of water and placed on the ion-exchange columns. When all the sample had entered the cellulose, either by gravity flow or by slight application of pressure, the sample was rinsed into the cellulose with several small portions (5 ml) of starting buffer. Approximately 20 ml of starting buffer were placed on the bed before the column was attached to the reservoir containing starting buffer.

Elution Techniques

Linear gradient elutions of cellulose ion-exchange columns were performed according to the recommendations of Peterson and Sober (1959). Two chambers of the Varigrad, one with 500 ml of starting buffer and the other with the same buffer containing 0.5 $\underline{M}$ NaCl, were used. The starting buffers in the DEAE-cellulose and the CM-cellulose columns were 1 m$\underline{M}$ Tris-phosphate buffer (pH 7.4) and 1 m$\underline{M}$ potassium phosphate buffer (pH 6.6), respectively. After sample application, the column first was eluted with starting buffer until the first protein fraction had been eluted and then with a linear

gradient. Effluent from the column was conducted to a Gilson absorption meter and the absorbance at 280 mμ was recorded continuously throughout the chromatographic run. Ten ml fractions were collected.

RESULTS

Effect of Sodium Chloride

During the separation and purification of the bean esterases

which hydrolyze phenyl propionate, sodium chloride was used to elute

the esterase fractions from both DEAE- and CM-cellulose columns.

Therefore, it was appropriate to study the effect of sodium chloride

on esterase activity. Water extract prior to purification was used

as the enzyme source. The results, shown in Table 1, reveal that

considerable inhibition occurs at 0.25 $\underline{M}$. Experiments, with 0.5 $\underline{M}$

sodium chloride in linear gradient elution, indicated that the con-

centration of salt in all the esterase fractions was less than 0.25 $\underline{M}$.

Hence, the fractions from the columns were assayed directly for

esterase activity without removing sodium chloride.

Effect of pH

The effect of pH on the esterase activity was determined using

phenyl propionate as substrate. Concentrations of sodium bicarbonate

buffers in the reaction mixtures were varied to obtain the desired pH

(Umbreit, Burris, and Stauffer, 1964). The results are shown in

Figure 9. The pH optimum lies at 7.2 and assays were performed at

this pH throughout this work.
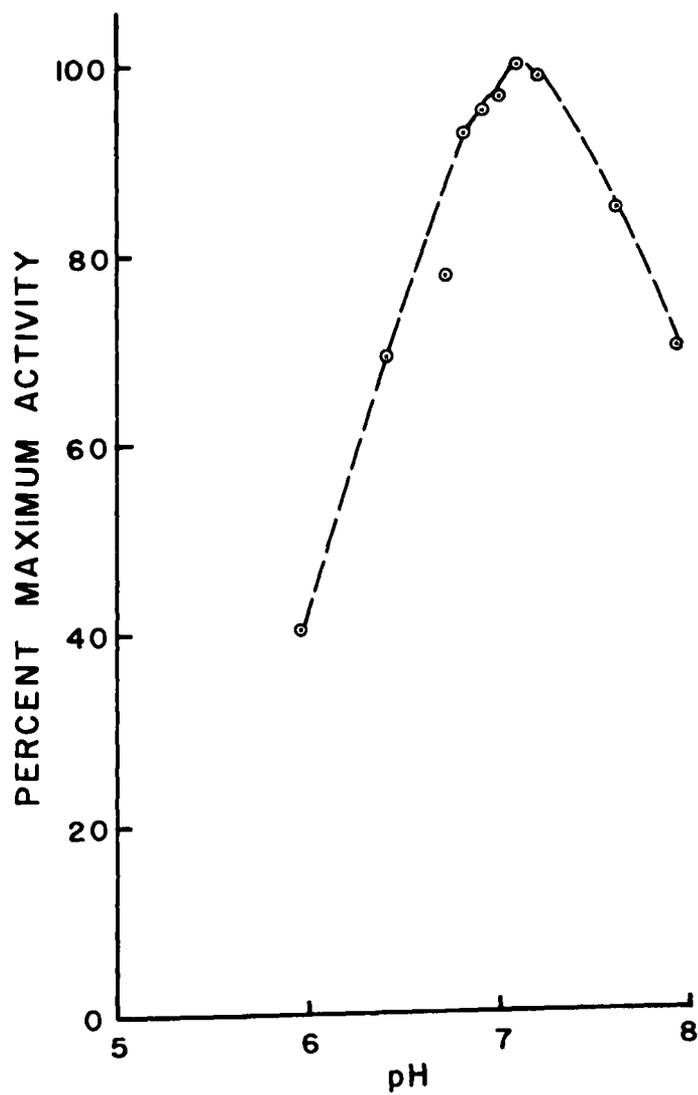
Figure 9. pH optimum of bean esterases.

Table 1. Effect of sodium chloride on bean esterase activity using phenyl propionate as substrate.

Concentration in final reaction mixture (m$\underline{M}$)	Percent Inhibition
2.5	0
5.0	0
25	3.8
50	7.0
250	33
500	41
1000	56

To determine the pH of buffers to be used in purification and separation procedures, the pH stability of the esterases was studied. Phenyl propionate was used as the substrate. Portions of bean extract were adjusted to various pH levels from 2.9 to 9.3 and were held for 46 hours at 4°C. Activities of the extracts, after readjustment to the original pH level (pH 6.5), are shown in Figure 10. The maximum stability appears to lie at pH 6.4. It is evident from these data that esterase activity was more stable in neutral and alkaline range than in the acidic range.

Polyvinylpyrrolidone (PVP) Treatment

Phenolic compounds, which are abundant in plant materials, may react reversibly with proteins by hydrogen bonding or irreversibly by oxidation, thus affecting the stability of enzymes (Loomis,
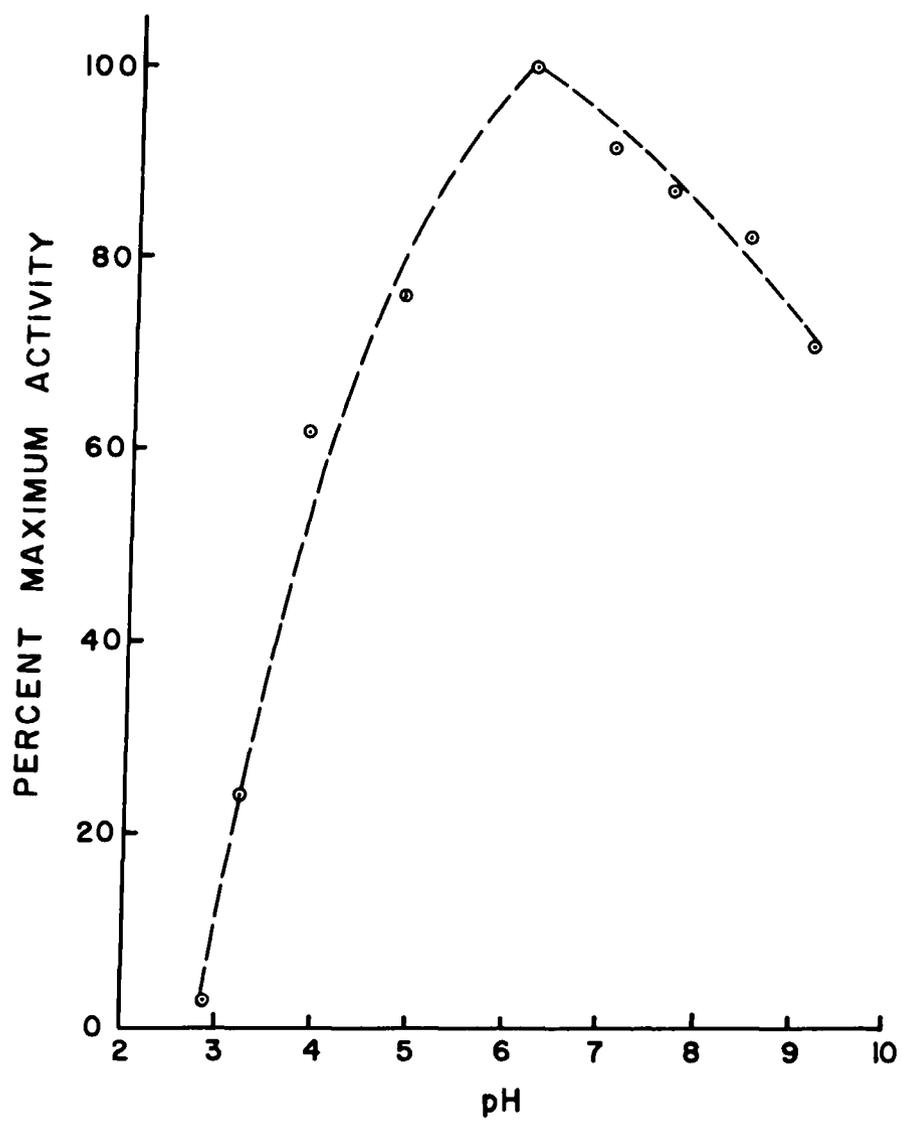
Figure 10. pH stability of bean esterases.

1968). The aqueous extract of the bean powder revealed a considerable amount of material absorbing at 260 mμ. This may have been due to the presence of nucleic acids and/or phenolic compounds. Phenolic compounds which would otherwise interfere with the fractionation of proteins, might have been effectively removed by treating the plant extract with PVP. Therefore, an attempt was made to remove phenolic compounds by extracting the enzyme in presence of PVP. Insoluble form of PVP, Polyclar AT[16] was purified by boiling for 10 minutes in 10 percent HCl and washing with water until free of Cl$^-$. The dried form of this material was used for the extraction.

Various levels of PVP (Table 2) were suspended in 20 ml volume of water. The suspensions were allowed to stand for 30 minutes at 5°C and centrifuged at 31,000 x G for 30 minutes. Resulting supernatants were used to determine 260 and 280 mμ absorbance and specific activity. Results shown in Table 2 indicate that even with the maximum amount of PVP, there is only slight removal of these compounds and a very small increase in specific activity. Therefore, the use of PVP did not offer an advantage in removing the 260 mμ absorbing material.

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[16]General Aniline and Film Corp.

Table 2. Effect of PVP on 260 and 280 mμ absorbing material and
esterase activity of the bean.

PVP* g	Absorbance** 280 mμ	Absorbance** 260 mμ	Specific Activity units/mg protein
0.0	39.6	60.0	0.29
0.6	37.2	55.2	0.32
0.8	36.6	55.2	0.32
1.0	36.6	54.0	0.32
1.5	33.6	48.0	0.35

*PVP used per g of dry bean powder

**Values calculated to 1:5 (w/v) aqueous bean extract

Protamine Sulfate Treatment

Protamine sulfate has been used to precipitate nucleic acids of
crude enzyme extracts (Felix, 1960). The method to estimate the
amount of protamine sulfate necessary for the precipitation reaction
was as follows: Seven tubes were set up containing from 0 to 5 ml
of two percent protamine sulfate solution (pH 6.5). Then 10.0 ml of
the original crude extract (1:5, w/v) were added to each tube. Fifteen
minutes after mixing, precipitates were removed by centrifugation
(12,000 x G for 10 minutes) and 0.1 ml of aliquots of the water-clear
supernatants were treated with a drop of the protamine sulfate solu-
tion. The lowest concentration of protamine sulfate, which did not
give a turbid reaction on the second treatment, was 1.0 ml of two
percent protamine sulfate. This concentration (0.2 percent) resulted

in a 50 percent decrease of 260 mμ absorbing material (Table 3).

However, the increase in concentration of protamine sulfate beyond

0.2 percent resulted in loss of enzyme activity. Therefore, 1.0 ml

of two percent protamine sulfate per ten ml of (1:5, w/v) aqueous

bean extract was used in this study. The same concentration of

protamine sulfate was found to remove the 260 mμ absorbing material

from pea extract (Montgomery, Norgaard and Veerabhadrappa, 1968).

Table 3. Effect of protamine sulfate on 260 and 280 mμ absorbing
material and esterase activity.

Percent Protamine Sulfate in Extract	Absorbance* 280 mμ	Absorbance* 260 mμ	Specific Activity units/mg protein
0.0	74.4	122	0.26
0.2	37.2	66	0.56
0.3	36.0	65	0.56
0.4	37.2	66	0.51
0.6	46.8	71	0.43
0.8	52.8	79	0.34
1.0	62.4	89	0.26

*Values calculated to 1:5 (w/v) aqueous bean extract.

Gel Filtration

Gel filtration of protamine sulfate treated extract on Sephadex

G-100 separated the esterase activity into three fractions (Figure 11).

Tubes 16 to 21 were combined and designated as fraction S_1.
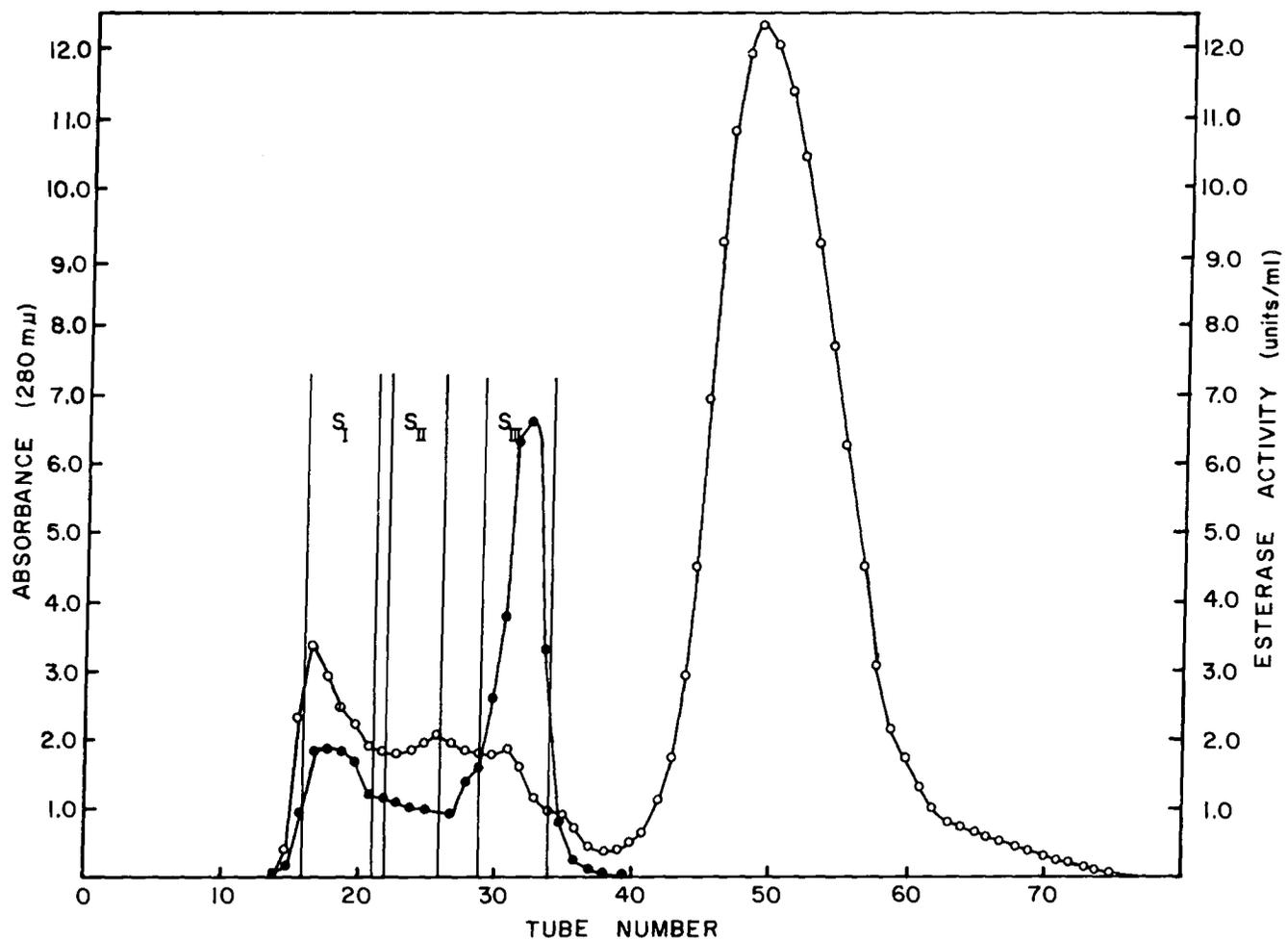
Figure 11. Gel filtration of protamine sulfate treated bean extract on Sephadex G-100. Equilibrated with 1 mM phosphate, pH 7.0, flow rate of about 15 ml per hour at 5°C. -●-●-●-, esterase activity (units/ml); -o-o-o-, absorbance at 280 mμ.

Appropriate tubes (22 to 26) in the plateau between the first and third

fractions were pooled and designated as fraction S_{II}. Similarly, the

combined tubes, 29 to 34, was designated as fraction S_{III}. All the

fractions were concentrated by lyophilization and stored dry at -20°C

until further investigation.

Each fraction along with the protamine sulfate treated extract

were subjected to electrophoresis on polyacrylamide gel. The

electrophoretic pattern (Figure 12) shows the presence of first group

of esterase activity in S_I, the first and second group of esterase

activity in S_{II}, and the third group with some contamination of the

first group in S_{III}. This observation indicates that the pattern of

separation on polyacrylamide gel correlated well with the results of

gel filtration with regard to their molecular size. The group of

esterase activity (first group) which was eluted first from Sephadex

gel was the slowest moving on polyacrylamide gel and those eluted

later migrated faster on the polyacrylamide-gel matrix.

Fractions S_I and S_{II} were eluted when the protein concentration

was higher and resulted only in 4 and 3 fold purification, respectively,

over that of crude extract (Table 4). On the other hand, fraction

S_{III} was eluted later when the protein concentration was lower

(Figure 11) and, hence, yielded a more purified preparation than the

other two fractions. Fraction S_{III} was purified 34 fold over that of

crude extract and the data presented in Table 4 show that this group
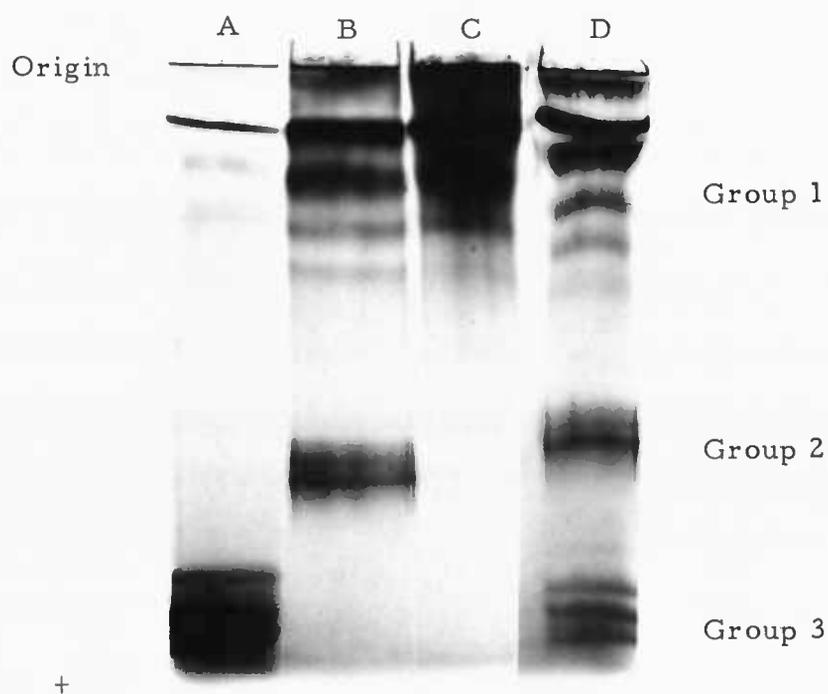
Figure 12. Electrophoretic pattern of bean carboxyl-
esterases after Sephadex gel filtration.
A, fraction S_{III}; B, fraction S_{II}; C,
fraction S_I; and D, bean extract before
fractionation. α-Naphthyl acetate was the
substrate.

Table 4. Data concerned with purification of bean carboxylesterases hydrolyzing phenyl propionate.

		Vol. (ml)	Total Units*	Total Protein (mg)	Specific Activity** (units/mg protein)	Yield (percent)	Purification (fold)
1.	Crude Extract	60	528	3024	0.17		
2.	Protamine-sulfate pptn	65	580	1930	0.30	110	1.8
3.	Sephadex G-100						
	a. Fraction S_I	63	104	202	0.70	19	4
	b. Fraction S_{II}	53	53	118	0.50	9	3
	c. Fraction S_{III}	53	195	75	5.3	35	34

*One activity unit represents one μ equivalent of substrate hydrolyzed per ml of enzyme solution.

**Most active tube of each fraction.

was the major constituent of the bean esterase activity. Although gel filtration of protamine sulfate treated extract did not yield highly purified preparations of S_I and S_{II}, an effective separation of the three groups of esterase activity was accomplished.

Ion-Exchange Chromatography

Chromatography of Fraction S_I on Fibrous DEAE-Cellulose

A preliminary trial was made in an attempt to separate and purify the esterase activity in fraction S_I by chromatography on fibrous DEAE-cellulose. Enzyme and protein elution data presented in Figure 13 indicate that the first protein peak, which was not retained by the exchanger, contained most of the enzyme activity. This observation suggests the possibility that these esterases were cationic in nature at the pH used (7.4). Since no active enzyme was eluted from the column after tube 20, protein elution pattern beyond tube 40 was not given in the Figure 13.

Tubes (7 to 9) containing enzyme activity were combined and subjected to electrophoresis on polyacrylamide gel. The electro-phoretic pattern (Figure 14) shows three esterase bands in this frac-tion, band 1 being most predominant. These results revealed no effective separation of the esterase bands and better chromatographic techniques might improve the fractionation of S_I.
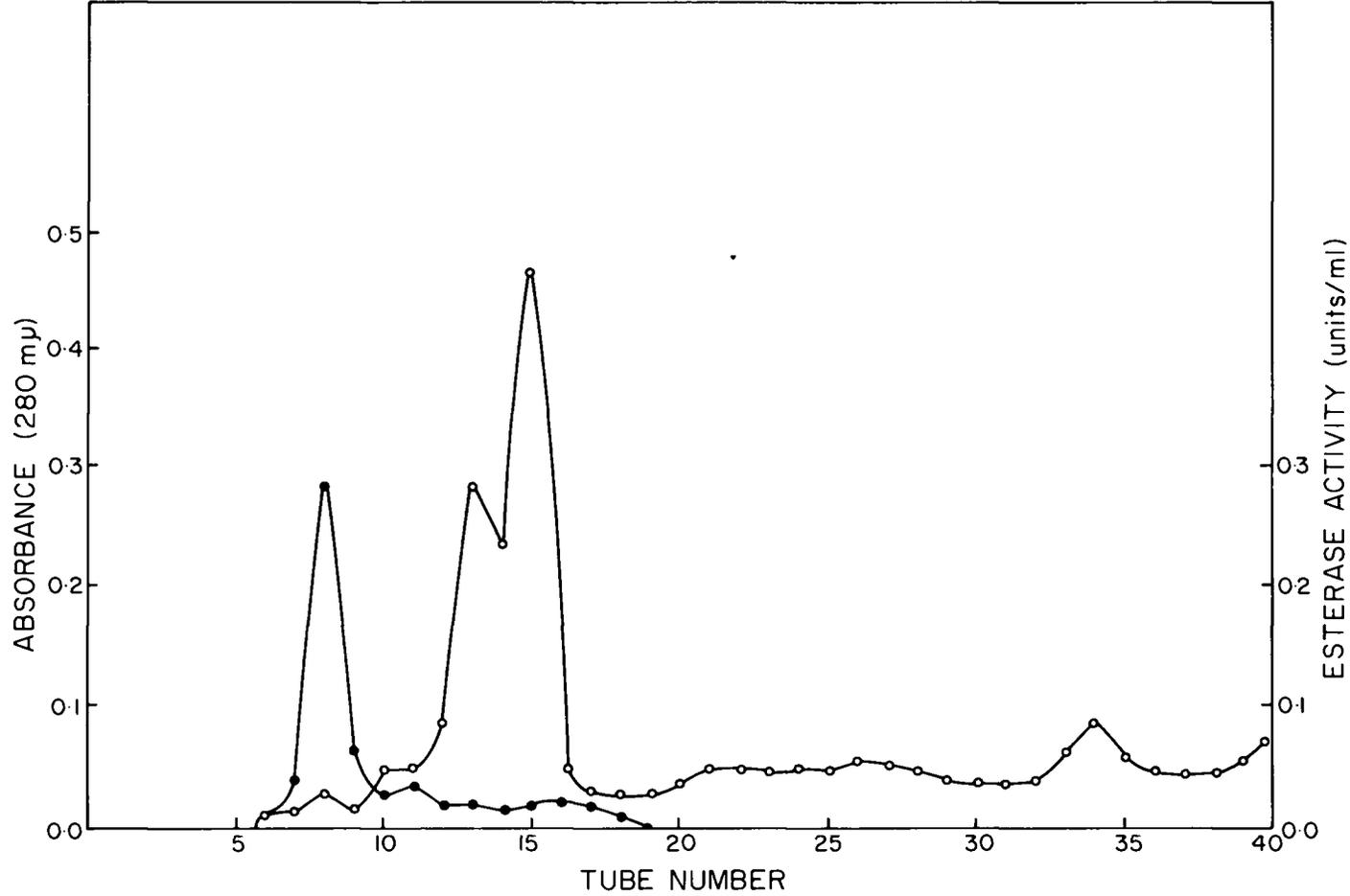
Figure 13. Chromatography of fraction S_I on DEAE-cellulose (fibrous). Equilibrated with 1 m$\underline{M}$ tris-phosphate, pH 7.4, flow rate of about 30 ml per hour at 5°C. -●-●-●-, esterase activity (units/ml); -o-o-o-, absorbance at 280 mμ.

Origin

+
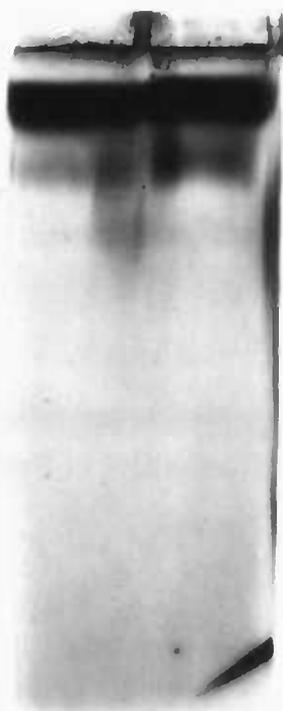
Figure 14. Electrophoretic pattern of tubes 7 to 9 from
fibrous DEAE-cellulose. α-Naphthyl acetate
was the substrate.

Chromatography of S_I on CM-Cellulose

Since S_I appeared cationic in nature, an attempt was made to fractionate these esterases on CM-cellulose. The elution pattern shown in Figure 15 indicates that the majority of the protein emerged from the column immediately and, in contrast to fibrous DEAE-cellulose pattern, only slight enzyme activity was associated with this protein peak. The greater portion of the enzyme activity was retained longer and was eluted when the protein concentration was lower. Thus, the column appeared to yield at this point better purified preparations than on DEAE-cellulose. The activity pattern revealed a partial separation of the enzyme activity into three fractions. Appropriate tubes of each fraction were combined and designated as CM_I, CM_{II} and CM_{III} (Figure 15). Purification data of these fractions are presented in Table 5.

Activity of the fraction CM_{III} was very low and no attempt was made to characterize it further. Fractions CM_I and CM_{II} were subjected to electrophoresis on polyacrylamide gel. The electrophoretic pattern of these fractions along with the unfractionated S_I is shown in Figure 16. Close examination of the gel revealed three esterase bands in CM_I and two esterase bands in CM_{II}. From the electrophoretic pattern (Figure 16) of CM_{II}, band 1 was more active than band 2. On the other hand, CM_I contained a less active band 1 than
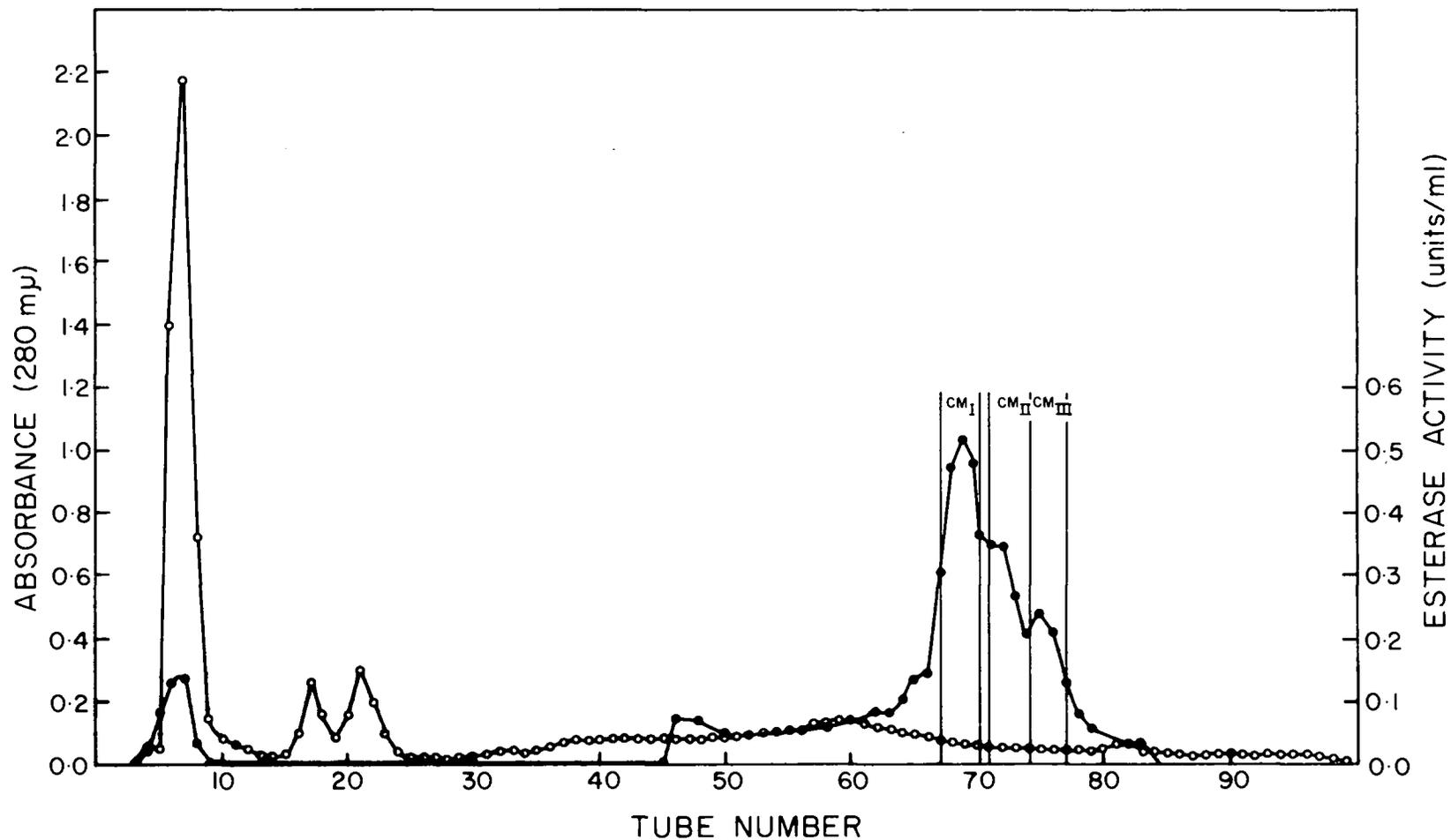
Figure 15. Chromatography of fraction S_I on CM-cellulose. Equilibrated with 1 m$\underline{M}$ phosphate, pH 6.6, flow rate of about 45 ml per hour at 5°C. -●-●-●-, esterase activity (units/ml); -o-o-o-, absorbance at 280 mμ.

Table 5. Data concerned with purification of Fraction S_I on CM-cellulose.

Fractions	Vol. (ml)	Total Units*	Total Protein (mg)	Specific Activity** (units/mg protein)	Yield (percent)	Purification (fold)
CM_I	50	19.9	18.2	8.0	35	47
CM_{II}	43	13	8.8	7.0	23	41
CM_{III}	44	7.3	8.1	4.8	13	28

*One activity unit represents one μ equivalent of substrate hydrolyzed per ml of enzyme solution.

**Most active tube of each fraction.

Figure 16. Electrophoretic pattern of fractions: A, CM_I; B, CM_{II}; and C, unfractionated S_I. α-Naphthyl acetate was the substrate.

CM_{II} and a slight activity from band 3. The absence of bands 4 and 5 from either of these fractions would suggest that these might be present in fraction CM_{III}, however, evidence for this assumption requires further examination of CM_{III} on polyacrylamide-gel electrophoresis. Although linear sodium chloride gradient elution of fraction S_I resulted in partial separation, a concave sodium chloride gradient might have resulted in better separation.

Chromatography of S_{II} on DEAE-Cellulose

Fraction S_{II} obtained from gel filtration contained both the first and second groups of esterase activity. Microgranular DEAE-cellulose was used to separate these groups of enzymes. A typical elution pattern is presented in Figure 17. Esterase activity was eluted in three fractions and were designated as DE_I, DE_{II}, and DE_{III}. According to Table 6, 66 percent of the total activity of fraction S_{II} was found in DE_I and was purified 170 fold over that of the crude extract. Since fractions DE_{II} and DE_{III} were eluted in areas where there were high concentrations of protein, purification was only 12 and 2 fold, respectively.

Identification of these fractions was carried out by polyacrylamide-gel electrophoresis. The level of activity was very low in the fraction DE_{III}, and an attempt was made to concentrate this fraction. After the fraction was dialyzed against water in the cold
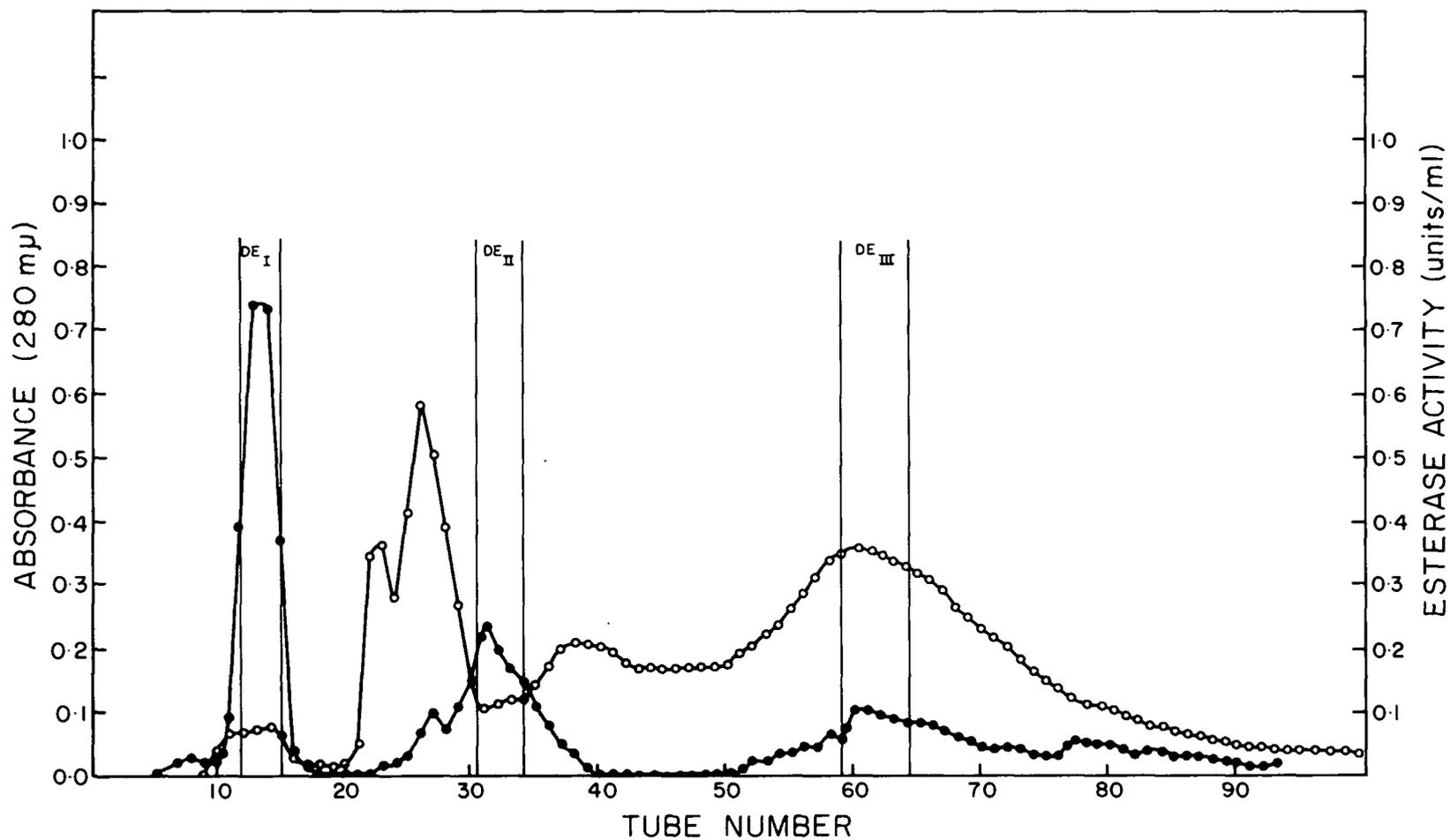
Figure 17. Chromatography of fraction S_{II} on DEAE-cellulose (microgranular). Equilibrated with 1 mM tris-phosphate, pH 7.4, flow rate of about 30 ml per hour at 5°C. -●-●-●-, esterase activity (units/ml); -o-o-o-, absorbance at 280 mμ.

Table 6. Data concerned with the purification of fraction S_{II} on DEAE-cellulose.

Fraction	Vol. (ml)	Total Units*	Total Protein (mg)	Specific Activity** (units/mg protein)	Yield (percent)	Purification (fold)
DE_I	44	30.6	2.5	28.9	41	170
DE_{II}	44	8.2	5.1	2.2	11	12
DE_{III}	66	6.0	22.9	0.3	8	2

*One unit activity represents one μ equivalent of substrate hydrolyzed per ml of enzyme solution.

**Most active tube of each fraction.

room for 3 days to remove sodium chloride, the fraction was

lyophilized and redissolved in water for electrophoresis. The zymo-

gram of the electrophoretic patterns of DE_I, DE_{II}, DE_{III} and unfrac-

tionated S_{II} is shown in Figure 18. It is evident from this figure that

the fraction DE_{III} contained only the second group of esterase activity.

Similarly, electrophoretic patterns of DE_I and DE_{II} revealed that

both fractions represent the first group of esterase activity, the

former containing bands 1, 2, and 3 and the latter containing bands

3, 4 and 5. Thus, band 3 was present in both fractions, DE_I and

DE_{II}, although both fractions were well separated from each other

in the chromatographic elution pattern (Figure 17).

It is interesting to note that when all these fractions were sub-

mitted to polyacrylamide-gel electrophoresis using AS naphthol

acetate as substrate, the second group of esterase activity was shown

to contain five distinct bands (Figure 19). Further it can be seen

that bands 1 and 2 of DE_I did not hydrolyze AS naphthol acetate,

whereas bands 3 and 4 of DE_{II} did, indicating that these two fractions

differed in the hydrolysis of AS naphthol acetate.

Chromatography of Fraction S_{III} on DEAE-Cellulose

Fraction S_{III} contained mainly the third group of esterase

activity and the chromatography of this fraction on microgranular

DEAE-cellulose is shown in Figure 20. The enzyme elution pattern
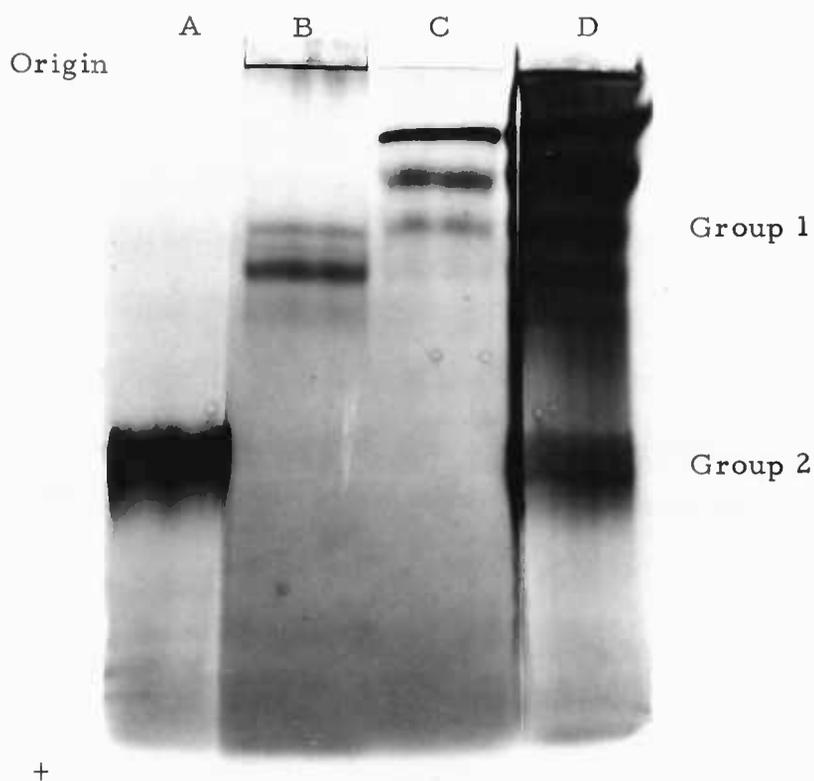
Figure 18. Electrophoretic pattern of fractions: A, DE_{III}; B, DE_{II}; C, DE_I; and D, unfractionated S_{II}. α-Naphthyl acetate was the substrate.
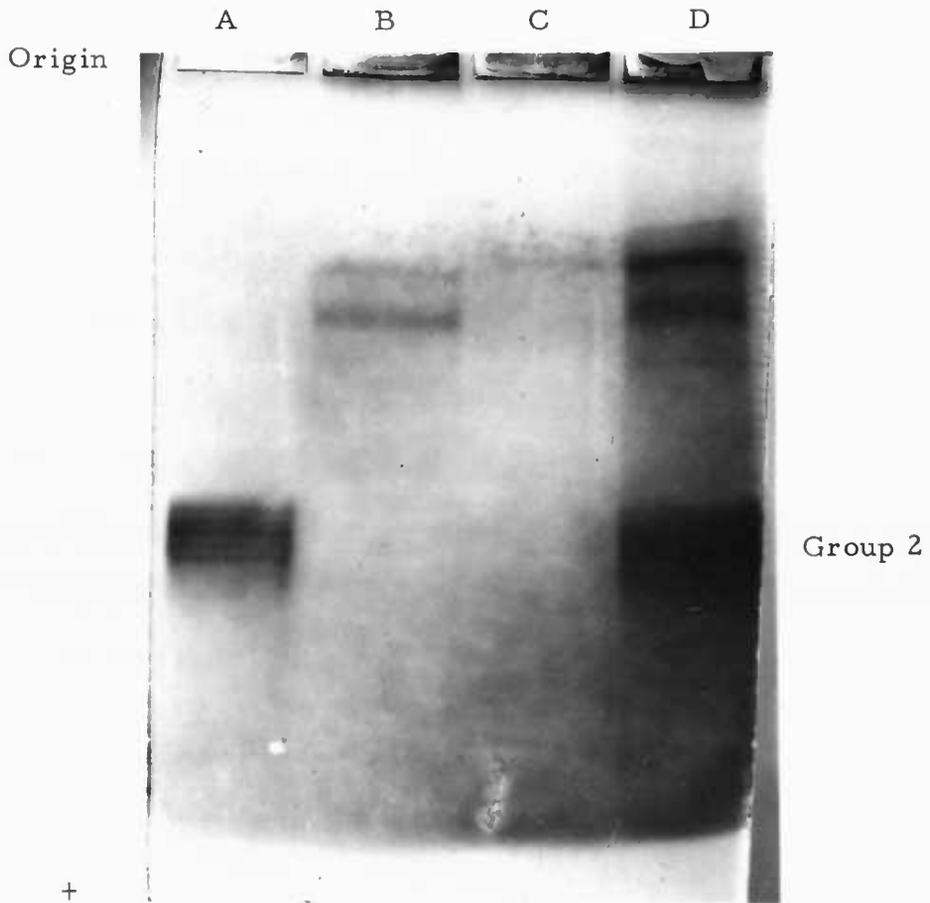
Figure 19. Electrophoretic pattern of fractions: A, DE_{III};
B, DE_{II}; C, DE_{I}; and D, unfractionated S_{II}.
AS naphthol acetate was the substrate.
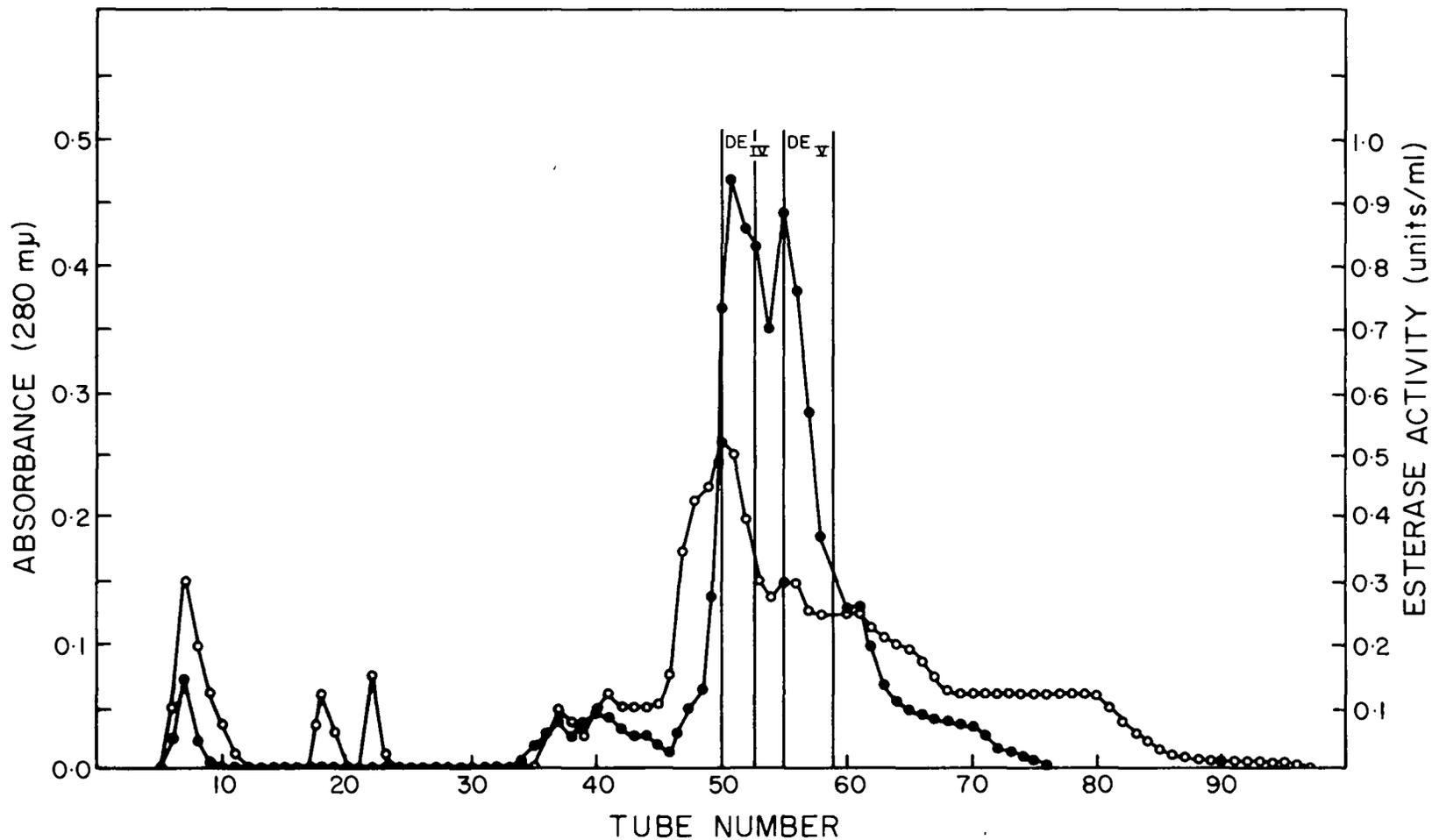
Figure 20. Chromatography of fraction S_{III} on DEAE-cellulose (microgranular). Equilibrated with 1 m$\underline{M}$ tris-phosphate, pH 7.4, flow rate of about 30 ml per hour at 5°C. -●-●-●-, esterase activity (units/ml); -o-o-o-, absorbance at 280 mμ.

revealed that the greater part of the activity emerged between the tubes 50 and 60 and was partially separated into two fractions of esterase activity, DE_{IV} and DE_V. The yield and purifications of these fractions are given in Table 7.

The extent and nature of separation can be visualized by subjecting these fractions to polyacrylamide-gel electrophoresis. Figure 21 shows that the four esterase bands of S_{III} were separated into two fractions, DE_{IV} and DE_V, each containing two esterase bands. Tube 54, which was midway between these fractions, contained two bands, one from each fraction. This would suggest that these four bands probably could exist as independent bands rather than doublets.

Substrate Specificity

Various fractions of esterase activity, obtained by DEAE- and CM-cellulose chromatography, were examined for their ability to catalyze the hydrolysis of some representative ester compounds. Nine substrates from three different families (triglycerides, naphthols, and phenols) were used to study the relative activity of the seven esterase fractions. The third fraction obtained from CM-cellulose (CM_{III}) was not used for substrate specificity because of the very low activity.

Results presented in Table 8 reveal the butyryl esters of triglyceride, sodium-2-naphthol-6-sulfonate and phenol were not

Table 7. Data concerned with purification of fraction S_{III} on DEAE-cellulose

Fraction	Vol. (ml)	Total Units*	Total Protein (mg)	Specific Activity** (units/mg protein)	Yield (percent)	Purification (fold)
DE_{IV}	44	35.8	8.0	5.0	9.2	30
DE_V	55	33.2	9.7	4.9	8.5	29

*One activity unit represents one μ equivalent of substrate hydrolyzed per ml of enzyme solution.

**Most active tube of each fraction.
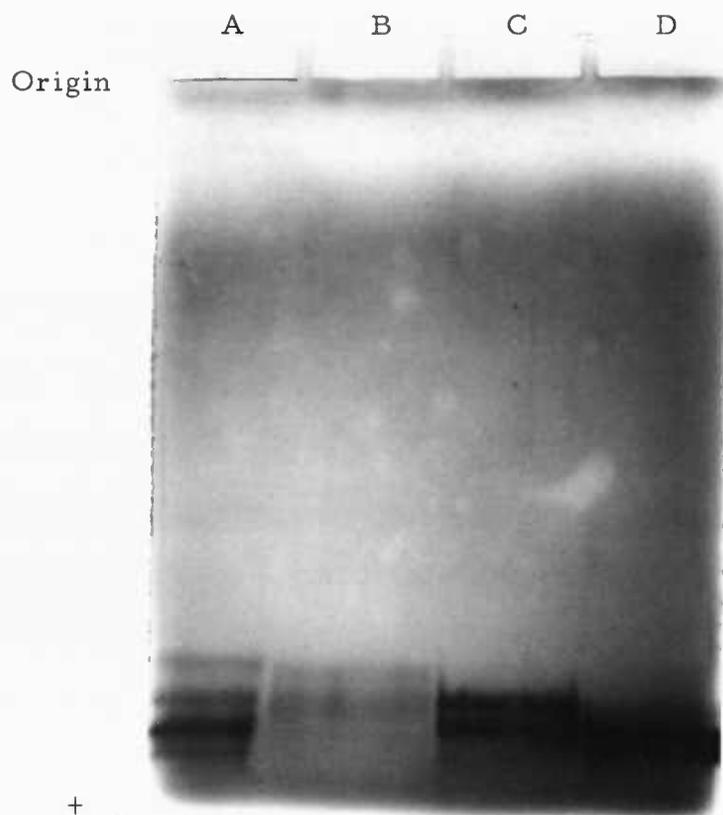
Figure 21. Electrophoretic pattern of fractions: A, unfractionated S_{III}; B, DE_{IV}; C, tube 54; and D, DE_V. α-Naphthyl acetate was the substrate.

Table 8. Relative activity* of esterase fractions toward various substrates.

Substrates	Crude Extract	CM_I	CM_{II}	DE_I	DE_{II}	DE_{III}	DE_{IV}	DE_V
Triacetin	13	27	15	9	25	38	33	55
Tripropionin	29	21	44	53	12	48	41	82
Tributyrin	11	6	9	8	0	0	30	30
Acetyl ester of Sodium-2-naphthol-6-sulfonate	11	79	38	0	84	16	12	17
Propionyl ester of Sodium-2-naphthol-6-sulfonate	17	140	77	12	85	40	13	39
Butyryl ester of Sodium-2-naphthol-6-sulfonate	14	1	2	3	9	6	22	55
Phenyl Acetate	107	125	128	106	107	73	104	66
Phenyl Propionate	100	100	100	100	100	100	100	100
Phenyl Butyrate	59	8	15	16	14	12	75	80

*Activity with phenyl propionate = 100.

appreciably hydrolyzed by fractions CM_I and CM_{II}. Both showed essentially the same activity in hydrolyzing acetyl and propionyl esters of phenols. However, their activities were different towards acetyl and propionyl esters of sodium-2-naphthol-6-sulfonate; the extent of hydrolysis by CM_I was approximately twice as great as CM_{II}. Acetyl and propionyl esters of triglycerides were not hydrolyzed to any appreciable extent, however, CM_{II} seemed to be twice as active as CM_I in hydrolyzing tripropionin. The very high relative activity of CM_I towards propionyl ester of sodium-2-naphthol-6-sulfonate, when compared to crude extract and all other fractions, is worth noting.

Both fractions DE_I and DE_{II} together comprise the electrophoretically slow moving group. The zymogram shown in Figure 18 reveals that these two fractions are different electrophoretically. It will be noted from Table 8 that both the fractions showed equal activity towards all phenol esters. However, an important distinction can be made between these two fractions by their activity towards acetyl and propionyl esters of triglycerides and sodium-2-naphthol-6-sulfonate. Fraction DE_I exerted very little activity with acetyl and propionyl esters of sodium-2-naphthol-6-sulfonate, while DE_{II} showed considerable activity towards both of these substrates. Similarly, differences in the hydrolysis of acetyl and propionyl esters of triglycerides also are indicated.

Fraction DE_{III} constitutes the second group of esterase activity which was intermediate in electrophoretic mobility. This fraction also had low activity towards the butyryl esters but differed from fractions DE_{I} and DE_{II} in specificity toward the propionyl esters.

In contrast to all other fractions, fractions DE_{IV} and DE_{V}, which together comprised the electrophoretically fast moving group, showed considerable activity towards butyryl esters of triglyceride, sodium-2-naphthol-6-sulfonate and phenol. Differences between these two fractions were evident since fraction DE_{V} was more active in hydrolyzing acetyl and propionyl esters of triglycerides and propionyl and butyryl esters of sodium-2-naphthol-6-sulfonate than DE_{IV}.

Inhibitor Studies

Organophosphorus inhibitors and PCMB have been used by various workers (Augustinsson, 1958, 1959, and 1961; Bergmann, Segal and Rimon, 1957) to identify esterases. Aldridge (1953a) distinguished two serum esterases which hydrolyzed the same substrate, by their sensitivity to organophosphorus inhibitors. A sigmoid curve was obtained when activity was plotted against the negative $\log_{10}$ of the molar inhibitor concentration (pI), indicating that one esterase was inhibited. Double sigmoid curves also have been obtained (Myers et al., 1957) suggesting the presence of two

esterases, which had different inhibitor sensitivities but hydrolyzed the same substrate. Therefore, the inhibitors, DFP, parathion, and PCMB, were used in the present study to establish the possible identity of the isolated esterase fractions.

The effect of parathion on each fraction and the crude bean extract is represented graphically in Figure 22. It is apparent from the inhibition pattern, that none of the fractions showed similar sensitivity towards parathion. Fractions CM_I and CM_{II} showed an inhibition pattern with parathion which indicated that CM_I was more resistant to parathion than CM_{II}. Closer examination of the inhibition curve (Figure 22B) indicates that there were at least two esterases present in CM_I. The most sensitive esterase was inhibited between pI 8 and 4 of parathion and was responsible for 35 percent of the activity. The second esterase was inhibited between pI 4 and 1 of parathion and accounted for 45 percent or more of the activity. Similarly, two esterases were present in CM_{II} and were inhibited at the same pI ranges. Therefore, these results indicated that each fraction contained two esterases, one resistant and the other sensitive to parathion. Whether the esterase sensitive to parathion present in CM_I was the same as the similar type esterase present in CM_{II}, but different in concentration, is not known. Electrophoretic patterns of CM_I and CM_{II} with α-naphthyl acetate as substrate (Figure 18) show that CM_{II} had more of band 1 than CM_I. These observations
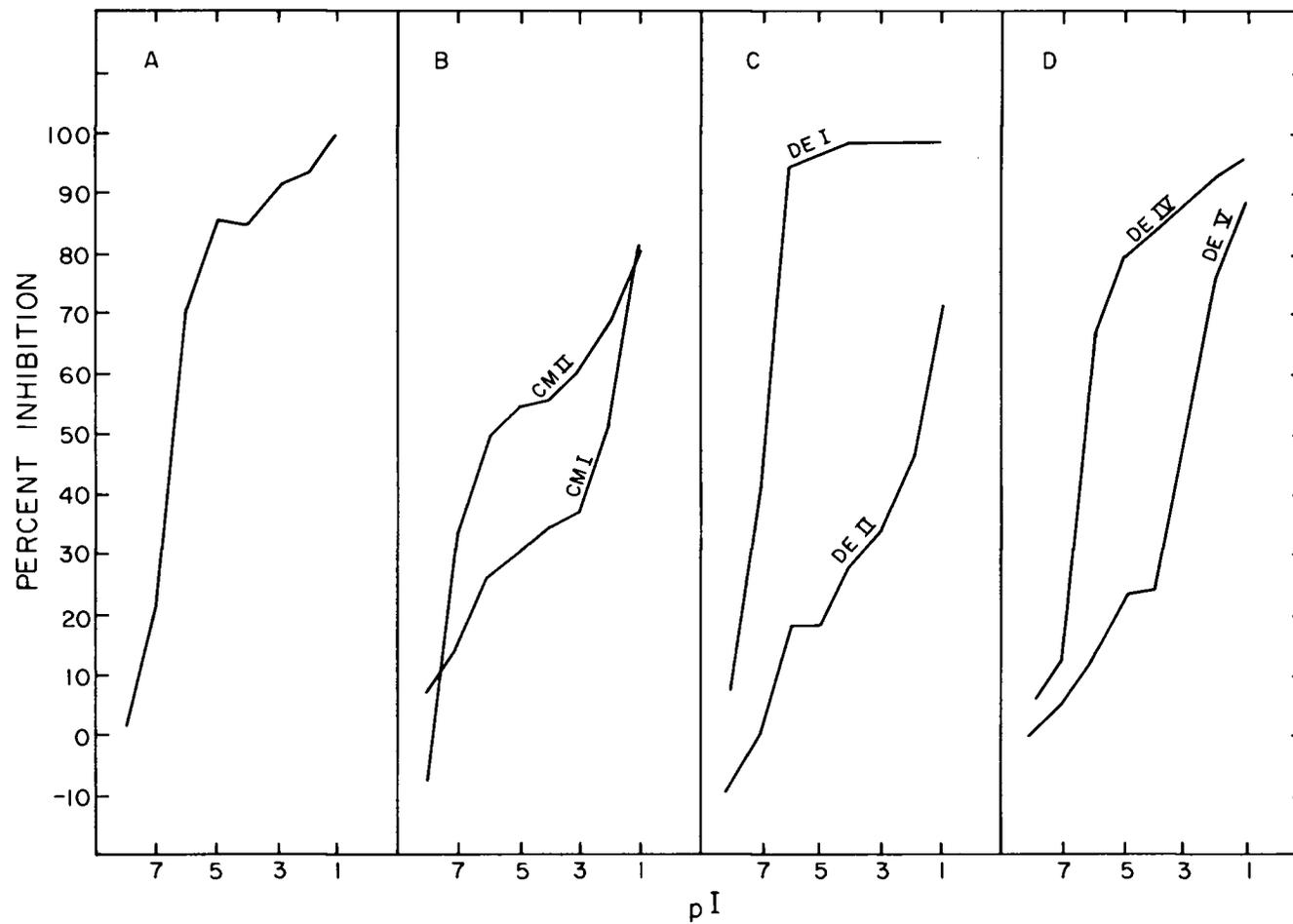
Figure 22. Inhibition with parathion of the hydrolysis of phenyl propionate by some purified fractions of bean carboxylesterases. Whole extract (A), fractions CM_I and CM_{II} (B), fractions DE_I and DE_{II} (C) and fractions DE_{IV} and DE_V (D).

would suggest that the more sensitive esterase was band 1 and the resistant esterase was band 2. However, fraction DE_I, which contained bands 1, 2, and 3 (Figure 18), appeared to have only the sensitive esterase present (Figure 22C). This phenomenon might be explained on the basis of Augustinsson's (1968) evidence that not all esterases were detected with α-naphthyl acetate. Therefore, esterases other than those that appeared on the gel might have caused these fractions to differ in their sensitivity to parathion when phenyl propionate was the substrate. These esterases probably were present in less concentration, or not active towards α-naphthyl acetate.

Several inhibition curves in Figure 22 did not reach 100 percent inhibition. In Figure 22B, the inhibition curves for CM_I and CM_{II} appeared to reach 80 percent inhibition. In Figure 22C, the curve for DE_{II} reached only 70 percent inhibition. These results suggest that there might be another esterase which was resistant to parathion. Complete inhibition may have resulted if the preparation of higher concentrations of parathion had been possible.

Fractions DE_I and DE_{II}, which together also constitute the first group of esterases, exhibited different type of curves with parathion (Figure 22C). These fractions not only differed in their sensitivity to parathion but also differed in their electrophoretic patterns (Figure 18). Fraction DE_I, being more sensitive to parathion than fraction DE_{II}, exhibited a single sigmoid curve indicating that all

three esterase bands (1, 2 and 3) showed similar sensitivity to parathion. Examination of the inhibition curve of DE_{II} (Figure 22C) indicated the presence of at least two esterases. The most sensitive esterase was inhibited between pI 8 and 6 and this being the minor component could be due to the presence of band 3. Therefore, bands 4 and 5 present in the fraction DE_{II} could possibly account for the major component of this fraction (resistant to parathion) which was inhibited in the pI range 5 to 1.

Inhibition curves in Figure 22D indicated that fractions DE_{IV} and DE_V differed in their sensitivity to parathion. In the previous section of this study, it was observed that at 10^{-4} $\underline{M}$ parathion, both DE_{IV} and DE_V showed equal intensity in color indicating that they do not differ in their sensitivity to parathion at this concentration (Figure 4). To the contrary, in Figure 22D, at 10^{-4} $\underline{M}$ parathion DE_V was inhibited 23 percent, whereas DE_{IV} was inhibited 83 percent. This discrepancy could be explained on the observation that each enzyme has different sensitivity to an inhibitor with different substrates (Putnam and Montgomery, 1968). These fractions showed different sensitivity to parathion when phenyl propionate was used as the substrate; however, these fractions might not exert any difference in their sensitivity to parathion with α-naphthyl acetate. Therefore, unless the same substrate was used for both electrophoresis and inhibitor studies, comparison is not justified.

Inhibition curves with DFP were shown in Figure 23. Fractions which were resistant to parathion were also resistant to DFP, however, the degree of difference in their resistance was greater in the case of parathion than with DFP. Unlike parathion, fractions CM_I and CM_{II} showed similar sensitivity to DFP, indicating that DFP was not as selective of an inhibitor of these fractions as parathion.

Similar studies carried out with PCMB (Figure 24) showed that each fraction differed in its sensitivity to PCMB although none of the fractions showed 100 percent inhibition. This would suggest that the purified fractions as well as the whole bean extract contained two esterases, one sensitive to PCMB, the other not. The similarity between the inhibition curves of DE_{IV} (Figure 24D) and the whole bean extract (Figure 24A) indicates that fraction DE_{IV} contained a major portion of the PCMB sensitive esterase activity. However, a certain portion of all fractions were sensitive to PCMB.
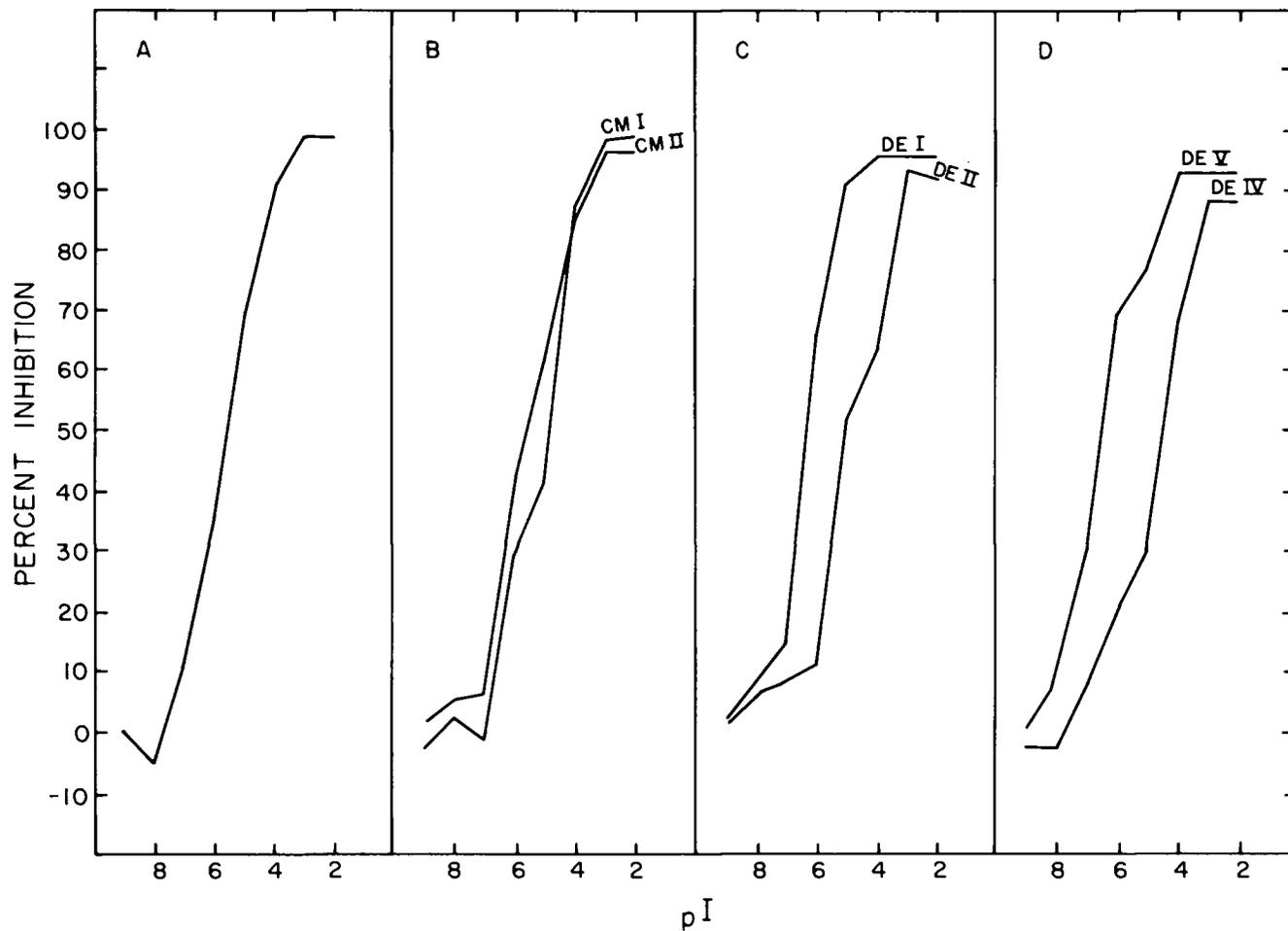
Figure 23. Inhibition with DFP of the hydrolysis of phenyl propionate by some purified fractions of bean carboxylesterases. Whole extract (A), fractions CM_I and CM_{II} (B), fractions DE_I and DE_{II} (C) and fractions DE_{IV} and DE_V (D).
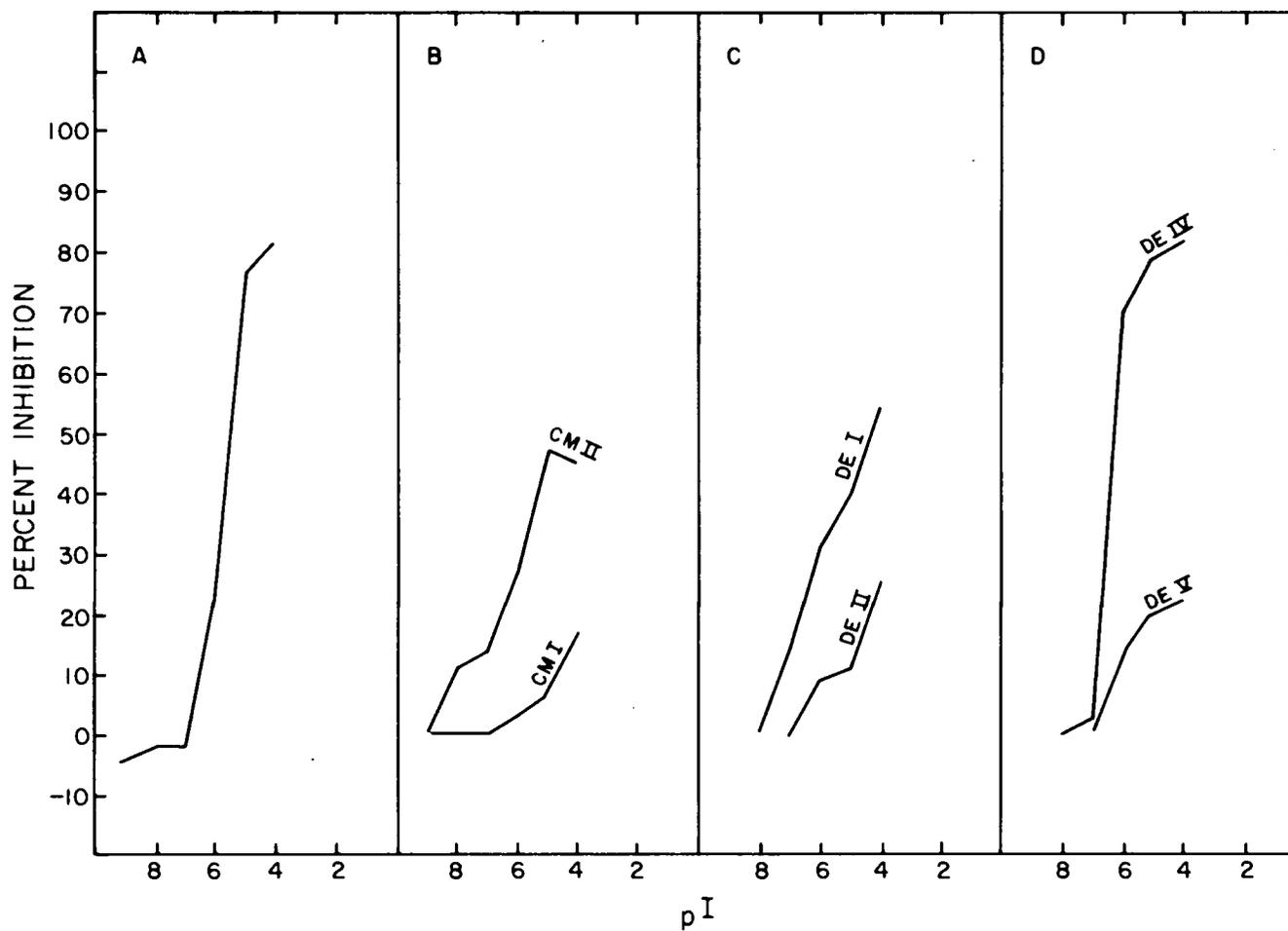
Figure 24. Inhibition with PCMB of the hydrolysis of phenyl propionate by some purified fractions of bean carboxylesterases. Whole extract (A), fractions CM_I and CM_{II} (B), fractions DE_I and DE_{II} (C), and fractions DE_{IV} and DE_V (D).

DISCUSSION

A recent report by Ecobichon (1968) described the isolation and purification of microsomal carboxylesterases from bovine liver. These were separated into a rapidly migrating band and a group of four slow moving bands of activity on starch-gel electrophoresis. By gel filtration on Sephadex G-200, bovine liver microsomal carboxylesterases were found to be of similar molecular size though differing in net electrical charge. In a similar manner, gel filtration of pea carboxylesterases on Sephadex G-100 showed that all of the esterase activity was eluted in one fraction (Montgomery, Norgaard, and Veerabhadrappa, 1968). These observations differed from the present study in that three fractions of bean esterase activity were eluted from gel filtration (Figure 11).

Results of gel filtration on Sephadex G-100 and subsequent electrophoretic examination of each fraction on polyacrylamide gel (Figures 11 and 12) suggest that these heterogenous carboxylesterases from beans are not of similar molecular size. Furthermore, gel electrophoresis of three fractions (S_I, S_{II} and S_{III}), obtained from gel filtration on Sephadex G-100, indicated the existance of variation in molecular size between the groups of bands, rather than individual bands. Of particular significance is the fact that the order of elution of these groups of bean esterases from the Sephadex G-100 column

was the precise inverse of the order in which these groups migrate in polyacrylamide-gel electrophoresis. That is, the group of esterase bands (first group) which was not retained on Sephadex gel appeared to move slowly on the polyacrylamide matrix and those eluted later (second and third group) were shown to move faster on polyacrylamide gel. Thus the results obtained with two different methods are closely correlated, and they suggest that the relative molecular sizes of the groups was first > second > third. This observation is not in agreement with either the bovine liver microsomal carboxylesterases (Ecobichon, 1968) or pea carboxylesterases (Montgomery, Norgaard, and Veerabhadrappa, 1968) where the difference appeared to be only in net charge.

It appears that, while being not similar in molecular size, the three groups of esterase activity have marked differences in net charges. This charge difference was obvious from the polyacrylamide-gel patterns and confirmed by the separation of these groups by chromatography on DEAE- and CM-cellulose. The nature of elution patterns, both in the case of anionic exchanger (DEAE-cellulose) and cationic exchanger (CM-cellulose), indicates that the first group of esterase activity was cationic, whereas, the second and third groups were anionic at the pH used (7.4) in this study. Hence, results obtained with both gel filtration and ion-exchange chromatography, suggest that bean esterases differ not only in the molecular size

but also in net charge.

Previous work (Montgomery, Norgaard and Veerabhadrappa, 1968) with pea carboxylesterases had revealed that two fractions from DEAE-cellulose showed similar substrate and inhibitor specificities even though each fraction contained two to three bands. This led to the suggestion that these two fractions were isozymes. Conversely, in the case of beans, groups of esterases which were alike in the hydrolysis of certain substrates and in sensitivity to certain inhibitors showed conspicuous differences on closer analysis. Thus fractions DE_{IV} and DE_V, otherwise similar in substrate specificity, differed in their sensitivity to the inhibitors parathion, DFP and PCMB. Fractions CM_I and CM_{II}, similar in the hydrolysis of phenol esters, showed conspicuous intra-group differences in that hydrolysis of acetyl and propionyl esters of sodium-2-naphthol-6-sulfonate was concentrated in fraction CM_I. Fraction DE_I was identical to DE_{II} in hydrolyzing phenol esters but differed to a considerable extent in hydrolyzing acetyl and propionyl esters of sodium 2-naphthol-6-sulfonate. The fractions of the third group, DE_{IV} and DE_V, differed from the rest of the groups in that the former hydrolyzed butyrate esters to a greater extent than the others. These variations in the properties of individual fractions suggest caution in their designation as isozymes, if the term is to be meaningful. Particularly, when the physiological substrate of these enzymes is unknown.

Originally, Markert and Moller (1959) stressed the limitations of the term "isozyme" to multiple molecular forms of an enzyme protein having the same enzymatic properties. They described similar affinities for eight substrates studied. However, Allen (1961) has shown that substrate specificities of lactic dehydrogenases of mouse tissue did vary. Markert and Appella (1961) in a subsequent paper recognized the applicability of the term "isozyme" to multiple forms differing in substrate specificity as well as in other properties. Similarly, two genetically controlled forms of an esterase present in an insect (Drosophila melanogaster) were shown to differ in their sensitivities to an organophosphorus inhibitor and heat (Wright, 1963). From the above observations, it might be speculated that isozymes exist with different specificities to substrates and inhibitors. Hence, bean carboxylesterases having variations and similarities in their sensitivity towards various substrates and inhibitors could probably be designated as isozymes.

In the study of multiple forms of enzymes, one must consider the possibility of artifacts produced in the course of extraction and purification. There are at least three kinds of artifacts in this category: column artifacts, extraction artifacts, and purification artifacts. These possibilities must be carefully examined before the decision is made as to the existence of multiple forms of an enzyme in plant material. Studies with D-glyceraldehyde-3-phosphate

dehydrogenase (Boross, Keleti, and Telegdi, 1960) and ribonuclease

(Shapiro and Parker, 1960) respectively, showed that column and

purification artifacts were responsible in some cases for the presence

of multiple forms. In the present case, identical elution patterns on

repeated chromatography rule out the possibility of a column artifact.

Identical electrophoretic patterns of esterase bands before and after

purification of the crude extract as well as other fractions indicate that

purification steps did not produce artifacts. Various extraction pro-

cedures were used to test the number of esterase bands present in

bean extract. Extraction with water and phosphate buffer (pH 7.4),

acid treated aqueous extract (pH 5.5), PVP treated aqueous extract,

and an extract treated with protamine sulfate all showed identical pat-

terns on polyacrylamide-gel electrophoresis. These observations

make it unlikely that any extraction artifacts are responsible for the

multiple forms of an enzyme.

Obviously, one would tend to hypothesize a model which explains

all the observed characteristics of the multiple forms of bean

esterases. Desborough and Peloquin (1968), as a result of genetic

studies on Solanum tubers, have suggested that the esterase molecule

in this plant might be a tetramer composed of one, two or three dif-

ferent types of monomers. These authors believe that the three

monomers could combine to yield 15 esterase isozymes. A similar

model has been proposed for lactate dehydrogenase isozymes which

have a tetrameric structure (Goldberg, 1966). Although this model would account approximately for the number of esterase bands present in beans, it would not seem to explain the differences in molecular size which exist between the groups of bean esterases. On the other hand, Smithies and Connell (1959) suggested that the heptoglobin molecule was made up of two types of subunits, but the total number of subunits per molecule was variable, so that separation of hepto-globins into several components by gel electrophoresis depended on differences in molecular size. Such a polymeric series was frac-tionated on Sephadex (Javid, 1964).

In order to explain the bean esterase isozyme patterns, a bio-chemical hypothesis, similar to the heptoglobin molecule, is pro-posed. This hypothesis is based on isozymes composed of two types of subunits although the number of subunits per molecule differs in each group. In order to account for the molecular size difference between the groups, the number of subunits assigned per molecule are five, four and three respectively, in the first, second and third groups. This hypothesized model is presented in Figure 25. Six bands are present in the first group, five in the second, and four in the third group. The number of esterase bands in the model does correlate well with the experimental results. However, only five bands, instead of six, were found (Figure 1) in the first group. The absence of one band may be due to its low concentration, or it may

BANDS

POSSIBLE
COMBINATIONS
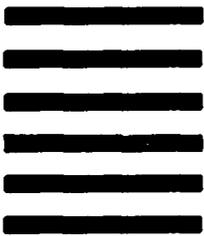
AAAAA
AAAAB
AAABB
AABBB
ABBBB
BBBBB

AAAA
AAAB
AABB
ABBB
BBBB

AAA
AAB
ABB
BBB

Figure 25. Hypothetical combinations of monomers A and B
representing esterase isozymes.

not have been active with the substrate (α-naphthyl acetate) used. Presence of another component in this group was postulated previously (page 82).

In view of the differences observed between the fractions in their sensitivity to inhibitors, an assumption that monomer A was sensitive and B was resistant to inhibitor, may have some merit. According to this assumption, fraction DE_{IV} containing two esterase bands AAA and AAB should be sensitive to inhibitor. Similarly, fraction DE_V containing two bands ABB and BBB should be resistant. This was indeed the case as was shown in Figures 22, 23 and 24. Although this model explains some of the important characteristics of bean esterases, the validity of this model rests entirely on precise biochemical analysis of each esterase band.

To explore, completely and conclusively, the question of isozymic nature of bean esterases, a study of the physicochemical nature of different esterases, including active center analysis for each of the individual components, is required. The current investigation revealed the complexities involved in the multiple forms of esterases and further indicated that with the proper selection of chromatographic techniques, it was possible to obtain individual esterases in purified form. Once, sufficient quantities of the purified esterases are obtained, physicochemical studies and active center

analysis will certainly lead to the clarification of isozymic nature of the multiple forms of esterases.

SUMMARY AND CONCLUSIONS

Aqueous extracts of peas and beans were subjected to zone electrophoresis on polyacrylamide gel. By the zymogram technique at least 14 bands of esterase activity in beans and seven bands of esterase activity in peas were separated. α-Naphthyl acetate, propionate, and butyrate and AS naphthol acetate were used as substrates to identify the esterases. Absence of activity with β-naphthyl laurate indicated the absence of lipase-type activity in both bean and pea aqueous extracts.

All of the heterogeneous esterases present in both beans and peas were classified as aliesterases or carboxylesterases (EC 3.1.1.1) based on their inhibition characteristics with certain organophosphorus compounds.

Carboxylesterases active toward phenyl propionate in protamine sulfate treated aqueous extract of the bean were separated into three fractions (S_I, S_{II} and S_{III}) by gel filtration on Sephadex G-100, indicating that they are not of similar molecular size. Subsequent analysis of each fraction by polyacrylamide-gel electrophoresis showed that fraction S_I contained the first group of esterase activity, fraction S_{II} possessed the first and second groups and third fraction S_{III} contained fast moving third group.

Each fraction from gel filtration was further separated by

ion-exchange chromatography on DEAE- or CM-cellulose. Preliminary studies with ion-exchange chromatography revealed that the first group was cationic while the second and the third groups were anionic at the pH used in this study.

Chromatography of the first group on CM-cellulose with sodium chloride elution resulted in three fractions CM_I, CM_{II} and CM_{III}. Esterase activity was separated into three fractions (DE_I, DE_{II} and DE_{III}) when S_{II} was chromatographed on DEAE-cellulose. Fraction DE_{III} was shown to contain only the second group of esterase activity. Sodium chloride linear gradient elution of fraction S_{III} on DEAE-cellulose resulted in two fractions (DE_{IV} and DE_V) and each fraction was shown to contain two esterase bands.

Inhibitor sensitivity determined with DFP, parathion and PCMB and substrate specificity towards nine substrates indicated that there were similarities and variations among each of the fractions.

The applicability of the term "isozyme" to bean esterases was discussed and a hypothetical model which accounted for the majority of the properties of these esterases has been proposed.

Although results observed in this study indicate that these complex forms of esterases were probably isozymes, conclusive evidence as to their isozymic nature is still to be established. Improved chromatographic techniques to separate these heteromorphs followed by structural analysis and physiochemical studies could lead to a

perspective view toward the challenging and intriguing concept of

multiple forms of enzymes. Once their identity is established, their

significance in the plant tissue could be understood.

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