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I am submitting herewith a thesis written by Hugh Oliver Jaynes entitled "Studies in Milk Lipase." I recommend that it be accepted for 9 quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Dairying.

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STUDIES OF MILK LIPASE

A THESIS

Submitted to
The Graduate Council
of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the degree of
Master of Science

by

Hugh Oliver Jaynes

June 1954

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CHAPTER I

INTRODUCTION

The processes involved in Cheddar cheese ripening are a mystery into which light is slowly being cast. It is generally accepted that raw milk cheese is ripened by enzymes native to the cheese milk and by microorganisms accidentally or purposely incorporated into the cheese. Pasteurization of milk for cheesemaking is a practice coming into wide acceptance and certainly has its merits. But the heat treatment of pasteurization inactivates many desirable microorganisms and destroys milk enzymes which could be important in cheese ripening. Pasteurized milk cheese could be made equal to the finest raw milk cheese if the agents responsible for flavor and body development in the aging cheese, which are destroyed by the heat treatment, could be added back to the milk after pasteurization. One of the agents destroyed by pasteurization is the lipolytic enzymes. It has long been believed that fat hydrolysis by a milk lipase was one phase of Cheddar cheese ripening. Therefore, it seemed desirable to investigate the lipase (or lipases) in milk which might be related to Cheddar cheese ripening.

CHAPTER II

REVIEW OF LITERATURE

Lipolysis has been recognized as a factor causing a desirable or an undesirable change in dairy products. Fouts and Weaver (8) and Weaver (58) have shown that from 10.4 to 14.3 per cent of the cows studied gave milk which became rancid in approximately 15 hours. According to Weaver (58) an objectionable rancid flavor was noted as being third in frequency in fluid milk. Hansen and Theophilus (18) and Jack, Tarassuk, and Scaramella (27) found the enzyme lipase responsible for rancid or other less easily defined off flavors in butter. Hlynka and Hood (26) showed that lipolytic activity caused rancid flavor to develop in raw milk Cheddar cheese. In 1935, Hileman and Courtney (23) recognized the presence of lipase in raw cream shipped for buttermaking to be responsible for a rancid flavor. Evidence has been presented by Lane and Hammer (41) that controlled amounts of the enzyme added to pasteurized milk used in making Cheddar cheese could influence the development of a desirable flavor in the finished product. Incomplete destruction of the enzyme by insufficient preheating prior to drying was found to cause dried whole milk powder to become rancid (17). Rice (47) found that as little as one part of raw milk added to three hundred sixty-six parts of preheated milk would cause rancidity to develop in sweetened condensed milk.

The presence of lipase in milk is now an accepted fact. In 1913 Eckles and Shaw (7) recognized a rancid flavor developing in cold-stored milk of certain cows. In 1922 Rice and Markley (48) proved the

presence of lipase in normal milk by incubating raw milk in a sugar saturated cream substrate. Rice (47) later showed the enzyme to be the cause of rancidity in sweetened condensed milk. Palmer (44) also showed that lipase was responsible for a bitter flavor defect in the milk of cows in late lactation.

The enzymatic hydrolysis of milk fat has been estimated in a variety of ways. When milk fat is hydrolyzed, free fatty acids and mono- and diglycerides are the products. The work of Johnson and Gould (26) has shown that water soluble fatty acids released from the fat by a lipase were capable of imparting a rancid flavor to milk fat. This fact formed the basis for organoleptic examination of milk as a means of detecting lipolysis. Several workers have used this method alone (8, 17, 18, 58). The work of Doan and Minster (5) and Tarassuk and Smith (55) has shown that the hydrolyzed fatty acids were surface active compounds, and when released in milk, tended to lower the surface tension. As hydrolysis of the fat proceeded, Tarassuk and Smith (55) found that the surface tension of the milk decreased from an original value of 50-52 dyne/cm.² to 37-38 dyne/cm.². Hetrick (22), working with homogenized milk, used decreases in surface tension per unit time as a measure of lipolysis and compared his results with a flavor test. Hlynka and Hood (24) correlated rancid odor defects in milk to decreases in surface tension. They found a positive correlation of 0.23, which they termed significant.

Several investigators have used titration methods to estimate lipase activity. These methods measured the free fatty acids released from the fat. The techniques which have been used differ mainly in the

method of obtaining the fatty acids for titration with a standard alkali. Herrington and Krukovsky (19) made fat acidity determinations by churning the fat, then dissolving butter oil in alcohol and titrating the acidity. Gould and Johnson (13, 14) recovered 97-98 per cent of the fatty acids from churned rancid fat by washing 50 grams of fat in an ether solution with six 20 ml. portions of 0.05N NaOH. They state that the free acids obtained from churned fat were water insoluble. Johnson and Gould (28) developed a solvent extraction method. The fat was extracted from milk using a 2:3 mixture of ethyl ether and Skellysolve F. The fat thus obtained was 30 per cent higher in acidity than fat obtained by churning, and it gave a higher percentage recovery of fatty acids. The recovery of volatile acids was also much higher than by churning. The same workers (29) found that the recovery of short chain fatty acids was further improved by acidifying the fat solution to pH 2.0 with H_2SO_4 .

Rice and Markley (48), and later Nair (43), titrated acidity directly without removing the fat from the fat-milk mixture. The method used was originated by Rice and Markley. It consisted of incubating raw milk in a substrate of boiled cream saturated with sucrose. Direct titrations with standard alkali of measured amounts of the incubation mixture were made at the beginning and end of the incubation time. The difference in the two titrations represented the extent of lipolysis. Bacterial growth was inhibited by the osmotic effect of sucrose. Peterson, Johnson, and Price (45), using tributyrin as a substrate, measured lipase activity by direct titration of a controlled amount of skim milk-tributyrin mixtures with standard alkali.

Removal of volatile fatty acids from lipolyzed fat by steam distillation has also been used. Roahen and Sommer (49) used this method for obtaining fatty acids from incubation mixtures of sucrose saturated cream and raw milk. Mattick and Kay (42) used steam distillation to remove hydrolyzed butyric acid from a tributyrin substrate acted upon by milk lipase.

Breaseale and Bird (3) compared four methods for measuring the acidity of butterfat. They used the AOAC method, Clarke's method, titration with 0.05 N alkali in petroleum ether - absolute alcohol solution of weighed amounts of fat, and a method in which the fatty acids were saponified, rehydrolyzed with H_2SO_4 , and extracted with a petroleum ether - absolute alcohol mixture after which the acids were titrated with 0.05 N alcoholic KOH. The last two methods described gave comparable results which were higher than either the AOAC or Clarke's method. To examine butter for lipolysis, Davies (4) simply weighed a portion of fat, dissolved it in ether, and titrated the fatty acids in the ether solution. Greenbank (16) recently reported a colorimetric method of determining lipase activity. He demonstrated the presence of a lipase, or esterase, in milk which would hydrolyze α -naphthyl esters of fatty acids. 2,6-dibromoquinone chloroimine combined with α -naphthol liberated by the lipase, imparting a purple color to the solution. The amount of color that developed in a given amount of time was measured quantitatively and was taken as an indication of the extent of ester hydrolysis by the enzyme. The author stated that one pound of raw milk mixed with two thousand pounds of heated milk could be detected by this procedure.

Another method for estimating lipase activity was presented by Archibald and Ortiz (1). They tested the activity of pancreatic lipase on Tween 20, an aqueous solution of a polyoxyalkylene derivative of sorbitan monolaurate which is soluble in water. However, the method was not applied to milk lipase.

There is some question in the literature as to whether the lipase in milk is a single enzyme or a mixture of many enzymes. Herrington and Krukovsky (19) postulated at least two enzymes on the basis of formaldehyde sensitivity. This was substantiated by the work of Peterson, Johnson, and Price (45) and Roahen and Sommer (49). The latter authors also showed the possibility of two enzymes by the fact that a part of the lipase activity of milk disappeared after 5 - 10 hours of holding. The lipase in milk has been found to hydrolyze a number of different fats and fatty acid esters. Gould (12) showed that milk lipase would hydrolyze butterfat, cottonseed oil, castor oil, coconut oil, corn oil, linseed oil, olive oil, margarine, hydrogenated cooking fat, and lard. Kelly (31, 33) found milk lipase able to hydrolyze tributyrin, tricaproin, triacetin, tripalmitin, butter oil, ethyl oleate, and diacetin. The rate of hydrolysis of the pure fats varied inversely with the length of the fatty acid chains. Reder (46), Mattick and Kay (42), Roahen and Sommer (49), and Peterson, Johnson, and Price (45) have also demonstrated the ability of milk lipase to hydrolyze tributyrin. Greenbank (16) has shown that the lipase in milk could hydrolyze α -naphthyl esters of fatty acids.

Chemical and physical conditions either limit or intensify the activity of enzymes. This is true of milk lipase. In 1932 Dorner and

Widmer (6) showed that homogenized milk became rancid much more rapidly at the phenolphthalein endpoint than at the reaction of normal milk. They also found that when the pH of milk was lowered with HCl, lipolysis was greatly inhibited. Since that time Roahen and Sommer (49) have found a pH optimum of 8.4 - 8.6 for raw cream, and Gould (12) demonstrated a pH optimum of 8.0 - 9.0 for the hydrolysis of butterfat homogenized into rennet whey from raw milk. Mattick and Kay (42), using a tributyrin substrate, found an optimum pH for lipase action at 8.2 - 8.7. Later Peterson, Johnson, and Price (45) demonstrated an optimum at pH 8.5 for the hydrolysis of tributyrin by milk lipase. The work of Lane and Hammer (41) indicated that lipase in dessicated bovine mammary tissue could be active at the pH of curing Cheddar cheese. Gould (12) and Peterson, Johnson, and Price (45) have found very little or no activity for milk lipase in the range of pH 5.0 - 5.5.

The effects of certain salts on the activity of milk lipase have also been studied. Davies (4) studied the effects of certain heavy metals on lipolysis in butter. Salts of the metals were incorporated into the butter with NaCl. His results were as follows:

Group	Grouped Samples	Per Cent Loss Lipase Activity
1.	Control Salted, Control Unsalted	0
2.	Sn 10,* Sn 50, Al 10, Al 50	5
3.	Fe 5, Co 5, Ni 5, Mn 5, Cr 5	15
4.	Mn 25, Fe 25, Cr 25, Ag 5	40

*numbers indicate ppm.

5.	Co 25, Ni 25, Cu 2, Ag 50	70
6.	Cu 10, past. control, PCu 2,* PCu 10, PFe 5, PFe 25	100

Hetrick and Tracy (22) and Gould (11) reported that copper had no significant effect on lipolysis in raw homogenized milk. However, Krukovsky and Sharp (40) published data which indicated that copper (in amounts up to 36 ppm) inhibited lipolysis in the presence of dissolved oxygen. In the absence of dissolved oxygen, copper had no inhibitory influence on the activity of lipase. Herrington and Krukovsky (19) showed that small amounts of copper (.2 to .4 ppm) reduced the activity of milk lipase by 20 per cent. Peterson, Johnson, and Price (45) found potassium cyanide, manganous sulfate, magnesium chloride, and ammonium chloride-ammonium hydroxide mixtures to inhibit lipase action at a pH of 8.5. Zinc chloride, sodium barbitol, phosphate buffer mixtures, and borate were not found to inhibit lipase action by these workers. Tuckey and Ruehe (57) have shown that NaCl can be a limiting factor in lipolysis. Their work showed that increasing the salt content of Cheddar cheese to 1.7 per cent or more prevented the development of a bitter flavor in the cheese. They believed the bitter flavor to be a result of lipolytic activity. Gould (11) reported inhibition of lipase action in homogenized raw cream by various concentrations of NaCl. His results were: two per cent NaCl inhibited 41 per cent of the lipase activity, 5 per cent NaCl inhibited 94 per cent, and 8 per cent NaCl in-

*P = pasteurized cream butter.

hibited 100 per cent of the lipase activity.

There is some disagreement in the literature concerning the effect of formaldehyde on milk lipase. The work of Roehen and Sommer (49), Herrington and Krukowsky (19), and Peterson, Johnson, and Price (45) showed that formaldehyde greatly reduced lipase activity in milk. Using the sensitivity of milk lipase towards formaldehyde, Herrington and Krukowsky (19) postulated two lipases in milk, one sensitive to formaldehyde and one not sensitive to it. On the other hand, Gould (11) and Tarassuk and Richardson (53) maintained that the addition of formaldehyde to milk did not inhibit lipase. In addition to formaldehyde, Peterson, Johnson, and Price (45) found cysteine to be slightly inhibitory to milk lipase. These workers further showed that chloral hydrate, toluene, merthiolate, sodium fluoride, and chloroform had no inhibitory effect on milk lipase under the conditions of their experiment. They also demonstrated that acid conditions greatly inhibited the activity of the enzyme. This substantiated Gould's work (12). In both experiments hydrochloric acid was used to lower the pH.

Kelly (32) found that adding pitocin to normal milk and milk which developed spontaneous rancidity consistently increased the hydrolysis of tributyrin by milk lipase. When longer chain substrates were used, the results were not consistent, in that the added pitocin might increase or decrease the amount of hydrolysis of the substrate. Hlynka and Hood (26) demonstrated that lipase was liable to proteolysis by rennet and pepsin. They found that the addition of extra amounts of rennet and/or pepsin to cheese milk prevented the development of rancid

flavor in Cheddar cheese when a commercial lipase (Pfanstiehl) was added to the cheese milk in small quantities. Garret, Hartman, and Arnold (9) and Tarassuk and Regan (52) reported that green feed contained a factor which limited the amount of lipase a cow secreted into her milk. This factor was not identified, although it was shown not to be carotene. Hansen and Theophilus (18) believed that feeding large amounts of dry feed, especially poor quality hay, was a factor responsible for increased lipolytic activity in milk.

It has been known for many years that milk lipase was inactivated by heat. Hetrick and Tracy (22) found that the rate of heat destruction of milk lipase followed a straight line curve on semi-log paper. This indicated a reaction of the first order. They stated that the speed at which milk was elevated to a given temperature was very important in determining the time required to inactivate the enzyme at that given temperature. For example, heating milk at 61.1°C . (142°F .) at the rate of 2.8°C . (5°F .) per minute inactivated the enzyme, whereas heating in a period of 5 seconds required a temperature of 85°C . (185°F .) to inactivate the enzyme. The speed of heating factor could be one explanation of the varying thermal destruction values cited for milk lipase. In a study of rancidity in whole milk powder, Greenbank and Wright (17) preheated milk for powder at 61.1°C . (142°F .), 66.7°C . (152°F .), and 72.2°C . (162°F .) for 30 minutes. The powder from the above treated milk became rancid in 112, 126, and 140 days, respectively, when stored at 30°C . (86°F .). Nair (43) could find no lipolysis taking place in whole milk powder made from milk preheated at 63° to 64.5°C . for 30 minutes. Dorner and Widmer (6) found lipase in homo-

genized milk to be inactivated by a heat treatment of $55^{\circ}\text{C}.$ for 20 minutes. Krukovsky and Herrington (36) investigated the thermal stability of milk lipase by observing lipolysis in butter from milk subjected to various heat treatments. At $43.3^{\circ}\text{C}.$ ($110^{\circ}\text{F}.$) two-thirds of the lipase activity was destroyed in 150 minutes. At $51.7^{\circ}\text{C}.$ ($125^{\circ}\text{F}.$) one-half the activity was destroyed in 20 minutes, but a measurable amount of activity was left after 150 minutes. At $60^{\circ}\text{C}.$ ($140^{\circ}\text{F}.$) activity was reduced one-half by momentary holding; it was measurable after 15 minutes but not 35 minutes. At $68.3^{\circ}\text{C}.$ ($155^{\circ}\text{F}.$) the lipase activity was scarcely measurable after instantaneous holding. The work of Doan and Minster (5) agrees approximately with the above figures. Mattick and Kay (42) reported that the ability of milk lipase to hydrolyze tributyrin was destroyed at much lower temperatures than those cited above.

Herrington and Krukovsky (19) have noted that lipolysis in freshly drawn milk was retarded by very rapid cooling of the milk. The rate of lipolysis in milk held at low temperatures after cooling was dependent on the rate at which the milk was cooled. Herrington and Krukovsky (21) found a critical temperature range in which an acceleration of the process was initiated by slow cooling. This range was 25° to $10^{\circ}\text{C}.$ Krukovsky and Herrington (35) also showed that lipolysis in milk could be accelerated by warming cold-aged milk to $30^{\circ}\text{C}.$ and recooling it immediately. The optimum temperature for lipolysis in milk treated in this manner was found to be 5° to $10^{\circ}\text{C}.$ Their explanation for this effect was that the rate of lipolysis was dependent on the crystalline state of the fat, which was changed by temperature manipulations. Krukovsky and Sharp (39) found this same relationship to be true and showed

that the rate of lipolysis in milk was also dependent on the melting point and degree of solidification of the fat. They also showed that lipolysis was modified by conditions at the fat-plasma interface. That changes in the adsorbed layer around fat globules was partly responsible for temperature acceleration of lipolysis was demonstrated. When butter fat with the normal adsorption membrane removed from around the fat globules was used as a substrate, hydrolysis by milk lipase increased with temperature. The reverse was found to be the case for milk fat with the normal adsorption membrane intact. Tarassuk and Richardson (53) noted the lipolysis accelerating effect of cooling milk. They suggested that cooling may change the permeability of the fat globule adsorption layer to lipase.

Other experiments have been carried out concerning temperature relationships of milk lipase. Roehen and Sommer (49) incubated sugar saturated cream with raw milk at 3° to 4°, 27°, and 37°C. The rate of lipolysis increased directly with temperature. Gould (11) found the same relationship with lipolysis in raw homogenized milk and cream. He used temperatures of -17.8°C. (0°F.), 1.67°C. (35°F.), 21.1°C. (70°F.); 21.1°C. (70°F.) gave the highest rate of lipolysis. Krukovsky and Herrington (37) found milk lipase to be active in butter at -1.1° to 0°C. (30° to 32°F.), but not at -15°C. (5°F.) or lower. Tarassuk and Richardson (53) have observed that holding fresh milk at 32° to 37°C. for 1 to 3 hours will prevent spontaneous development of rancidity. Weaver's work (58) substantiates this by showing that raw milk samples held 15 hours at 4.4°C. (40°F.) became more rancid than duplicate samples held the same length of time at 21.1°C. (70°F.).

The process of homogenization or agitation has been shown to accelerate the rate of lipolysis in raw milk (2, 5, 6, 10, 15, 22, 25, 38, 49, 53, 56). Hlynka and Hood (25) demonstrated that agitation of milk at 30°C. (86°F.) dispersed the fat globules. They suggested that increased lipolysis in agitated milk might be explained by the increase of the fat-liquid interface area and by an alteration of the surface properties of the fat brought about by agitation. Tarassuk and Jack (51) stated that all physical processes (such as homogenization and agitation) which accelerate the action of lipase in milk have one property in common; they lead to disruption, partial displacement, or distortion of the natural adsorption layer on the fat globules. Experimentation by Tarassuk and Richardson (54) has borne out this postulation. They found that a replacement of the natural adsorption layer of the fat globules by other surface active material promoted the hydrolysis of the fat in raw milk or cream.

In 1932 Dorner and Widmer (6) published work dealing with the effects of homogenization on the development of rancidity in milk. Portions of this work have been quoted above. They found that rancidity developed most rapidly in raw milk homogenized at 40°C. and held at this temperature. Gould (10) homogenized milk at 21.1°C. (70°F.), 40.6°C. (105°F.), 46.1°C. (115°F.), 51.7°C. (125°F.), and 62.8°C. (145°F.). When stored at -1.1° to 4.4°C. (30° to 40°F.) for 72 hours, all samples showed some increase in fat acidity. The maximum amount of hydrolysis occurred in the range of 40.6° to 46.1°C. (105° to 115°F.) homogenizing temperature. The work of Babcock (2) indicated that the optimum homogenization temperature for the development of lipolysis was

30° to 40°C. Trout and Scheid (56) homogenized raw milk at temperatures of 4.4°C. (40°F.), 15.6°C. (60°F.), 26.7°C. (80°F.), 37.8°C. (100°F.), 48.9°C. (120°F.), and 60°C. (140°F.). When homogenized at 4.4° and 15.6°C. (40° and 60°F.), the milk fat was not dispersed and rancidity did not develop. From 26.7° to 60°C. (80° to 140°F.), the milk fat was dispersed, rancidity did develop, and increases in acidity were observed after 24 hours storage below 4.4°C. (40°F.). They homogenized raw milk five times at 4.4°C. (40°F.) under a pressure of 5000 pounds per square inch. This treatment failed to disperse the fat, and after holding 96 hours, no increases in acidity or changes in flavor could be shown. This indicated that milk fat had to be in the liquid state for homogenization of the fat, with its effect on lipolysis, to take place.

Several investigators (25, 38, 49, 53) have shown that shaking, or mild agitation, had an accelerating effect on lipolysis in raw milk. It was believed that effects similar to those induced by homogenization were brought about by agitation.

Krukovsky (34) observed that milk lipase was inactivated by exposure of raw milk to direct sunlight. His data indicated that the process was one of oxidation. Ninety minute exposures of deoxygenated milk in sunlight reduced lipolytic activity by only ten per cent.

Tarassuk and Henderson (50) have presented data showing that the development of spontaneous rancidity in milk can be prevented by diluting susceptible milk with four parts of normal milk. For maximum effect, this should be done before or immediately after cooling the fresh-drawn milk.

A number of reports have shown that advanced lactation is a major

factor in the secretion of milk high in a naturally active lipase (7, 8, 18, 23, 44, 52, 58). Others (20, 42, 49) have found no relationship or an inverse relationship between advance lactation and milk high in lipase. Seasonal variations in the lipase content of milk have been attributed in part to advanced lactation (8, 18, 23). Kelly (33) has shown a relationship between estrus and the lipase content of milk. The greatest amount of lipase activity was observed a few days before estrus. During the period of heat, lipolytic activity decreased, then increased slowly until the next estrus cycle.

During their study of homogenization effects on rancidity, Dorner and Widmer (6) attempted to ascertain which portion of the milk carried lipase. Their conclusion was that it accompanied the casein, or that an activator for the enzyme accompanied the casein. Mattick and Kay (42) believed the lipase was present in the aqueous rather than the fatty portion of milk. Using milk made from 40 per cent cream and skim milk, where one or the other had been pasteurized, Gould (11) found that most of the enzyme was in the skim milk. He also compared the lipolytic activity of skim milk and rennet whey on melted butter oil, finding again that most of the enzyme was in the skim milk.

CHAPTER III

METHODS

In this investigation the method of Peterson, Johnson, and Price (45) was used, with modifications, for the estimation of lipase activity in raw skim milk. The procedure consisted of incubating raw skim milk with a tributyrin substrate and measuring increases in titratable acidity after a thirty minute interval. The method was designed to operate at a pH of 8.5. To a 10 ml. sample of raw skim milk 2 ml. of 0.6 N sodium diethyl barbiturate buffer was added. The pH of this mixture was then adjusted to 8.5 with 5 N acid or base. The solution was placed in a water bath at 40°C. and allowed to come to this temperature, after which 0.2 ml. of tributyrin was added. After shaking vigorously for one minute, a 5 ml. aliquot was removed and titrated. The remaining solution was incubated 30 minutes at 40°C., shaken 30 seconds, and a second 5 ml. aliquot removed and titrated. The difference in the two titrations represented hydrolysis of tributyrin. The titration was carried out as follows: The 5 ml. aliquot was pipetted into a 50 ml. Erlenmeyer containing 5 ml. of a 0.02 per cent solution of thymolphthalein in 95 per cent alcohol. After adding 2 ml. of ether, the solution was titrated to a definite blue with 0.1 N alcoholic NaOH, using a microburette calibrated to 0.01 ml.

Certain modifications of the procedure given above were found to be necessary in the work reported in this paper. The titrating procedure and temperature of incubation listed above were used throughout. An incubation time of 1½ hours was used. Incubations were made in a

Precision Scientific Company model C-2 constant temperature water bath with a rated accuracy of $\frac{1}{2}^{\circ}\text{C}$. Adjustments of pH were made with 85 per cent lactic acid. All pH measurements were made with a glass electrode, using a Beckman Model H pH meter. A limited number of lipase activity tests were made in the pH range between the pH of normal milk and pH 8.5. In these tests sodium barbital and/or phosphate buffer was used as recommended by Peterson, Johnson, and Price (45). In all tests made at or below the pH of normal milk no buffer was used. During the first part of the work, pH adjustments were made as follows: Eighty-five per cent lactic acid was added to the raw skim milk, bringing it to the desired pH. The samples were then placed in a refrigerator for a period of approximately 30 minutes and the pH readjusted. It was found, however, that this period was not of sufficient length to allow the pH to reach a final constant value. Thereafter, pH adjustments were made in the afternoon with 85 per cent lactic acid, and the samples were stored at 4°C . overnight. The succeeding day, the pH was readjusted and lipase activity tests carried out. All pH adjustments were made at approximately 40°C . During the first ten experiments, control samples with no substrate added were incubated and titrated to check for acidity increases due to bacterial action. In the remainder of the work this practice was discontinued.

Milk from a variety of sources was used. A number of the samples were separated in an International Centrifuge, Size 1, Type SB, at 2000 rpm for 10 minutes. With this procedure it was difficult to obtain as low fat a skim milk as produced by the De Laval cold milk separator in the University of Tennessee Creamery, so the majority of samples used

were separated at the University of Tennessee Creamery.

The tributyrin, tricaproin, tricaprylin, and ethyl butyrate obtained from Eastman Kodak Company were used as substrates. Commercial rennet extract was obtained from Marschall Dairy Laboratory, Inc. for the experiments with rennet whey.

CHAPTER IV

RESULTS

The main object of this research was to establish the presence or absence of a lipolytic enzyme in milk which was active below the pH of normal milk so that additional information might be gained about Cheddar cheese ripening. Since milk contains materials which act as buffers, it was deemed unnecessary to add additional buffers to the milk when making pH adjustments in the range of pH 6.6 to approximately pH 5.0. The adjustments of pH were made with lactic acid since it is the major acid produced by the culture bacteria in the manufacture of Cheddar cheese. When pH adjustments were made with 85 per cent lactic acid, or other acids used in the experiments, the pH tended to rise slowly upon cool-aging. Since the pH intervals for the lipolysis tests were made in 0.10 units, it was necessary to hold the pH of the milk to as constant a value as possible during the incubation period. To obtain pH values which would hold constant, pH adjustments were made with the acid, the adjusted milk stored 18 to 24 hours at 4°C., warmed to 40°C., and a second pH adjustment made with the addition of the same acid. After the second adjustment was made the pH remained constant for the next 24 hours of cool-aging. Table I shows typical pH changes which took place in a sample of raw milk. The first column shows the initial pH of the adjusted milk; the second column shows the pH of the milk after being stored at 4°C. for 18 hours; the third column shows the pH after the second adjustment; the fourth column shows the pH of the readjusted restored milk after an additional storage for 24 hours.

TABLE I

PH CHANGES DURING COOL-AGING
OF ACIDIFIED MILKS

Adjusted pH	pH After 18 Hours ^a	Readjusted pH	pH After Next 24 Hours ^a
6.63	6.63		6.61
6.50	6.60	6.50	6.50
6.40	6.47	6.40	6.38
6.30	6.35	6.30	6.31
6.20	6.30	6.20	6.19
6.10	6.20	6.10	6.12
6.00	6.12	6.00	6.01
5.90	6.08	5.90	5.90
5.80	6.00	5.80	5.82
5.70	5.94	5.70	5.71
5.60	5.83	5.60	5.64
5.50	5.75	5.50	5.55
5.40	5.69	5.40	5.43
5.30	5.61	5.30	5.30
5.20	5.48	5.20	5.20
5.10	5.35		

^aStorage at 4°C.

Figure 1 shows the effect of pH on lipolysis of tributyrin. The values given represent the average of 10 trials. The same general relationship between lipase activity and pH was found to hold even when milks from different sources were used. Two definite systems of lipolysis of tributyrin were found; one with an optimum at pH 6.3 and the other at pH 5.4. Lipase activity was evident throughout the area of pH 5.0 to 5.7 and pH 6.0 to 6.6. Very slight activity, and sometimes none at all, was found in the range of pH 5.7 to 6.0. Figures 2, 3, 4, 5, and 6 show the pH curves of lipolysis for different milks. These were as follows: Figure 2--University of Tennessee Creamery Producer X-100; Figure 3--University of Tennessee Creamery Producer X-77; Figure 4--University of Tennessee Creamery Producer X-90; Figure 5--Pet Dairy Products Producer R-17; Figure 6--mixed milk from the University Herd. It will be noted that with the majority of these samples lipase tests were not conducted at close intervals between pH 6.0 and 6.6. A number of experiments have been conducted, however, which were included in the average pH curve, and which substantiated the presence of an enzyme whose optimum was at pH 6.3. In this series of tests samples were incubated with no substrate added to check for possible increases in titratable acidity due to bacterial action. Titration increases were never found in these samples.

Previous workers (4, 45, 57) have shown that certain salts have marked effects on the activity of milk lipase. Table II shows the effect of adding the major salts normally found in milk as chlorides and lactates. The salts were added to raw skim milk as 1.0 M aqueous solutions in such quantities as to give 0.1 M concentration in the milk.

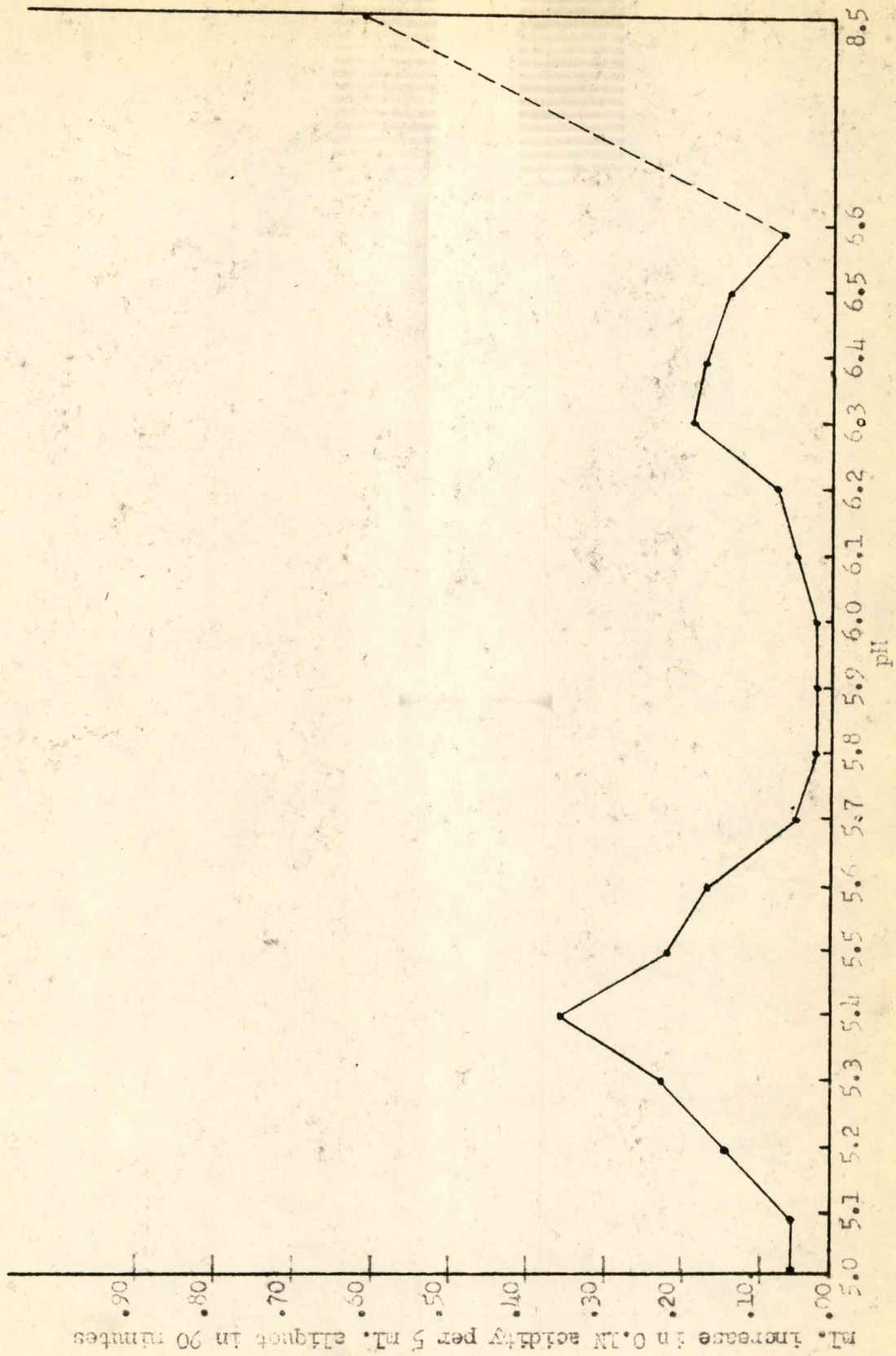


Figure 1. Effect of pH on lipolysis. Average of ten experiments.

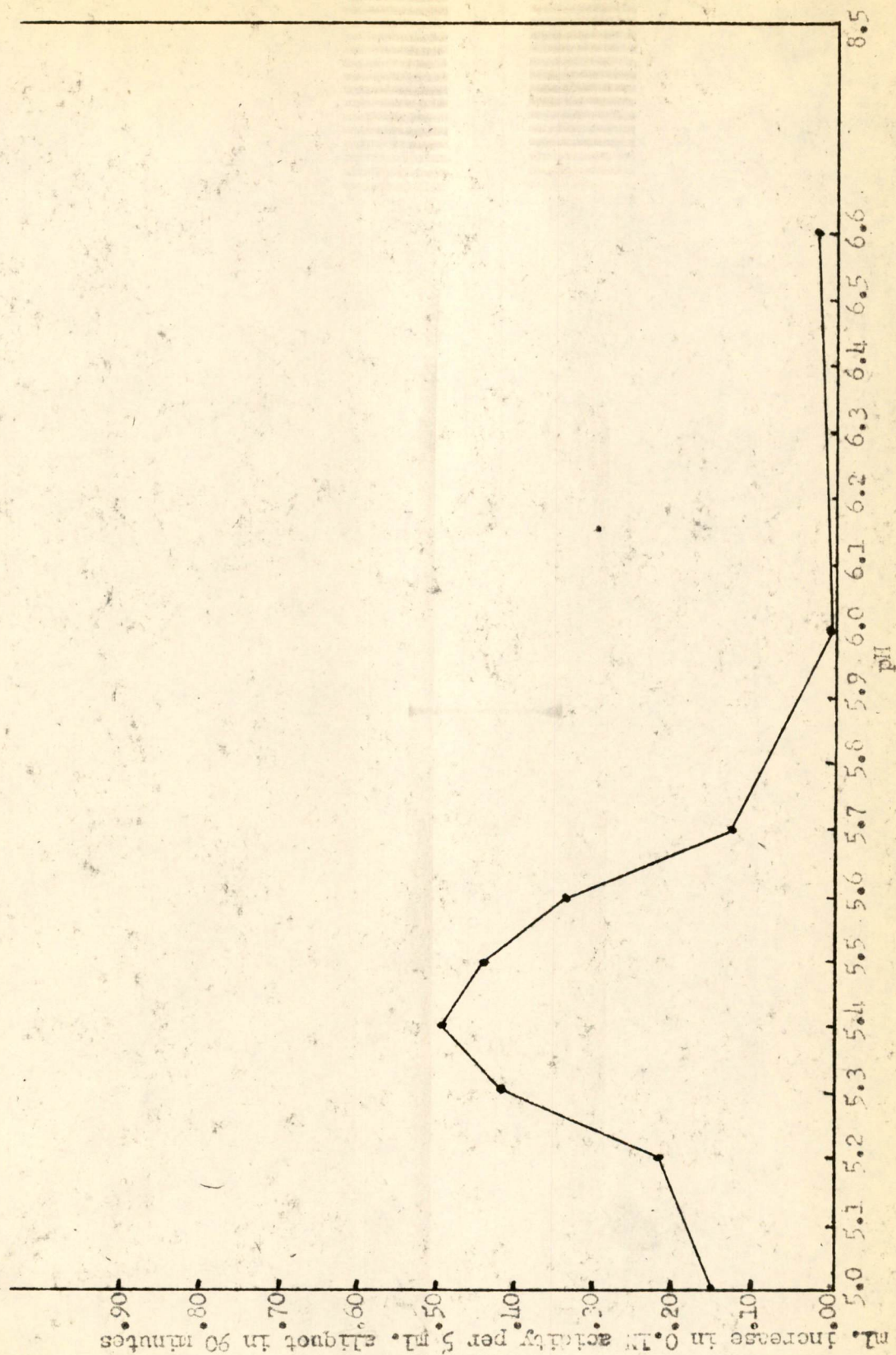


Figure 2. Effect of pH on lipolysis. Milk from University Creamery, Producer X-100.

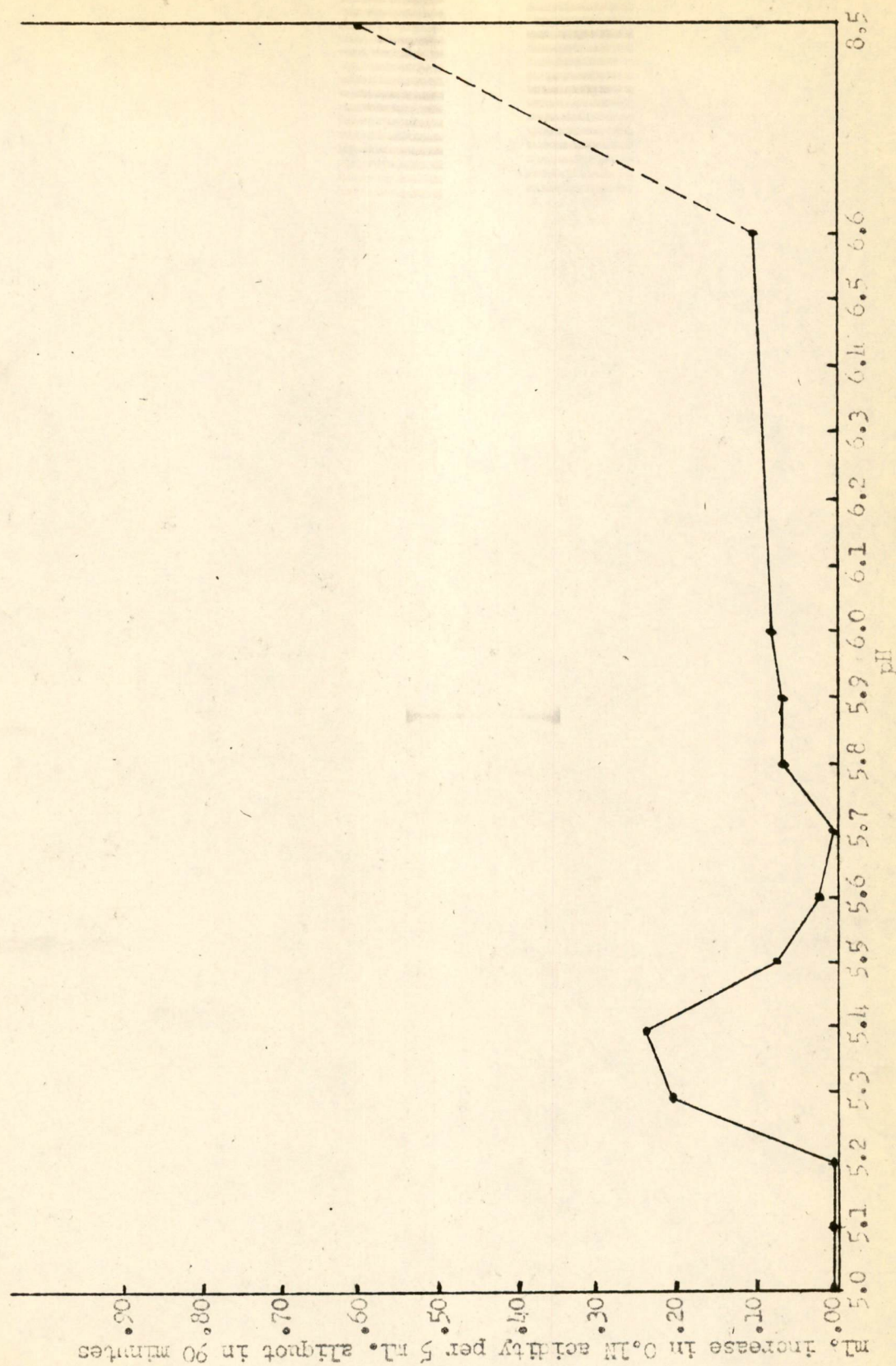


Figure 3. Effect of pH on lipolysis. Milk from University Creamery, Producer X-77.

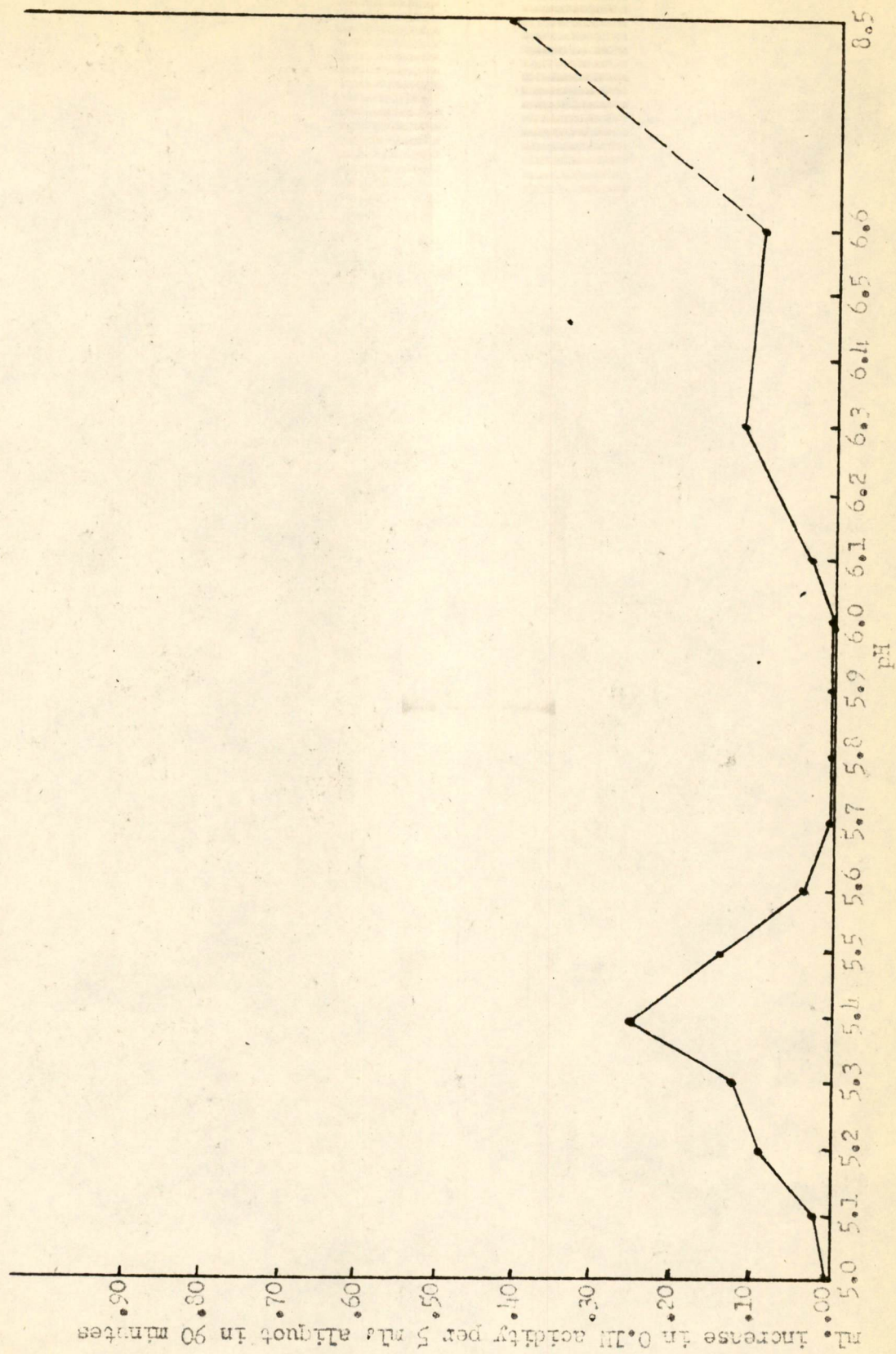


Figure 4. Effect of pH on lipolysis. Milk from University Creamery, Producer M-90.

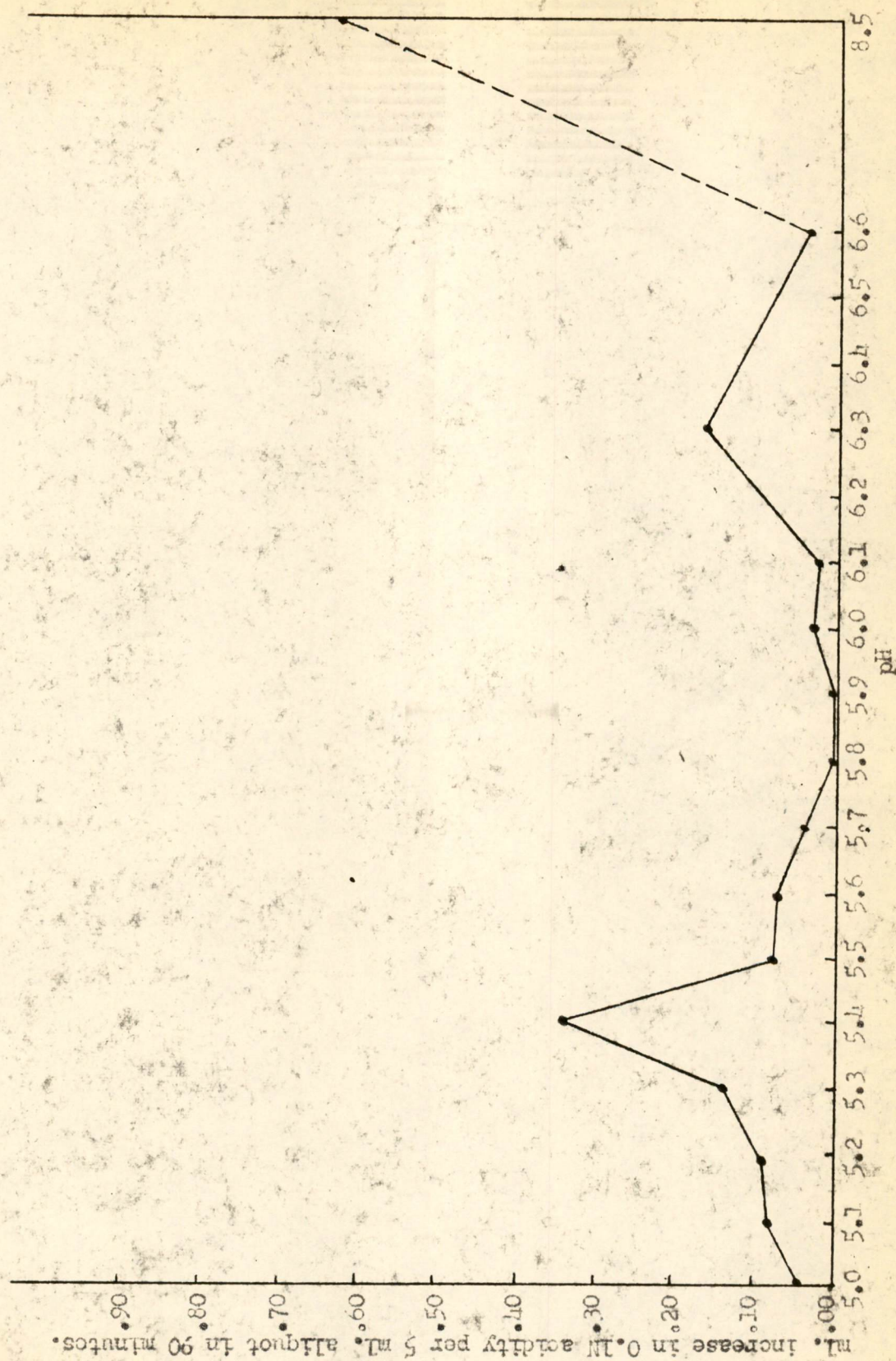


Figure 5. Effect of pH on lipolysis. Milk from Fet Dairy Products, Producer R-17.

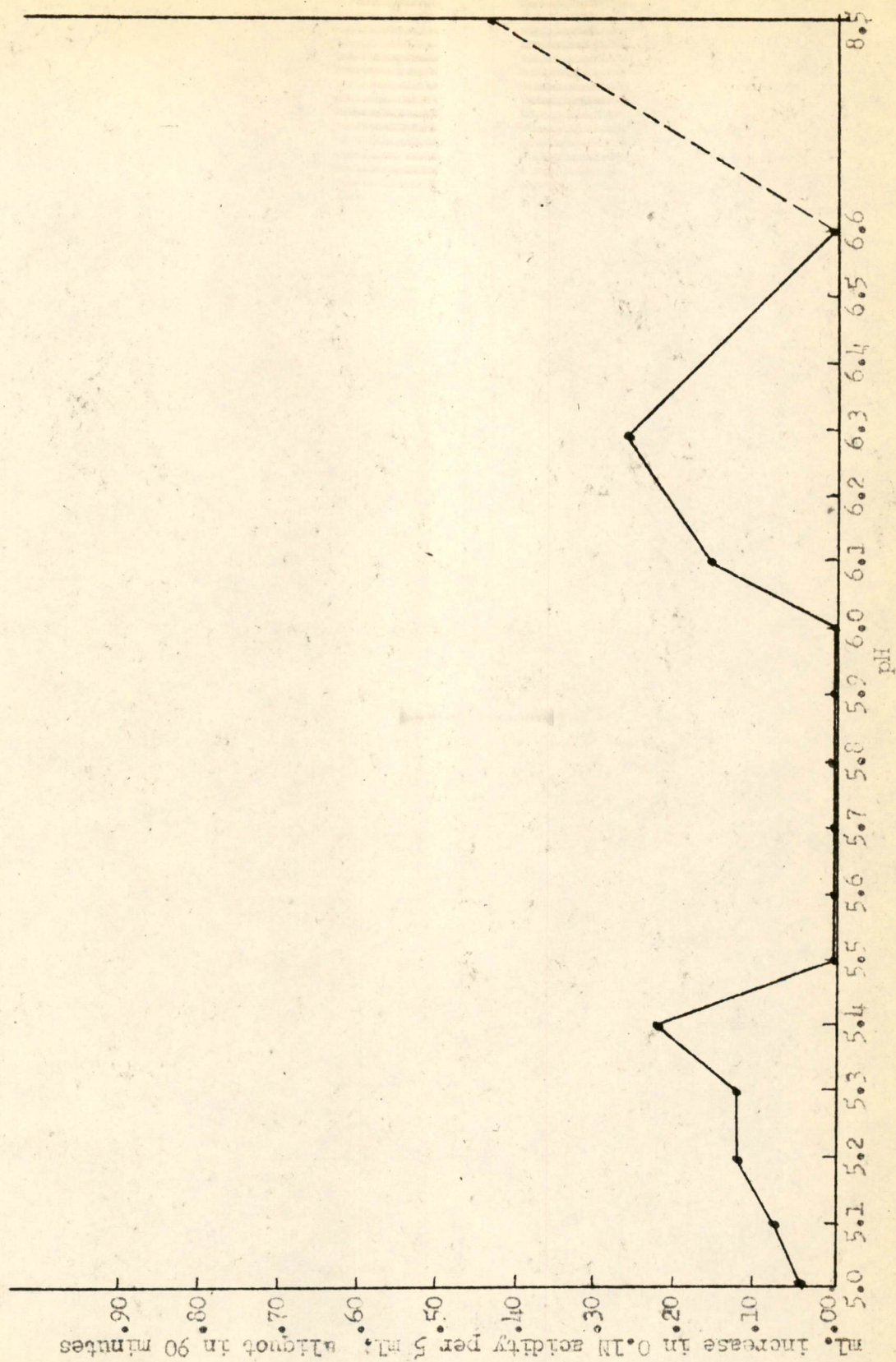


Figure 6. Effect of pH on lipolysis. Mixed milk from the University Herd.

TABLE II

EFFECTS OF CERTAIN SALTS ON LIPOLYSIS
OF TRIBUTYRIN BY MILK LIPASE

Salt Used	Molar Concentration	Titration Differences ^a pH 6.3	Titration Differences ^a pH 5.4
(Experiment I)			
Control	-	.33	.385
CaCl ₂	0.1	.12	.22
MgCl ₂	0.1	.27	.56
NaCl	0.1	.06	.07
KCl	0.1	.145	.27
(Experiment II)			
Control ^b	-	.19	.35
Ca lactate	0.1	.17	.34
Mg lactate	0.1	.17	.39
Na lactate	0.1	.10	.15
K lactate	0.1	.13	.21

^aTitration differences represent tributyrin hydrolysis by lipase. They are in terms of ml. 0.1 N acid released in 90 minutes by 5 ml. of milk held at 40°C. The term "titration difference" is used throughout in this same sense.

^bDifferent milk used.

After adding the salts to the milk the pH was then adjusted to pH 6.3 and 5.4 with 85 per cent lactic acid, the mixture stored 18 to 24 hours at 4°C., the pH readjusted to pH 6.3 or 5.4, and lipase activity tests conducted. In a preliminary experiment ZnSO_4 and MnCl_2 were used. When the 0.1 M concentration ZnSO_4 was added to the milk, it precipitated the casein completely; the pH was around 4.7. When 0.1 M MnCl_2 was used, a brown precipitate formed during titration which masked the thymolphthalein endpoint. For these reasons the two salts were not used in the experiments reported. CaCl_2 , MgCl_2 , NaCl , and KCl were used since these four cations make up the majority of the normal salts found in milk. CaCl_2 was found to lower lipase activity at both pH 5.4 and pH 6.3. MgCl_2 had no significant effect at pH 6.3 but may be stimulatory at pH 5.4. NaCl showed marked inhibition of lipolysis at both pH levels used. KCl was inhibitory at pH 5.4 and markedly inhibitory at pH 6.3. As a check on these results, the same four cations were tested at pH 6.3 and 5.4 using the lactate salts. These results also appear in Table II. Similar results were obtained with the exception of the calcium salt. Ca lactate was not significantly inhibitory at either pH level.

The lipase activity optima of pH 6.3 and pH 5.4 were established using lactic acid to make pH adjustments. A series of experiments were carried out to determine the effect on lipolysis at these pH levels using other acids to make pH adjustments. The pH adjustments and lipase activity tests were carried out exactly as described above with the exception that lactic acid was replaced with another acid. Acids used were 95 to 98 per cent sulfuric acid, concentrated hydrochloric, glacial

acetic, 85 per cent phosphoric, and a saturated solution of citric acid. These acids were added in high concentration to minimize dilution of the milk. Results from these experiments appear in Table III. This tabulation represents the average of four experiments. Acetic, hydrochloric, and sulfuric acid almost completely inhibited lipase activity at pH 6.3 and 5.4, as compared to a lactic acid. Citric and phosphoric acid were also inhibitory, but not nearly to the extent of acetic, hydrochloric, and sulfuric.

The effect of pH on the hydrolysis of four different substrates by milk lipases was investigated. The substrates employed were tributyrin, tricaproin, tricaprylin, and ethyl butyrate, which were added to the milk at the rate of 2 per cent by volume. Figure 7 shows the effect of pH on the hydrolysis of the three triglycerides by milk lipase. The pH curves were similar in shape with the exception that tricaprylin was hydrolyzed between pH 5.7 and 5.9 to a greater extent than the other two triglycerides. Figure 8 compares the hydrolysis of tributyrin with that of ethyl butyrate. The experiments shown by the two graphs were conducted using different sample of raw skim milk. It was noted that ethyl butyrate was hydrolyzed at pH limits of the two lipases, but in the case of the pH 5.4 optimum system the enzyme was capable of hydrolyzing the ester at a higher pH range. This may account for the small amount of tributyrin hydrolysis previously noted in Figure 1 between pH 5.7 and 6.0.

Peterson, Johnson, and Price (45) reported that formaldehyde inhibited lipolysis of tributyrin by about 75 per cent. An experiment was set up to determine whether or not formaldehyde inhibited milk lipase

TABLE III

EFFECTS OF CERTAIN ACIDS ON HYDROLYSIS
OF TRIBUTYRIN BY MILK LIPASE

Acid Used	Titration Differences pH 6.3	Titration Differences pH 5.4
Lactic	.33	.47
Citric	.18	.31
Phosphoric	.13	.25
Acetic	.01	.02
Hydrochloric	.005	.01
Sulfuric	.01	.015

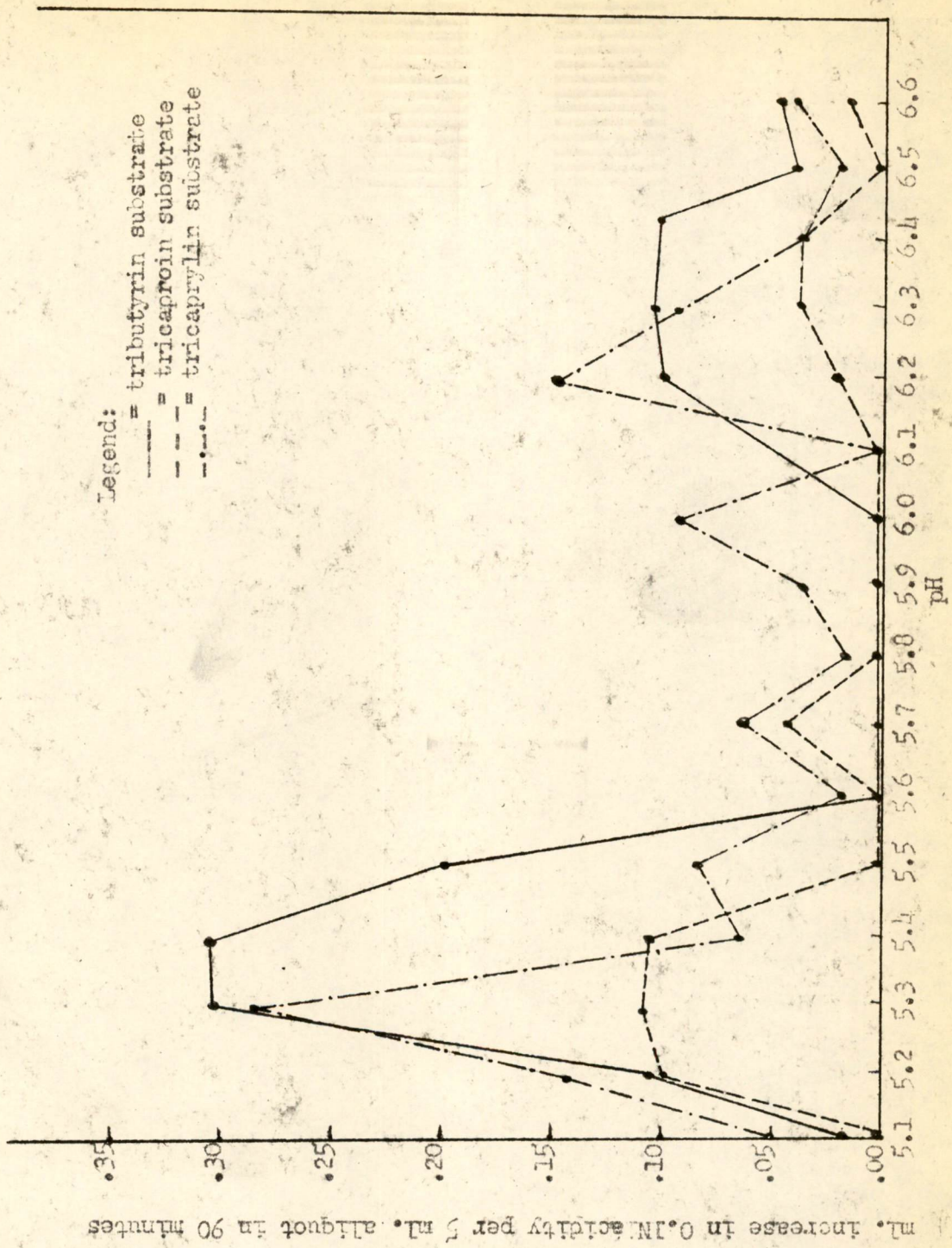


Figure 7. Effect of pH on triglyceride hydrolysis by milk lipase.

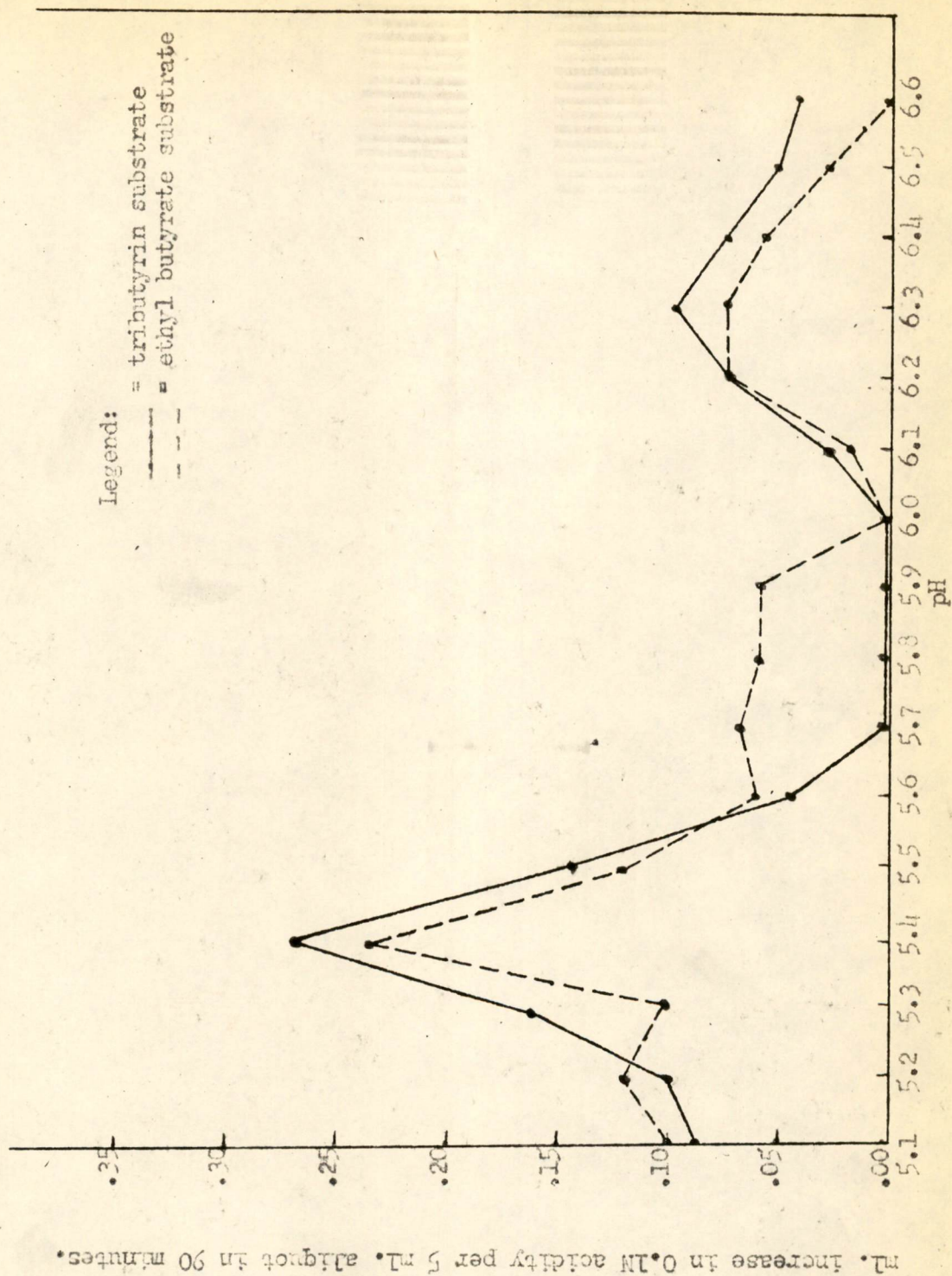


Figure 8. Effect of pH on hydrolysis of tributyrin and ethyl butyrate by milk lipase.

and esterase active between pH 5.1 and pH 6.6. Formaldehyde (37 per cent) was added to raw skim milk at the rate of 2 grams per liter. A portion of the same skim milk was retained with no formaldehyde added. PH adjustments were made on the two samples and lipase activity was determined by the method previously described. Tributyrin and ethyl butyrate (2 per cent added by volume) were used as substrates. Figure 9 shows the results of this experiment. The addition of formaldehyde to the milk reduced the activity of both the lipase and the esterase in skim milk. The formaldehyde also reduced the area of the pH 5.4 optimum esterase system so that the upper limit was only pH 5.7 instead of the 5.9 to 6.0 as observed for the untreated skim milk.

Various investigators have demonstrated that the lipase in milk is associated with casein. Others have found that a part of the enzyme accompanied the whey after milk was coagulated with rennin. A series of experiments were carried out to determine whether the lipases and esterases active in milk at pH 6.3 and 5.4 were associated with the casein or with the whey proteins of skim milk. In the first experiment a sample of raw skim milk was adjusted to pH 6.3 with 85 per cent lactic acid and precipitated with concentrated rennet extract (5cc. added per 1000 cc. milk) at 30°C. A portion of the original skim milk was retained. Portions of that milk and the rennet whey (filtered) were adjusted to pH 6.3 and 5.4 with 85 per cent lactic acid, stored overnight at 4°C., the pH readjusted, and lipase activity tests carried out. Table IV shows the results. The samples of skim milk at pH 6.3 and 5.4 hydrolyzed tributyrin; the whey samples at the same pH did not. A possibility that the enzyme might be adsorbed on the filter paper

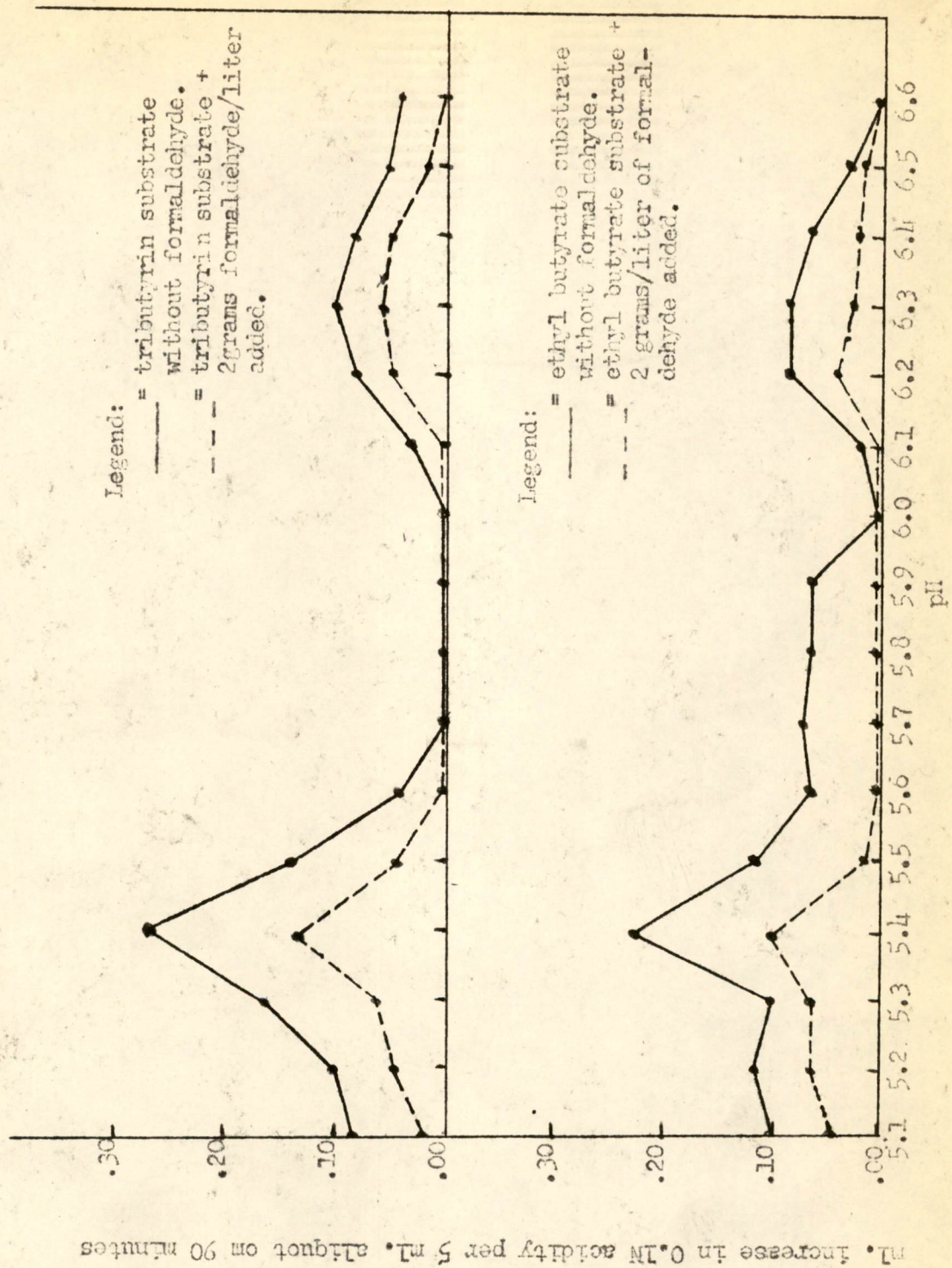


Figure 9. Effect of formaldehyde on milk lipase and esterase between pH 6.6 and pH 5.1.

TABLE IV

HYDROLYSIS OF TRIBUTYRIN BY SKIM MILK
AND RENNET WHEY (FILTERED)

Sample	Adjusted pH	After Storage pH	Titration Differences
Raw Skim Milk	6.3	6.36	.23
Raw Skim Milk	5.4	5.58	.52
Rennet Whey	6.3	6.30	.00
Rennet Whey	5.4	5.40	.00

existed, so a second experiment was conducted, similar to the one described above, in which a part of the rennet whey was filtered and a part was drained from the curd without filtering. Both tributyrin and ethyl butyrate were used as substrates. Table V shows that the raw skim milk hydrolyzed both these substrates at pH 6.3 and 5.4, while neither filtered nor unfiltered whey had any hydrolytic activity. This indicated that the lipases and esterases active in the raw skim milk at pH 6.3 and 5.4 were combined with rennet coagulated paracasein.

In a third experiment, a sample of raw skim milk was divided into two parts. One portion was precipitated by adjusting the pH to 4.7 with 85 per cent lactic acid at 25°C., and the other portion was held as a control. The acid whey from the coagulated milk showed a pH of 4.62 after draining. A part of this whey was filtered and another part retained without having been filtered. The pH of the skim milk was adjusted to pH 6.3 and 5.4 with 85 per cent lactic acid. Portions of the filtered and unfiltered whey were adjusted to pH 6.3 and 5.4, using a heavy suspension of $\text{Ca}(\text{OH})_2$. All samples were stored overnight at 4°C., the pH readjusted, and lipase activity tests conducted. The results appear in Table VI. No lipase or esterase activity was demonstrated in either of the whey samples, while it was present in the skim milk controls. The purpose of the fourth experiment was to ascertain whether or not the lipase and esterase active in milk at pH 6.3 and 5.4 was irreversibly denatured by acid precipitation of the casein. A sample of raw skim milk was precipitated with 85 per cent lactic acid. Portions of the whey from this were adjusted to pH 6.3 and 5.4 with $\text{Ca}(\text{OH})_2$. A portion of the curd from the precipitated milk was drained

TABLE V

HYDROLYSIS OF TRIBUTYRIN AND ETHYL BUTYRATE
BY SKIM MILK, FILTERED RENNET WHEY,
AND UNFILTERED RENNET WHEY

Sample	Substrate	Adjusted pH	After Storage pH	Titration Differences
Raw Skim Milk	Tributyrin	6.3	6.38	.07
Raw Skim Milk	Ethyl Butyrate	6.3		.04
Raw Skim Milk	Tributyrin	5.4	5.61	.31
Raw Skim Milk	Ethyl Butyrate	5.4		.31
Filtered Whey	Tributyrin	6.3	6.31	.00
Filtered Whey	Ethyl Butyrate	6.3		.00
Filtered Whey	Tributyrin	5.4	5.42	.00
Filtered Whey	Ethyl Butyrate	5.4		.00
Unfiltered Whey	Tributyrin	6.3	6.30	.00
Unfiltered Whey	Ethyl Butyrate	6.3		.00
Unfiltered Whey	Tributyrin	5.4	5.40	.00
Unfiltered Whey	Ethyl Butyrate	5.4		.00

TABLE VI

HYDROLYSIS OF TRIBUTYRIN AND ETHYL BUTYRATE BY
SKIM MILK, FILTERED LACTIC ACID WHEY,
AND UNFILTERED LACTIC ACID WHEY

Sample	Substrate	Adjusted pH	After Storage pH	Titration Differences
Raw Skim Milk	Tributyrim	6.3	6.38	.00
Raw Skim Milk	Ethyl Butyrate	6.3		.00
Raw Skim Milk	Tributyrim	5.4	5.65	.29
Raw Skim Milk	Ethyl Butyrate	5.4		.20
Filtered Whey	Tributyrim	6.3	6.32	.00
Filtered Whey	Ethyl Butyrate	6.3		.00
Filtered Whey	Tributyrim	5.4	5.40	.00
Filtered Whey	Ethyl Butyrate	5.4		.00
Unfiltered Whey	Tributyrim	6.3	6.30	.00
Unfiltered Whey	Ethyl Butyrate	6.3		.00
Unfiltered Whey	Tributyrim	5.4	5.49	.01
Unfiltered Whey	Ethyl Butyrate	5.4		.00

of whey and resuspended in enough distilled water containing $\text{Ca}(\text{OH})_2$ to give approximately the same concentration of resuspended curd as originally existed in the milk. Portions of the curd suspension were adjusted to pH 6.3 and 5.4 with lactic acid. Control samples of the original skim milk were adjusted to pH 6.3 and 5.4 with 85 per cent lactic acid. All samples were stored overnight and lipase activities carried out the succeeding day after the usual adjustments of pH. Results appear in Table VII. The resuspended curd showed lipase and esterase activity comparable to the original skim milk. No activity was shown by the acid whey samples. It is interesting to note the pH changes with cool-aging of skim milk and whey as shown by Tables IV, V, VI, and VII. In all cases the pH of skim milk increased more than that of whey.

Throughout the experimental work reported, an incubation temperature of 40°C . was used. This was the temperature recommended by Peterson, Johnson, and Price (45) for the hydrolysis of tributyrin at pH 8.5. An experiment varying incubation temperatures with the lipases active at pH 6.3 and 5.4 was conducted. Tributyrin was used as the substrate; pH adjustments and lipase activity tests were conducted as previously described with incubation temperature as the variable factor. The results of this experiment are shown by Figure 10. The temperature curves for the two lipases with optima at pH 6.3 and 5.4 were roughly parallel. The pH 6.3 system had an optimum temperature at $37\frac{1}{2}^\circ\text{C}$.; the optimum for the pH 5.4 system was 40°C .

TABLE VII

HYDROLYSIS OF TRIBUTYRIN AND ETHYL BUTYRATE
BY SKIM MILK, LACTIC ACID WHEY, AND
RESUSPENDED LACTIC ACID CURD

Sample	Substrate	Adjusted pH	After Storage pH	Titration Differences
Raw Skim Milk	Tributyrim	6.3	6.36	.07
	Ethyl Butyrate	6.3		.06
	Tributyrim	5.4	5.78	.11
	Ethyl Butyrate	5.4		.10
Acid Whey	Tributyrim	6.3	6.33	.00
	Ethyl Butyrate	6.3		.00
	Tributyrim	5.4	5.70	.00
	Ethyl Butyrate	5.4		.00
Resuspended Acid Curd	Tributyrim	6.3	6.40	.06
	Ethyl Butyrate	6.3		.06
	Tributyrim	5.4	5.52	.10
	Ethyl Butyrate	5.4		.10

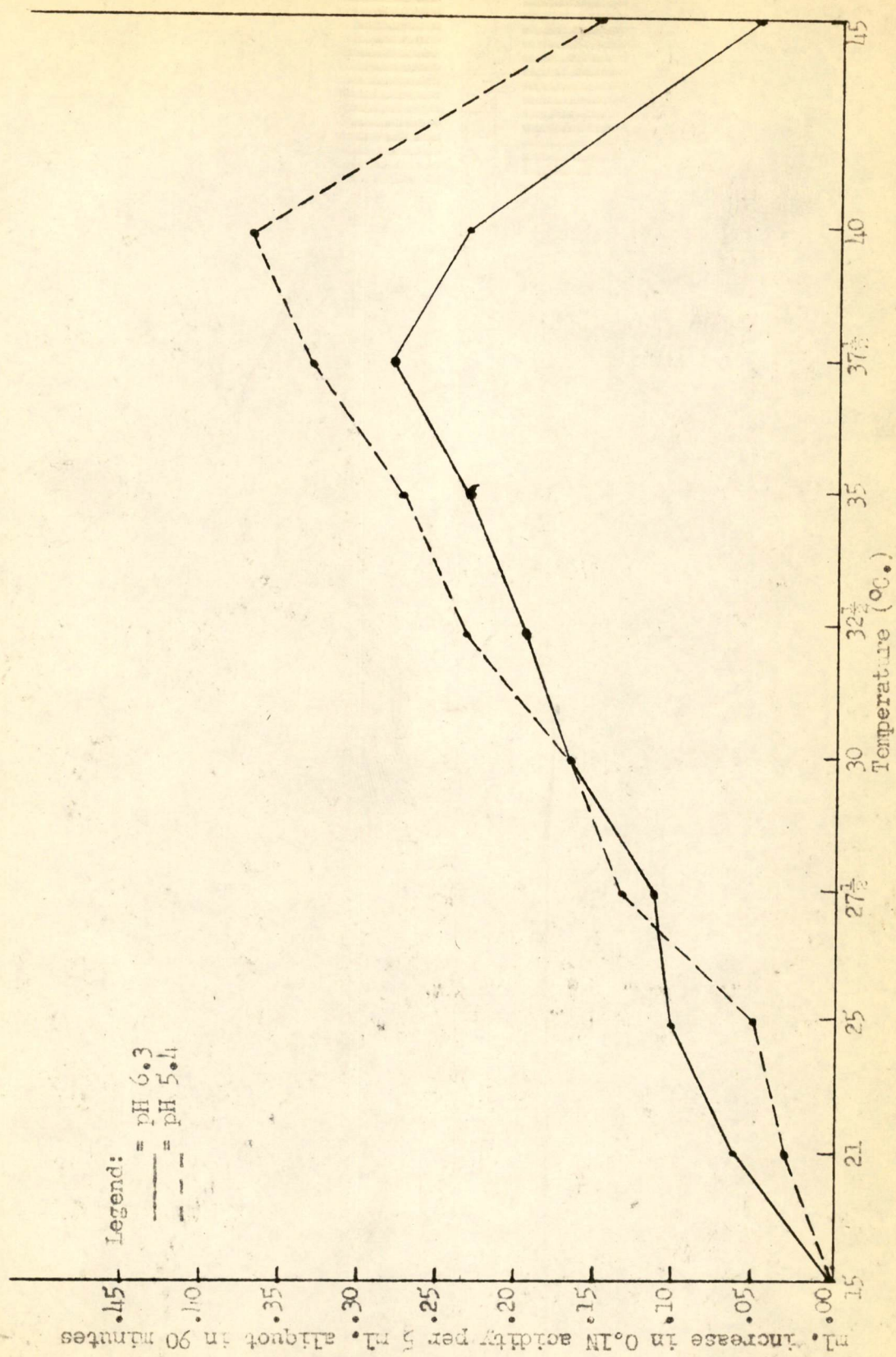


Figure 10. Effect of temperature of incubation on the rate of tributyrin hydrolysis by milk lipase.

CHAPTER V

DISCUSSION

The findings of many earlier workers related to lipase activity in raw milk have been confirmed and extended. Two definite areas of lipolytic activity have been observed in skim milk between the pH of 6.6 and 5.0, with one optimum at 6.3 and another at pH 5.4 when tributyrin was used as a substrate. The same optima were observed for the three triglycerides used in this study; therefore, it may be assumed from the work of Gould (12) that other triglycerides would behave similarly. Whereas the amount of lipase activity varied from milk to milk, the optimum activity of the two enzymes was always observed at pH 6.3 and 5.4.

There were two areas of esterase activity in the skim milk when ethyl butyrate was used as a substrate. Figure 8 shows the esterase areas to be similar to the two lipase areas. In the case of the optimum pH 5.4 system the area of hydrolysis was extended to an upper limit of pH 6.0, whereas in the case of the lipase within the same activity area, the upper limit of hydrolysis was pH 5.7.

The addition of formaldehyde to the skim milk reduced the activity of the lipase and the esterase systems. In the case of the esterase system the formaldehyde restricted the breadth of the pH 5.4 optimum system leaving the general shape of a lipase system corresponding to the same pH area. This indicated the possibility that the esterase was inactivated by the addition of formaldehyde. Therefore, the lipase and esterase were capable of utilizing either the triglyceride or

the ethyl butyrate. In the pH 6.3 system the effect of the added formaldehyde was more severe, with the lipase enzyme almost completely inactivated and the esterase reduced to about half its original activity. In this system it may easily be the lipase that is inactivated and the small amount of hydrolysis of triglyceride was in reality the esterase using the triglyceride.

The choice of acid used to lower the pH of the skim milk was found to be of the utmost importance. Previous workers (6, 12, 45) have used acids other than lactic acid and have reported very little, if any, lipolytic activity within the pH range of 6.6 to 5.0. Hydrochloric, sulfuric, and acetic acid as shown in Table III reduced the enzymatic activity to almost zero, whereas phosphoric and citric acids retarded the activity about fifty per cent. The addition of the cations common to milk affected the activity of the two lipase systems. Sodium and potassium retarded the activity, whereas calcium and magnesium in the form of lactates caused very little change. When the chlorides of the above salts were used, the activity was depressed in all cases which indicated the effect of the chloride ion as well as the monobasic ions. The exception to the above was the magnesium chloride which was stimulatory in the pH 5.4 active system.

One of the most interesting observations in these experiments was the change of the pH of acidified milk upon cool-aging. When the acidified skim milks were stored at 4°C., the pH always rose. The lower the pH of the milk, the greater was the rise when cool-aged for 18 to 24 hours. This effect was observed regardless of the acid used to make the pH adjustments. The experiments dealing with acid and rennet whey

indicated that the casein was responsible for a major part of the effects. The pH of the whey always rose less than the corresponding skim milk.

Generally the enzyme systems in milk have been associated with the whey proteins. However, the lipases in this study can be quantitatively removed with the casein either by the addition of lactic acid to the isoelectric point of the casein or by rennet coagulation. This point may be of considerable significance in the ripening of Cheddar cheese. In the manufacture of raw milk Cheddar cheese a considerable amount of lipolytic activity has been associated with the development of a typical Cheddar cheese flavor (Lane and Hammer (41)). The development of a rancid cheese would indicate a milk high in lipase of the pH 5.4 optimum system was used, and since it is almost totally carried with the cheese curd, it would be present in the cheese. Now, if the amount of salt added to the cheese was reduced, the flavor might also develop. Tuckey and Ruehe (57) claimed that there must be at least 1.70 per cent salt in the finished cheese to control this factor. In pasturized milk cheese this is not a factor as the enzymes are reported to be inactivated by normal pasturizing temperature.

CHAPTER VI

SUMMARY

1. Two lipase systems have been demonstrated in raw skim milk; one was found to have an optimum at pH 6.3 and the other an optimum at pH 5.4. Lipolysis was evident throughout the areas of pH 5.0 to pH 5.7 and from pH 6.0 to pH 6.6.

2. Esterase activity was also found at the above pH areas, and in addition in the area of pH 5.7 to pH 6.0.

3. The lipases active at pH 6.3 and 5.4 were inhibited by NaCl and KCl.

4. Lactic acid was the only pH depressant used which did not inhibit the pH 6.3 and 5.4 lipases. Other acids used were sulphuric, hydrochloric, acetic, phosphoric, and citric.

5. Formaldehyde (2 grams added per 1000 ml. milk) decreased both lipase and esterase activity.

6. The lipases and esterases studied were found to be quantitatively removed from skim milk by precipitation of the casein using rennet extract or by adjusting the skim milk to pH 4.7 with lactic acid.

7. The optimum temperature for the enzyme active at pH 6.3 was found to be $37\frac{1}{2}^{\circ}\text{C}$. The optimum temperature for the enzyme active at pH 5.4 was found to be 40°C .

8. The pH of acidified, cool-aged skim milk was found to rise over a 24 hour period following the pH adjustment. After a second adjustment was made, the pH of the readjusted skim milk tended to change very little.

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