THE PURIFICATION AND PROPERTIES OF
HORSE LIVER ESTERASE

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the
Graduate School of The Ohio State
University

By

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The Ohio State University
1956

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Introduction

The term "esterase" describes an enzyme which hydrolyzes an ester linkage. The hydrolysis of methyl butyrate, which forms methanol and butyric acid in the presence of liver esterase, is an example of an enzyme catalyzed ester hydrolysis.

\[
\text{Liver esterase: } \text{H}_2\text{O} + \text{C}_3\text{H}_7\text{C}=\text{O}+\text{CH}_3 \xrightarrow{\text{esterase}} \text{CH}_3\text{OH} + \text{C}_3\text{H}_7\text{COH}
\]

Many esterases are named by coupling the term "esterase" to the name of the substrate. Acetylcholine esterase and cholesterol esterase are examples of this nomenclature. Because the esterase found in the liver hydrolyzes many esters rather than a single ester, it has been described as "non-specific esterase" by Stotz (1) or "ali-esterase" by Richters and Craft (2). Though it will act on triglycerides, liver esterase is not a lipase, because, according to Willstater (3), this hydrolysis takes place at a much slower rate than the hydrolysis of the esters of the lower fatty acids. It would be more accurate to describe the enzyme discussed in this investigation as a "methyl butyrase," since only this substrate has been used in testing the purity of the enzyme. The enzyme is not specific for methyl butyrate, and so will be described, henceforth, as simply "esterase."
In this investigation, horse liver esterase was purified and the properties of the purified material studied. The methods used were: extraction of the enzyme from a commercial liver preparation; fractionation of the extracted protein with ammonium sulfate, alcohol, alcohol plus zinc, phosphate gel; and isoelectric fractionation. The behavior of liver esterase upon thermal denaturation, dialysis, lyophilization, electrophoresis, and ultracentrifugation was studied. The question of the possible existence of coenzymes was also investigated.

Following the purification, hydrolytic properties of the enzyme were determined. The substrate requirements, the effect of hydrogen ions upon esterase activity, and other enzymatic constants were measured by an accurate titrimetric method.

It was not because of any unique quality of the substrate, nor for the possible requirements for any particular co-factor, but rather for the absence of these, that purification of liver esterase was investigated. If enzymatic catalysis is to be studied for itself, then a model should be chosen that is general rather than specific in nature. Esterase, which catalyzes the hydrolysis of well known compounds, is such an enzyme. The enzyme must be highly purified before it can be meaningfully investigated.
Historical

The enzyme of the liver that hydrolyzes esters was first recognized as an esterase rather than a lipase by Willstatter (3) in 1924. Previously, the enzyme was believed to be similar to the lipase of the pancreas (Kastle and Loerenhart, 4, 5). Following recognition of this enzyme as an esterase the liver esterases of many species were investigated; pig by Rozengart, et al (6), beef by Baman, et al (7), horse by Mohamed (8), Burch (9) and Conners, et al (10), rat by Adridge (11), mouse by Omachi, et al (12) and guinea pig by Harrer and King (13). These enzymes were found to have different properties and each required an individual purification procedure.

Three procedures have been described for the purification of horse liver esterase. A crystalline product was isolated by Mohamed (8), but when an attempt was made to repeat this crystallization by Burch (9) and by Conners, et al (10), it was found that the crystalline material lost activity on recrystallization.
Methods

A. Assay

1. Enzyme

Esterase activity may be assayed by a variety of methods which depend on the physical properties of the ester solution or on the presence of the products of ester hydrolysis. According to Rona and Michaelis (14), modified stalagmometric and dilatometric techniques are suitable for the determination of esterase activity. The usual method for the assay of esterase activity is to measure either the alcohol or the acid produced by the ester hydrolysis. Colorimetric measurements are frequently employed for the alcohol determination. The alcohol, which is usually aromatic, can be coupled to a diazo dye or it may be colored itself. An example of diazotization given by Seligman and Nachlass (15) is the hydrolysis of β-naphthyl laurate to β-naphthol and subsequent coupling of this alcohol to tetraazotized diorthoanisidine. In the hydrolysis of p-nitrophenyl acetate, Huggins (16) measured the color of the p-nitrophenol directly.

Several methods for determining the acid produced by the ester hydrolysis have also been described. In the manometric technique, the enzyme and the substrate were incubated in a
bicarbonate buffer under an atmosphere of carbon dioxide and nitrogen. As employed by Conners, et al. (10), carbon dioxide was liberated as acid was produced and the increase in pressure was measured monometrically, in a Warburg manometer. This technique has been criticized by Burch (9), because the volatile substrate distilled into the enzyme solution and also because of Murray's report (17) that carbon dioxide inhibits the enzyme reaction.

Two titrimetric techniques have been established for measuring the acid directly. In one method the ester is incubated with the enzyme for a definite period of time; and then, the acid produced is titrated. A buffer is usually added to the solution to decrease the change in pH as the acid is produced, according to Ammon and Jaarma (18). One disadvantage of the procedure, as it was originally described by Harrer and King (13), is that it requires thirty minutes for a measurable amount of acid to be produced. Aside from the inconvenience of this period of time, the rate of reaction may not be at its maximum over the entire thirty minutes. The principle advantages of this method are its simplicity and lack of requirements for elaborate apparatus.

In the second titrimetric technique, the acid is titrated continuously as it is produced. Rates of reaction are measured
directly in this procedure. In the original description of this method by Willstatter, et al (19), bromthymol blue was used as an internal indicator. It has been pointed out by Bamann (20) that the internal indicator may inhibit the enzyme. This difficulty is obviated by the use of the glass electrode, demonstrated by Glick (21). Burch (9) has described an apparatus employing both a glass electrode and an amplifying potentiometric circuit. This continuous titration technique is a more accurate one, but does require more elaborate apparatus.

Both of these two titrimetric methods for determining the enzyme activity were employed in this investigation. For rapid, routine analyses, required in testing the effects of the factors involved in any of the purification procedures, a method was developed by which enzyme activity can be measured in less than five minutes. A potentiometric apparatus similar to that designed by Burch (9) was found to be suitable for more exact determination of the amount of activity in the purified material.

In the rapid method the degree of ester hydrolysis was measured after a fixed period of time. A 25 ml. Erlenmeyer flask containing varying amounts of 1% (v/v) methyl butyrate substrate, enzyme solution, and a sodium diethyl barbiturate-diethyl barbituric acid buffer, ionic strength 0.05, pH 8.0, was placed in a
constant temperature bath maintained at 38°C. After a short period of time, usually two minutes, the enzyme was inactivated by adding 1 ml. of a 5% (v/v) phenol solution, and the mixture poured into a 50 ml. beaker. The flask was rinsed out with 10 ml. of distilled water which was also poured into the beaker. The solution was then titrated with 0.02 N HCL from a 1.0 ml. microburet to a pH 7.0 end point measured with a Leeds and Northrup pH meter while the solution was stirred magnetically. An accurate end point was obtained at a pH of 7.0 because this pH was outside the range of effective barbiturate buffering action.

To avoid the effect of the protein alone on the buffer, the enzyme solution was added first to the solution being tested, and then an equal amount was added to a control solution, which was identical to the test solution except that it already contained phenol to inactivate the enzyme. Both the test and control solutions were at the bath temperature before the enzyme was added. During the incubation period of the test solutions the control solution was titrated to a pH of 7.0. After the amount of hydrochloric acid required for the test solution was measured, it was subtracted from the amount of acid needed by the control. This difference was then equivalent to the butyric acid formed, since at this pH butyric acid is completely ionized.
In this procedure every step was duplicated in the test and control solutions except that the test solution was kept in the constant temperature bath a few minutes longer than in the control solution. Previous experiments have shown that there was no appreciable hydrolysis of methyl butyrate under these conditions.

This rapid method was an easily performed assay requiring little apparatus and having several advantages over other procedures. It was a quick method since the time consumed for one experiment, both incubation and titration, was usually less than five minutes. Methods used by Conners (10) and Mohamed (8) required at least one-half hour for completion, while fifteen minutes were needed by Burch's procedure (9). A short reaction time insures a maximal rate of reaction which is essential for determination of the amount of enzyme present.

No elaborate apparatus was required for the titration since this was performed following, not during, the incubation. Phenol was employed to inhibit the enzyme. Parathion has been shown by Hofstee (22) to be a more efficient inhibitor, but it has the disadvantage of being highly poisonous. The use of a pH meter insured accurate end points at any pH required. One final advantage to this technique was the range of activities that could be determined.
Suitable choices of buffer concentration and incubation time permitted the measurement of very concentrated or very dilute enzyme solutions. While the initial choice of buffer concentration and incubation time might not be the best, the rapidity of the assay made it possible to repeat the experiment without undue loss of time.

The most serious disadvantage of the rapid technique was the pH change that occurred during incubation. While the buffer reduced this change to a minimum, some pH change cannot be prevented. According to Burch (9), the activity of the enzyme decreased 15% when the pH of the solution was changed from 8.0 to 7.0. Conners, et al (10) and Kistiakowsky and Mangelsdorf (23) observed a 50% decrease in activity over the same pH range with their preparations. Any change in enzyme activity due to pH change was interpreted as a lower amount of enzyme in the rapid assay technique. Another disadvantage was that as the incubation times were decreased, an error in recording the time of incubation was increased. Despite these disadvantages, duplicate determinations usually agreed within less than 5%.

The potentiometric continuous titration technique was a more accurate measure of enzyme assay than the rapid method. In the potentiometric method, the acid produced by the hydrolysis of the
ester was neutralized as rapidly as it was formed. The rate of addition of the alkali was proportional to the rate of acid production and consequently of ester hydrolysis.

The apparatus for this enzyme assay, which was patterned after that designed by Burch (9) consisted of two parts: a pair of electrodes capable of detecting the potential difference of solutions of varying pH, and an electronic amplification device. As is shown in Figure 1, this apparatus had two cells, each containing an inlet and outlet tube, a grounded silver chloride electrode, and a Leeds and Northrup Model 1199-30 glass electrode. In addition, the reaction cell contained a mercury-sealed stirrer and had an ultramicroburet attachment. A potential difference which depended upon pH was observed between ground and glass electrode in both of the cells.

This part of the apparatus differed from that described by Burch (9) in two respects. Two silver chloride electrodes were used rather than one electrode connected to both cells by salt bridges. In place of large pyrex tubes, 50 ml. beakers were used as cells. Rubber stoppers were fitted inside the beakers, and wax was poured over the top of the stoppers with the electrodes in place. The total volume of the actual cell was thus reduced to 10 ml., or one-fifth the size of the cells described by Burch (9).
Figure 1.

Potentiometric Esterase Assay Apparatus - Standard Cell

NOTE:
The reference cell is identical with this except that it lacks the ultramicroburst and stirrer.
The electronic part, illustrated in Figure 2, was built according to Burch's design (9) with the exception that an American made vacuum tube 6B8G was substituted for the British pentode EBL 31. The battery supply was changed correspondingly. This apparatus detected the very weak current from a Leeds and Northrup Model 1199-30 glass electrode by receiving the electrode output on a floating grid, A, which mediated the current flow through the vacuum tube. The vacuum tube controlled the current flow in the rest of the circuit. The internal resistance, B, could then be adjusted so that the charge on the suppressor grid, C, just balanced the effect of the floating grid. This prevented current flow in the tube, as was indicated on the microammeter. Before the floating grid was connected to either of the two glass electrodes, the internal resistance, D, was adjusted so that no current passed through the vacuum tube. The charge on the suppressor grid was then exactly equivalent to the voltage from the standard cell. The floating grid was next connected to the glass electrode of the test cell. When the potential difference from this electrode was the same as that from the standard cell, the microammeter indicated no current flow. When the pH of the test cell changed as the ester was hydrolyzed, the electrical output of the glass electrode also changed, and the microammeter indicated current flow. Base
Figure 2.

Potentiometric Esterase Assay Apparatus - Electronic Section

- Reference Cell
- Suppressor Grid
- Floating Grid
- Standard Cell

Connections:
- 67.5 volts
- 3 volts
- 1.5 volts
was then added to return the pH to its original value; i.e., when the microammeter indicated no current flow. The record of the rate of addition of base was then equivalent to the rate of enzyme action.

A. Assay

2. Protein

The biuret method for determining protein content is both rapid and, as modified by Gornall, et al (24), accurate. In this method sodium potassium tartarate is included with copper sulfate and sodium hydroxide as a copper complexing agent. This reagent is reported to keep indefinitely and to agree with Kjeldahl N determinations, assuming the 6.25 factor, within 0.1 gram % protein.
B. Purification

1. Starting Material

The first problem in the purification of horse liver esterase was the choice of a starting material. Fresh tissue, the usual source of enzymes, has been extracted with water by Fodor (25) and by Falconer and Taylor (26) and glycerol by Simmonds (27) and Glick (21) and according to Ammon and Jaarma (18), also converted to a press juice. Falconer and Taylor (26) found that the highest yield with aqueous extracts of pig liver was obtained with an extraction medium at pH 9. According to Bamann (28), esterase can be recovered from a minced tissue brei following a few days of alkaline autolysis without antiseptics, although a high degree of infection was encountered on repetition of this process by Burch (9), by Conners, et al (10) and by Falconer and Taylor (26). Dried, fat-free tissue powders have also been used as a source of liver esterase. These powders may be prepared by treatment of the fresh liver with acetone, according to Burch (9), acetone-ether, according to Ammon and Jaarma (18), or with chlorinated short-chain hydrocarbons in the VioBin\textsuperscript{1} process. The VioBin patent (29) states that a high degree of activity was retained in this preparation.

1. The VioBin Corporation, Monticello, Illinois
A commercial preparation of horse liver manufactured by the VioBin Company was used in this investigation as the source of the enzyme. This material was designated as Jayron Powder, Raw Liver Substance. The manufacturer's label describes it as "Mammalian Liver, 1:4 1/2, processed in the raw state, dessicated and defatted at 37° C. Autolysis is inhibited to retain enzyme activity." It was a fine brown powder which had no odor of putrefaction, either wet or dry.

B. Purification

2. Extraction

Three variables were considered in the extraction of esterase from VioBin liver powder: pH, temperature, and powder concentration. The extraction time chosen was 24 hours. This was probably insufficient for quantitative extraction considering Simmond's report (27) that 34 months was not long enough to completely remove all the enzyme from the insoluble portion. Double distilled water was the extraction medium. A paste was first made from the VioBin powder, and water was then added with stirring. The solution was covered with Saran Wrap plastic sheet to reduce bacterial infection, and was stored either at room or refrigerator temperature for 24 hours.
B. Purification

3. Isoelectric Precipitation

The technique generally employed in isoelectric precipitation consists of first adjusting the pH, allowing time for equilibration, and then separating the insoluble enzyme from the foreign protein by centrifuging. Since esterase was found to be unstable at pH 3, where it is insoluble, the foreign protein rather than the enzyme was removed by isoelectric precipitation. In this procedure the cold solution obtained after aqueous extraction followed by centrifugation was stirred magnetically while 0.5 N HCL was slowly added. The pH of the solution was measured with a Leeds and Northrup pH meter. After the pH had been adjusted to 4.5 - 5.0, the solution was stored in the refrigerator for two hours and then centrifuged. After separation of the insoluble protein, the pH of the solution was adjusted to 6.0 with 0.1 N NaOH since the enzyme is more stable at this than at the lower pH.

Rapidity and simplicity of operation are the advantages of this purification process. Two factors limiting the effectiveness of this method are the anion-cation type of protein interaction brought about by the pH change, and the mechanical occlusion of the enzyme by the precipitate. This procedure has been employed previously with horse liver esterase, but it was combined with the
thermal denaturation of the contaminating protein so that data are not available concerning the effect of isoelectric precipitation alone.

B. Purification

4. Thermal Denaturation

Three variables are involved in the thermal denaturation of the protein contaminating the enzyme protein: the temperature attained, the time of incubation at this temperature, and the composition of the solution. The first two variables reflect the dependence of denaturation on both time and temperature. The last is a consequence of the different behavior of proteins in the presence of salts and of other proteins.

The effect of heat was tested on aqueous extracts of Vio-Bin liver powder. These extracts were employed because it has been reported by Conners, et al (10) that the purified enzyme is heat labile. In these experiments the solutions were adjusted to the desired pH, centrifuged if precipitation occurred, and then subjected to the heat treatment. The test tube containing the enzyme solution was placed in boiling water bath and the solution stirred rapidly. One minute after the solution reached the proper temperature it was placed in an ice bath.
Table 1 shows the results of various treatments on horse liver esterase solution by other investigators. It is interesting to note that in the procedures reported, the solution temperatures were 55° and 63° C., while the purified enzyme was heat sensitive with partial loss of activity occurring at 30° C.

Table 1

Thermal Denaturation of Horse Liver Esterase

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Burch (9)</td>
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<tr>
<td></td>
<td>Conners et al (10)</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
</tr>
<tr>
<td>Temperature °C.</td>
<td>55</td>
</tr>
<tr>
<td>Time at Temperature in minutes</td>
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</tr>
<tr>
<td>Salts Present</td>
<td>none</td>
</tr>
<tr>
<td>Activity Increase Factor</td>
<td>4.1</td>
</tr>
<tr>
<td>Per Cent Recovery</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>0.145 M phosphate buffer</td>
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<tr>
<td></td>
<td>2.0</td>
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<td></td>
<td>75</td>
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B. Purification

5. Ammonium Sulfate Fractionation

Fractional precipitation with ammonium sulfate is a time-honored method for the separation of the components of a protein mixture. There are several advantages in the use of this salt. The anion is more effective than the other anions tested in
precipitating protein as the Hofmeister series quoted by Gortner (30) shows.

Anions: sulfate > citrate > tartrate > acetate > chloride > nitrate > iodide

Cations: Li⁺ > Na⁺ > K⁺ > NH₄⁺ > Rb⁺ > Cs⁺

While several cations are more effective than the ammonium ion in precipitating protein, the solubility in water (70.6 gm. % at 0° C., 103.3 gm. % at 100° C., Lange, 31) is one reason for its use in precipitating esterase. Also the pH of the concentrated solution approximates the isoelectric point of many proteins. Finally, ammonium sulfate does not inhibit liver esterase. The nitrogen content of the ammonium ion is the principle disadvantage of this fractionation since this may interfere with protein determinations. Another disadvantage is found in the large temperature coefficient of solubility of the salt.

This salt has been employed in the purification of liver esterase from several species. The less soluble proteins are usually precipitated first, and then, by increasing the salt concentrations, the enzyme is precipitated. Burch (9) reported horse liver esterase was precipitated between 28 and 40 gm. % ammonium sulfate, while Conners, et al (10) reported it to be precipitated between 36 and 46 gm. %. Both procedures resulted in a substantial purification of the enzyme (Table 2).
Table 2

Increase in Activity by Ammonium Sulfate Fractionation

<table>
<thead>
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<td>Range in Salt Concentration</td>
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<td>gm./100 ml.</td>
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<td>pH</td>
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<td>Increase in Activity Factor</td>
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</tr>
<tr>
<td>Per Cent Recovery</td>
<td>80</td>
</tr>
</tbody>
</table>

The technique used in this investigation was to add a weighed amount of the solid salt with stirring and then allow 24 hours for equilibration before centrifuging to remove the precipitate. Dialysis of the precipitated enzyme against distilled water in the refrigerator removed the salt. The protein solution was judged salt free when the dialysate did not show any precipitate in the presence of lead nitrate. After no lead sulfate precipitate could be seen the solution was dialyzed against five to ten times its volume of water over a period of 24 hours to insure salt removal. During this time the water was changed from three to six times. In some of the experiments the dialysis sacs were stirred to increase the rate of attainment of equilibrium. A small amount of insoluble precipitate resulting from dialysis was removed by centrifugation and decantation.
No correction was made in the calculations for either the volume occupied by the salt or for the amount of salt removed with the precipitated protein since this was an empirical purification.

B. Purification

6. Fractionation with Organic Solvents

The organic solvents most often employed in the fractional precipitation of protein solutions are ethyl alcohol and acetone. While other organic solvents, such as ethyl ether, dioxane, and tetrahydrofuran have been used in enzyme purification according to Colowick and Kaplan (32), they do not satisfy the requirements of a satisfactory fractionating agent. These requirements are that the solvent must be sufficiently soluble in water to allow a suitable concentration range, and that the solvent or impurities in the solvent must not denature the enzyme. Ethyl ether is not a good fractionating agent since it is only slightly soluble in water. Dioxane and tetrahydrofuran are frequently contaminated with peroxides which are very active in protein denaturation. Methyl alcohol has been proposed as a precipitating agent by Pillemer and Hutchinson (33), because it is less active in denaturing proteins than either ethyl alcohol or acetone. By maintaining sufficiently low temperatures and by limiting the time of contact,
protein fractionation with ethyl alcohol or acetone does not lead to much loss in enzyme activity. The marked change in solubility of the protein with a change in temperature makes the use of a constant temperature mandatory. This change in solubility can be a useful fractionating parameter.

Table 3 illustrates the previous fractionation of horse liver esterase with ethyl alcohol and with acetone. Increasing the temperature caused an increase in the amount of acetone required for enzyme precipitation.

Table 3
Fractionation of Horse Liver Esterase with Ethyl Alcohol and with Acetone

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractionating Agent</td>
<td>Mohamed (8)</td>
</tr>
<tr>
<td>Concentration Range (v/v %)</td>
<td>Burch (9)</td>
</tr>
<tr>
<td>Temperature °C.</td>
<td>Conners et al (10)</td>
</tr>
<tr>
<td>Increase in Activity Factor</td>
<td></td>
</tr>
<tr>
<td>Per Cent Recovery</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
</tr>
<tr>
<td>Ethyl Alcohol</td>
<td>Alcohol</td>
</tr>
<tr>
<td>45 - 62</td>
<td>0 - 43</td>
</tr>
<tr>
<td>- 10</td>
<td>- 6</td>
</tr>
<tr>
<td>1.6</td>
<td>2.4</td>
</tr>
<tr>
<td>67 - 95</td>
<td>58</td>
</tr>
<tr>
<td>7.2</td>
<td>6.6</td>
</tr>
</tbody>
</table>

It is not known exactly how the organic solvent acts in lowering the enzyme solubility, but it is believed that the solvent may lower
the dielectric constant and in this way increase the coulombic attractive forces between the unlike charges of the protein molecules. This will lead to aggregation and precipitation. Another mechanism is the attraction of water by the solvent, thus producing a dehydrating effect on the protein. Since the effect of these factors is unpredictable, the exact concentrations of solvent required for enzyme precipitation must be determined by experiment.

Both alcohol and acetone were investigated in the fractionation of impure solutions of liver esterase. The solutions tested were those obtained upon the completion of dialysis following ammonium sulfate fractionation. In this method the cold alcohol or acetone was added to the solution, and after a few hours to insure equilibration, the solution was centrifuged. It was observed that the precipitate did not separate readily on centrifugation. An International centrifuge was used for the acetone fractionation and a Servall table model centrifuge for the alcohol separations. Capped centrifuge tubes were employed to prevent evaporation of the solvent during the centrifugation. The precipitated protein was tested for activity following exhaustive dialysis.

B. Purification

7. Fractionation by Heavy Metal Ions

Fractional precipitation by heavy metal ions has long been known as an excellent method for enzyme purification. Low
temperature and pH control are requirements for this procedure, especially when the metal displaces protons. This proton displacement is believed to be the mechanism of precipitation by the metal ion since this will affect the isoelectric point of the protein. Calcium, according to Colowick and Kaplan (32), mercury by Warburg and Peterson (34), copper by Scott (35) and zinc ions by Isliker and Antoniades (36) have been found appropriate for various proteins. Only one of these ions, copper, has been used on horse liver esterase. This was employed in the procedure of Conners, et al (10). In this precipitation, the yellow brown color associated with the enzyme was removed and a clear, colorless solution was obtained. No other method has been found to remove this color. Unfortunately, 78% of the enzyme activity was lost in the procedure.

In repeating this procedure, cupric acetate was added both in solution and in the solid state to the cold enzyme solution. After one hour the solutions were centrifuged. The resultant supernatant solutions were exhaustively dialyzed against distilled water. The entire procedure was performed at 15° C. in the refrigerator.
B. Purification

8. Dialysis

While dialysis is not usually considered a method of purification, in the isolation of esterase from dried liver preparations it may be considered such. Burch (9) reported a loss of 15% of the total nitrogen with no loss in activity upon dialysis. This corresponds to a 10% increase in specific activity.

Dialysis was employed at two stages of the purification process: following ammonium sulfate and following zinc-alcohol fractionation. In some of the experiments the dialysis sac was attached to the glass rod of a stirring motor and in this way rotated through the solution. This decreased the time required for reaching equilibrium between the contents of the dialysis sac and the surrounding solution.

B. Purification

9. Zinc Alcohol Fractionation

Since the combination of zinc acetate and ethyl alcohol had been found to be so successful in the fractionation of the serum proteins by Edsal, et al (37), its use was investigated in the purification of horse liver esterase. The solution tested was obtained by ammonium sulfate fractionation of aqueous extracts of VioBin liver powder. Alcohol, precooled to -10°C, was added to a
series of solutions, each cooled to 0° C. and containing from 0 to 50 mM zinc acetate. After keeping the solutions at 4° C. for from one to three hours, they were centrifuged at that temperature. The supernatants solutions were analyzed immediately or following dialysis, since this treatment had no effect on enzyme activity. The second series of experiments is treated at -10° C. The enzyme activity was restored to the precipitate by dialysis to remove alcohol and by treatment with Amberlite IRC 100 cation exchange resin to remove zinc.

B. Purification

10. Adsorbents

The reversible adsorption of proteins is an effective purification method because different proteins show different degrees of adsorption. Kaolin and alumina by Willstatter (3) and tricalcium phosphate by Burch (9) have been employed in the purification of liver esterase. Kaolin and alumina were used in the purification of pig liver esterase. The enzyme was adsorbed from a solution at pH 4.2 - 4.4 and eluted from the adsorbent by ammonia water. This procedure was reported to yield a 10-fold increase in specific activity.

In the adsorption of horse liver esterase on tricalcium phosphate gel, according to Burch's procedure (9), the pH was 5.5 - 6.0.
The enzyme was eluted by sodium sulfate yielding a 3.6-fold increase in specific activity. This procedure was repeated in this investigation after the VioBin extract had been fractionated with ammonium sulfate. The gel was prepared according to the method of Keilin and Hartree (38) and used within a month of preparation. A sample of the gel was heated at 110° C. to determine the dry weight. After the proper amount of gel was added, the solution was stirred and then kept in the refrigerator for equilibration. The gel was separated from the supernatant solution by centrifugation and immediately eluted with sodium sulfate. In the elution process, the gel was suspended in the eluting solution, stirred for one hour and then separated again by centrifugation. This process was repeated with either the same or increased salt concentration.
C. Units

The units used to express esterase activity depend on the methods employed in the measurements of enzyme activity. A "butyrase" unit has been defined by Willstatter and Memmen (42) as the amount of enzyme which at a pH 8.6 causes a 20 drop diminution of the number of drops of a saturated solution of tributyrin in 50 minutes when measured in a stalagmometer. When the activity was measured manometrically by Conners, et al (10), the activity was expressed as μl CO₂/min. The volume units are expressed in terms of carbon dioxide because this gas is liberated from the bicarbonate buffer by acid produced in ester hydrolysis. Optical density measurements have been used by Seligman (15) and by Huggins (16) with colored products. The optical readings are compared with reference tables prepared from known amounts. In this and in the manometric procedure, the fraction of substrate hydrolyzed can be determined from the calibration. A unit used by Falconer (26), who measured the pH drop with a glass electrode, was the amount of enzyme sufficient to lower the pH by 0.0001 unit per minute. Titrimetric methods, as employed by Mohamed (8), can lead directly to units of acid produced as 0.1 N acid/min. The formol titration has also been used by Fodor (25) to follow the appearance of acid from the hydrolyzed ester. These units were micromoles NaOH/hr./mg. enzyme nitrogen. Finally, an
"esterase unit" has been defined, according to Ammon and Jaarma (18), as the amount of enzyme, measured by the hydrolysis of methyl butyrate that splits 25% of the ester in 60 minutes at pH 8.9.

In this investigation the esterase activity is reported as milliequivalents of ester hydrolyzed per minute per milligram of protein. These units were derived from titrimetric measurements of the amount of acid produced from ester hydrolysis.
D. Properties

The comparable physical and enzymatic properties determined on the two previously purified preparations of horse liver esterase are illustrated in Table 4.

Table 4

Properties of Purified Horse Liver Esterase Preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Electrophoretic Mobility x 10^-5 cm^2/sec./volt</th>
<th>Per Cent Enzyme</th>
<th>Michaelis-Menten Constant mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burch (9)</td>
<td>4.2 at pH 7.5</td>
<td>95% on 0.77% solution</td>
<td>2.32</td>
</tr>
<tr>
<td>Conners (10)</td>
<td>3.4 at pH 7.0</td>
<td>70%</td>
<td>22.</td>
</tr>
<tr>
<td>Kistiakowsky (23)</td>
<td></td>
<td></td>
<td>2.48</td>
</tr>
</tbody>
</table>

The electrophoretic mobility at pH 7.0 was 3.4 X 10^-5 cm^2/sec./volt and at pH 7.5 was 4.2 X 10^-5 cm^2/sec./volt. Either bicarbonate inhibition or loss of the volatile substrate was suggested by Burch (9) as the cause of the difference in the Michaelis-Menten constants (2.2 X 10^-2 and 2.32 X 10^-3 M). In addition to these data, Conners, et al (10) reported an Arrhenius activation energy of 4,400 cal./mole. Burch (9) measured the extinction coefficient in a 1 cm. cell and reported the following values per mg. nitrogen per milliliter of solution: 600 μ, 0.039; 500 μ, 0.092;
Bourshell and Webb (39), using an enzyme preparation purified by Burch (9), calculated a molecular weight of 100,000 based on the uptake of a radioactive phosphate inhibitor.

Enzymatic properties of the protein may be studied by measuring the velocity of the catalyzed reaction. By this method Burch (9) found that methyl butyrate was hydrolyzed about twice as fast as ethyl butyrate which was hydrolyzed approximately 15 times as rapidly as ethyl acetate. The pH optimum was found by Burch (9), by Conners, et al (10) and by Titchener (40) to be 8.0. A recent publication of Kistiakowsky and Mangelsdorf (23) indicated that the enzyme was most active from pH 8.5 to pH 9.5. According to these authors the previous reports of optimum activity at pH 8 did not differentiate between a lowered enzyme activity and enzyme denaturation.

In this investigation the sedimentation constant was determined in a Spinco Model E analytical ultracentrifuge over a 3-fold range of enzyme concentration (3.5 to 10 mg./ml. protein). The protein sedimentation is observed by the schlieren optical system in this instrument. The refractive index gradients, which are caused by the proteins sedimenting, are reproduced as peaks above a base line. Each component in a protein mixture will
sediment at a different speed and each will produce a change in
the refractive index of the solution. The schlieren lens system
records this as an individual peak for each component (Figure 2).
The sedimentation constant is determined from the rate of travel
of the peak across the photographic plate. Reference marks are
provided by two dark bars on the photographic plate (white on the
print) which come from light passing through two holes in the
counterbalance cell in the rotor. According to the manufacturer's
instruction bulletin, the distance from the center of rotation to
the outer edge of the first index is 5.70 cm. A traveling micro-
comparator was employed to measure the distance between this
index and both sides of a peak. The average of these readings,
after dividing by the magnification factor, 2.158, is added to
5.70 cm. to give the distance of the peak from the center of ro-
tation. In this way the distance from the center of rotation is
known for each time the peak was photographed.

Sedimentation constants are by convention converted to the
value which would have been determined if the temperature were
20° C. This correction is made by multiplying the time of each
exposure by the ratio of the viscosity at experimental temperature
determined before and after each centrifugation and at 20° C.
Finally, the corrected time is plotted versus the logarithm of
Figure 3
Typical Schlieren Patterns in the Ultra centrifuge
the distance traveled (Figure 3). The sedimentation constant can then be calculated, knowing the speed of rotation in revolutions per minute (RPM) from the slope of the curve. This procedure is based on the definition of the sedimentation constant

\[ s = \frac{dx}{dt} \frac{2}{x \omega^2} \quad \frac{d \ln x}{\omega^2 dt} \]

where "s" is the sedimentation constant, "x" is the distance traveled in centimeters, "t" is the time in minutes, and \( \omega^2 \) is the angular velocity in radians per second. Radians per second can be converted to revolutions per second by

\[ \omega^2 = \left( \frac{2 \text{ RPM}}{60} \right)^2 \]

The sedimentation constant is then given by

\[ s = 2.303 \frac{60}{(2 \omega \text{RPM})^2} \cdot \frac{d \log x}{dt} \]

The sedimentation constant at infinite dilution is obtained by plotting the sedimentation constants at known concentrations versus the concentration. Extrapolation to zero protein concentration then results in the sedimentation constant at infinite dilution (Figure 4).

Electrophoretic mobilities were measured in the semi-micro cell of an Aminco Stern apparatus. Protein migration is observed by a schlieren lens system in this instrument. The
Figure 4
Determination of Sedimentation Constant

\[ \log x \]

\[ \text{cm.} \]

\[ \text{Time in Minutes} \]
Figure 5
Determination of Sedimentation Constant at Infinite Dilution

Sedimentation Constant
$\rho 10^{-12}$ sec.

mg/ml. Protein
distance traveled by the protein was determined by measuring
the distance from the schlieren peaks to the edge of the photographic
emulsion (Figure 5). Both a traveling microcomparator and en-
largements of the plates were used for this. The mobility was then
calculated from the expression defining electrophoretic mobility:

\[ \mu = \frac{\Delta x}{\Delta t} \cdot \frac{q}{i} \cdot \frac{c}{1.91} \cdot \frac{13.45}{i} \]

where "u" is the mobility, "\( \Delta x \)" is the distance the protein migrated,
"\( \Delta t \)" is the time required for migration, "q" is the cross sectional
area in square centimeters, "c" is the equivalent conductance per
centimeter, and "i" is the amperage. Two correction factors were
applied to this expression for this experiment. One is to remove
the magnification in the optical system \( (1/1.91) \) and the second is
an instrument constant relating the position of the cell in one of
two positions \( (13.45) \). The final expression is given below.

\[ \mu = \frac{\Delta x}{\Delta t} \cdot \frac{13.45}{1.91} \cdot \frac{q}{i} \cdot \frac{c}{i} \]

The equivalent conductance of the solution was measured by an
Industrial Instruments Inc. Conductivity Bridge Model IRC 1 B.
The expressions "\( x \)" and "\( t \)" were determined from a graph
of "\( x \)" versus "\( t \)" (Figure 6).

The metal content of the purified preparation was determined
from the emission spectra observed with a concave reflecting
Figure 6

Typical Schlieren Pattern

in the Electrophoresis Apparatus

descending —> ascending
Figure 7

Determination of Electrophoretic Mobility

\[ \text{Time in Minutes} \]
The enzyme preparation was digested with sulfuric acid to concentrate any metals present. After evaporating most of the acid, the residue was placed into a hole drilled into the carbon electrode.

The adsorption spectra was measured in a Beckman Spectrophotometer Model D U. A Beckman Spectrophotometer Model B was employed in measurements in the range 390 m\(\mu\) - 600 m\(\mu\), in the early purification stages. Horizontal paper electrophoresis was employed to measure the degree of homogeneity of the enzyme preparation. The Michaelis-Menten constant and the effect of pH on the rate of reaction was determined with the potentiometric assay apparatus.
Experimental Results

A. Assay

1. Enzyme

The data for a typical activity analysis by the rapid method are shown in Table 5. The propagated error calculated for this experiment is also shown in Table 5. The finest division that can be read on the buret is 0.01 ml. In a 2 minute assay this corresponds to an enzyme activity of $1 \times 10^{-4}$ meq./min./ml., or $5 \times 10^{-5}$ meq./min./ml. for a 4 minute experiment. The propagated error for a 2 minute analysis may be of the same order of magnitude as the actual measurement. For this reason activity measurements were not accepted unless the difference in the control and test sample buret readings was at least 0.1 ml. The maximum possible propagated error with the above limitation is then reduced to 11%. The lower limit of detectable enzyme activity was then $1.0 \times 10^{-3}$ meq./min./ml. for a 2 minute assay. Lower enzyme activities were measured by lengthening the time of incubation or increasing the size of enzyme sample. The upper limit of activity determination was set by the buffer strength. The acid required to neutralize the control had to be equal to, or greater than, the acid liberated in the control sample. As the activity approached this limit a further error was introduced.
Table 5

Typical Calculation for Rapid Titrimetric Esterase Assay

<table>
<thead>
<tr>
<th>Reagents:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 5 (v/v) % phenol</td>
<td></td>
</tr>
<tr>
<td>2. 0.020 N HCl</td>
<td></td>
</tr>
<tr>
<td>3. Sodium diethylbarbiturate-barbituric acid buffer, u=0.05: pH 8.0</td>
<td></td>
</tr>
<tr>
<td>4. 1 (v/v) % methyl butyrate</td>
<td></td>
</tr>
<tr>
<td>5. Enzyme solution: 1.0 ml.</td>
<td></td>
</tr>
</tbody>
</table>

Sample | pH | ml. HCl required to adjust pH to 7 | Time
---|---|---|---
Control | 7.90 | 2.44 | 2 minutes
Test | 7.46 | 1.68 | 0.76 ml. of 0.02 N HCl equivalent to the butyric acid liberated by ester hydrolysis

\[
0.76 \text{ ml.} \times 0.02 \text{ N} = 7.6 \times 10^{-3} \text{ meq./min./ml. enzyme}
\]

2 minutes

Propagated Error
1. Error in buret reading = 0.01 ml.
2. Error in time-3 seconds-time required for addition and mixing
3. Error in normality of HCl = 1%
4. Error in methyl butyrate concentration = 1%

\[
\% \text{ Probable Error} = 100 \sqrt{\frac{(\text{Errors})^2}{\text{Value}}} = 100 \sqrt{\frac{0.01^2 + 3^2}{0.76} + \frac{0.01^2}{120} + 0.01^2}
\]

since as the hydrogen ion concentration was increased, the enzyme activity was decreased. For this reason an arbitrary limit of 1.5 ml. difference in the titration of the control and test samples was established. Any enzyme sample in which this difference exceeded 1.5 ml. was diluted and the activity assay repeated.
The error in the addition of phenol was minimized by treating the two samples in an identical manner so that any possible error would be compensated.

In the potentiometric method the rate of enzyme action was determined from the rate of addition of base. The data recorded in a typical analysis (Table 6) were: the normality of the base, the volume and concentration of the substrate, the amount of enzyme added, and the time and amounts of base added to the reaction cell. A graph showing the amount of alkali added versus time was drawn for each analysis (Figure 8). The slope of the line was then the rate of addition of base, or the rate of acid liberation. Table 6 illustrates the calculations required to convert the units from ml. base added/min./ml. to meq./min./ml.
Table 6
Typical Calculation for Potentiometric Esterase Assay

1. 0.02 N NaOH
2. 5 ml. of 1 (v/v) % methyl butyrate
3. 1.0 ml. enzyme solution

<table>
<thead>
<tr>
<th>Clock Time</th>
<th>ul NaOH</th>
<th>Time</th>
<th>ul NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:51:00</td>
<td>140</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4:51:30</td>
<td>260</td>
<td>0.50</td>
<td>120</td>
</tr>
<tr>
<td>4:52:05</td>
<td>320</td>
<td>1.08</td>
<td>180</td>
</tr>
<tr>
<td>4:52:25</td>
<td>390</td>
<td>1.42</td>
<td>250</td>
</tr>
<tr>
<td>4:52:40</td>
<td>450</td>
<td>1.67</td>
<td>310</td>
</tr>
<tr>
<td>4:53:10</td>
<td>530</td>
<td>2.17</td>
<td>390</td>
</tr>
<tr>
<td>4:53:35</td>
<td>610</td>
<td>2.58</td>
<td>470</td>
</tr>
<tr>
<td>4:54:05</td>
<td>700</td>
<td>3.08</td>
<td>560</td>
</tr>
<tr>
<td>4:54:35</td>
<td>800</td>
<td>3.58</td>
<td>660</td>
</tr>
<tr>
<td>4:55:10</td>
<td>900</td>
<td>4.17</td>
<td>760</td>
</tr>
<tr>
<td>4:55:50</td>
<td>1000</td>
<td>4.83</td>
<td>860</td>
</tr>
</tbody>
</table>

The slope of the graph is 183 ul/min./ml.

\[
\text{ul} \times 10^{-3} \text{ml} = 10^{-3} \text{meq.} / \text{min.} / \text{ml.}
\]

183 ul/min./ml. becomes 1.83 \times 10^{-1} \text{meq.} / \text{min.} / \text{ml.}
Figure 8
Typical Potentiometric Assay
A. Assay

2. Protein

The biuret reagent prepared according to Gornal (24) was used to determine protein content. A dialyzed, salt-free, concentrated solution of serum albumin was the primary standard. Its nitrogen content was determined by the micro-Kjeldahl method. Optical densities of the biuret solution were measured at 540 μm in a 1.0 Beckman cuvette with a Model B Beckman Spectrophotometer. A standard curve was drawn from the analysis of serum albumin for each preparation of the biuret reagent, and the protein content of the enzyme solutions was read from this. Figure 4 is one of the primary standard graphs.

![Figure 4: Biuret Primary Protein Standard](image-url)
B. Purification

1. Starting Material

The three lots of VioBin liver powder purchased during the course of this investigation were found to be similar in specific activity. Analysis of a sample of VioBin powder obtained 30 months previously indicated that it was only one-fourth as active per mg. protein as the recently purchased liver powder (Table 7). The difference in activity could have been due to a variation in the quality of the VioBin liver powder or, more probably, it could have been due to a slow denaturation of the enzyme protein.

Table 7

Effect of Time on Stability of Esterase as Determined by Measurements on Aqueous Extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity $\times 10^{-3}$ meq./min./mg. protein</th>
<th>Activity $\times 10^{-3}$ meq./min./ml.</th>
<th>Protein mg./ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recently Purchased</td>
<td>2.2</td>
<td>14</td>
<td>6.4</td>
</tr>
<tr>
<td>Purchased 30 mo. 0.55</td>
<td>3.2</td>
<td>5.8</td>
<td></td>
</tr>
</tbody>
</table>

B. Purification

2. Extraction

The enzyme was extracted from the VioBin liver powder with double distilled water. The variables investigated included the
powder concentration, the extraction temperature (room or refrigerator), and the pH of the extraction solution. Table 8 illustrates the results of varying the powder concentration from 2.5 gm. % to 20 gm. %. The highest activity was observed in the extraction at 20 gm. % protein. When the effect of temperature was tested in a comparison of extraction at room and refrigerator temperature (28° and 15° C.), it was found that a more active solution was obtained from the room temperature extraction (Table 9). It was also observed that this treatment, without any antiseptic agent present, yielded a preparation highly infected with bacteria. To decrease this contamination, all subsequent preparations were kept covered with Saran Wrap plastic sheet at refrigerator temperature even though the yield obtained under these conditions was slightly lower than that obtained at room temperature.

Table 8

Aqueous Extraction of Esterase from Several Concentrations of VioBin Powder

<table>
<thead>
<tr>
<th>Per Cent Powder</th>
<th>Activity x 10^-3 meq./min./ml</th>
<th>Protein mg./ml</th>
<th>Specific Activity x 10^-3 meq./min./mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>2.9</td>
<td>2.3</td>
<td>1.3</td>
</tr>
<tr>
<td>10.</td>
<td>13.</td>
<td>6.5</td>
<td>2.0</td>
</tr>
<tr>
<td>15.</td>
<td>19.</td>
<td>10.</td>
<td>1.8</td>
</tr>
<tr>
<td>20.</td>
<td>29.</td>
<td>12.</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Extraction performed over a 24 hour period at pH 7.
Table 9

Extraction of Esterase at Room and Refrigerator Temperature

<table>
<thead>
<tr>
<th></th>
<th>Room Temperature</th>
<th>Refrigerator Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$x 10^{-3}$ meq./min./mg.</td>
<td>2.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$x 10^{-3}$ meq./min./ml.</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg./ml.</td>
<td>6.4</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Extraction performed over a 24 hour period at pH 7.

The effect of pH was investigated by adjusting the hydrogen ion concentration of the liver powder suspension after the water had been added. Both the enzyme and the contaminating protein were found to be extracted better at the higher rather than lower pH (Table 10). Less of the enzyme was recovered at pH 9 than at a lower pH, but this is probably due to enzyme denaturation. The amount of protein extracted was found to increase as the pH was raised over the entire pH range tested.
Table 10

Effect of pH on Esterase Extraction

<table>
<thead>
<tr>
<th>pH</th>
<th>Protein Concentration (mg./ml.)</th>
<th>Activity Milliliter (x 10^-3 meq./min./ml.)</th>
<th>Activity Milligrams Protein (x 10^-3 meq./min./mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>3.3</td>
<td>6.7</td>
<td>2.0</td>
</tr>
<tr>
<td>6.5</td>
<td>5.2</td>
<td>10.</td>
<td>1.9</td>
</tr>
<tr>
<td>7.0</td>
<td>6.5</td>
<td>13.</td>
<td>2.0</td>
</tr>
<tr>
<td>8.0</td>
<td>8.8</td>
<td>13.</td>
<td>1.5</td>
</tr>
<tr>
<td>9.0</td>
<td>9.0</td>
<td>8.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Extraction performed over a period of 24 hours.

The final choice of experimental conditions for the extraction of liver esterase was that the aqueous extraction be made from a 20 gm.% VioBin suspension at 15° C. and at pH 7. This was found to recover 52% of the activity in the VioBin powder.

B. Purification

3. Isoelectric Precipitation

The behavior of liver esterase on isoelectric precipitation was investigated by adjusting the pH of a series of solutions from pH 6.0 to 2.8 with 0.5 N HCl. It was observed (Table 11) that 95% of the activity was lost from the supernatant of the solution at pH 3.2. The precipitated protein did not dissolve and the enzyme activity was not regained when the hydrogen ion concentration was decreased to pH 6.0. From these data it was concluded that the enzyme was inactivated at pH 3.2.
It was also observed in this experiment that the specific activity of the solution at pH 4.8 was 37% higher than of the solution at pH 6.0. This was the result of the precipitation of contaminating protein, as the decrease in total soluble protein indicates (Table 11). Liver esterase could, therefore, be purified by isoelectric precipitation of the impurities.

Table 11

Analysis of the Supernatant Solution in the Isoelectric Precipitation of Horse Liver Esterase

<table>
<thead>
<tr>
<th>pH</th>
<th>Total Activity meq./min.</th>
<th>Total Protein mg.</th>
<th>Specific Activity $\times 10^{-3}$ meq./min./mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>0.50</td>
<td>460</td>
<td>1.1</td>
</tr>
<tr>
<td>4.8</td>
<td>0.37</td>
<td>350</td>
<td>1.5</td>
</tr>
<tr>
<td>4.4</td>
<td>0.31</td>
<td>260</td>
<td>1.5</td>
</tr>
<tr>
<td>3.9</td>
<td>0.11</td>
<td>210</td>
<td>1.4</td>
</tr>
<tr>
<td>3.6</td>
<td>0.02</td>
<td>190</td>
<td>0.59</td>
</tr>
<tr>
<td>3.2</td>
<td>0.02</td>
<td>180</td>
<td>0.10</td>
</tr>
<tr>
<td>2.8</td>
<td>0.02</td>
<td>170</td>
<td>0.14</td>
</tr>
</tbody>
</table>

The isoelectric precipitation of impurities was tested on the aqueous extract of VioBin powder. As Table 12 shows, this treatment increased the specific activity from 1.3 fold to 1.7 fold while the loss of total activity ranged from 2.5% to 20%.
Table 12

Isoelectric Precipitation of Contaminating Protein from Aqueous Extracts of VioBin Liver Powder

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pH</th>
<th>Total Activity x 10^{-2} meq./min.</th>
<th>Total Protein mg.</th>
<th>Specific Activity x 10^{-3} meq./min./mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.8</td>
<td>4.0</td>
<td>4.3</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>4.8</td>
<td>3.9</td>
<td>2.6</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
<td>8.4</td>
<td>9.4</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>7.8</td>
<td>4.7</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>7.0</td>
<td>6.2</td>
<td>4.7</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>4.9</td>
<td>6.0</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>6.2</td>
<td>15.</td>
<td>7.0</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>12.</td>
<td>4.2</td>
<td>2.8</td>
</tr>
</tbody>
</table>

B. Purification

4. Thermal Denaturation

The effect of three factors was investigated in this procedure: pH, temperature, and added ammonium sulfate. In testing the effect of the hydrogen ion concentration the solutions were heated to 60°C. Enzyme activity was lost in all of these solutions, but, as Table 13 shows, less was lost at pH 6.0. This indicates that the enzyme was most stable with respect to thermal denaturation at this pH.
Table 13

Effect of pH on the Thermal Denaturation of Contaminating Protein

<table>
<thead>
<tr>
<th>pH</th>
<th>Original Activity $x 10^{-3}$ meq./min./mg. protein</th>
<th>Final Activity $x 10^{-3}$ meq./min./mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>2.7</td>
<td>1.1</td>
</tr>
<tr>
<td>6.0</td>
<td>2.2</td>
<td>1.5</td>
</tr>
<tr>
<td>7.0</td>
<td>2.3</td>
<td>1.2</td>
</tr>
<tr>
<td>8.0</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>9.0</td>
<td>1.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The solutions were heated to 60° C.

The next factor examined was the temperature of the heated solutions. The enzyme was stable only at the lowest temperature tested, 55° C. (Table 14). Heating the solution to this temperature did not increase the specific activity.

Table 14

Effect of Temperature on the Thermal Denaturation of Contaminating Protein

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Activity $x 10^{-3}$ meq./min./mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2</td>
</tr>
<tr>
<td>55°</td>
<td>2.2</td>
</tr>
<tr>
<td>59°</td>
<td>2.0</td>
</tr>
<tr>
<td>60°</td>
<td>1.5</td>
</tr>
<tr>
<td>63°</td>
<td>1.4</td>
</tr>
<tr>
<td>67°</td>
<td>0.9</td>
</tr>
</tbody>
</table>

The pH of the solution was 6.0.
Since all previous experiments had been performed with dialyzed, salt-free preparations, the effect of added ammonium sulfate was tested. As shown in Table 15, 5% ammonium sulfate exerted a protective action on the enzyme. This table also indicates that no gain in specific activity was observed.

Table 15

Effect of Ammonium Sulfate on the Thermal Denaturation of Contaminating Protein

<table>
<thead>
<tr>
<th>Time Heated</th>
<th>Control 10^{-3}\text{meq./min.}/\text{mg. protein} x 10^{-3}\text{meq./min.}/\text{mg. protein}</th>
<th>5\text{gm. % Ammonium Sulfate}</th>
</tr>
</thead>
<tbody>
<tr>
<td>One minute</td>
<td>2.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Three minutes</td>
<td>1.9</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The original specific activity was $2.5 \times 10^{-3} \text{meq./min./mg. protein}$. The solution was heated to $55^\circ \text{C.}$ at pH 5.5.

From these data, it was concluded that under these conditions, thermal denaturation is not a satisfactory procedure for the purification of horse liver esterase from VioBin powder.

B. Purification

5. Ammonium Sulfate Fractionation

Aqueous extracts of VioBin liver powder were fractionated either directly after the aqueous fractionation or following isoelectric precipitation of contaminating protein. The results of an aqueous extraction fractionation are illustrated in Figures 5 and 6. The enzyme was recovered in the fraction precipitating
Figure 10
Fractionation of the Total Activity by Ammonium Sulfate

Total Activity

-1
X 10 milliequiv.

min.

Gram Per Cent Ammonium Sulfate
Figure 11
Fractionation of the Specific Activity by Ammonium Sulfate

Specific Activity $8 \times 10^{-3}$ meq./min./mg. protein

Gram Per Cent Ammonium Sulfate
between 30 and 35 gm. % ammonium sulfate since although only 55% of the total activity was found in this fraction, the specific activity was 4.3 times that of the starting material.

In subsequent purifications the enzyme was fractionated over a 5 gm. % interval of salt concentration at 30-35, 32-36, and 32-37 gm. %. The increase in specific activity ranged from 3 to 14 times that of the starting material.

B. Purification

6. Organic Solvent Fractionation

The precipitation of esterase by acetone and alcohol was found to be temperature dependent. This is illustrated in Figure 12 which shows two acetone fractionations of esterase. In both experiments the solutions were kept in the refrigerator until centrifugation, but in the second experiment greater care was taken to keep the solution cold. All subsequent operations were performed in constant temperature cold rooms either at 4° C. or -10° C. Figure 13 shows the results of acetone fractionation at 4° C. at pH 6.5.

The effect of ammonium sulfate on acetone fractionation of liver esterase is shown in Figure 14. The salt markedly lowered the amount of acetone required to precipitate the enzyme.
Figure 12

Acetone Fractionation of Horse Liver Esterase at Refrigerator Temperature

Specific Activity

$\times 10^{-3}$ mg/min/mg protein

Volume Per Cent Acetone
Figure 13
Acetone Fractionation of Horse Liver Esterase at 4°C.

Specific Activity

$10^{-3}$ meq/min/mg protein

Volume Per Cent Acetone
Figure 14
Effect of Ammonium Sulfate on the Acetone Fractionation of Horse Liver Esterase

Specific Activity $x 10^{-3}$ meq./min./mg. protein

25% Ammonium Sulfate

Trace of Ammonium Sulfate

Volume Per Cent Acetone
With either acetone or alcohol fractionation, the major problem involved is denaturation of the enzyme. The effects of both time and temperature must be determined, since denaturation depends on both factors. The influence of temperature is reflected in Table 16, which contains a comparison of fractionation at 4°C and at -10°C. Ninety-two per cent of the total activity was lost at 4°C, while 47% was lost at -10°C. This loss in total activity was decreased to 11%, also shown in Table 16, by decreasing the time of fractionation from 36 to 8 hours.

These data indicate that organic solvents are suitable for the fractionation of liver esterase. The experimental conditions, time, temperature and salt concentration must be carefully controlled to insure enzyme activity.
Table 16

Ethyl Alcohol Fractionation

<table>
<thead>
<tr>
<th>Per Cent Ethyl Alcohol</th>
<th>4°C 48 hours</th>
<th>-10°C 36 hours</th>
<th>-10°C 8 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific Activity</td>
<td>Per Cent Total Activity</td>
<td>Specific Activity</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$ x meq./min./mg. Protein</td>
<td></td>
<td>$10^{-3}$ x meq./min./mg. Protein</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
<td>20</td>
<td>4.4</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>25</td>
<td>5.0</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>30</td>
<td>5.0</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>31</td>
<td>6.0</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>40</td>
<td>6.0</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>50</td>
<td>6.0</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Residue</td>
<td>2</td>
<td>27</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>54</td>
<td>21</td>
</tr>
<tr>
<td>Lost</td>
<td>92</td>
<td>46</td>
<td>11</td>
</tr>
</tbody>
</table>
B. Purification

7. Cupric Acetate Fractionation

The results of two series of cupric acetate fractionations are shown in Tables 17 and 18. The two series of experiments differ in the protein concentrations and in the specific activities of the starting material. In both series at least 89% of the activity was not recovered. In the second series (Table 18), the specific activity of the remaining enzyme had been increased by 1.7 fold in the most active sample.

Table 17

Fractionation of Horse Liver Esterase by Cupric Acetate

<table>
<thead>
<tr>
<th>Cupric Acetate mg./ml.</th>
<th>Protein mg./ml.</th>
<th>Activity $x 10^{-3}$ meq./min./mg. protein</th>
<th>Per Cent Activity Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.2</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>1.9</td>
<td>0.30</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>1.8</td>
<td>0.67</td>
<td>8.5</td>
</tr>
<tr>
<td>12</td>
<td>2.1</td>
<td>0.62</td>
<td>9.2</td>
</tr>
</tbody>
</table>
Table 18

Fractionation of Horse Liver Esterase by Cupric Acetate

<table>
<thead>
<tr>
<th>Cupric Acetate (mg./ml.)</th>
<th>Protein (mg./ml.)</th>
<th>Activity ($x \times 10^{-3}$ meq./min./mg. protein)</th>
<th>Per Cent Activity Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.</td>
<td>10.</td>
<td>100</td>
</tr>
<tr>
<td>3.0</td>
<td>0.60</td>
<td>17.</td>
<td>11.</td>
</tr>
<tr>
<td>4.9</td>
<td>0.75</td>
<td>15.</td>
<td>4.9</td>
</tr>
<tr>
<td>8.6</td>
<td>0.38</td>
<td>16.</td>
<td>3.7</td>
</tr>
<tr>
<td>12.0</td>
<td>0.8</td>
<td>9.5</td>
<td>3.4</td>
</tr>
</tbody>
</table>

B. Purification

8. Dialysis

The effect of dialysis on the specific activity of the enzyme was investigated for two reasons. This procedure has been shown to be useful after aqueous extraction of dried liver powder. Also, dialysis was used in this investigation after both ammonium sulfate and zinc-alcohol fractionation. The data shown in Table 19 are the results of two factors found in dialyzing liver esterase. The two solutions tested were extracted from VioBin liver powder at pH 7 and 8. The increase in specific activity of the solution extraction at pH 8 on dialysis illustrates the loss of dialyzable substances, probably polypeptides, which gave a positive biuret test. Since more of these compounds are extracted at pH 8 than at pH 7, this effect is greater at the higher pH. The decrease in
specific activity after dialysis of a solution obtained by aqueous extraction at pH 7 was a consequence either of the instability of the enzyme in solutions of low ionic strength, or of the passage of the enzyme through the dialysis membrane. On several occasions it was observed that on prolonged dialysis the esterase solution would deposit a fine, yellow-brown precipitate. This may be evidence of protein denaturation.

Table 19

Effect of Dialysis of Enzyme Solutions Extracted from VioBin Liver Powder at pH 7 and 8

<table>
<thead>
<tr>
<th></th>
<th>Undialyzed</th>
<th>Dialyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7</td>
<td>3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>pH 8</td>
<td>2.7</td>
<td>2.9</td>
</tr>
</tbody>
</table>

B. Purification

9. Zinc Alcohol Fractionation

Liver esterase solutions purified through the ammonium sulfate stage were fractionated at two temperatures: 4°C and -10°C. Analysis of the supernatant solutions at 4°C indicated that zinc acetate in small amounts precipitated the enzyme, while larger amounts of the zinc salt increased the enzyme solubility. The results of the analyses of the activities of the supernatant solutions (Figure 15) indicated that a considerable degree of
Figure 15

Activities of Supernatant Solutions from Zn and Alcohol Enzyme Precipitation

Maximum Zn (C₂H₅OH)₂/yr

Supernatant Activity

25% Alcohol
30% Alcohol
No Alcohol
purification could be obtained if the enzyme could be recovered from the precipitate. Electrodialysis; dialysis versus water, glycine, or Versene (tetrascodium ethylendiaminetetraacetate); and the treatment with Amberlite cation exchange resin IRC 100 yielded solutions low in enzyme activity. Lyophilization of the precipitate to remove alcohol also failed to yield an active powder.

The enzyme activity was recovered when the zinc-alcohol enzyme solution was maintained at \(-10^0\text{C}\). instead of \(4^0\text{C}\). Dialysis at \(4^0\text{C}\). was used to remove the alcohol and treatment with Amberlite cation exchange to remove the zinc ion. The amount of activity recovered varied from 75 to 87\%. The results of a series of fractionations are shown in Figure 16. The specific activity of an already highly purified sample was increased from 11 to 13\% by this treatment.

B. Purification

10. Adsorbents

Tricalcium phosphate gel was investigated in both the adsorption of the enzyme from solution and in the elution of the enzyme from the gel. The method was tested on a solution after ammonium sulfate fractionation and dialysis. As shown in Table 20, analysis of the supernatant solution indicated that the enzyme was preferentially adsorbed from the solution and that 84\% of the
Figure 16
Zinc Alcohol Fractionation

Specific Activity $10^3$ mae/min/mg protein 0.9

Millimoles $\text{Zn (C}_2\text{H}_3\text{O}_2)_2$/liter
enzyme was removed from the solutions. Figures 17 and 18 illustrate the recovery of the enzyme by sodium sulfate elution of the gel, and the increased activity of the eluted solutions.

Table 20

Adsorption of Esterase on Tricalcium Phosphate Gel at pH 7.5

<table>
<thead>
<tr>
<th>Per Cent unadsorbed</th>
<th>Per Cent Adsorbed</th>
<th>Mg. Protein Dry wt. gel mg.</th>
<th>Activity in Supernatant x 10^{-3} meq./min./mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>8.4</td>
</tr>
<tr>
<td>56</td>
<td>44</td>
<td>0.7</td>
<td>7.2</td>
</tr>
<tr>
<td>16</td>
<td>84</td>
<td>0.81</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The principal disadvantage of this procedure was the low yield of recovered enzyme. While 84% of the enzyme was removed from solution by the gel, the combined activity of the five eluting solutions was only 36% of the amount adsorbed onto the surface of the gel. Though the average specific activity of the eluted solutions was increased by 10%, the overall recovery was only 31%. If only the most active of the five eluting solutions (50% more active per mg. protein than the original solution) were retained, the final yield was 9.6%. These results do not necessarily indicate that the phosphate gel procedure was not a suitable one, but rather that under the conditions examined, it was not a very rewarding one.
Figure 17

Elution of the Total Activity from Tricalcium Phosphate Gel

![Graph showing the elution of total activity from tricalcium phosphate gel.

The x-axis represents eluted fractions, with values 1, 2, 3, 4, and 5.

The y-axis represents total activity in milliequivalents per minute (mEq/min), with values 0.00, 0.10, 0.20, and 0.30.

- Fraction 1: 0.09 mEq/min
- Fraction 2: 0.31 mEq/min
- Fraction 3: 0.37 mEq/min
- Fraction 4: 0.18 mEq/min
- Fraction 5: 0.14 mEq/min]
Figure 18
Elution of the Specific Activity from Tricalcium Phosphate Gel

Specific Activity

$10^{-3} \text{ mcpm/min/mg.6 protein}$

Eluted Fraction
C. Properties

The sedimentation constant at infinite dilution was calculated by plotting the sedimentation constants (3.82, 3.74 and 3.58 x $10^{-13}$ sec.) versus the experimental protein concentrations (3.5, 5.9 and 10 mg./ml.) and extrapolating the curve to zero protein concentration (Figure 4 and 5). The sedimentation constant at infinite dilution obtained by this procedure was 3.9 x $10^{-13}$ sec. In the solution containing the highest protein concentration an impurity was detected sedimenting ahead of the major component. This fraction had a sedimentation constant of 7.2 x $10^{-13}$ sec. By planimetric analysis of the area under each of the two peaks it was found that approximately 85% of the solution was the major fraction and 15% the minor fraction.

The electrophoretic mobilities determined at pH 7.7 and 8.5 were 4.8 and 6.4 x $10^{-5}$ cm.$^2$/sec./volt respectively. A representative plate is shown in Figure 6. In the experiment at pH 8.5, a second component was detected. On analysis with a planimeter, it was found that this fraction made up approximately 18% of the total protein.

The emission spectra from approximately 20 mg. of lyophilized and sulfuric acid digested sample is shown in Figure 19. A large amount of sodium is present with trace quantities of zinc and iron.
Figure 19

Emission Spectrum of Horse Liver Esterase

Iron Control
Figure 20 illustrates the adsorption spectrum of an aqueous solution of the purified esterase. Aside from the tyrosine-tryptophan peak at 275-280 μ, the only distinguishing feature of the curve is a small hump at 405 μ. The extinction coefficients per milligram protein are listed in Table 21.

<table>
<thead>
<tr>
<th>Wave Length (μ)</th>
<th>Extinction Coefficient in 1.0 cm. cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>260</td>
<td>7.93</td>
</tr>
<tr>
<td>280</td>
<td>11.8</td>
</tr>
<tr>
<td>300</td>
<td>2.21</td>
</tr>
<tr>
<td>350</td>
<td>0.390</td>
</tr>
<tr>
<td>405</td>
<td>0.252</td>
</tr>
<tr>
<td>450</td>
<td>0.124</td>
</tr>
<tr>
<td>500</td>
<td>0.072</td>
</tr>
<tr>
<td>600</td>
<td>0.032</td>
</tr>
</tbody>
</table>

The yellow color of the enzyme was observed to migrate in with the major fraction in electrophoresis on paper. A tracing of the brom phenol blue dyed paper (Figure 21) made with Spinco Analytrol Model RA also indicates contaminating fractions. One of these migrates in advance of the major constituent while the second trails this fraction. A comparison with a normal human serum pattern (Figure 22) shows that the first fraction migrates with the speed of the albumin fraction. The presence of the second
Figure 20

Adsorption Spectra of Horse Liver Esterase

[Graph showing optical density vs. wavelength]
Figure 21

Paper Electrophoresis Tracing of Horse Liver Esterase

Figure 22

Paper Electrophoresis of Normal Human Serum
contaminating fraction is shown by the elevated region behind the major peak. According to the instrument, the albumin fraction makes up 15%, the major component 75%, and the last fraction 10% of the total. These measurements are only approximate both because of incomplete separation of the proteins and because of irreversible adsorption of the protein on the paper.

The enzyme activity was determined by the potentiometric method over the pH range 8 - 10 to determine whether or not the activity diminished at pH values above 8. The results, shown in Table 22, indicate that the enzyme was more active at pH 8.0 than at pH 9 and 10.

Table 22

Effect of pH on Horse Liver Esterase Activity

<table>
<thead>
<tr>
<th>pH</th>
<th>Specific Activity x 10^{-2} meq./min./mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1.6</td>
</tr>
<tr>
<td>9</td>
<td>1.4</td>
</tr>
<tr>
<td>10</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The Michaelis-Menten constant was determined at pH 8 over a 40-fold range of substrate concentration. Figure 23 illustrates the Lineweaver-Burk (41) plot used to calculate Km. The maximum velocity was 3.3 x 10^{-2} meq./min./mg. protein and the Michaelis-Menten constant was found to be 5.2 mM.
Figure 23
Lineweaver-Burk Graph of Horse Liver Esterase Activity
Discussion

A. Assay

For rapid, routine analyses, any of the several colorimetric or titrimetric procedures previously described would be adequate. The titrimetric method employed in this investigation for rapid analyses, though not extremely accurate, had the advantage of not requiring elaborate experimental apparatus.

The ideal method for precise activity measurements would include provision for maintaining a constant enzyme environment with respect to salt, substrate and hydrogen ion concentration. Since this is impossible to achieve experimentally, the usual practice is to measure only the initial reaction rate and in this way reduce the complication of a varying ionic atmosphere. It would appear from these considerations that some variation of the constant pH technique must be employed for accurate esterase assay. The variation in activity with pH precludes the use of methods not maintaining this factor constant. Other experimental variables, such as a constant temperature and salt concentration and the absence of carbon dioxide, must also be carefully controlled. In this investigation, following the procedure of Burch (9), the reaction vessel was maintained in a constant temperature bath and the atmosphere over the reaction solution was either carbon dioxide-free air or nitrogen. The potentiometric
device for detection of pH change was found to be very sensitive both to pH change and to any electrical disturbance. A good contact for the instrument case and the bath is essential.

Kistiakowsky and Mangelsdorf (23) recently reported a conductometric method for determining esterase activity. One disadvantage of the technique described by these authors is that the pH was allowed to change during the course of the reaction. It might be advantageous to retain the conductometric principle while maintaining the solution at a constant pH by the continuous addition of base. The final question in this case is whether the initial rate of the reaction is affected more by a change in pH or by a change in ionic strength. Pending further investigation it would seem that the ionic strength variation is the lesser of the two factors affecting the enzyme activity.
B. Purification

While the goal of any purification scheme is the attainment of both complete purification and complete recovery, in practice usually one or the other of those aims is not achieved. In this investigation, purity was the desideratum and purification methods were tested primarily for selectivity rather than for enzyme recovery. In the laboratory, this principle meant that the change in specific activity was the standard by which all procedures were judged.

An abundant supply of starting material was essential for this kind of investigation. This was the primary reason for the use of the commercial preparation of horse liver powder. The disadvantage of not knowing precisely the previous history of the liver powder was overcome by the advantages of its low cost ($10.00 per lb.) and the ease of acquiring it in 5 and 10 lb. lots.

Aqueous extraction of the liver powder at pH 5-7 was employed following the same principle of maintaining as high a specific activity as was possible. Extraction with salt solutions at pH 8 would have solubilized more esterase, but this treatment would also lower the specific activity of the extract by dissolving more of the contaminating protein. Probably the low salt content of the aqueous extracts was the reason for the failure of thermal
denaturation to increase the specific activity of the solution. While Burch (9) reported an increase in specific activity by thermal denaturation, her procedure also included an isoelectric precipitation of contaminating protein, so that the increase was due to either or both processes.

The most effective single procedure was ammonium sulfate fractionation. By this procedure both specific activity and the protein concentration were increased. Most of the other purification procedures, fractionation by organic solvents, cupric acetate, zinc-alcohol, and tricalcium phosphate gel were investigated on solutions purified by ammonium sulfate fractionation.

Since the starting material was already purified, the increase in specific activity was less in these purification procedures than with the previous methods. Also, since the recovery of esterase had been low, it was necessary, if possible, to prevent further loss in the remaining procedures. Among the remaining purification methods tested, fractionation by tricalcium phosphate gel and cupric acetate resulted in approximately 10% yields of enzyme. These low yields do not indicate that the procedures are not useful, but rather that they are not very productive with liver esterase at this stage of purification.
Another consideration that became apparent at this time was the need for sterilization of the solutions. To reduce bacterial contamination the extracts were refrigerated under Saran Wrap plastic sheet as much as possible during the fractionation. The solutions were not turbid from bacterial growth, but an odor of putrefaction could be detected upon prolonged dialysis of some solutions. Fractionation either with organic solvents or with zinc plus alcohol controlled this contamination. These procedures also allowed a recovery of most of the enzyme. The zinc-alcohol procedure had the further advantage of decreasing the length of time in which the protein was in the alcoholic solution.

In the final purification procedure (Table 23), a 20 gm. % suspension of VioBin liver powder was extracted at pH 7 with double distilled water. Then, after 24 hours of extraction, some of the contaminating proteins were precipitated at their isoelectric pH by adjusting the pH to 4.8, and removed by centrifugation. Other contaminating proteins were precipitated with 31 gm. % ammonium sulfate and also removed by centrifugation. The enzyme was then precipitated at pH 7 by 35 gm. % ammonium sulfate. This precipitate was exhaustively dialyzed and precipitated with zinc acetate (6 mM/1) from a solution containing 6.5 mg./ml. protein and 20 (v/v) % ethyl alcohol. The final enzyme
solution obtained after dialysis at 4°C and treatment with Amberlite IRC 100 cation exchange resin was 160 times as active per mg. protein as the starting material. The yield, based on VioBin liver powder, was 9.8%. The final material had a light yellow color in solution. Lyophilization yielded a grey-brown powder.

Table 23
Method of Purification of Horse Liver Esterase

<table>
<thead>
<tr>
<th>Method</th>
<th>Specific Activity ($\times 10^{-3}$ meq./min./mg. protein)</th>
<th>Per Cent Recovery</th>
<th>Activity Increase Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>VioBin powder</td>
<td>0.098</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>20 gm. % aqueous extraction</td>
<td>0.89</td>
<td>42</td>
<td>9.1</td>
</tr>
<tr>
<td>Isoelectric precipitation at pH 4.8</td>
<td>1.7</td>
<td>39</td>
<td>17</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation between 31 and 35 gm.% salt</td>
<td>12</td>
<td>12</td>
<td>120</td>
</tr>
<tr>
<td>Zinc alcohol fractionation 6 mM/l, zinc acetate 6.5 mg./ml. protein</td>
<td>16</td>
<td>9.8</td>
<td>160</td>
</tr>
</tbody>
</table>
C. Properties

The emission spectra indicated traces of zinc and iron. On the basis of the size of the sample digested (20 mg.) and the faintness of the bands, it is believed that the zinc is an accidental contamination. Some of the iron may also be a contaminant, but some is probably due to hematin adsorbed on the protein surface, as shown by the adsorption spectrum.

There are two peaks in the adsorption spectrum: the peak at 275-280 μ due to adsorption by aromatic rings, and the peak at 405 μ due to hematin, according to Burch (9). This second peak may account for the iron observed in the emission spectrum, since it is in the region of the Soret band (400 μ) of hematin. No correlation between the enzyme activity and the adsorption at 405 μu was observed. Also, albumins separated from esterase by ammonium sulfate fractionation were found to exhibit this adsorption peak. This indicates that the adsorption is probably non-specific and is due rather to the nature of the protein itself.

Recently, Sober, et al (42) in fractionating human serum isolated a fraction containing serum esterase, which had an adsorption peak at 407 μ. The authors also report that the value obtained from the ratio of the extinction coefficients at 280 μ to that at 407 μ was about 10. A value of 7.4 was calculated from Burch's data (9) for the ratio of the extinction coefficients.
at curve lengths 280 m\(\mu\) to 405 m\(\mu\). This corresponds to a value of 43 obtained in this investigation. It would be interesting to know whether or not serum esterase and liver esterase are the same enzyme.

Another possibility is that this adsorption is a characteristic of globulins of this type. There might be some correlation between this adsorption and the unusually high extinction coefficient observed at 280 m\(\mu\). Since this adsorption is due to the presence of the aromatic amino acids it is possible that these structures aid in the adsorption of the hematin.

The sedimentation constant calculated at infinite dilution may be used, according to Greenberg (43), to calculate a minimum molecular weight. The expression given below is derived on the basis of an unsolvated sphere, which will sediment faster than any other mathematic model.

\[
M^2 = \frac{162 \pi^2 N^2 \eta^3 V^3 s^3}{(1 - V \rho)^3}
\]

In this expression "\(N\)" is Avogadro's number, "\(\eta\)" is the viscosity in poises, "\(V\)" is the partial specific volume, "\(s\)" is the sedimentation constant at infinite dilution, and "\(\rho\)" is the density of the solution. If a partial specific volume of 0.75 is assumed, which is similar to the value determined for a great many proteins, according to Neilands and Stumpf (44), then the expression for the minimum
molecular weight becomes 40,000. The expression

\[ M = \frac{RTs}{D(1 - V\rho)} \]

where "T" is the temperature in degrees Kelvin, may be used to calculate a molecular weight. If a diffusion constant of 7.0 is assumed, the molecular weight is 54,000. A diffusion constant of 7 was assumed because, according to Greenberg (43), human globulin has sedimentation constants and diffusion constants of 3.8 and 7.1, and the values for bovine \( \beta \) lactoglobulin are 3.2 and 7.3. This latter molecular weight is probably a better approximation because the enzyme is undoubtedly solvated.

Electrophoresis of the 5 gm. % enzyme solution yielded mobilities at pH 7.7 and 8.5. If these values and the mobilities according to Burch (9) and Conners, et al (10) are graphed versus pH (Figure 24), it may be seen that the isoelectric pH of the enzyme is approximately 5.4. This extrapolated curve is bent upward because this is the usual shape of horse serum protein curves, as reported by Abramson, et al (45). If 5.4 is the isoelectric pH, then it may be possible to remove protein contaminants by separatory electrophoresis in the neighborhood of this pH.

A comparison of the paper electrophoresis tracings of liver esterase and human serum shows that the enzyme migrated at a
Figure 24
Isoelectric pH from Electrophoretic Mobilities
rate less than that of the \( \alpha_1 \) globulin, but faster than that of the \( \alpha_2 \) globulin fraction. Paper electrophoresis also revealed a contaminating protein that migrated at the same rate as the albumin fraction. In both ultracentrifugation and Tiselius electrophoresis, globulin-like contamination had been observed, but this was the first observation of an albumin-like contamination.

From these results and a comparison of the composition determined by the measurement of the area under the schlieren curves and paper electrophoresis tracings, it is estimated that the solution contained from 75 to 80% of the enzyme and that the contaminants were similar to serum albumin and to \( \beta \) and \( \gamma \) globulin. Further purification could be obtained by sedimenting the globulin fraction (sedimentation constant approximately 7 according to Greenberg, 43) and then employing electrophoresis to separate the albumin fraction from the enzyme. On the basis of the width of the schlieren peaks, the protein which would remain would not be homogeneous. For several reasons it would be interesting to further investigate the degree of heterogeneity of the enzyme within the one peak. One of these reasons is the often repeated question of the possible existence of several esterases. It is within the realm of possibility that in place of one "non-specific liver esterase" there are several similar enzymes having varying
degrees of specificity. Kistiakowsky and Mangelsdorf (23) report that their kinetic data can be explained on the basis of two active centers per enzyme or of two independent enzymes. A second consideration, recently discussed by Ogsten (46), is the concept of whether or not all the particles in a biologically defined, and enzymatically defined, material are necessarily all of the same size and shape. The heterogeneity of the esterase that caused the broad peaks in the schlieren optical system may be inherent in the biological synthesis of this enzyme. An answer to this question would be of interest also in view of the known function of the liver in the synthesis of some of the serum proteins. If the heterogeneous protein can be separated into definite, although arbitrary, homogeneous fractions, then the catalytic function could be investigated more precisely than with a mixture of catalysts. From these considerations it may be seen that further purification and characterization of horse liver esterase would be very much desired.

The activity of the purified enzyme was investigated over the pH range 8 - 10 to determine whether or not the pH optimum reported by Conners, et al (10) would be observed or whether the broad plateau in activity from pH 9.5 to 11, reported by Kistiakowsky and Mangelsdorf (23) would be measured. In essential
agreement with Conners, et al (10), the enzyme was most active at pH 8.0, but the activity did not decrease with increasing pH nearly as rapidly as reported by these authors. Kistiakowsky and Mangelsdorf (23) accounted for this difference in behavior by their report of a decrease in enzyme activity when the enzyme was allowed to remain in an alkaline solution. In an attempt to prevent this inactivation in this investigation, the enzyme was not placed into the alkaline solution until the moment the reaction was started. No adequate explanation has been offered for this difference in experimental results.

The Michaelis-Menten constant determined in this investigation of 5.2 mM was greater than the values reported by Kistiakowsky and Mangelsdorf (23) of 2.4 mM, by Burch (9) of 2.32 mM, and of 3.14 mM by Schwert and Glaid (47), who employed an enzyme prepared by the method of Conners, et al (10). This value is lower than the 22 mM Km reported by Conners, et al (10), which was determined in a concentrated buffer solution.
Summary

Horse liver esterase was purified from a commercial dried liver preparation (VioBin liver powder). The purification procedure included: aqueous extraction of a 20 gm. % VioBin powder; isoelectric precipitation of some of the contaminating protein at pH 4.8; ammonium sulfate fractionation between 31 and 35 gm. % of the salt; and zinc-alcohol fractionation with 6 mM/l. zinc acetate and 20 (v/v) % ethyl alcohol. The enzyme prepared by this procedure was 160 times as active per mg. protein as the original starting material. The overall yield was 9.8%.

For assay of the enzyme, two different titrimetric procedures were employed. For rapid, routine analyses, a method was developed by which enzyme activity can be measured in less than 5 minutes. Duplicate determinations by this method usually agreed within 5%. A potentiometric method in which alkali was continually added to maintain a constant pH was employed for precise determinations of enzyme activity.

As shown by boundary spreading in the sedimentation and electrophoretic analyses, the purified preparation was a mixture of heterogeneous proteins that were very similar in both sedimentation constants and electrophoretic mobilities. The weight average sedimentation constant at infinite dilution was $3.9 \times 10^{-13}$ sec. Electrophoresis at pH 7.7 and 8.5 yielded
mobilities of 4.8 and 6.4 cm$^2$/sec./volt respectively. From this and data in the literature an isoelectric pH of approximately 5.4 was determined. From ultracentrifugation measurements, Tiselius and paper electrophoresis, the preparation consisted of 75 to 80% of one component. The optimum pH for enzyme activity was 8.0. The Michaelis-Menten constant for the esterase measured over a 40 fold range of enzyme concentration was 5.2 mM.
Bibliography


25. Fodor, P.J., Enzymologia, 14, 397 (1951).


34. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1922).


Autobiography

I, Edward Bradford Titchener, was born in Cambridge, Massachusetts, July 15, 1927. I received my secondary school education in the public schools of Columbus, Ohio. The first year of my undergraduate training was obtained at Ohio State University. Following eighteen months in the Army, I completed my undergraduate work at the University of Michigan, from which I received the degree Bachelor of Science in 1951. From Ohio State University, I received the degree Master of Science in Physiological Chemistry in 1954. I have been employed by the Department of Physiological Chemistry during the years 1953 - 1955 as a laboratory assistant and by the Department of Medicine during the year 1955-1956 as a research assistant.