THE LIPASE(S) AND LIPIDS OF COAGULASE POSITIVE STAPHYLOCOCCUS AUREUS

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Dharam Vir Vadehra

1966

This is to certify that the

thesis entitled

The lipase(s) and lipids
of coagulase positive
Staphylococcus aureus

presented by

Dharam Vir Vadehra

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science and
Microbiology and Public
Health

Laurence A. Harmon - FSc
Donald L. Zaleff MPH
Major professor

Feb 25, 1966

Date _____

O-169

THE LIPASE(S) AND LIPIDS OF COAGULASE POSITIVE *STAPHYLOCOCCUS AUREUS*

By

Dharam Vir Vadehra

AN ABSTRACT OF A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Departments of
Food Science and
Microbiology and Public Health

1966

ABSTRACT

THE LIPASE(S) AND LIPIDS OF COAGULASE POSITIVE *STAPHYLOCOCCUS AUREUS*

By Dharam Vir Vadehra

The activity of the lipase(s) of two strains of coagulase positive *Staphylococcus aureus* was determined in milk fat incubated at 15, 22 and 30 C for 8 days. Hydrolysis of fat was measured by acid degree values. Neutral lipids were separated into component groups on a florisil column. Free fatty acids were determined by temperature programmed gas chromatography. The acid degree values were 25-50% greater at 22 than at 15 C and 4-7 times greater at 30 than at 22 C. The lipases liberated as much as 0.48 g of fatty acids per g of fat during 8 days incubation at 30 C. The enzyme showed a predilection for the palmitic acid-glycerol bond. Addition of fatty acids C_4 to C_{18} inclusive to inoculated sterile milk caused inhibition of *S. aureus* as follows: (a) complete at 0.05 and 0.10% concentration of C_{10}, and (b) partial at 0.05 and complete at 0.10% concentration of C_8. The samples showing inhibition were negative for peptonization, coagulase, and change in pH. Addition of oleic and stearic acid to sterile milk inoculated with *S. aureus* resulted in an increase in nonprotein nitrogen, and the C_4 to C_{12} acids caused a decrease in protease activity.

A lipase-rich fraction was isolated from the cell-free supernatant of a 24 hr broth culture of *S. aureus* B-120. The organisms were grown in trypticase soy broth at 37 C with continuous agitation. Initial concentration of lipase was achieved by precipitation of the

cell-free supernatant with equal volumes of absolute ethanol. This fraction was further purified by differential precipitation at pH 8.6 and 4.3. Subsequent purification using Sephadex G-200 and BioGel 300 yielded a preparation which showed a 350-450 fold increase in specific activity when compared to the original cell-free supernatant. Lipase production by the cells appears to be affected by air supply, agitation, age and pH of the cultures during growth. The optimum pH for lipase production at 37 C was about 8.0; however, another optimum was found at pH 9.0 suggesting the possibility of more than one lipase. The presence of air was essential for lipase production and maximum lipase activity was produced by 5 day old cells. The purified lipase had an optimum pH of 8.5 at 37 C. The electrophoretic mobility was -7.78×10^{-5} cm^2/volt/sec. The sedimentation coefficient for the two peaks was 2.85 and 8.5, respectively, and the molecular weight was 100,000. The purified lipase was capable of hydrolyzing a variety of natural oils and fats. The amount of free fatty acids liberated from these fats and oils was similar except in the case of hydrogenated soybean oil (iodine value < 3) in which the activity was reduced to one-third as compared to natural oils and fats. Gas chromatographic analysis of a hydrolyzed synthetic triglyceride with palmitic, stearic and oleic acid at the α, β and α' positions, respectively, indicated that the enzyme is capable of hydrolyzing the glycerol-fatty acid bonds at all three positions. The yield was 40% palmitic, 20% stearic and 39% oleic acids.

Formaldehyde, mercaptoethanol, cysteine, glutathione, and terramycin had an inhibitory effect on the lipase activity while

hydrogen peroxide, streptomycin and sodium taurocholate had a stimulatory effect on the activity.

During storage the enzyme lost less than 10% of the activity over a period of 21 days at 4 and -23 C. Storage at 4 C or freezing was the preferred method of preserving the lipase activity of the purified preparation, as compared to lyophilization where as much as 40% of the activity was lost.

The enzyme lost 7% of the activity when heated for 30 min at 50 C and was completely destroyed after 30 min at 70 C.

The cells of S. aureus contained 12.4% nitrogen, 4.5% phosphorus, approximately 4% lipids, 78% moisture, and on ignition yielded 13% ash. Fractionation of the total lipids yielded 77% phospho and 23% neutral lipids. Diglyceride was the major component of the neutral lipids, constituting approximately 45% of the total. Lecithin was the predominant phospholipid, constituting approximately 46-52% of that fraction. Analysis of phospho and neutral lipids for constituent fatty acids by gas chromatography revealed similar fatty acid composition in both of these components.

THE LIPASE(S) AND LIPIDS OF COAGULASE POSITIVE *STAPHYLOCOCCUS AUREUS*

By

Dharam Vir Vadehra

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Departments of
Food Science and
Microbiology and Public Health

1966

Dedicated

to

Mr. and Mrs. Sri Ram Vadehra

ACKNOWLEDGEMENTS

The author wishes to thank all with whom he has been associated during the course of his study in this country and who were instrumental in making his stay a pleasant and memorable one.

Special thanks to to the following persons:

Dr. L. G. Harmon, Professor of Food Science, for his guidance, encouragement and counsel in academic and personal matters. His philosophy of life will be a constant afflatus throughout the time to come.

Dr. Harold L. Sadoff, Professor of Microbiology, for acting as joint major professor and giving his valuable time for discussions and preparation of the manuscript.

Dr. J. Robert Brunner, Professor of Food Science, for allowing use of his laboratory facilities and for his enriching philosophical and scientific discussions.

Dr. Richard V. Lechowich, Department of Food Science, for his help in the preparation of the manuscript and willingness to share his laboratory facilities.

Dr. Hans A. Lillevik of the Department of Biochemistry for serving on the guidance committee.

Dr. Bernard S. Schweigert, Chairman, Department of Food Science, for providing financial assistance.

Miss Delores M. Fang for her secretarial help.

Last but not least the author thanks his parents Mr. and Mrs. Sri Ram Vadehra for their sacrifices and constant inspiration for a higher education, and to whom this thesis is respectfully dedicated.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

OBJECTIVES OF THE RESEARCH

Whole milk is inferior to skimmilk as a substrate for the growth of Staphylococcus aureus. Since the only difference between the two substrates is fat, then either fat per se or its derivatives are deleterious to the growth of the organism. The first part of this dissertation therefore is concerned with the elucidation of the nature of the changes produced in milk fat during growth of S. aureus. The effect of added fatty acids on the growth and enzyme activity of S. aureus was studied using skimmilk as a substrate.

After studying the gross changes produced by S. aureus lipase, the isolation and purification of the enzyme was deemed desirable. The factors affecting the production and activity of this enzyme were also investigated.

During the investigation of changes in the lipid portion of milk caused by growth of S. aureus, the lipids of this organism were also extracted along with the milk fat. The S. aureus lipids were unimportant in the compositional changes of milk fat as they represented only 0.002% of the total lipid extracted, but the qualitative and quantitative analyses of S. aureus lipids were considered worthy of investigation.

INTRODUCTION

The lipases are ubiquitous in nature being present in all biological systems. Several microorganisms possess strong lipolytic enzymes. The lipases exist both extracellularly and intracellularly but exolipases have been investigated more thoroughly because they are easier to obtain. The lipases are biologically important enzymes in the degradation of fats and oils to make available the fatty acids for energy and nutrition in cellular metabolism. The beneficial effect of lipases during the ripening of various varieties of cheese has been amply demonstrated by research workers. Excessive amounts of liberated fatty acids can also have a detrimental effect on the growth and fermentation ability of the organisms.

Lipases have many commercial uses. In foods, butter oil is being lipolysed under controlled conditions to develop a specific type of aroma used extensively in certain bakery products. Lipases could also be used to alter the composition of the natural fats and oils to meet changing consumer demands. A combination of several lipases with different specificities might be necessary to achieve this goal. In human medicine, lipases are being tried to lower the blood lipid level. The great concern and confusion of professional and nonprofessional people about dietary fats and their possible relationship to coronary diseases have opened new avenues for the use of lipases. It is possible that lipases in association with cholesterases and phospholipases could be used to modify lipid composition. Lipases could also have a possible use in the mobilization of depot fats and fat around the cardiac muscles.

A possible relationship between pathogenicity and lipase

production by Staphylococcus aureus has been suggested since S. aureus is the only pathogenic organism known to produce a detectable exolipase (Davies, 1954).

Recent studies on microbial lipases indicate that some of these lipases have a significant difference in their specificity as compared to classical pancreatic lipase. With the improved instrumentation during the past decade, the interest in fundamental and applied research on lipolytic enzymes has been renewed.

The purpose of the investigation reported herein was therefore to: a) study the effect of growth of S. aureus on milk fat and the effect of fatty acids on the growth of S. aureus; b) isolate, purify and characterize the exolipase of S. aureus; c) determine the chemical composition of S. aureus with special emphasis on lipid composition.

LITERATURE REVIEW

During the past several years there has been an extensive investigation of the physiology of staphylococci. This renewed interest has been brought about by both the medical profession and dairy and food bacteriologists interested in the resistance of the staphylococci to antibiotics and heat. Several theses have been written on the thermal resistance of staphylococci in milk including those by Busta (1963), Walker (1964), and Zottola (1965). The subject of thermal resistance of the staphylococci in certain foods has been thoroughly reviewed by Angelotti et al. (1960). Ecology is important in the growth, death and fermentation ability of most organisms including staphylococci. During 1962-1964 Peterson et al. published a series of papers entitled "Staphylococci in Competition". They studied naturally occurring mixed populations in precooked frozen food and the total number and proportion of staphylococci in mixed cultures capable of growth in artificial media. The effect of pH, salt, sugar, eggs and lipids on pure and mixed cultures of staphylococci has also been investigated by Peterson et al. (1962a,b and 1964a,b,c). Walker (1964) observed that skim-milk was superior to whole milk as a medium for the growth of staphylococci. Free fatty acids liberated from the whole milk were suggested as the possible cause of inhibition of S. aureus. Some earlier work by Costilow and Speck (1951) on streptococci showed that some of the fatty acids are inhibitory to the growth of the streptococci. The liberation of these fatty acids in biological systems results from the action of lipolytic enzymes on triglycerides.

Lipases. There is some confusion about the definition of "lipase" and "esterase". Bier (1955) suggests that this confusion arises from the fact that both groups are usually nonspecific in their action and the nature of either the fatty acids or the alcohol residue has a secondary effect, influencing only their rate of hydrolysis. Several workers have used the term lipase synonymously with esterase. According to Desnuelle (1961a), a true lipase is one which splits emulsified esters of glycerin and fatty acids with long chains, such as triolein or tripalmitin. This definition is arbitrary except that it has a physiological basis. Marchis-Mouren et al. (1959) suggested that a lipase hydrolyses di and monoglycerides as well as triglycerides although the rates are in decreasing order of the saturation of the glyceride molecule. On the other hand, Overbreek (1957) and Desnuelle (1961a) prefer to use the term aliesterase or simply esterase for the "so called lipases" which act on solutions of triglycerides with short chain fatty acids. "Wheat germ lipase" for example, in the true sense of the word, is an esterase, while pancreatic lipase is a true lipase.

The importance of lipases in digestion and metabolism has been amply demonstrated. Their role in the food industry is two fold involving both beneficial and detrimental effects. In spite of their fundamental importance, our understanding of these enzymes is rather meager.

The lipase subjected to the most extensive study is the one produced by the pancreas. This enzyme has been purified by Willstätter and Waldschmidt-Leitz (1923), Meyer et al. (1947), Boissonnas (1948), and more recently by Sarda et al. (1957), Marchis-Mouren (1959) and

Coleman (1963). The interest in pancreatic lipase has been from the physiological and pathological standpoint and there is an excellent review by Desnuelle (1961a).

Microbial lipases. Our knowledge on microbial lipases is limited and scattered. Goldman and Rayman (1952) reported that fungi and some bacteria produce lipases of importance in the food industry. Most of the work on bacterial lipases has been done on crude preparations and concerns the comparison of relative rates of hydrolysis of various natural and synthetic triglycerides. Shipe (1951) studied the relative specificity of lipases from Penicillium roqueforti and Aspergillus niger. The enzymes studied were intracellular lipases extracted from the mycelia. The optimum temperature, pH, effect of calcium and effect of several inhibitors were also reported. Fukumoto et al. (1963) and Iwai et al. (1964a,b) crystallized lipase from A. niger and have studied the enzyme physically and chemically. The comparisons of the lipolytic activity of the various microorganisms have limited validity since the assay conditions, time and temperature of incubation, difference in solubility, ease and stability of emulsion, and the size of fat globules have an effect on the rate of hydrolysis and the assay method. At least five or six different methods have been extensively used for assaying lipase activity. These methods involve one of the several basic techniques such as using a) chromogenic substrates, Foster et al. (1955) and Greenbank (1950); b) manometric techniques, Willart and Sjösstrom (1959); c) reduction of surface tension, Dunkley and Smith (1951); d) spectrophotometric determination, Börgstrom (1957) and e) the pH stat method of Marchis-Mouren

et al. (1959) and Parry et al. (1965). Recently (1962) the International Commission on Enzyme Nomenclature has defined a lipase unit and recommended standardized techniques for measuring activity.

Bacterial lipases have not been studied extensively. Members of the genera Pseudomonas, Achromobacter are prolific producers of lipases. Nashif and Nelson (1953a,b,c) published on the lipase of Pseudomonas fragi. Factors affecting the lipase production and the action of this lipase on cream and butter as well as some of the characteristics of the enzyme were described. Mencher and Alford (1965) isolated a lipase-rich fraction from P. fragi. Shahani et al. (1964) isolated an intracellular lipase-rich fraction from Achromobacter lipolyticum while Khan et al. (1964) reported the existence of an extracellular lipase in the same organism. Earlier work of Richards and ElSadek (1949) and Wilcox et al. (1955) on the action of microbial lipase was done on milk fat. They applied the techniques of differential volatility and paper chromatography to steam distillates and suggested that differences in specificities existed among the lipases of various organisms.

Alford et al. (1964) in an extensive survey tested over 73 microorganisms for their ability to produce lipase. Using lyophilized cell-free supernatants they reported that the specificity of microbial lipases falls into three main categories: a) attacks primarily the α position, b) attacks the α, β or α' positions, and c) attacks the oleic acid linkage irrespective of position. The third type of activity is shown by Geotrichum candidum. They claim this type of activity is unusual because the enzyme specificity is primarily for

the acid and the position is of secondary importance. Jensen et al. (1965) have confirmed this report.

The interest in the study of staphylococcal lipase dates back to the work of Gillepsie and Alder (1952) and Alder et al. (1953) who associated the "egg yolk factor" with virulence. They also suggested that this egg yolk factor has lipase activity. However, Rosendal (1962) presented evidence to the contrary showing no relationship between the egg yolk factor and virulence. Burns and Holtman (1960) suggested a possible relationship of the egg yolk factor to pathogenicity. Blobel et al. (1961) isolated the egg yolk factor and later Shah and Wilson (1963, 1965) studied some of its characteristics. This factor has been reported to have lipase activity but it is not clear if it is lipase per se.

Renshaw and SanClemente (1964) reported on partial purification of staphylococcal lipase but the characteristics have not yet been determined.

Lipid composition of microbial cells. One of the earliest references to bacterial lipids was that of Nencki (1886). However, interest in the study of bacterial lipids was dormant until Anderson (1943) began his studies on the lipids of Mycobacterium tuberculosis to determine if any correlation existed between lipid composition and pathogenicity. Woodbine (1959) in a review of microbial lipids emphasized the importance of microorganisms as a potential source of human food. Most of the research on bacterial lipids has been performed to find an answer to intrinsic scientific inquiry and mode of synthesis of lipids.

The amount of lipids present in bacteria varies from 8-40%

(Larson and Larson, 1922). The quantity depends on the method employed for extraction of lipids and the growth medium. Salton (1953) reported that ether extraction before or after methanol treatment removes only one-third of the lipids isolated from the cell walls of Escherichia coli. For complete release and extraction of lipids, hydrolysis with 6N HCl at 100 C for several hr is recommended by Luria (1960). This method is less applicable when the individual composition of various labile components is desired. Larson and Larson (1922) also reported that the presence of glycerol in the growth medium increased the lipid content of most organisms by 3-5 fold. Cook and Woodbine (1958) using Aspergillus ustus showed that agitation during growth had a beneficial effect on lipid production. Kates (1964) has reviewed the literature on bacterial lipids but not much information is available on the lipids of S. aureus. Macfarlane (1962) and Polonovski et al. (1962) have recently studied the lipids of S. aureus but report somewhat conflicting data.

EXPERIMENTAL PROCEDURES AND RESULTS

Growth and Action of S. aureus on Milk Fat

The work reported describes the effect of a) growth of S. aureus on milk fat and b) the presence of fatty acids on the growth and enzyme activity of S. aureus in skimmilk.

Two strains of S. aureus were used in this study. The strain B-120, responsible for a food posioning outbreak, was obtained from the Northern Regional Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois. Strain S-1 was isolated from milk from a cow in the Michigan State University dairy herd, which had clinical staphylococcal mastitis. These strains had the typical characteristics of S. aureus as described by Breed et al. (1957).

Growth of S. aureus. Both the B-120 and S-1 strains of S. aureus were used to study the gross changes produced in the lipid fraction of milk. However, during the latter part of the study only strain B-120 was used.

The organisms were grown in sterile cereal milk (milk containing 10% milk fat). Five hundred milliliter quantities of milk were dispensed in one liter Erlenmeyer flasks and sterilized at 121 C for 20 min. The inoculum was grown either in sterilized cereal milk or trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Maryland) at 32 C for approximately 18 hr. Enough inoculum was added to give a final population of approximately 1,000,000 cells per ml in the substrate. The cultures were incubated for a period of 8 days at 15, 22 and 30 C. One flask of

medium was inoculated with a culture grown for 18 hr in trypticase soy broth. This flask was incubated at 30 C and treated the same as the other samples. Uninoculated samples were incubated along with the inoculated samples and served as controls. Another flask with added inoculum was frozen and stored at -23 C during the period of study.

Fifty milliliter samples were withdrawn aseptically from each flask immediately after inoculation and mixing, and at 24 hr intervals thereafter up to 8 days. The number of organisms per ml of sample was determined according to Standard Methods (1960) using plate count agar (Difco Laboratories, Detroit, Michigan).

The data obtained are summarized in Table 1. The temperature had a profound effect on the rate of growth. At 30 C the maximum population occurred within 48 hr and then declined during the remainder of the incubation period. However, at 22 and 15 C there was a continuous gradual increase in population through the 8 day incubation period. The variation in the number of viable cells at the end of the incubation period is noteworthy.

pH changes during growth. The pH of each sample was determined using a Beckman glass electrode pH meter Model H-2. The changes observed in pH during the 8 day incubation period of cereal milk inoculated with S. aureus B-120 and S-1 are recorded in Table 2.

Determination of acid degree value. The milk fat was extracted with a separatory funnel from the lyophilized samples using 50 ml of absolute ethyl alcohol, followed by the same amount of ethyl ether and finally taken up in 50 ml of hexane. The solvents were removed from the lipid extract under vacuum using a Rinco evaporator with an

Table 1. Number of organisms per ml in cereal milk inoculated with S. aureus and incubated at 15, 22, and 30 C for 8 days

Strain of organism	Incubation temperature	Days of incubation								
		0	1	2	3	4	5	6	7	8
		Count per ml (in millions)								
B-120	15	6.4	81	270	300	420	500	860	1200	990
	22	6.2	110	23	540	530	850	1000	1200	-
	30	6.1	1700	2300	760	580	290	160	48	72
S-1	15	10.0	86	210	330	250	400	710	910	980
	22	8.2	140	390	550	430	710	960	1200	1200
	30	8.2	890	1100	630	160	680	25	23	12
None (Control)	30	-	< 1	-	-	< 1	-	-	-	< 1

- not determined

Table 2. Changes in the pH of cereal milk inoculated with S. aureus and incubated at 15, 22, and 30 C for 8 days

Strain of organism	Incubation temperature	Days of incubation								
		0	1	2	3	4	5	6	7	8
		pH								
B-120	15	6.10	6.40	6.50	6.50	6.55	6.40	6.40	6.20	6.40
	22	6.25	6.55	6.45	6.30	6.10	6.05	6.10	6.10	6.35
	30	6.20	5.85	5.35	5.20	5.15	5.05	5.00	5.10	5.05
S-1	15	6.30	6.30	6.35	6.40	6.35	6.40	6.35	6.30	6.30
	22	6.30	6.25	6.35	6.30	6.40	6.15	6.05	6.00	6.00
	30	6.20	5.90	5.50	5.35	5.35	5.25	5.15	5.20	5.30
None (Control)	30	6.30	-	-	6.20	-	-	6.25	-	6.25

- not determined

air driven motor and a laboratory aspirator. The evaporation was performed at 60 C. The extracted lipids were usually cloudy at this point and were centrifuged at 10,000 X g for 15 min. The final drying of the extracted lipids was done on a steam bath under a constant stream of nitrogen.

The extracted and dried milk fat was tested for rancidity using the method proposed by Thomas et al. (1955). The rancidity was expressed as acid degree value which is defined as ml of 1N methanolic potassium hydroxide required to titrate 100 g of fat.

The method consisted of taking 1 ml of melted milk fat in a syringe and dissolving in 5 ml of fat solvent consisting of hexane and N-propanol. The titrant was 0.025 N methanolic potassium hydroxide and the indicator was 1% alcoholic phenolpthalein. The fat solvent alone was titrated to obtain the blank reading. The acid degree value (ADV) was calculated using the following equation.

$$ADV = \frac{\text{ml KOH used for sample - ml KOH used for blank x normality x 100}}{\text{weight (g) of fat in the sample}}$$

In these calculations 1 ml of milk fat was considered as 0.90 g.

The degree of hydrolysis of milk fat during the growth of S. aureus in cereal milk at various incubation temperatures was measured by acid degree value. The data are shown in Table 3.

The acid degree value increased as the incubation time progressed at all temperatures and there was a positive correlation between the acid degree value and temperature. The acid degree value was only 25-50% greater at 22 C than at 15 C but at 30 C the acid degree value was 4-7 times greater than at 22 C. These results indicate a definite preference for the production and/or action of

Table 3. Acid degree values of fat extracted from milk inoculated with S. aureus and incubated as indicated

Inoculum	Incubation temperature	Days of incubation								
		0	1	2	3	4	5	6	7	8
		Acid degree value								
B-120 grown in trypticase soy broth	30	3.60	11.25	33.61	48.89	54.17	58.23	73.61	90.47	90.58
B-120 grown in milk	15	3.25	4.86	6.61	7.31	7.31	9.58	10.42	11.81	12.50
	22	3.37	5.81	7.42	9.72	12.78	14.53	15.54	16.94	18.34
	30	3.60	25.97	51.39	78.36	lost	102.78	112.22	116.94	118.19
S-1 grown in milk	15	2.40	4.00	-	-	6.51	-	-	-	10.50
	22	2.51	5.10	-	-	10.20	-	-	-	14.20
	30	2.35	18.20	-	-	46.42	-	-	-	78.52

- not determined

lipase at 30 C by the enzyme(s). The data also indicate that lipolytic activity varies considerably between strains, with strain B-120 being almost twice as active as strain S-1. The cells activated in cereal milk were more lipolytic than those activated in broth.

Analysis of the milk lipids. Cells of S. aureus S-1 were grown in milk as described previously. The samples removed initially and thereafter every 24 hr were not lyophilized; however, the lipids were extracted in the usual manner. The extracted lipids were dried and 1 g was dissolved in 20 ml of N-pentane. The free fatty acids and the remaining portion of the neutral lipids were separated using Amberlite (Mallinckrodt Chemical Works, St. Louis, Missouri) IRA-400 resin which had been prepared in the following manner. Ten grams of resin were stirred with 25 ml of 1N NaOH for 5 min. The base was removed by washing several times with water. This was followed by dehydration of the resin using absolute ethanol. The alcohol was finally replaced by N-pentane.

The dissolved fat was applied to the resin and stirred with a magnetic stirrer for 6 min. The supernatant was removed and the resin was washed two additional times with 25 ml of N-pentane. The washings were collected, filtered and concentrated under vacuum. The final drying was done on a hot plate under a constant stream of nitrogen. The residue of neutral lipids was weighed. The free fatty acids which were adsorbed by the resin were calculated by difference. The neutral lipids were further separated into various fractions using Florisil column chromatography as recommended by Carroll (1961). Florisil (activated magnesium silicate manufactured

by Floridin Co., Tallahassee, Florida) as supplied was deactivated by mixing 7 g of water with 100 g of florisil in a tightly stoppered Erlenmeyer flask and allowed to set overnight. A 20 x 1.6 cm column with a water cooled jacket was used. The preparation of the column, application of the sample, and the solvents for elution were exactly the same as suggested by Carroll (1961). The solvents were evaporated and the quantities of various fractions determined gravimetrically. The separation of cholesterol esters and triglycerides was not very sharp, so they are reported as a single fraction.

The result of fractionation of the hydrolyzed fat on a florisil column is shown in Table 4. The data show an increase in the lipolytic activity, evidenced by an increase in the amount of free fatty acids as the time increased from 0-8 days and the temperature increased from 15-30 C. Also, the amount of tri and diglycerides decreased in approximate proportion to the increase in free fatty acids as the temperature increased and the time progressed. The quantities of monoglycerides and cholesterol remained uniform or showed only slight fluctuations during incubation.

Esterification of fatty acids. The free fatty acids adsorbed to the resin were converted into their methyl esters by the method of Hornstein et al. (1960). The methanolic hydrochloric acid used for esterification was prepared by adding concentrated sulfuric acid into concentrated hydrochloric acid and bubbling the liberated gas into absolute methyl alcohol. After an increase of 5-10% in weight, the bubbling was stopped and the mixture stored at a temperature of 4 C. The methanolic hydrochloric acid was generally used within a week and was discarded after 10 days. Twenty five milli-

Table 4. Components of lipids extracted from cereal milk inoculated with S. aureus strain S-1 and incubated as indicated

Compound	Noninoculated control	Temperature (C) 15			22			30		
		Time in days								
	0	1	4	8	1	4	8	1	4	8
		mg per g of fat								
Free fatty acids	50	120	165	225	170	230	330	255	380	480
Monoglycerides	0	5	0	0	5	5	5	10	10	5
Diglycerides	0	40	20	10	50	5	5	50	10	10
Triglycerides*	910	770	700	640	700	615	540	615	490	445
Cholesterol	40	60	60	60	30	50	40	50	40	40
Percentage recovered	100	99.5	94.5	96.5	95.5	90.5	92.0	98.0	96.0	98.0

* Includes cholesterol esters

liters of methanolic hydrochloric acid were added to the resin and stirred for 25 min. The supernatant was removed and the process repeated twice with 15 ml of fresh methanolic hydrochloric acid, and stirred for 5 min. Fifteen milliliters of deionized water were added to the supernatant and the esters were extracted with 50 ml of N-pentane. The extraction was repeated twice with 20 ml of N-pentane. The combined N-pentane extracts were washed with water to remove any acid present and then dried over anhydrous sodium sulfate. The solvent was evaporated and the samples analysed on a temperature programmed F and M gas chromatograph (Model 500, F and M Corporation, Avondale, Pennsylvania). The temperature was programmed at the rate of 7.9 C/min over a range of 80-200 C. Twenty microliters of the evaporated samples were injected into the column. The final volume of the evaporated material and the amount of the material injected for analysis by gas-liquid chromatography was the same in all cases for ease of comparison. Known quantities of pure fatty acids were mixed and esterified on the resin in exactly the same way as the lipolyzed fat. The quantitation of the unknown samples was done by comparison to the standard and known mixture of fatty acids. The data obtained from the chromatographic analysis are shown in Table 5. These data include only the fatty acids with 10 or more carbon atoms. Lower chain fatty acids were determined in a separate experiment.

Measurement of volatile fatty acids. During the preparation of the samples for gas chromatographic analysis, extensive losses of volatile acids made it apparent that another method of measuring these volatile acids was needed. The rapid distillation method of

Table 5. Amount of free fatty acids liberated by S. aureus strain S-1 when grown in cereal milk under the conditions indicated

Acid	Noninoculated control	Temperature (C) 15			22			30		
		Time in days								
	0	1	4	8	1	4	8	1	4	8
		mg of fatty acids per g of fat								
Capric	0.34	0.64	0.87	3.32	0.78	1.78	4.14	1.86	4.65	6.17
Lauric	0.52	0.81	1.13	2.00	1.02	2.26	5.52	2.59	6.05	8.22
Myristic	0.32	2.24	3.83	7.61	3.16	4.49	14.68	7.94	19.30	24.38
Palmitic	3.00	4.46	7.80	13.91	7.35	16.40	44.80	21.13	46.67	53.83
Stearic	1.50	2.07	2.50	3.93	1.98	4.58	13.14	8.0	17.49	26.90
Oleic	2.23	4.54	6.86	9.54	4.90	9.33	22.77	12.18	29.93	42.97

Kosikowski and Dahlberg (1946) was adapted for this work. The method involved digesting and hydrolyzing 10 g of inoculated cereal milk with magnesium sulfate and sulfuric acid. The volatile materials were distilled over with precaution being taken not to exceed a temperature of 110 C and to collect the same quantity each time. Since alcohol was used to wash the condenser, both the water soluble and water insoluble acids were measured by this method.

The volatile acidities were measured as the ml of base required to neutralize the acids. The data are presented in Table 6. These results indicate a progressive increase in lipolysis as the temperature increased from 15-30 C and the incubation period increased from 0-8 days.

Effect of free fatty acids on growth of S. aureus. The effect of free fatty acids on growth of S. aureus strain S-1 was determined using a skimmilk substrate. The skimmilk was reconstituted from non-fat dry milk solids to give a final concentration of 10% solids. The saturated fatty acids C_4 through C_{18}, and oleic acid, were added in 0.01, 0.05 and 0.1% concentrations to reconstituted skimmilk. The mixtures were homogenized at 500 psi. The mixture was recycled once again to achieve greater homogeneity. The milk was sterilized, inoculated with S. aureus and incubated at 30 C for 8 days. During the incubation period samples were drawn aseptically every 24 hr and were analysed for changes in pH and population. The coagulase test was performed on the samples by the tube method described by Chapman et al. (1941). The population was measured by Standard Methods (1960) using plate count agar.

The changes in the number of organisms per ml of the cereal

Table 6. Volatile fatty acids recovered from 10 g of cereal milk (10% milk fat) inoculated with S. aureus strain S-1 and incubated as indicated

Incubation temperature (C)	Days of incubation			
	0	1	4	8
	ml of 0.1N KOH required to titrate volatile fatty acids			
15	0.5	1.33	1.92	2.15
22	0.5	1.74	2.18	2.97
30	0.3	3.50	5.47	7.28

milk inoculated with S. aureus and containing various concentrations of fatty acids are shown in Fig. 1. The log of the count is plotted against time. None of the fatty acids tested were inhibitory at 0.01% concentration. Caprylic acid was the most effective in suppressing the growth of the organisms causing complete permanent inhibition at 0.1% concentration and temporary inhibition at 0.05% concentration. The other saturated fatty acids (C_4 to C_{18}) and oleic acid added in concentrations of 0.01, 0.05 and 0.1% had no effect on the growth of S. aureus strain S-1 in skimmilk (Tables 7, 8, 9).

Measurement of nonprotein nitrogen. The effect of fatty acids on the proteolytic activity of S. aureus was determined. At the end of 8 days of incubation, 1 ml aliquot of milk sample was withdrawn and added to 5 ml of 10% trichloracetic acid. The trichloracetic acid soluble nonprotein nitrogen was determined in the supernatant using the method of Lowry et al. (1951). The results were expressed as micrograms of tyrosine liberated. The quantitation was done using a standard curve prepared with L-tyrosine.

The data are presented in Table 10. The short chain fatty acids C_6 to C_{12} inclusive caused partial inhibition of proteolysis of skimmilk when added to the skimmilk in concentrations of 0.05 and 0.10%. Butyric acid caused some inhibition at 0.10%.

Effect of fatty acids on coagulase activity of S. aureus. The skimmilk with added fatty acids and inoculated with S. aureus was tested for coagulase activity. The results were recorded as positive (+), negative (-), or doubtful (±). The data obtained at the end of the 8th day of incubation at 30 C are summarized in Table 11.

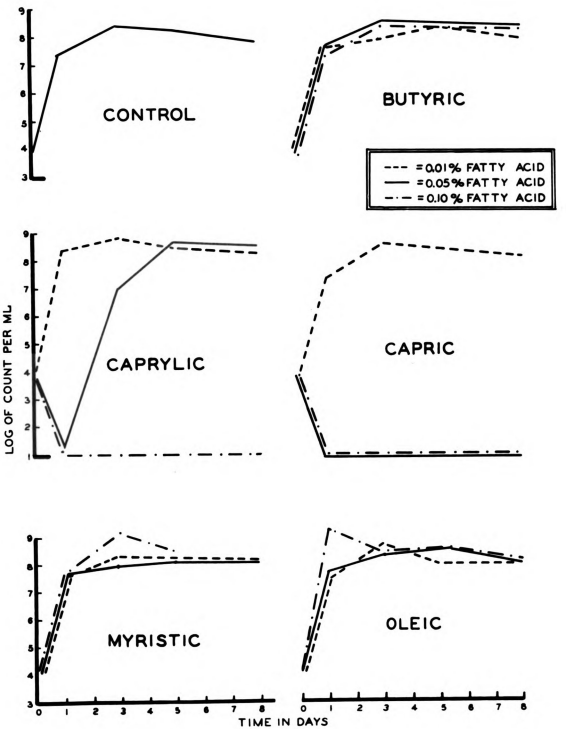

Fig. 1. Changes in population of S. aureus strain S-1 when grown in skimmilk containing 0.01, 0.05 and 0.10% of the fatty acids indicated.

Table 7. Population per ml when S. aureus strain S-1 was grown at 30 C in skimmilk containing 0.01% free fatty acids

Fatty acids added	Days of incubation				
	0	1	3	5	8
	S. aureus per ml				
Butyric	5500	4.5×10^7	9.2×10^7	20×10^7	10×10^7
Caproic	8100	22×10^7	48×10^7	17×10^7	16×10^7
Caprylic	6000	27×10^7	52×10^7	31×10^7	20×10^7
Capric	6500	5.5×10^7	41×10^7	22×10^7	16×10^7
Lauric	6600	3.5×10^7	32×10^7	30×10^7	27×10^7
Myristic	9400	4.0×10^7	22×10^7	21×10^7	16×10^7
Palmitic	12000	3.0×10^7	40×10^7	28×10^7	16×10^7
Stearic	14000	4.0×10^7	23×10^7	37×10^7	14×10^7
Oleic	14000	3.1×10^7	48×10^7	10×10^7	10×10^7
None (control)	6000	5.0×10^7	41×10^7	38×10^7	16×10^7

Table 8. Population per ml when S. aureus strain S-1 was grown at 30 C in skimmilk containing 0.05% free fatty acids

Fatty acids added	Days of incubation				
	0	1	3	5	8
	S. aureus per ml				
Butyric	5,700	5.0×10^7	43×10^7	44×10^7	30×10^7
Caproic	9,300	6.0×10^5	29×10^7	49×10^7	38×10^7
Caprylic	6,000	-	-	-	-
Capric	6,000	-	-	-	-
Lauric	6,100	10×10^6	3.0×10^6	35×10^6	7.0×10^6
Myristic	6,500	5.0×10^7	12×10^7	14×10^7	13×10^7
Palmitic	18,000	37×10^7	32×10^7	28×10^7	18×10^7
Stearic	14,000	55×10^7	20×10^7	31×10^7	24×10^7
Oleic	9,000	5.1×10^7	23×10^7	39×10^7	36×10^7
None (Control)	6,500	5.4×10^7	41×10^7	39×10^7	16×10^7

- no growth (inhibition)

Table 9. Population per ml when S. aureus strain S-1 was grown at 30 C in skimmilk containing 0.1% free fatty acids

Fatty acids added	Days of incubation				
	0	1	3	5	8
	S. aureus per ml				
Butyric	5,000	$2x10^7$	$25x10^7$	$24x10^7$	$23x10^7$
Caproic	4,200	$28x10^7$	$61x10^7$	$60x10^7$	$21x10^7$
Caprylic	6,300	-	$10x10^6$	$50x10^7$	$36x10^7$
Capric	6,000	-	-	-	-
Lauric	6,000	$1x10^7$	$12x10^7$	$11x10^7$	$2x10^7$
Myristic	6,400	$4x10^7$	$150x10^7$	$38x10^7$	contaminated
Palmitic	5,000	$3x10^7$	$25x10^7$	$24x10^7$	$22x10^7$
Stearic	13,000	$107x10^7$	$26x10^7$	$41x10^7$	$18x10^7$
Oleic	9,000	$200x10^7$	$30x10^7$	$42x10^7$	$15x10^7$
None (control)	9,000	$4x10^7$	$40x10^7$	$31x10^7$	$10x10^7$

- no growth inhibition

Table 10. Amount of tyrosine liberated when skimmilk containing added fatty acids was inoculated with S. aureus strain S-1 and incubated 8 days at 30 C*

Fatty acid added	Concentration of fatty acid (%)		
	0.01	0.05	0.1
	mg of tyrosine per ml of milk		
Butyric	1.00	1.08	0.75
Caproic	1.17	0.96	0.36
Caprylic	1.25	0.38	0.29
Capric	1.05	0.27	0.27
Lauric	1.55	0.75	0.80
Myristic	0.86	lost	0.89
Palmitic	1.30	1.30	1.34
Stearic	0.92	1.19	1.19
Oleic	0.96	1.07	1.17

*Two samples which did not contain fatty acids were used as controls. Noninoculated skimmilk and skimmilk inoculated with S. aureus and incubated 8 days yielded 0.27 and 0.91 mg of tyrosine per ml of milk, respectively.

Table 11. Coagulase activity of skimmilk containing fatty acids, inoculated with S. aureus strain S-1 and incubated at 30 C for 8 days*

Fatty acid added	Concentration of fatty acid (%)		
	0.01	0.05	0.10
Butyric	+	+	+
Caproic	+	+	+
Caprylic	+	+	-
Capric	+	-	-
Lauric	+	-	±
Myristic	+	+	+
Palmitic	+	+	+
Stearic	+	+	+
Oleic	+	+	+

*Samples of skimmilk containing no added fatty acids were coagulase positive when inoculated with S. aureus and coagulase negative when not inoculated.

All of the samples were coagulase positive, indicating substantial growth of S. aureus, except: a) those which contained 0.1% of C_8, C_{10} and C_{12} acids, and b) those which contained 0.05% of C_{10} and C_{12} acids.

Effect of fatty acids on the pH production by S. aureus in skimmilk. The skimmilk with added fatty acids was inoculated with S. aureus and incubated at 30 C. Samples were withdrawn after 1, 3 and 5 days of incubation and the changes in pH observed. The data are summarized in Table 12. A direct relationship existed between pH and inhibition of the growth of S. aureus, with the highest pH prevailing in those samples in which growth of S. aureus was inhibited.

Effect of fatty acids on coagulability of skimmilk inoculated with S. aureus. The growth of S. aureus in skimmilk produces enough acidity to cause the precipitation of casein and hence the coagulation of milk. However, when S. aureus was grown in skimmilk with added fatty acids at 30 C it was observed that all samples did not show coagulation. The inoculated samples were examined at 0, 1, 3, 5 and 8 days of incubation for visual signs of coagulation. The data are shown in Table 13 with coagulation indicated by (+) and noncoagulation as (-).

Table 12. Changes in pH when skimmilk containing added fatty acids was inoculated with S. aureus strain S-1 and incubated at 30 C

Fatty acid added	Concentration % of fatty acid	Hr of incubation 24	72	120
		pH		
None		6.3	5.8	5.1
Butyric	0.01	6.3	5.9	5.4
"	0.05	6.2	5.6	5.0
"	0.10	6.6	5.8	5.3
Caproic	0.01	6.3	6.1	5.2
"	0.05	6.3	5.7	5.2
"	0.10	5.8	5.5	5.1
Caprylic	0.01	6.3	5.6	5.1
"	0.05	6.1	6.2	5.9
"	0.10	6.0	6.0	5.9
Caproic	0.01	6.3	5.9	5.8
"	0.05	6.2	6.1	6.1
"	0.10	6.1	6.1	6.1
Lauric	0.01	6.3	6.0	5.5
"	0.05	6.2	6.3	6.0
"	0.10	6.2	6.2	5.8
Myristic	0.01	6.2	5.8	5.3
"	0.05	6.2	5.9	5.1
"	0.10	6.3	5.9	5.3
Palmitic	0.01	6.2	5.9	5.2
"	0.05	6.2	5.8	5.2
"	0.10	6.1	5.9	5.1
Stearic	0.01	6.2	5.8	5.2
"	0.05	6.1	5.7	5.2
"	0.10	6.0	5.7	5.0
Oleic	0.01	6.2	5.8	5.1
"	0.05	6.1	5.8	5.3
"	0.10	6.0	5.7	5.2

Table 13. Coagulability of skimmilk containing added fatty acids when inoculated with S. aureus strain S-1 and incubated at 30 C for 8 days

	Days of incubation														
	0			1			3			5			8		
	Concentration of fatty acid (%)														
Fatty acid added	.01	.05	.10	.01	.05	.10	.01	.05	.10	.01	.05	.10	.01	.05	.10
Butyric	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Caproic	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+
Caprylic	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-
Capric	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Lauric	-	-	-	-	-	-	-	-	-	+	-	-	+	±	-
Myristic	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+
Palmitic	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Stearic	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Oleic	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+

+ = coagulation

- = noncoagulation

± = doubtful coagulation

Isolation and Purification of Lipase

S. aureus strain B-120 was used as a source of lipase since preliminary work showed that this strain produced a very active lipase. A culture grown for 18 hr at 37 C in 10 ml of trypticase soy broth was used to inoculate approximately 1,250 ml quantities of trypticase soy broth in two liter Erlenmeyer flasks. The cells were grown at 37 C for 24 hr with continuous agitation using a rotary shaker at approximately 150 rev/min.

Preparation of the cell-free supernatant. At the end of incubation, the cells were removed from the medium in a refrigerated Servall centrifuge operated at 8,000 X g for 20-30 min. The supernatant was collected and assayed for lipase activity. The cells were washed at least twice with normal saline (0.9% sodium chloride), centrifuged and then resuspended in distilled water and lyophilized. The lyophilized cells were stored at -23 C.

Assay for lipase. In preliminary work, cream containing 35% fat was used as the substrate and the reaction mixture was incubated at 37 C for 1 hr. The liberated fatty acids were titrated with alcoholic potassium hydroxide to the phenolpthalein end point. However, this method was not reproducible and was deemed unsatisfactory.

The method adopted was reported in the Worthington catalog (1964) and used olive oil as the substrate. An emulsion was prepared by blending a mixture of 20 ml of olive oil with 165 ml of 5% gum acacia and 15 g of ice in a Waring blender for a total of 10 min (5 min at low speed and 5 min at high speed). Then the following were pipetted into a 150 ml beaker to form a substrate

for the enzyme:

Olive oil emulsion		5.0 ml
Sodium taurocholate	15 mg/ml	2.0 ml
Sodium chloride	3 M	2.0 ml
Calcium chloride	0.075 M	1.0 ml
Deionized water		5.0 ml

One milliliter of the enzyme solution was added to this substrate mixture and the pH adjusted to 8.0. The reaction mixture was incubated in a reciprocating shaker bath at 37 C for 10 min. At the end of incubation the mixture was electrometrically titrated to pH 8.0 with 0.01N KOH. The 10 min incubation period included the titration time. The lipase activity was defined in terms of "units", with a unit being equal to one micromole of acid produced per mg of the protein in 1 min at 37 C under the specified conditions.

Measurement of protein concentration. The protein concentration was determined by the method of Lowry et al. (1951) with certain modifications. The solution of $CuSO_4 \cdot 5H_2O$ and potassium sodium tartrate were added directly to the sodium carbonate solution and the phenol reagent was diluted 1:1 with water. Lysozyme 3X crystallized was used to prepare a standard curve for quantitation. Protein concentration was plotted against optical density measured at 660 mμ and the data are shown in Fig. 2.

Lipase production. During earlier investigations it was found that cultures which were not shaken during incubation did not produce lipase. The effect of several physical and chemical factors influencing lipase production by S. aureus is described below:

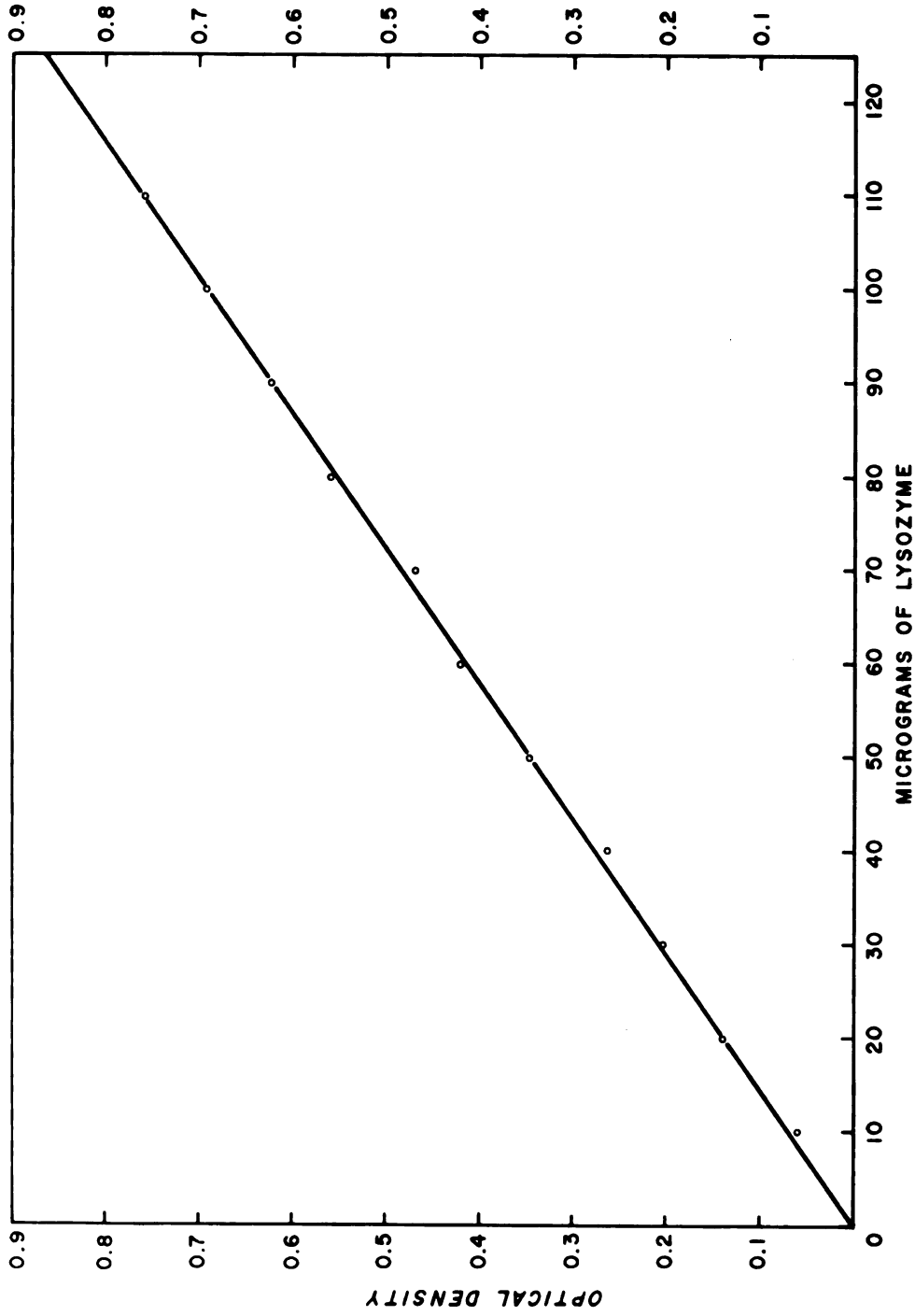

Fig. 2. Standard curve indicating protein concentration (lysozyme) vs optical density at 660 mμ using the method of Lowry et al. (1951).

a) rate of agitation and atmosphere, b) age of culture, c) pH, and d) added chemicals.

Effect of agitation and air. Four two liter Erlenmeyer flasks containing 1,000 ml of sterile trypticase soy broth were inoculated with S. aureus and incubated at 37 C under the conditions indicated below: a) without agitation, b) with agitation, c) with agitation and under vacuum, and d) without agitation but with sterile air supplied through filter tubes. The vacuum in flask in method (c) was created by using a laboratory aspirator operated at maximum water flow for 30 min. At the end of this period the exit tube was sealed. After 1, 2 and 3 days of incubation a portion of the medium was removed aseptically and the cells were removed by centrifugation at 8,000 X g for 20-30 min. The supernatant was assayed for lipase activity.

Effect of age. The cells were grown in trypticase soy broth for a maximum period of 7 days at 37 C under the following conditions: a) continuous agitation throughout the incubation period, b) agitation during the first 24 hr of incubation, and c) no agitation during incubation. Samples were withdrawn at regular intervals. The cells were removed by centrifugation and the supernatant was assayed for lipase. The data in Table 14 indicate that continuous agitation of the S. aureus cells resulted in a progressive increase in the lipolytic activity of the cell-free supernatant when the organism was grown in trypticase soy broth. The maximum activity was achieved with extracts from cells incubated 5 days, followed by somewhat lower activity from cells incubated 7 days at which time when the experiment was terminated. However, when the cultures

were agitated for the first 24 hr only, followed by quiescent incubation (Table 14, column 2) there was only a slight increase in the lipolytic activity on the second day and this was followed by a decrease in activity during the remainder of the incubation period. There was no evidence of lipase activity in the extracts from cells grown in a still culture.

Lack of enzyme activity in still cultures of S. aureus grown in trypticase soy broth at 37 C (Table 14) indicated that either air or agitation was necessary for the production (release) of lipase by S. aureus. An experiment was conducted to find which of these two factors was responsible for the presence of lipase in the cell-free supernatant. The data in Table 15 show an increase in lipase activity with an increase in the age of S. aureus cells in all cases. As shown in Tables 14 and 15 the extract from the non-agitated cultures had no lipase activity. There was only slight lipase activity when the growth medium was agitated under vacuum. However, when air was bubbled into the growth medium, the cell-free supernatant showed an amount of lipase activity similar to the activity of the cell-free supernatant obtained from cells grown with continuous mechanical agitation.

Effect of pH on lipase activity. Fifty milliliter portions of trypticase soy broth were adjusted to an initial pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 10.0 with 0.1N NaOH or HCl. The medium was autoclaved in 125 ml Erlenmeyer flasks and inoculated with an 18 hr culture of S. aureus. The organisms were grown in the above medium for 24 hr in a reciprocating shaker-water bath at 37 C. The medium was centrifuged and the supernatant

Table 14. Effect of age and agitation on the activity of lipase produced by S. aureus strain B-120 when grown in trypticase soy broth at 37 C. (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0.)

Days of incubation at 37 C	Conditions for growth of cells		
	No agitation	Agitation for first 24 hr	Continuous agitation
	ml of 0.01N NaOH used to titrate free fatty acids liberated from olive oil		
1	0.0	1.3	1.3
2	0.0	1.8	2.8
3	0.0	1.1	3.8
4	0.0	1.3	4.2
5	0.0	1.3	4.5
6	0.0	1.3	4.0
7	0.0	1.4	4.1
Noninoculated broth (Control)	0.0	0.0	0.0

Table 15. Effect of air and agitation on the activity of lipase produced by S. aureus strain B-120 grown in trypticase soy broth at 37 C. (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0.)

Days of incubation at 37 C	Conditions for growth of cells			
	No agitation	Mechanical agitation under vacuum	Continuous mechanical agitation	No mechanical agitation but bubbled with filtered air
	ml of 0.01N NaOH used to titrate free fatty acids liberated from olive oil			
1	0.0	0.3	1.3	1.0
2	0.0	0.5	2.7	2.7
3	0.0	0.6	3.6	3.2

assayed for lipase activity. The cells were washed with deionized water and transferred to preweighed aluminum evaporating dishes. The cells were dried at 110 C for 24 hr and the dry weight determined.

The lipase activity of the cell-free supernatant obtained from S. aureus grown in trypticase soy broth at 37 C at different pH between 4.0 and 10.0 is shown in Table 16. Also recorded in Table 16 is the dry weight of the S. aureus cells. Visual observations were made during the growth of the cells and at the end of the incubation period. There was no turbidity at pH 4.0 and 4.5 and only slight turbidity at 5.0, 9.5 and 10.0. Interestingly, growth in the form of clumps was observed between pH 6.5 and 8.5 while clumps were not observed at other pH values and most of the cells settled to the bottom. The data in Table 16 are plotted in Fig. 3 which shows that there were two optima for lipase production, one at pH 8.0 and the other at pH 9.0. The optimum for cell production ranged from pH 7.0 to 9.0 (Table 16, column 4).

Effect of added chemicals on lipase production. Previous work indicated that staphylococcal lipase may be an inducible enzyme (Table 3). Experiments were conducted to elucidate this phenomenon. One or two percent of glycerol, Tween 65, sorbitan monooleate polyoxyethylene (Tween 80), olive oil, and corn oil were added to trypticase soy broth and incubated at 37 C without agitation. The cell-free supernatant obtained at the end of incubation was assayed for lipase activity. The lipids from the supernatant containing olive oil and corn oil were extracted three times with N-pentane. The solvent was evaporated and the hydrolyzed oil titrated to the

Table 16. Effect of pH on activity of lipase produced by S. aureus strain B-120 grown in trypticase soy broth for 24 hr at 37 C. (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0)

Initial pH of the medium	Final pH of the medium	ml of 0.01N NaOH required to titrate free fatty acid liberated from olive oil	mg of S. aureus cells recovered per 50 ml of the medium
4.00	4.05	0.20	8.6
4.50	4.65	0.60	7.5
5.00	4.90	0.60	29.0
5.50	4.95	0.65	40.9
6.00	5.50	1.25	53.5
6.50	6.35	4.05	60.0
7.00	6.70	5.90	77.7
7.50	6.95	6.70	87.1
8.00	7.25	7.30	84.1
8.50	7.45	5.00	70.4
9.00	7.75	7.10	68.0
9.50	7.80	1.70	36.7
10.00	7.95	0.90	20.2

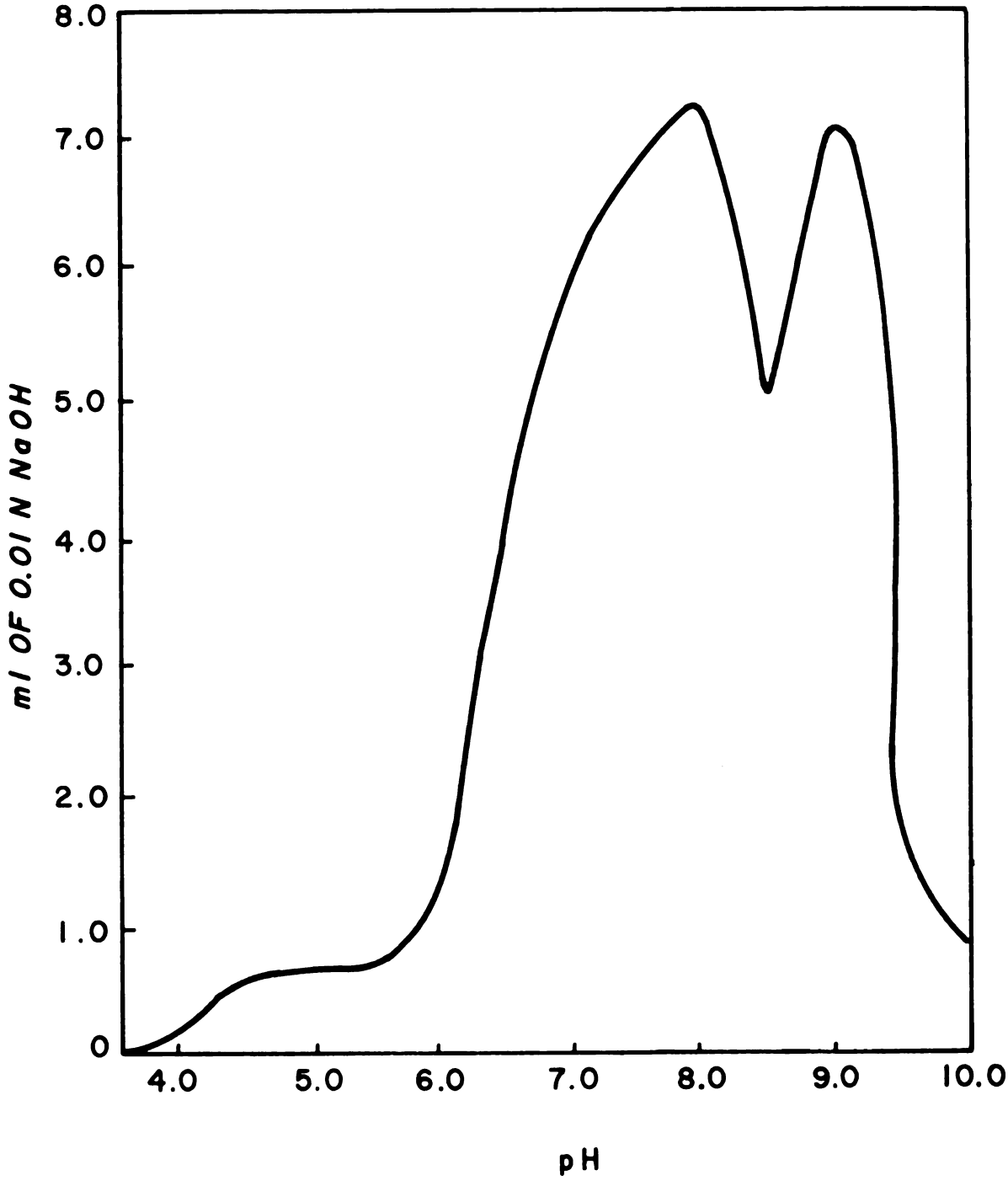

Fig. 3. Milliliters of 0.01N NaOH required to neutralize the free fatty acids formed by the lipolytic enzymes produced by S. aureus strain B-120 grown in trypticase soy broth at 37 C at the pH indicated. (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0.)

phenolpthalein end point with 0.025N NaOH. The acid degree value was calculated as described previously.

The data obtained are presented in Table 17. Apparently none of the added chemical substances had a stimulating effect on the lipase production by S. aureus under the conditions of this experiment. However, when the lipids were extracted from the cell-free supernatant and titrated to the phenolpthalein end point with 0.025N KOH to determine acid degree value, they showed extensive lipolysis.

Presence of intracellular lipase. Five grams of the cells obtained from still cultures were washed in a Waring blender (1 min at slow speed and 1 min at high speed). The cells were separated by centrifugation and disintegrated by sonification for 5 min at 7-9 amps. The cell debris and intact cells were removed by centrifugation at 20,000 X g for 20 min. The supernatant from the washings in the blender and the cell-free supernatant from sonification were analyzed for lipase activity. The supernatant obtained after S. aureus cells were washed in a Waring blender and centrifuged showed no lipase activity when tested on olive oil. The results were also negative with the cell-free supernatant obtained from sonified cells. These observations suggest that under the conditions prevailing in this experiment there was no evidence for the existence of an intracellular lipase in S. aureus B-120.

Concentration of staphylococcal lipase. The extracellular nature of the staphylococcal lipase and the presence of this enzyme in the growth medium makes it mandatory to concentrate the lipase into smaller volumes of liquid for convenience of handling. Several

Table 17. Effect of chemical agents on activity of lipase produced by S. aureus strain B-120 grown as indicated at 37 C for 24 hr. (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0.)

Basal medium Trypticase soy broth (TSB)	Micromoles of acid produced per mg of protein in the growth medium	Acid degree value
TSB, agitated	0.3-0.5	-
TSB, stationary	0.0	-
TSB + 1% Tween 65, stationary	0.0	-
TSB + 1% Tween 80, stationary	0.0	-
TSB + 2% olive oil, stationary	0.0	15.4
TSB + 2% corn oil, stationary	0.0	12.2
TSB + 1% glycerol, stationary	0.0	-

- not determined

methods were tried and are described below. All operations were performed at 4 C or lower, unless otherwise specified and all fractions were analyzed for protein content and lipase activity, as described previously.

Ammonium sulfate precipitation. The cell-free supernatant was precipitated with 40, 50, 60, 70, 80 and 100% ammonium sulfate. The precipitates were dissolved in deionized water and dialyzed. The supernatants were first dialyzed against tap water followed by pervaporization and dialysis against deionized water.

The data in Table 18 indicate the activity of lipase precipitated with various concentrations of ammonium sulfate. In the interest of clarity and valid comparison the lipase activity is presented in "specific units". A specific unit is defined as the micromoles of free fatty acids produced per mg of the protein in the enzyme solution under the experimental conditions.

The lipase activity in the supernatant showed a progressive decrease as the percent concentration of ammonium sulfate was increased above 40% saturation. Lipase activity in the precipitate increased as the concentration of ammonium sulfate increased from 40-80% saturation. At 100% saturation there was a decrease in activity probably due to denaturation of the enzyme protein.

Precipitation with acid. Zolli and SanClemente (1963) reported that lipase can be precipitated from the cell-free supernatant by dialyzing against acetate buffer at pH 4.3 containing 10% v/v ethyl alcohol. Direct precipitation of the supernatant was tried in this experiment with some success. The precipitate obtained by adding 10% v/v of absolute ethyl alcohol to the cell-free supernatant

Table 18. The lipase activity of various fractions obtained by precipitation of the cell-free supernatant obtained from S. aureus strain B-120 with different concentrations of ammonium sulfate. (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0.)

Percent saturation of ammonium sulfate	Lipase activity (specific units)	
	Precipitate	Supernatant
40	8-10	8-10
50	15-18	4- 7
60	12-17	2- 5
70	15-16	0- 4
80	17-20	1- 4
100	15-18	-

- not determined

at pH 4.3 had a lipase activity of 13 units which was lower than the activity of the precipitate obtained with ammonium sulfate (80% saturation).

Precipitation with ethyl alcohol and acetone. The cell-free supernatant was precipitated with 40, 50, 60 and 70% ethyl alcohol. The mixture was constantly stirred during addition of alcohol. The precipitate was dissolved in water while the supernatants were dialyzed against deionized water. The process was repeated using acetone as a precipitant. The precipitates and supernatants obtained were tested for lipase activity and the data obtained are shown in Table 19. The data indicate that the precipitation with a 50% concentration of alcohol gave the most lipase activity per mg of protein and as the concentration of alcohol increased to 70% the activity of the precipitate decreased.

The results obtained with acetone precipitation were similar to those obtained with alcohol, except 50% acetone gave a precipitate with slightly lower activity than the one obtained with 50% alcohol.

A comparison of the various methods indicates that precipitation with 50% alcohol was the preferable method for initial concentration. The data showing the optimum concentrations for each of the various methods are summarized in Table 20.

Purification of staphylococcal lipase. The precipitate removed from the cell-free supernatant with 50% ethyl alcohol showed the most activity and this crude preparation was further purified by the following procedure;

Table 19. Lipase activity of the fractions resulting from treating the cell-free supernatant obtained from S. aureus with absolute ethyl alcohol. (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0.)

Percentage of alcohol	Lipase activity (specific units)	
	Precipitate	Supernatant
40	10-12	5-6
50	24-28	0-1
60	14-16	0
70	10-12	0

Table 20. A comparison of the various methods for the initial concentration of staphylococcal lipase. (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0.)

Method of precipitation	Units of lipase activity	
	Precipitate	Supernatant
80% ammonium sulfate	17-20	1-4
10% v/v ethanol at pH 4.3	11-16	4-5
50% v/v ethyl alcohol	24-28	0-1
50% v/v acetone	22-24	0-2

Step 1. The alcohol precipitate was dissolved in deionized water and the pH adjusted to 8.6. The mixture was cloudy and was centrifuged for 30 min at 1000 X g.

Step 2. The pH of the supernatant obtained from Step 1 was slowly adjusted to pH 4.3 with 0.1N HCl. The resulting precipitate was removed by centrifugation at 18,000 X g for 30 min.

Step 3. Use of column chromatography using Sephadex G-200.

The columns were packed with Sephadex or BioGel according to the directions of Flodin (1962). The height of the packed material was generally 15 times the internal diameter of the various columns. The packed columns were checked with blue dextran 2000 for uniformity of packing and void volumes. The buffer solution was passed through the packed column for at least 12 hr before chromatographing the protein solution. A 1-2% solution of the protein in tris (hydroxymethyl) amino methane-hydrochloric acid buffer pH 8.1, $\Gamma/2 = 0.1$ was used to charge the column. The buffer was prepared according to the method of Long (1961). The columns were eluted with tris buffer pH 8.1, $\Gamma/2 = 0.1$ at room temperatures. The eluant was passed through an ultraviolet monitor and the fractions collected every 20 min using an automatic fraction collector. The chromatographic pattern obtained with Sephadex G-200 is shown in Fig. 4. The tube numbers were plotted against protein concentration and lipase activity.

Step 4. The fractions showing maximum lipase activity from Step 3 were pooled and concentrated by

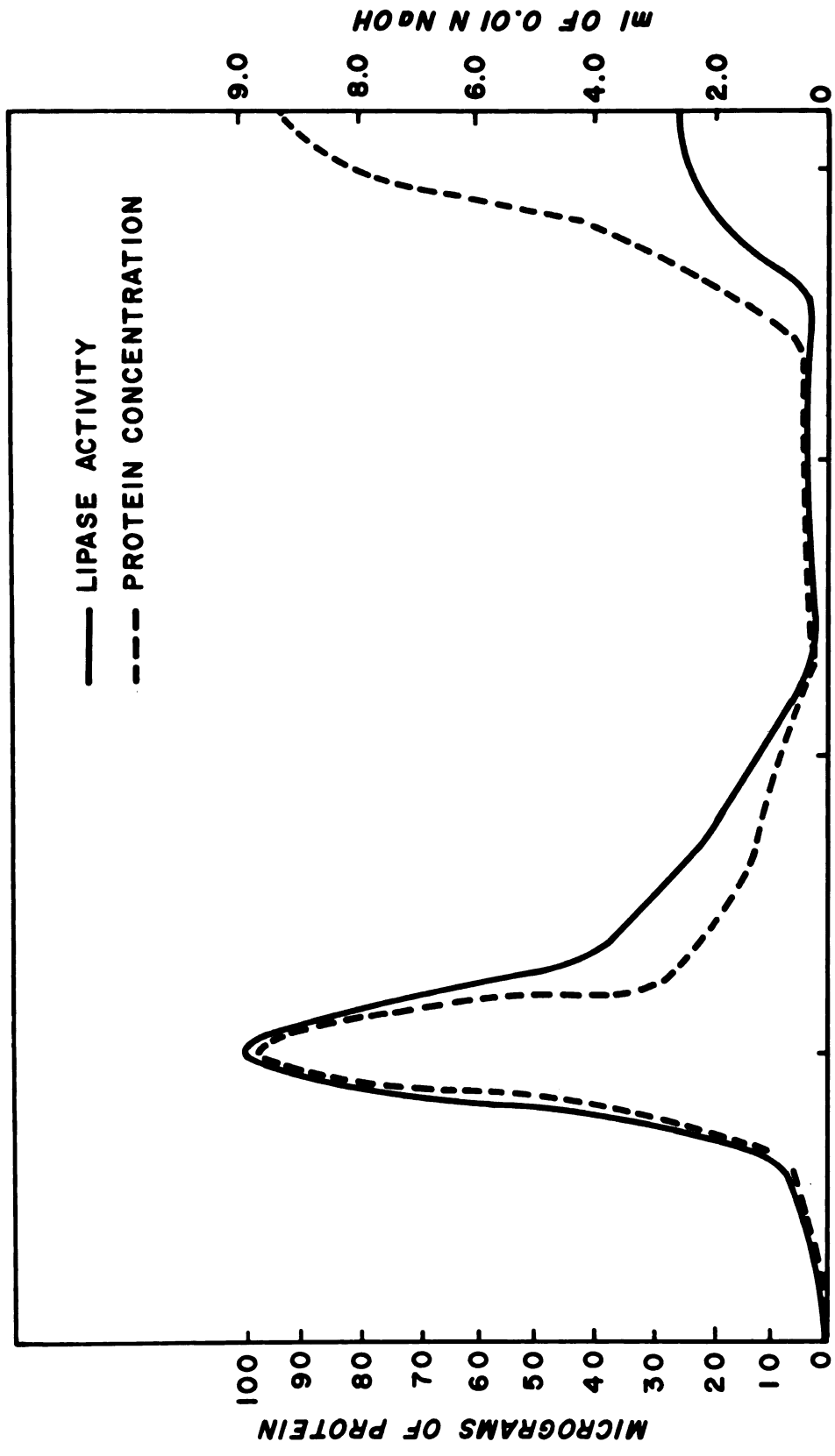

Fig. 4. An elution diagram of a lipase preparation on Sephadex G-200 using tris buffer pH 8.1, $\Gamma/2 = 0.1$. Lipase concentration is represented as micrograms of protein and the lipase activity as ml of 0.01N NaOH required to titrate free fatty acids liberated by the lipase in olive oil emulsion for 10 min at 37 C and pH 8.0.

pervaporation. The concentrated material was then chromatographed using a 40x2.3 cm tube packed with BioGel 300. The chromatographic pattern is shown in Fig. 5. Most of the lipase was eluted in the first peak, though the second peak showed some lipolytic activity. A direct relationship between protein concentration and lipase activity was observed in the fractions obtained under the first peak.

A schematic flow sheet for the isolation procedure is shown in Fig. 6. The data showing the degree of purification during the various steps of purification are summarized in Table 21. The final purified lipase preparation possessed 350-450 times more lipolytic activity per mg of protein than the cell-free supernatant.

Characterization of the Enzyme Preparation

The lipase-rich fraction isolated by the procedure outlined above was assayed at different pH values and temperatures to determine the optimum conditions for lipolytic activity. The storage stability and thermal inactivation at various temperatures were determined. The action of this enzyme on various natural and synthetic substances was also ascertained. In addition, the inhibitory or stimulatory effect of various chemical agents on lipase activity was investigated and the electrophoretic mobility, sedimentation coefficient and molecular weight were determined.

Optimum pH for lipase action. One milliliter of the enzyme solution was added to the substrate and the pH adjusted to one of the following values: 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0,

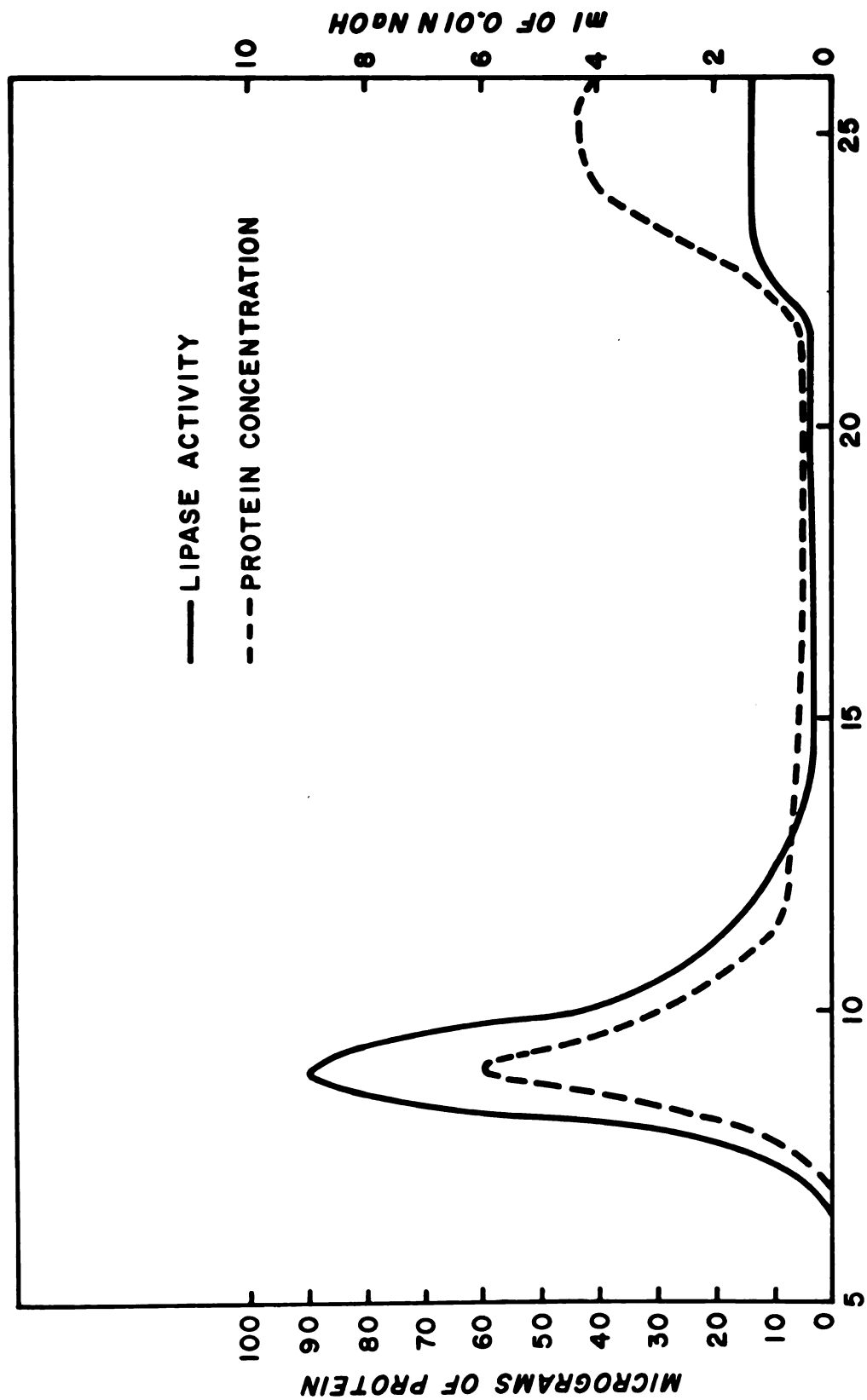

Fig. 5. An elution diagram of a lipase preparation on BioGel 300 using tris buffer pH 8.1, $\Gamma/2 = 0.1$. Lipase concentration is represented as micrograms of protein and the lipase activity as ml of 0.01N NaOH required to titrate free fatty acids liberated by the lipase in olive oil emulsion for 10 min at 37 C and pH 8.0.

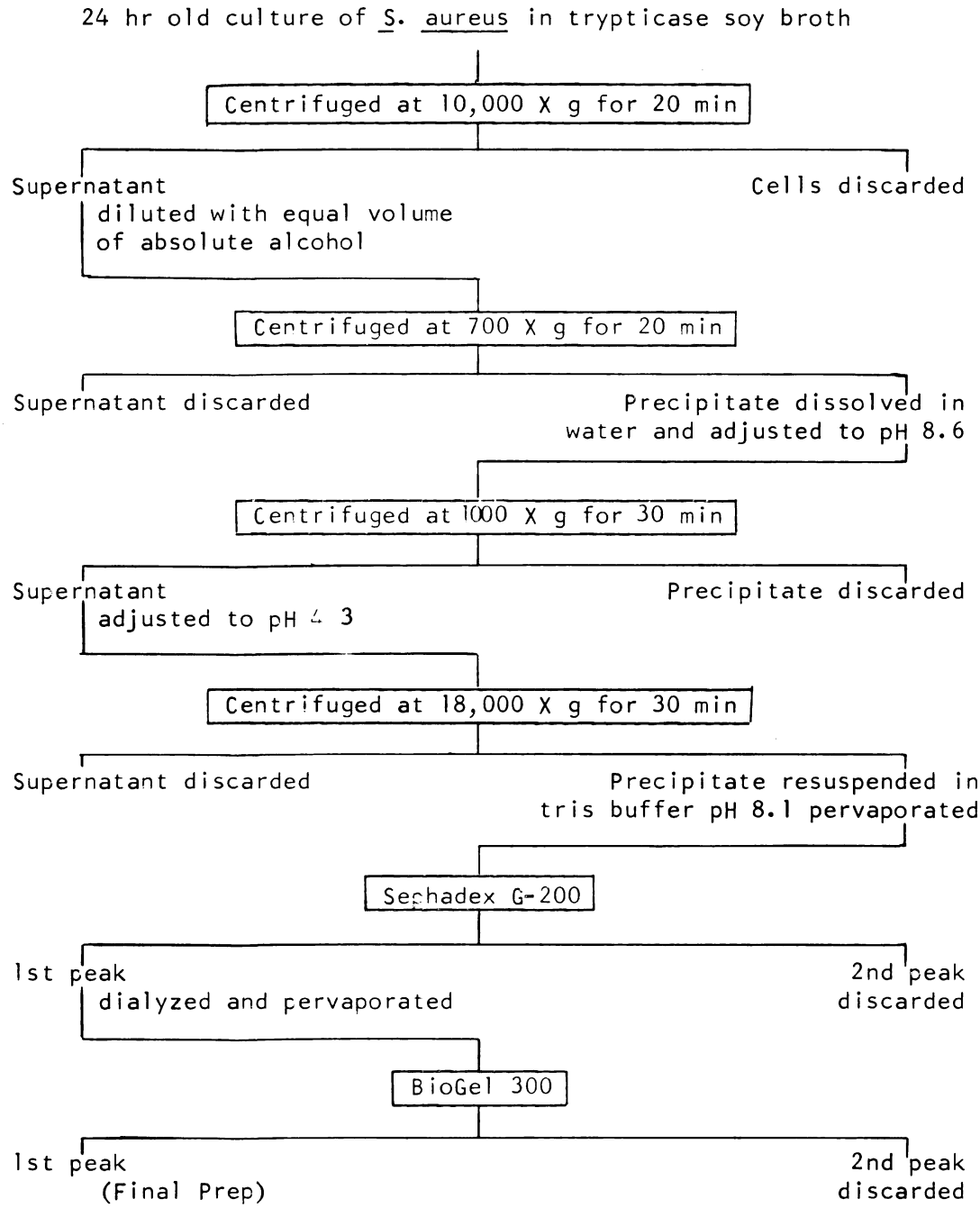

Fig. 6. Flow sheet for the isolation and purification of staphylococcal lipase.

Table 21. Activity of the staphylococcal lipase (strain B-120) obtained from the cell-free supernatant at each state of fractionation. (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0.)

Stage of fractionation	Specific activity (units)		Degree of purification
	Precipitate	Supernatant	
cell-free supernatant	0.3	-	1
50% alcohol	25.0	2	83
pH 8.6	6.0	32	106
pH 4.3	60.0	-	200
Sephadex G-200	95.0	12	316
BioGel 300	135.0	5	450

- not determined

9.5, 10.0, 10.5 and 11.0. The reaction mixture was incubated at 37 C for 10 min. At the end of incubation, the pH was adjusted back to the original value with 0.01N NaOH.

The milliliters of 0.01N NaOH required to titrate were plotted against pH and the data are shown in Fig. 7. The enzyme appeared to have a single pH optimum around 8.5. However, the enzyme was active over a wide range of pH mostly on the alkaline side of neutrality.

Optimum temperature for lipase activity. The optimum temperature for the action of the lipase preparation was determined at pH 8.0 using olive oil as the substrate. The enzyme activity was determined at 5, 15, 25, 30, 35, 37, 40, 45, 50, 55, 60, 65 and 70 C. The substrate was conditioned at the test temperature before the enzyme solution was added. The activity represented as ml of 0.01N NaOH required to neutralize the liberated fatty acids was plotted against the incubation temperature and the graph obtained is shown in Fig. 8. The optimum temperature under experimental conditions was around 45 C. There was little activity at 70 C.

Storage stability of purified lipase. The lipase preparation was stored at 37, 32, 22, 16, 4 and -23 C for a maximum period of 21 days. One milliliter aliquots were assayed for lipase activity. The percentage of loss of activity was plotted against the storage time and the results are indicated in Fig. 9. The high degree of stability of this enzyme is shown by the fact that only 7% of the activity was lost after 21 days of storage at 4 C. At 37 C the enzyme preparation lost 50% of its activity after 21 days of storage.

Thermal inactivation of the lipase. Thermal destruction studies

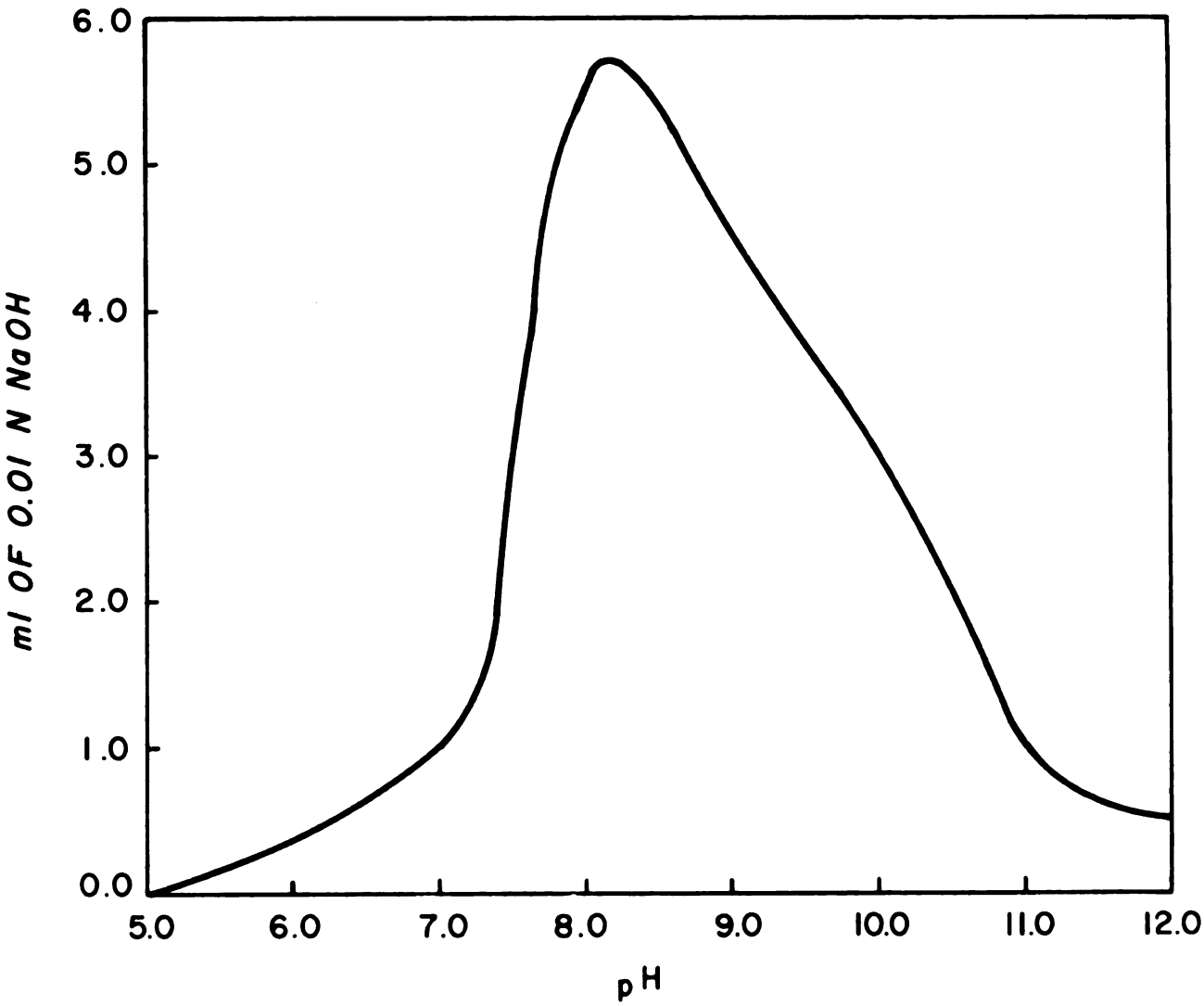

Fig. 7. Milliliters of 0.01N NaOH used to titrate the fatty acids liberated by the action of staphylococcal lipase in olive oil emulsion for 10 min at 37 C at various pH values.

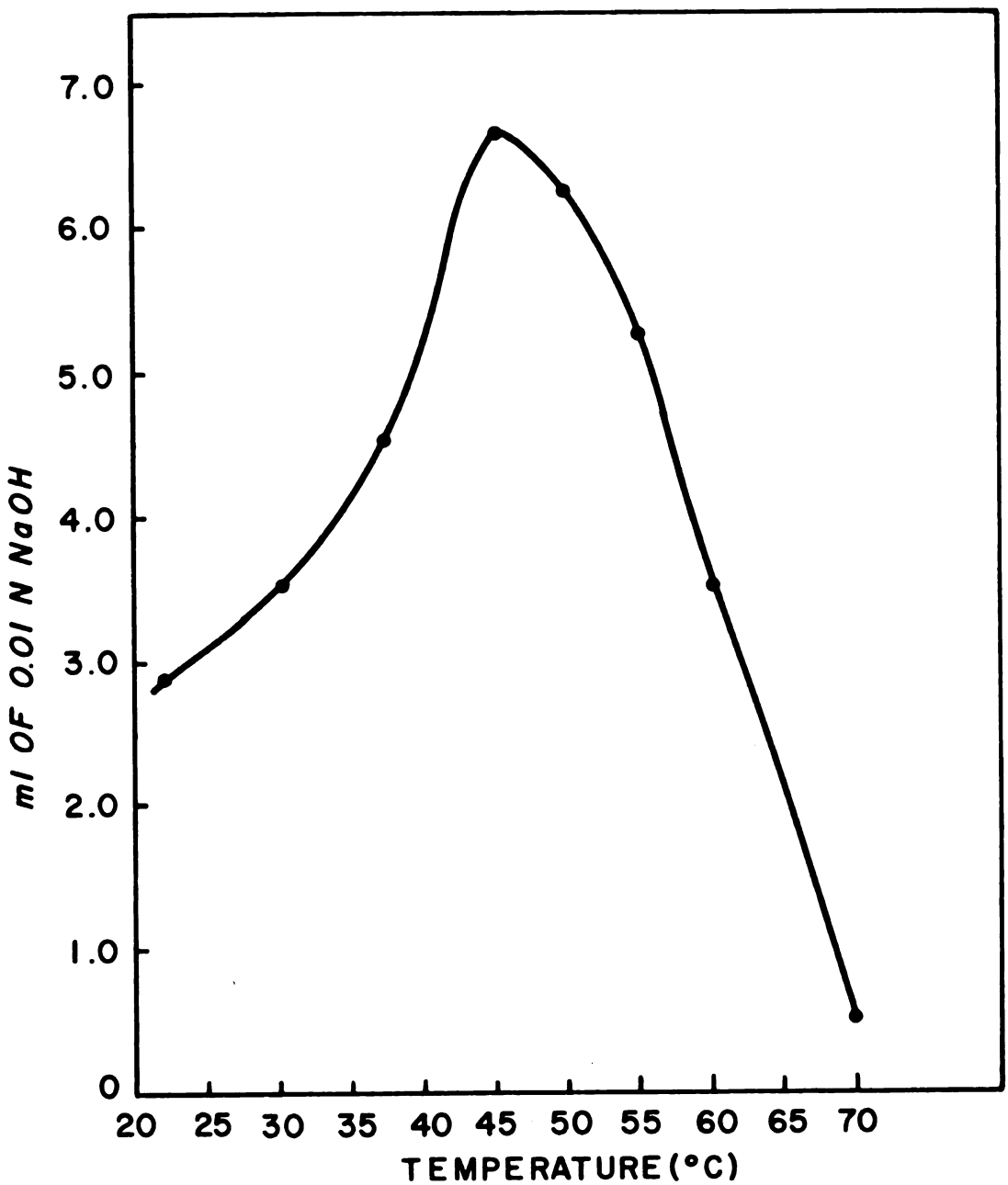

Fig. 8. Milliliters of 0.01N NaOH required to titrate the free fatty acids liberated from olive oil by staphylococcal lipase after 10 min at pH 8.0 at various incubation temperatures.

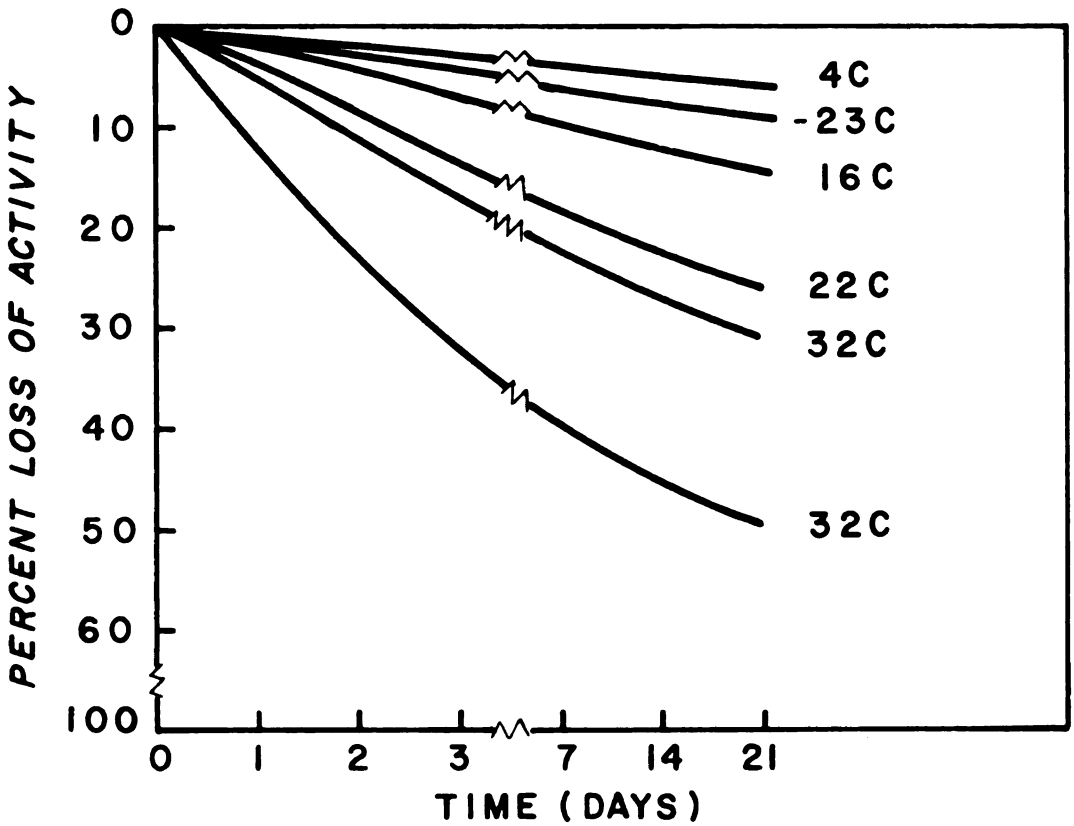

Fig. 9. Percent loss of activity of staphylococcal lipase when stored at various temperatures for the times indicated.

were performed using tris buffer pH 8.1, $\Gamma/2 = 0.1$ as the heating menstrum. The design of the apparatus used for thermal resistance studies was based on the unit devised by Malin (1953) and modified by Walker (1964). The enzyme solution was heated at 50, 55, 60, 65 and 70 C. The samples were withdrawn every 5 min for 30 min and were immediately cooled in pre-chilled tubes.

The lipase activity was determined for the heated aliquots and corresponding unheated controls and the percentage loss of activity in the heated samples was calculated. Fig. 10 shows a plot of the percent loss of activity vs time. The percent loss of activity was also plotted against the heating temperature and the graph is shown in Fig. 11. The enzyme lost 6% of the activity after exposure to 50 C for 30 min and 95% of the activity when heated at 70 C for 30 min.

Action on various natural fats and oils. The lipase activity of the enzyme preparation was tested on several oils and fats including olive oil, soybean oil, safflower oil, corn oil, cottonseed oil, butter oil, lard, turkey fat, beef tallow and hydrogenated soybean oil. The hydrogenated soybean oil was flaky and solid at room temperature and had an iodine value of less than three. Animal fat from the fresh tissue was extracted with chloroform-methanol (2:1). The ability of staphylococcal lipase to act on a wide variety of natural oils and fats is shown by the data in Table 22. The staphylococcal lipase did not show any preference for animal or vegetable fats or oils. It is noteworthy that the emulsion of hydrogenated soybean oil (iodine value < 3) was also hydrolyzed by this enzyme.

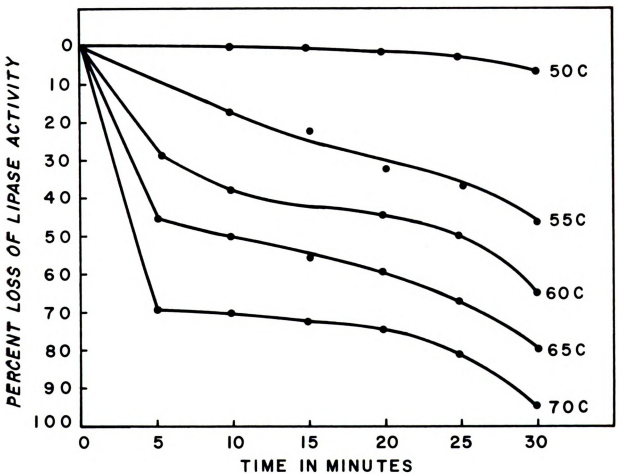

Fig. 10. Percent loss in the activity of staphylococcal lipase heated in tris buffer at pH 8.1 at the various times and temperatures indicated.

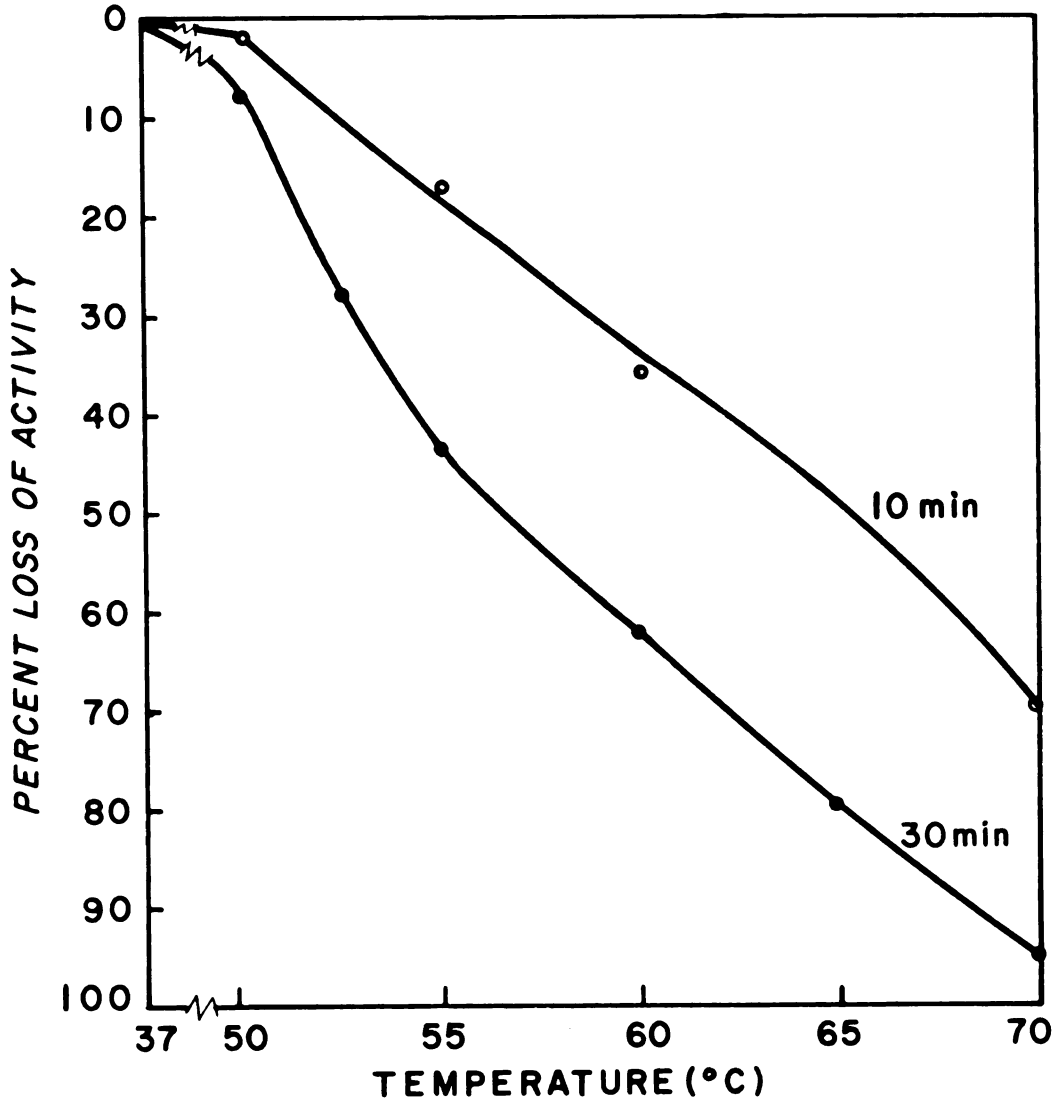

Fig. 11. Percent loss of staphylococcal lipase activity when heated in tris buffer at pH 8.1 at the various temperatures for the time indicated.

Table 22. Amount of free fatty acids liberated from various natural lipids by purified staphylococcal lipase (strain B-120). (Lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0.)

Lipid	ml of 0.01N NaOH required to titrate free fatty acids
Olive oil	4.10
Soybean oil	3.40
Safflower oil	4.25
Corn oil	4.20
Cottonseed oil	3.30
Butter oil	4.00
Lard	3.30
Turkey fat	3.80
Beef tallow	4.10
Hydrogenated soybean oil	1.40

Action of lipase on a synthetic triglyceride. The only synthetic triglyceride tested had palmitic, stearic and oleic acids at α, β, and α' positions. Since only a small quantity of this glyceride was available, the following procedure was adopted to prepare an emulsion. The following ingredients were added in a sonifier tube.

Synthetic triglyceride	50.0 mg
10% gum acacia solution	0.2 ml
0.075 M calcium chloride	0.2 ml
tris buffer pH 8.1	2.0 ml
Deionized water	5.0 ml

The tube was then placed in a water bath maintained at 65 C and the mixture sonified at 7-9 amps for 4 min. One milliliter of the enzyme preparation was added to the above emulsion and the reaction mixture incubated at 37 C for 1 hr. The hydrolyzed glycerides were extracted with N-pentane and analyzed for constituent fatty acids by gas chromatography. Thin layer chromatographic analysis was also performed at the end of the incubation period. The technique used was essentially as given in the standard texts of Bobbit (1963), Randerbath (1963) and Stahl (1965). The solvent used for development was ether, hexane, and acetic acid mixed in the proportion of 30:90:1, respectively. Concentrated sulfuric acid was used to develop the spots. The percentages of the three free fatty acids were as follows: palmitic, 40%; stearic, 21%; oleic, 39%. The analysis of hydrolyzed fat on thin layer chromatography showed only faint spots for mono and diglycerides, with triglycerides and free fatty acids predominating.

Effect of chemical agents. The effect of several chemical compounds on the lipase activity was determined using olive oil as the substrate. In each trial a control titration was performed on a substrate containing the enzyme but no additive. The percentage of stimulation or inhibition was calculated from the titration value obtained on the control. The assay for lipase was performed by adding 1 ml of the enzyme solution to the substrate mixture described previously.

The chemicals used in the experiments were mostly oxidizing agents (hydrogen peroxide), reducing agents (glutathione, cysteine and ascorbic acid), or agents which are known to effect -SH or S-S bonds (2-mercaptoethanol, iodoacetic acid, N-ethyl maleimide and p-chromomercuribenzoate). Certain antibacterial agents (formaldehyde, penicillin, streptomycin, aureomycin, terramycin, phenol and potassium cyanide) were also tested for their effect on lipase activity. The following chemical agents (control excluded) were used in the reaction mixture.

Hydrogen peroxide. The enzyme-substrate mixture was mixed with 0.1, 0.2, 0.3, 0.4, 0.5, 0.8 and 1.0 ml of a 35% hydrogen peroxide solution. The pH was adjusted to 8.0.

Formaldehyde. Quantities varying from 0.1 to 1.0 ml of 40% formaldehyde solution (formalin) were added to the enzyme-substrate mixture to study the effect on enzyme activity.

2-Mercaptoethanol. One-tenth, 0.2, 0.3, 0.4, 0.5, 0.8 and 1.0 ml of 2-mercaptoethanol were added to the enzyme-substrate mixture to determine the effect on lipase activity.

Glutathione, cysteine and ascorbic acid. A 5% solution

of each of the three components was prepared. Aliquots of 0.5, 1.0, 2.0, 3.0 and 5.0 ml were added to the enzyme-substrate mixture to determine the effect of enzyme activity.

Iodoacetic acid, N-ethylmaleimide and p-chloromercuribenzoate. The effect of these compounds on lipase activity was determined by adding 0.5, 1.0, 2.0, 3.0 and 5.0 ml of a 5% aqueous solution of each of these compounds to the reaction mixture.

Penicillin, streptomycin, aureomycin and terramycin. A 5% stock solution of each of these antibiotics was prepared in deionized water. One, 2, 3 and 5 ml of the antibiotic solution were added to the enzyme-substrate mixture to determine the effect of these antibiotics on enzyme activity.

Phenol and potassium cyanide. The effect of a 5% solution of these reagents was studied using 0.5, 1.0, 2.0, 3.0 and 5.0 ml of inhibitor in the reaction mixture.

The data in Table 23 indicate that the lipase activity was stimulated by hydrogen peroxide and inhibited by formaldehyde and 2-mercaptoethanol. The data in Table 24 show that reducing agents inhibited the lipase activity. Antibiotics had a variable influence with terramycin inhibiting activity, streptomycin and aureomycin stimulating activity, and penicillin causing no appreciable change. Chemical compounds such as iodoacetic acid, p-chloromercuribenzoate, phenol and potassium cyanide inhibited the lipase activity.

Free boundary electrophoresis. A 1% solution of the lyophilized enzyme preparation was prepared and dialyzed against veronal buffer at pH 8.6, $\Gamma/2 = 0.1$ for 12 hr with constant stirring at 4 C. The Tiselius electrophoresis apparatus, Model 38 manufactured by

Table 23. Effect of hydrogen peroxide, formaldehyde and 2-mercaptoethanol on lipase activity measured in olive oil emulsion for 10 min at 37 C and pH 8.0

	Quantities of chemical agent (ml)							
	0	0.1	0.2	0.3	0.4	0.5	0.8	1.0
Chemicals used	ml of 0.01N NaOH required to titrate free fatty acids							
Formaldehyde	4.4	3.4	2.1	1.8	1.6	1.2	0.8	0.1
Hydrogen peroxide	4.4	5.3	6.2	7.1	7.0	5.9	4.5	4.2
2-mercaptoethanol	4.5	3.6	2.7	1.8	1.0	0.0	0.0	0.0

Table 24. Effect of various chemicals on lipase activity of *S. aureus* measured in olive oil emulsion for 10 min at 37 C and pH 8.0

Chemicals used	Quantities of a 5% solution of chemical agents (ml)					
	0	0.5	1.0	2.0	3.0	5.0
	ml of 0.01N NaOH required to titrate free fatty acids					
Glutathione	3.0	2.3	0.4	0.0	0.0	0.0
Cysteine	3.0	1.3	0.4	0.0	-	-
Ascorbic acid	3.0	-	2.0	1.5	-	0.9
Iodoacetic acid	3.3	2.6	1.7	-	-	0.0
n-ethylmaleimide	3.1	3.3	3.0	3.1	3.3	3.1
p-chloromercuribenzoate	3.2	2.6	1.5	-	-	0.0
Penicillin	3.1	-	2.8	2.8	3.0	-
Streptomycin	3.0	-	3.3	4.2	5.7	6.6
Aureomycin	3.0	-	4.2	5.8	-	7.2
Terramycin	3.3	-	2.7	1.0	0.7	-
Phenol	3.2	2.8	2.3	-	-	1.4
Potassium cyanide	3.1	1.2	0.0	-	-	-

- not determined

Perkin-Elmer Corporation was used for analysis employing a 2 ml cell. The electrophoretic mobility (μ) was calculated using the following equation

$$\mu = \frac{d\ A\ K}{t\ I\ R\ m}$$

where d = the distance traveled in cm; A = the cross sectional area of electrophoretic cell; K = the conductivity cell constant; t = time in seconds; I = current in amps; R = resistance in ohms; and m = the magnification factor of the optical system. The initial and final pictures of free boundary electrophoretic analysis at the end of 3,000 seconds are shown in Fig. 12. The enzyme preparation showed a single peak at pH 8.6 using a veronal buffer system, and can be considered electrophoretically homogereous.

Sedimentation coefficient and molecular weight. The enzyme preparation was analyzed using a Spinco model E, analytical ultracentrifuge. The sedimentation velocity and sedimentation equilibrium were determined at 50,790 and 12,000 rpm, respectively. The calculations were made using the standard texts of Svedberg and Pedersen (1952), Schachman (1959) and Williams (1963).

The sedimentation velocity and molecular weight were determined using a 1% enzyme solution at 20 C and the sedimentation pattern is shown in Fig. 13. The sedimentation velocity determination (Fig. 13) suggests that the enzyme preparation is heteroger in regard to the size of the protein molecules. The electrophoretic mobility, sedimentation coefficient and molecular weight of the highly purified preparation are shown in Table 25.

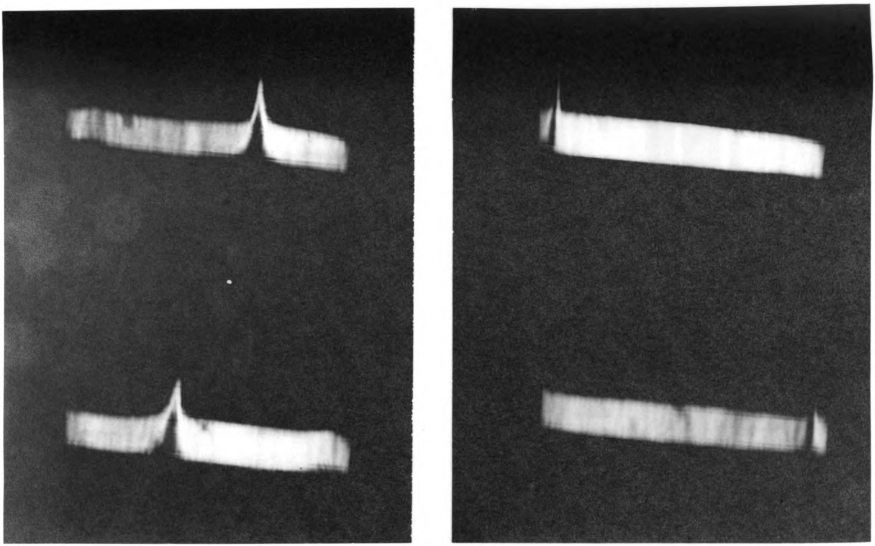

Fig. 12. Free boundary electrophoretic pattern of purified staphylococcal lipase in veronal buffer at pH 8.6, $\Gamma/2 = 0.1$ after 3,000 seconds of electrophoresis.

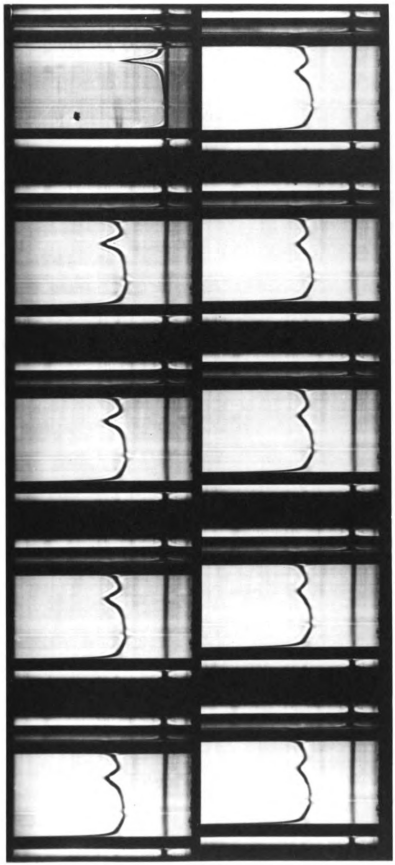

Fig. 13. Sedimentation velocity pattern of 1% lyophilized purified staphylococcal lipase solution in veronal buffer at pH 8.6, $\Gamma/2 = 0.1$ centrifuged at 50,790 rpm at 20 C.

Table 25. Physical characteristics of the purified staphylococcal lipase preparation in veronal buffer pH 8.6, $\Gamma/2 = 0.1$.

Physical characteristic	Value
Electrophoretic mobility	
Ascending	-7.78×10^{-5} cm^2/volt/sec
Descending	-8.12×10^{-5} cm^2/volt/sec
Sedimentation coefficient	
1st peak	2.85 Svedberg
2nd peak	8.50 Svedberg
Molecular weight	100,000

Chemical Composition of S. aureus Cells

S. aureus B-120 was grown in sterile trypticase soy broth with and without 3% glycerol for 24 hr at 37 C with continuous agitation. At the end of incubation the cells were removed by centrifugation and washed as previously described. The washed and packed cells were analyzed for moisture and then lyophilized for use in other experiments.

Determination of moisture. One gram of wet packed cells was weighed into an aluminum dish. The sample was then placed in a vacuum oven maintained at a temperature of 100 C. Dishes were weighed every 12 hr until a constant weight was obtained.

Determination of nitrogen. The lyophilized cells were dried to constant weight in a vacuum oven at 100 C. The dried cells were analyzed for nitrogen using the microkjeldahl procedure described in Official Methods of Analysis of the Association of Official Agricultural Chemists (AOAC), (1960). The percentage of protein was calculated from the percent nitrogen using a factor of 6.25.

Determination of phosphorus. The total phosphorus present in the dried cells was determined by the method of Sumner (1944). The cells were digested with 10N H_2SO_4 for 45 min at approximately 130 C. Complete oxidation was achieved by the addition of hydrogen peroxide. Excess hydrogen peroxide was avoided since it interferes with color development.

Determination of ash. The direct ashing procedure as recommended by AOAC (1960) was used. The cells were weighed into an ashing dish that had been previously ignited and cooled. Ignition was

performed in a muffle furnace at 550 C for 24 hr.

Determination of total lipids. The lyophilized cells were analyzed for total lipids by the following procedures. The cells were hydrolyzed with 6N HCl at 100 C for several hours followed by extraction with N-pentane. Five grams of cells were heated with 100 ml of absolute methanol for 15 min at 65 C. The mixture was brought to room temperature and stirred with 200 ml of chloroform for 20 min. The mixture was then filtered through a fine sintered glass disc funnel. The residue was again subjected to the above extraction procedure with chloroform-methanol. This method of extraction is essentially similar to one given by Vorbeck and Marinetti (1965). The combined solvents were evaporated and the crude lipid extract dissolved in N-pentane. The water soluble material was removed by the method of Folsh et al. (1957). The lipids were either analyzed immediately or stored at -20 C for not over 2 weeks.

The data on the nitrogen, phosphorus, ash, total lipids and moisture content of S. aureus B-120 are shown in Table 26. The data presented show no significant difference in the chemical composition of the cells grown with and without glycerol.

Separation of neutral and phospholipids. The total lipid extract was separated into phospho and neutral lipids using silicic acid as recommended by Hanhan (1960). The silicic acid slurry was poured into a sintered glass disc funnel. The sample was poured on to the silicic acid and eluted with 250 ml of chloroform. Subsequently, the phospholipid fraction was eluted with 250 ml of absolute methanol. The counter-current principle of Galanos and Kopoulas (1962) was also

Table 26. Nitrogen, phosphorus, ash, total lipids and moisture content of cells of S. aureus strain B-120 grown as indicated for 24 hr at 37 C

Chemical component	Cells grown in trypticase soy broth	Cells grown in trypticase soy broth +3% glycerol
	Percent	
Nitrogen	12.4	12.6
Phosphorus	4.5	4.2
Ash	13.1	12.9
Total lipids		
a) 6N HCl hydrolysis	4.2-4.8	4.6-5.2
b) Extraction method	3.9-4.5	4.4-5.1
Moisture	78-82	81-82

used to isolate the polar and nonpolar lipids from crude extract.

The crude lipid fraction obtained by the solvent extraction method of Vorbeck and Marinetti(1965) was separated into phospho and neutral lipids. The results obtained with the silicic acid method and countercurrent distribution method of separation are shown in Table 27. The results obtained with the two methods were similar.

Thin layer chromatograph of neutral and phospholipids. Thirty grams of silica gel G were suspended in 60 ml of water and the slurry was applied to precleaned 20x20 cm glass plates in 0.5 mm thickness using the Research Specialties Co. (RSCo) applicator. The plates were allowed to dry overnight at room temperature and activated at 100 C for at least 2 hr. Five to 10 mg of the sample dissolved in benzene were applied by means of a capillary tube about 2.5-4.0 cm from the edge of the plate. The weight of the sample applied was calculated by the difference between the initial and final weight of the total sample. Prior to weighing, the solvent was dried in a vacuum oven at 40 C for 2 hr. Initially the split plate technique was tried for quantitation in which one-fourth of the plate was sprayed for spotting and the other three-fourths of the plate was used for quantitation. Later, the split plate technique was discarded in preference to monitoring the plates by ultraviolet (UV) lamp. The results obtained by UV diagnosis were confirmed by spraying the plates with sulfuric acid in case of neutral lipids, and the color reagent of Ditmer and Lester (1964) in case of phospholipids. After the efficacy of UV monitoring was established, this method was used exclusively for identification of both phospho

Table 27. Phospho and neutral lipid composition of the lipids of S. aureus strain B-120 using the silicic acid and counter-current distribution methods of separation

Separation method	Phospholipids	Neutral lipids
	Percent	
Silicic acid	74-81	21-27
Counter-current distribution	72-78	24-29

and neutral lipids. Once the identification was established, the silica gel strips containing the sample were stripped from the plate and washed with chloroform when determining phospholipids, and with diethyl ether when determining neutral lipids. The silica gel and the solvent were passed through an ultrafine porosity sintered glass disc funnel and the solvent evaporated under vacuum at a temperature somewhat below the boiling point of the solvent.

Analysis of neutral lipids. The neutral lipids were separated into six component fractions by thin layer chromatography as described above. The solvent mixture used for development contained hexane, diethyl ether and acetic acid in the ratio of 79:21:1, respectively. The quantities of the various fractions were determined using a gravimetric procedure. The various fractions were dissolved in a definite volume of the solvent. An aliquot or the whole amount was evaporated in a teflon cup at 40 C until completely dry. The cups were then put in a vacuum oven at 40 C for 1 hr. At the end of this period the samples were weighed using a Cahn electrobalance and the percentage of each fraction calculated. The data obtained are shown in Table 28.

Analysis of phospholipids. The procedure used was similar to that described under neutral lipids, except that the percentage of each fraction was calculated on the basis of the amount of phosphorus. The phosphorus analysis was performed using the method of Sumner (1944). Table 29 summarizes the data obtained.

Analysis of fatty acids. The fatty acid composition of the phospho and neutral lipids was determined using the method of McGinnis and Dugan (1965). An F and M gas chromatograph was used for

Table 28. Composition of the neutral lipid fraction extracted from S. aureus strain B-120 when separated into various components as indicated using thin layer chromatography

Component	Percent
Free fatty acids	3-7
Monoglycerides	15-20
Diglycerides	42-48
Triglycerides	12-18
Cholesterol	16-20
Cholesterol esters	8-12

Table 29. Composition of the phospholipid fraction extracted from S. aureus strain B-120 when separated into various components as indicated using thin layer chromatography

Component	Percent
Lecithins	46-52
Cephalins	28-32
Sphingomyelins	18-20

separation of fatty acids. The temperature used for analysis was 200 C. The gas chromatograms of the two fractions are shown in Figs. 14 and 15, respectively. The two components appear to have similar fatty acid composition.

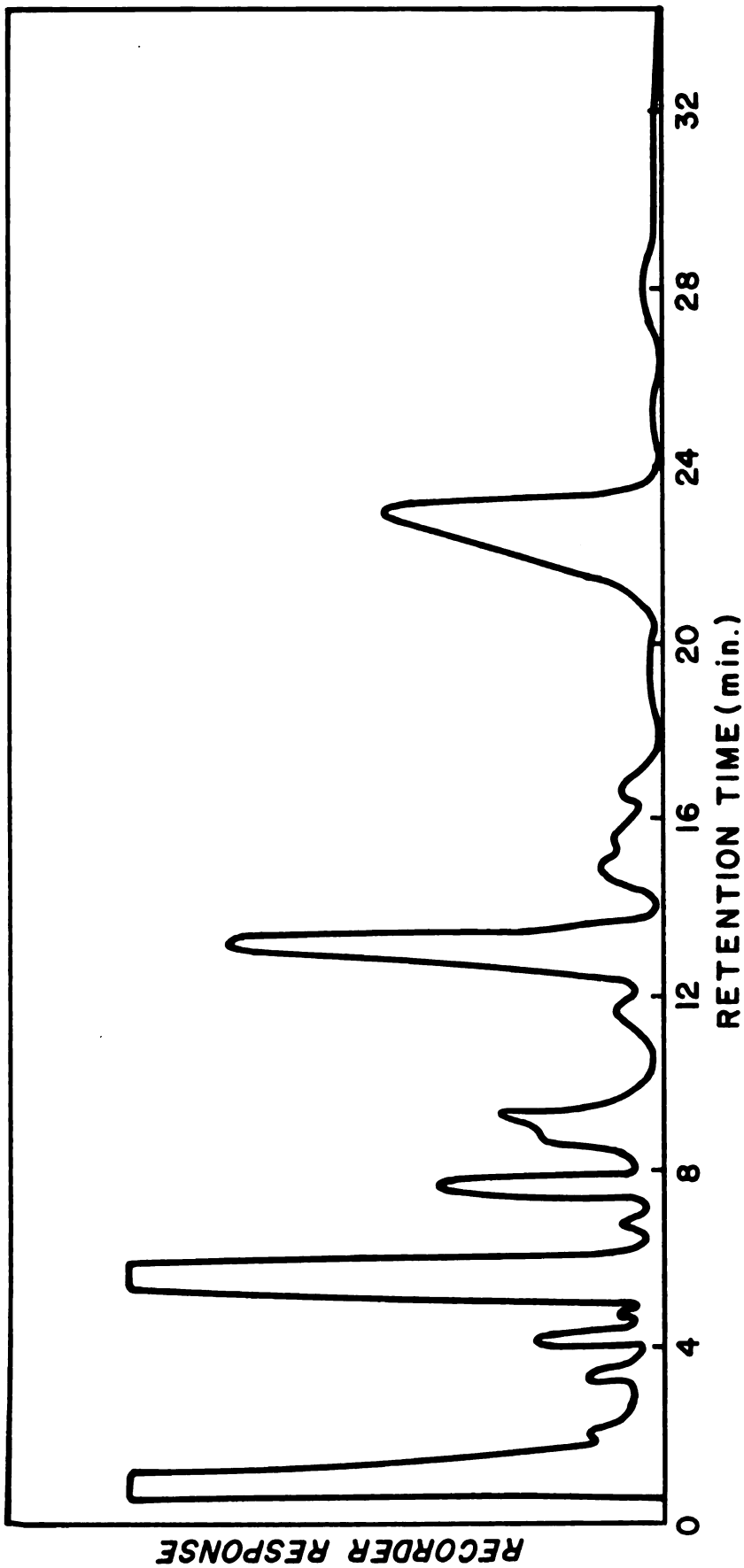

Fig. 14. The gas chromatographic pattern of methyl esters of fatty acids of phospholipids extracted from cells of S. aureus B-120.

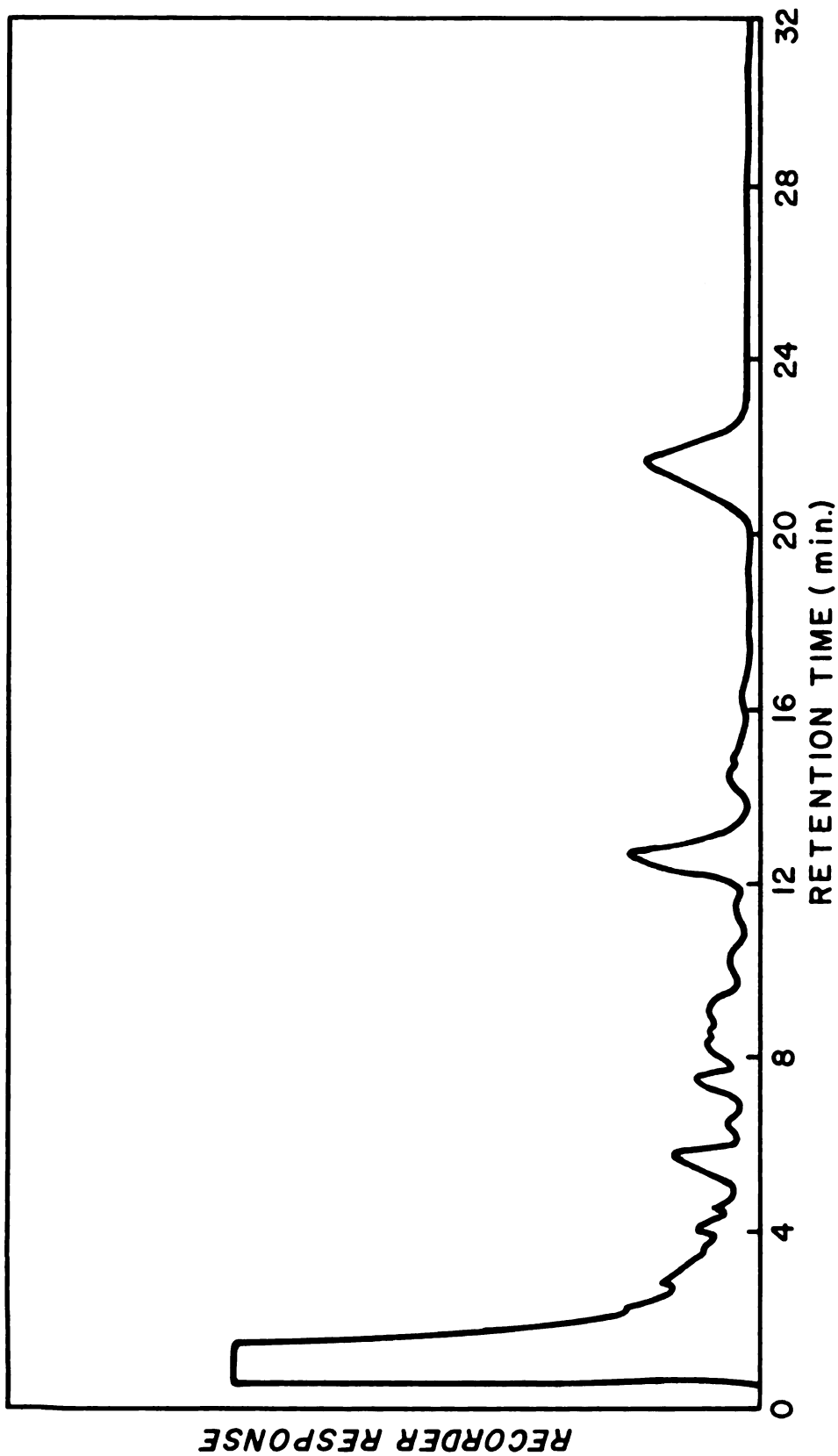

Fig. 15. The gas chromatographic pattern of the methyl esters of fatty acids of neutral lipids extracted from cells of S. aureus B-120.

DISCUSSION

The two strains of S. aureus used in this study were similar in their growth and pH pattern when inoculated into cereal milk. At 30 C a population of approximately 2.3 billion was reached in about 2 days while 7 days were required to reach similar populations at 15 and 22 C. The pH of the inoculated cereal milk did not change appreciably during incubation at 15 C for 8 days. The samples incubated at 22 C showed a slight decrease in pH while at 30 C the pH decreased approximately one unit during 8 days of incubation.

The lipolytic enzyme system of S. aureus was active on milk fat as indicated by the increase in free fatty acids during incubation of inoculated samples. This enzyme system appears to be extracellular since appreciable lipolysis occurred during the first day of incubation at 30 C. At 15 and 22 C the lipolysis was slower but showed a progressive increase during incubation. Presumably autolysis is minimal during the first 24 hr and lipolysis during that period is due to an extracellular enzyme. However, the intracellular lipases, if present, could contribute to the lipolytic activity. The autolysis and release of lipase might be a factor after the cells go into the stationary or death phase and previous work done by Walker (1964) suggests that the death phase starts after about 3-4 days of incubation at 30 C with the strains used in this study.

There was greater lipolysis by the cells grown in cereal milk, than by those grown in broth, when inoculated into cereal milk. The

potency of the lipase(s) of S. aureus is further established from the calculations based on the total amount of fatty acid liberated. The maximum amount of free fatty acid liberated was 0.48 g per g of fat in the inoculated cereal milk when incubated at 30 C for 8 days. The lipid portion of milk contains approximately 85% fatty acids which are bonded to glycerol. Calculations based on the total amount of fatty acids present indicate that about 56.4% of the milk fat in cereal milk was hydrolyzed by S. aureus during 8 days of incubation at 30 C. The gas chromatographic analysis of the lipolyzed milk fat showed that the fatty acid composition was similar to the hydrolyzed milk fat. Milk fat contains an average of about 32% oleic acid and 20% palmitic acid as reported by Jenness and Patton (1959). The data summarized in Table 5, however, show a higher percentage of palmitic acid than any other fatty acids which suggests that the lipase from S. aureus has a preference for the palmitic acid-glycerol bond. These data, however, did not include results of analysis involving short chain fatty acids. The volatile fatty acids were determined (Table 6) but the method used for their determination did not differentiate the various individual acids. No correlation can be made between volatile acidity and fatty acids determined by gas chromatography. In fact, the volatile acidity values include acetic, propionic, butyric, caproic, caprylic and a substantial portion of C_{10}, C_{12} and C_{14} fatty acids. Kosikowski and Dahlberg (1946) have estimated that approximately 79, 46 and 16% of the C_{10}, C_{12} and C_{14} acids, respectively, are included in the determination used for volatile acids.

Gas chromatographic analysis was performed on the total lipids

extracted from cereal milk inoculated with S. aureus. The possibility exists that lipids from autolyzed cells of S. aureus may have contributed minor amounts of fatty acids to the values reported in Table 5, particularly in samples withdrawn toward the end of the incubation period and at the higher temperatures. Analyses showed that S. aureus cells contain about 4% lipid on a dry weight basis; therefore, the amount of free fatty acids contributed by the autolyzed cells of S. aureus is very small, if any.

The inhibitory effect of the C_8, C_{10} and C_{12} acids on the growth of S. aureus is apparently bacteriostatic rather than bactericidal. Costilow and Speck (1951) also reported that these fatty acids in concentrations of 0.05-0.10% were inhibitory to Streptococcus lactis. It would appear that these fatty acids are general inhibitory agents and their exact mode of action may be of great interest. The conclusion that these acids are bacteriostatic is supported by the fact that increases in population frequently followed periods of inhibition. The exact mode of action of fatty acids in functioning as inhibitors is speculative. The fatty acids could accomplish inhibition by reducing the surface tension, or coating the bacterial surfaces. Also, they could selectively attack the permease, thus blocking the flow of nutrients and/or preventing the release of extracellular enzymes. The inhibition of S. aureus or inactivity of the extracellular enzyme is evidenced by a decrease in proteolysis, negative coagulase test, and stability of pH.

The decrease in proteolysis in the presence of C_4 to C_{10} fatty acids and the increase in proteolysis caused by stearic and oleic

acid suggests that the influence of these acids is related to the activity or amount of the enzyme systems. This decrease or increase in proteolysis was independent of population.

The lack of lipase activity in the cell-free supernatant from still cultures of S. aureus B-120 and the presence of lipase in the agitated cultures suggests that agitation increases lipase production. The presence of lipase in the cell-free supernatant from agitated culture is probably due to the effect of incorporated air on the cells. The cell-free supernatant from cultures grown under vacuum and agitation did not show any lipase activity indicating that the lipase production (release) is not due to a mechanical effect alone. However, the cultures supplied with a constant stream of air but not mechanically agitated showed considerable lipase activity. The cells showed no evidence of an ectocellular lipase, since lipolytic activity was not detectable in the washings obtained from the packed cells when subjected to agitation in the Waring blender with normal saline for 2 min. Also, the intracellular milieu lacked lipase activity. Thus it appears that the lipase is an extracellular enzyme, the production of which seems to be related to the air supply in the growth medium. The lipase activity of the cell-free supernatant was greater when agitation was achieved with a reciprocating shaker as compared to a rotary shaker. This difference is probably due to the greater amount of air incorporated into the medium by the former method. The amount of lipase activity in the cell-free supernatant was influenced by the age of the cells. At 37 C the activity increased as the incubation progressed during the first 5 days. A slight decrease occurred

on the 6th and 7th days. Possibly during the later stages of incubation the autolysis of the cells and the release of intracellular enzymes contributed to the total lipase activity. However, this does not seem plausible since no intracellular lipase activity was detected at the end of 24 hr of incubation. It is still possible that the intracellular enzymes may have been produced and released during the later stages of incubation.

The lipase from the cell-free supernatant was easily precipitated by several methods such as ammonium sulfate, alcohol, acetone and pH adjustment. Most of the reagents had an optimum concentration for maximum lipase activity. Any further increase in the concentration of the salt or the solvent decreased the specific activity of the enzyme, presumably due to protein denaturation or to precipitation of nonenzymic protein. Precipitation of the cell-free supernatant with equal volumes of absolute ethanol was the preferred method in this work since it completely removed the lipase from the cell-free supernatant and also yielded maximum specific activity. The precipitate so obtained was flocculent and settled out after several hours. The precipitate obtained at pH 4.3 was highly active (200 units/mg of protein), while the supernatant was almost devoid of activity. This enzyme could be precipitated repeatedly at pH 4.3 indicating that this pH may be its isoelectric point. Desnuelle (1961b) reported that the isoelectric point of pancreatic lipase was pH 5.2. The precipitate at pH 4.3 was further purified using molecular sieving techniques. The two fractions obtained using Sephadex G-200 were distinctly different. Most of the activity was concentrated in the first fraction which

suggests that this enzyme is a high molecular weight protein. A plot of protein concentration and lipase activity against tube numbers (fraction numbers) shows that there is a tailing of the first peak, indicating a heterogeneous material. However, this tailing was minimized when the fractions from the first peak were concentrated and rechromatographed using BioGel 300. The plot shows a straight line relationship between protein concentration and enzyme activity which is indicative of a homogeneous preparation. No further purification was attempted. This purified lipase was tested for its homogeneity by several other methods. Free boundary electrophoresis analysis of this preparation in veronal buffer, pH 8.6, $\sqrt{\ }/2 = 0.1$ indicates this preparation is electrophoretically homogeneous. An analysis for sedimentation velocity of the preparation showed two components, a major component and a rapidly sedimenting minor component which were approximately in 95 and 5% concentrations, respectively. The identity of either of these components as a lipase has not been established unequivocally. It is possible that the minor component may be some of the acrylamide particles which might have passed through the sintered glass disc during column chromatography.

The purified lipase had an optimum pH of 8.6; however, it showed activity over a pH range 6-12. The optimum pH is similar to the optimum for pancreatic lipase (pH 8-9) as reported by Börgstrom (1957). An optimum of pH 9.0-9.2 has been reported for milk lipase by Chandan and Shahani (1964). Shah and Wilson (1965) reported an optimum pH of 8.2 for the egg yolk factor which they claim was a lipase. The optimum temperature for the action of the staphylococcal

lipase was determined to be 45 C and the enzyme was active over a wide temperature range. The optimum temperature for the activity of milk lipase as reported by Chandan and Shahani (1963a) is approximately 37 C despite the fact that a considerable amount of enzyme is inactivated at this temperature.

The crude staphylococcal lipase preparation and the purified lipase appear to be very stable at various storage temperatures used in these experiments. The cell-free supernatant lost less than 10% of the original activity when kept frozen for more than 6 months. The purified enzyme lost 7% of the activity after 21 days of storage at 4 C. When stored at 37 C the enzyme lost only 12% of the activity during the first 24 hr and about 50% of the activity at the end of 21 days. These results are in contrast to results reported by Chandan and Shahani (1963b) who observed a complete loss of activity of milk lipase within 24 hr at 37 C. Besides showing a high degree of stability during storage, the staphylococcal lipase is very heat resistant. For example, heating at 50 C for 30 min decreased the activity of the enzyme only 6% and it lost 46, 65 and 80% of its activity when heated for 30 min at 55, 60 and 65 C, respectively. The high degree of thermal stability raises the question whether some of the rancidity observed in pasteurized dairy products could be caused by residual enzyme activity.

The enzyme did not show any particular preference for different substrates. All the natural oils and fats tested were hydrolyzed to a similar extent with the exception of hydrogenated soybean oil (iodine value < 3) in which case the activity was reduced

by two-thirds. This enzyme when tested for activity on a palmitic, stearic and oleic acid synthetic triglyceride was found to hydrolyze the fatty acids in all the three positions α, β and α'. This is in contrast to pancreatic lipase which attacks α and α' positions only. These observations on staphylococcal lipase were similar to the ones reported by Alford et al. (1964). Even though staphylococcal lipase attacks all three positions there appears to be slight preference for the α and α' positions as compared to the β position, the ratios being 40:21:39 for α, β and α' positions, respectively. Since the enzyme substrate was incubated for a period of 1 hr there could be some acyl migration. Another important fact about the enzyme appears to be the "all or none" phenomenon; that is if the enzyme attacks a triglyceride then that triglyceride will be completely digested into its constituent fatty acids.

The enzyme activity was inhibited by reducing agents while oxidizing agents stimulated the activity. Mercaptoethanol, cysteine and glutathione inhibited the activity while hydrogen peroxide stimulated the activity. Perhaps -SH groups are not required by the enzyme or possibly the disulfide bonds are necessary for enzyme action. Similar results were reported by Wills (1960) on pancreatic lipase. However, Chandan et al. (1963) believe that milk lipase is dependent on the sulfhydral group for the activity. Whether the disulfide bonds are involved or present in staphylococcal lipase has not been determined.

The action of antibiotics on staphylococcal lipase was varied. Penicillin and terramycin inhibited the activity while streptomycin and aureomycin had a stimulatory effect. The exact mode of action

or the nature of inhibition or stimulation is not known. These observations on the effect of the antibiotics on staphylococcal lipase are in contrast with the work of Shahani and Chandan (1962) who reported that all of these antibiotics had an inhibitory effect on milk lipase.

Under the experimental conditions employed for sedimentation equilibrium analysis, staphylococcal lipase had a molecular weight of 100,000. The S_{20} of the enzyme preparation was 2.85 and 8.5 for the two components. Assuming a globular protein and using the equation $S = M^{2/3} (3.29 \times 10^{-16})$, a sedimentation coefficient of approximately 7.0 is required for a molecule with molecular weight of 100,000. The molecular weights corresponding to S_{20} of 2.85 and 8.5 are 28,000 and 130,000, respectively, which suggests that the enzyme may not be a globular protein. The elution properties of the enzyme with molecular sieving technique using Sephadex G-200 and BioGel 300 definitely suggest that the enzyme is a high molecular weight entity.

S. aureus cells have approximately the same chemical composition as other bacterial cells. Addition of 3% glycerol to the growth medium did not change the chemical composition of the cells, except for a slight increase in the total lipid content. However, this is in contrast to the results reported by Larson and Larson (1922) in which they found five-fold increase in the lipid content of S. albus when grown in the presence of glycerol.

Macfarlane (1962) reported that S. aureus cells contain 25 mg of lipids per g of dry weight and this value is slightly lower than

the value of 40 mg/g obtained in this study. The lower value obtained by Macfarlane may be due to the solvent system used for extraction. The method of extraction has a definite influence on the amount of lipid extracted from the cells. The data reported here showed that hydrolyzing the cells with 6N HCl gave a higher amount of lipids than heating the cells with methanol followed by extraction with chloroform. The extent of heating of the cells in methanol also had a definite influence on the amount of extractable lipids. As the length of heating time increased up to 30 min the amount of lipid recovered increased. Macfarlane (1962) reported a ratio of 1 part neutral lipid to 5 parts of phospholipids in S. aureus cells. The lipid fraction of the S. aureus cells examined in the work reported herein contained 74-81% phospholipids and 21-27% neutral lipids. A high concentration (42-48%) of diglycerides was present in the neutral lipid fraction. This confirms the data of Macfarlane (1962) who reported 51% of diglycerides in the neutral lipids extracted from S. aureus.

The phospholipid fraction contained 46-52% lecithin, 28-32% cephalin and 18-20% sphingomyelin. The fatty acid composition of the phospho and neutral lipids was similar.

SUMMARY

Two coagulase positive strains of Staphylococcus aureus, B-120 and S-1, were used to determine the effect of a) their growth on the milk fat, b) added fatty acids on the growth of the cells, and c) the effect of fatty acids on the activity of enzymes produced by the cells. The lipase liberated from S. aureus strain B-120 was isolated, purified and characterized. The lipid composition of this strain was also determined.

Approximately 10^6 cells per ml grown in either a) cereal milk or b) trypticase soy broth were inoculated into sterile cereal milk. The inoculated medium was incubated at 15, 22 and 30 C for a maximum of 8 days.

The effect of added fatty acids on growth was determined using sterile reconstituted skimmilk as the substrate. Saturated fatty acids C_4 through C_{18} and oleic acid were added at 0.01, 0.05 and 0.1% concentrations.

The two strains of S. aureus showed similar results regarding pH, population and acid degree value. The cells grown in trypticase soy broth showed lower lipolytic activity than those grown in cereal milk. Maximum lipolytic activity was observed at 30 C and increased with time, with 0.48 g of free fatty acids being liberated per g of fat at the end of 8 days of incubation at 30 C. Palmitic acid was the most predominant fatty acid liberated by the lipase(s) of S. aureus. There was a progressive decrease in triglycerides with only minor changes in mono and diglycerides as well as cholesterol. The fatty acids which caused partial or complete inhibition

of the growth of S. aureus were C_8 and C_{10} at 0.05 and 0.10% concentrations. The samples showing inhibition were also negative for coagulase activity, peptonization and change in pH. The fatty acids varied in their effect on liberation of nonprotein nitrogen. The addition of oleic and stearic acid to sterile skimmilk inoculated with S. aureus caused an increase in the amount of nonprotein nitrogen while the C_4 to C_{12} acids caused a decrease in protease activity.

The isolation, purification and characterization of the lipase of S. aureus was studied using strain B-120. Several factors influenced the production of lipase by S. aureus cells. Agitation of the cultures during incubation increased the lipolytic activity of the cell-free supernatant as compared to unagitated cultures which did not show any activity. The addition of Tweens, glycerol and oils to trypticase soy broth did not increase the lipase activity of unagitated cultures but the incorporated lipids were hydrolyzed. Maximum lipase activity was observed when the cells were 5 days old and the pH of the medium was 8.0 or 9.0. Precipitation of lipase from the cell-free supernatant was best achieved with equal volumes of absolute ethanol. Further purification was achieved using differential pH precipitation at 8.6 and 4.3. The final purification was achieved with Sephadex G-200 and BioGel 300. The final purified preparation showed a 350-450 fold increase in specific activity as compared to the cell-free supernatant. The preparation showed a single peak on a Tiselius free boundary electrophoretic pattern with a mobility of -7.78×10^{-5} cm/volt/sec. The ultracentrifugal analysis for sedimentation velocity indicated that the purified preparation

had two components; a major component with S_{20} = 2.85 and a minor, fast moving component with a S_{20} = 8.5. The molecular weight of the preparation was determined using sedimentation equilibrium data and an approximate value of 100,000 was obtained. The enzyme preparation was active over a wide range of pH and temperature, with optima being 8.5 and 45 C, respectively. A storage temperature of 4 and -23 C was found to be superior for preserving the enzyme activity of the purified preparation. In general there was a decrease in activity as the storage temperature increased. The enzyme was relatively heat stable. Less than 10% of the activity was lost when exposed to 50 C for 30 min; however, a 30 min exposure at 70 C destroyed 95% of the activity. The enzymes were active on all the lipid substrates tested and the activity was similar in all cases except the hydrogenated soybean oil where activity was reduced to one-third. The gas chromatographic analysis of enzyme degraded synthetic triglyceride indicated that the enzyme was capable of attacking the α, β, and α' positions. The effect of several chemicals tested on lipase activity was varied. Formaldehyde, mercaptoethanol, cysteine, glutathione and terramycin were inhibitory while hydrogen peroxide, streptomycin and sodium taurocholate had a stimulatory effect.

The chemical composition of S. aureus B-120 was determined on 24 hr old cells which were grown in trypticase soy broth with continuous agitation at 37 C. The cells were washed and lyophilized prior to analysis. The cells contained 12.4% nitrogen, 4.5% phosphorus, approximately 4% lipids, 78% moisture and 13% ash. The total lipids were separated into phospho and neutral lipids using

silicic acid. Both phospho and neutral lipids were further fractionated into the constituent parts using thin layer chromatography and the total fatty acid composition determined by gas chromatography. Analysis of neutral lipids showed diglycerides to be the predominant fraction approximating 45% while free fatty acids represented only 5%. Lecithins, cephalins, and sphingomyelins were approximately 50, 30 and 20%, respectively, of the total phospholipids. Both phospho and neutral lipids had similar fatty acid composition.

LITERATURE CITED

Alder, V. G., W. A. Gillespie, and G. Herdan. 1953. Production of opacity in egg yolk broth by staphylococci from various sources. J. Pathol. Bacteriol. 66:205-210.

Alford, J. A., D. A. Pierce, and F. G. Suggs. 1964. Activity of microbial lipases on natural fats and synthetic triglycerides. J. Lipid Res. 5:390-394.

American Public Health Association. 1960. Standard Methods for the Examination of Dairy Products. 11th ed. American Public Health Association, New York.

Anderson, R. J. 1943. Lipids of Mycobacterium tuberculosis. Yale J. Biol. Med. 15:311-316.

Angelotti, R., M. J. Foter, and K. H. Lewis. 1960. Time-temperature effects on salmonella and staphylococci in foods. II. Behavior of warm holding temperatures, thermal death studies. Tech. Rep. F60-5. R. A. Taft Sanitary Engineering Center, U.S. Public Health Service, Cincinnati, Ohio.

Association of Official Agricultural Chemists. 1960. Official Methods of Analysis. 9th ed. Association of Agricultural Chemists, Washington 4, D. C.

Bier, M. 1955. Lipases, p. 627-642. In S. P. Colwick and N. O. Kaplan [ed]. Methods in Enzymology. Vol. 1. Academic Press, Inc., New York.

Blobel, H., D. B. Shah, and J. B. Wilson. 1961. Serological studies of the egg yolk factor isolated from coagulase positive staphylococci. J. Immunol. 87:285-289.

Bobbit, J. M. 1963. Thin Layer Chromatography. Reinhold Publishing Co., New York.

Boissonnas, R. A. 1948. Esterase II. Purification partielle de la lipase de porc. Helvetica. Chim. Acta. 31:1577-1583.

Borgström, B. 1957. Determination of pancreatic lipases in human small intestinal content. Scandinav. J. Clin. Lab. Invest. 9:226-229.

Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's Manual of Determinative Bacteriology. 7th ed. Williams and Wilkins, Baltimore, Maryland.

Burns, J., and D. F. Holtman. 1960. Biochemical properties of virulent and avirulent staphylococci. Ann. N. Y. Acad. Sci. 88:1115-1124.

Busta, F. F., and J. J. Jezeski. 1963. Effect of sodium chloride concentration in an agar medium on growth of heat shocked Staphylococcus aureus. Appl. Microbiol. 11:404-407.

Carroll, K. K. 1961. Separation of lipid classes by chromatography on florisil. J. Lipid Res. 2:135-141.

Chandan, R. C., and K. M. Shahani. 1963a. Purification and characterization of milk lipase. I. Purification. J. Dairy Sci. 46:275-283.

Chandan, R. C., and K. M. Shahani. 1963b. Purification and characterization of milk lipase. II. Characterization of the purified enzyme. J. Dairy Sci. 46:503-509.

Chandan, R. C., and K. M. Shahani. 1964. Milk lipases. A Review. J. Dairy Sci. 47:471-480.

Chandan, R. C., K. M. Shahani, R. H. Hill and J. J. Scholz. 1963. Purification and characterization of milk lipase. III. Sedimentation coefficient, diffusion coefficient and molecular weight. Enzymolgia 26:87-95.

Chapman, G. H., C. Berens, and M. H. Stiles. 1941. The coagulation of plasma by staphylococci. J. Bacteriol. 41:431-435.

Coleman, M. H. 1963. A rapid lipase purification. Biochem. Biophys. Acta. 67:146-147.

Cook, D. J., and M. Woodbine. 1958. The reduction of the biochemical oxygen demand of whey by controlled mould growth. J. Appl. Bacteriol. 21:vii (Abstr.)

Costilow, R. N., and M. L. Speck. 1951. Inhibition of Streptococcus lactis in milk by fatty acids. J. Dairy Sci. 34:1104-1110.

Davies, D. M. 1954. A study of diffusible lipase produced by staphylococci and of its immunological activity. J. Gen Microbiol. 11:37-44.

Desnuelle, P. 1961a. Pancreatic lipase. A Review. Advances in Enzymology. 23:129-161.

Desnuelle, P. 1961b. The Enzymes of Lipid Metabolism. Pergamon Press, New York.

Ditmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin layer chromatograms. J. Lipid Res. 5:126-127.

Dunkley, W. L., and L. M. Smith. 1951. Hydrolytic rancidity in milk. IV. Relationship between tributyrinase and lipolysis. J. Dairy Sci. 34:940-947.

Flodin, P. 1962. Dextran gels and their application in gel filtration. Pharmacica. Uppsala.

Folsh, J. M., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Foster, T. L., C. Jensen, and E. Plath. 1955. A photometric method for estimating the lipase content of milk. J. Dairy Sci. 38: 764-774.

Fukumoto, J., M. Iwai, and Y. Tsujisaka. 1963. Studies on lipase. I. Purification and crystallization of a lipase secreted by Aspergillus niger. J. Gen. Appl. Microbiol. 9:353-361.

Galanos, D. S., and V. M. Kapoulas. 1962. Isolation of polar lipids from triglyceride mixtures. J. Lipid Res. 3:134-136.

Gillespie, W. A., and V. G. Alder. 1952. Production of opacity in egg yolk media by coagulase positive staphylococci. J. Pathol. Bacteriol. 64:187-200.

Goldman, M. L., and M. M. Rayman. 1952. Hydrolysis of fats by bacteria of the Pseudomonas genus. Food Res. 17:326-337.

Greenbank, G. R., and P. A. Wright. 1950. A colorimetric determination of lipase. J. Dairy Sci. 33:396 (Abstr.)

Hanhan, D. J. 1960. Lipide Chemistry. John Wiley and Sons, Inc., New York.

Hornstein, I., J. A. Alford, L. E. Elliot and P. F. Crowe. 1960. Determination of free fatty acids in fat. Anal. Chem. 32: 540-542.

International Union of Biochemistry. 1961. Report of the commission on enzyme nomenclature. p. 104. Pergamon Press, New York.

Iwai, M., Y. Tsujisaka, and J. Fukumoto. 1964a. Studies on lipase II. Hydrolytic and esterifying actions of crystalline lipase of A. niger. J. Gen. Appl. Microbiol. 10:13-22.

Iwai, M., Y. Tsujisaka, and J. Fukumoto. 1964b. Studies on lipase III. Effect of calcium ion on the action of crystalline lipase of A. niger. J. Gen. Appl. Microbiol. 10:87-93.

Jenness, R., and S. Patton. 1959. Principles of Dairy Chemistry. John Wiley and Sons, Inc., New York.

Jensen, R. G., J. Sampugna, J. G. Quinn and D. L. Carpenter. 1965. Partial analysis of milk triglycerides with aid of a lipase system highly specific for unsaturated fatty acids. J. Dairy Sci. 48:767. (Abstr.)

Kates, M. 1964. Bacterial lipids. p. 17-90. In P. Faoletti and D. Kritchevsky [ed]. Advances in Lipid Research. Vol. 2. Academic Press, Inc., New York.

Khan, J. M., R. C. Chandan, C. W. Dill, and K. M. Shahani. 1964. Production and properties of extracellular lipase of Achromobacter lipolyticum. J. Dairy Sci. 47:675. (Abstr.)

Kosikowski, F. V., and A. C. Dahlberg. 1946. A rapid distillation method for determining the volatile fatty acids in cheese. J. Dairy Sci. 29:861-871.

Larson, L. W., and Larson, W. F. 1922. Factors governing the fat content of bacteria and the influence of fat on pellicle formation. J. Infec. Diseases. 31:407-415.

Long, C. 1961. Biochemists Handbook. D. VanNostrand Co., Inc. Princeton, New Jersey.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Luria, S. E. 1960. The bacterial protoplasm: composition and organization. p. 1-34. In J. C. Gunsalus and R. Y. Stanier [ed]. The Bacteria. Vol. I. Academic Press, Inc., New York.

Macfarlane, M. G. 1962. Lipid components of Staphylococcus aureus and Salmonella typhimurium. Biochem. J. 82:40P. (Abstr.)

Malin, B. 1953. Studies of the heat resistance of bacterial spores. Ph.D. thesis. Univ. of Illinois. (L.C. Card No. Mic.A. 53-108) University Microfilms. Ann Arbor, Michigan. (Dis. Abstr. 13:6)

Marchis-Mouren, G., L. Sarda, and P. Desnuelle. 1959. Purification of hog pancreatic lipase. Arch. Biochem. Biophys. 83:309.

McGinnis, G. W., and L. R. Dugan, Jr. 1965. A rapid low temperature method for preparation of methyl esters of fatty acids. J. Am. Oil Chemists' Soc. 42:305-307.

Mencher, J. R., and J. A. Alford. 1965. Purification of the lipase of Pseudomonas fragi. Bacteriol. Proc. 78. (Abstr.)

Meyer, K. H., E. H. Fischer, and P. Bernfield. 1947. Sur les enzymes amylolytiques (1) L'isolement de l α' amylase depancrease. Helv. Chim. Acta. 30:64-78.

Nashif, S. A., and F. E. Nelson. 1953a. The lipase of Pseudomonas fragi. I. Characterization of the enzyme. J. Dairy Sci. 36: 459-470.

Nashif, S. A., and F. E. Nelson. 1953b. The lipase of Pseudomonas fragi. II. Factors affecting lipase production. J. Dairy Sci. 36:471-480.

Nashif, S. A., and F. E. Nelson. 1953c. The lipase of Pseudomonas fragi. III. Enzyme action in cream and butter. J. Dairy Sci. 36:481-488.

Nencki, M. 1886. Uber die Spaltung der Saureester der Aromatischen Verbindungen im Organismus und Durch das Pankreas. Arch. Exp. Path. Pharmak. 20:595-599.

Overbreck, G. A. 1957. Fat splitting enzymes in blood. Clin. Chem. Acta. 2:1-8.

Perkin-Elmer Corporation. 1951. Instruction Manual Portable Tiselius Electrophoresis Apparatus Model 38. The Perkin-Elmer Corporation, Norwalk, Connecticut.

Parry, R. M., R. C. Chandan, and K. M. Shahani. 1965. Rapid and sensitive assay for milk lipase. J. Dairy Sci. 48:766 (Abstr.)

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1962a. Staphylococci in competition. I. Growth of naturally occurring mixed populations in precooked frozen foods during defrost. Appl. Microbiol. 10:16-22.

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1962b. Staphylococci in competition. II. Effect of total numbers and proportion of staphylococci in mixed cultures on growth in artificial culture medium. Appl. Microbiol. 10:23-30.

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1964a. Staphylococci in competition. III. Influence of pH and salt on staphylococcal growth in mixed populations. Appl. Microbiol. 12:70-76.

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1964b. Staphylococci in competition. IV. Effect of starch and kind and concentration of sugar on staphylococcal growth in mixed populations. Appl. Microbiol. 12:77-82.

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1964c. Staphylococci in competition. V. Effect of eggs, eggs plus carbohydrates and lipids in staphylococcal growth. Appl. Microbiol. 12:83-86.

Polonovski, J., R. Wald, and M. Paysant-Diament. 1962. Les Lipides de Staphylococcus aureus. Ann. Inst. Fasteur. 103:32-42.

Randerbath, K. 1963. Thin Layer Chromatography. Academic Press, Inc., New York.

Renshaw, E. C., and C. L. SanClemente. 1964. Studies on a lipase prepared from Staphylococcus aureus. Bact. Proced. p. 61 (Abstr.)

Richards, T., and G. M. El-Sadek. 1949. The nature and quantity of fatty acids produced in butterfat by the action of microorganisms. J. Dairy Res. 16:46-52.

Rosendal, K. 1962. Correlation of virulence of staphylococci from patients with bacteraemia with various laboratory indices. p. 570-574. In N. E. Gibbons [ed]. Recent Progress in Microbiology. Univ. of Toronto Press, Canada.

Salton, M. R. J. 1953. Studies on the bacterial cell wall. IV. The composition of the cell walls of some gram positive and gram negative bacteria. Biochem. et Biophys. Acta. 10:512-520.

Sarda, L., G. Marchis-Mouren, M. J. Constantin, and P. Desnuelle. 1957. Sur quelques essais de purification de la lipase pancreatique. Biochem. Biophys. Acta. 23:264.

Schachman, H. K. 1959. Ultracentrifugation in Biochemistry. Academic Press, Inc., New York.

Shah, D. B., and J. B. Wilson. 1963. Egg yolk factor of Staphylococcus aureus. I. Nature of the substrate and enzyme involved in the egg yolk opacity reaction. J. Bacteriol. 85:516-521.

Shah, D. B., and J. B. Wilson. 1965. Egg yolk factor of Staphylococcus aureus. II. Characterization of lipase activity. J. Bacteriol. 89:949-953.

Shahani, K. M., and R. C. Chandan. 1962. Sensitivity of milk lipase to antibiotics. J. Dairy Sci. 45:1178-1183.

Shahani, K. M., L. Sarda, P. Desnuelle, and E. Azoulay. 1964. Partial purification and standardization of technique of assay of intracellular lipase of Achromobacter lipolyticum. J. Dairy Sci. 47:675. (Abstr.)

Shipe, W. F. 1951. A study of the relative specificity of lipase produced by Penicillium roqueforti and Aspergillus niger. Arch. Biochem. Biophys. 30:165-179.

Stahl, E. 1965. Thin Layer Chromatography. A laboratory hand book. Academic Press Inc., New York.

Sumner, J. B. 1944. A method for colorimetric determination of phosphorus. Science, 100:413-414.

Svedberg, T., and K. P. Pedersen. 1952. The Ultracentrifuge. Oxford Univ. Press, London.

Thomas, E. L., A. J. Nielsen and J. C. Olson. 1955. Hydrolytic rancidity of milk. A simplified method for estimating the extent of its development. Am. Milk Rev. 17:50-52.

Vorbeck, M. L., and G. V. Marinetti. 1965. Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography. J. Lipid Res. 6:3-6.

Walker, G. C. 1964. The growth and thermal resistance of coagulase positive Staphylococcus aureus in selected substrates. Ph.D. thesis. Michigan State University, East Lansing, Michigan.

Wilcox, J. C., W. O. Nelson, and W. A. Wood. 1955. The selective release of volatile acids from butterfat by microbial lipase. J. Dairy Sci. 38:775-781.

Willart, S., and G. Sjöstrom. 1959. The influence of sodium chloride upon the hydrolysis of milk fat in milk and cheese. Proc. 15th Intern. Dairy Congr. 3:1482-1484.

Williams, T. W. 1963. Ultracentrifugal Analysis in Theory and Experiment. Academic Press Inc., New York.

Wills, E. D. 1960. The relation of metals and -SH groups to the activity of pancreatic lipase. Biochim. Biophys. Acta. 40: 481-485.

Willstätler, R., and E. Waldschmidt-Leitz. 1923. Uber Pankreaslipase. Zweite Abhandlung uber Pankreasenzyme. Z. Physiol. Chem. 125:132-198.

Woodbine, M. 1959. Microbial fat. Microorganisms as potential fat producers. p. 179-254. In D. J. D. Hockenhull [ed] Progress in Industrial Microbiology. Vol. 1. Interscience Publishers, New York.

Worthington Biochemical Corporation. 1964. Enzymes. Enzyme Reagents. Worthington Biochemical Corporation, Freehold, New Jersey.

Zolli, Z., Jr., and C. L. SanClemente. 1963. Purification and characterization of staphylocoagulase. J. Bacteriol. 86:527-535.

Zottola, E. A. 1965. Thermal destruction studies on Staphylococcus aureus in milk using short time exposure. (Dis. Abstr. 25: 4915-4916).