STUDIES ON BOVINE SKIMMILK LIPASE AND ITS RELATIONSHIP TO CERTAIN MILK PROTEINS

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CHAPTER I

INTRODUCTION

Hydrolytic rancidity describes a flavor in food products caused by the presence of free fatty acids, especially those of shorter chain-length ($\leqslant C_{12}$). These fatty acids result from the hydrolytic cleavage of triglycerides by an enzyme called lipase. In milk, rancid flavors usually are undesirable and detract from the market value of the product. In fact, the flavor can become so intense that the milk is unfit for human consumption. In recent years the increased use of pipeline milkers has brought about a considerable increase in the incidence of rancidity. While some of this increase has been reduced by improved processing and handling techniques (e.g. equipment modification, cooling procedure, etc.), the basic information needed for the control of this enzyme has not been elucidated.

Another reason for the study of this enzyme is the recent increased popularity of certain cheeses and confectionary items. The desirable flavor of these products is thought to be partly due to the controlled development of rancid flavor. Thus, a knowledge of the factors involved in the development and control of this flavor would be of practical importance.

Lipase functions in body metabolism through its role in the breakdown of triglycerides to fatty acids. This facilitates their transfer through cellular membranes. After digestion, the fats are stored as

triglycerides in the adipose tissue. These triglycerides must be broken down to fatty acids before they can be transported to the liver for degradation and for synthesis. Lipase is an essential and perhaps controlling step in this transfer, thus the level of lipase activity may play an important role in body fat metabolism and could be related to various metabolic disorders.

A lipoprotein lipase, requiring a protein acceptor for the fatty acids released, has been reported in bovine skimmilk. Whether this enzyme is the same as that normally associated with rancid flavor is not known. A study of whether a protein acceptor is needed for lipolysis to occur in milk might indicate whether these enzymes are the same. A fraction of milk protein, called kappa-casein, has been reported by some to be either the enzyme lipase or closely associated with it. Other workers have used sialic acid as an indirect measurement of kappa-casein. However, a study of the correlation between lipase activity and kappa-casein has not been reported. The purification and study of lipases is desirable because it would contribute to our understanding of the properties and functions of these enzymes.

The objectives of this research were: (a) to study the emulsification requirements of bovine skimmilk lipase, (b) to quantitatively measure and correlate the lipase activity with the sialic acid content of bovine skimmilk, and (c) to separate and study the protein(s) responsible for lipase activity in bovine skimmilk.

CHAPTER II

LITERATURE REVIEW

The serial classification number for lipase, according to the suggestions of the Commission on Enzymes of the International Union of Biochemists (44), is 3.1.1.3. In this number the first digit 3, represents the class of hydrolases; the second digit 1, represents the subclass of enzymes acting on ester bonds; the third digit 1, represents the sub-subclass of carboxylic ester hydrolases; and the final digit 3, represents the enzyme glycerol ester hydrolase - the systematic name for lipase. A somewhat similar enzyme, esterase (E.C. 3.1.1.1) has been distinguished from lipase, in that it will hydrolyze esters in solution, while lipase will only hydrolyze an ester emulsion. Most of the free fatty acids produced in milk result from the action of lipase.

Distribution and Purification of Milk Lipase(s)

Two excellent reviews on milk lipase (6) and lipolysis (28) were published in 1964. According to Chandan and Shahani (6), Dorner and Widner (12) were the first to report that milk lipase was associated with the casein fraction of milk. There now is ample evidence that the principal lipolytic activity of cows milk is associated with the casein fraction (23, 43, 49). However, there is considerable disagreement as to the specific location of lipase activity within casein. In 1956, Harper, *et al*. (24) reported that lipase activity was associated with

the casein fraction of freeze-dried raw skimmilk. In 1959, Patton (43) proposed the theory that lipase was casein *per se* rather than a minor contaminant of casein. However, two groups of authors (46, 57) have since demonstrated that lipolytic activity was associated with the alpha (α) - casein fraction of milk. The α-casein, however, is a highly heterogeneous mixture of at least three components: α_s-, lambda (λ) -, and kappa (K) - caseins (35) and both α_s- and K- caseins have been shown to exist in several polymorphic forms (31, 40, 55).

Purified lipase preparations have been obtained by several workers. These preparations include lipase from: clarifier slime (4), rennet casein extracts (18, 20), frozen-thawed skimmilk (20), and fat globule membranes (34). However, none of these lipase-active preparations have been shown to be α-casein or a component thereof. Using diethylaminoethyl (DEAE) cellulose chromatography, Yaguchi and Tarassuk (57) suggested that the majority of the lipase activity in milk was associated with K-casein and that rennin splits K-casein into lipase-active para-casein and a lipase-inactive glycomacropeptide. However, the question as to whether milk lipase is K-casein *per se*, or only a part of the K-casein complex is still unresolved. Recently, Fox, *et al.* (17), attempted to disassociate lipase activity from K-casein with dimethyl-formamide (DMF). They reported that approximately 50% of the total lipase activity was soluble in half saturated $(NH_4)_2SO_4$ after being equilibrated against a 30% DMF buffer. The specific activity of the supernatant of the $(NH_4)_2SO_4$ was seven to eight times higher than that of the original skimmilk. Thus, lipase activity, perhaps, could be separated from K-casein. Further work from the same laboratory (18, 42), reported the isolation and purification of a lipase from skimmilk

which appeared to be a distinct minor protein moiety different from any of the other known milk proteins. The molecular weight (M.W.) of their isolate was 210,000, as estimated by Sephadex G-200.

Gaffney et al. (20) separated water extracts of rennet casein into eight fractions with a DEAE-cellulose column and into three fractions using Sephadex gel filtration. These authors theorized that the wide variation in specific activities of the different fractions suggested that a single lipase was absorbed to varying degrees on the various casein components and that caseins per se are not lipases. Their findings show the enzyme to be associated with other proteins in addition to K-casein. In later work from the same laboratory (21), lipase-rich fractions of skimmilk, frozen-thawed skimmilk and the water-extract of rennet casein were obtained by Sephadex gel filtration. Molecular weights of the compounds with lipase activity from these three sources were all less than 10,000.

In 1963, Chandan, et al. (5) isolated a bovine milk lipase from separator slime. Using sedimentation-diffusion and osmotic pressure techniques, they reported a M.W. of approximately 7,000. This is the lowest M.W. for any enzyme thus far reported in the literature. Using Sephadex G-100 and G-200 gel filtration columns, Downey and Andrews (14) demonstrated four tributyrinases (lipases) from individual cow's milk. Molecular weights of 62,000, 75,000 and 112,000 were estimated for three of them. The fourth was very small, M.W. $<$ 10,000, and they stated that this might be the same enzyme isolated by Chandan and Shahani (4). Korn (34) has reported a lipase in milk which requires a lipoprotein substrate. Although he used the buttermilk from churned cream as the enzyme source, Korn reported most of the enzyme was

located in the skimmilk. The relationship between this enzyme and the others reported in this review is not clear.

Lipase Assay

Several methods have been used to assay lipase activity in milk. In general, lipase or its source is incubated with some lipid substrate and the free fatty acids resulting from the enzyme action are measured. One method, used by several workers, involves the extraction of free fatty acids from the lipolyzed substrate with organic solvents (19) and titration of these acids with a standard alkali. Another technique, called the pH-stat method (37, 41), involves lipolysis of a fat-emulsion and continuous titration of the liberated free fatty acids to maintain a constant optimum pH.

Milk fat or tributyrin are the substrates used by most workers. Milk fat is the natural substrate, but due to its inconsistent composition Montgomery and Forster (39) and others (11, 14) favor the use of tributyrin in lipase assays. Dunkley and Smith (11) have shown a statistically significant positive correlation between results obtained when the two substrates were compared. Wills, in Advances in Lipid Research (54), has stated that "tributyrin appears to be the substrate of choice." Desnuelle (9) believes that a prerequisite for a substrate for lipase assay is an insoluble ester that is properly emulsified. He recommends the use of 10% gum arabic for emulsion of tributyrin.

The effect of pH on lipase hydrolysis rates is a result of its effects on the enzyme, and on the emulsified substrate. Various pH optima for lipase activity have been reported. Albrecht and Jaynes (2) demonstrated the presence of acid lipases in raw skimmilk with pH

optima at 5.4 and 6.3. However, the usual pH optima vary between 8.0 and 9.5. Schwartz et al. (45) using lyophilized skimmilk as the enzyme source and pasteurized-homogenized cream as the substrate, observed a maximum activity at pH 8.5. Frankel and Tarassuk (19) reported that the pH optimum of milk lipase varied with the substrate used. They found an optimum of 8.8 to 9.1 for milk fat and 8.7 to 8.9 for tributyrin.

With a few exceptions, lipases are most active within the temperature range of 30 to 40 C. The optimum temperature most often reported for lipase assay is 37 C. Jensen (28) has used 38 C for milk fat as the substrate. However, Downey and Andrews (14) reported using 25 C for their enzyme assay procedure and Stadhouders and Mulder (47) recommend that a temperature of 15 C be used in lipase assay work. They based this on the fact that at 37 C milk lipase activity, during a one hour incubation period, will be affected by heat inactivation of the enzyme. However, as Shahani (6) has pointed out, at 15 C the physical state of high melting substrates like milk fat may not be conducive to optimum lipase action. Incubation of reaction mixtures for one hour has been generally accepted as the optimum time when milk fat is used as the substrate. A shorter time has been recommended when tributyrin is the substrate (19).

Lipase activity has been reported in various terms, including acid degree value, free fatty acid titer, surface tension decrease, and CO_2 volume. This has made it difficult to compare the results of different laboratories. More recently, however, reports in the literature have conformed to the recommendations of the Commission on Enzymes of the International Union of Biochemistry (44). Their definition of a lipase

unit is the amount of enzyme which will liberate one micromole of acid per unit of time from a standard substrate under specific test conditions. Thus, a lipase unit is the μmoles of fatty acid liberated per min, per ml of enzyme preparation, or per mg of protein.

Relationship of Sialic Acid to Lipase Activity

Sialic acids are substituted derivatives of neuraminic acid with N-acetyl and N-glycolyl being the most frequent substituted groups. According to Gottschalk (22), sialic acids function as components of hormones, enzymes, antibodies, cellular membranes and other biological structures. The K-casein fraction of bovine milk protein contains a glycomacropeptide which can be released by the action of rennin (1, 30). The peptide and glucide portions of this glycopeptide have been characterized, and it has been shown that the glucide portion contains a sialic acid (1) present solely as N-acetyl-neuraminic acid. This acid appears to be fundamental to the micellar structure of casein in milk (33).

Sialic acid determinations have been used as a measure of K-casein in sedimented fractions of whole casein. Sullivan et al. (48), using differential centrifugation of raw skimmilk, demonstrated a linear increase in the concentration of sialic acid as progressively more casein was centrifuged from the milk. Marier et al. (38) have shown that the only fraction in skimmilk containing any appreciable amount of sialic acid, other than K-casein, is the proteose-peptone fraction. Kappa-casein and the proteose-peptone fraction are separated on precipitation of casein at pH 4.5.

A method was developed in 1958 by Warren (51) which uses thiobar-

bituric acid to measure the sialic acid content of various biological materials. This method has been used by a number of workers (25, 26, 38). Using a modification of Warren's method, Marier et al. (38) concluded that sialic acid measurements can be used as an index of the intact K-casein content of α_s-K-casein mixtures, as well as an index of the K-casein concentration of whole acid casein. These workers obtained values of 0.26-0.59% sialic acid in washed whole acid casein indicating that the proportion of K-casein is variable. The sialic acid content of K-casein appears to be close to 2.3%; on this basis the proportion of K-casein in whole milk varies between 11-26%. This agrees with the work of Waugh and von Hippel (52) who have reported the relative amounts of the casein fractions in skimmilk to be about 55% α-casein, 30% β-casein and 15% K-casein. Yaguchi et al. (58) found that most of the sialic acid is concentrated in the kappa fraction and that is where the greatest lipase activity is located. They found the "correlation between sialic acid and lipase activity to be good." These authors did not, however, report a correlation study of these two factors. Hill (25) reported that the proportion of sialic acid in K-casein varied depending upon the method of preparation, ranging from 0.79% (8) to 2.4% (1) with the acid precipitation method giving the smaller values. However, Marier et al. (38) found that approximately 90% of the sialic acid was released from K-casein by acid precipitation. If this fraction of the skimmilk influences lipase, perhaps this could explain some of the variations between cows, that is evident in the study of the activity of this enzyme.

CHAPTER III

EXPERIMENTAL PROCEDURE

Emulsification Requirements

In this study tributyrin (99% pure)[1], was used as the substrate and lipase activity was compared when this substrate was emulsified with gum arabic[2] and with bovine serum albumin (BSA)[2]. The tributyrin-gum arabic substrate contained 7.5 g tributyrin and 10.0 g gum arabic. This was diluted to 100 ml with deionized water and emulsified in a "laboratory hand homogenizer[3]." The tributyrin - BSA substrate was a similar emulsion, but 15.0 g of BSA was substituted for the gum arabic.

A total of 120 milk samples were obtained for this part of the study by sampling three individual cows once or twice each week for 16 weeks. Fifteen of the samples were analyzed in duplicate with each emulsifier (a total of 60 observations). These data made a complete block of values for all three cows over five sampling periods. All of the cows used in this and subsequent studies were housed, fed and handled under similar conditions. The ration used throughout the experiments consisted of alfalfa hay and sorghum silage fed ad lib and a

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[1]Less than 1% free fatty acids by titration, balance assumed to be glycerides. Sample saponified with KOH and methyl esters of released fatty acids identified as > 99% butyric by GLC.

[2]Sigma Chemical Company, St. Louis, Missouri.

[3]Fisher Scientific Company, Houston, Texas.

pelleted concentrate fed according to production response.

The milk samples were cooled to < 5 C immediately after collection and assayed within 2-3 hrs. The milkfat was removed by centrifugation and 25 ml of the resulting skimmilk were added to an erlenmeyer flask containing 25 ml of tris buffer at pH 8.5 and 2.0 ml of one of the two substrate preparations. The samples were incubated for one hour in a shaking water bath at 37 C, then the reaction was stopped by adding 10% HCl to reduce the pH to < 2.0. A blank titer at zero time was obtained by preparing a duplicate sample to which the acid was added prior to the skimmilk (the enzyme source). This blank was not incubated, but otherwise was handled in the same way as the reaction mixture. To obtain the free butyric acid released during the reaction the samples were extracted twice with a 1:2:2 mixture of ethanol, ethyl ether and petroleum ether. The ether extracts were combined and titrated with 0.02N alcoholic potassium hydroxide using thymolphthalein indicator. This extraction procedure has been shown to recover 90% of the butyric acid (27). The data were expressed as lipase units per ml of skimmilk, with a lipase unit being defined as the amount of enzyme required to liberate one μ mole of butyric acid per minute of reaction time.

Sialic Acid Content vs Lipase Activity

In this portion of the study lipase activity was measured using the gum arabic substrate. Eighty-one milk samples were obtained for this work by collecting 27 samples from each of three cows once or twice weekly for 17 weeks. These cows were all bred and in the latter half of their lactation at the time sampling began. The animals were five to eight years of age and none of them had any known disease condition

during the experiment. The skimmilk was assayed for sialic acid content by a modification of the procedure used by Marier et al. (38). Their procedure was an adaption of Warren's method of thiobarbituric acid assay for sialic acids (51).

A. Casein Preparation

The procedure was as follows: to each of two conical centrifuge test tubes, 5 ml of raw skimmilk were added along with 0.25 ml of 1 N HCl. The solutions were mixed by inverting the stoppered tubes, then they were centrifuged for one min at 1500 rpm. The supernatant was discarded, and 5 ml of 0.1N sodium acetate buffer (pH 4.5) were added to each tube and the casein precipitates resuspended to wash them. The samples were then centrifuged as before and the supernatant discarded. These washing procedures were repeated three times and the casein then was reprecipitated.

Five milliliters of 0.1N H_2SO_4 were added to each tube of casein (pH $\cong$ 2.0), the protein resuspended and the tubes incubated for 45 min in an 80 C water bath with occasional mixing to hydrolyze and release the sialic acid. After this hydrolysis period, the tubes were cooled to room temperature, and 0.5 ml of 1 N NaOH was mixed into each tube (resulting pH $\cong$ 4.5). The samples were then centrifuged for 2 min at 3000 rpm and the supernatant containing the hydrolyzed sialic acid was decanted and saved. The casein pellet was then washed with 5 ml of 0.1N sodium acetate buffer and this washing added to the other supernatant.

The volume of the combined supernatants was recorded and 0.1 ml of chloroform was added to each tube. The tubes were shaken and allowed

to set for 5 min, then centrifuged for one min at 3000 rpm, to remove the last traces of "cloudiness" from the solutions. Aliquots of 0.5 ml were removed from each of the two tubes of supernatant (duplicates) for sialic acid analysis.

B. Thiobarbituric Acid Assay of Sialic Acids

The following solutions were used: (a) sodium periodate (meta) 0.2M, in 9M phosphoric acid; (b) sodium arsenite, 10 percent in a solution of 0.5M sodium sulfate - 0.1N H_2SO_4; (c) thiobarbituric acid, 0.6 percent in 0.5M sodium sulfate; and (d) sialic acid (N-acetyl-neuraminic acid)[1] 0.25 μmole/ ml.

Solutions (a) and (b) were stable for more than a month at room temperature, while solution (c) was not, and a fresh sample was prepared each week. Solution (d) used for standard curves, was unstable even at 5 C and showed progressive deterioration, probably due to hydrolysis, which resulted in less color per unit weight. However, the crystalline material was stable at -20 C, thus a standard curve determination was made only from freshly prepared solutions of sialic acid.

To assay for sialic acid, 0.5 ml of the supernatant from each hydrolyzed sample, plus 0.5 ml of distilled water was placed in a test tube. Next, 0.2 ml of the periodate solution was added, the tubes were shaken and allowed to set at room temperature for 20 min to allow for hydrolysis of the sialic acid. Two milliliters of the arsenite solution were added to neutralize the periodate and the tubes were shaken until a yellow-brown color disappeared. Six milliliters of the thiobarbitu-

[1]Sigma Chemical Company, St. Louis, Missouri.

ric acid solution was then added, the tubes shaken, capped with glass marbles to act as condensors, and heated in a boiling water bath for 15 min to allow color development. The tubes were then removed and cooled in ice water for 5 min to room temperature (21-23 C). The contents of each tube were transferred into short, round-bottom screwcap test tubes and 8.0 ml of cyclohexanone were added to extract the color. The tubes then were capped, shaken for 10-15 sec, and centrifuged for 3 min at 1500 rpm. Using a hypodermic syringe, 5-6 ml of the upper (cyclohexanone) phase was transferred to a cuvette. The optical densities were determined at 549 mμ in a Beckman model B spectrophotometer. The concentration was determined from a standard curve (Fig. 1) and, after correcting for the dilution factor, expressed as μg of sialic acid per ml of skimmilk.

Separation And Study of Lipase-active Protein Fractions

The enzyme source for this part of the study was fresh mixed-herd skimmilk and (unless otherwise stated) lipase activities were determined using tributyrin emulsified with gum arabic.

A. Kinetic Characterizations of Skimmilk Lipase

A series of kinetic studies were performed to determine optimum assay conditions for this lipase. Unless otherwise stated, the reaction pH was 8.5 and the temperature was 37 C. All results are averages of 2 to 5 determinations.

Incubation time - Lipase activities were measured after 10, 15, 30, 45 and 60 min with a zero time sample used as a blank. Individual samples were prepared for each incubation time, rather than withdrawing

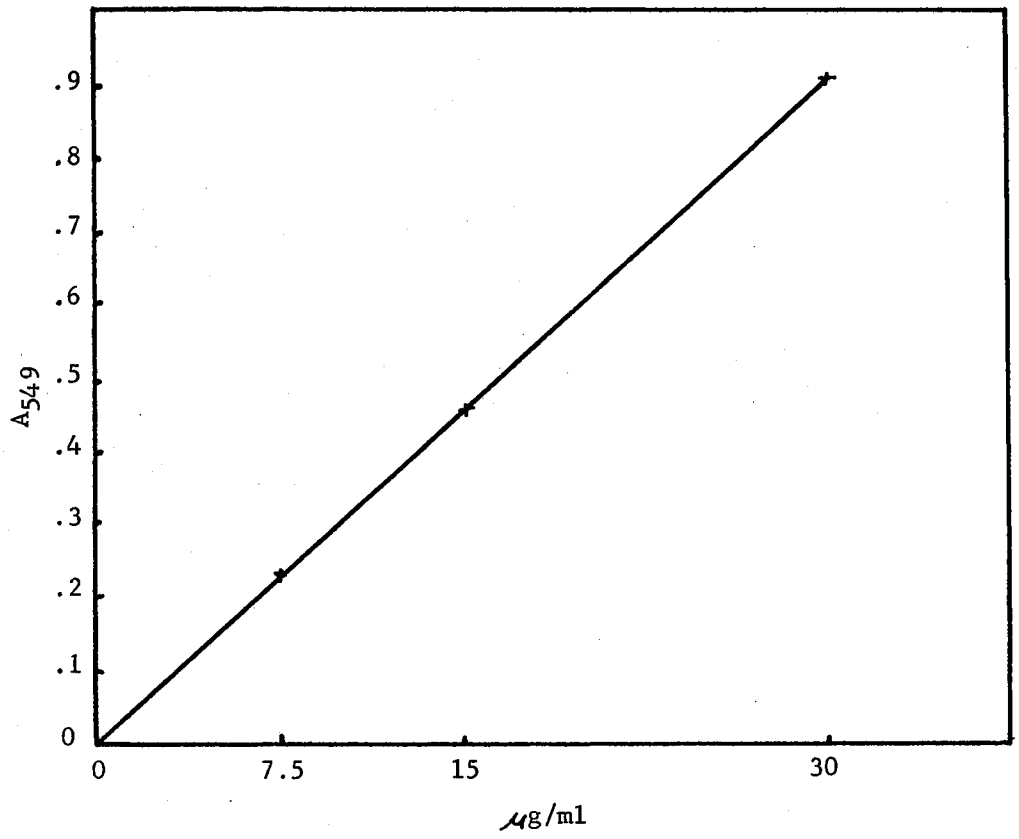

Fig. 1. Standard curve for sialic acid determinations.

aliquots from one large sample.

Enzyme to substrate ratio - In order to determine the optimum enzyme-substrate combination, the following ratios of enzyme to substrate (in ml) were used; 12.5:1, 2.5:1 and 1:1. All samples were combined in 25 ml of tris buffer and incubated for 15 min.

Substrate concentration - Four levels of substrate; 2.5, 5.0, 7.5 and 10.0 ml were used, while holding the enzyme level constant at 5 ml and incubating the samples for 15 min. The maximum velocity (V_m) and Michaelis constant (K_m) were determined, both from a plot of velocity (V) vs substrate concentration (S) and from an Eadie procedure plot (15) i.e. S/V vs S.

pH - The optimum pH for lipase activity was determined by measuring the activity of the lipase in 5 ml of skimmilk at intervals of 0.5 pH units between pH 7.0 and 9.5. Tris buffer was adjusted to the desired pH by adding NaOH or HCl. Skimmilk was added to 25 ml of each of the pH adjusted buffers, the samples were allowed to equilibrate at the particular pH for 15 min at 37 C, then 5 ml of substrate were added and the reaction mixture incubated for 15 min.

Temperature - Lipase activities were determined at 20, 25, 30, 37 and 45 C. Five milliliters of skimmilk in one flask and 5 ml of substrate plus 25 ml of tris buffer in another flask were equilibrated to the temperature used for 10 min. They then were combined and incubated for 15 min. In addition to determining the optimum temperature, these results also were used to construct an Arrhenius plot from which the energy of activation for the reaction was calculated.

Different substrates - To determine the relative activity of skimmilk lipase toward different substrates, the activity (lipase units/ml)

obtained with tributyrin was compared to that obtained when using milkfat and triolein as substrates. These substrates were used at the same concentration as the tributyrin (7.5%) and the same concentration of gum arabic (10%) was used for emulsification. The samples were incubated for 15 min using 5 ml of skimmilk, 5 ml of substrate, 25 ml of tris buffer at pH 8.5, and a temperature of 37 C.

B. Column Chromatography Studies

The proteins in skimmilk were separated on Sepharose Type 4-B, packed according to the manufacturer's recommendations (50). The column bed was 30.0 by 2.5 cm (length x diameter) and was operated at room temperature. One milliliter of skimmilk, diluted to 5 ml with phosphate buffer (0.02M, pH 7.0) was allowed to enter the column bed before the eluent (phosphate buffer) was added. The column flow rate was approximately 60 ml/hr and 5 ml fractions were collected. The protein content of these fractions was measured at 280 mμ.

Skimmilk proteins also were separated on a Sephadex G-200 column which was packed by the method of Flodin (16). The column size was 61.0 by 2.5 cm, it was operated at 4 C and had a flow rate of 20 ml/hr. Ten milliliter fractions were collected and phosphate buffer was used as the eluent. The sample consisted of 25 ml of skimmilk mixed with an equal amount of cold saturated $(NH_4)_2SO_4$ and held overnight at 4 C. This sample was then centrifuged, the precipitate resuspended, made up to 25 ml in phosphate buffer, and the sample dialyzed for 48 hr against phosphate buffer. One milliliter of this dialyzed sample was added to the column.

Void volumes for both the Sepharose and Sephadex G-200 columns

were determined using Blue Dextran 2000 (a high M.W. dextran with a blue chromophore).

A DEAE-cellulose column (exchange capacity 1.0 meq/g) was used to separate skimmilk proteins. This column was prepared according to the method of Yaguchi et al. (56), except that N_2 pressure was not applied. The column bed size was 15 by 5 cm, the flow rate was 200 ml/hr and the column was operated at 4 C. A 25 ml sample of skimmilk was dialyzed overnight against phosphate buffer and placed on the column. The eluents used were: a) 3.3 M urea, b) 3.3 M urea plus 0.8 M NaCl (all in 0.02 N. phosphate buffer) and, c) 0.25 M NaOH.

Protein contents of the skimmilk were determined by the Kjeldahl method using a conversion factor of 6.38. The protein contents of the various column fractions were estimated by the method of Lowry et al. (36) with absorbancy measured at 725 mμ.

C. Purification Studies

A modification of the procedure of Fox and Tarassuk (18) was used to obtain a purified skimmilk lipase (Fig. 2). These modifications included: a) filtering the sample through Sephadex G-25 (7), b) concentrating it with Lyphogel[1], and c) using a stepwise NaCl gradient to elute the proteins from the DEAE-cellulose column. Jeso's procedure (29) for $(NH_4)_2SO_4$ fractionation, which corrects for temperature, was used to determine the amount of $(NH_4)_2SO_4$ to add. Some of the peaks obtained from the DEAE-cellulose column were further studied by concentrating them with Lyphogel and rechromatographing them on Sephadex

[1]Gelman Instrument Company, Ann Arbor, Michigan.

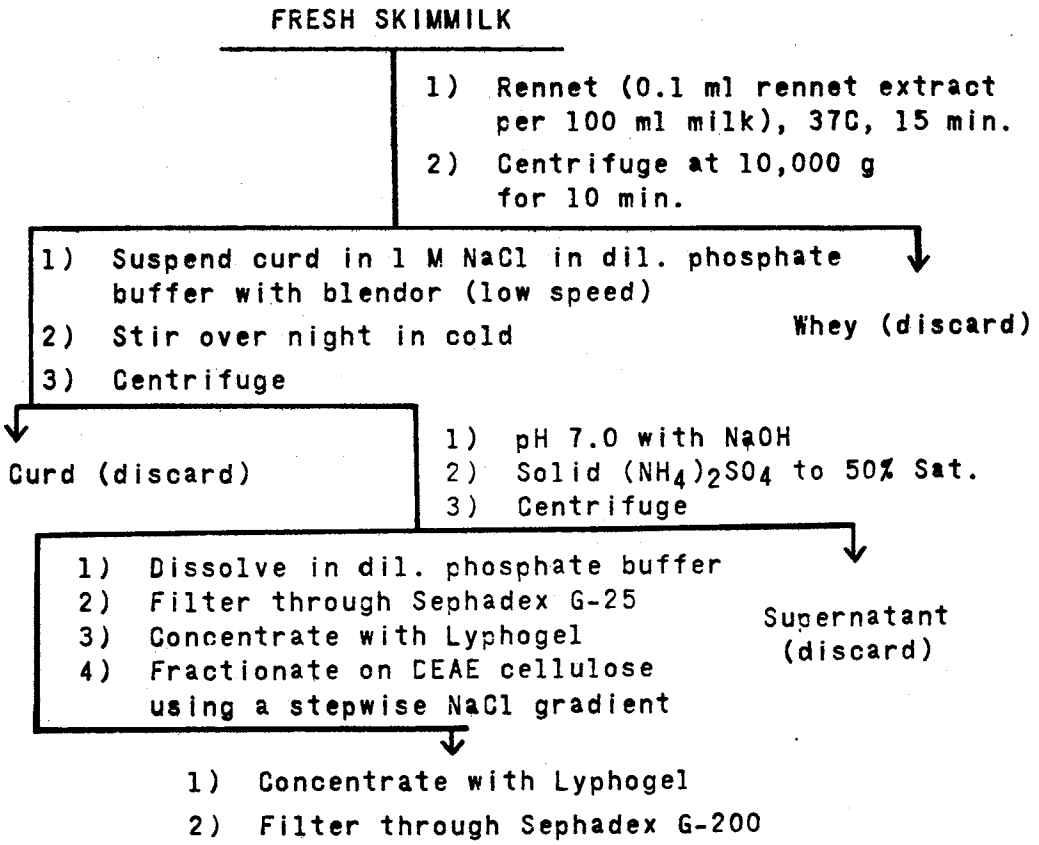

Fig. 2. Flow diagram of procedure used to purify skimmilk lipase.

G-200.

The DEAE-cellulose column (65.0 by 2.5 cm) used for this part of the study was operated at 4 C, at a flow rate of approximately 100 ml/hr and fractions of 10.5 ml were collected. In each column chromatography experiment, approximately 1.25 grams of protein were added to the column at a concentration of 12-18 mg/ml. These proteins were eluted by a stepwise NaCl gradient.

Polyacrylamide gel electrophoresis was performed by the method of Blattler (3), using a 7.0% polyacrylamide gel and 0.0065 M sodium phosphate buffer (pH 7.0). The electrophoretograms were stained by Amido black in a 5:5:1 mixture of water, methanol and acetic acid.

Ultraviolet adsorption spectra were obtained on some of the purified protein fractions using a Beckman DB spectrophotometer and recorder.

CHAPTER IV

RESULTS AND DISCUSSION

Emulsification Requirements

Lipase activities of bovine skimmilk were compared using BSA and gum arabic as emulsifiers for the tributyrin substrate. Lipase activities of these samples ranged from 0.29 to 0.68 lipase units/ml (Table I). The means for the BSA and gum arabic analysis were 0.491 and 0.494 units/ml, respectively. A "t" test indicated the difference between these values was not statistically significant ($P > 0.50$). Coefficients of variation of 14.87 and 14.37%, plus standard deviations of 0.0066 and 0.0065 units/ml provided further evidence about the similarity of these data.

An analysis of variance (Table II) showed significant differences among cows and days ($P < 0.01$). These results agreed with literature reports that lipase activity varies greatly from day to day within individual animals as well as between animals (53). No significant differences were found between emulsifiers or any of the interactions involving emulsifiers ($P > 0.10$). The mean square for duplicates was low in relation to the other sources of variation indicating that the analyses were reproducible within rather narrow limits.

Since there was no significant difference between lipase activities when BSA or gum arabic were used, the two compounds apparently performed similar functions in the reaction mixture. Since gum

TABLE I

LIPASE ACTIVITIES OF BOVINE SKIMMILK, USING BSA[a] OR GA[b] TO EMULSIFY THE SUBSTRATE.

Date	Cow No. 721		Cow No. 775		Cow No. 807	
	BSA	GA	BSA	GA	BSA	GA
	Lipase units/ml[c]					
8- 8	.54	.56				
8-12	.53	.54				
8-15	.45	.47				
8-19	.42	.41				
8-20	.48	.49				
8-21	.32	.33				
8-22	.32	.33				
8-23	.39	.30				
8-24	.40	.51				
8-26	.38	.40				
8-29	.46	.47				
8-30	.55	.41	.34	.59	.33	.45
9- 3	.54	.59	.52	.54	.40	.51
9- 5	.42	.48	.46	.49	.41	.44
9- 6	.42	.41				
9- 9	.45	.46	.52	.52	.39	.45
9-10	.49	.49				
9-11	.45	.46				
9-12	.48	.49	.56	.56	.45	.45
9-13	.50	.53				
9-17	.51	.52				
9-18	.38	.37				
9-19	.31	.46			.39	.36
9-20	.47	.40	.55	.59	.45	.44
9-24	.29	.29	.56	.50	.45	.47
9-25	.43	.42				
9-27	.42	.44	.61	.64	.49	.50
10- 1	.42	.43	.59	.61	.44	.42
10- 2	.51	.53	.54	.58	.45	.47
10- 4	.44	.43	.53	.54	.52	.43
10- 8	.49	.44	.58	.47	.53	.44
10- 9	.50	.48	.53	.50	.46	.45
10-11	.48	.48	.59	.68	.53	.56
10-15	.47	.50	.58	.58	.47	.44
10-16	.51	.54	.53	.55	.56	.59
10-18	.54	.53	.58	.60	.51	.42
10-22	.51	.49	.55	.56	.60	.52
10-23	.48	.48	.47	.49	.52	.46
10-29	.47	.47	.56	.52		

TABLE I (Cont.)

Date	Cow No. 721		Cow No. 775		Cow No. 807	
	BSA	GA	BSA	GA	BSA	GA
	Lipase units/ml					
10-30	.53	.54	.42	.40		
11- 6	.57	.59	.39	.43	.43	.48
11- 7	.57	.57	.45	.47	.54	.47
11- 8	.50	.45	.45	.47	.48	.47
11-12[d]	.58	.57	.58	.57	.56	.57
	.58	.57	.59	.58	.60	.59
11-13[d]	.52	.49	.56	.51	.49	.50
	.51	.48	.57	.56	.48	.49
11-19[d]	.48	.48	.48	.50	.56	.58
	.52	.56	.52	.34	.65	.60
11-20[d]	.57	.55	.55	.56	.61	.59
	.56	.54	.48	.47	.58	.56
11-22[d]	.38	.47	.48	.45	.51	.50
	.40	.57	.48	.47	.48	.49

[a] Bovine Serum Albumin.

[b] Gum Arabic.

[c] Amount of enzyme required to liberate one μm butyric acid/ minute of reaction time.

[d] Duplicate analyses obtained on these samples were used for analysis of variance in Table II.

TABLE II

ANALYSIS OF VARIANCE OF DUPLICATE ANALYSIS FOR SAMPLES FROM THREE COWS ON FIVE SAMPLING DAYS

Source	df	SS	MS	F
Total	59	0.20121		
Blocks	1	0.00016	0.00016	0.13[b]
Emulsifiers (E)	1	0.00237	0.00237	1.88[b]
Cows (C)	2	0.01424	0.00712	5.64[a]
Days (D)	4	0.07593	0.01898	15.05[a]
E x C	2	0.00329	0.00165	1.31[b]
E x D	4	0.00503	0.00126	1.00[b]
C x D	8	0.04597	0.00575	4.56[a]
C x D x E	8	0.01653	0.00207	1.64[b]
Error (duplicates)	29	0.03769	0.00129	

[a] $P < 0.01$.

[b] Not significant $P > 0.10$.

arabic is generally accepted as functioning solely as an emulsifier, then BSA is probably just serving to emulsify the substrate also. However, it is possible that BSA is operating in a dual role of slightly emulsifying the substrate, and forming a lipoprotein complex with it.

Sialic Acid Content And Lipase Activity

Sialic acid contents of the skimmed milk ranged from 49.0 to 154.0 μg/ml (Table III) and the means were 113.4, 100.9 and 74.5 μg/ml for cows 488, 453 and 650, respectively (Table IV). Duplicate sialic acid analyses were very close to each other as shown by the small mean squares of blocks ($P > 0.10$) in an analysis of variance (Table V). The AOV also showed a statistically significant difference ($P < 0.01$) among cows and days, as well as for the cows x days interaction. Kiermeir and Freisfeld (32) have reported that sialic acid levels in milk vary widely from day to day, fluctuating between 122 and 286 μg/ml of milk, with a mean of 171 μg/ml. Downey and Andrews (14) and Marier et al. (38) have reported 96 and 87 μg/ml, respectively. Thus, the values in this study, though somewhat lower than those of Kiermeir and Freisfeld, are in reasonable agreement with the other literature reports.

Lipase activities showed considerable day to day variation ranging from 0.21 and 0.64 lipase units/ml. However, the means did not change appreciably when comparing the beginning to the end of the study. Average means and standard deviations were 0.425 ± 0.087, 0.499 ± 0.086 and 0.534 ± 0.067 units/ml for cows 488, 453 and 650, respectively. Since statistical analysis of the lipase assay was treated in the previous section it was not deemed necessary to repeat this analysis in this part of the study.

TABLE III

SIALIC ACID CONTENT AND LIPASE ACTIVITY OF SKIMMILK FROM THREE COWS

Date	Cow No. 488			Cow No. 453			Cow No. 650		
	Sialic acid[a]		Lipase[b]	Sialic acid		Lipase	Sialic acid		Lipase
4- 1	92	94	.40	91	94	.45	56	55	.57
4- 4	100	100	.43	116	112	.45	51	50	.62
4- 8	94	93	.42	87	94	.36	52	49	.59
4-15	96	96	.21	104	107	.36	58	60	.57
4-18	116	112	.43	112	116	.47	75	77	.57
4-22	88	89	.40	99	98	.28	64	63	.42
4-25	92	94	.55	92	90	.40	61	61	.55
5- 2	87	88	.45	94	103	.55	72	68	.57
5- 6	90	95	.47	81	79	.55	69	69	.53
5- 9	85	85	.49	94	99	.55	69	62	.55
5-13	127	127	.30	91	91	.53	74	70	.55
5-20	122	88	.43	117	117	.38	81	81	.51
5-23	116	117	.49	111	111	.40	94	94	.42
5-30	106	112	.51	110	113	.55	76	75	.53
6-10	114	114	.49	104	104	.47	79	75	.42
6-13	120	118	.53	107	102	.51	72	74	.51
6-17	118	120	.51	98	98	.53	77	80	.55
6-20	118	120	.42	92	120	.38	85	80	.61
6-24	118	120	.38	85	98	.43	66	69	.57
7- 1	123	123	.30	110	110	.36	75	76	.45
7- 8	154	154	.49	106	106	.45	91	90	.64
7-11	127	127	--	96	99	.40	76	81	.51
7-15	134	134	.34	107	117	.30	87	89	.45
7-18	131	134	.55	97	110	.61	80	81	.61
7-22	134	134	.40	96	104	.40	88	88	.62
7-25	128	134	.28	80	76	.47	88	85	.43
7-29	134	131	.38	107	111	.53	104	99	.49

[a]Duplicate analyses as μg sialic acid per milliliter of skimmilk.

[b]Lipase units per milliliter of skimmilk.

TABLE IV

MEANS AND STANDARD DEVIATIONS FOR SIALIC ACID CONTENTS IN SKIMMILK OF THREE COWS

Cow No.	Number of duplicate observations	μg/ml of skimmilk	
		Mean	Std. Dev.
488	27	113.4	18.0
453	27	100.9	10.2
650	27	74.5	12.7

TABLE V

ANALYSIS OF VARIANCE FOR SIALIC ACID CONTENTS OF SKIMMILK

Source	df	SS	MS	F
Total	161	74,929		
Blocks	1	17	17	0.92[b]
Cows (C)	2	42,527	21,263	1149.00[a]
Days (D)	26	18,868	726	39.20[a]
D x C	52	12,032	231	12.50[a]
Error	80	1,485	18.5	

[a] $P < 0.01$.

[b] Not significant $P > 0.10$.

The lipase activities and sialic acid levels fluctuated widely during this 17 week study, both between samplings (days) and between animals (Fig. 3). In two of the cows, No. 488 and 650, the sialic acid levels seemed to increase toward the end of lactation, however, this was not the case with cow 453 where sialic acid levels at the end of the study were not appreciably different from those at the beginning.

Limited segments of the data in Fig. 3 could be chosen where lipase activities and sialic acid contents were positively correlated, and other segments could be chosen which would illustrate a negative relationship. However, when all the data were considered the correlation coefficients for these two variables were -0.10, -0.22 and -0.29 for cows No. 488, 453 and 650, respectively. These results may explain some of the differences found in the literature as to the relationship between these two variables. In support of these findings is the recent report by Patel et al. (42), that skimmilk lipase is a distinct separate minor protein moiety different from any other known protein of milk. Thus, lipase apparently is not K-casein per se, and lipase activity can not necessarily be predicted from K-casein values.

Separation and Study of Lipase-Active Protein Fractions

A. Kinetic Characterizations of Skimmilk Lipase

A plot of velocity (V), in lipase units/ml of skimmilk vs time (t), in Fig. 4, showed that this reaction was first order for approximately 15 min. Although total lipase activity continues to increase throughout the hour, the reaction rate remains almost constant after the first 15 min. On the basis of these findings a standard time of 15 min was selected for all lipase assays in this work. This length

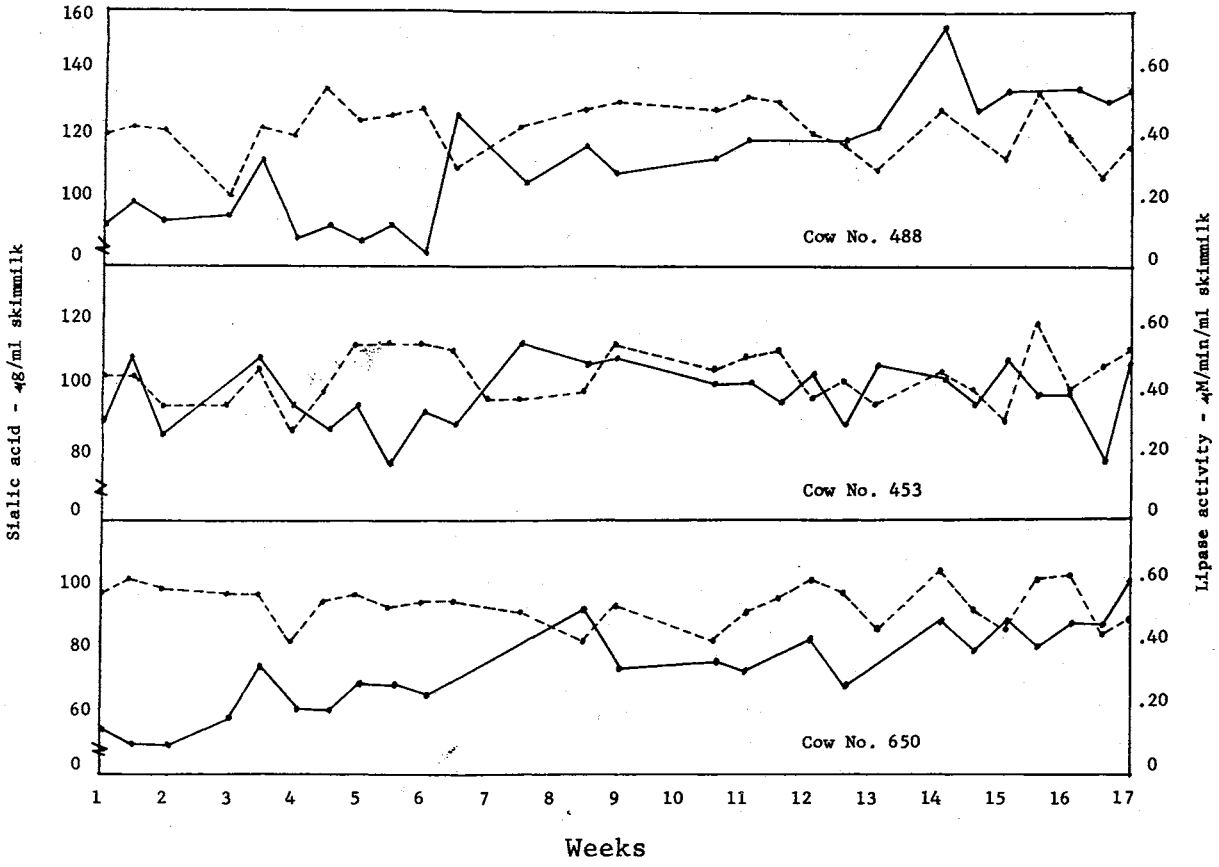

Fig. 3. Sialic acid content (——) and lipase activity (----) of three cows sampled once or twice a week for 17 weeks.

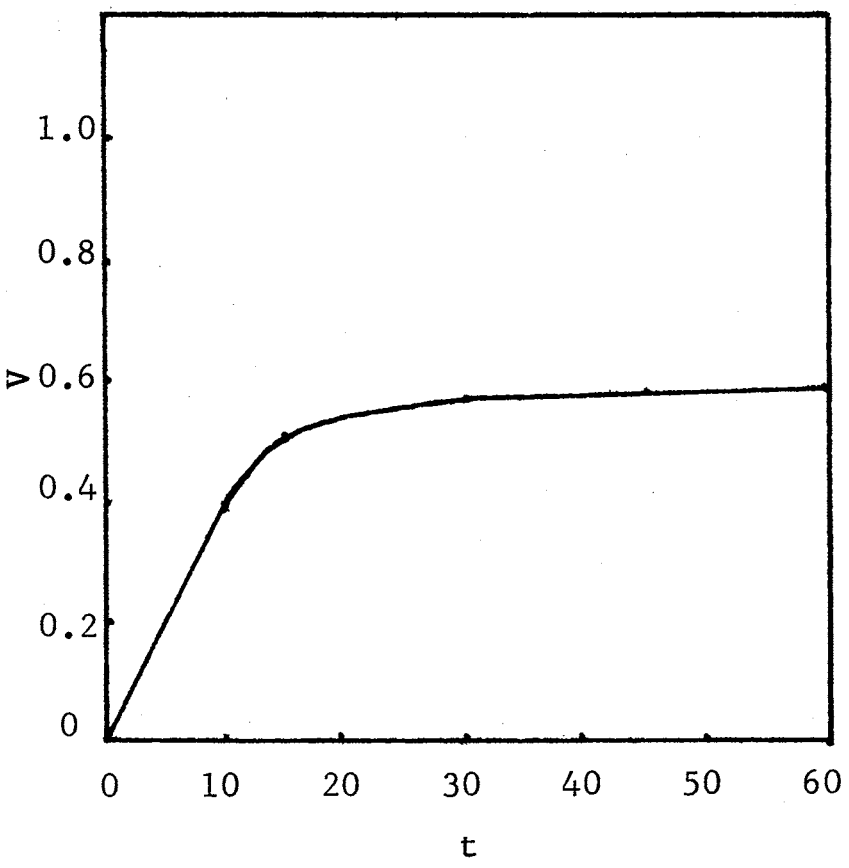

Fig. 4. Reaction velocity (V) in lipase units per ml of skimmilk vs reaction time (t) in minutes, at pH 8.5 and 37 C.

of time is in agreement with the recommendation of Frankel and Tarassuk (19); however, some workers using the pH stat technique have assayed for only 5 min (14).

Of the three mixtures used, a 1:1 ratio of skimmilk and substrate gave maximum lipase activities (Table VI), and this ratio was confirmed by later work (Fig. 5). Thus, at a 1:1 ratio the reaction was zero order with respect to substrate concentration, and maximum velocity (V_m) could be determined, since substrate concentration (S) was not limiting the reaction rate. The V_m from this plot (Fig. 5) was approximately 1.0 lipase units/ml and the Michaelis constant (K_m) approximately 1.6 ml. A plot of this data by the Eadie procedure (Fig. 6) gave a V_m of 1.35 lipase units/ml and a K_m of 1.0 ml (15). This latter procedure, according to Dowd and Riggs (13) is statistically superior to Lineweaver-Burk and Hofstee plots, which have been used by many workers to express this kind of data.

Judging from the symmetry of the curve, the pH optimum (Fig. 7) for this enzyme was approximately 8.5. The enzyme is quite active over the pH range of 8.0 to 9.0, but the activity drops off sharply outside this range. Only one pH optimum was observed between pH 7.0 and 9.5. This is in agreement with the data of Schwartz et al. (45), but is slightly lower than the pH optimum of 8.7-8.9 reported by Frankel and Tarassuk (19).

The peak formed by graphing temperature (T_c) vs percent of maximum lipase activity (Fig. 8) was much narrower than that formed by the pH data. The optimum temperature was 37 C, which is the temperature used by a number of other workers (11, 19, 45). The activity at this temperature was considerably higher than would have been observed at

TABLE VI

LIPASE ACTIVITY OF CERTAIN ENZYME-SUBSTRATE RATIOS[a]

Skimmilk	Substrate[b]	Lipase units/ml
ml	ml	
25	2	0.505
5	2	1.006
5	5	1.160

[a]All in 25 ml of tris buffer at pH 8.5.

[b]Tributyrin (7.5%) emulsified with 10% gum arabic.

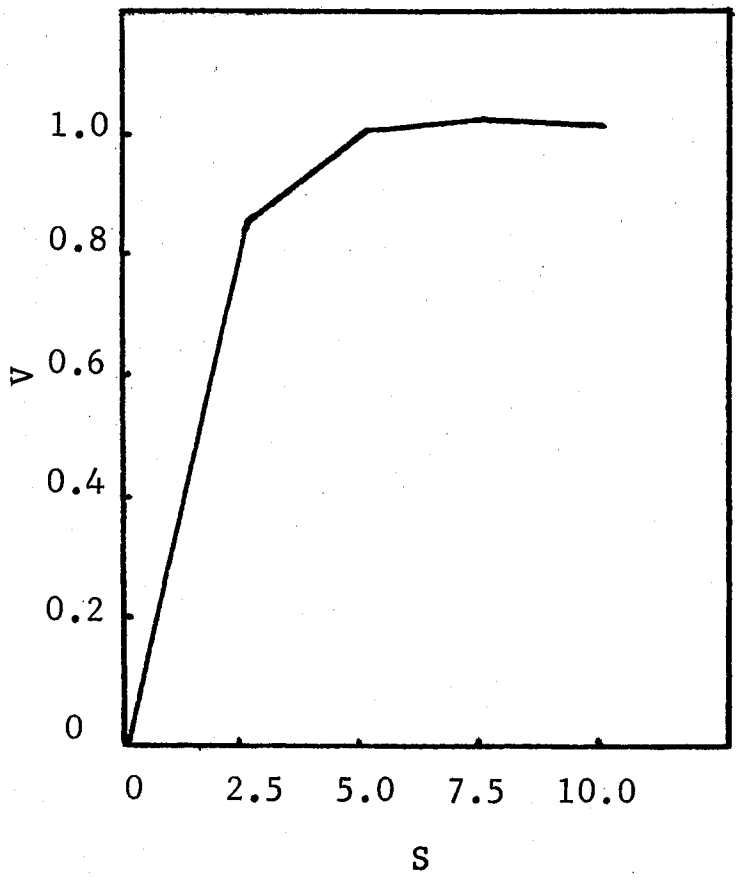

Fig. 5. Reaction velocity (V) vs substrate concentration (S) at pH 8.5 and 37 C.

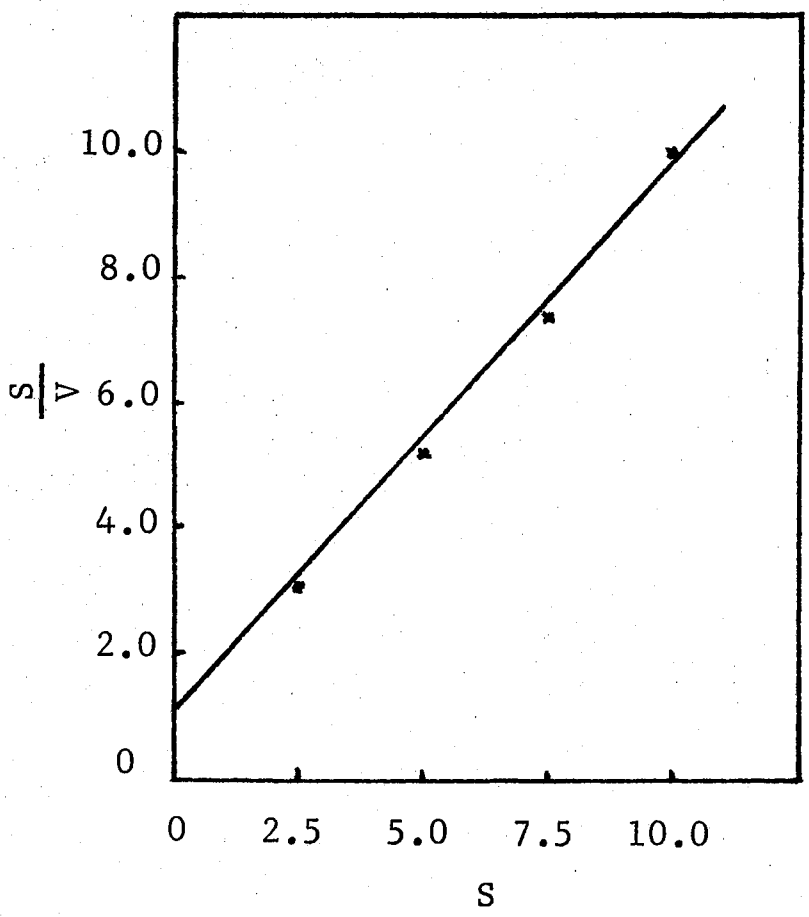

Fig. 6. "Eadie" graph of reaction velocity (V) and substrate concentration (S) data.

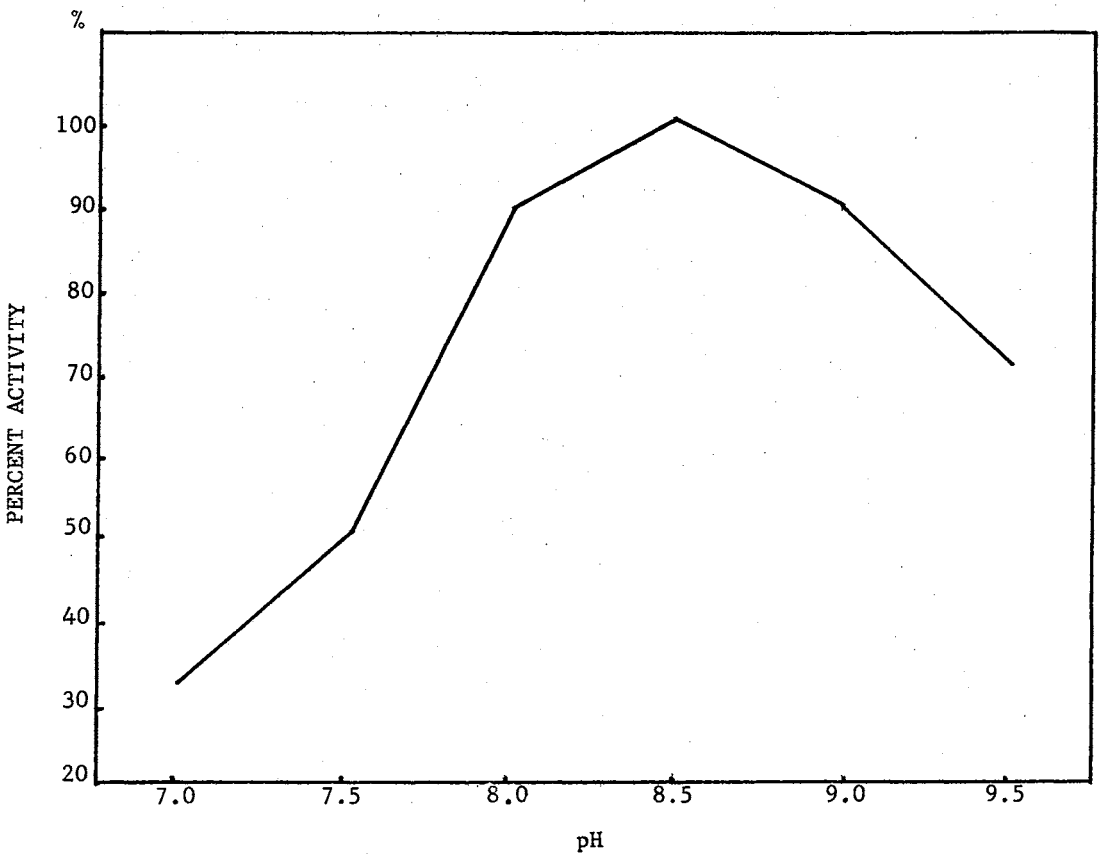

Fig. 7. Percent of maximum lipase activity at selected pH values. Reaction mixture buffered with tris at 37 C.

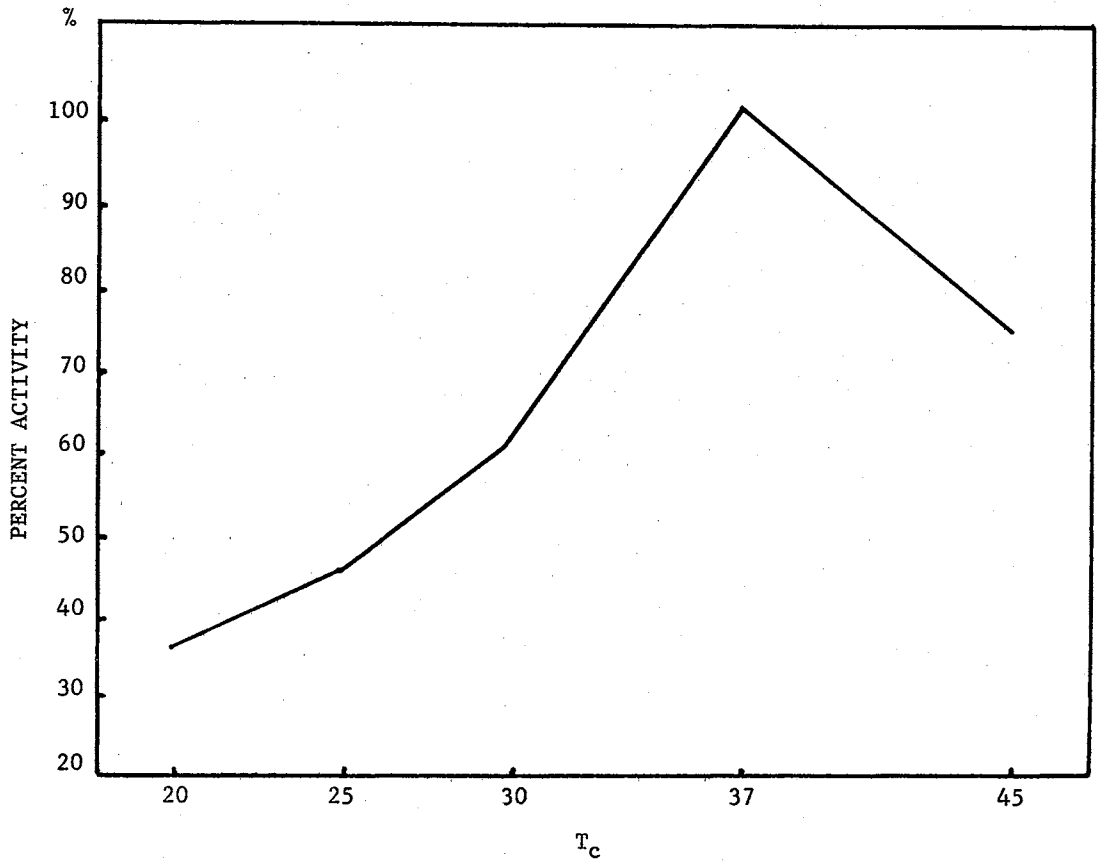

Fig. 8. Percent of maximum lipase activity at selected temperatures at pH 8.5.

25 C as Downey and Andrews used in their study (14). Milk lipase is quite heat labile as evidenced by the marked reduction in activity at 45 C. Data from this temperature study (Table VII) were used to determine the energy of activation. From an Arrhenius plot (Fig. 9) this energy of activation was calculated to be 11,100 calories for the lipolytic activity in milk toward tributyrin. This was calculated by inserting numbers representing the slope of the line in Fig. 9 in the following equation:

$$\log \frac{V_2}{V_1} = \frac{\Delta E_a}{2.3R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right).$$

In this equation V_1 and V_2 are velocities at T_1 and T_2, respectively, ΔE_a is the energy of activation, and R is the universal gas constant.

Lipase activities measured against tributyrin substrates were more than three times as large as those measured against a milkfat substrate (Table VIII) and over six times as high as those obtained with a triolein substrate. These data are in agreement with most of the literature reports that lipase hydrolyzes short-chain fatty acids, e.g. tributyrin, faster than long-chain fatty acids e.g. triolein. Milkfat, which would contain a mixture of fatty acid chain lengths, would be expected to be hydrolyzed at an intermediate rate.

B. Column Chromatography Studies

The void volume for the Sepharose column (Fig. 10) was 35-40 ml, thus the first peak was excluded from the gel matrix and did not enter the Sepharose beads. Peaks 1 and 2 were resolved, however, peaks 2 and 3 were not as well separated and the graph indicates that another peak might be buried between these two.

TABLE VII

DATA USED TO DETERMINE ENERGY OF ACTIVATION

T_c	T_k[a]	$\frac{1}{T_k} \times 10^{-3}$	$V \times 10^2$	$\text{Log}(V \times 10^2)$
20	293	3.41	34.0	1.532
25	298	3.36	42.6	1.628
30	303	3.30	56.5	1.752
37	310	3.23	94.8	1.977
45	318	3.14	70.4	1.847

[a]Temperature in degrees Kelvin.

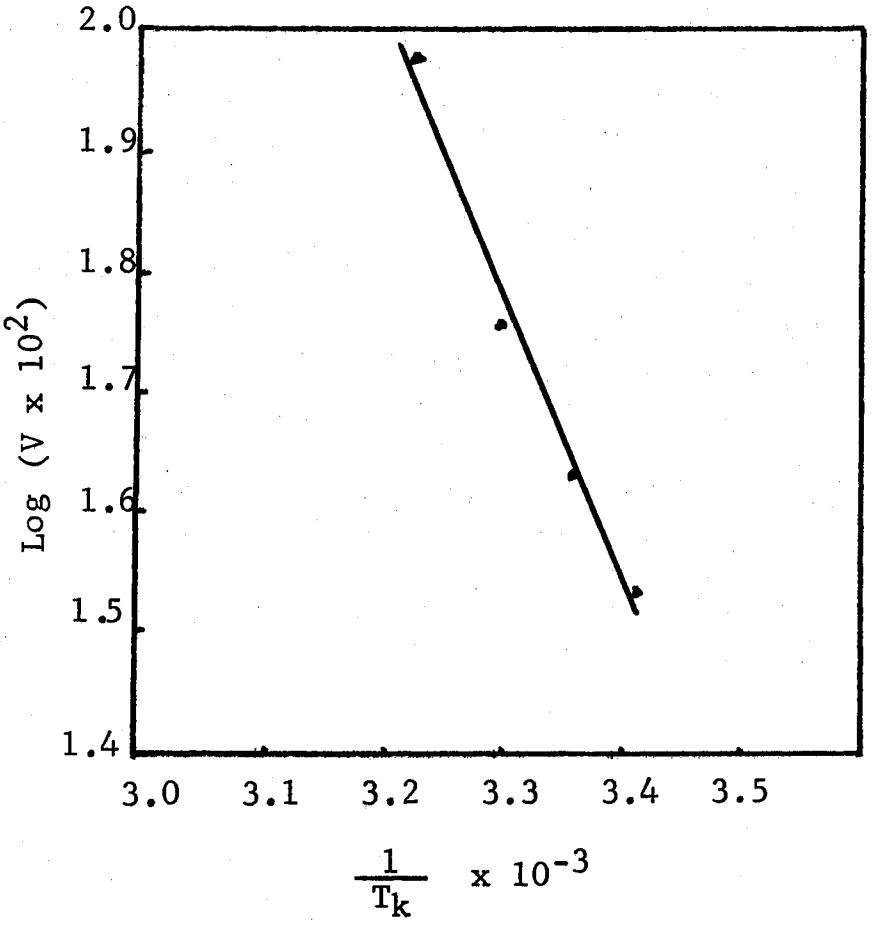

Fig. 9. Arrhenius plot of temperature data (Table VII).

TABLE VIII

LIPASE ACTIVITY MEASURED WITH DIFFERENT SUBSTRATES

Substrate	Lipase units/ml
Tributyrin	0.888
Milk fat	0.267
Triolein	0.134

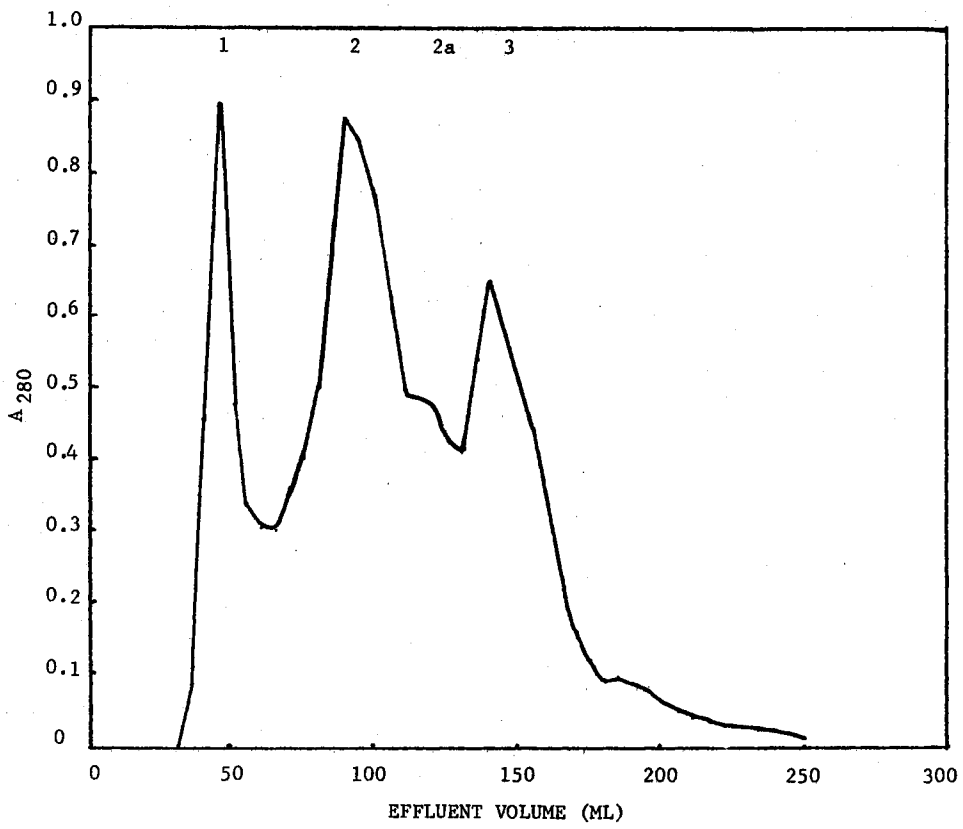

Fig. 10. Protein patterns of skimmilk filtered through Sepharose 4-B. A 1 ml sample was applied to a 30 x 2.5 cm column and eluted with phosphate buffer (0.02M, pH 7.0) at the rate of 20 ml/hr at 4 C.

Efforts to use a larger sample in order to assay for enzyme activity in the effluent from this column were not successful. When the column size was increased from 30.0 x 2.5 cm to 36.0 x 4.0 cm, resolution at 4 C was greatly decreased and most of the sample was eluted in the void volume.

Separation of skimmilk proteins on a Sephadex G-200 column gave two large distinct peaks plus two or three smaller less well resolved ones (Fig. 11). The void volume for this column was 80 ml, so the protein in the first peak was excluded from the gel. The pattern from this gel is very similar to that reported by Yaguchi and Tarassuk (59) for separation of skimmilk on Sephadex. They reported 5 peaks, but stated that the fifth peak was a dialyzable nonprotein material, so it would have been removed from this sample before putting it on the column. According to Yaguchi and Tarassuk (59), the first peak contains a mixture of K-, α_s and β-caseins. The second peak contains α_s and β-caseins, but no K-casein; while the third peak contains β-lactoglobulin, and the fourth α-lactalbumin.

No lipase activity could be detected in any of the peaks eluted, probably due to the small sample size applied to the column. Larger columns with larger samples had slow flow rates and were impractical, since any increase in lipase activity resulting from larger protein fractions would have been offset by decreasing lipase activity because of the increase in time.

In the protein pattern from the DEAE-cellulose column (Fig. 12) peak 1 was probably in the void volume, i.e. not held to the column. Peaks 2 and 3 appear to correspond to β - and α_s-casein as reported by Dumas (10), while peak 4, which required NaOH for elution, was probably

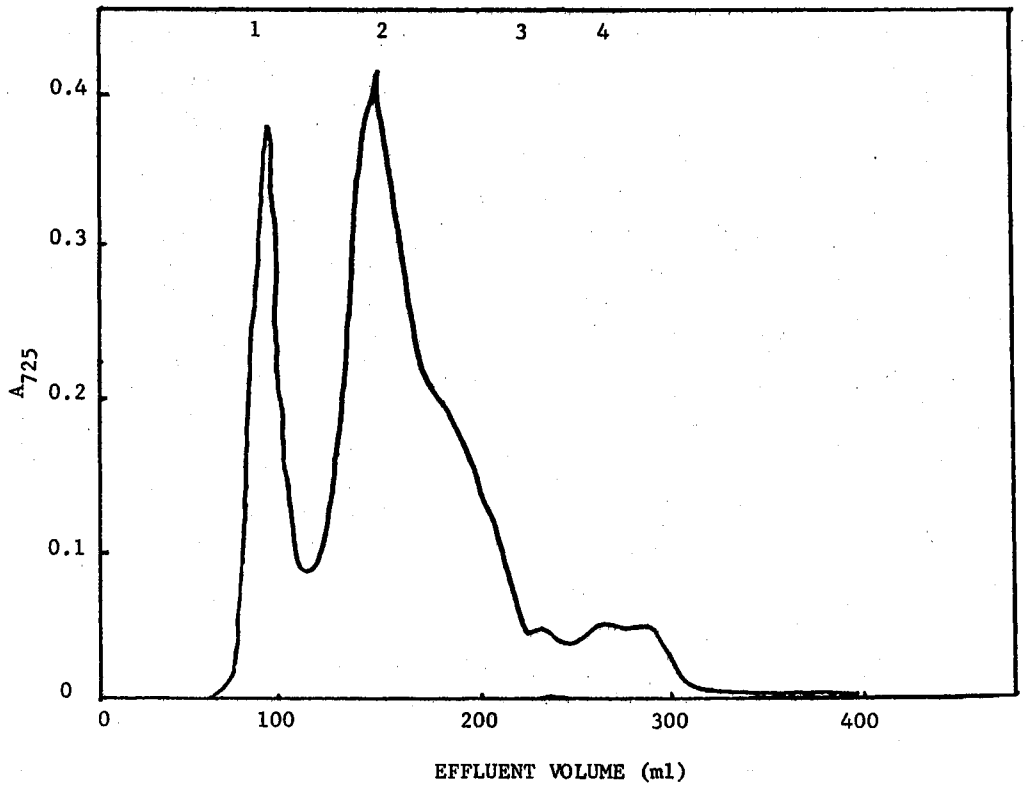

Fig. 11. Protein pattern of skimmilk filtered through Sephadex G-200 A 1 ml sample was applied to a 61 x 2.5 cm column and eluted with phosphate buffer (0.02M, pH 7.0) at the flow rate of 20 ml/hr at 4 C.

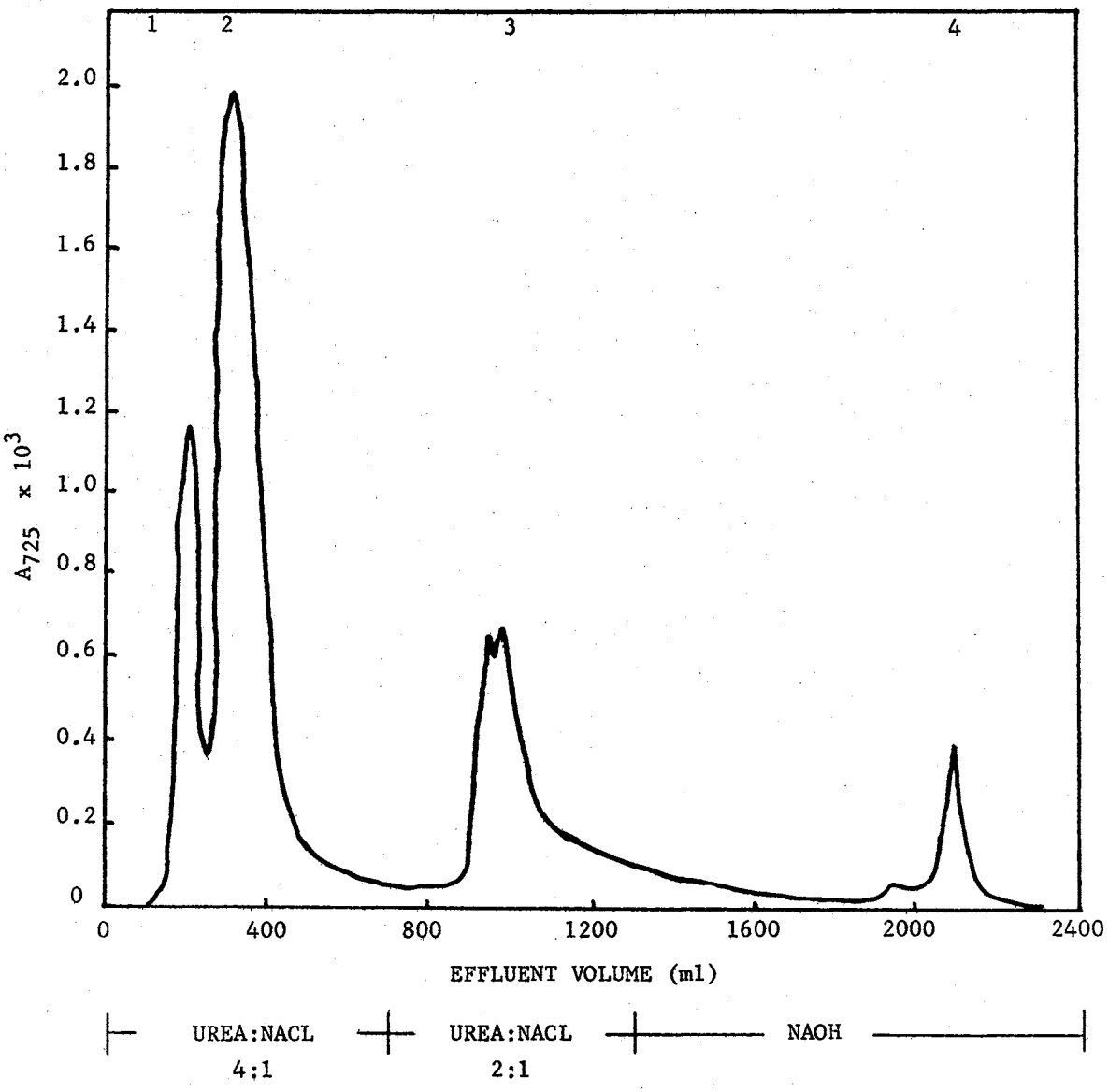

Fig. 12. Protein pattern of skimmilk filtered through DEAE-cellulose. A 25 ml sample was applied to a 15 x 5 cm column and eluted by increasing stepwise gradients of urea and NaCl in phosphate buffer, followed by 0.25M NaOH, at the rate of 200 ml/hr at 4 C.

K-casein.

None of these fractions had any appreciable amount of lipase activity. However, peak 4 did cause hydrolysis (saponification) of the tributyrin substrate. Sodium hydroxide at the same concentration used for elution of peak 4 (0.25M) gave the same amount of hydrolysis.

C. Purification Studies

Table IX summarizes the degree of purification and recovery obtained at each step of the protein preparation. This procedure gives a higher percent purification and specific activity for the DEAE-cellulose step, but a lower yield of total activity than that reported by Fox and Tarassuk (18).

Five levels of NaCl, from 0.0 to 0.4M at 0.1M intervals, were used to elute the purified proteins from a DEAE-cellulose column (Fig. 13). Four main peaks and two minor ones were observed. The two minor peaks, 2a and 3a, were due to inadvertent mixing of some of the NaCl levels, thus disrupting the stepwise elution. A small amount of lipase activity was associated with the first peak (probable void volume), however, based on specific activity, this activity was of little consequence. Most of the lipase activity was in the leading edge of the peak eluted by the 0.3M NaCl solution (peak 3) with some activity appearing in peak 4 (eluted with 0.4M NaCl). Approximately 82% of the lipase applied to the column was recovered.

The effluent from the leading edge of peak 3 was concentrated by Lyphogel and 1.0 ml was filtered through a 40.0 x 1.6 cm Sephadex G-200 column (Fig. 14) at a flow rate of 10 ml/hr at 21-23 C. The void volume was 20 ml and this was the point at which the protein began to be

TABLE IX

PURIFICATION STEPS FOR SKIMMILK LIPASE

Fraction	Act. (Units/ml)	Protein (mg/ml)	Spec. Act. (Units/mg)	Yield %	Purification
Skimmilk	1.0	35	.0286	100	1.0
NaCl Supernatant	0.78	5	.1550	63	5.5
G-25-Filtrate	2.39	15	.1600	23	5.6
DEAE-Cellulose	1.26	1	1.2600	7	44.0

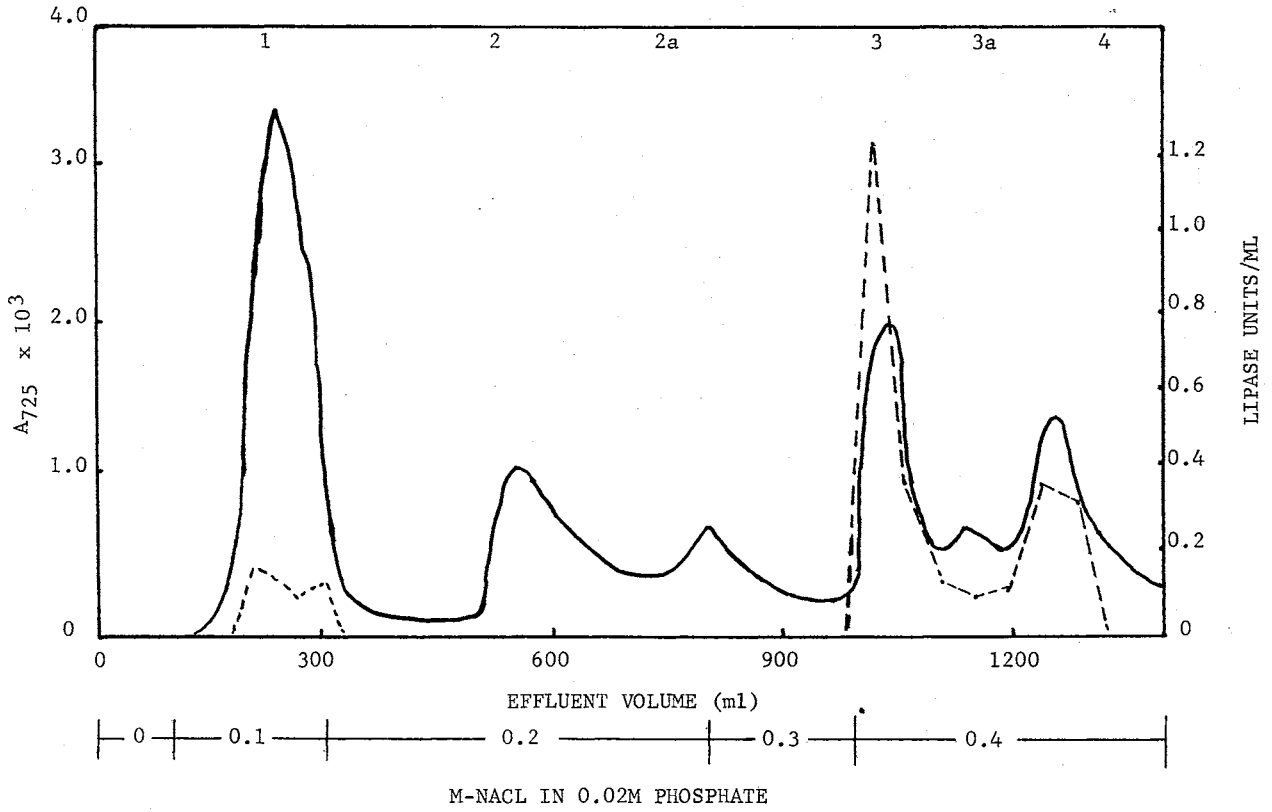

Fig. 13. Lipase activity (----) and protein patterns (——) from skimmilk filtered through DEAE-cellulose. I. Protein precipitated by 50% saturated $(NH_4)_2SO_4$ from a 1 M NaCl extract of rennet curd. Approximately 1.25 g protein applied to a 65 x 2.5 cm column and eluted with increasing stepwise NaCl gradients in 0.02M phosphate buffer. Fractions of 10.5 ml collected at the rate of 100 ml/hr at 4 C.

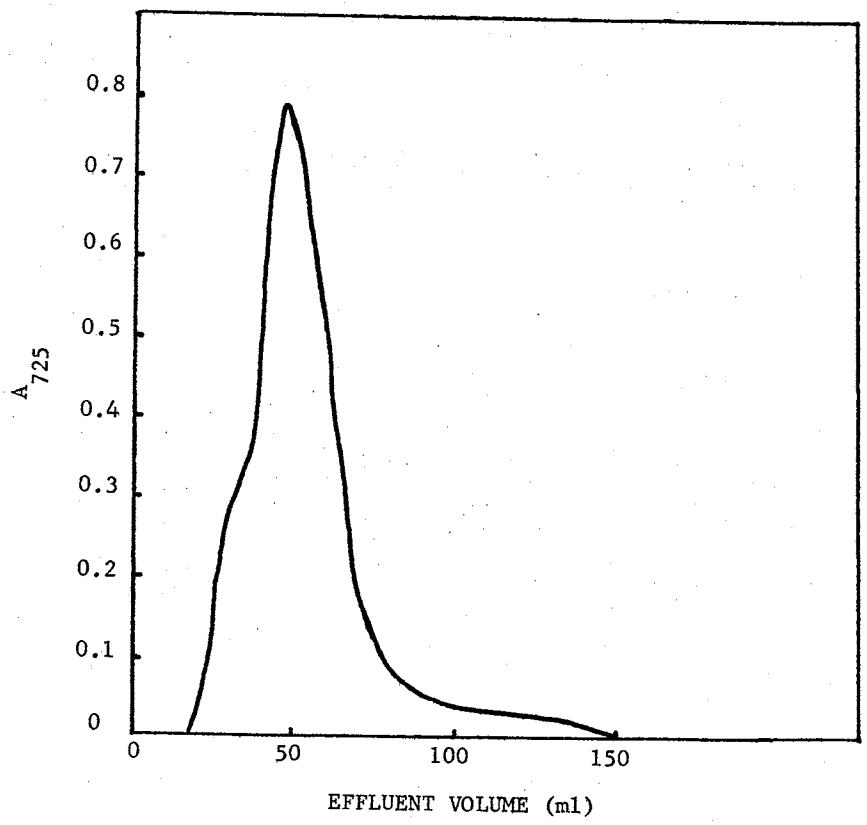

Fig. 14. Chromatograms of peak 3 from DEAE-cellulose column (Fig. 13) on Sephadex G-200. A sample of 6 mg protein was applied to the 40 x 1.6 cm column and eluted at the rate of 10 ml/hr with 0.02M phosphate buffer. Fractions of 5 ml were collected at 21-23 C.

eluted from the column, however, the swollen leading edge of this peak would seem to indicate that the molecular weight of this protein is very close to the exclusion limits of this gel, i.e. 200,000. This is in agreement with the 210,000 molecular weight reported by Patel et al. (42) for their isolate.

In Fig. 15, under the same chromatography conditions, again four major peaks and one minor one (peak 5) were obtained. Peak 5 was due to increasing the NaCl gradient to 0.5M. Again most of the lipase activity was in peaks 3 and 4. The specific activity of these two peaks was about the same and they may be the same protein with microheterogeneity accounting for the difference in displacement from the ion-exchange column. In an effort to study this possibility, the NaCl gradient was changed to that shown in Fig. 16 where the NaCl level was increased directly from 0.2M to 0,5M. This fraction as well as some of the earlier ones were concentrated with Lyphogel then anaylzed by polyacryamide gel electrophoresis.

Duplicate electrophoretograms of certain protein peaks from the DEAE-cellulose column graphed in Fig. 13 are shown in Fig. 17. Slots 13-a in this gel are bovine serum albumin (BSA), which was used as a marker. The original sample applied to the DEAE-cellulose column (slots 13-S) appears to contain 7 or 8 bands. Slots 13-3, from peak 3, which contained the majority of the lipase activity, show two major bands. However, slots 13-4 (from peak 4) appear to contain only one band. Fig. 18 shows certain peaks from the DEAE-cellulose column in Fig. 15, which was similar to that graphed in Fig. 13. Slot 15-a again is BSA and slot 15-S is the original sample. The protein concentration in slot 15-3 (from peak 3) was too low to be seen in the photograph. Slot

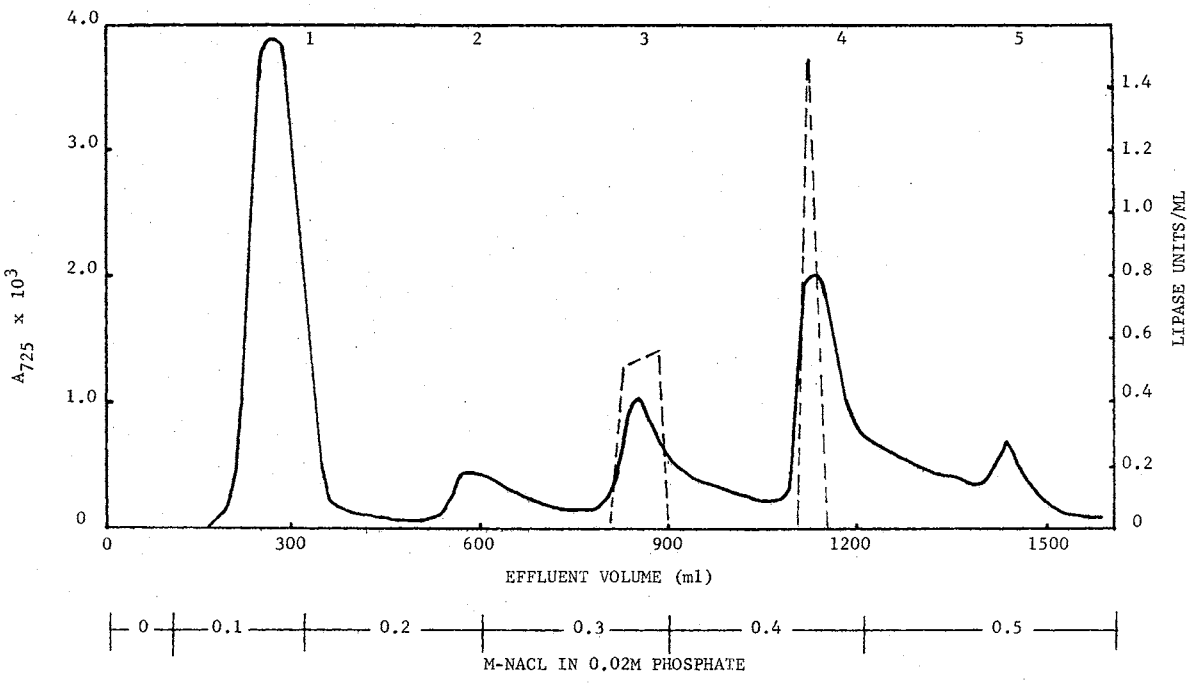

Fig. 15. Lipase activity (----) and protein patterns (——) from skimmilk filtered through DEAE-cellulose. II. NaCl gradient modified from that in Fig. 13.

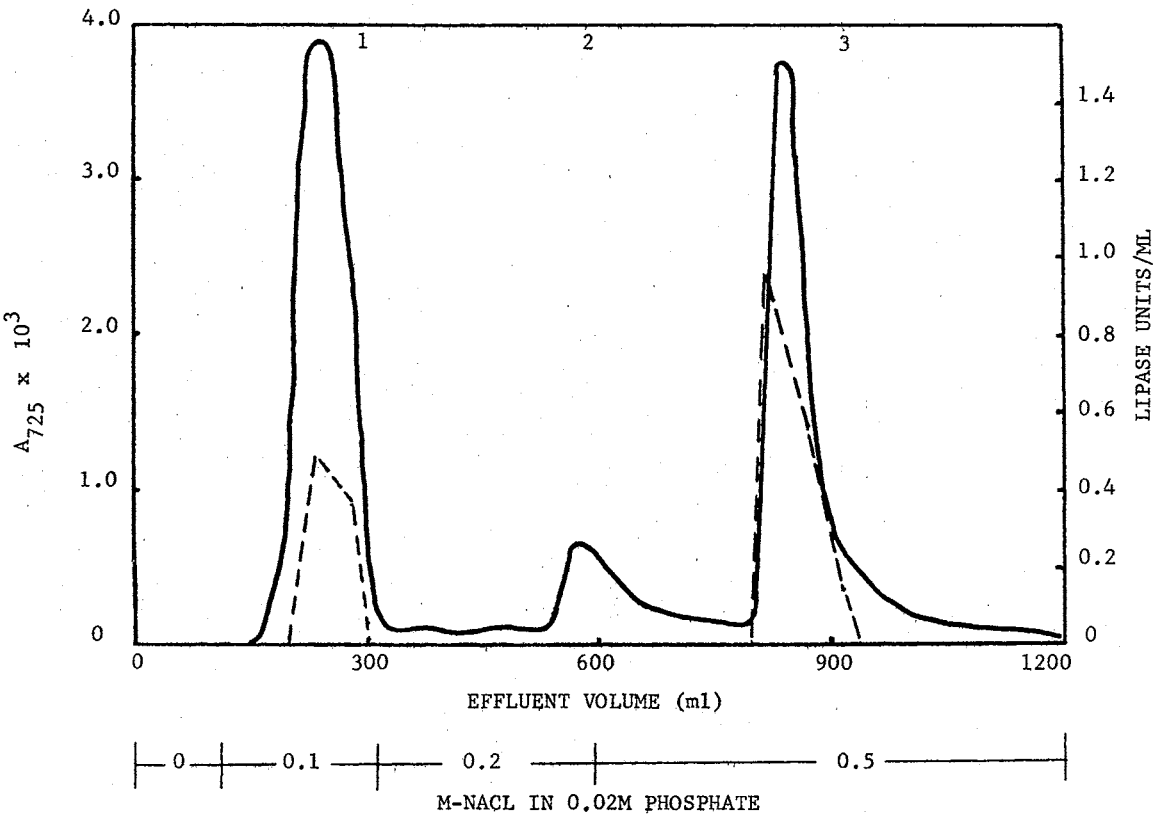

Fig. 16. Lipase activity (----) and protein patterns (——) from skimmilk filtered through DEAE-cellulose. III. NaCl gradient modified from that in Fig. 13 and 15.

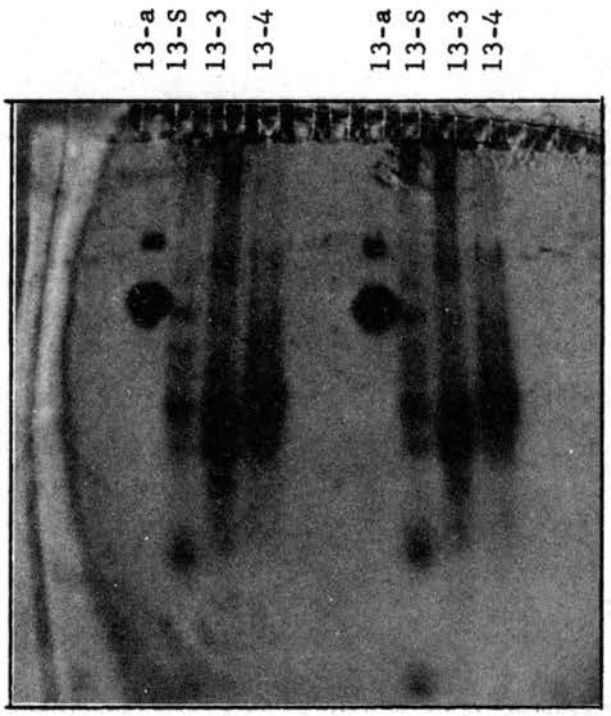

Fig. 17. Polyacrylamide gel electrophoretogram of certain protein peaks from DEAE-cellulose (Fig. 13). Slot Nos. 13-a, Bovine serum albumin; slot Nos. 13-S, original sample applied to the DEAE-cellulose column; slots Nos. 13-3 and 13-4 are from peaks 3 and 4, respectively.

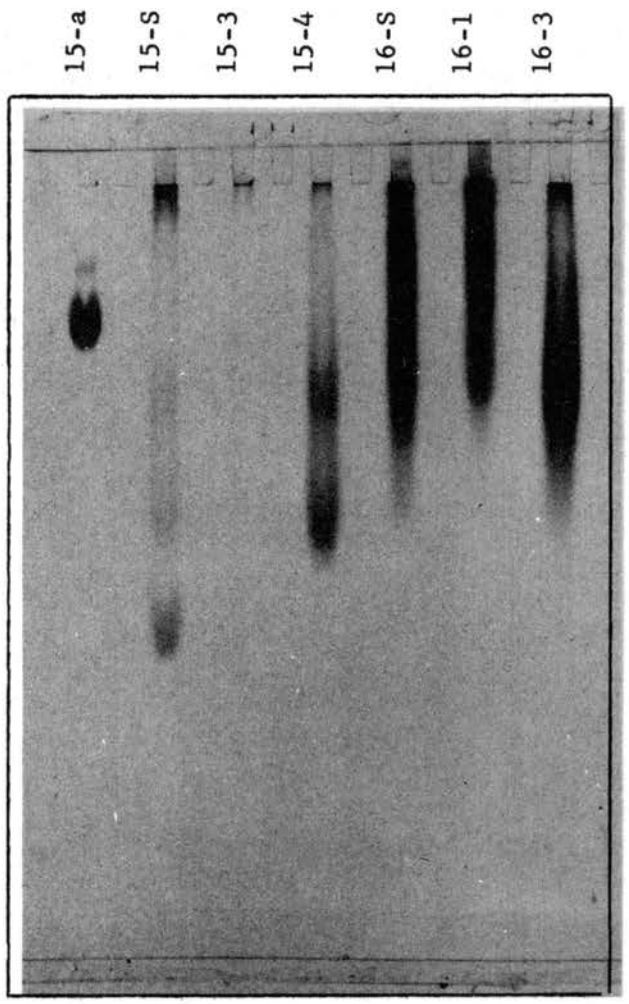

Fig. 18. Polyacrylamide gel electrophoretogram of protein peaks from DEAE-cellulose columns (Fig. 15 and 16). Slot No. 15-a, Bovine serum albumin; slot No. 15-S, original sample applied to DEAE-cellulose column. Slot Nos. 15-3 and 15-4 are from peaks 3 and 4, respectively of Fig. 15. Slot Nos. 16-S, 16-1 and 16-3 are the original sample, peaks 1 and 3, respectively from Fig. 16.

15-4 (from peak 4) contains two bands, rather than one band seen in peak 4 on the previous gel (slot 13-4). Protein samples from the DEAE-cellulose column in Fig. 16, which had a modified NaCl gradient, also are shown in Fig. 18. The proteins in these slots (16-S, 16-1, 16-3) were too concentrated, or the voltage was too low, for the proteins to be well resolved. However, one could distinguish a difference between the original sample (16-S) and the proteins in peak 1 (slot 16-1), which was in the void volume, and peak 3 (slot 16-3), which contained most of the lipase activity. The proteins in the latter slot were not sufficiently resolved to determine if more than one protein fraction was present. An ultraviolet absorption spectrum of this peak gave a typical protein spectrum with a maximum absorption at 279 mμ and minimum at 253 mμ with A_{279}/A_{253} equal to 1.63. Thus, this material was confirmed as largely protein with no appreciable quantities of other biological materials (lipids or carbohydrates) present.

These results indicate that the protein peaks which contain lipase activity (peaks 3 and 4), in some cases, appear to contain only one major protein band, but in others they exhibit two major bands. Assuming approximately equal electrical charges on these proteins and on the BSA, the proteins in peaks 3 and 4 have a higher mobility than BSA and thus a lower molecular weight. Based on a molecular weight of 70,000 for BSA, the molecular weights of these proteins are probably in the range of 50,000. Thus, the proteins in peak 3 of M.W. $\cong$ 200,000 (from the Sephadex determination - Fig. 14) probably has undergone some breakdown into at least two types of subunits. Four of these subunits, with M.W. $\cong$ 50,000 would give a molecular weight close to that found by Patel *et al.* (42) and to the 200,000 estimated in this work. Addition-

al work will be necessary to determine if all or only part of these subunits are necessary for lipase activity.

CHAPTER V

SUMMARY AND CONCLUSIONS

Lipase activities of bovine skimmilk were compared using bovine serum albumin (BSA) and gum arabic as emulsifiers for the tributyrin substrate. A total of 120 milk samples were obtained for this work by sampling three individual cows once or twice each week for 16 weeks. Fifteen of these samples were analyzed in duplicate with each emulsifier (a total of 60 observations). A "t" test indicated no difference between the mean lipase values for these two emulsifiers ($P > 0.50$). An analysis of variance on duplicate samples showed differences among cows and days ($P < 0.01$). No differences were found between emulsifiers ($P > 0.10$). Thus, BSA and gum arabic apparently performed similar functions in the reaction mixture.

Twenty-seven milk samples were obtained from each of three cows and these 81 samples were assayed for sialic acid contents (in duplicate) and lipase activities. There were large variations in sialic acid content among cows and sampling periods ($P < 0.01$). The average sialic acid contents of the milk from these three cows were 113, 101 and 75 μg/ml of skimmilk. Correlation coefficients for sialic acid contents vs lipase activities for the three cows were -0.10, -0.22 and -0.29. These low correlations would indicate that lipase activities can not be predicted from K-casein values (measured indirectly as sialic acid).

A series of kinetic studies were performed to determine optimum

assay conditions for this lipase. The enzymatic reaction was determined to be first-order for approximately 15 min. The V_m was approximately 1.35 lipase units/ml and the K_m was 1.0 ml of substrate. The optimum pH and temperature were 8.5 and 37 C respectively and the energy of activation was calculated to be 11,100 calories.

The milk proteins containing lipase activity were concentrated using rennin and $(NH_4)_2SO_4$ precipitation plus a Sephadex column and Lyphogel. These concentrated proteins were then separated on DEAE-cellulose columns and analyzed by gel electrophoresis and Sephadex chromatography for purity and molecular weight estimations.

The lipase activity was largely concentrated into two DEAE peaks (No. 3 and 4). This protein had a M.W. $\cong$ 200,000, which separated into at least two types of subunits on polyacrylamide gels. These subunits had a M.W. in the range of 50,000.

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