1958

The Detection and Determination of Esters.

Mohd. Mohsin Qureshi
Louisiana State University and Agricultural & Mechanical College

THE DETECTION AND DETERMINATION OF ESTERS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by
Mohd. Mohsin Qureshi
M.Sc., Aligarh University, 1944
August, 1958

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation and gratitude to Dr. Philip W. West under whose guidance this research was carried out.

He is grateful to Dr. James G. Traynham for supplying him with a number of esters and for his many helpful suggestions. The financial support given to him by the Continental Oil Company is gratefully acknowledged.

He offers his sincere thanks to Miss Magdalena Usategui for her valuable suggestions and her ungrudging help during the course of this investigation. Dr. Anil K. Mukerjee and Dr. Bharat R. Sant were very helpful in the latter part of this work, for which the author thanks them very much.

ABSTRACT

An enzymatic method for the detection of esters has been developed. The use of various esterases as an analytical tool has been examined. It has been found that lipase can be used for the detection of esters under specified conditions.

Ion exchange resins have been utilized for the quantitative determination of esters in microgram quantities. The method gives satisfactory results in the analysis of dilute solutions.

It has been found possible to titrate solutions of molybdate and tungstate as dilute as 0.00025M by the use of high frequency titrations employing solutions of lead salts as the titrant.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

PART I

DEVELOPMENT OF A GENERAL CLASS TEST FOR ESTERS

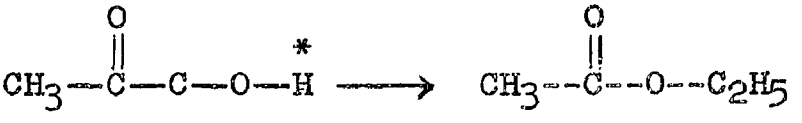
Chapter I

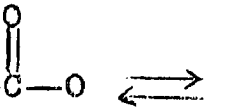
A Review of the Different Methods for the Detection of Esters

A. Introduction

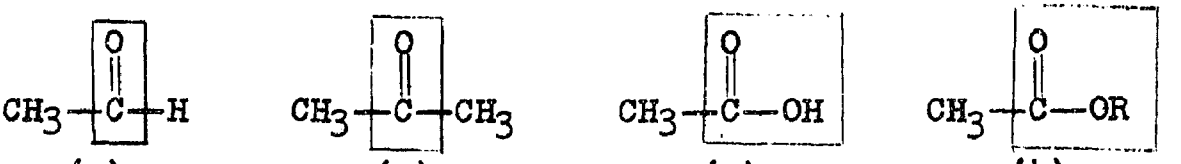
Esters form one of the most important classes of organic compounds. They occur widely in nature and are intimately associated with innumerable modern industries. They find widespread use as flavoring agents, as solvents and in perfumes. Despite their importance, very few methods for the detection of esters, as a class, are known.

In the widest sense, an ester is defined as a compound derived from an acid--defined in the Arrhenius sense --by replacing its active hydrogen atoms by organic radicals. These organic radicals may be aliphatic, aromatic, alicyclic or heterocyclic in nature. In a limited sense, the word ester is applied to the derivatives of organic acids only.



Here the reactive hydrogen atom of the acid marked by an asterisk has been replaced by a relatively inert ethyl group. The functional group of esters is a resonance stabilized  group in which the carbonyl group has lost much of its reactive properties.

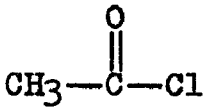
In order to appreciate fully the difficulties involved in the detection of esters, let us concentrate our attention on four classes of organic compounds represented by formulae (1-4).



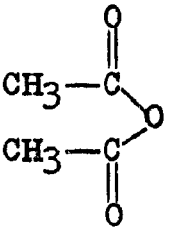
(1) (2) (3) (4)

In the class of organic compounds represented by formula (1), the reactivity of the carbonyl group is enhanced by the presence of a reactive hydrogen atom and by the absence of resonance. Such compounds enter into a number of chemical reactions and are very easy to detect. In compounds of class (2) the hydrogen atom is no longer present but the absence of resonance maintains the reactive character of the carbonyl group. In class (3) compounds, the presence of perfect resonance suppresses the reactivity of the carbonyl group but at the same time helps in the ionization of a proton which offers a ready means of detection. When we consider the compounds represented by (4), we notice two important things. The characteristic group in this class is stabilized owing to resonance between the carbon atom of the carbonyl group and the two oxygen atoms, and we no longer have the easily ionizable hydrogen atom. It is, therefore, not surprising that the detection of esters forms one of the more difficult tasks in qualitative

organic analysis. The difficulties in detection are further increased by the existence of other closely related substances such as acid halides and anhydrides given by the formulae (5) and (6).



(5)



(6)

From what has been said it is clear that there are two general courses open for the detection of esters. Either we may hydrolyze the ester into the acid and the alcohol and regard the hydrolysis as a test for the presence of an ester, or we may choose such conditions and reagents that the carbonyl group only of the ester is affected. The latter course is the more difficult owing to the reasons set forth above. It is, therefore, no wonder that the classical method of detection has depended upon hydrolysis and that new approaches have been extremely rare.

B. Various Methods for the Detection of Esters

For special purposes, special methods have been devised for the detection of esters. It is neither

necessary nor practicable to enumerate all the methods used. A selection of such methods will be given to help in understanding the different possible approaches. Lanthanum has been used as a specific reagent for the detection of acetate (50, 59). Formates can be converted to formic acid which on reduction gives formaldehyde. The methods for formaldehyde are then applicable (31, 39, 40, 62). The esters of aromatic acids can be saponified and the acids then detected by testing for functional groups other than the carboxylic group. If such a group is not already present, it can be introduced into the molecule. Thus benzoic acid is nitrated, reduced, diazotized and coupled with N-(1-naphthyl) ethylenediamine hydrochloride (27). In a mixture of hydrocarbons, esters of glycerol may be detected by the acrolein test for glycerine (29). Esters of dicarboxylic acids are detected by melting with resorcinol (34). Infrared spectra have also been used for the qualitative detection of organic compounds (6, 10).

Methods Based on Hydrolysis

The hydrolysis of esters is catalyzed by acids as well as by alkalies. The alkaline hydrolysis is preferred because then the reaction goes to completion. For the successful application of this method it is necessary that the classification tests are applied in proper sequence, otherwise the conclusions may be erroneous. To take an

example, benzaldehyde and benzylbenzoate would give the same end products on treatment with NaOH.

$$C_6H_5CHO + 2NaOH \longrightarrow C_6H_5COONa + C_6H_5CH_2OH$$

$$C_6H_5CH_2OOCC_6H_5 + NaOH \longrightarrow C_6H_5COONa + C_6H_5CH_2OH$$

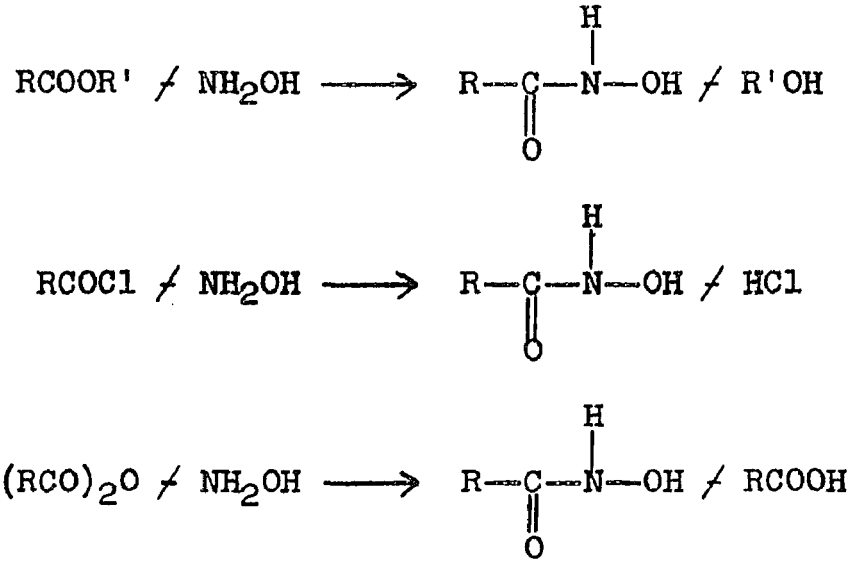
Not all esters hydrolyze easily and modifications have been introduced to remedy this defect (77). In some cases an acid is preferable for the hydrolysis of the ester.

Sometimes the alcohol produced by hydrolysis is oxidized and the product of oxidation is detected. The limit of detection of the ester then depends upon the limit of detection of the aldehydes so formed.

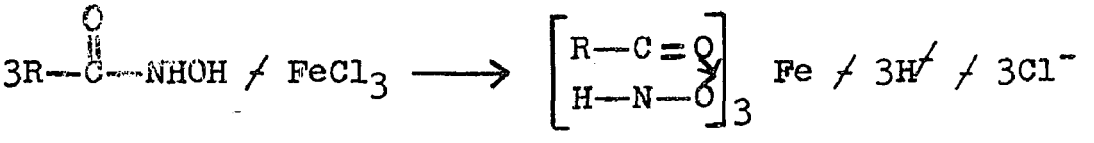
The Hydroxamic Acid Test

The introduction of the hydroxamic acid test has been of great practical utility in the detection of esters. The method was first developed by Feigl in 1934 (30, 35). The general adoption of the test was facilitated by Davidson's article in the Journal of Chemical Education (21). The test was carefully evaluated and three useful modifications of the test were introduced by Buckles and Thelen (16). Recently a method has been developed for the detection of esters on Florisil utilizing this test (73).

The test is based on the well known reaction of hydroxylamine with derivatives of carboxylic acid.



Hydroxamic acid produced by these reactions can be detected by adding acidified ferric chloride. A deep magenta color is produced as a result of this reaction. The reaction may be formulated as follows:



In the following two tables are summarized the scope and limitations of the hydroxamic acid test and its three modifications A, B and C.

TABLE I

Limitations and Scope

of the Hydroxamic Acid Test

Compounds	Test A	Test B	Test C
Most Esters and Lactones	Yellow	Deep Magenta	Yellow
Hydrogenated cottonseed oil, glyptal resin, polymethyl methyl acrylate	Yellow	Medium magenta	Yellow
Isobutyl acid phthalate and sec. octyl acid phthalate	Yellow	Weak tests	Yellow
Oxalates or methyl acetate with oxalic acid	Yellow	Require more $FeCl_3$	Yellow
Et.CO_3, Et. carbamate, Et. chloroformate, Me-p-toluene-sulfonate, Me_2SO_4, Et_2SO_4, $EtNO_3$, Bu_3PO_4	Not mentioned	Negative tests	Not mentioned
CCl_3CHO, benzotrichloride Compds. with $CCl_3C(OH)$- as $CCl_3COH(CH_3)_2$ give false tests*	Not mentioned	Deep magenta	Not mentioned
$CHCl_3$, $CHBr_3$, CBr_4, $CBr.Cl_3$, CCl_3COOH	Not mentioned	Medium magenta	Not mentioned
Acetic, succinic, maleic, phthalic and 3-nitrophthalic anhydride	Not mentioned	Deep magenta	Deep magenta

* David Davidson, J. Chem. Ed., 17, 81 (1940).

Compounds	Test A	Test B	Test C
$CHCl_3$, $CHBr_3$, CBr_4, $CBrCl_3$, CCl_3COOH	Not mentioned	Medium magenta	Not mentioned
Acetic, succinic, maleic, phthalic and 3-nitrophthalic anhydrides	Not mentioned	Deep magenta	Deep magenta
Benzoyl and p-phenyl-benzoyl chlorides	Not mentioned	Deep magenta	Deep magenta
Formic and phthalic acids	Not mentioned	Deep magenta	Deep magenta
Lactic acid	Negative	Deep magenta	Negative
HCHO, ØCHO, p-hydroxybenzal-dehyde, m-nitrobenzaldehyde, vanillin, anisaldehyde	Not clear	Weakly positive tests	Not clear
Formamide	Not mentioned	Deep magenta	Not mentioned
Acetamide, formanilide, benza-mide, diacetylhydrazine, phthalhydrazide	Not mentioned	Medium magenta	Not mentioned
Diacetamide, succinimide phthalimide, nitrophthalimide	Not mentioned	Deep magenta	Not mentioned
Acetonitrile and propionitrile	Not mentioned	Medium magenta	Not mentioned
Isocyanates	Not mentioned	Weak or negative	Medium magenta
Nitromethane, 2-Nitropropane, 2-nitro-1-butanol, 2-Nitro-1-chlorobutane		Gave deep red solu-tion even without hydroxylamine	

TABLE II

Modifications A, B and C

of the Hydroxamic Acid Test

Test	
A	Dissolve a drop or a few crystals of the compound to be tested in one ml. of 95% alcohol and add one ml. of one N hydrochloric acid. Note the color produced when one drop of 10% ferric chloride is added to the solution.
B	Mix one drop or several crystals of the compound to be tested with one ml. of .5 M hydroxylamine hydrochloride in 95% ethyl alcohol. Add .2 ml. of 6 N aqueous sodium hydroxide, heat the mixture to boiling, and after the solution has cooled slightly, add 2 ml. of one N HCl. If the solution is cloudy, add 2 ml. of 95% ethyl alcohol. Observe the color produced when one ml. of 10% ferric chloride solution has been added. If the color formed by the drop of ferric chloride solution does not remain when it is mixed into the test solution, keep adding the reagent dropwise until the observed color pervades the entire test solution. Usually only one drop of the reagent solution is necessary. Compare the color produced by Test A. A positive test will be a distinct burgundy or magenta color as compared with the yellow observed when the original compound is tested with ferric chloride in the presence

Test	
B (con't)	of acid. It is best to observe the color of the test solution within 5 minutes after adding the ferric chloride solution.
C	If Test B is positive, repeat it without adding sodium hydroxide and with only one ml. of the hydrochloric acid for acidification.

A positive test for esters is obtained when we obtain a deep magenta color in Test B and do not get this color in Test C. Thus, two tests have to be run. The test is given only with esters of carboxylic acids. A positive test is also given by most aldehydes and such common substances as chloroform and chloral hydrate. Common nitrogen compounds such as acetamide and benzamide also give positive tests. It will thus be apparent that though the test is useful its utility is severely restricted by the many limitations imposed upon it. However, it is often useful as a means of avoiding lengthy saponification tests. The hydroxamic test has been dealt with at length partly because it is the best qualitative test for esters (19) and partly because many standard text books omit any mention of this test (65, 69, 88, 89).

Esters have long been used for the qualitative and quantitative determination of the activities of esterases. For this purpose, phenyl esters (112), nitrophenyl esters (2) and esters of salicylic acid have been widely used. Phenol is determined colorimetrically after diazotization while salicylic acid is detected and determined with ferric chloride (64) or by spectrophotometric methods (49). A beautiful spot test has been described by Sastri and Sreenivasya (83): A drop of the substrate and a drop of the test liquid are mixed on a strip of fat-free filter paper. The paper is left (together with necessary controls)

for 30 to 60 minutes in a moist atmosphere under a bell jar. Methyl red is used as the indicator. A red color indicates the presence of the enzyme. A cup plate method has also been described for the detection of the activity of various enzymes (18).

In view of the considerations set out above, it was considered desirable to develop another class test for esters, which may not be as long and tedious as the saponification test and may not suffer the same limitations as the hydroxamic acid test. Since enzymes are known to be more or less specific in their actions and since no effort has been recorded to use the enzymes for the analytical detection of esters, it was considered fruitful to look into this approach. The present work was undertaken to find the scope and limitations of the utilization of enzymes as an analytical tool for the detection of carboxylic esters as a class.

Chapter II

Enzymatic Detection of Esters

A. Enzymes and Their Properties

Enzymes are biological catalysts which speed up chemical reactions. They are much more effective than inorganic catalysts and their activity is proportional to the concentration of the enzyme. Most enzyme catalyzed reactions do not occur at an appreciable rate in the absence of the enzyme so that a plot of rate versus enzyme concentration is a straight line passing through the origin.

Enzymes are largely protein in nature although some of the enzymes have a non-protein part also. The structural unit of a protein is a long chain arising from the condensation of a number of amino acid residues. The simplest amino acid is glycine $NH_2CH_2 \cdot COOH$. A protein formed from glycine will have the structure

$$—NH \cdot CH_2 \cdot CO—NH \cdot CH_2 \cdot CO—NH \cdot CH_2 \cdot CO—.$$

The —NH·CO— linkage characteristic of proteins is known as the peptide linkage. The naturally occurring proteins largely consist of such chains made up of α-amino acids which are derivatives of glycine of the general formula

$$NH_2—\underset{\displaystyle R}{\underset{|}{CH}}—COOH.$$

There are usually a number of different amino acids within a single protein molecule. Not much is known about the order in which the acids are arranged in such molecules nor is the configuration taken up by the chain known.

Many of the characteristic properties of proteins and therefore of enzymes arise from the very large sizes of the molecules. Other characteristic properties of enzymes are due to their electrical nature. Proteins bear a number of positive and negative charges which arise from the presence of carboxyl (—COOH), amino ($—NH_2$) and other groups in the molecule. In fairly neutral solutions, these groups are in the ionized state so that a protein molecule may contain a number of positive and negative charges. These charges tend to bind the ions that are added to a solution of the protein, and enzymatic action is frequently related to such binding of ions. In acidic solutions, the ionization of the —COOH group is suppressed while the amino group is in the form of $—NH_3^+$. The protein is therefore positively charged.

Similarly, in basic solutions the proteins will be negatively charged owing to the formation of $—COO^-$ groups. At some intermediate pH--known as the isoelectric point characteristic of each protein--the number of positive and negative charges on the molecule will be equal and the molecule will have no net charge.

When an enzyme is made up of a protein and a non-protein part, these two parts are known respectively as apoenzyme and prosthetic groups. When the prosthetic group is readily separated from the apoenzyme, it is known as coenzyme. In some cases it is found that certain ions are attached to a protein. If these ions are removed, the enzyme loses its activity. Such ions are called activators.

Enzymes are also characterized by the specificity which they exhibit towards their substrates. When an enzyme will catalyze the reaction with only one compound, we speak of absolute specificity. A group specificity is shown by the enzyme when it acts on a number of compounds having a particular required grouping. Stereochemical specificity results when the enzyme acts upon only one stereochemical form. The lowest degree of specificity is shown when the enzyme acts upon a certain type of linkage irrespective of the environments of the linkage. For instance, lipases hydrolyze any type of carboxylic ester irrespective of the acid or the alcohol involved. The rates of reaction, however, differ with different types of esters.

A rate may be expressed either in terms of a decrease in the concentration of the reactant or an increase in the concentration of the product. The important factors on which the rate of enzyme reactions depends are:

1. The pH of the solution.
2. The substrate concentration.
3. The temperature.
4. The concentrations of any inhibitors present.

Influence of pH

In the majority of cases the rate is found to pass through a maximum as the pH is varied and a curve of the type shown in Figure 1 results. If the solution is taken too far towards the basic or acidic side away from the optimum pH, the enzyme loses its activity. However, within a fairly narrow optimum the pH can be changed back and forth and no permanent effects are obtained. One of the explanations for the type of pH curve shown in Figure 1 may be that the active enzyme has the form $NH_3^+ \sim COO^-$. The result of addition of acid or base to this active enzyme may then be depicted as follows:

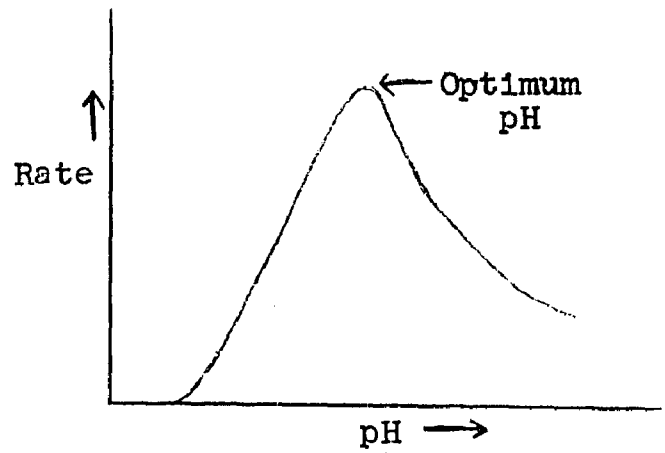


Figure 1
Denaturation

$$NH_2 \sim\!\!\sim COO^- \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} NH_3^+ \sim\!\!\sim COO^- \underset{OH^-}{\overset{H^+}{\rightleftharpoons}} NH_3^+ \sim\!\!\sim COOH$$

more basic Inactive — Active — more acidic Inactive

This idea that the active form of the enzyme is an acid-base pair in the protein has been useful in understanding other aspects of enzyme mechanism also as we shall see a little later.

Influence of Substrate Concentration

The most common relationship which exists between the substrate concentration and rate of enzyme reaction is given in Figure 2. At very low substrate concentrations the rate increases linearly with substrate concentration, i.e., the kinetics are first order. At high substrate concentrations the rate becomes independent of substrate concentration, the reaction being of zero order. When this type of behavior is observed, the curve can usually be fitted to an equation of the form

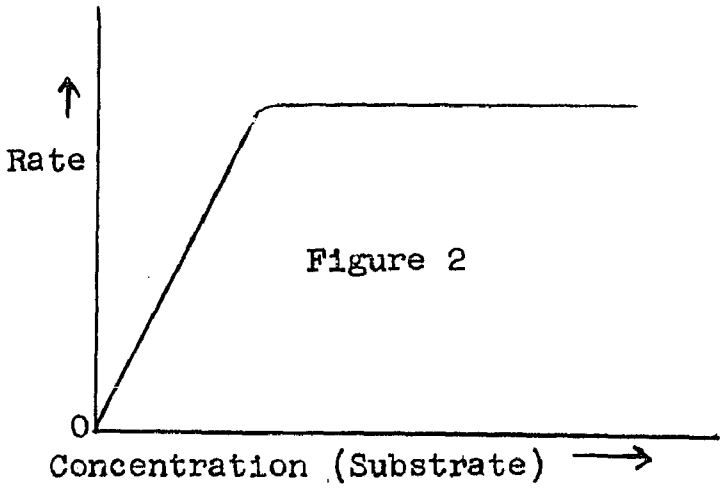


Figure 2

$$v = \frac{a[S]}{1 + h[S]}$$

where a and h are constants. [S] represents the concentration of the substrate in moles per liter and v is the rate. This type of behavior was first interpreted theoretically by Michaelis and Menten (28). The essence of their theory

is that the enzyme reaction proceeds in two stages:

$$E + S \rightleftharpoons X \qquad (1)$$

The first stage represents the formation of a complex between enzyme and substrate.

$$X \rightleftharpoons P + E \qquad (2)$$

The second stage is the decomposition of the complex to give the enzyme and the products. When the substrate concentration is very low the concentration of the complex is directly proportional to the concentration of the substrate. However, when all the enzyme is tied up with the substrate to form a complex, then any increase in the concentration of the substrate will not affect the rate of enzyme reaction.

The Michaelis-Menten law can be derived rather simply. The equilibrium equation for reaction (1) is

$$\frac{[X]}{[E][S]} = K \qquad (2)$$

Total concentration of enzyme $[E_o] = [E] + [X]$ where $[E]$ represents the concentration of the free enzyme. Equation (2) then becomes

$$\frac{[E]}{(E_o - [X])\,[S]} = K \quad \text{or} \quad [X] = K\,[S]\,[E_o] - K\,[X]\,[S]$$

$$\text{or} \quad [X] + K\,[X]\,[S] = K\,[S]\,[E_o]$$

$$\text{or} \quad [X]\,(1 + K\,[S]) = K\,[S]\,[E_o]$$

$$\text{or} \quad [X] = \frac{K\,[S]\,[E_o]}{1 + K\,[S]}$$

However, the rate of formation of products is proportional to $[X]$; i.e.,

$$v = K_2\,[X]$$

$$= \frac{K_2\,K\,[S]\,[E_o]}{1 + K\,[S]} = \frac{a\,[S]}{1 + h\,[S]}$$

The Effect of Inhibitors and of Temperature

A word may also be said about the effect of inhibitors. Inhibitors are of two types: competitive and non-competitive. Competitive inhibitors are allied to the substrate in structure. They, therefore, compete with the substrate for the active sites on the enzymes. Therefore the degree of inhibition brought out by a certain concentration of the inhibitor decreases with an increase in the concentration of the substrate. In the case of non-competitive inhibitor the degree of inhibition is not concentration dependent.

As in the case of pH, the rate versus temperature curve also shows an optimum value. However, the study of temperature effect is complicated by the fact that at

higher temperatures inactivation starts coming into play. If proper account is taken of inactivation and the reaction is studied within a small range of temperature, the famous law of Arrhenius has been found to apply almost invariably

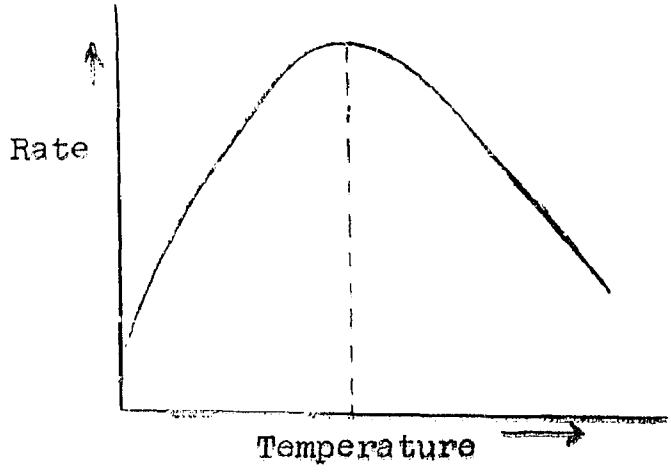


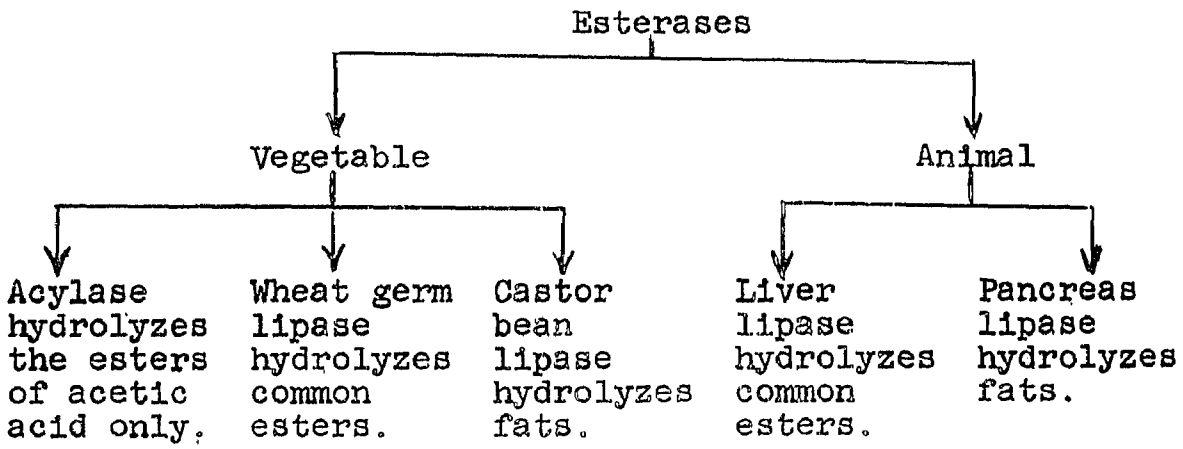
Figure 3
Effect of Temperature on Activity

$$K = A \cdot e^{-E/RT}$$

where K is the rate constant and R is the gas constant.

B. Esterases and Their Characteristics

As is apparent from the name, the esterases hydrolyze esters in general. They hydrolyze fats, common esters and esters of sulfuric and phosphoric acids. We shall, however, be concerned more with the enzymes which hydrolyze the esters of carboxylic acids. It is possible to classify the esterases either according to the source of their origin or according to the esters which they hydrolyze. A useful classification from our standpoint is given here.



Lipases

The term lipase is used to include those esterases which hydrolyze fats better than the common esters. They are enzymes of low specificity in the sense that they hydrolyze to a significant extent the esters of acids which are not true fatty acids and alcohols other than glycerol. Such esters we have included under the name "common esters." Lipases are generally found in pancreas and in plants.

Esterases Proper or Aliesterases

These enzymes hydrolyze the common esters much better than the fats. It does not mean that they hydrolyze every common ester; but in general they would hydrolyze methyl butyrate, for instance, much better than glyceryl stearate. It was found that one gram pancreas powder corresponds to the activity of 10,600 grams liver powder in the hydrolysis of olive oil, to the activity of 100 grams liver powder in hydrolysis of tributyrin and to the activity of 4 grams in the hydrolysis of methyl

butyrate. Richter and Croft proposed the name Aliesterases for these esterases which hydrolyze simple aliphatic esters and glycerides. Some of the more common esterases are the following: 1. steapsin, 2. wheat germ lipase, 3. liver lipase, 4. glandular lipase. We shall discuss the first three in some detail.

1. Steapsin

Steapsin, the commercial name given to pancreas lipase, hydrolyzes fats much faster than other esters. Many of the studies made on this lipase have been done on oils and fats. Since the enzyme is commercially available, details will not be given concerning its preparation. It may be prepared by the method of Willstatter and coworkers (9, 106, 107, 113). It is purified by adsorption on aluminum hydroxide (105) or Kaolin (114).

a. Optimum conditions

The pH optima of the esterases lie between 5 and 9 (59). In the case of pancreatic lipase it is about 8. The optimum conditions of temperature and pH depend on the substrate, state of purity of enzyme, buffers, methods of assay, etc. In the case of pancreatic lipase, the optimum temperature for most substrates is about 37°.

The optimum pH for the hydrolysis of lower triglycerides is around pH = 7. For the higher triglycerides it is shifted to pH = 8.8. However, for the hydrolysis of acid esters of dicarboxylic acids, such as succinic and

malonic esters, hydrolysis takes place only in acidic media around pH = 5. The optima for lipases of other animal tissues are listed by Ammon (3).

b. Activators

Albumin and calcium ions activate the action of lipase (104). The activating influence of $CaCl_2$ was investigated by Schonheyder and Volqvartz (84).

Calcium salts increase the activity of the enzyme in homogeneous as well as in heterogeneous systems on both sides of the optimum pH. Whereby on the acid side, the range of the hydrolytic activity was found to be broadened by one pH unit (84). This effect can be ascribed in part to the precipitation of calcium salts of inhibiting higher fatty acids, but the same mechanism cannot account for the activation of the hydrolysis of lower fatty acids. It appears more probable that this is a case of specific action of calcium ions on the enzyme itself similar to the now well established mechanism in the case of trypsin (13).

Salts of bile acids also activate the action of lipase. The findings of Wills (103) strongly suggest that the sole function of bile salts is confined to substrate emulsification and is not involved in an activation mechanism. According to Fodor (32), the role of bile salts in activating the hydrolysis of esters by pancreatic lipase is not a specific one but, rather, involves changes in surface tension at the ester-water interface subsequent

to ester hydrolysis. Emulsification of the ester by bile salts may result in increased or decreased ester hydrolysis.

The activation of the lipase effect of the pancreatic enzyme by amino acids is a striking phenomenon (25, 104a). The L form of Leucylglycylglycine exerts the greatest influence on pancreatic lipase (36). It was shown by Dawson (115) that amino acids accelerate the hydrolysis in alkaline or neutral media but not in acidic solutions of ethyl butyrate and olive oil by pancreatic lipase. The effect of temperature on lipase activity has been determined by Schwartz (86) and Sizer (93).

Yamamoto studied the effect of a number of amino acids on the action of lipase. He found that di- and tricarboxylic acids such as succinic, malic and citric acids also show activating effects similar to some amino acids. He favors the view that these activators act by regenerating active lipase from inactivated enzyme rather than by affording protection against alkaline inactivation (108).

c. Specificity

It has been established that the hydrolyzability of glycerides by lipase increases with the number of fatty acid residues on the glyceride and with the chain length of the fatty acid. Unsaturated fatty acids of greater chain length increase the hydrolyzability of the glyceride (85). Balls and Matlack (5) investigated the

hydrolysis of stearic acid esters by pancreatic lipase; the various alcohols caused appreciable differences in the hydrolysis. Figure 4 summarizes the results of their experiments. Lactic acid esters and mandelic acid esters are among the hydroxy acids which are hydrolyzed well.

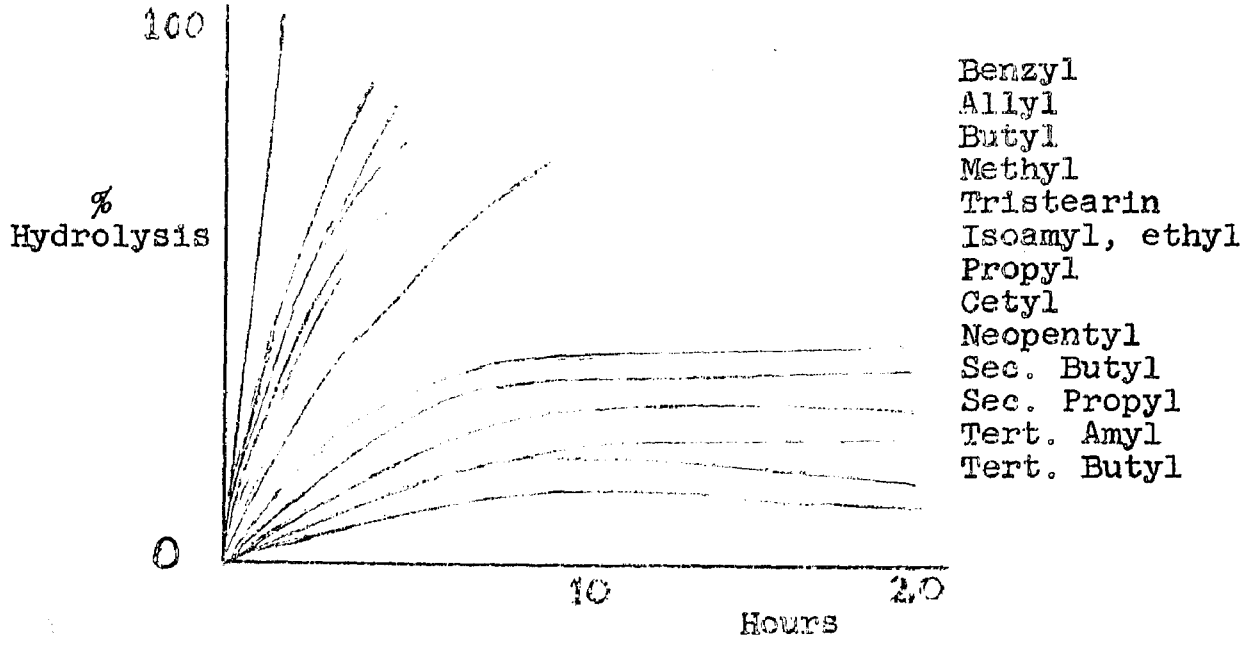


Figure 4
Hydrolysis of Stearic Acid Esters by Pancreatic Lipase

Only one alcohol residue is generally hydrolyzed off from the esters of dibasic acids. Thus the monoesters of oxalic, succinic, phthalic and fumaric acids are not considered hydrolyzable (52). Among the higher members of the series, the diesters as well as the monoesters are saponified. At pH values of 5.0 or lower, the monoesters of succinic and malonic acids can also be hydrolyzed (53).

Mattson and Beck (64) have shown conclusively that under their conditions hydrolysis proceeds stepwise from

triglyceride to 1,2-diglyceride and then to 2-monoglyceride, and then the action of pancreatic lipase ceases. According to Baaman and Rendlen (6) the reason substrates like succinic acid monoester are so very slowly hydrolyzed by esterase may be sought for in the presence of the electrically charged carboxylate groups in the molecule.

It has been shown that esterases do not hydrolyze the lactones (7, 96). If a lactone is added to an ester then the hydrolysis of the ester is inhibited, showing thereby that the lactones combine with the enzyme as such. Coumarin and santonin behave likewise. The behavior of lactides and other ester-like anhydrides is irregular (96). Urethans which may be considered as esters of carbamic acid are not hydrolyzed by esterases (95).

2. Wheat Germ Lipase

Wheat germ lipase has not been studied as thoroughly as pancreatic lipase. Singer has given a method for the preparation of this lipase (90). Singer and Hofstee tried the esters, methyl, ethyl, propyl, butyl acetates and ethyl formate. They found that calcium chloride did not affect the activity of the enzyme (91). Zero order reactions with simple esters as methyl, ethyl and propyl acetates and ethylene glycol diacetates were observed. At 38°C. the pH of the optimum stability of the enzyme was 6.8 and the apparent pH optimum was at 7.4 (92). Gauron, et at., (35a) have studied the effect of

substituents on the hydrolysis of phenyl acetate by wheat germ lipase. The result obtained with substituents of varying electronegativity is not in disagreement with their idea that the induced variation in the electrophilic character of the carbonyl carbon of the substrate can influence the strength of the enzyme-substrate complex and the rate of hydrolysis. They have presented a simple exposition of what is commonly regarded as a reasonable mechanism.

3. Liver Esterase

Liver esterase is in the strict sense not a lipase but an esterase proper. It is present in most livers. It may be isolated rapidly and quantitatively from fresh liver by allowing the minced brei to autolyze for several days in an alkaline medium (8). Safwat Mohamed (81) claims to have crystallized it from horse liver. A good method for the preparation of this esterase has been given recently (20) although the method is not applicable to beef liver which has a much lower esterase activity than horse liver (51). The optimum pH is 8.0 and can be established using a borate buffer. The action of liver esterase from sheep is inhibited by an excess of the ester (66, 71). Alcohols also inhibit the action of the liver lipase (55). It is not appreciably affected by ascorbic acid (68).

C. Experimental

The following section outlines the experimental approach used and the general conclusions obtained during the studies of the enzymatic detection of esters.

Preparation of Reagents

1. Lipase Solution

Pancreatic lipase (steapsin) is weighed and the necessary amount of distilled water is added to make a 10 per cent solution. The contents are thoroughly stirred with a glass rod until a homogeneous emulsion is formed. A drop of toluene is added and the emulsion is again thoroughly shaken. The lipase solution lasts for 24 hours even if it is kept at the ordinary temperatures (approximately 26°C.).

2. Methyl Red

Methyl red is prepared according to the method given in Kolthoff and Sandell (55a).

3. Ammonium Hydroxide

The ammonium hydroxide solutions need not be accurate. They are prepared by calculating the normality on the basis of the data given on the bottle of ammonium hydroxide. By successive dilutions the various dilute solutions are made.

Method

Three drops of the lipase solution are added to 3 drops of the neutral substance to be tested. A drop of methyl red is added. If the solution becomes pink, it is made yellow again by the addition of the least possible quantity of ammonium hydroxide. The appropriate amount can be found by proceeding systematically from ammonium hydroxide of higher concentration to that of lower concentration. The intensity of the pink color also gives a clue in this case.

If more than 3 drops of N/10 NH_4OH are required to make the original substance (3 drops) neutral to methyl red, then the substance is either not an ester or it has been highly hydrolyzed. It is most probably an acid, an anhydride or an acid halide.

After neutralization has been done the contents are shaken with hand vigorously for 3 minutes. A change of color from yellow to light orange or pink indicates the presence of an ester. Another drop of indicator is added if the change in color is not clearly visible. It is not always necessary but it is sometimes preferable owing to the fact that the indicator is preferentially soluble in the organic phase. However, it is always better to check by adding another drop of indicator.

Interferences

1. Acids, Anhydrides, Acid Halides

As has already been indicated, the acids, anhydrides and acid halides also give this test. However, their presence is easily recognized by the fact that they need more than 3 drops of N/10 NH_4OH for neutralization and by the fact that the pink color appears very fast. A little practice with known substances helps very much in understanding the difference in the behavior. Another important distinction is that they will give a pink color with methyl red even without the use of lipase.

2. Nitro and Bromo Compounds

Nitroethane, nitrobenzene and bromobenzene also give this test. In such a case it is useful to take 20% emulsion of the unknown in water and to repeat the test with lipase. A positive test is obtained in the case of an ester while all the three above mentioned compounds give a negative test.

3. Benzaldehyde

Benzaldehyde owing to rapid autoxidation also gives a positive test.

4. Esters of Secondary and Tertiary Alcohols

As the branching of the alcoholic part of the ester increases, the hydrolysis of the ester decreases and the test becomes less sensitive. However, simple esters of secondary alcohols as isopropyl acetate and the esters of

dibasic acids of secondary alcohols as isopropyl phthalate give a positive test with lipase.

5. Benzyl Benzoate, Ethyl Cinnamate and Ethyl Salicylate

Among the common esters, these three esters did not give a positive test with lipase. Methyl and ethyl benzoates are easily hydrolyzed but not benzyl benzoate, while cinnamates and salicylates do not appear to respond to this test.

D. Detection of Esters in Pure Form

The experiments described in the following sections were carried out to evaluate the scope and limitations of the enzymatic method for the detection of esters. In the selection of substances for the study of interferences, two points were kept in view: (1) preference was shown to substances which are more commonly encountered in qualitative organic analysis and (2) as far as possible representatives of all the common types of organic substances were included.

Nitrobenzene--owing to its common occurrence and its especially troublesome nature--was more thoroughly studied. The technique developed in this case was also useful in dealing with other interferences. Unless otherwise stated, the procedure used in these two sections is the one described in Section C, Chapter II. The substances

used were either commercial samples or the more highly purified substances available in the market. Unless otherwise stated, no effort was made to purify them further.

The following abbreviations have been frequently used.

E	for	Ethyl acetate (pure)
L	for	10% solution of steapsin in distilled water
D.W.	for	Distilled water
I	for	Methyl red. If the amount of the indicator used is not specified, then "I" refers to one drop of the indicator.

Any other abbreviations used are mentioned in the experiments concerned. Thus if the letter "B" appears after benzene in the form "Benzene (B)," it means that "B" has been used in that experiment as a symbol for benzene.

Experiment No. 1

The Ester Test With Pure Acetone, Ethyl Alcohol, Ethyl Acetate and Acetic Acid

	Ethyl Alcohol	Acetone	Ethyl Acetate	Lipase Solution	Acetic Acid
Initial Color	Yellow	Yellow	Yellow	Yellow	Yellow
After 3 Minutes	Yellow	Yellow	Orange	Yellow	Yellow

Method

The pure substances were first made neutral to methyl red. Then to 3 drops of the neutral substance, 3 drops of 10% lipase were added. A drop of methyl red was then added; and if on shaking an orange or pink color developed, it was changed to yellow by the addition of the least amount of most dilute ammonium hydroxide necessary.

Conclusions

Acetone, ethyl alcohol and acetic acid do not give a positive ester test.

Experiment No. 2

Test for Esters in Dilute Solutions

	3 Drops of L + 3 Drops of D.W. + I	3 Drops of L + 3 Drops of 10% E + I	3 Drops of L + 3 Drops of 10% Alcohol + I
Initial Color	Yellow	Yellow	Yellow
Color After 12 Minutes	Yellow	Orange	Yellow
Color After 30 Minutes	Yellow	Reddish Orange	Yellow
Color After One Hour	Yellow	Red	Yellow

Method

The slightly acidic (methyl red) lipase solution was mixed with the other substances together with the indicator and ammonium hydroxide was added drop by drop until each solution turned yellow and did not change color upon thorough shaking.

Conclusions

A 10% solution of ester requires about 12 minutes to develop a positive test. A 10% alcohol solution does not give a positive test.

Experiment No. 3

The Ester Test With Acetaldehyde (A) and Acetone

	5 Drops of 10% E, 5 Drops of L / I	5 Drops of D. W., 5 Drops of L / I	5 Drops of 10% A, 5 Drops of L / I	5 Drops of 10% Acetone, 5 Drops of L / I
Initial Color	Yellow	Yellow	Yellow	Yellow
Color After 30 Minutes	Orange	Yellow	Yellow	Yellow
Color After One Hour	Reddish Orange	Yellow	Yellow	Yellow

Preparation of Acetaldehyde Solution

One ml. of acetaldehyde was dissolved in distilled water and the solution made up to 10 ml. A drop of methyl red was added and ammonium hydroxide drop by drop until a yellow color was obtained.

Conclusions

Ten percent acetaldehyde and 10% acetone after neutralization do not give the ester test.

Experiment No. 4

The Ester Test With Acetamide, Ether and Glycerine (G)

	5 Drops of 10% E / 5 Drops of L / 1 Drop of I	5 Drops of 10% Acetamide / 5 Drops of L / 1 Drop of I	5 Drops of D. W. / 5 Drops of L / 1 Drop of I	5 Drops of Ether / 5 Drops of L / 1 Drop of I	5 Drops of 10% L / 5 Drops of 10% G / 1 Drop of I	Temp.
Initial Color	Yellow	Yellow	Yellow	Yellow	Yellow	25°C.
Color After 45 Minutes	Orange	Yellow	Yellow	Yellow	Yellow	25°C.
Color After 11 Hours	Red	Yellow	Yellow	Yellow	Yellow	25°C.

Conclusions

Acetamide, ether and glycerine do not give a positive test with the lipase solution.

Experiment No. 5

Ester Test With Toluene (T), Chloroform, Bromobenzene

and Carbon Tetrachloride

	3 Drops E 3 Drops D.W. 3 Drops L 1 Drop I	3 Drops T 3 Drops D.W. 3 Drops L 1 Drop I	3 Drops $CHCl_3$ 3 Drops D.W. 3 Drops L 1 Drop I	3 Drops ØBr 3 Drops D.W. 3 Drops L 1 Drop I	3 Drops CCl_4 3 Drops D.W. 3 Drops L 1 Drop I
Initial Color	Yellow	Yellow	Yellow	Yellow	Yellow
Color After 10 Minutes	Light Orange	Yellow	Yellow	Yellow	Yellow
Color After 30 Minutes	Orange	Yellow ring & light yellow soln.	Upper layer almost color-less	Yellow	Yellow

Conclusions

Toluene, chloroform, bromobenzene and carbon tetrachloride do not give a positive test with lipase under the conditions of the experiment.

Experiment No. 6

The Ester Test With Acetophenone, Benzyl Alcohol, Nitrobenzene, Nitroethane

	3 Drops D.W. 3 Drops Benzyl Alcohol 3 Drops L 1 Drop I	3 Drops D.W. 3 Drops Acetophenone 3 Drops L 1 Drop I	3 Drops D.W. 3 Drops Nitrobenzene 3 Drops L 1 Drop I	3 Drops D.W. 3 Drops Nitroethane 3 Drops L 1 Drop I	3 Drops D.W. 3 Drops Ester 3 Drops L 1 Drop I
Inital Color	Yellow	Yellow	Yellow	Yellow	Yellow
Color After 10 Minutes	Yellow	Yellow	Yellow	Slightly Orange	Light Orange
Color After 3 Hours	Yellow	Yellow	Yellow	Slightly Orange	Reddish Orange

Conclusions

Nitroethane gives a positive test while benzyl alcohol, acetophenone and nitrobenzene give a negative test.

Experiment No. 7

Ester Test With Urea, Acetanilide, Aniline, Ethylamine and B-Naphthol

5 Drops Urea 5 Drops L 2 Drops I	5 Drops Acetanilide 5 Drops L 2 Drops I	5 Drops Aniline 5 Drops L 2 Drops I	5 Drops Ethylamine 5 Drops L 2 Drops I	5 Drops B-Nap. 5 Drops L 2 Drops I
After adding 1 drop of N/10 NH_4OH, the solution turns yellow and stays yellow.	Acetanilide and lipase gives a pink color with the indicator but on adding 1 drop of N/10 NH_4OH the solution turns yellow.	No positive test.	No positive test.	A pink color develops. On adding 2 drops of N/10 NH_4OH the color vanishes and stays as such even on keeping.

Urea = 10% in water

Acetanilide = 10% in Alcohol

Aniline (C.P.) = Used without Distillation

Ethylamine 100% = Used without Distillation

Conclusions

Urea, acetanilide, aniline, ethylamine and B-naphthol do not interfere in the ester test.

E. Test for Esters in the Presence of Other Compounds

Experiment No. 8

To Detect the Ester in the Presence of Ether

	(1)	(2)	(3)	(4)
	3 Drops E 3 Drops L 3 Drops D.W. 1 Drop I	3 Drops E 3 Drops L 3 Drops Ether 1 Drop I	3 Drops L 3 Drops Ether 3 Drops D.W. 1 Drop I	3 Drops L 6 Drops D.W. 1 Drop I
Initial Color	Yellow	Yellow	Yellow	Yellow
Color on shaking after 5 minutes	Dark Orange	Light Orange	Yellow	Yellow
After 30 minutes add 1 drop of indicator and some D.W. to test tube (2)	Pink	Dark Orange Notice lower layer	Yellow	Yellow

Remarks

When the second drop of indicator is added after 10 minutes of shaking, a beautiful red ring is formed at the bottom of the second test tube only.

Conclusions

It is possible to detect an ester in the presence of an equal amount of ether in 5 minutes.

Experiment No. 9

To Detect Ester in the Presence of Ether

	5 Drops of 10% E 5 Drops of L 1 Drop of I	5 Drops of Ether 5 Drops of L 1 Drop of I	5 Drops of D.W. 5 Drops of L 1 Drop M.R.	5 Drops of 10% E 5 Drops of Ether 5 Drops of L 1 Drop I
Initial Color	Yellow	Yellow	Yellow	Yellow
After 40 minutes	Orange	Yellow	Yellow	Add a few drops of distilled water; the layer becomes pink.

Conclusions

It is possible to detect the ester in 10 times of ether in 40 minutes. The ester is taken in the form of 10% solution in distilled water.

Experiment No. 10

To detect the ester in the presence of benzene (B)

	3 Drops E 3 Drops B 3 Drops L 1 Drop I	3 Drops E 3 Drops D.W. 3 Drops L 1 Drop I	3 Drops D.W. 3 Drops B 3 Drops L 1 Drop I	6 Drops D.W. 3 Drops L 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow
Color after shaking 10 minutes	Upper layer Orange Lower layer Orange	Orange	Upper layer Yellow Lower layer Yellow	Yellow

Conclusions

(i) Ester can be tested in the presence of an equal amount of benzene in 10 minutes.

(ii) It is much easier to notice the orange or pink color of the lower layer after shaking for 10 minutes only. However, if it is allowed to stand longer, the upper layer also becomes distinctly different.

Experiment No. 11

To Study the Effect of Benzene (B) on the Hydrolysis of the Ester if the Hydrolysis is allowed to Proceed for a Long Time

	5 Drops B 5 Drops 10% Ester 5 Drops L 1 Drop I	5 Drops D.W. 5 Drops Ester 5 Drops L 1 Drop I	10 Drops D.W. 5 Drops Ester 1 Drop I	5 Drops Benzene 5 Drops L 1 Drop I	5 Drops 10% Ester 5 Drops L 1 Drop I	5 Drops D.W. 5 Drops L 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
After 12 hours	A pink ring at the top	Color-less	Color-less	A yelowish orange ring at the top	A pink ring at the top	Color-less

Conculsion

(1) The solution has become colorless because lipase has been spoiled. It might be a good test for lipase. If the lipase is good, a drop of methyl red is not decolorized. However, if lipase is not good, the color of methyl orange is destroyed in a minute.

(2) Benzene does not retard the action of the lipase on the ester.

(3) The presence of benzene helps in prolonging the life of lipase.

Experiment No. 12

Detection of Ester in the Presence of an Equal Amount of Acetamide (A)

	3 Drops E 3 Drops L 3 Drops D.W. 1 Drop I	3 Drops E 3 Drops L 1 Drop I .15 gms. A	6 Drops D.W. 3 Drops L 1 Drop I	3 Drops D.W. 3 Drops L 1 Drop I .15 gms. A
Initial color	Yellow	Yellow	Yellow	Yellow
Color after 5 min. shaking	Orange	Orange	Yellow	Yellow
After 10 min. shaking	Reddish Orange	Pink	Yellow	Yellow

Conclusions

(1) It is possible to detect an ester in the presence of an equal amount of acetamide.

(2) The color in the presence of acetamide is more reddish than in its absence. This is in harmony with the fact that acetamide acts as an activator of pancreatic lipase.

Experiment No. 13

Detection of Esters in the Presence of Glycerine

	3 Drops E 3 Drops Glycerine 3 Drops L 1 Drop I	3 Drops E 3 Drops L 3 Drops D.W. 1 Drop I	3 Drops L 3 Drops Glycerine 3 Drops D.W. 1 Drop I	3 Drops L 6 Drops D.W. 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow
After shaking for 5 minutes	Orange Darker than with pure ester	Orange	Yellow	Yellow
After one hour	Pink Darker than with pure ester	Pink	Yellow	Yellow

Conclusions

(1) It is possible to detect the ester in the presence of an equal amount of glycerine.

(2) Glycerine appears to activate the hydrolysis of ethyl acetate.

Experiment No. 14

Detection of Ethyl Acetate in the Presence of Glycerine (G)

	(1) 5 Drops 10% G 5 Drops 15% E 5 Drops 10% L 1 Drop I	(2) 5 Drops L 5 Drops 10% G 1 Drop I	(3) 5 Drops 10% E 5 Drops L 1 Drop I	(4) 5 Drops L 10 Drops D.W. 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow
After 40 minutes	Orange Darker than in Col. 3	Yellow	Orange	Yellow
After 6 hours	Dark Orange Darker than in Col. 3	Yellow	Dark Orange	Yellow

Conclusions

(1) It is possible to detect 15% ethyl acetate in the presence of 10% glycerine in 40 minutes.

(2) Glycerine appears to activate the hydrolysis of ethyl acetate.

Experiment No. 15

To Detect the Ester in the Presence of Sucrose (S)

	3 Drops E .15 gms. S 3 Drops L 1 Drop I	3 Drops E 3 Drops Lipase 3 Drops D.W. 1 Drop I	.15 gms. S 3 Drops L 3 Drops D.W. 1 Drop I	3 Drops L 6 Drops D.W. 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow
Color after shaking for 5 minutes	Orange	Orange	Yellow	Yellow

Conclusion

It is possible to detect the ester in the presence of an equal amount of sucrose in 5 minutes.

Experiment No. 16

To Test for Ester in the Presence of Dextrose (D)

	3 Drops E 3 Drops L .15 gms. D 1 Drop I	3 Drops E 3 Drops L 3 Drops D.W. 1 Drop I	3 Drops L .15 mgs. D 3 Drops D.W.	3 Drops L 6 Drops D.W.
Initial color	Yellow	Yellow	Yellow	Yellow
Color after shaking for 5 minutes	Orange	Orange	Yellow	Yellow

Conclusion

It is possible to detect 3 drops of an ester (.15 ml. approximately) when mixed with .15 gm. of dextrose.

Experiment No. 17

To Test for Ester in the Presence of Propionaldehyde (P)

	3 Drops E 3 Drops L 3 Drops D.W. 1 Drop I	3 Drops E 3 Drops L 3 Drops P 1 Drop I	3 Drops L 3 Drops P 3 Drops D.W. 1 Drop I	3 Drops L 6 Drops D.W. 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow
Color after shaking 5 min.	Orange	Light Orange	Yellow	Yellow
After 10 min.	Orange	Light Orange	Yellow	Yellow
After 30 min.	Reddish Orange	Orange	Very Light Orange	Yellow

Conclusions

(1) It is possible to detect the ester mixed with an equal amount of aldehyde.

(2) After about half an hour the solution containing only lipase and propionaldehyde starts acquiring slight orange color. Hence it is preferable to look for change in color within 15 minutes of shaking.

Experiment No. 18

Detection of Ester in the Presence of Acetaldehyde (A)

	(1)	(2)	(3)	(4)	(5)	(6)
	3 Drops E 5 Drops A 1 Drop 50% $NaHSO_3$ 3 Drops L 1 Drop I	3 Drops E 5 Drops A 1 Drop 10% $NaHSO_3$ 3 Drops L 1 Drop I	3 Drops E 5 Drops A 1 Drop D.W. 3 Drops L 1 Drop I	3 Drops E 6 Drops D.W. 3 Drops L 1 Drop I	3 Drops L 9 Drops D.W. 1 Drop I	1 Drop 50% $NaHSO_3$ 5 Drops A 3 Drops D.W. 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
After 5 min.	Orange	Orange	Orange	Darker orange than others	Yellow	Yellow
After 1 hour	No difference in color of these three. Orange	Orange	Orange	Red	Colorless	Yellow
After 18 hrs.	The orange color diminishes from (1) to (4). (1) is the darkest.			Red	Colorless	Yellow

Conclusions

(1) It is possible to detect the ester in the presence of about 20% acetaldehyde in 5 minutes.

(2) Under the conditions of experiment, no difference could be detected between the colors of tests (1), (2), (3), and (4) during the first one hour of hydrolysis. Hence there does not appear to be any advantage in adding sodium bisulphite.

(3) The effect of acetaldehyde is visible in the fact that the intensity of the color in (5) is higher than in the first four.

(4) Sodium bisulphite does not significantly become acidic after it has been neutralized.

(5) The most appropriate amount of sodium bisulphite is almost equal to the amount of acetaldehyde present because the color in Column (1) is darkest.

(6) The beneficial effect of sodium bisulphite is felt after hydrolysis has proceeded.

Experiment No. 19

To Detect the Presence of Ethyl Acetate in the Presence of Propionaldehyde (A)

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	3 Drops E 5 Drops L 5 Drops 10% A 1 Drop 50% S 1 Drop I	3 Drops E 5 Drops L 5 Drops 10% A 2 Drops 50% S 1 Drop I	3 Drops E 5 Drops L 5 Drops 10% A 3 Drops 50% S 1 Drop I	3 Drops E 5 Drops L 5 Drops 10% A 1 Drop I	3 Drops D.W. 5 Drops L 5 Drops 10% A 1 Drop 50% S 1 Drop I	3 Drops D.W. 5 Drops L 5 Drops 10% A 2 Drops 50% S 1 Drop I	3 Drops D.W. 5 Drops L 5 Drops 10% A 3 Drops 50% S 1 Drop I	3 Drops E 5 Drops L 7 Drops D.W. 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
After 10 min.	Orange	Light orange	Light orange	Orange	Yellow	Color-less	Color-less	More reddish orange than (1)
After ½ hour.	Orange	Light orange	Light orange	Orange	Yellow	Color-less	Color-less	Red
After 45 min.	Orange	Color-less	Color-less	Orange	Yellow	Color-less	Color-less	Red
After 24 hrs.	Red	Color-less	Color-less	Orange	Yellow	Color-less	Color-less	Red

S = Sodium bisulfite

Conclusions

This experiment appears to confirm the results of the previous experiment, i.e.

(1) The presence of aldehyde does decrease the intensity of the color in Columns (4) and (8).

(2) The addition of sodium bisulfite equivalent to the amount of aldehyde present does not affect the color of the indicator. (1) and (4) are of the same intensity.

(3) After 24 hours the color of the solution containing sodium bisulfite becomes red. Hence the effect of sodium bisulfite is not felt in one or 2 hours but later.

(4) Even after 24 hours the solution in Column (5) remains yellow. Therefore it follows that the solution containing aldehyde and sodium bisulfite in equivalent amounts does not become acidic even on keeping for 24 hours if it has been made neutral at the start of the experiment.

(5) The optimum amount of sodium bisulfite is equal to the amount which is equivalent to the aldehyde present. If more sodium bisulfite is added it reacts with the indicator and destroys its color.

Experiment No. 20

To Investigate the Action of Sodium Bisulfite on Methyl Red in the Presence and Absence of Aldehydes

	5 Drops L 1 Drop 50% S 5 Drops 10% P 1 Drop I	5 Drops L 1 Drop 50% S 5 Drops D.W. 1 Drop I	5 Drops L 1 Drop D.W. 5 Drops 10% P 1 Drop I	5 Drops D.W. 1 Drop 50% S 5 Drops 10% P 1 Drop I	5 Drops D.W. 1 Drop 50% S 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow
After 5 min.	Yellow	Yellow	Yellow	Yellow	Pink
After ½ hour.	Yellow	Colorless	Yellow	Yellow	Colorless

S = Sodium bisulfite P = Propionaldehyde

Conclusion

In the absence of aldehyde sodium bisulfite reacts with the indicator and makes it colorless.

Experiment No. 21

To Test for the Ester in the Presence of Ethyl Alcohol (E.A.)

	3 Drops E 3 Drops L 3 Drops D.W. 1 Drop I	3 Drops E 3 Drops L 3 Drops E.A. 1 Drop I	3 Drops D.W. 3 Drops L 3 Drops E.A. 1 Drop I	6 Drops D.W. 3 Drops L 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow
After 5 min.	Orange	Orange	Yellow	Yellow

Conclusions

It is possible to detect ethyl acetate in the presence of an equal amount of ethyl alcohol.

NOTE: Methyl alcohol gives the same results as ethyl alcohol.

Experiment No. 22

To Test for Ester in the Presence of Benzyl Alcohol (B.A.)

	3 Drops E 3 Drops B.A. 3 Drops L 1 Drop I	3 Drops E 3 Drops D.W. 3 Drops L 1 Drop I	3 Drops B.A. 3 Drops D.W. 3 Drops L 1 Drop I	6 Drops D.W. 3 Drops L 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow
Color after shaking for 5 minutes	White turbidity with pink droplets	Orange	White turbidity with pink droplets	Yellow

Conclusions

(1) It is not possible to detect ethyl acetate in the presence of an equal amount of ordinary benzyl alcohol which is not freshly distilled.

(2) The interference may be due to the fact that benzyl alcohol contains small amounts of benzaldehyde which on autoxidation gives benzoic acid.

(3) The presence of benzyl alcohol may be suspected in a preliminary test since even without lipase it gives pink droplets whereas the ester after reacting with lipase for 5 minutes gives only an orange color.

Experiment No. 23

To Detect the Presence of Ethyl Acetate in the Presence of Toluene (T)

	3 Drops E 3 Drops T 3 Drops L 1 Drop I	3 Drops D 3 Drops D.W. 3 Drops L 1 Drop I	3 Drops D.W. 3 Drops T 3 Drops L 1 Drop I	6 Drops D.W. 3 Drops L 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow
Color after 5 min. after adding one drop of I	Light orange Notice the color of the lower aqueous layer	Orange	Yellow	Yellow
Color after 10 min. after adding 1 drop of I	Orange Notice the color the lower aqueous layer	Reddish orange	Yellow	Yellow

Conclusions

It is possible to detect the presence of ester in an equal amount of toluene in 10 minutes.

Experiment No. 24

Detection of Ester in Different Amounts of Toluene (T)

	(1)	(2)	(3)	(4)	(5)
	3 Drops E 3 Drops T 3 Drops L 1 Drop I	3 Drops E 2 Drops T 3 Drops L 1 Drop I	3 Drops E 1 Drop T 3 Drops L 1 Drop I	3 Drops E 3 Drops D.W. 3 Drops L 1 Drop I	3 Drops D.W. 3 Drops T 3 Drops L 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow
After ½ hr.	Orange ring Light orange solution	Orange ring Light orange solution	Orange ring Light orange solution	Orange	Yellow ring Light yellow solution
After 22 hrs.	Lowest aqueous layer is pink	Lowest layer more pink than (1)	Lowest layer more pink than (2)	Pink	Lowest aqueous layer is yellowish

Conclusions

(1) Toluene does inhibit the action of lipase on the ester. As the concentration of toluene increases the hydrolytic action of the lipase decreases. The orange color is in the order of 4, 3, 2, 1, 5. (4) is pink and (5) is yellow.

(2) It is best to observe the lower aqueous layer.

(3) As found earlier it is possible to detect the ester in the presence of an equal amount of toluene.

Experiment No. 25

Detection of Ester in the Presesce of Chloroform (C)

	(1) 3 Drops E 3 Drops C 3 Drops L 1 Drop I	(2) 3 Drops E 2 Drops C 1 Drop L 1 Drop I	(3) 3 Drops E 1 Drop C 3 Drops L 1 Drop I	(4) 3 Drops D.W. 3 Drops E 3 Drops L 1 Drop I	(5) 3 Drops C 3 Drops L 3 Drops D.W. 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow
Color after 8 minutes	Upper layer light orange	Upper layer more orange than (1)	Upper layer more orange than (2)	More orange than (3)	Upper layer very light yellow
Color after 8 hours	Upper layer light pink	Upper layer more pink than (1)	Upper layer more pink than (2)	Red	Upper layer is yellow

Conclusions

(1) It is possible to detect the ester in the presence of an equal amount of chloroform in 8 minutes.

(2) It is best to look at the color of the upper layer.

Experiment No. 26

Detection of Esters in the Presence of Bromobenzene (B)

	(1) 3 Drops E 3 Drops B 3 Drops L 1 Drop I	(2) 3 Drops E 2 Drops B 3 Drops L 1 Drop I	(3) 3 Drops E 1 Drop B 3 Drops L 1 Drop I	(4) 3 Drops E 3 Drops D.W. 3 Drops L 1 Drop I	(5) 3 Drops D.W. 3 Drops B 3 Drops L 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow
Color after 10 min.	Upper layer light orange	Upper layer is more orange than (1)	Upper layer is more orange than (2)	More orange than (3)	Slightly orange /less than (1)/

Conclusions

(1) It is possible to detect ester in the presence of bromobenzene even if the ester is present in an equal quantity. For this, it is necessary to compare with pure bromobenzene.

(2) Bromobenzene offers slight interference in the detection of pure compounds.

Experiment No. 27

To Detect the Esters in the Presence of Carbon Tetrachloride (C)

	(1)	(2)	(3)	(4)	(5)
	3 Drops E 3 Drops C 3 Drops L 1 Drop I	3 Drops E 2 Drops C 3 Drops L 1 Drop I	3 Drops E 1 Drop C 3 Drops L 1 Drop I	3 Drops E 3 Drops D.W. 3 Drops L 1 Drop I	3 Drops D.W. 3 Drops C 3 Drops L 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow
Color after shaking for 10 minutes :	Upper layer is light orange	Upper layer is more orange than (1)	Upper layer is more orange than (2)	More orange than (3)	Yellow
After Keeping for 12 hours	Upper layer is pink	Upper layer is pink	Upper layer is pink	Pink	Yellow

Conclusions

(1) It is possible to detect the ester in the presence of an equal amount of carbon tetrachloride.

(2) There is no interferences from carbon tetrachloride.

Experiment No. 28

Detection of Esters in the Presence of Purified Acetophenone (A.P.) and Benzyl Alcohol (B) (Acetophenone was Purified by Shaking With Sodium Carbonate and Then Distilling it. Benzyl Alcohol was Purified by Redistilling it.)

	(1)	(2)	(3)	(4)	(5)	(6)
	3 Drops E 3 Drops A.P. 3 Drops L 1 Drop I	3 Drops E 1 Drop A.P. 3 Drops L 1 Drop I	3 Drops A.P. 3 Drops L 3 Drops D.W. 1 Drop I	3 Drops E 3 Drops B 3 Drops L 1 Drop I	3 Drops E 1 Drop B 3 Drops L 1 Drop I	3 Drops D.W.. 3 Drops B 3 Drops L 1 Drop I
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Color after shaking for 10 min. and allowing to stand for 3 min.	Upper layer is slightly orange	Upper layer is more orange than (1)	No upper layer is formed	Upper layer is light orange	Upper layer is more orange than (4)	Yellow solution

Procedure

Shake thoroughly, add lipase, and N/25 ammonium hydroxide until the solutions become yellow. Now shake for another 10 minutes. Allow to settle for 3 minutes.

and notice the color of the upper layers. Absence of upper layers denotes the absence of ethyl acetate. In the case of benzyl alcohol I could arrange them in the order of increasing amounts of benzyl alcohol without looking at the labels.

Conclusions

(1) When acetophenone and benzyl alcohol are freshly distilled they do not interfere in the detection of ethyl acetate.

(2) It is possible to detect the ester very easily when benzyl alcohol or acetophenone are 33% of the ester present.

Experiment No. 29

Detection of Ethyl Acetate in the Presence of an Equal Amount of Benzyl Alcohol (B.A.)

	5 Drops 10% B.A. 5 Drops L 5 Drops 10% E. 1 Drop I	5 Drops 10% B.A. 5 Drops L 5 Drops D.W. 1 Drop I
Initial color	Yellow	Yellow
Color after 1½ hrs.	Pink	Yellow

Conclusions

It is possible to detect the presence of ethyl acetate in an equal amount of benzyl alcohol, if 10% emulsions in distilled water are used. But in this case the time taken is 90 minutes.

Experiment No.30

Detection of Ethyl Acetate in the Presence of Nitrobenzene (N.B.)

	(1) 3 Drops N.B. 3 Drops E. 3 Drops L 1 Drop I	(2) 2 Drops N.B. 3 Drops E. 3 Drops L 1 Drop I	(3) 1 Drop N.B. 3 Drops E. 3 Drops L 1 Drop I	(4) 3 Drops N.B. 3 Drops L 1 Drop I 3 Drops D.W.
Initial color	Yellow	Yellow	Yellow	Yellow
Shake for 10 minutes	Upper layer almost colorless	Upper layer less orange than in (3)	Upper layer light orange	Upper layer colorless
Now add another drop of I and a drop of N/10 NH_4OH	Upper layer orange	Upper layer orange	Upper layer orange	Upper layer colorless

Conclusions

(1) Under the ordinary conditions of experiment it is possible to detect the presence of ethyl acetate in the presence of 33% of nitrobenzene.

(2) It is possible to modify the experiment so that ethyl acetate can be detected in the presence of an equal amount of nitrobenzene.

Experiment No. 31

Detection of Ethyl Acetate in the Presence of Nitrobenzene (N.B.)

	4 Drops N.B. 3 Drops E. 3 Drops L 1 Drop I 2 Drops D.W.	5 Drops N.B. 3 Drops E. 3 Drops L 1 Drop I 1 Drop D.W.	6 Drops N.B. 3 Drops E. 3 Drops L 1 Drop I	6 Drops N.B. 3 Drops D.W. 3 Drops L 1 Drop I
Add a drop of N/10 NH_4OH after mixing together	Yellow	Yellow	Yellow	Yellow
Shake for 10 min. Add 1 drop of indicator	Upper layer light orange	Upper layer light orange	Upper layer light orange	Upper layer yellow

Conclusions

It is possible to detect ethyl acetate in the presence of twice the amount of nitrobenzene.

Experiment No. 32

Detection of Ethyl Acetate in the Presence of Nitrobenzene (N.B.)

	(1) 7 Drops N.B. 3 Drops E. 3 Drops L 1 Drop I	(2) 8 Drops N.B. 3 Drops E 3 Drops L 1 Drop I	(3) 9 Drops N.B. 3 Drops E 3 Drops L 1 Drop I	(4) 9 Drops N.B. 3 Drops L 1 Drop I
Initial color	Upper layer yellow	Upper layer yellow	Upper layer yellow	Upper layer yellow
After shaking for 10 minutes add 1 drop of indicator	Upper layer light orange	Upper layer light orange	Upper layer light orange	Upper layer yellow

Conclusions

(1) It is possible to detect ethyl acetate in the presence of thrice the amount of nitrobenzene.

(2) It is not desirable to equalize the volumes in Columns (1) through (4) by adding distilled water. The experiment performed in this way did not succeed.

Experiment No. 33

Detection of Ethyl Acetate in the Presence of Nitrobenzene (N.B.) at a Higher Temperature.

	3 Drops N.B. 3 Drops E. 3 Drops L 1 Drop I	2 Drops N.B. 3 Drops E. 3 Drops L 1 Drop I 1 Drop D.W.	1 Drop N.B. 3 Drops E. 3 Drops L 1 Drop I 2 Drops D.W.	3 Drops N.B. 3 Drops D.W. 3 Drops L 1 Drop I	Temp. 50°C. ±1°C.
Initial color	Yellow	Yellow	Yellow	Yellow	"
After shaking for 5 min. add 1 drop of I	Upper layer light orange	Upper layer light orange	Upper layer light orange	Upper layer yellow	"
After ½ hrs.	Upper layer light orange	Upper layer light orange	Upper layer light orange	Upper layer yellow	"

Conclusions

It is possible to decrease the time of shaking to 5 minutes if the experiment is performed at 50°C.

Experiment No. 34

To Find the Limit of Detection of Ethyl Acetate in the Presence of Nitrobenzene (N.B.)

	3 Drops E. 10% in N.B. 3 Drops L 1 Drop I	3 Drops E. 20% in N.B. 3 Drops L 1 Drop I	3 Drops N.B. 3 Drops L 1 Drop I	Temp.
Initial color	Yellow	Yellow	Yellow	25°C.
Shake for 10 min. Add 1 drop of I	Upper layer yellow	Upper layer light orange	Upper layer yellow	25°C.
Color after 3 hrs.	Upper layer orange	Upper layer orange	Upper layer yellow	25°C.

Prodecure

After shaking for 3 minutes, another drop of indicator and another drop of N/25 NH_4OH was added. This gave the initial yellow color.

Conclusions

(1) It is possible to detect 20% ethyl acetate in nitrobenzene.

(2) If the proportion is less than 20% the minimum time necessary for detection is 3 hours.

F. The Ester Test with Different Esters

The experiments described herein were undertaken to extent the enzymatic method of detection of different types of esters. Esterases other than lipase have also been studied with this point in view. The procedure used is the same as described in section C of this chapter. Any modifications in the procedure have been indicated in the text.

Experiment No. 35

The Ester Test with Different Esters

	Phenyl benzoate in 10% alcohol	Benzyl acetate	Amyl butyrate	Ethyl cinnamate	Ethyl aceto-acetate	Ethyl oxalate	Ethyl α-Bromo-isobutyrate
Initial color	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Color after 10 min. at room temp. 25°C.	Deep yellow	Buff color	Orange	Yellow	Orange	Pink	Deep yellow

Conclusions

(1) Phenyl benzoate, ethyl cinnamate and ethyl α-Bromoisobutyrate give a negative test.

(2) Performing the hydrolysis at a higher temperature is not useful.

(3) According to the intensity of the color developed the rates of hydrolysis appear to be in this order: Ethyl oxalate > Acetoacetic ester > Benzyl acetate > Amyl acetate.

Experiment No. 36

Ester Test with Different Esters

Ester	Initial color	Color after 3 minutes shaking	Further change in color
Allyl acetate	Yellow	Yellow	Light orange after 16 min.
Ethyl acetate	Yellow	Yellow	Light orange after 11 min.
Methyl acetate	Yellow	Yellow	Light orange after 30 min.
Benzyl acetate	Yellow	Light orange in 1 min.	Color as before
Vinyl acetate	Yellow	Orange in 1 min.	Color as before
Ethyl acetoacetate	Yellow	Light orange in 1 & pink in 3	Color as before
Ethyl bromoacetate	Yellow	Light orange	Color as before
Ethyl propionate	Yellow	Light orange	Color as before
Ethyl oxalate	Yellow	Light orange	Color as before
Ethyl malonate	Yellow	Orange	Color as before
Methyl benzoate	Yellow	Upper layer is milky white Lower layer is orange	Color as before

Methyl salicylate	Yellow	Lower layer is orange Upper layer is yellowish white	Color as before
Ethyl phthalate	Yellow	Upper layer is yellowish white Lower layer is orange	Color as before
Amyl butyrate	Yellow	Light pink	Color as before
N-Amyl succinate	Yellow	Light pink	Color as before
Ethyl anisate	Yellow	Yellow	Color as before
Ethyl adipate	Yellow	Light orange	Color as before
Ethyl fumarate	Yellow	Light orange	Color as before

Conclusions

(1) Ethyl anisate does not respond to the ester test.

(2) It is better to take 3 drops of the ester rather than one drop of the ester.

(3) Methyl benzoate, methyl salicylate and methyl phthalate give very light orange colors.

Experiment No. 37

To Study the Effect of Horse Serum on a Number of Esters

Ester	Initial color	Color after 30 minutes	Color after shaking overnight	Conclusion
Benzyl benzoate	Yellow	Yellow	White turbidity in upper aqueous layer.	-
Ethyl anisate	Yellow	Orange	White turbidity in upper aqueous layer.	?
Ethyl propionate	Yellow	Orange	Light pink	+
Phenyl benzoate	Yellow	Light orange	Very pink	+
Benzyl acetate	Yellow	Orange	Light orange turbidity	+
Ethyl cinnamate	Yellow	Yellow	Very slight turbidity	-
Ethyl acetate	Yellow	Orange	Light pink	+
Methyl salicylate	Yellow	Yellow	Almost white turbidity	-
Methyl benzoate	Yellow	Light orange	Light orange turbidity Some precipitation	+
Methyl acetate	Yellow	Orange	Pink	+

Conclusions

(1) Horse serum appears to hydrolyze low molecular weight esters better than high molecular weight esters.

(2) Benzyl benzoate, ethyl cinnamate and methyl salicylate are not hydrolyzed by horse serum.

Experiment No. 38

Experiments with a Mixture of Horse Serum and 10% Steapsin in Equal Proportions

Ester	Initial color	Color after 10 minutes	Conclusion
Amyl butyrate	Yellow	Light pink	+
Ethyl phthalate	Yellow	Upper layer white	-
Benzyl benzoate	Yellow	Upper layer colorless	-
Ethyl anisate	Yellow	Light pink	+
Ethyl propionate	Yellow	Light orange	+
Phenyl benzolate	Yellow	Pink	+
Benzyl acetate	Yellow	Pink	+
Ethyl cinnamate	Yellow	Upper layer white	-
Ethyl acetate	Yellow	Light orange	+
Methyl salicylate	Yellow	Upper layer white	-
Methyl benzoate	Yellow	Light orange	+
Methyl acetate	Yellow	Pink	+

Conclusions

(1) The results are the same as with horse serum alone.

(2) Ethyl phthalate, benzyl benzoate, ethyl cinnamate and methyl salicylate give negative results.

Experiment No. 39

Experiments on Wheat Germ Lipase with Different Esters

Ester	Initial color	Color after 20 min.	Color after 50 min. (Blank)	Color after 50 min. (Ester)
Ethyl acetate	Yellow	Pink ppt.	Yellow	Pink
Methyl benzoate	Yellow	Yellow	Yellow	Upper layer light pink
Methyl salicylate	Yellow	Yellow	Yellow	Upper layer light pink
Benzyl acetate	Yellow	White ppt.	Yellow	Red
Phenyl benzoate	Yellow	Pink ppt.	Yellow	Light orange
Ethyl cinnamate	Yellow	Yellow	Yellow	Yellow
Methyl acetate	Yellow	Orange ppt.	Light orange solution	Pink ppt.
Ethyl propionate	Yellow	Orange ring	Yellow	Pink ppt.
Ethyl anisate	Yellow	Yellow	Yellow	Light pink
Benzyl benzoate	Yellow	Yellow	Yellow	Light pink
Ethyl phthalate	Yellow	Yellow	Yellow	Light pink
Amyl butyrate	Yellow	Upper layer yellow	Yellow	Yellow

Conclusions

(1) Hydrolysis under the conditions of the experiment appears to increase with decrease in molecular weight. Methyl acetate is the most hydrolyzed ester and amyl butyrate is the least hydrolyzed.

(2) Benzyl acetate and phenyl benzoate are also significantly hydrolyzed.

Experiment No. 40

The Hydrolysis of Esters by Wheat Germ Lipase

Ester	Shake for 3 min. & add 1 drop of indicator	Add 4 drops of distilled water before shaking
Methyl benzoate	Pink	Pink
Methyl salicylate	Yellow	Yellow
Nitrobenzene	Pink	Yellow

Conclusions

(1) Methyl benzoate is hydrolyzed by wheat germ lipase but not methyl salicylate.

(2) Nitrobenzene interferes but if 20% emulsion in distilled water is used there is no interference from nitrobenzene.

Experiment No. 41

Action of Lipase on Esters of Branched Chain Alcohols

Ester	Initial color	Color after 10 min.	Color after 1 hr.	Color after 15 hrs.
Ethyl acetate	Yellow	Light orange	Red	Red
Isopropyl benzoate	Yellow	Yellow	Yellow	Pink
Isoamyl salicylate	Yellow	Yellow	Yellow	Yellow
Isobutyl acetate	Yellow	Light orange	Orange	Red
Isopropyl propionate	Yellow	Yellow	Light orange	Pink
Isobutyl formate	Yellow	Light orange	Orange	Red

Conclusions

(1) Salicylate is very difficult to hydrolyze and does not give satisfactory results.

(2) It is not true to say that lipase is specific for the primary alcoholic group only. It hydrolyzes esters of secondary alcohols also.

Experiment No. 42

Experiments of the Hydrolysis of Esters of Dibasic and Tribasic Acids

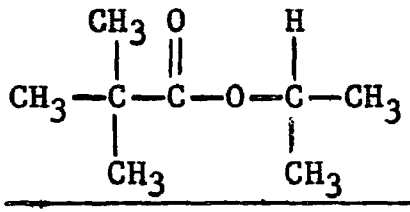
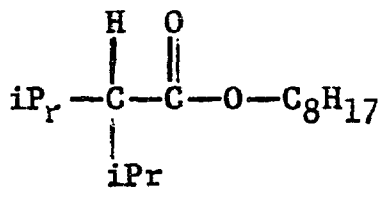
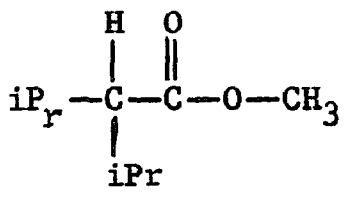
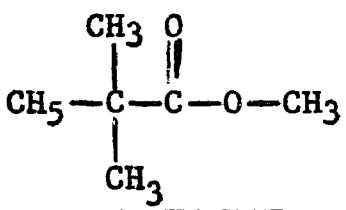
Ester	Original color	Color after 1 min.	Color after 3 min.
Ethyl citrate	Yellow	Light orange	Pink
n-Butyl citrate	Yellow	Light orange	Pink
Dimethyl phthalate	Yellow	Light orange	Pink

Conclusions

The esters of dibasic and tribasic acids hydrolyze very quickly.

Experiment No. 43

The Ester Test with Sterically Hindered and Substituted Esters

	Steapsin	Glandular lipase
	After 3 min. negative test. After 12 hrs. dark orange	After 3 min. negative test. After 12 hrs. dark orange.
	After 3 min. negative test. After 12 hrs. light orange.	After 3 min. negative test. After 12 hrs. light orange.
	After 3 min. negative test. After 12 hrs. negative test.	After 3 min. negative test. After 12 hrs. negative test.
	After 3 min. negative test. After 12 hrs. dark orange.	After 3 min. negative test. After 12 hrs. dark orange.

$CN \cdot CH_2 \cdot COOEt$	Hydrolysis within a min.	Hydrolysis within a min.
Sec-octyl trimethyl acetate	After 3 min. negative test. After 12 hrs. light orange	After 3 min. negative test. After 12 hrs. light orange.

Conclusions

(1) Branching does hinder the hydrolysis.

(2) For the purpose of the test all the esters except No. 5 gave negative results.

(3) The cyanide group increases hydrolysis very much.

G. Comparison Between Various Esterases:

Effect of Esterases on Different Esters from an Analytical Point of View.

Preparation of Different Solutions

(1) Pancreatic lipase 5%. N/10 NH_4OH was added until the pH changed from 6.00 to 6.20.

(2) Wheat germ lipase 5%. N/10 NH_4OH was added until its pH changed from 6.00 to 6.20.

(3) Horse serum. N/10 HCl was added drop by drop until the pH fell from 7.50 to 6.20.

(4) Distilled water. Its pH was 7.50 but it was not necessary to carry out an adjustment because distilled water itself lacks the capacity to establish the pH of a system.

Experiment No. 44

Comparison Between Pancreatic, Wheat Germ & Horse Serum Lipases.

	Pancreatic Lipase (P.L.)	Wheat Germ Lipase (W.G.)	Horse Serum (H.S.)	Remarks
Methyl acetate	Pink color	Light orange	Light orange	P.L. > W.G. > H.S. Results confirmed on repetition.

Ethyl acetate	Light orange	Light orange	Light orange	Order of color after 4 hrs. P.L.>W.G.>H.S.
Vinyl acetate	Light orange	Pink	Light orange	W.G.>H.S.>P.L.
Bornyl acetate	After 12 hrs. light orange	After 12 hrs. pink	After 12 hrs. light orange	W.G.>H.S.>P.L.
Benzyl acetate	Light orange	Light orange	Hydrolysis negligible	P.L.>W.G.>H.S.
Ethyl bromoacetate	Light orange	Pink	Orange	W.G.>H.S.>P.L.
Ethyl propionate	Orange	Light orange	Light orange	P.L.>H.S.>W.G.
Ethyl butyrate	Orange	Orange	Orange	After 30 min. P.L.>H.S.>W.G.
Amyl butyrate	Light pink	Light orange	Light orange	P.L.>W.G.>H.S.
Methyl benzoate	Light orange	Light orange	Light orange	P.L.>H.S.>W.G.>D.W.
Methyl salicylate	3 min.	3 min.	3 min.	P.L.>H.S.~W.G. Negative test
Ethyl phthalate	3 min.	3 min.	3 min.	P.L.>H.S.>W.G. Negative test

Ethyl cinnamate	Light pink	Light orange	Light orange	P.L.$>$H.S.$>$W.G. Positive test with P.L.
Benzyl benzoate	12 hrs.	12 hrs.	12 hrs.	P.L.$>$H.S.$\sim$W.G. Negative test
Phenyl benzoate	1 hr.	1 hr.	Pink in $\frac{1}{2}$ hr.	H.S.$>$P.L.$\sim$W.G.
Ethyl anisate	----	----	----	P.L.$>$W.G.$\sim$H.S. Negative test
Ethyl oxalate	Quick hydrolysis	Quick hydrolysis	Quick hydrolysis	P.L.$\sim$W.G.$\sim$H.S.
Ethyl aceto-acetate	Orange	Orange	Orange	P.L.$>$H.S.$>$W.G.
Ethyl malonate	Light orange	Light orange	Pink	H.S.$>$P.L.$>$W.G.
Ethylene diacetate	Pink	Pink	Pink	H.S.$>$W.G.$>$P.L.
Isopropyl phthalate	Quick hydrolysis	Quick hydrolysis	Quick hydrolysis	

Note (i) Where no time is mentioned the color refers to 3 minutes.

(ii) 3 drops of vinyl acetate (stabilized, of Eastman Kodak) needed 1 drop of N/5 NH_4OH and 1 drop of N/7.5 NH_4OH.

Experiment No. 45

To Compare Lipase of Pancreas Glands with Steapsin

Procedure

The lipase solutions used were 10% in distilled water. Three drops of ester were added to 3 drops of lipase. A drop of methyl red was then added, followed by a drop of N/25 NH_4OH to make the solution yellow.

Ester	Steapsin after 10 min.	Gland lipase after 10 min.	Remarks
Ethyl acetate	Light orange	Light orange	Both work equally well.
Methyl benzoate	Light pink	Light orange	Steapsin works better than gland lipase.
Benzyl acetate	Pink	Pink	Both work equally well.
Phenyl benzoate	Yellow	Pink	Gland lipase gives a positive test while steapsin gives a negative test.

H. Effect of Inhibitors and Activators

The Effect of Inhibitors & Activators on Hydrolysis of Esters

The following experiments were undertaken to supplement the conclusions reached from qualitative work on the effect of acetaldehyde on hydrolysis and to explore the utility of the use of calcium chloride. In this section L = Lipase; D.W. = Distilled water.

Experiment No. 46

To Study the Effect of Acetaldehyde (A) on the Hydrolysis of Amyl Acetate (E) in the Absence of a Buffer in 72 hrs. at 25°C ±2°C.

E	A	L 10%	D.W.	N/10 KOH	N/10 KOH
2 ml.	1 ml.	2 ml.	10 ml.	2 ml.	2 ml.
2 ml.	1 ml.	x	12 ml.	1.90 ml.	5.60 ml.
2 ml.	x	2 ml.	11 ml.	1.50 ml.	1.50 ml.
2 ml.	x	x	13 ml.	0.15 ml.	0.15 ml.
x	1 ml.	2 ml.	12 ml.	1.20 ml.	1.20 ml.
x	1 ml.	x	14 ml.	1.05 ml.	1.00 ml.

Experiment No. 46 (Part 2)

E	A	L	D.W.	Time	N/10 KOH	N/10 KOH	N/10 KOH	Mean
2 ml.	1 ml.	2 ml.	10 ml.	72 hrs.	2.30	2.00	2.10	2.10
2 ml.	1 ml.	x	12 ml.	72 hrs.	2.00	2.30	1.70	2.00
2 ml.	x	2 ml.	11 ml.	72 hrs.	1.70	1.80	1.50	1.66
2 ml.	x	x	13 ml.	72 hrs.	0.15	0.05	0.05	0.08
x	1 ml.	2 ml.	12 ml.	72 hrs.	1.25	1.25	1.80	1.25
x	1 ml.	x	14 ml.	72 hrs.	1.10	1.00	1.05	1.05

Method

The ethyl acetate, acetaldehyde and lipase were kept in glass stoppered Erlenmeyer flasks with occasional shaking. Acetaldehyde was transferred by cooling the acetaldehyde solution and the transferring pipete to $0^{o}C$.

Discussion

The purpose of this experiment was to study the effect of acetaldehyde on the hydrolysis of amyl acetate in the absence of a buffer system and at ordinary temperature.

The experiments show that there is a definite inhibition on the hydrolysis of amyl acetate by the presence of aldehyde. This inhibition may be due to the decrease of pH by the air oxidation of acetaldehyde. This has to be studied further in the presence of a buffer system.

Hydrolysis of amyl acetate in the presence of acetaldehyde = 2.1 ml. of N/10 KOH.

Hydrolysis of amyl acetate in the absence of acetaldehyde = 1.66 ml.

During the same time oxidation of acetaldehyde in the presence of lipase = 1.25 ml.

Therefore, inhibition = 1.66 / 1.25 — 2.1

= 2.91 — 2.1 = .81 ml.

Experiment No. 47

To Study the Effect of Acetaldehyde on the Hydrolysis of Amyl Acetate in the Presence of Buffered System (B)

	A in ml.	E in ml.	B in ml.	1% L in ml.	D.W. in ml.	N/10 KOH used in ml.
(1)	1	2	2	2	10	11.4,11.4,11.3
(2)	x	2	2	2	11	10.2,10.3,10.0
(3)	1	2	2	x	12	10.3,* 9.7,9.5
(4)	1	2	2	2	14	9.7,9.7,9.7
(5)	1	x	2	2	12	10.1,10.1,10.3
(6)	x	2	2	x	13	9.3,9.2,9.2

Reaction time - 36 hrs. Room temperature - 25°C. ± 2° * Rejected

Method

Acetaldehyde, amyl acetate, the buffer and lipase were kept in glass stoppered Erlenmeyer flasks. The buffer used was ammonium chloride ammonium hydroxide buffer of pH - 8.8.

The flasks were kept at room temperature with occasional shakings. The titration was done with alcoholic KOH using bromothymol blue as the indicator.

Discussion

Hydrolysis in the presence of aldehyde = (1)-(5) = 11.36—10.16 = 1.20 ml.

Hydrolysis in the absence of aldehyde = (2)-(6) 10.16—9.23 = .93 ml.

It therefore follows that the presence of acetaldehyde does not inhibit the hydrolysis of amyl acetate in a buffer system.

Experiment No. 48

To Study the Effect of Calcium Chloride (C) on the Hydrolysis of Amyl Acetate in the Absence of Buffer

	E	5% L	2% C	N/10 KOH used	Average	Temp.	Time
(1)	2 ml.	2 ml.	2 ml.	4.3,4.3,4.35	4.35	30°C.	4 hrs.
(2)	x	2 ml.	2 ml.	3.05,3.05,2.55	2.88	30°C.	4 hrs.
(3)	2 ml.	2 ml.	x	5.1,5.5,5.1	5.23	30°C.	4 hrs.
(4)	x	2 ml.	2 ml.	.1,.2,.2	0.17	30°C.	4 hrs.
(5)	2 ml.	x	x	.1,.1,.1	0.10	30°C.	4 hrs.
(6)	x	2 ml.	x	2.3,2.6,2.7	2.53	30°C.	4 hrs.
(7)	x	x	2 ml.	.1,.1,.1	0.10	30°C.	4 hrs.

Method

Amyl acetate, lipase, calcium chloride were all kept in glass stoppered Erlenmeyer flasks in a thermostat. They were occasionally shaken. After 4 hrs., 20 cc. of isopropyl alcohol were added and the amount of fatty acids was determined by titration with .1 N KOH, bromothymol blue being used as the indicator. Since

all the digestion mixtures in a comparative set of experiments could not be titrated at the same time, it was necessary in order to stop the enzyme action to keep the flasks immersed in crushed ice during the interval between removal from bath and titration.

Discussion

The data show that the presence of calcium chloride in an unbuffered system inhibits the hydrolysis of amyl acetate. It also brings about the following points:

(1) Comparison of (2) and (6) shows that lipase reacts with calcium chloride with the liberation of hydrogen ions.

(2) Actual hydrolysis in the presence of calcium chloride = (1)—(2) = 4.35—2.88 = 1.47 ml. of KOH

(3) Actual hydrolysis in the absence of calcium chloride = 5.23—2.53—.1 = 2.60 ml. of KOH

(4) Actual hydrolysis of ester in the absence of lipase = .1 ml. of KOH

Experiment No. 48 (Part 2)

E	5% L	2% C	Vol. of N/10 KOH used	Average	Temp.	Time
2 ml.	2 ml.	2 ml.	5.45,5.85,5.2	5.50	30°C.	3 hrs.
x	2 ml.	2 ml.	4.05,3.75,3.5	3.76	30°C.	3 hrs.
2 ml.	2 ml.	D.W.(2 ml.)	6.20,6.25,6.25	6.23	30°C.	3 hrs.
2 ml.	D.W.(2 ml.)	2 ml.	.22,.28,.15	0.22	30°C.	3 hrs.
2 ml.	D.W.(2 ml.)	D.W.(2 ml.)	.15,.15,.15	0.15	30°C.	3 hrs.
x	2 ml.	D.W.(2 ml.)	4.00,3.95,4.1	4.02	30°C.	3 hrs.
x	D.W.(2 ml.)	2 ml.	.15,.1,.15	0.12	30°C.	3 hrs.

Indicator

Thymolphthalein

Discussion

The method used and the results obtained were similar to the first set.

Experiment No. 48 (Part 3)

Amyl Acetate	Lipase	$CaCl_2$	Vol. of N/10 KOH used	Average	Temp.	Time
2 ml.	2 ml.	2 ml.	4.63,4.75,4.50	4.62	30°C.	3 hrs.
x	2 ml.	2 ml.	3.38,3.4,3.35	3.37		
2 ml.	2 ml.	x	5.6,5.4,5.6	5.53		
x	2 ml.	2 ml. water	3.30,3.30,3.5	3.36		

Indicator

Phenolphthalein

Method

As in 1st set.

Discussion

In all the three sets we have got consistent results showing that the presence of calcium chloride inhibits the hydrolysis of amyl acetate in an unbuffered system.

Experiment No. 49

To Study the Effect of Time on pH of Different Solutions

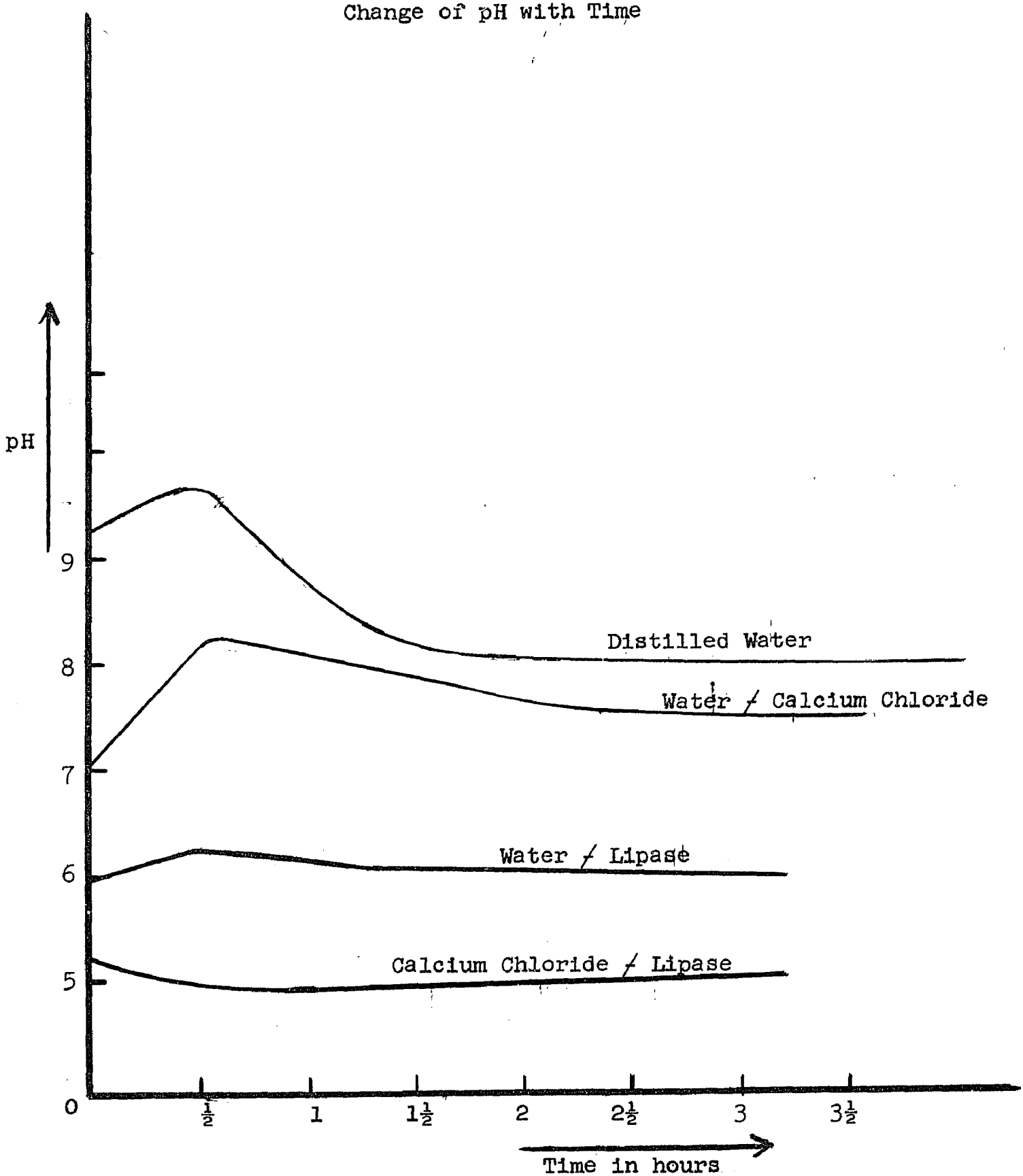
Time in min.	10 ml. of 5% L 10 ml. of 2% C	10 ml. of 2% $CaCl_2$ 10 ml. of D.W.	20 ml. of D.W.	10 ml. of 5% L 10 ml. of D.W.
0	5.20	7.00	9.30	6.00
30	4.90	8.15	9.60	6.20
65	4.90	8.00	8.30	6.10
90	4.95	7.90	8.10	6.10
105	4.95	7.60	8.10	6.10
150	5.00	7.50	8.00	6.05
180	4.90	7.50	8.00	6.05

Discussion

With D.W. the increase of pH may be due to dissolution of glass and decrease in pH due to the absorption of carbon dioxide from the atmosphere. The increase in pH of lipase solution is not significant due to its buffering action. Decrease in pH due to interaction between L and $CaCl_2$ overcomes the effect of dissolution. See Graph #1.

GRAPH NO. 1

Change of pH with Time



Experiment No. 50

To Find the Effect of Calcium Chloride Alone and With Albumin on the Hydrolysis of Amyl Acetate by Steapsin in a Buffered System

2% C	5% L	E	NH_4Cl-NH_4OH buffer	D.W.	N/10 KOH	Albumin
2 ml.	2 ml.	2 ml.	2 ml.	2 ml.	19.0,18.5	x
x	2 ml.	2 ml.	2 ml.	4 ml.	16.1,16.3	x
2 ml.	2 ml.	x	2 ml.	4 ml.	15.80,15.80	
2 ml.	2 ml.	2 ml.	2 ml.	x	19.4,19.3	2 ml.
2 ml.	x	x	x	x	5.2,5.2	x

Temperature - 30°C. pH - 8.8 Digestion period - 4 hrs.

Method

The method used in this case was that of Willstatter, Waldschmidt-Leitz, Ber., 54, 2988 (1921). To the contents of the glass stoppered Erlenmeyer flasks were added 100 ml. of absolute alcohol and 25 ml. of ether. The titration was then done with alcoholic KOH using thymolphthalein as the indicator.

Discussion

It appears from the comparison of (1) and (2) as if calcium chloride helps in the hydrolysis of amyl acetate. But when (5) also is taken into consideration it follows that calcium chloride inhibits the hydrolysis of amyl acetate. This inhibition may result from the fact that there is a lowering of pH when calcium chloride reacts with lipase. Comparison of (1) and (4) shows that albumin helps in the hydrolysis of amyl acetate.

Chapter III

Scope and Limitations of the Enzymatic Method
Results of Running Unknowns

The enzymatic method for the detection of esters is simple and in most cases rapid. Interferences from most classes of organic compounds are negligible. The principal interferences arise from specific compounds such as nitrobenzene, nitroethane, benzaldehyde, benzyl alcohol and acetophenone. In most of these cases the interferences are removed if the test is made using a 20% aqueous emulsion of the unknown. Acids and anhydrides do not interfere, while the interference due to acid halides is quickly detected with a little practice. In order to test the reliability of the method, a number of unknowns were run. The results are given below.

No.	Substance Given	Substance Found
1	Acetone	Ester absent
2	Ethyl alcohol	Ester absent
3	Chloroform	Ester absent
4	Ethyl acetate	Ester present
5	Toluene	Ester absent
6	Propionaldehyde	Ester absent
7	Ethyl acetate	Ester present
8	Sucrose	Ester absent
9	Urea	Ester absent

10	Acetamide	Ester absent
11	Glycerine	Ester absent
12	Ethyl acetate	Ester present
13	Dextrose	Ester absent
14	Ethyl acetate	Ester present
15	Diethyl ether	Ester absent
16	Nitrobenzene	Ester absent
17	Acetic anhydride	Ester absent
18	Benzoyl chloride	Ester absent
19	20% Acetanilide in alcohol	Ester absent
20	Ethyl propionate	Ester present
21	Benzaldehyde	Ester present
22	Benzoic acid in alcohol	Ester absent
23	Methyl benzoate	Ester present
24	Nitroethane	Ester present
25	10% Propionaldehyde	Ester absent
26	Amyl butyrate	Ester present
27	Acetophenone	Ester absent
28	Benzaldehyde	Ester absent
29	Benzyl chloride	Ester absent
30	Methyl salicylate	Ester present
31	10% Acetamide in water	Ester absent
32	Acetic anhydride	Ester absent
33	Benzyl acetate	Ester present
34	Bromobenzene	Ester present
35	Ethyl phthalate	Ester absent

36	Urea in water	Ester absent
37	Acetone	Ester absent
38	Benzaldehyde	Ester absent

Discussion

It will be apparent that the unknowns include most of the organic substances met in qualitative analysis. The test was performed only once and it was not repeated with 20% emulsions in water. Hence it is understandable why some times positive tests were obtained even when the ester was not present. In no case was the ester missed. Even in the case of methyl salicylate, which does not hydrolyze very well, a positive test was obtained. The esters used were: ethyl acetate, ethyl propionate, methyl benzoate, benzyl acetate, methyl salicylate, ethyl phthalate and amyl butyrate. They are fairly representative of aliphatic and aromatic esters.

Chapter IV

Comparison with the Hydroxamic Test

The hydroxamic acid test is the most useful test so far known for the detection of esters. Therefore, a comparison was made of the hydroxamic acid test with the enzyme test. Broadly speaking, the following points need consideration.

1. The hydroxamic acid test gives a positive test with lactones while the enzyme test gives a negative test.

2. The hydroxamic acid test gives a positive test with many trichloro compounds. The enzymatic test does not give a positive test with chloroform.

3. The hydroxamic acid test gives positive tests with most of the nitrogen compounds. The enzyme test gives a negative test with acetamide.

It will thus be clear that the hydroxamic acid test is somewhat limited in its application when compared to the enzyme test. The enzyme test is moreover simpler and does not require that three tests be run at the same time. Unfortunately most of the weak spots in the enzymatic method are also weak spots in the hydroxamic acid test. Thus nitroethane and benzaldehyde give positive tests in both cases.

Table III

Comparison of Hydroxamic Acid and Enzymatic Tests

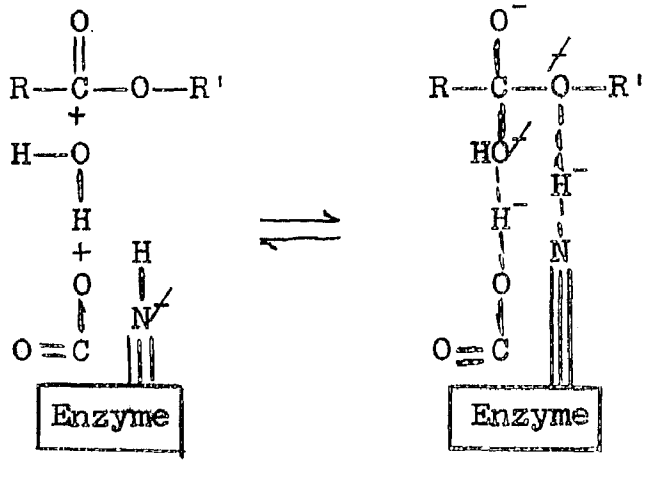
Basis of Comparison	Hydroxamic Acid Test	Enzymatic Test
Number of test usually performed	Three tests are recommended	Usually only one test is necessary
Effect of time	It is best to notice the color within 5 min. The color may diminish with time.	The color is noticed after 3 min. The color increases with time.
Tests with lactones	Gives a positive test	Gives a negative test
Tests with esters of inorganic acids	Negative test	Negative test
Tests with chloral hydrate and other compounds containing $CCl_3C(OH)$- grouping	Positive test	Not studied
Tests with chloroform, bromoform, carbon tetrabromide, trichloro bromomethane, trichloroacetic acid	Positive test	Chloroform gives a negative test
Tests with formaldehyde, benzaldehyde, p-hydroxybenzaldehyde, m-nitrobenzaldehyde, vanillin, anisaldehyde	Weakly positive tests	Weakly positive tests
Formamide	Positive test	Not studied

Basis of Comparison	Hydroxamic Acid Test	Enzymatic Test
Most amides	Positive tests	Negative tests
Acid esters	Weak tests	Weak tests
Lactic acid	Positive test	Positive test
Acetonitrile and propionitrile	Positive test	Not studied
Nitromethane, 2-nitropropane, 2-nitro-1-butanol, 2-nitro-1-chloro-butane	Positive test even without hydroxamic acid	Not studied

Chapter V

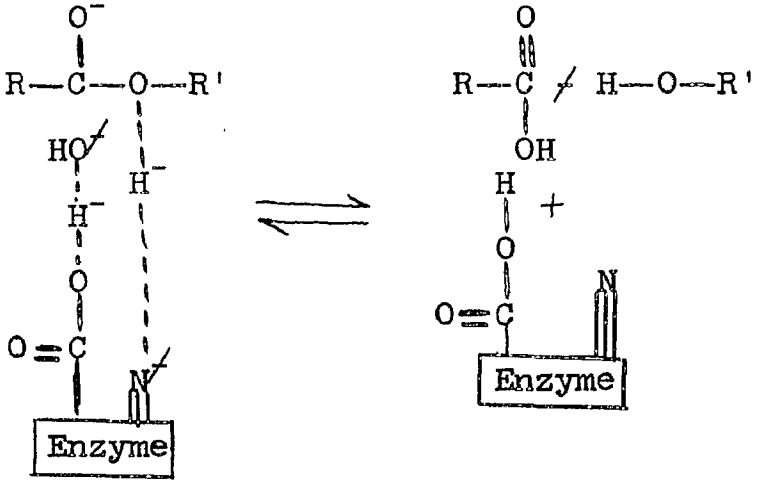
Mechanism of Enzymatic Hydrolysis of Esters

The mechanism of hydrolysis by enzymes is not yet fully known. The simplest way is to consider enzymes as bifunctional catalysts as proposed by Laidler (59a). According to this hypothesis, the active form of the enzyme contains two groups: (1) $-NH_4^+$ and (2) $-COO^-$. The $-NH_4^+$ group on the protein approaches the alcoholic oxygen of the ester and simultaneously the $-COO^-$ group approaches the carbonyl carbon atom. It is further assumed that a water molecule remains between the $-COO^-$ group and the carbonyl carbon atom of the ester. The $-COO^-$ group tends to attract a proton from the water molecule. As the bond between hydrogen and oxygen of the water molecule is weakened, a new bond is simultaneously formed between the carbon atom of the carbonyl group and the oxygen of the water molecule. The process may be represented as follows:



Since it has been established that hydrogen bonds can exist in stable compounds, the ester-enzyme complex can be quite stable. This mechanism also explains the high efficiency of enzymes as catalysts. This results from their bifunctional nature according to this postulation. The fact that the increase in substrate concentration increases the rate of hydrolysis only up to a certain point is also understandable. As the amount of the substrate increases the amount of the complex also increases and when all the enzyme has been tied up any further increase in the amount of substrate should not be of any help in increasing the rate of hydrolysis.

The enzyme substrate complex may be stable with respect to enzyme and the substrate, but at the same time it may readily break down into products as follows:



The mechanism proposed above is of a general nature. It does not imply that always the $-NH_4^+$ and $-COO^-$ groups

are involved. Nevertheless it is a useful hypothesis and helps us in predicting many things. For instance, we can make the following general predictions:

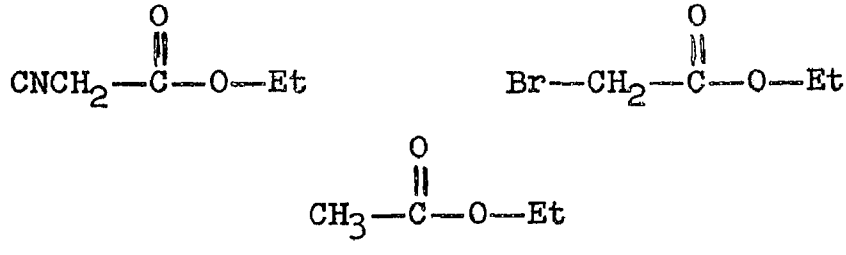
1. Any substituent which helps to increase the electropositive character of the carbonyl carbon atom may help in increasing the rate of hydrolysis and vice versa.

2. Any substituent which helps to increase the electronegative character of the alcoholic oxygen atom may help in increasing the rate of hydrolysis and vice versa.

3. Since only the approach of the proton is depicted to the alcoholic oxygen atom, the R' radical should have less steric effect on the hydrolysis than the R radical.

Let us now try to analyze the trends in hydrolysis which have been observed in the previous investigation.

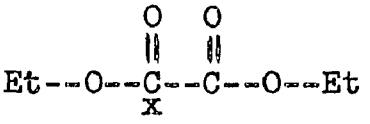
1. It has been found that ethyl bromoacetate and ethyl cyanoacetate are hydrolyzed much faster, for instance, than ethyl acetate.



This can be explained on the basis that the methyl group is a weak electron-releasing group which decreases the electropositive character of the carbonyl carbon atom. While the bromo- and cyano- groups are electron attracting

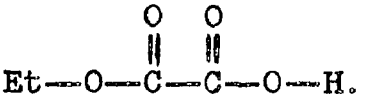
groups which increase the electropositive character of the carbonyl carbon atom by pulling away electrons from it.

2. It has also been observed that the esters of dicarboxylic and tricarboxylic acids are hydrolyzed very easily. To be specific let us consider the case of diethyl oxalate.

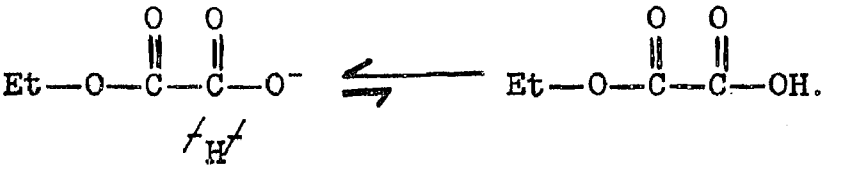


The electron-attracting carbonyl group (marked x) attracts electrons away from the carbonyl group (unmarked) thus increasing the positive character of the carbon atom. This may also help in weakening the —O—Et bond. Thus the hydrolysis of the esters of dibasic acids is very fast.

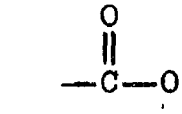
3. The acid esters of the dibasic acids are not easily hydrolyzed. Hydrolysis takes place if the pH is shifted to 5 whereas normally pH = 8 is the optimum pH for the lipase. Taking again the example of oxalic acid, the acid ester has the formula



When the pH is on the basic side, the ester exists mostly in the ionic form



When the ester esists in the ionic form the charged



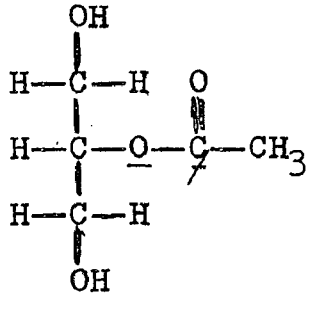
group becomes a strongly electron-releasing group thus decreasing the electropositive character of the carbonyl carbon atom of the ester. It is also possible that the negatively charged carboxyl group offers a better site for the proton to attach itself than the alcoholic oxygen atom.

However, when the pH is decreased to 5, the equilibrium is shifted to the right side

We do not have a charged electron-releasing group and hence the hydrolysis takes place easily.

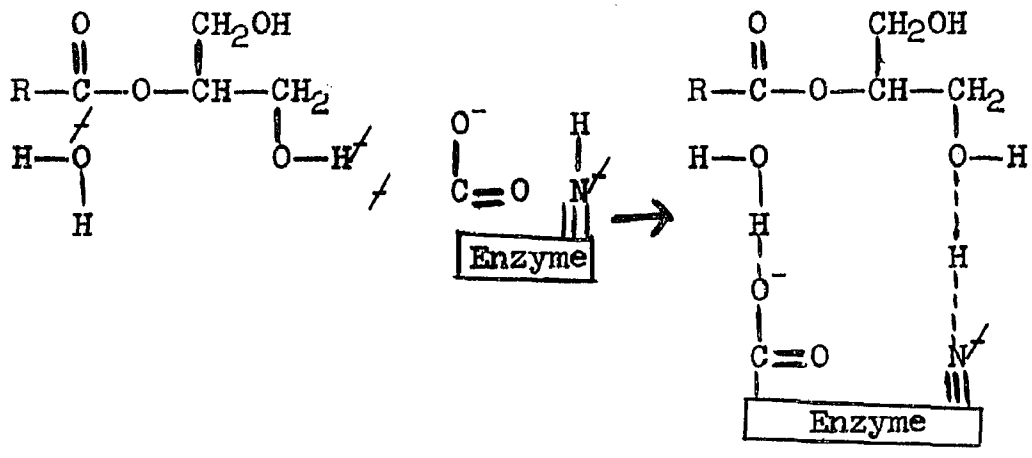
4. It has been established conclusively that lipase hydrolyzes the triglycerides to 1,2-diglyceride and then to 2-monoglyceride. The action of pancreatic lipase then ceases. It has been taken to imply that lipase action is specific for primary hydroxyl groups. That this is not true is shown by our findings that lipase hydrolyzes many esters of secondary alcohols.

A suspicion has been expressed by the reviewer in the annual review of Biochemistry (86a) that the ester bond at the secondary hydroxyl group might not have been hydrolyzed because Mattson and Beck did not use bile salts as an activator. Activators do not usually initiate reactions. The function of bile salts in particular is not to act as an activator but only as an emulsifier. It appears more probable that the reason is to be found neither in the specificity of lipase for the ester bond of the primary hydroxyl group nor in the absence of activators but in the functional characteristics of the compound

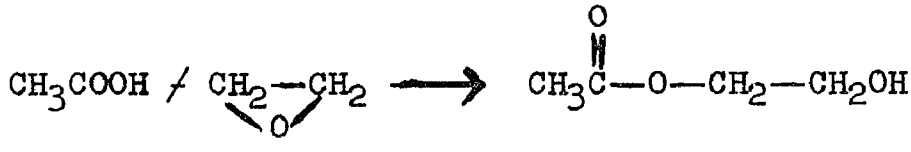


2-monoglyceride

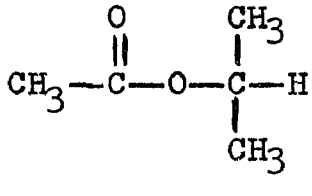
Here the terminal hydroxyl groups offer a better site for the proton of the $-NH_4^+$ group rather than the oxygen atom of the ester bond. This might be depicted as follows:



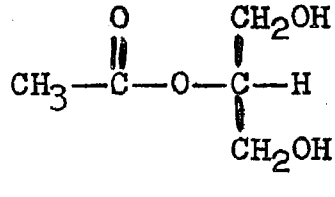
If such an explanation is true it is to be expected that glycol monoacetate will not be hydrolyzed by the lipase even though it does not contain the ester bond of a secondary hydroxyl group. It is proposed to prepare the ester by the action of ethylene oxide on acetic acid and to test this hypothesis



It may also be remarked in this connection that isopropyl acetate is very easily hydrolyzed which lends weight to the reasoning that it is not the secondary nature of the ester bond which is responsible for the lack of activity of the lipase.



Isopropyl Acetate



2-monoglyceride

From what has been said above it should not be implied that the factors discussed above are the only ones to be considered. The steric requirements which allow a close fitting of the enzyme and the substrate for the formation of the enzyme-substrate complex are also extremely important. Even when the enzyme-substrate complex may be formed it is possible that this complex may not easily break up. However, a detailed consideration of such factors requires more knowledge about the structure of the lipases and a detailed study of the kinetics of hydrolysis. Another important consideration may be the products of hydrolysis. It has been found during the present investigation that salicylates in general are much less hydrolyzed than the benzoates. A reasonable explanation may be that salicylic acid has a strong inhibiting effect on the activity of lipase and this does not allow the hydrolysis to proceed to any significant degree.

Chapter VI

Scope for Further Work

One of the main limitations of the enzymatic method is the question of time. The enzymatic reactions are usually slow and depend to a very large extent on the concentration of the enzyme and the substrate. If the time of reaction is increased it can compensate for dilution effects and some exceedingly beautiful tests are obtained. However, there is another factor which has to be borne in mind--the impurities in the lipase exert a buffering action and even when hydrolysis proceeds to a sufficient extent the change in pH is not significant. The indicator is therefore not affected to allow the detection of the ester. One answer to this problem may be in the use of pure lipases. Such an approach does not appear to be feasible at present. It is not known when we would be able to obtain pure lipases or that their use will be economically feasible. Recently Safwat Mohammed reported the preparation of a pure lipase but his claim has been contested by others who say that purified lipase is more active than the crystalline lipase of Safwat Mohammed. A reasonable approach may be the use of ion exchange columns to purify the lipase from its buffering impurities. This would be cheap and can be done in a short time. It is quite possible that if the buffering impurities are removed the sensitivity of the test may be significantly increased

and some esters which need a long time to give a positive reaction may do so in a short time with such a lipase. Such an approach may be more useful from the analytical point of view.

In the present investigation attention has been concentrated on the use of those lipases which are commercially available. For this reason steapsin, wheat germ lipase, horse serum and gland lipase have been used. It is apparent that the availability of the reagents is an important consideration for the usefulness of a test. However, it is also known that liver lipases hydrolyze common esters much better than the pancreatic lipase. It may be useful, therefore, to investigate the use of liver lipase for this test instead of steapsin.

Though it does not always happen, it is usually found that the substrates and the enzymes responsible for their hydrolysis occur side by side in nature. The fragrance of the flowers is mostly due to esters. It is not unreasonable to suppose that the flowers may contain esterases also which may be of interest from the analytical point of view. Some preliminary experiments done, in this connection, did not give positive results, possibly because the esterases are present in flowers in very minute quantities and may require different treatments. As far as is known to us, no effort has been reported in this direction.

An encouraging study has been published by W. N. Aldridge (2) in which he claims to have isolated an aromatic lipase. It is not clear whether this lipase acts on the esters of (1) aromatic alcohols and aliphatic acids, or (2) aliphatic acids and aromatic acids, or (3) aromatic acids and aromatic alcohols or on all of them. It will be interesting to investigate it further to see whether it hydrolyzes salicylates, cinnamates and benzyl benzoate. In that case we would mix this lipase with steapsin and it is possible that this mixture may give better results.

PART II

QUANTITATIVE DETERMINATION OF ESTERS IN MICROGRAM QUANTITIES

Chapter VII

A Review of the Different Methods for the Determination of Esters

The difficulties mentioned in connection with the detection of esters are also inherent in the methods of their determination. Two general procedures are available in this case also: (1) To hydrolyze the ester into the acid and the alcohol and then to determine either the acid or the alcohol formed and (2) To allow the esters to react under such conditions that as far as possible only the ester group may react. The very fact that even today the first method is the most widely used of all methods for the determination of esters is a confession of the truth that acids are easier to determine than the esters. The various methods for the determination of esters have been critically reviewed recently (45). Hence no effort will be made to go into details. Only the more important points regarding the various methods will be mentioned. The entire discussion will be limited to carboxylic esters only.

The Saponification Method

The earliest method used for the determination of esters is the saponification method. The method is based on the principle that the esters of the general formula RCOOR' can be hydrolyzed in the neutral, acidic or alkaline

medium. The alkaline medium is preferred. As in this case, the reaction goes to completion.

$$RCOOR' + OH' \longrightarrow RCOO' + R'OH$$

The method was first used analytically by Koettstorfer in 1878 (58). Since then numerous modifications have been proposed. The improvements have been concerned mostly in the choice of solvents. It has been found advantageous in certain cases to use higher boiling solvents (77, 94). If the esters are difficult to saponify, alkoxides are preferable to alkali hydroxides (12, 56, 57, 60, 72). Some water should be present in the saponification mixture containing an alkoxide (72). Another important advance in this method was the use of double-indicator titration by Riemann (78). In this method, a 5 gm. sample of oil or fat is saponified; the saponification mixture is cooled and titrated to neutral point with phenolphthalein and then after adding 10 ml. of benzene, the reaction mixture is titrated to the green end point using bromothymol blue as the indicator. The acid added between the two end points is equivalent to the soap formed and also to the potassium hydroxide which reacted with the oil during saponification. In this procedure it is unnecessary to know either the exact volume of the potassium hydroxide used or its concentration. The reaction which takes place while titrating from one end point to another may

be represented as follows:

$$RCOO^- + K^+ + H^+ + Cl^- \longrightarrow RCOOH + K^+ + Cl^-$$

For the quantitative saponification of dark colored substances, an ingenious method was proposed by McIlhiney (67). It consists in measuring the alkali used in the reaction by determining the ammonia which it will liberate from an ammonium salt. Electrometric titration methods for determining the saponification value of dark colored substances were first satisfactorily developed by Demarest and Rieman (26). The method has also been applied in the micro and semimicro scale. Five 32 mg. samples are used by Furter (33). A 25 ml. flask with a fused-on condenser was designed. Alcoholic potassium hydroxide was added from a special weighing pipet. Titration with IN sulfuric acid was carried out with a microburette using α-Naphtholphthalein as an indicator. Results were accurate within 5%. The microsaponification technique devised by Van Etten (70) required .05 milliequivalents.

Limitations of the Saponification Method

The saponification method is open to a large number of interferences. Anything which reacts with alkalies is liable to interfere. Acids and anhydrides should be determined separately and corrections should be applied for them. Amides and nitriles are also hydrolyzed by the

alkali to give the corresponding carboxylic acids. Lactones offer another interference. The same is true for lactams. Certain alkali-labile groups, e.g., sulfones, are hydrolyzed by strong alkali and consequently interfere. Aldehydes and other carbonyl compounds also react with alkalies and must be eliminated by suitable means (77). The variable attack of the alkali on the glass is another factor which has to be considered. In addition, such factors as ester transposition and absorption of carbon dioxide during the titration should also be taken into consideration. The method has been applied to milligram quantities but does not appear to have been used for microgram quantities of esters.

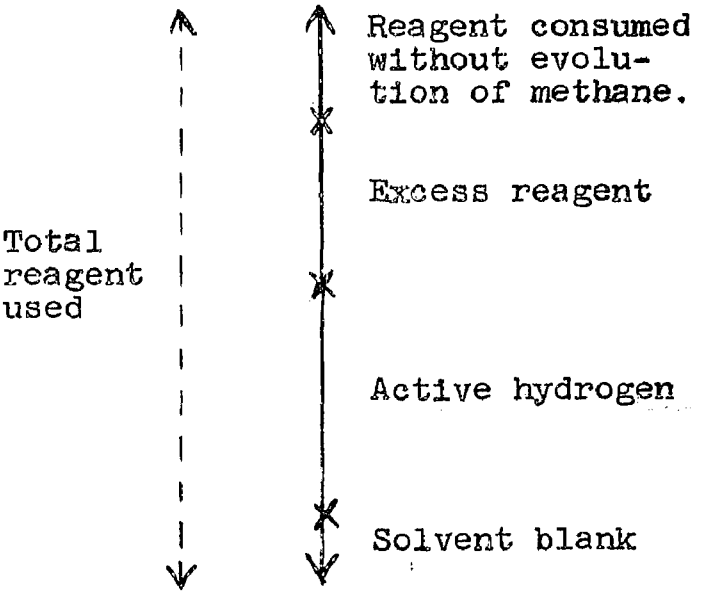
Reaction with Grignard Reagent

The Grignard reaction depends upon the principle that a Grignard reagent, usually CH_3MgI, reacts with substances containing an active hydrogen atom with the liberation of methane and with the substances without an active hydrogen atom the reagent is consumed but no methane is evolved.

$$ROH + CH_3MgI \longrightarrow CH_4 + ROMgI$$

$$RCOOR' + 2CH_3MgI \longrightarrow R-\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{\overset{|}{\underset{|}{C}}}}-OMgI + R'OMgI$$

Thus when a given weight of the reagent is allowed to react with an unknown weight of the ester, it is possible to find the weight of the unreacted reagent by a subsequent treatment with a substance containing active hydrogen atoms, usually aniline. The principles of the method are explained in the adjoining diagram which is a modified version of that given by K. G. Stone (97).



The analytical procedure using the Grignard reaction has resulted from the pioneering work of Zerewitinoff (111). A suitable apparatus was devised by Kohler and associates (55b) in which the total Grignard reagent consumed is measured. The apparatus has been improved by other workers (63, 109).

The use of Grignard reaction for the determination of esters is of very limited value. Aldehydes, ketones, halogen compounds, nitriles, isonitriles and acid halides all behave like esters. Carboxylic acids are unique in the respect that they evolve methane and also react further without evolution of methane. The practical difficulties, despite the improved apparatus, are considerable. Grignard

reagent is extremely sensitive to water. As a result, the apparatus, the inert gas used to fill the system and all reagents must be very carefully prepared and stored so that the blank may be as small as possible. With low molecular weight esters, the results with Grignard reaction are accurate to 2% but in complex cases they may vary 10% from the theoretical. The utility of the method lies in its non-selective character. It may be used where more selective methods cannot be applied, e.g., in the study of mineral oil deterioration where a measure of all the oxidation products of the oil is desired.

Reaction with Lithium Aluminum Hydride

The basic principles and the limitations of lithium aluminum hydride are similar to those of Grignard reagent. It reacts with substances containing active hydrogen atoms.

$$2H_2O + LiAlH_4 \longrightarrow LiAlO_2 + 4H_2$$

Lithium aluminum hydride is an outstanding substance as a reducing agent because it is strong and at the same time may be used at room temperature without special equipment.

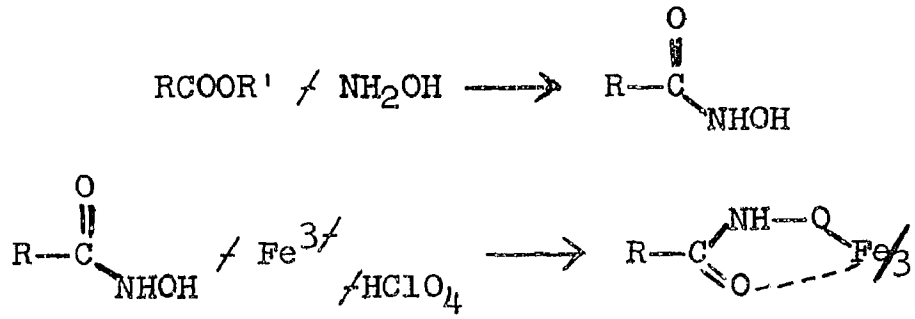
$$2RCOOR' + LiAlH_4 \longrightarrow LiAl(OR')_2(OCH_2R)_2$$

Like the Grignard reagent, lithium aluminum hydride is not specific, not generally applicable, but useful in certain cases. The working procedure of Zaugg and Horrom

(110) is perhaps the most satisfactory. Both acidimetric (47) and potentiometric methods have been described but as yet have not been widely adapted.

Colorimetric Methods

There are not many reactions of esters which result in colored products or in products which may form colored compounds. In addition, most ester reactions tend to be equilibrium processes. The colorimetric estimation of esters is, therefore, not easy. Feigl was the first to develop a spot test for esters which has already been described. It is encouraging to find that this qualitative procedure has been developed into a quantitative tool by Hill (48) and Keenan (54).



Water provides a slight interference, especially in the first step. Hence the first step must be carried out in a dry solvent. Feigl had used HCl and $FeCl_3$ in the second step. Hill showed that if $HClO_4$ and $FeClO_4$ are used the complex follows Beer's law.

The method was extensively studied by Thompson (98) who made a study of the variables of the two reactions

involved and adapted it to the estimation of volatile esters. A determination of esters in the presence of anhydrides has been described by R. F. Goddu and associates (38). They found that when refluxing with hydroxylamine is done in alkaline medium both esters and anhydrides are determined. If refluxing is done in neutral medium only, anhydrides react. The esters are found by difference. The optimum concentration range is 10^{-3}M to 10^{-2}M. Acids, most amides and nitriles do not interfere. M. H. Hack (43) applied the method for the determination of fatty acid esters in lipide extracts. 2-3 Micromoles of ester can be accurately determined by this method. A useful modification of the method has been used by O. A. Guagnini and E. E. Vonesch (42) for the determination of formic esters. The formic esters are converted into formohydroxamic esters by treatment with hydroxylamine in an aqueous alcoholic ammoniacal medium. Under the specified conditions, other carboxylic esters, carboxylic acids, amides, nitriles, imides and acid chlorides do not interfere. The method is sensitive to 5 micrograms or 1 p.p.m. Other esters (x10,000) cause no interference.

The colorimetric method is simple, relatively rapid and versatile. Most of the work reported in the literature is restricted to aliphatic substances. R. T. Hall and W. E. Shaeffer (45) report that the method is successful in the case of aromatic esters also. However,

more work is necessary before the full value of the method can be estimated. The question of interferences has also not been fully looked into. Lactones and anhydrides interfere because they are measured as esters. The interference due to anhydrides can be removed by performing the first reaction in neutral and then in alkaline medium as shown by R. F. Goddu and associates (38). Nothing has been reported about the interference of carbonyl compounds; but since they also react with hydroxylamine, they should also cause some interference.

Physical Methods

A number of esters have been determined polarographically. Such a determination is not due to the presence of the ester grouping but due to the presence of a reducible group in the acidic or the alcoholic part of the molecule. Maleates and fumarates (28), acrylates (99), methacrylates (100), phthalates (102), nitrobenzoates (74) and vinyl esters (87) have been determined polarographically.

The investigations reported on the determination of esters by ultraviolet and infrared spectroscopy are limited but interest has been shown in this field in the past few years and the technique is likely to become more important in the future.

The determination of esters by infrared spectroscopy has been based on absorption of the carbonyl group. Any other carbonyl group may cause interference. Each system must be studied to determine possible interference and to confirm the validity of infrared measurement. Acids and aldehydes both give large interferences with ester and ketone measurements (4). An important advantage is that only 20 mgs. of the sample are required and this is recovered after the test (1).

Discussion

Of the various methods for the determination of esters, two methods stand out with regard to their utility in this field. The methods are: (1) The saponification method and (2) The hydroxamic acid test method. The difficulties encountered in their determination arise from the fact that the esters do not have an individuality of their own, if such a term may be used in scientific literature. There are a large number of compounds having a carbonyl group which interfere—whether we use a chemical method or a physical method. The number of reactions which give rise to colored products are also few. In the saponification procedure the conditions are so drastic that a wide variety of organic compounds react. If the conditions for saponification or hydrolysis could be made less drastic, interference from many types of compounds

could be eliminated. It is surprising that no effort has been made in this direction. No analytical procedure has been reported in which hydrolysis of esters has been accomplished in neutral medium. It is well known that enzymes hydrolyze esters under very mild conditions. They are also specific as far as the ester group is considered. And thus elimination of such common interferences as acids, anhydrides, amides, aldehydes and ketones can be accomplished. It was with this object in view that we developed the qualitative method for the enzymatic detection of esters. Such a method, if it can be converted into a quantitative tool, will be valuable in many respects.

Ion exchangers are another class of organic substances which can hydrolyze esters under mild conditions. They are not as specific as enzymes but they are commercially available and simple in their manipulation. It was, therefore, considered desirable to look into the possibilities of such an approach and with this point of view the present investigation was undertaken.

Chapter VIII

Ion Exchange Method for the Determination of Esters

A. Basic Principles of Ion Exchange

The study of ion exchange started about a hundred years ago with the classical papers of Thompson and Way. A very important step was taken in the development of the ion exchange methods when Adams and Holmes of England prepared synthetic organic ion exchangers. Valuable work was also done by Griessbach (41) and Samuelson (82).

The commercial resins represent a compromise in which the cross linking is neither too much nor too little. They may be regarded as an adsorbent or as a homogeneous solution according to the problem at hand. Their behavior with simple inorganic ions is governed primarily by electrostatic forces and it is a good approximation to regard the resin as existing in a concentrated aqueous solution (23, 37). If, however, the influent contains an organic non-electrolyte interfacial and adsorption effects become important. In such a case the resin may be considered as a solid adsorbent in contact with aqueous solution. Adsorption equations now become applicable (22). In non-ideal cases both the effects mentioned above play their part and the affinity of the ion for the resin may depend on electrostatic factors such as charge and effective size as well as on Van der Waal's forces (24).

With a cation exchange reaction such as

$$BR + A^{+} \longrightarrow AR + B^{+}$$

the following generalizations may be made:

1. Ion-exchange reactions are stoichiometric. For each gram equivalent of A^{+} taken up by the resin, a gram equivalent of B^{+} is given up to the solution. This equivalence may sometimes be unobservable owing to other effects.

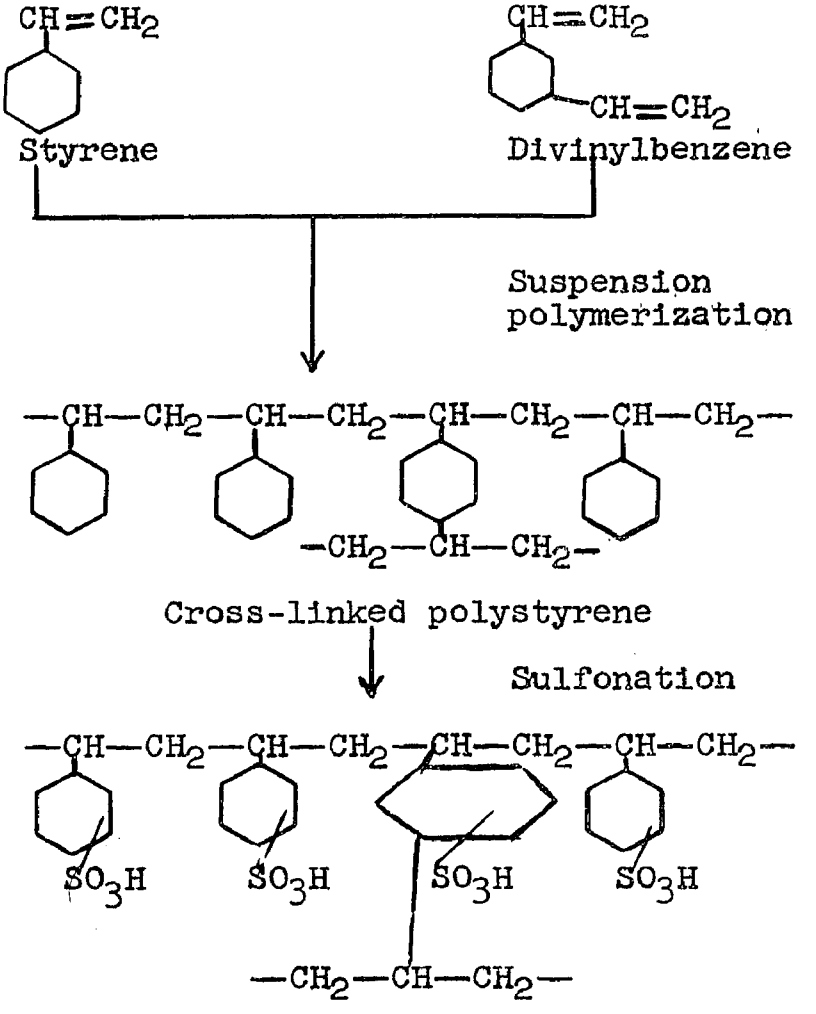
2. Ion-exchange reactions are in general reversible. That is to say, the reaction mentioned above proceeds both ways. It is possible to shift the equilibrium to the right by using a very large concentration of A^{+} or by choosing A^{+} such that it has much greater attraction for the resin than B^{+}. Similarly, if B^{+} ions are removed from the solution, the forward reactions will proceed to completion.

3. Ion-exchange resins show no hystereses. The ionic composition of a resin in a given solution will always be the same no matter from which side the equilibrium is approached. If this is not so, it may be due to the fact that equilibrium has not been attained (44).

4. All the acidic (or basic) groups in an ion exchange resin are accessible as exchange sites for small ions.

The total exchange capacity for small ions, therefore, does not depend on particle size.

Ion exchange resins consist of a cross-linked polymer network to which are attached ionizable groups. The ionizable groups may be acidic or basic, strong or weak. Sulfonic acid group is a strongly acidic group while a carboxyl group is a weakly acidic group. An example of a strongly acid cation is given below.



Sulfonated cross-linked polystyrene

Owing to the presence of a large number of polar groups, the ion exchange resins are strongly hydrophilic.

They swell and shrink by the adsorption and desorption of water. Their solubility in water is prevented by the cross-linked network. The ion-exchange behavior depends largely on the nature of groups present. The characteristic behavior of ionizable groups in simple compounds is reproduced in the resins also, as can be verified by titrating the resins with an acid or base. A sulfonic acid cation resin appears to be fully ionized. A carboxylic resin behaves as a weak acid and its theoretical capacity can be achieved in alkaline solution but in acid solution the capacity is negligible. The same is true of the basic resins. Weakly basic resins cannot be used for the absorption of weak acids since the latter are ionized appreciably only in acid solutions. Strongly basic resins absorb weak acids such as carbon dioxide and acetic acid.

The salts of strongly acidic or strongly basic resins are stable and do not hydrolyze on washing. The salts of weakly acidic and weakly basic resins are unstable and hydrolyze slowly when washed with water.

The distribution of ions is not solely determined by the ion exchange reaction. If dry resin in the hydrogen form is added to the solution of an acid the resin absorbs water and, also, acid diffuses into the resin. Since water is preferentially absorbed, the acid concentration of the surrounding solution increases (11, 23, 75). Non-electro-

lytes or undissociated electrolytes may be appreciably adsorbed by the resin (40a).

One of the most important properties of ion exchange resins is that in general they show selective adsorption. They adsorb different ions to different extents. Thus for univalent cations we have

$$\frac{[B_R]}{[A_R]} = \frac{K_A^B [B_S] r_B}{[A_S] r_A}$$

where B_R, A_R, B_S and A_S represent the concentration of the two ions in the resin and the solution. A and B are the corresponding activity coefficients and K_A^B is the relative affinity of the two ions for the resin.

The relative affinity of a pair of ions remains constant only for a narrow range of concentration. The organic ions are subject to large variations in this respect. They are very strongly held when their amount is very small but if their concentration increases their affinity for the resin decreases since large ions cannot be accommodated easily within the resin framework.

B. Use of Ion Exchange Resins for the Determination of Esters

1. Principle

The ion exchange resins act as catalysts. Thus we have

$$CH_3COOC_2H_5 + H_2O \xrightarrow{H^+} CH_3COOH + C_2H_5OH$$

The acetic acid liberated during the hydrolysis decreases the pH. Since the reaction is a reversible one, it takes a long time and requires sometimes higher temperature to go to completion. However, if the conditions of the experiment are the same the degree of hydrolysis of the ester will also be the same. Thus for each ester there will be a lowering of pH depending upon its concentration. The decrease in pH can be related to the concentration of the ester through a calibration curve.

It is possible to use anion exchange resins as well as cation exchange resins for hydrolysis. However, in the anion exchange resins, the acid liberated in hydrolysis will be taken up by the resin to form water as follows:

$$ROH + CH_3COOH \longrightarrow CH_3COOR + H_2O$$

where R represents the non-hydroxylic part of the anion exchange resin. Since it is much easier to detect an acid than an alcohol, the cation exchange resins are to be preferred.

2. Theoretical Limitations

a. Taking the case of ethyl acetate as an example, we have

$$CH_3COOC_2H_5 + H_2O \longrightarrow CH_3COOH + C_2H_5OH$$

$$\underset{(a - x)}{CH_3COOH} \longrightarrow \underset{x}{CH_3COO^-} + \underset{x}{H^+}$$

$$\frac{x^2}{a - x} = K_a$$

which gives $\log x = \frac{1}{2} \log K_a + \frac{1}{2} \log (a - x)$;

if x is very small, $a - x \approx a$

and $-\log x = -\frac{1}{2} \log K_a - \frac{1}{2} \log a$

or $\quad pH = -\frac{1}{2} \log K_a - \frac{1}{2} \log a.$

Let us suppose that when we change the concentration from a to a/y, then the pH changes from pH to pH'. Then we have

$$pH' - pH = \tfrac{1}{2} \log y.$$

However, the change in pH which can be determined on the pH meter is 0.1 pH units, which gives

$$0.1 = \tfrac{1}{2} \log y$$

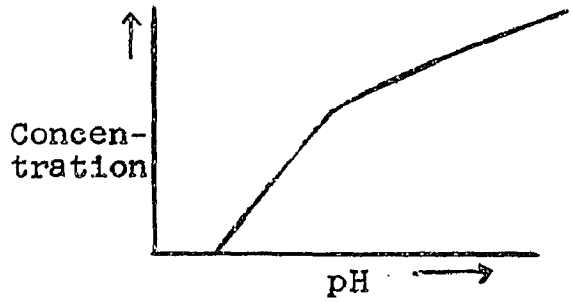
$$\text{or} \quad y = 3.$$

This shows that theoretically when the concentration changes three times the change in pH is only 0.1 pH units. Hence it may not be possible to detect a change in two solutions if their concentrations have a ratio of less than three.

b. The second limitation arises from the fact that

$$pH = -\tfrac{1}{2} \log K_a - \tfrac{1}{2} \log a$$
$$= b \log c,$$

which gives a curve of the type shown. Thus it is clear that there will be a greater change in pH with concentration at lower concentrations than at higher concentrations and the accuracy of the determination will increase as the concentration decreases.



C. EXPERIMENTAL

The present section describes the experimental approach used for the quantitative determination of esters with the help of ion exchange resins. The experiments were set up to check the reproducbility of the resins and the effect of common interferences on the hydrogen ion concentration of the effluent. Ethyl acetate was used as a representative of common esters in evaluating the experimental approach and the cation exchange resin was invariably used to catalyze the hydrolysis. Unless otherwise stated the procedure was the same as given below.

Reagents and Instruments:

(1) Beckman Model B pH meter

(2) 60 mesh or 40 mesh Amberlite I.R. 120 cation exchange resin.

(3) A small ion exchange column (4'5" long & 4" in diameter) attached to a separatory funnel.

(4) Deionized water

Procedure:

The resin was regenerated and sieved to obtain the proper mesh. A glass column was filled with the resin using cotton plugs to hold the resin bed in place. Deionized water was then passed through the resin and the instantaneous pH taken. The effluent was collected in

3 ml. portions until the pH became constant. Ten milliliters of the ester of a known concentration was then added to the column by means of a separatory funnel. The effluent was again collected in pH meter beakers and the pH of each increment was noted. This procedure was repeated with a number of known samples. With each sample the lowest pH (an average of 4 runs) was used in preparing the calibration curve. The unknown sample was then treated in the same manner as used in preparing the calibration curve. The lowest pH obtained with the unknown was noted and its concentration was then found from the curve. The same source of water was used for the unknown as for the known samples. The rate of flow of knowns and unknowns through the exchange bed was made as comparable as possible and pH determinations were likewise made using standardized technics.

Experiment No. 51

Determination of the Sensitivity of the Ion Exchange Method

Method:

Twenty mesh Amberlite I.R. 120 was regenerated, crushed and separated into different grades with the help of a sieve. The resin was placed in a simple ion-exchange column and washed with distilled water until the effluent reached a constant pH level of four. Then 10 ml. of the ethyl acetate solution of a known strength was added and the column was washed again with 20 ml. of distilled water. The following results were obtained.

Flow rate - 8 drops per minute.

Flow rate - 2 drops per minute.

Serial number	Volume of effluent	pH of the effluent with 1 gm per liter ethyl acetate	pH of the effluent with 10 mgm per liter ethyl acetate	pH of the effluent with 100 γ per liter ethyl acetate	pH of the effluent with 50 γ per liter ethyl acetate
1	3 ml	4.8	5.1	5.0	5.0
2	"	5.0	5.0	5.0	5.0
3	"	4.8	4.95	5.0	5.0
4	"	4.7	4.7	5.0	4.9
5	"	4.8	4.5	5.0	4.9
6	"	4.5	4.4	4.98	4.85
7	"	3.8	4.4	4.95	4.78
8	"	3.75	4.4	4.92	4.75
9	"	4.0	4.6	4.92	4.62
10	"	3.8	4.65	4.92	4.62
11	"	3.9	4.70	5.0	4.70
12	"	4.05	4.85	5.0	4.75

13	"	4.4	4.8	5.0	4.75
14	"	4.4	4.8		
15	"	4.4	4.8		
16	"				

Discussion

The results show that with the rate of flow at 8 drops per minute it is possible to detect 100γ per liter of ethyl acetate. The minimum pH obtained for

1 gm per liter ethyl acetate = 3.75

10 mgms per liter ethyl acetate = 4.4

100γ per liter ethyl acetate = 4.92

pH of the distilled water = 5.05

However, when the drop time was increased to 2 drops per minute it was found possible to detect 50γ per liter of ethyl acetate.

Conclusion

Sensitivity of the test under the best conditions is 50γ per liter.

Experiment No. 52

Effect of Flow Rate on the pH of Deionized Water on Passing Through a Cation Exchange Column

Procedure

Deionized water was passed through a cation exchange column and the effect of different flow rates on pH was noted.

Resin = 40 mesh		Resin = 20 mesh	
Rate of flow	pH	Rate of flow	pH
36 drops per minute	4.60	11 drops per minute	4.55
24 drops per minute	4.65	3 drops per minute	4.60
6 drops per minute	4.68	2 drops per minute	4.75
4 drops per minute	4.70	1 drop per minute	4.75

Conclusion

Two drops per minute is the maximum speed suitable for the attainment of equilibrium in 20 mesh resins. With 40 mesh beds rates up to 6 drops per minute may be used.

Experiment No. 53

To Determine the Sensitivity of the Test Using the Resins in the Powder Form

Part 1: Washing of Resins

Procedure

Resins of 20 mesh, 40 mesh, 60 mesh and 100 mesh were washed with water till the pH of the wash was the same as the pH of the distilled water. Prior to this treatment the resins had been regenerated with 4 M HCl. The washed resins were allowed to dry at room temperature (25-30°C) for three days. After they were dry 500 mgms of each was taken in a separate beaker and to them 20 ml. of distilled water was added. In a separate beaker 10 ml. of distilled water was also kept so as to serve as a check for any change due to absorption of gases from the atmosphere. Approximately after 24 hours the pH was recorded. The water was thrown away and fresh distilled water was added to all of the resins. This procedure was repeated for 12 days.

Part 2: Hydrolysis of Ethyl Acetate

The resins were again washed and dried. Ester solutions of different concentrations were treated with 50 mgms of 40 mesh resin and the pH was noted after shaking for 2 and 4 min.

Ester Concentration	T = 0 minutes	T = 2 minutes	T = 4 minutes
100 γ	5.5	5.1	5.00
50 γ	5.5	5.15	5.08
20 γ	5.5	5.2	5.08
Distilled water	5.5	5.20	5.11

Remarks

It is possible to detect up to 50 γ per liter ethyl acetate.

Conclusion

Even after washing for 12 days the resins give out acids and hence it is necessary to use blanks. The above results show that if the concentration of the ester is decreased below 50 γ per liter the behavior of the solution is the same as that of distilled water. Ther is not much difference between the behavior of distilled water and 25 γ ester solution. The same results [illegible] by the column method.

	pH After 12 hours	pH After 1½ days	pH After 3 days	pH After 12 days
Distilled water	4.9	4.9	4.9	5.3
100 mesh resin	4.5	4.3	4.4	4.8
60 mesh resin	4.55	4.4	4.5	4.9
40 mesh resin	4.55	4.4	4.5	5.0
20 mesh resin	4.55	4.4	4.6	5.2
Folins' permulit	4.9	4.55	4.72	5.3

Experiment No. 54

To Study the Interference due to Alcohol

Concentration of Alcohol = 10 ml. per liter

Number	Volume of effluent	pH of effluent	Number	Volume of effluent	pH of effluent
1	3 ml.	5.25	1	3 ml.	4.90
2	"	5.22	2	"	4.75
3	"	5.25	3	"	4.75
4	"	5.20	4	"	4.62
5	"	5.00	5	"	4.50
6	"	4.90	6	"	4.50
7	"	4.90	7	"	4.50
8	"	4.90	8	"	4.48
9	"	4.90	9	"	4.50
10	"	4.85	10	"	4.50

Conclusion

10 ml. per liter of alcohol solution decreases the pH by about .4 units.

Experiment No. 55

To Study the Effect of Passing the Same Deionized Water Again and Again Through the Same Column

Number of times water is passed through the column	pH
0	5.6
1	5.5
2	5.30
3	5.25
4	5.15
5	5.10

$$\frac{\text{Total fall in pH units}}{\text{Number of times}} = \frac{0.5}{5.0} = 0.1 \text{ pH units}$$

Conclusion

The average decrease in pH is 0.1 unit in passing deionized water through cation exchange column.

Experiment No. 56

To Study the Interference due to Acetone

pH of deionized water = 5.10

pH of the acetone solution prepared in this water after passing through the column = 4.88

∴ Difference in pH = .22 pH units

Strength of Acetone solution = 10 ml. per liter

Since from our last experiment the result of passing deionized water once through the column was to decrease the pH by 0.1 pH unit, the actual decrease due to acetone was only 0.12 pH units which is not significant considering the high concentration of the acetone solution.

Conclusion

Interference due to acetone is negligible.

Experiment No. 57

To Study the Interference due to Acetaldehyde

Part I

Strength of acetaldehyde solution = 10 ml. per liter

pH of aldehyde solution = 3.7

The column was first washed until an almost constant pH was obtained and then 80 ml. of acetaldehyde solution was added. The column was again washed twice with 100 ml. of deionized water each time. The effluent was collected in the 5 ml. pH meter beaker and the pH determined.

pH in washing the column	pH after adding aldehyde	pH in 1st washing after adding aldehyde	pH in 2nd washing after adding aldehyde	Minimum pH
4.80	5.12	3.72	4.55	
4.80	5.05	3.72	4.55	
4.95	4.00	3.72	4.75	
5.00	4.00	3.90	4.80	
5.00	3.75	4.20	5.10	3.62
5.00	3.65	4.70	4.90	
5.10	3.62	4.85	4.70	
5.15	3.72	4.90	4.70	
	3.60	4.75	5.10	
	3.62	4.70	5.10	
		4.55	5.10	
		4.55		

Part II (Repetition)

Concentration of Acetaldehyde Solution = 10 ml per liter

pH of the Acetaldehyde Solution = 3.62

pH before adding acetaldehyde in washing the column	pH after adding acetaldehyde	pH of 1st washing	pH of 2nd washing	Minimum pH
4.20	4.90	3.55	4.80	
4.55	4.90	3.70	4.95	
4.75	3.90	3.70	5.00	
4.82	3.70	3.90	5.00	3.55
4.90	3.65	4.20	5.12	
4.85	3.72	4.50	5.20	
4.85	3.70	4.55	5.10	
4.90	3.62	4.70	5.08	
4.90		4.72	5.08	
4.90		4.75		
4.92		4.75		
4.92		4.82		
4.90		4.82		
		4.80		
		4.80		
		4.72		
		4.70		
		4.68		
		4.68		

Conclusion

Acetaldelyde does not interfere in this method of determination of esters. In the first case the pH of the acetaldelyde solution was 3.7 and the lowest pH obtained after passing the aldelyde solution through the column was 3.62 which is what should be expected. In the second case also the pH of the aldelyde solution is 3.62 and the lowest pH recorded = 3.55. The expected pH would have been 3.52 taking into consideration the fact that even in passing deionized water an average decrease in pH is 0.1 pH units.

Experiment No. 58

To Study the Interference due to Formaldehyde

Concentration of formaldehyde = 10 ml. per liter

pH of formaldehyde solution = 4.1

Washing prior to the addition of formaldehyde	pH after adding formaldehyde	1st washing after adding formaldehyde	2nd washing after adding formaldehyde	Lowest pH
4.65	5.20	3.90		
4.95	4.70	4.00		
5.20	4.30	3.92		
5.10	4.00	4.35	Not necessary	
5.15	3.98	4.90		
5.18	3.92	5.18		3.82
5.05	3.90	5.28		
5.15	3.90	5.35		
5.12	3.90	5.35		
5.12	3.82	5.32		
5.12	3.90	5.35		
5.12		5.40		
5.15		5.50		
		5.42		
		5.50		
		5.40		
		5.45		
		5.40		

Conclusion

There is a decrease of 0.18 pH units in this case. This shows that there is a slight interference from formaldehyde.

Experiment No. 59

To Study the Interference due to Urea

Concentration of urea solution = 7.6 gms. per liter

pH of urea solution = 6.0 pH of distilled water = 5.00

f the washing	pH after adding urea solution	pH after the first washing	Lowest pH
5.35	4.35	4.10	
5.25	4.35	4.10	
5.10	4.45	4.25	
5.12	4.10	4.60	
5.10	4.10	5.00	
5.05	4.10	5.00	4.05
5.10	4.05	4.98	
5.10	4.05	4.95	
4.95	4.05	5.00	
5.05	4.10	5.10	
5.00	4.15	5.10	

Conclusion

Urea interferes considerably. It is probable that the urea is hydrolyzed by the cation exchanger to give ammonia and carbon dioxide which results in the formation of ammonium carbonate or bicarbonate. The cation exchanger takes up the ammonium ions and releases an equivalent amount of hydrogen ions which lowers the pH.

$$NH_2\ CO\ NH_2 + H_2O \longrightarrow 2NH_3 + CO_2$$

$$NH_3 + H_2O \longrightarrow NH_4OH$$

$$CO_2 + H_2O \longrightarrow H_2CO_3$$

$$NH_4OH + H_2CO_3 \longrightarrow (NH_4)_2\ CO_3$$

$$(NH_4)_2\ CO_3 \xrightarrow[\text{exchanger}]{\text{cation}} H_2CO_3$$

Experiment No. 60

To Study the Interference due to Glucose.

Concentration of the glucose solution = 1 gm per liter

pH of the glucose solution = 2.95

Washing of the column	pH after adding glucose solution	First washing	Second washing	Lowest pH	Inference
5.20	5.12	2.80	4.20		
5.01	5.05	2.90	4.20		
5.05	4.65	2.90	4.40		
5.25	3.50	2.90	4.40		
5.10	3.20	3.00	4.50	2.80	Glucose does
5.12	3.10	3.05	4.40		not interfere
5.12	3.00	3.12	4.50		
5.05	2.95	3.10	4.40		
5.00	2.90	3.35	4.50		
5.05	2.92	3.38	4.50		
	2.90	3.40	4.60		
	2.90	3.60	4.60		
	2.80	3.60	4.90		
	2.90	3.75	4.90		
	2.90	3.82	4.80		
		4.10	5.00		
		4.10	5.00		

Experiment No. 61

To Study the Interference due to Cane Sugar

pH of the sugar solution = 5.5

Concentration of sugar solution = 1 gm per liter

Washing of the column	pH after adding sugar solution	First subsequent washing	Lowest pH
5.25	5.15	4.82	
5.25	5.15	4.80	
5.10	4.99	5.20	4.82
5.05	4.88	5.50	
5.25	4.88	5.40	
5.38	4.82		
5.20	4.90		
5.30	4.90		

Conclusion

Cane sugar solution causes considerable interference. The cause of this interference may lie in the presence of small quantities of salts which react with the cation exchanger to release hydrogen ions.

Experiment No. 62

To Study the Effect of Using Larger Amounts of Solution for Analysis

Procedure

Deionized water was passed through the column until the pH became constant, then the ester solution was added and the pH of the effluent was noted. Concentration of the ethyl acetate solution = 2 ml. per liter and resin = 60 mesh.

pH on using 10 ml. of ethyl acetate solution	pH on using 20 ml. of ethyl acetate solution	pH on using 10 ml. of ethyl acetate solution	pH on using 30 ml. of ethyl acetate solution	Lowest pH
5.30	5.20	5.40	5.25	
5.25	5.05	5.45	4.50	
4.50	4.83	5.00	4.00	
3.80	3.70	3.90	4.00	
3.70	3.50	3.70	3.70	
3.90	3.70	3.90	3.50	for 10 ml. of ethyl acetate - 3.70
4.00	3.80	4.00	3.45	
4.10	4.00	4.20	3.45	for 20 ml. of ethyl acetate - 3.50
4.30	4.15	4.35	3.80	
4.50	4.30	4.50	3.75	for 30 ml. of ethyl acetate - 3.45
4.65	4.50	4.60	3.75	
4.80	4.62	4.70	4.00	
4.90	4.70	4.80	4.40	
4.90	4.75	4.90	4.70	
4.90	5.00	4.95	5.00	
		5.00	5.10	

Conclusion

It appears from this experiment that there is no advantage in using larger volumes of solution for analysis. On the other hand, washing takes more time if a larger amount of solution is used for analysis.

Experiment No. 63

To Study the Effect of Passing the Deionized Water Twice Through the Same Cation Exchange Column.

pH on passing deionized water once	pH on passing deionized water again	Inference
4.70	4.6	Even if the deionized water is passed twice through the cation exchange column the pH still varies. However, the range in both cases is the same.
4.70	4.6	
4.60	4.4	
4.62	4.6	
4.70	4.6	
4.70	4.7	
4.50	4.45	ΔpH = (4.70 - 4.40) = 0.30 pH units
4.40	4.40	
4.60	4.48	
4.70	4.45	
4.70		
4.65		
4.65		
4.50		

Experiment No. 64

To Study the Effect of Washing the Resin with Alcohol on the Consistency of the pH

Procedure

The 40 mesh resin was washed several times with alcohol. The alcoholic solution turned yellow showing that the resin dissolved to some extent. The resin was then repeatedly washed with deionized water. When deionized water was now passed through the cation exchange column the following pH were obtained:

pH of the effluent	Inference
5.90	
5.70	
5.50	Washing with alcohol does not
5.60	
5.60	appear to help. The pH change = (5.90 - 5.50)
5.58	
5.60	= 0.4 pH units
5.60	
5.60	
5.55	
5.55	
5.60	
5.55	

Experiment No. 65

To Check the Reproducibility of the Alcohol Washed 40 mesh Resin

pH after passing through the column	pH without passing through the column	Instant pH of effluent	pH of effluent after 5 minutes
5.10	5.55	5.18	5.15
5.15	5.60	5.12	5.30
5.20	5.55	5.25	5.25
5.20	5.50	5.20	5.22
5.20	5.52	5.15	5.16
5.20	5.52	5.20	5.38
5.20	5.52		
5.15	5.49		
5.20	5.52		
5.15	5.52		
5.20	5.52		
5.30	5.49		
5.30	5.58		

Conclusions

The pH of the distilled water varies by about 0.1 pH units. If the distilled water is passed through the column its pH varies by 0.2 pH units. Keeping the solution for 5 minutes is of no help since in this case the pH varies by 0.28 units.

Experiment No. 66

To Study the Effect of Shaking and Standing on the pH of the Deionized Water

Procedure

The deionized water was taken from the deionizing apparatus and kept in the pH meter beaker without shaking for half an hour. In the second set of experiments, after keeping the deionized water in the pH meter beaker, the beaker was shaken and then allowed to stand.

Results

It was found that in all cases the pH of the deionized water on standing first goes down and then goes up. This effect becomes more pronounced with shaking. With each shaking the pH goes down by 0.1 pH units and then rises up. It is therefore suggested that in taking the pH of the deionized water the number of shakings and the time taken in recording the pH must be the same as far as possible.

Time in minutes	Change in pH on shaking	pH before shaking	pH after shaking	pH 1 min. after shaking	pH 2 min. after shaking	pH 3 min. after shaking
0	5.00	5.50	5.30	5.40	5.45	5.50
1	4.95					
2	4.95	5.50	5.30	5.40	5.45	5.50
3	4.98	5.50	5.30	5.40	5.45	5.50

Experiment No. 67

To Prepare Calibration Curves for the Different Concentrations of Ethyl Acetate from 1 ml Per Liter to 0.0025 ml Per Liter and to Run the Unknowns

Procedure

The column was washed till the pH of the effluent became constant. 10 ml of the ester solution was added to the separatory funnel. Now the effluent was allowed to run at the top speed. The pH was taken by collecting the effluent in the pH meter beaker. The electrodes were dried with cotton each time. The solution whose pH was to be taken was filled in the pH meter beaker. The electrodes were immersed in the beaker and the beaker was shaken for a while to rinse the electrodes and to make the solution in the pH meter beaker homogeneous. The instantaneous pH was taken. When the liquid was about to leave the stop-cock of the separating funnel the stop-cock was closed. The separatory funnel was washed with 10 ml of distilled deionized water. The washing was continued till the pH of the effluent became constant. The lowest pH was taken for each particular determination. With each particular concentration of the ester the experiment was repeated till three concurrent results were obtained. The data for the preparation of calibration curves and the results of running the unknowns are given below:

	Ester Concentration per liter	Lowest pH	pH of the unknown	Concentration of the unknown found	Concentration of the unknown given
Calibration Curve No. 1	1 ml	3.86			
	0.5 ml	4.15	4.15	0.5 ml per liter	0.75 ml per liter
	0.33 ml	4.24	4.24	0.35 ml per liter	0.35 ml per liter
Calibration Curve No.2	0.1 ml	4.45			
	0.05 ml	4.55	4.50	0.076 ml per liter	0.08 ml per liter
	0.033 ml	4.80			
	0.025 ml	4.84			
	0.01 ml	5.00			
Calibration Curve No.3	0.0075 ml	4.70			
	0.005 ml	4.95			
	0.0025 ml	5.01	5.00	0.0031 ml per liter	0.0031 ml per liter
	0.001 ml	5.20			

Conclusion

It is possible to determine the esters accurately from the calibration curve when the concentretion is less than 0.5 ml per liter. See graphs 2-4 for calibration curves 1-3.

GRAPH NO. 2

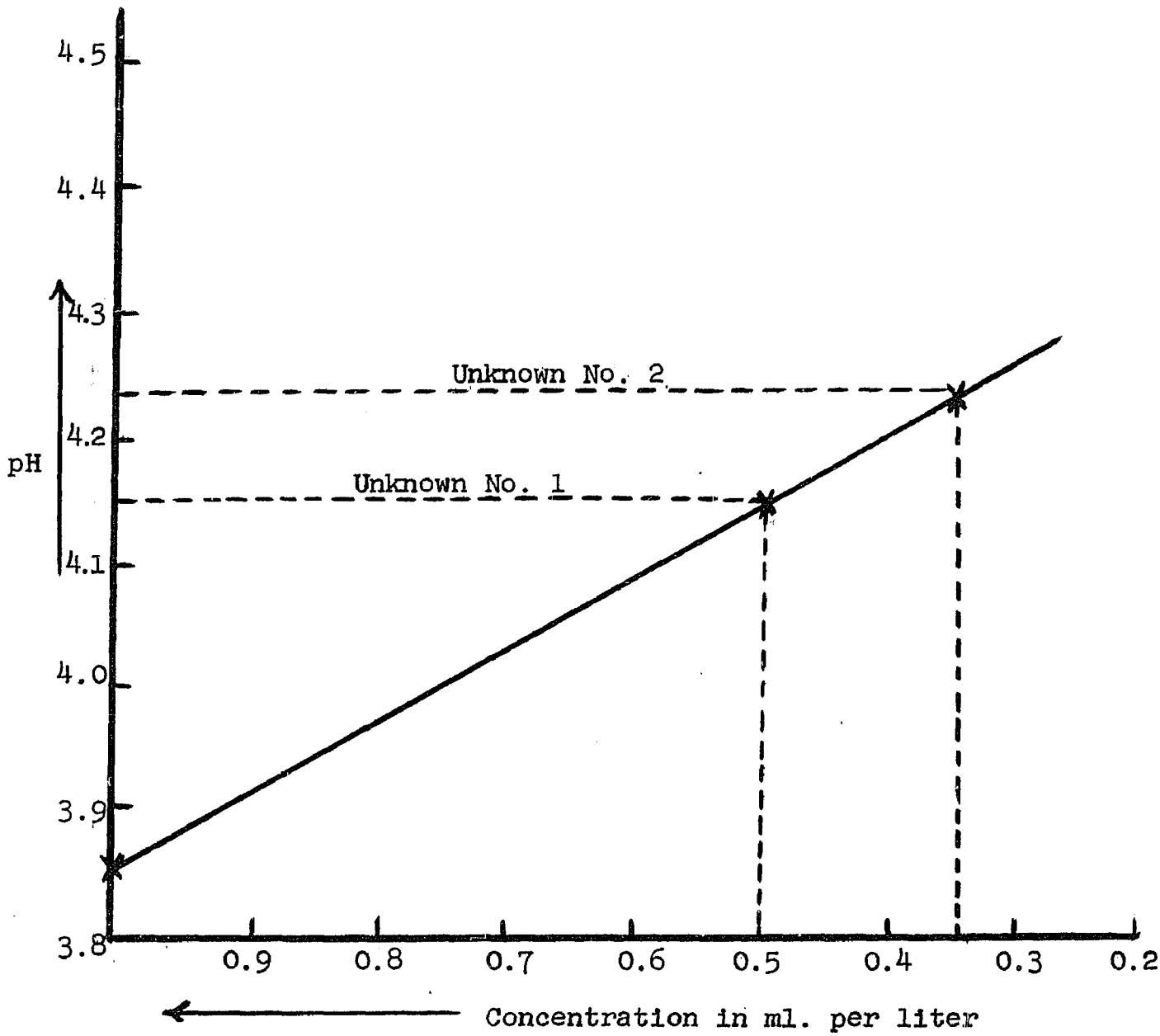
Calibration Curve No. 1

Unknown No. 1: Correct concentration of ethyl acetate - 0.75 ml. per liter

Concentration of ethyl acetate found - 0.5 ml. per liter

Unknown No. 2: Correct concentration of ethyl acetate - 0.35 ml. per liter

Concentration of ethyl acetate found - 0.35 ml. per liter

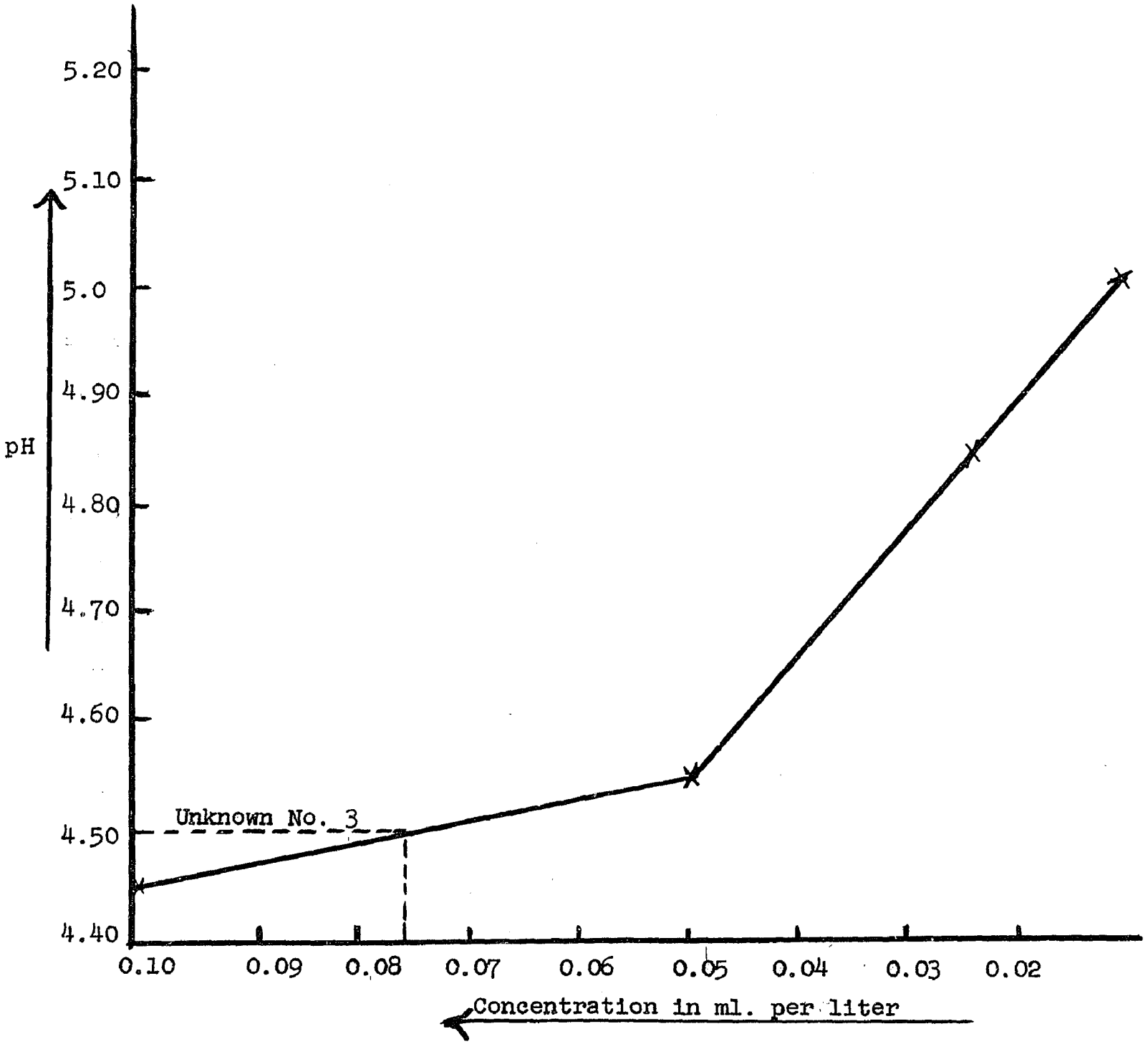


GRAPH NO. 3

Calibration Curve No. 2

Unknown No. 3: Correct concentration of ethyl acetate - 0.08 ml. per liter

Concentration of ethyl acetate found - 0.076 ml. per liter

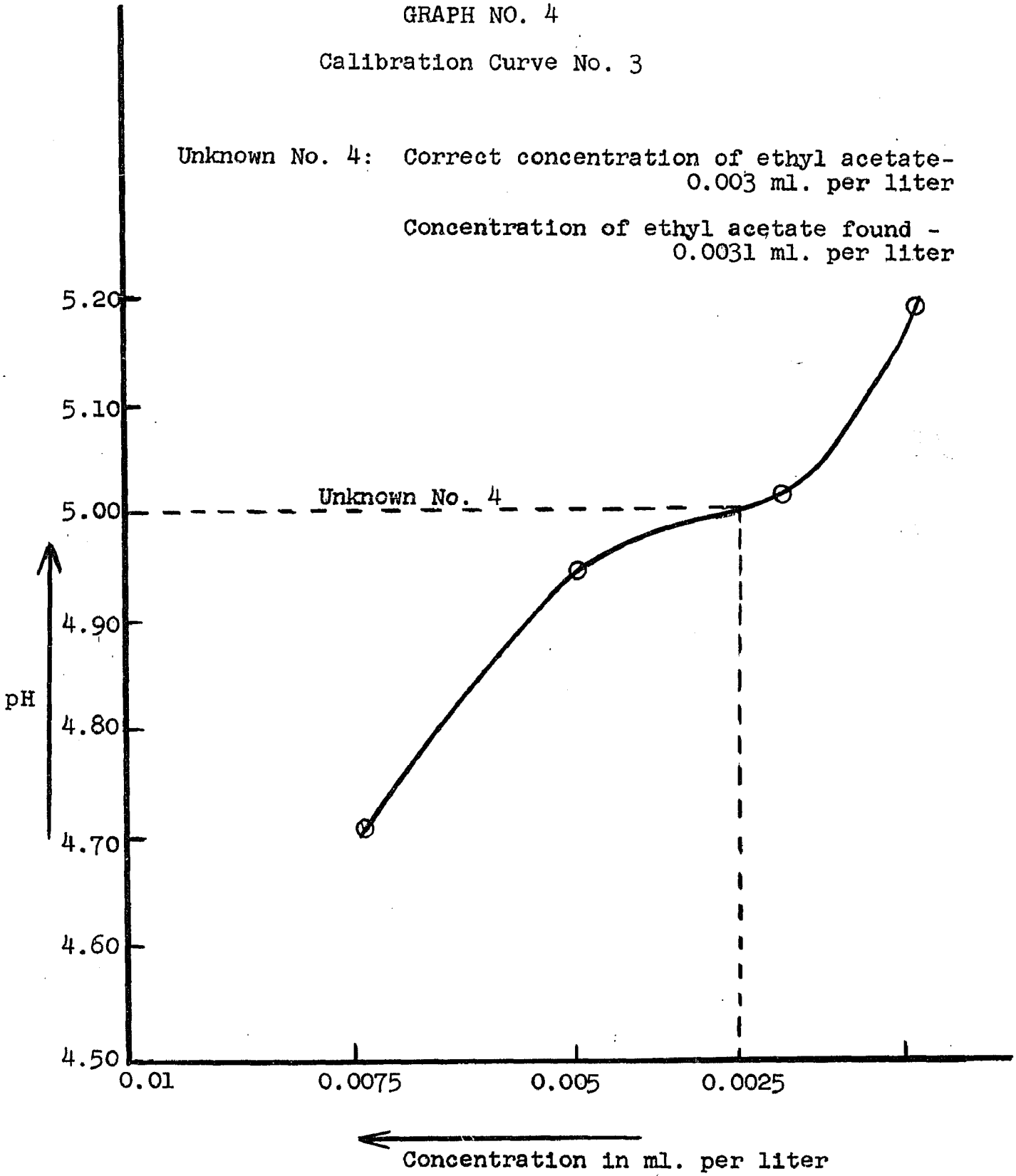


GRAPH NO. 4

Calibration Curve No. 3

Unknown No. 4: Correct concentration of ethyl acetate- 0.003 ml. per liter

Concentration of ethyl acetate found - 0.0031 ml. per liter



D. Scope and Limitations of the Method

The method used is simple and relatively rapid. After the preparation of the calibration curve it takes about 10 minutes to obtain the concentration of the unknown. In its present form, the method appears to be suitable for quantities smaller than 0.5 ml. per liter. When the concentration is less than 0.5 ml. per liter, the accuracy is 3%. In this determination, acetaldehyde, acetone and glucose do not interfere. However, urea interferes due to hydrolysis. Any inorganic impurities will also interfere. Interference from formaldehyde is negligible. It is not easy to determine an ester in the presence of aldehydes and ketones in such small quantities. As far as is known no such method has been reported. When the use of chemical means is done to remove the interference due to aldehydes and ketones by forming oximes the ester concentration used is about 4%. In those cases where the concentration of the ester is less than 4%, this method can be of use. It can be used to detect esters up to 50 γ per liter.

Out of the four unknowns which were determined by this method, the concentration of 3 samples whose strength was below 0.5 ml. per liter was found within 3% of the true concentration (cf. Graphs 2-4).

It is necessary for this method to succeed that the source of water for the preparation of the sample and the unknown be the same. Otherwise changes in the cationic strength of the distilled water may give rise to errors. It is also necessary that the same column and the same conditions be used for the sample as for the unknown.

It is obvious that the success of the method depends upon two factors: (1) In the determination of pH and (2) In the reproducibility of the resin behavior. The pH of pure water is so sensitive and subject to so many interferences that its determination is not an easy task. Similarly, the resin behavior is sometimes anamolous. Its behavior, however, improves on washing.

This method is unique in two respects: (1) It is the first method used for the detection of esters in which hydrolysis is done under mild conditions and (2) It is not necessary for the success of this method that the reaction may go to completion. However, to improve the utility of the method, further work on the reproducibility of the resins is necessary. It will also be of interest to perform the hydrolysis in an inert atmosphere, to use water as pure as possible and to use polyethylene apparatus. Under such circumstances only the error due to non-reproducibility of the resins will remain. The method may be tried in non-aqueous solvents with other esters.

By using longer ion exchange columns it may be possible to get better results for higher concentrations of the ester. Similarly, the use of higher temperature may also prove helpful in this case.

PART III

HIGH FREQUENCY TITRATIONS OF TUNGSTATE AND MOLYBDATE

Chapter IX

High Frequency Titrations of Tungstate and Molybdate with Lead Salts

A. Introduction

High frequency titrations are particularly useful in precipitation reactions. The absence of direct contact between electrodes and solutions is a very definite advantage. In this respect they are superior to conductometric titrations in which errors sometimes arise through variations in the cell constant during the performance of the titration because very fine precipitates tend to adhere to the platinum black of the electrodes. However, owing to non-linear response or poor sensitivity in certain regions, it is necessary to have proper choice of titration conditions. Each case has to be investigated individually. It is important to select sample size and end point volume so that the end point conductance will correspond to the conductance on the steep part of the response curve. The response is not generally a simple function of the fundamental properties of solution (specific conductance and dielectric constant) but usually depends on both.

The high frequency measurements have been used to determine the composition of chemical systems and the end point of titrations. West and coworkers (101) made an excellent use of this method to determine traces of water

in alcohols. Hara and West (45a-d) used the high frequency technique in the study of EDTA complexes with several metal ions and for the determination of organic functional groups in aqueous and non-aqueous solvents. Blaedel and Malmstadt (14, 15) used it for the argentimetric and mercurimetric titrations of chloride and thiocyanate. The present investigation was carried out to determine the possibility of titrating very dilute solutions of sodium molybdate and tungstate with standard lead solutions.

B. Experimental

Instrument: A Sargent Model V Oscillometer operating at 110-120 volts and 60 cycles was used in these experiments. High frequency titrations were carried out in the large 100 ml. cell.

Reagents: One tenth molar aqueous solutions of reagent grade lead nitrate, lead acetate, sodium molybdate and sodium tungstate were prepared and standardized by the classical methods. More dilute solutions were prepared with subsequent dilution of these solutions.

Procedure: The instrument was allowed to warm up for about an hour. The cell was cleaned and dried. After the cell was placed in the cell holder,

the needle was brought to zero by means of the zero adjuster. After this, a measured volume of tungstate or molybdate solution was transferred to the cell and the total volume brought to 100 ml. by further dilution with distilled water. The needle was again brought to zero by adjusting the capacitance of the instrument and the initial reading noted. Small increments of lead acetate or nitrate solution were added, the solution was stirred thoroughly with a thin glass rod and the readings taken. The titration was continued beyond the end point and the experimental value was obtained by plotting a graph and finding the point of intersection.

Results: The capacitance readings obtained by the titration of sodium molybdate and sodium tungstate are given in Tables 4-7. The end points were obtained by plotting ΔCp against the volume of the titrant. In the titration of sodium molybdate with lead acetate, the capacitance first decreases and then there is a sharp increase after the end point (cf. Graph 5). In the titration of sodium tungstate with lead nitrate, the capacitance remains initially constant and

then rises continuously beyond the end point (cf. Graphs 6 and 7). It was not found possible to titrate solutions more dilute than 0.0002M. In such a case the addition of 2 ml. of absolute alcohol was very helpful. When the lead acetate and sodium molybdate solutions were acidified with acetic acid to a change of color in methyl orange, the titration was not feasible. In Table 8 the theoretical end points are compared with the end points obtained experimentally.

TABLE 4

Titration of 0.0004 M Sodium Molybdate Against .1 M Lead Acetate

	Volume of .1 M lead acetate	Reading of oscillometer	ΔCp.
1	0	22750	0
2	.05	22710	-40
3	.1	22670	-80
4	.2	22565	-185
5	.33	22448	-302
6	.40	22380	-370
7	.45	22512	-240
8	.50	22660	-110
9	.55	22818	+68
10	.65	23192	+442
11	.70	23236	+486
12	.75	23360	+610
14	.85	23700	+950

TABLE 5

Titration of 0.0002 M Sodium Molybdate Against .05 M Lead Acetate

	Volume of .05 M lead acetate	Reading of oscillometer	ΔCp.
1	0	22590	0
2	.1	22545	-45
3	.2	22528	-62
4	.3	22485	-105
5	.45	22500	-90
6	.50	22535	-55
7	.60	22647	+57
8	.65	22735	+240

TABLE 6

Titration of 0.001 M Sodium Tungstate Against .1 M Lead Nitrate

Serial No.	Vol. of .1 M lead nitrate	Oscillometer reading	ΔCp.
1.	0 ml.	25,250	0
2.	.8 ml.	25,255	5
3.	.9 ml.	25,255	5
4.	1.0 ml.	25,255	5
5.	1.05 ml.	25,365	115
6.	1.1	25,595	345
7.	1.15	25,750	500
8.	1.20	25,895	645

TABLE 7

Titration of 0.00025 M Sodium Tungstate Against .05 M Lead Nitrate

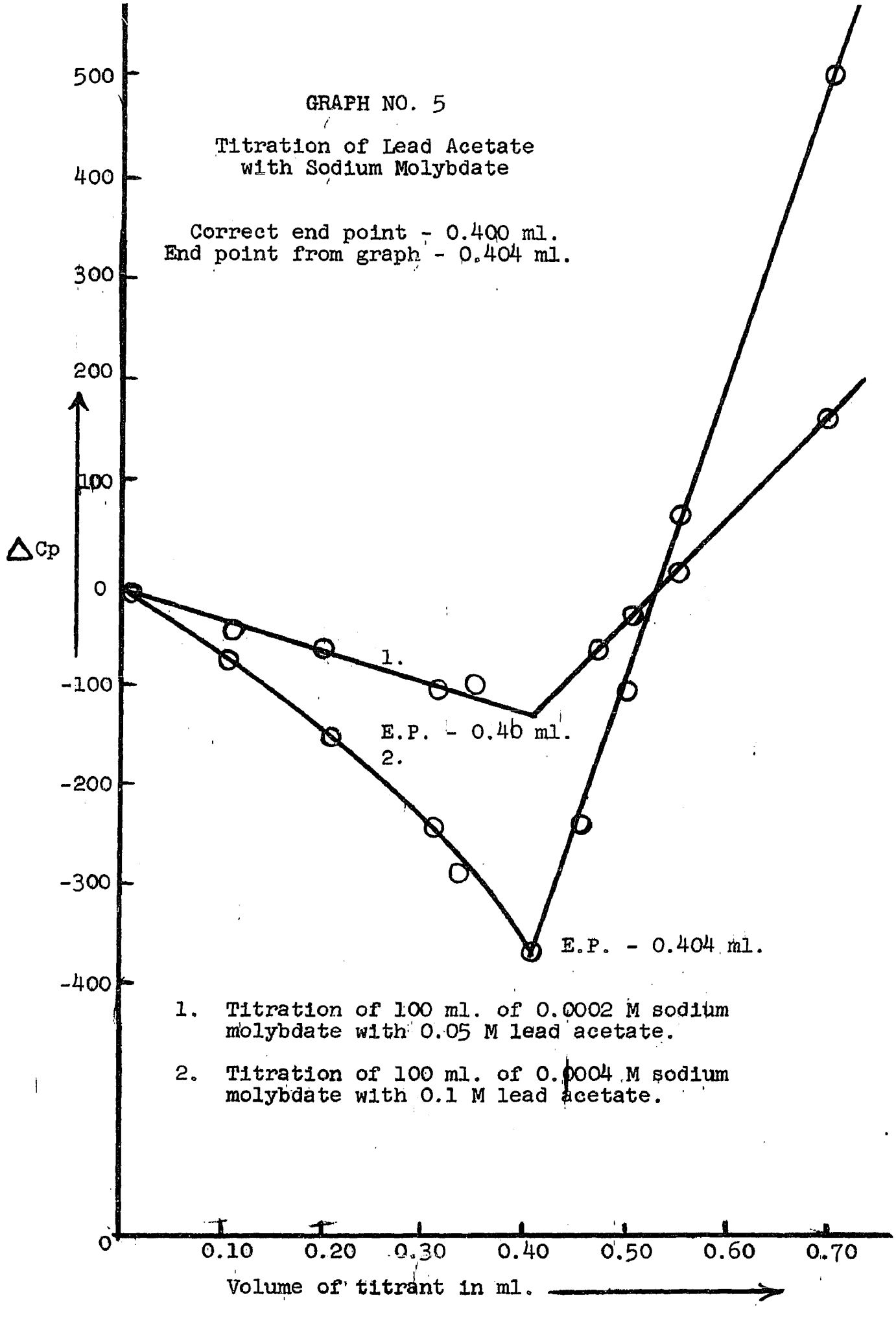
Serial No.	Vol. of .05 lead nitrate	Oscillometer reading	Δ Cp.
1.	0 ml.	22,287	0
2.	.1 ml.	22,287	0
3.	.2 ml.	22,287	0
4.	.3 ml.	22,287	0
5.	.4 ml.	22,287	0
6.	.5 ml.	22,287	0
7.	.6 ml.	22,515	238
8.	.7 ml.	22,765	478
9.	.8 ml.	23,050	763
10.	.9 ml.	23,226	933
11.	1.0	23,600	1,313
12.	1.1	23,900	1,613

TABLE 8

Comparison of the Theoretical end Points with the

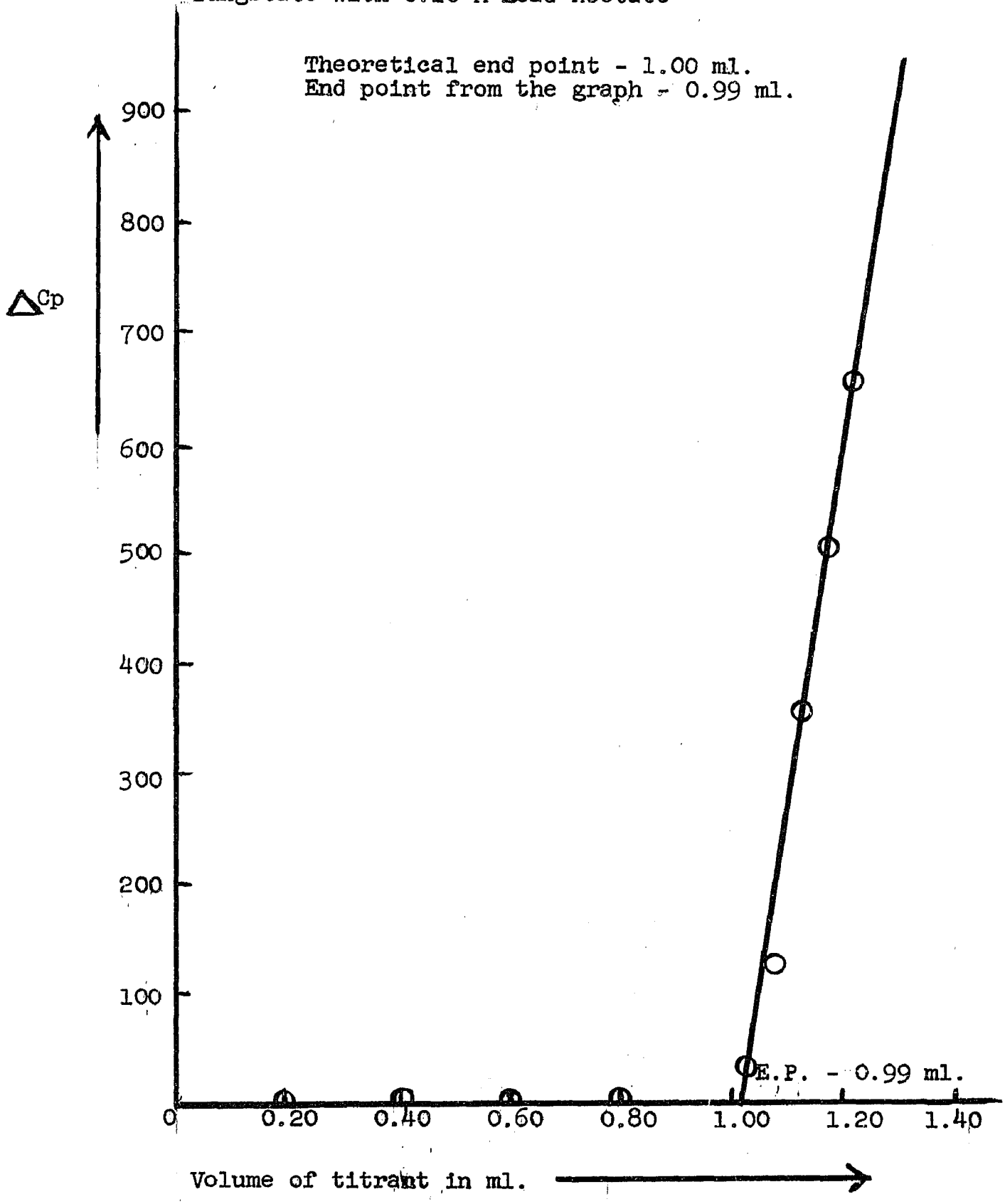
end Points Obtained Experimentally

	Titration performed	Theoretical end point	Experimental end point	Error
1.	100 ml. of 0.0004 M sodium molybdate with .1 M lead acetate	0.4 ml.	0.404 ml.	1.0%
2.	100 ml. of 0.0002 M sodium molybdate with .05 M lead acetate	0.4 ml.	0.404 ml.	1.0%
3.	100 ml. of .001 M sodium tungstate with .1 M lead nitrate	1.0 ml.	0.99 ml.	1.0%
4.	100 ml. of .00025 M sodium tungstate with .05 M lead nitrate	0.5 ml.	0.5 ml.	0.0%


GRAPH NO. 5
Titration of Lead Acetate
with Sodium Molybdate
Correct end point - 0.400 ml.
End point from graph - 0.404 ml.
500
400
300
200
100
0
-100
-200
-300
-400
ΔCp
1.
2.
E.P. - 0.4b ml.
E.P. - 0.404 ml.
1. Titration of 100 ml. of 0.0002 M sodium molybdate with 0.05 M lead acetate.
2. Titration of 100 ml. of 0.0004 M sodium molybdate with 0.1 M lead acetate.
0
0.10
0.20
0.30
0.40
0.50
0.60
0.70
Volume of titrant in ml.

GRAPH NO. 6

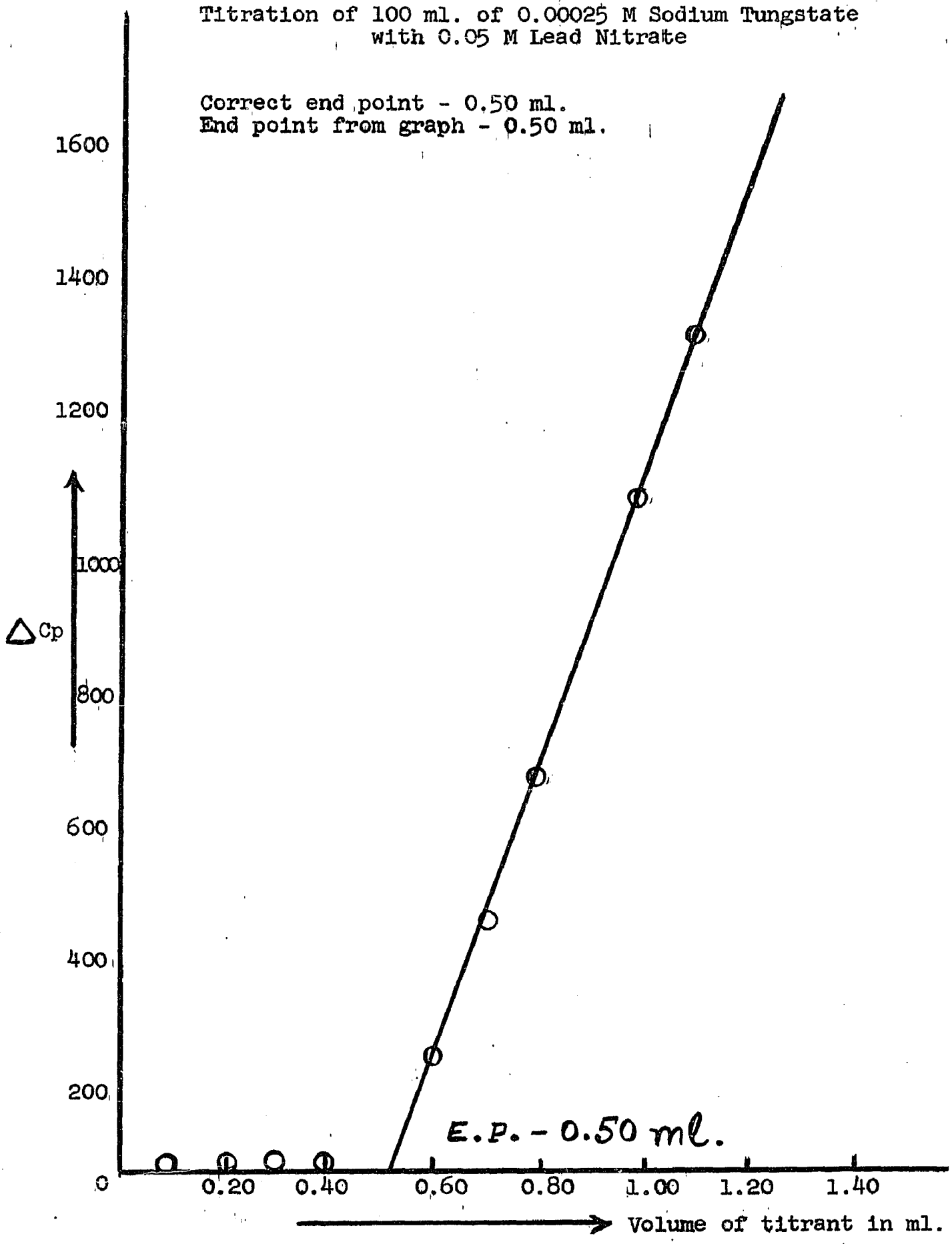
Titration of 100 ml. of 0.001 M Sodium Tungstate with 0.10 M Lead Acetate



GRAPH NO. 7

Titration of 100 ml. of 0.00025 M Sodium Tungstate with 0.05 M Lead Nitrate

Correct end point - 0.50 ml.
End point from graph - 0.50 ml.



Discussion: Titrations of molybdate and tungstate with lead salts are difficult to perform. Adsorption indicators are used, the pH has to be adjusted and the solution sometimes has to be heated (17, 76). Ringbom (79) has described a method in which tungstates are brought to a pH of 8.5-9 and titrated hot using bromothymol blue as an indicator while molybdates are adjusted initially to pH 9-9.5 and titrated with phenol red as an indicator. Rother and Jander (80) showed that it is possible to titrate accurately sodium molybdate and sodium tungstate with lead salts conductometrically. However, in this method the pH of the solutions has to be adjusted and the method has been applied to strong solutions only.

The high frequency titrations can be done successfully in neutral solutions. Heating is also not required. The method is quick and simple, and the results are accurate within one per cent. Even in dilute solutions the end points are sharp. From the results obtained by this method it is clear that it is possible to titrate solutions as dilute as 0.00025M with accuracy. In such low

concentrations, titrimetric and conductometric methods have not been applied successfully; and the high frequency titration becomes especially useful.

The difference in the nature of the two curves can be explained qualitatively thus: Since the mobility of the acetate ion is less than the mobility of the nitrate ion hence in the titration with lead acetate we obtain an initial decrease in the capacitance while in the titration with lead nitrate the capacitance stays constant until the end point is reached and then it rises sharply.

REFERENCES

1. Ahlers, N. H. E., and W. G. McTaggart, Analyst, 79, 70-6 (1954).

2. Aldridge, W. N., Biochem. J. (London), 57, 692 (1954).

3. Ammon, R., Handbuch der Enzymologie, Nord and Weidengahen eds., Akademische Verlags gesselschaft, Leipzig, 1940, p. 350.

4. Anderson, J. A., and W. D. Seyfried, Anal. Chem., 20, 998 (1948).

5. Balls, A. K., and M. B. Matlack, J. Biol. Chem., 123, 679 (1938).

6. Bamann, E., and E. Rendlen, Z. physiol. Chem., 238, 133 (1936).

7. Bamann, E., and M. Schmeller, Z. physiol. Chem., 194, 14 (1931); E. Bamann, E. Schweizer, and M. Schmeller, ibid., 222, 121 (1933).

8. Bamann, E., J. N. Mukerjee, and L. Vogel, Z. physiol. Chem., 229, 15 (1934).

9. Bamann, E., Ber., 62, 1538 (1929).

10. Barnes, et al., Anal. Chem., 20, 402 (1948).

11. Bauman, W. C., and J. Eichorn, J. Am. Chem. Soc., 70, 2370 (1948).

12. Beythien, A., Pharm. Zentrahalle, 38, 850-2 (1897).

13. Bier, M., and F. F. Nord, Arch. Biochem. & Biophys., 31, 335 (1951).

14. Blaedel, W. J., and H. V. Malmstadt, Anal. Chem., 23, 471 (1951).

15. Blaedel, W. J., and H. V. Malmstadt, Anal. Chem., 22, 1410 (1950); 1413 (1950).

16. Buckles, R. E., and C. J. Thelen, Anal. Chem., 22, 676 (1950).

17. Candea, C., and I. G. Murgulescu, Ann. Chim. anal. Chim. appl., 18, 33 (1936); through Chem. Abstracts, 30, 2133 (1936).

18. Castren, E., Svensk Farm. Tidskr., 60, 871-8 (1956); Chem. Abstracts, 51, 6751 (1957); Farm. Aikakauslehti, 66, 2-11 (1957).

19. Cheronis, N. D., and J..B. Entrikin, Semimicro Qualitative Organic Analysis, 2nd Edition, 1957.

20. Connors, W. M., A. Phil, A. L. Dounce, and E. Stotz, J. Biol. Chem., 184, 29-36 (1950).

21. Davidson, D., J. Chem. Education, 17, 81 (1940).

22. Davies, C. W., and G. G. Thomas, J. Chem. Soc., 2624 (1951); 1607 (1952).

23. Davies, C. W., and G. D. Yeoman, Trans. Faraday Soc., 49, 968, 975 (1953).

24. Davies, C. W., Biochem. J. (London), 45, 38 (1949).

25. Dawson, R. E., Biochem. J. (London), 21, 398 (1927).

26. Demarest, J. V., and W. Riemann, III, Ind. Eng. Chem., Anal. Ed., 3, 15-7 (1931).

27. Dickens, F., and Joan Pearson, Biochem. J., 48, 216-21 (1951).

28. Elving, P. J., and C. Teitelbaum, J. Am. Chem. Soc., 71, 3916-21 (1949).

29. Feigl, F., V. Anger, and O. Frehden, Mikrochemie, 15, 18 (1934).

30. Feigl, F., Spot Tests, Vol. II, 4th Edition, 1954, p. 171.

31. Fenton, H. J. H., and H. A. Sisson, Proc. Cambridge Phil. Soc., 14, 385 (1908).

32. Fodor, P. J., Arch. Biochem., 30, 197 (1951).

33. Furter, M., Helv. Chim. Acta, 21, 601-13 (1938).

34. Feigl, F., Spot Tests, Vol. II, 4th Edition, 1954, p. 175.

35. Feigl, F., V. Anger, and O. Frehden, Mikrochemie, 15, 12 (1934).

35a. Gauron, O., C. J. Grelecki, and M. Duggan, Arch. Biochem. & Biophys., 44, 455 (1953).

36. Gertler, W., Fermentforschung, 15, 171 (1938).

37. Glueckauf, E., Proc. royal Soc., 1952, (A) 214, 207; J. F. Duncan, Proc. royal Soc., 1952 (A) 214, 344.

38. Goddu, R. F., N. F. LeBlanc, and C. M. Wright, Anal. Chem., 27, 1251-5 (1955).

39. Grant, W. M., Anal. Chem., 20, 267-9 (1948).

40. Grant, W. M., Ind. Eng. Chem., Anal. Ed., 19, 206-7 (1947).

40a. Gregor, H. P., F. C. Collins, and M. Pope, J. Colloid Science, 6, 304 (1951).

41. Griessbach, R., Angew. Chem., 52, 215 (1939).

42. Guagnini, O. A., and E. E. Vonesch, Mikrochim. Acta, 12, 1767-73 (1956) (in Spanish) through Anal. Abstracts, July 1957.

43. Hack, M. H., Arch. Biochem. & Biophys., 58, 19-23 (1955).

44. Hale, D. K., D. I. Packham, and K. W. Pepper, J. Chem. Soc., 844 (1953).

45. Hall, R. T., and W. E. Schaefer, Organic Analysis, Vol. II, Interscience Publishers, Inc., New York, 1954, pp. 19-70.

45a. Hara, R., and P. W. West, Anal. Chim. Acta, 11, 264 (1954).

45b. Hara, R., and P. W. West, Anal. Chim. Acta, 12, 72 (1955).

45c. Hara, R., and P. W. West, Anal. Chim. Acta, 13, 189 (1955).

45d. Hara, R., and P. W. West, Anal. Chim. Acta, 14, 280 (1956).

45e. Hara, R., and P. W. West, Anal. Chim. Acta, 12, 285 (1955).

46. Harmes, Anal. Chem., 25, 1140 (1953).

47. Higuchi, T., and D. A. Zuck, J. Am. Chem. Soc., 73, 2676-9 (1951).

48. Hill, U. T., Anal. Chem., 19, 932-33 (1947); 18, 317-9 (1946).

49. Hofstee, B. H. J., Science, 114, 128 (1951); J. Biol. Chem., 199, 357 (1952).

50. Hutchens, J. O., and B. M. Kass, J. Biol. Chem., 177, 571-5 (1949).

51. Kaplan, C., Methods in Enzymology, Vol. I, pp. 657-9.

52. Kastle, J. H., Am. Chem. J., 27 481 (1902); A. A. Kristman and H. B. Lewis, J. Biol. Chem., 47, 495 (1921); Kohler, E. P., J. F. Stone, Jr., and R. C. Fuson, J. Am. Chem. Soc., 49, 3181-9 (1927).

53. Kastle, J. H., Am. Chem. J., 27, 481 (1902); A. A. Christman and H. B. Lewis, J. Biol. Chem., 47, 495 (1921); D. A. McGinty and H. B. Lewis, ibid., 67, 567 (1926); A. Lourtieg and C. E. Cardini, Anales farm. y bioquim. Buenos Aires, 18, 59 (1947); ibid., 18, 54 (1947).

54. Keenan, A. G., Can. Chem. Process Inds., 29, 857-8 (1945).

55. Kernot, J. C., and H. W. Hills, Z. physiol. Chem., 222, 11 (1933).

55a. Kolthoff, I. M., and E. B. Sandell, Text Book of Quantitative Inorganic Analysis, 1938, p. 427.

55b. Kohler, E. P., J. F. Stone, Jr., and R. C. Fuson, J. Am. Chem. Soc., 49, 3181-9 (1927).

56. Kossel, A., and M. Kruger, Z. physiol. Chem., 15, 320-1 (1891).

57. Kossel, A., and K. Obermuller, ibid., 14, 599-601 (1890); through J. Chem. Soc. Abstracts, 58, 1474 (1890).

58. Koettstorfer, J., Z. anal. Chem., 18, 199, 431 (1879).

59. Kruger, Deodata, and Erich Tschrich, Ber., 628, 2776-83 (1929).

59a. Laidler, K. J., and M. L. Barnard, J. Am. Chem. Soc., 74, 6099 (1952).

60. Lewkowitsch, J., Chemical Technology and Analysis of Oils, Fats and Waxes, 5th Ed., Vol. I, Macmillan, London, 1913, pp. 107, 380.

61. Lintner, C. J., R. H. Schleif, and T. Higuchi, Anal. Chem., 22, 534-8 (1950).

62. MacFayden, D. A., J. Biol. Chem., 158, 107-33 (1945).

63. Maginnity, P. M., with J. B. Cloke, Anal. Chem., 20, 978-9 (1948).

64. Mariani, A., and P. B. Camponovo, Acta argentina fisiol. y fisipatol., 1, 683 (1951).

64a. Mattson, F. H., and L. W. Beck, J. Biol. Chem., 219, 735 (1956).

65. McElvain, M., The Characterization of Organic Compounds, The Macmillan Company, 1953.

66. McGuire, G., and K. G. Falk, J. Biol. Chem., 105, 373 (1934).

67. McIlhiney, P. C., J. Am. Chem. Soc., 16, 408-10 (1894).

68. Meezon, T. I., Med. exptl (Ukraine), No. 2, 1-6 (1940); Meezon, T. I., and K. M. Schleifer, Biochem. J. (Ukraine), 16, 215-46 (1940).

69. Milton, R. F., and W. A. Waters, Methods of Quantitative Micro-Analysis, 2nd Ed., 1955.

70. Mitchell, J., Jr., and D. M. Smith, Anal. Chem., 22, 746-50 (1950).

71. Murray, D. R. P., Biochem. J., 24, 1890 (1930).

72. Obermuller, K., Z. physiol. Chem., 16, 152-9 (1892).

73. Oneal, F. B., and J. Carlton, Anal. Chem., 30, 1051 (1958).

74. Page, J. E., J. W. Smith, and G. G. Waller, J. Phys. & Colloid Chem., 53, 545-62 (1949).

75. Pepper, K. W., D. Reichenberg, and D. K. Hale, J. Chem. Soc., 3129 (1952).

76. Rachinstein, Z., and N. Korbov, Z. anal. Chem., 104, 192 (1936).

77. Redemann, C. E., and H. J. Lucas, Ind. Eng. Chem., Anal. Ed., 9, 521-2 (1937).

78. Riemann, W., III, Ind. Eng. Chem., Anal. Ed., 15, 325-6 (1943).

79. Ringbom, A., Thesis, Abo, Finland, 1934, Chem. Abstracts, 29, 3261 (1935).

80. Rother and Jander, Z. angew. Chem., 43, 930 (1930).

81. Safwat, M. M., Acta Chem. Scand., 2, 90 (1948).

82. Samuelson, O., Ingen Vetensk Akad. Handl., No. 179, 1945.

83. Sastri, B. N., and M. Sreenivasya, Mikrochemie, 14, 159-60 (1934).

84. Schonheyder, F., and K. Volqvartz, Acta Physiol. Scand., 10, 62 (1945).

85. Schonheyder, F., and K. Volqvartz, Enzymologia, 11, 178 (1943-5).

86. Schwartz, B., J. Gen. Physiol., 27, 113 (1943).

86a. Schwimmer, S., Annual Review of Biochemistry, 26, 65 (1957).

87. Shiro, U., Japan Analyst, 5(9), 499-502 (1956).

88. Shriner, R. L., R. C. Fuson, and D. Y. Curtin, The Systematic Identification of Organic Compounds, 4th Ed., 1956.

89. Siggia, S., Quantitative Organic Analysis Via Functional Groups, 2nd Ed., 1954.

90. Singer, T. P., J. Biol. Chem., 174, 11 (1948).

91. Singer, T. P., and B. H. J. Hofstee, Arch. Biochem., 18, 229 (1948).

92. Singer, T. P., and B. H. J. Hofstee, Arch. Biochem., 18, 245 (1948).

93. Sizer, I. W., Advances in Enzymology, 3, 35 (1948).

94. Smith, W. C., Ind. Eng. Chem., Anal. Ed., 16, 416 (1944).

95. Stedman, E., and E. Stedman, Biochem. J., 25, 1147 (1931).

96. Steensholt, G., Acta Physiol. Scand., 5, 103 (1943).

97. Stone, K. G., Determination of Organic Compounds, McGraw-Hill Company, Inc., New York, 1956, p. 103.

98. Thompson, A. R., Australian J. of Sci. Research, A 3, 128-35 (1950).

99. Wawzonek, S., and J. H. Fossum, Communication to International Polarographic Congress, Prague, 1951; through Coll. Czechoslov. Chem. Communs., 15, 1107-1208 (1950).

100. Wawzonek, S., R. C. Reck, W. W. Vaught, Jr., and J. W. Fan, J. Am. Chem. Soc., 67, 1300-2 (1945).

101. West, P. W., P. Senise, and T. S. Burkhalter, Anal. Chem., 24, 1250 (1952).

102. Whitnac, G. C., and E. St. C. Gantz, Anal. Chem., 24, 1060-1 (1952).

103. Wills, E. D., Biochem. J. (London), 60, 529 (1955).

104. Willstatter, R., and F. Memmen, Z. physiol. Chem., 133, 229 (1924).

104a. Ibid., 138, 216 (1924).

105. Willstatter, R., and H. Kraut, Ber., 56, 149 (1923).

106. Willstatter, R., J. Graser, and R. Kuhn, Z. physiol. Chem., 123, 1 (1922).

107. Willstatter, R., and E. Waldschmidt-leitz, Z. physiol. Chem., 125, 132 (1923); ibid., 217 (1925).

108. Yamamoto, T., J. Biochem. (Japan), 38, 147 (1951).

109. Zaugg, H. E., and W. M. Lauer, Anal. Chem., 20, 1022-6 (1948).

110. Zaugg, H. E., and B. W. Horrom, Anal. Chem., 20, 1026-9 (1948).

111. Zerewitinoff, T., Ber., 40, 2023 (1907); 41, 2233 (1908); 42, 4802 (1909); 43, 3590 (1910); 47, 1659, 2417 (1914).

112. Am. J. Clin. Pathol., 24, 99 (1954).

113. See ref. 107; see also p. 315, Zur Geschichte der Adsorption von Enzymen.

114. See ref. 107, p. 182.

115. Biochem. J. (London), 21, 398-403 (1927).

VITA

MOHD. MOHSIN QURESHI was born on March 7, 1924 in Aligarh (INDIA). He passed the High School examination in 1938. He received the degree of Bachelor of Science from the University of Allahabad (INDIA) in 1942. He then entered the Muslim University, Aligarh (INDIA) where the degree of Master of Science in Chemistry was conferred upon him in 1944. He was appointed as a lecturer in Chemistry in this University where he was teaching before he came to the Louisiana State University.

He was accepted as a graduate student in Chemistry in Louisiana State University in September 1955. Now he is a candidate for the degree of Doctor of Philosophy in Chemistry.

EXAMINATION AND THESIS REPORT

Candidate: Mohd Mohsin Qurishi

Major Field: Chemistry

Title of Thesis: The Detection and Determination of Esters

Approved:

Philip W. West
Major Professor and Chairman

Richard J. Russell
Dean of the Graduate School

EXAMINING COMMITTEE:

Marion G. Day, Jr.

Vincent E. Parker

H. B. Williams

S. P. McGlynn

Date of Examination:

July 29, 1958