LIPASE OF STAPHYLOCOCCUS AUREUS: ITS PURIFICATION AND CHARACTERIZATION

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ABSTRACT

LIPASE OF STAPHYLOCOCCUS AUREUS: ITS PURIFICATION AND CHARACTERIZATION

by Earl C. Renshaw, Jr.

This investigation was concerned with the purification and characterization of a lipase isolated from Staphylococcus aureus, P.S. 71. The extra-cellular enzyme was isolated and purified some 2000-fold by acid-ethanol precipitation at -4 C, followed by molecular sieving through Sephadex G-200, treatment with activated carbon and a final passage through DEAE Sephadex A-50, eluting with 0.6 M sodium chloride. The purified enzyme preparation, of high molecular weight, was free of other enzymatic activities initially present (coagulase, phosphatase, alpha-hemolysin, fibrinolysin and deoxyribonuclease), but was not completely homogeneous as electrophoretic and gel diffusion techniques indicated the presence of a trace impurity.

Lipolytic activity was assayed by electrometric titration of liberated free fatty acids from an emulsified olive oil substrate using the method of Tietz et al. (Am. J. Clin. Pathol. 31:148, 1959) with certain modifications. These included an increase in buffering capacity and the addition of calcium acetate to accelerate the reaction, thereby reducing the time of incubation to one hour. Flowing steam was used to terminate the reaction

as neither ethanol nor acetone in concentrations up to 50% stopped lipolysis.

The enzyme has an optimal temperature of 40 C and is extremely stable at room temperature or lower but is completely inactivated at 60 C for one hour. The optimal pH for activity was 8.2, but the optimal pH for stability in tris buffer was 9.0. Calcium salts are required as fatty acid acceptors, with calcium acetate the most active. Substrate specificity and inhibitor studies characterize the enzyme as a true lipase. The enzyme readily hydrolyzes both egg yolk and human plasma agar.

Agglutinating, precipitating and neutralizing antibodies were readily produced in mice, rabbits and cows when the enzyme was injected with Freund's adjuvant.

LIPASE OF *STAPHYLOCOCCUS AUREUS*: ITS PURIFICATION AND CHARACTERIZATION

By

Earl C. Renshaw, Jr.

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INTRODUCTION

Lipases are widespread in nature, occurring in animals, insects, plants, bacteria and molds. In animals they are found in various tissues and organs, especially in the milk, pancreas, and gastric mucosa. They are found in plants and seeds, such as the castor bean, sunflower, oats and in wheat germ. In bacteria, they are found in some species of the enterobacteriaceae, such as Serratia and Proteus; as well as in some of the pseudomonads, Aeromonas, Vibrio, Clostridium, mycobacter and staphylococci. They are also found in such fungi as Penicillium, Aspergillus and Candida.

Lipases are a biologically important group of enzymes since they are associated with fat metabolism in animals and plants. They are also of significance in the food industry as they produce undesirable rancid flavors in milk and other food products, as well as being essential for the development of desired flavors in certain cheeses.

Since little is known about lipases in general, and microbial lipases in particular, the present investigation was undertaken to isolate, purify and characterize a lipase from Staphylococcus aureus.

REVIEW OF LITERATURE

Lipolytic Enzymes

Lipolytic enzymes catalyze the hydrolysis of ester linkages in lipids with the release of the constituent alcohol and acid moieties. These enzymes have been classified in various ways and the literature has not been consistent in terminology. They are classified as esterases, using such ill-defined terms as aliesterases, simple esterases, and lipases. Indiscriminate use of these terms and the overlapping specificities of these enzymes have resulted in much confusion. In general, one differentiates between lipase and aliesterase or simple esterase on the basis of their relative preferential specificity (Nachlas and Seligman, 1949). The term lipase is usually used for those enzymes attacking fatty acid esters with a long carbon chain, and whose natural substrates are oils and fats. Esterases or aliesterases, however, hydrolyze simple short chain aliphatic esters. This division, however, is only relative as both groups are unusually non-specific in their action. Another basis for the classification of fatty acid esterases concerns the solubility of the substrate (Desnuelle, 1961). Esterases act on substrates in solution while lipases act predominantly on undissolved

substrates. This separation is mostly a matter of convenience as the usual kinetic treatment does not apply directly to undissolved substrates. Since it has not been proved as yet that lipases are completely inert towards dissolved substrates, it is not to be implied that the two categories refer to different modes of action. However, no reports have been found which indicate the existence of enzymes that attack dissolved and undissolved substrates with equal readiness.

Lipase Assay

Several methods are employed for the assay of lipolytic activity. In general, the enzyme is incubated with oil, simple triglycerides or other substrates, and the free fatty acids liberated by enzymatic action are measured.

One of the most commonly used methods involves the direct titration of an olive oil emulsion with standard alkali after a suitable incubation period (Tietz et al., 1959; MacDonald and LeFave, 1962).

Another method, called the pH-stat method, involves the lipolysis of an emulsion at the optimum pH value (Marchis-Mouren et al., 1959). The free fatty acids which are liberated are continuously titrated with standard alkali to maintain a constant optimal pH. The titration value per unit time is a direct measurement of the concentration, since the reaction is of zero order.

A third method involves the use of chromogenic substrates, such as B-naphthol esters of fatty acids (Nachlas and Seligman, 1949; Kramer et al., 1964). These esters upon hydrolysis yield or can be converted into a colored product for colorimetric estimation, as the intensity of color is directly proportional to lipase activity.

A fourth method employs Warburg manometric techniques (Drummond and Tager, 1959). The fatty acids liberated through lipolytic activity under standard conditions react with $NaHCO_3$ and the CO_2 produced is measured.

A fifth method, a rapid phototurbidimetric assay has been recently devised (Vogel and Zieve, 1963). This is based on the observation that during the hydrolysis of a finely emulsified pure triglyceride, a clearing of the emulsion takes place which is proportional to the enzyme concentration.

A sixth method is based upon the hydrolysis of fluorescein esters catalyzed by lipase (Kramer and Guilbault, 1963). The rate of change in the fluorescence of the solution due to the production of the fluorscein is measured and correlated with enzyme activity.

Since the substrates acted on by lipase are, in general, water-insoluble, the reaction takes place in a heterogeneous system and hence, the accessibility of the enzyme for the substrate plays a decisive role in lipolysis (Desnuelle, 1961). Thus, it is to be expected that the degree of emulsification and the presence of surface-active agents or other substances

which affect the nature of the lipid-water interface are important factors influencing the rate of hydrolysis.

Olive oil is the most commonly used natural substrate for lipase determination, but many other esters and triglycerides, both natural and synthetic, are used. These include many oils and fats such as cottonseed, castor, coconut, corn and linseed oils; margarine, lard and hydrogenated cooking fats; as well as pure triglycerides, the tweens and esters of p-nitrophenyl or 2-naphthol-6-sulfonic acid. However, the use of soluble esters and chromogenic substrates in the lipase assay has been condemmed by Desnuelle (1961), who observed that purified pancreatic lipase acts only on fatty acid esters in the insoluble emulsified state and not on those esters in solution. In contrast, he found esterases acted only on soluble esters in solution and not on insoluble emulsified esters.

The optimal pH for lipase activity varies with the substrates and buffer systems used, as well as with the source of enzyme. Pancreatic lipase has a pH range of 7-9. The optimum was found to increase from pH 7 with triacetin to pH 8.8 with tristearin, while for olive oil the optimum was just above 8 (Roe and Byler, 1963). Plant and mold lipases have lower pH optima than animal lipases. Castor bean lipase has an optimum at pH 4.0-4.2 (Ory et al., 1960), and Aspergillus lipase has its optimum at pH 5.6 (Fokumoto et al., 1963). However, Soya bean lipase

has its optimum at pH 8.2-8.4 (Perl and Diamant, 1963). Leptospiral lipase has an optimum at pH 7 (Patel et al., 1964), while Mycoplasma has its at pH 7.5-8.0 (Rottem and Razin, 1964). The pH optimum of milk lipase was found to be 9.0-9.2 (Chandan and Shahani, 1964).

Most animal lipases have temperature optima around 40 C. Human serum lipase has an optimum temperature of 37.5 C, while pancreatic lipase has its optimum at 45 C (Kramer et al., 1963). Milk lipases are rather heat sensitive, being extremely unstable at 20-45 C, but have their optima at 37 C (Chandan and Shahani, 1963). Microorganisms show optimal activity between 25-40 C (Alford and Elliott, 1960), Aspergillus at 25 C (Fokumoto et al., 1963), leptospira at 30 C (Patel et al., 1964) and most bacteria near 40 C (Alford and Elliott, 1960). Some microorganisms even show lipolytic activity within three weeks at -29 C (Alford and Pierce, 1961).

Lipolysis is very time dependent as zero order kinetics occur only for a short period during hydrolysis. This is due mainly to the strong fatty acids produced and the inability to maintain a stable emulsion of the right particle size. The kinetics of undissolved systems can be based on the total available surface of the undissolved substrate instead of the substrate concentration. With a sufficient amount of finely emulsified substrate, all of the enzyme is adsorbed on the substrate surface (Desnuelle, 1961).

At the present time there exists no uniformity in defining a lipase unit or in the reporting of data (Chandan and Shahani, 1964). Different workers have employed different conditions for determining the enzymatic activity and have used different terms in expressing the activity, such as acid degree, free fatty acid titer, or volume of CO_2 liberated. Therefore, there is no way to compare or interconvert the units of lipase activity. According to the recommendation of the Commission on Enzymes of the International Union of Biochemistry (1961), a lipase unit may be defined as the amount of enzyme which will liberate one micromole of acid per unit time from a standard substrate under specific conditions. However, the standard substrate or conditions have not as yet been agreed upon.

Factors Affecting Lipase Activity

Many substances are known which will show an activating or an inhibiting effect on lipolytic activity. It has long been known that bile products activate or accelerate pancreatic lipolysis, but the mechanism of action still remains obscure (Melcer and Lazo-Wasem, 1964). Some authors have attributed the accelerating effect of bile acids to substrate emulsification and lowering of surface tension.

Calcium and magnesium salts are also known to accelerate pancreatic lipolysis (Constantin et al., 1960). This activation has been attributed to the ability to remove the strongly inhibitory fatty acids by forming insoluble soaps.

The formation of insoluble soaps also prevents the fatty acids from reforming ester linkages, and therefore shifts the reaction in the direction of hydrolysis. However, others have found calcium and magnesium salts to have no accelerating effect on human serum lipase, in fact calcium salts in high concentration were found to inhibited the reaction (MacDonald and LeFave, 1962).

Lipase is a very stable enzyme and is resistant to most enzyme inhibitors (Long, 1961). Sulfhydryl inactivating agents such as p-chloromercuri-benzoate, iodoacetate and potassium cyanide all inhibit lipase, but this may be reversed by cysteine (Ory et al., 1960; Chandan and Shahani, 1963). Heavy metals also inhibit lipase while organophosphorus compounds have no effect on lipase, but they are very effective esterase inhibitors in low concentration (Chandon and Shahani, 1964).

In the hydrolysis of lipids by lipase, the enzyme may show three types of specificity: substrate, fatty acid, and positional.

Studies on the substrate specificity of lipase are complicated by two major factors: (1) the presence of esterases, and (2) the degree of emulsification of the substrate. The first difficulty may be overcome by the use of specific esterase inhibitors such as the organophosphorus compounds which have no effect on lipase. The second problem, however, is more difficult to control as a suitable emulsion is very difficult to prepare and

maintain. Since lipases hydrolyze oils and fats as well as various simple and mixed glycerides, substrate specificity appears to be very low, if at all (Chandan and Shahani, 1964).

Lipases in general show very little fatty acid specificity (Jensen et al., 1964 a,b,c). However, some microbial lipases show specificity for oleic and linoleic esters (Alford et al., 1964) and in some, the rate of lipolysis is directly related to the degree of unsaturation (Alford and Pierce, 1961).

Positional specificity is, however, an important factor in lipase activity. Pancreatic and milk lipases attack the 1,3-position rapidly and the 2-position only very slowly, if at all (Jensen et al., 1964 a,b,c). Lipases from microorganisms may be divided into three classes according to positional specificity (Alford et al., 1964): (1) those which attack the 1,3-position like pancreatic lipase (many microorganisms); (2) those which attack the 1,2- or 2,3-positions (Staphylococcus and Aspergillus); and (3) those which will attack all three positions (Geotrichum).

Staphylococcal Lipases

Eijkman (1901) in his classical paper on the diffusible enzymes of microorganisms was the first person to suggest that staphylococci possessed lipolytic enzymes. Wells and Corper (1912) confirmed this observation and noted that the staphylococci were more active in this

respect than several other bacterial species. Until the last few years there has been little available information concerning the properties of staphylococcal lipases. Staphylococci have both lipases and esterases, with the esterases prevailing over the lipases (Pernice, 1961). As no clear cut distinctions between these two groups of enzymes appear to have been made by earlier authors, many workers were probably measuring esterase instead of lipase activity.

In a systematic survey of over 1,000 strains of bacteria in 18 genera, the English worker, Davies (1954), showed that the staphylococci were the only potentially pathogenic bacteria which produced a diffusible or exo-lipase. Since the publication of this report, there has been a renewed interest in bacterial lipases, and in staphylococcal lipases in particular.

Several agar plate methods have been employed for the qualitative determination of lipase activity of microorganisms. Sierra (1957) developed a simple test to detect lipolytic activity by incorporating water-soluble tweens in nutrient media. Colonies showing lipolytic activity produced visible halos due to the formation of crystals of calcium salts of the liberated fatty acids. Davies (1954) employed emulsified horse fat as a substrate and Nile blue sulfate as an indicator of acid production. Lipolytic colonies produce a blue zone around the colony while the rest of the plate remains pink. Hugo and Beveridge (1962)

used a similar indicator, Victoria blue, and various oils and fats as substrates.

Another method employing emulsified fats and oils in nutrient agar uses saturated aqueous copper sulfate as an indicator for lipolysis. Lipolysis is then shown by precipitation of blue cupric soaps around the colony (Berry, 1933; Stewart, 1965). Egg yolk agar media has been employed by many workers (Carter, 1960; Willis and Turner, 1962; Lowbury and Collins, 1964) for the detection of staphylococcal lipolysis. On this medium lipolytic activity produces both an opacity and clearing around the colony.

For the quantitative determination of lipolytic activity, fats and oils are emulsified and added to broth. After incubation, the fatty acids are either extracted with ether-acetone and then titrated with standard alkali (Stewart, 1956), or titrated directly without extraction (Hugo and Beveridge, 1962).

Elek (1959) states that over 99.5% of coagulase-positive human strains of staphylococci are lipolytic, but only 75% of animal strains produce the enzyme. Vogelsang et al. (1962) in testing over 1700 coagulase-positive staphylococcal strains found 70% to be lipase positive, while only about 10% gave a positive lipase reaction from 100 coagulase-negative strains. They also found that the great majority of lipase-negative strains were in phage group III and most of these were resistant to penicillin.

Lowbury and Collins (1964) in their study of the egg yolk reaction of staphylococci isolated from burns, found egg yolk negative strains to be more common in phage group IiI and more commonly resistant to penicillin, tetracycline and erythromycin, as well as being more resistant to the action of mercuric chloride.

Kasarov and Bajljozow (1963) found about 70% of coagulase-positive human strains of Staphylococcus aureus to have lipase activity, while only a few animal strains had such activity.

In studying the lipolytic activity of several microbial lipases Alford and co-workers (1964) found staphylococcal lipase to be specific for attacking the 1- and 2-positions of natural fats and triglycerides. Staphylococcal lipase was also found to have a slight preference for oleic over stearic linkages. Vadehra and Harmon (1965) however, found staphylococcal lipase to attack all three positions with a preference for the palmitic linkage. In their earlier investigations of lipolytic activity of microorganisms at low temperature, Alford and Pierce (1961) found staphylococcal lipase to be active during three weeks at -29 C. They also found that the ability of staphylococcal lipase to attack the B-esterified position was nullified by lowering the temperature below -18 C.

Willis and Turner (1962), as well as Stewart (1965), studied the relationship of staphylococcal lipolysis and

and pigmentation. The former authors found that staphylococci growing on nutrient agar media containing 1% glycerol monoacetate showed colonial pigmentation to be greatly enhanced, and at least two color types of coagulase-positive staphylococci, yellow and orange, could be detected. Colonies of coagulase-negative staphylococci were usually porcelain-white, and no white colonies of coagulase-positive strains were observed. The latter worker also found that biosynthesis of pigment was accentuated by the presence of fats, fatty esters or organic acid radicals, and that the main pathway of pigment synthesis in Staphylococcus aureus involves acetyl coenzyme A of the citric acid cycle.

Two groups of investigators have isolated and purified lipases from Staphylococcus aureus, one group studying the egg yolk factor, the other the enzyme producing free fatty acids from human plasma. Shah and Wilson (1963, 1965) investigated the egg yolk factor of Staphylococcus aureus. They found that the egg yolk factor was a lipase which required calcium ions as a fatty acid acceptor. Heart infusion broth was the best medium for growth and the enzyme was purified from cell-free culture filtrates by acid precipitation, two cycles of ethanol fractionation, and zone electrophoresis (Blobel et al., 1961). The enzyme had an optimal pH of 7.8 and hydrolyzed oils as well as triglycerides.

Weld and co-workers (1963 a, b) reported that many strains of Staphylococcus aureus elaborate a diffusible

extracellular lipolytic substance that produced visible plaques in large amounts on 10% plasma agar. These plaques were found to consist primarily of octadecenoic acid, both free and in triglycerides. Lesser amounts of other fatty acids were also present, and they postulated that probably a lipase was acting on the lipids present in human plasma. O'Leary and Weld (1964) isolated and purified this enzyme approximately 225-fold from culture extracts by ammonium sulfate precipitation, ethanol precipitation, gel filtration, and DEAE-cellulose chromatography. They found this lipolytic enzyme to be quite stable, with broad tolerances in both pH and temperature. The optimal pH was 8.0 to 8.2 with a temperature optimal range of 33 to 39 C. The principle substrate was found to be a plasma lipoprotein and calcium ion was the only cofactor required.

At present there is no direct evidence that lipase plays any significant role in the pathogenicity of staphylococci (Elek, 1959), but there are some authors who believe that it may be related to virulence in some manner. Bruni (1939) was the first to suggest a relationship between pathogenicity and lipolytic activity. He found that staphylococcal strains isolated from lesions had greater lipolytic activity than those strains which were non-pathogenic. However, he used tributyrin as the substrate for lipolytic activity and it is now known that this substrate measures esterase activity much more readily than lipase activity. In a later paper (Bruni, 1961), a review

on the possible relationship between lipolysis and virulence of staphylococci, he concludes that lipolysis is an index of virulence, if not actually a factor of virulence. In passage through rabbits he was able to show that a gradual increase in virulence was accompanied by an increase in lipolytic activity, and also that the reverse was true.

Gillespie and Alder (1952) reported that most strains of coagulase-positive staphylococci of human origin produced an opacity due to lipolysis when grown in egg yolk broth, while coagulase-negative strains caused no change in the broth. Burns and Holtman (1960) noted that all cultures isolated from lesions of undisputed staphylococcal etiology gave a positive egg yolk opacity reaction, and suggested a possible association of the egg yolk factor with virulence.

Lowbury and Collins (1964) found a strong association between penicillin resistance and a negative egg yolk reaction. These strains are particularly common in superficial lesions, such as burns, impetigo and open wounds, while egg yolk positive strains are more commonly associated with invasive infections and those causing deep wounds. Nathsarma (1963), however, did not find a relationship between the egg yolk reaction and source of the organism, antibiotic resistance, or phage typability.

Davies (1954) refers to the fact that lipolytic strains tend to be more active biochemically than non-lipolytic ones, and Stewart (1965) suggests that the power to metabolize

simple fats may be related to the readiness with which these organisms colonize and persist in sebaceous sites in the skin.

O'Leary and Weld (1964) suggest that the formation of free fatty acids in human plasma by staphylococci might be significant at the molecular level in pathological processes. Since free oleic acid is capable of uncoupling oxidative phosphorylation in mammalian cells, the liberation of free oleic acid in the circulation by staphylococcal lipase could possibly cause quite serious effects. In this connection a report of Fusillo and Weiss (1963) shows that the severity and fatality of staphylococcal infection in mice was markedly enhanced by the injection of certain human sera.

MATERIALS AND METHODS

Cultures and Their Maintenance

Propagating strains of Staphylococcus aureus of the International-Blair series (Blair and Carr, 1953) as well as six strains of bovine origin (Seto and Wilson, 1958) were used in these studies. In addition, over 100 strains of coagulase-positive and coagulase-negative staphylococci isolated from both human and animal sources were also used. Stock cultures were maintained on trypticase soy agar slants at 4 C and were transferred approximately every two months.

Assay Methods

Lipase.--Lipase activity was qualitatively estimated by the method of Sierra (1957). This was done by cultivation on plates of trypticase soy agar containing 1% tweens and 0.01% calcium chloride. Three tweens were used as substrates for lipolysis. Tweens are water-soluble high fatty acid esters of a polyoxyalkylene derivative of sorbitan, and differ only in the particular fatty acid component. Tween 20 is a lauric acid (C_{12}) ester; Tween 40, a palmitic acid (C_{16}) ester; and Tween 80, an oleic acid (C_{18}) ester. Lipolytic activity is indicated by a visible halo around the colonies. This halo is due to crystals of calcium salts of the fatty acids liberated by lipolysis of the tweens.

Quantitative determination of lipase activity was made by a modification of the method of Tietz et al. (1959). An olive oil emulsion was prepared by emulsifying intermittently in a Waring blender a solution containing 0.2 gm sodium benzoate, 7 gm gum arabic, 100 ml distilled water, and 100 ml of purified olive oil. This emulsion was then re-emulsified with 0.1 M calcium acetate and 1.0 M Tris-HCl buffer [tris (hydroxymethyl) aminomethane], pH 8.3, in a ratio of 3:3:1 to produce the working substrate. Two large test tubes were used for each assay. One tube was the test, which contained 9 ml of the working substrate plus 1 ml of enzyme solution. The other tube or blank contained only the 9 ml of substrate. Each tube was thoroughly mixed and incubated in a water bath at 40 C for 1 hour. One ml of enzyme solution was then added to the blank tube, and both tubes were then placed in flowing steam or boiling water for 5-10 minutes to stop the reaction. The contents of each tube were transferred to 100 ml beakers, and the tubes rinsed with 5 ml of 95% ethanol. The contents of both beakers were then titrated electrometrically with N/20 NaOH to a pH of 10.5. By calculating the difference between the control and test values, the units of lipase activity were recorded as the number of ml of N/20 NaOH used to neutralize the liberated free fatty acids. If lipase activity exceeds 4 units, the enzyme solution must be re-run on dilutions as the reaction is of zero order only up to 4 units.

Total Protein.--Quantitative determination of total protein was made by the Folin-Ciocalteu micro modification as described in Kabat and Mayer (1961). Crystalline bovine albumin[1] was prepared so that values on the standard curve ranged from 50 to 300 µg per tube. Protein samples were placed in tubes and enough distilled water added to bring the volume to 2.0 ml. To each tube was added 6.0 ml of a 12.5% solution of anhydrous sodium carbonate and 1.0 ml of a 0.1% solution of copper sulfate. After standing at room temperature for one hour, 1.0 ml of 1/3 Folin-Ciocalteu phenol reagent (1 part freshly diluted with 2 parts of distilled water) was then added slowly with constant mixing, and the tubes allowed to stand an additional 20-30 min. at room temperature. Optical density readings were made at 750 mµ on a Bausch and Lomb Spectronic 20 spectrophotometer or a Beckman DU-spectrophotometer. Total protein per ml of sample was determined from a calibrated chart currently prepared.

Concentration and Purification of Lipase

Staphylococcus aureus, P.S. 71 was used for purification as it was the highest producer of lipase in the group. Five hundred ml of culture in log phase growth were added to each of two 10-liter Florence flasks each containing 8 liters of brain heart infusion or trypticase soy broth.

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[1]Nutritional Biochemicals Corp., Cleveland, Ohio.

The flasks were incubated for four to seven days at 37 C on a rotary shaker (ca 150 cycles/min). The organisms were removed by use of a Serval continuous-flow superspeed centrifuge[1] (Model KSB-1) at a flow rate of 50 ml/min. The supernatant fluid was slowly adjusted to pH 4.1 with glacial acetic acid, and cooled to -4 C in a cold bath. Cold ethanol (95%) was slowly added to a final concentration of 10% (v/v). Precipitation was allowed to occur at -4 C for 24-48 hrs. The acid-alcohol precipitate was then collected by continuous flow superspeed centrifugation and the precipitate extracted four to six times with 100 ml amounts of 0.1 M tris-HCl buffer, pH 8.0. The extracts were combined, dialyzed overnight against running tap water, and concentrated to a volume of about 50 ml by dialysis against polyvinylpyrrolidone[2] (PVP). The concentrated solution was then dialyzed against distilled water overnight and the insoluble precipitate removed by centrifugation.

The supernatant fluid was added to a column (4 x 40 cm) of Sephadex[3] G-200 and eluted from the column with the 0.1 M tris buffer, pH 8.0, according to the procedures of Porath (1960) and Hallander (1963). The eluate was collected in 10-ml fractions, and protein and lipolytic activity were

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[1]Ivan Sorvall, Inc., Norwalk, Connecticut.

[2]Oxford Laboratories, Redwood City, California.

[3]Pharmacia Fine Chemicals, Inc., Piscataway, New Market, New Jersey.

determined for each fraction. Fractions containing lipolytic activity were pooled and concentrated against PVP as previously described. This concentrated solution was then recycled through the Sephadex G-200 column as before and those fractions containing the highest specific activity were again pooled. Activated carbon (Darco[1]) was then added (10 gm/100 ml), the suspension mixed on a magnetic stirrer for 10 to 20 minutes, filtered and centrifuged. To the supernatant fluid there was added activated DEAE-Sephadex A-50 (50 gm wet wt/100 ml). The lipolytic enzyme was eluted with 0.1 M tris buffer, pH 8.0 containing 0.6 M sodium chloride. The active enzyme was then concentrated by dialysis against PVP to a volume of approximately 30 ml, and dialyzed extensively against 0.1 M tris buffer, pH 8.0. This concentrated enzyme preparation was then stored at -20 C.

Characterization of Lipase

Stability studies.--Temperature stability studies were done by placing aliquots of purified lipase solution into test tubes and incubating at 50, 56 and 60 C. Two ml aliquots were removed from each tube at 15 minute intervals, immediately cooled in an ice bath and a lipase determination done on each.

The pH stability of the purified enzyme was determined by adjusting 5 ml samples to pH 6.0, 7.0, 8.0, and 9.0 with

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[1]Atlas Powder Co., New York, N. Y.

0.1 N NaOH or HCl. The total volume was adjusted to 6 ml with distilled water. After incubation at 56 C for 15 minutes, the samples were assayed for lipase activity.

Substrate specificity.--To determine substrate specificity nine complex natural oils as well as thirteen synthetic esters were tested. The natural oils were emulsified and used as in the lipase assay method. The synthetic esters were dissolved or emulsified with distilled water to a concentration of 5 per cent (wt/vol) and used in the lipase assay as previously described.

Accelerators.--Various salts and other substances were tested for their role as accelerators or fatty acid acceptors. These were made up in distilled water to give a final concentration in the assay of approximately 4×10^{-4} and 4×10^{-3} M. A lipase assay was done on each and the control consisted of distilled water in place of the salt solutions.

Inhibitors.--The following inhibitors were dissolved in distilled water at various concentrations, usually 10^{-3} and 10^{-5} M: Parathion, Eserine, isopropanol, sodium fluoride, iodoacetic acid, sodium arsenate, Triton WR-1339,[1] and various metals and trace elements. To 1.5 ml of each inhibitor was added 1.5 ml of purified enzyme solution. After incubation of these mixtures at 40 C for 1 hr, lipase activity was measured as previously described.

Electrophoretic analysis.--Small scale electrophoresis using cellulose acetate strips was performed as described by

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[1] Rohm and Haas Co., Philadelphia, Pennsylvania.

Smith (1960). Oxoid[1] strips (2.5 x 12 cm) were impregnated by floating on the surface of the desired buffer solution. The strips were then removed and lightly blotted with filter paper and applied across the bridge gap of a Universal apparatus.[2] Using a micropipette, 0.01 ml sample was applied to the strip as a narrow zone. The time interval varied with the conditions, for example, when barbitone buffer (pH 8.6, ionic strength 0.07) and a current of 0.4 ma/cm were used, optimal results were obtained in 2 hr. Immediately after removal of the strips from the electrophoretic apparatus, they were "fixed" by immersion into 5% trichloracetic acid for 20 min. Protein was stained by either 0.2% Ponceau S[3] in 3% aqueous trichloracetic acid, or 0.002% Nigrosin[4] in 2% acetic acid solution. Staining was rapid with Ponceau S and gave satisfactory results in 10-20 min. With Nigrosin, overnight immersion of the strips was necessary. After staining, the strips were transferred to a washing solution (5% aqueous acetic acid) until the background appeared colorless. The strips were dried by placing between paper towels and pressing with weighted glass plates. To locate lipase activity, strips were cut lengthwise immediately upon removal from the electrophoretic apparatus. One half of the

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[1]Consolidated Laboratores, Inc., Chicago Heights, Illinois.

[2]Shandon Scientific Co., London, England.

[3]Allied Chemical Corp., New York, New York.

[4]Allied Chemical Corp., New York, New York.

strip was stained for protein and the other half immediately placed upside down on a tween agar plate. After a suitable incubation period, lipase activity was indicated by an opaque area beneath the strip. This area was then compared to the strip stained for protein.

Acrylamide gel electrophoretic analysis was performed on the purified preparation exactly as described by Davis (1964). The disc electrophoresis was performed in small (7 x 0.5 cm) columns of polyacrylamide gel consisting of 3 sections: (1) a large-pore anticonvection gel into which the purified lipase sample is introduced; (2) a large-pore spacer gel in which the sample is electrophoretically concentrated; and (3) a small-pore gel in which the sample is separated electrophoretically and by molecular sieving. Electrophoresis was in tris-glycine buffer, pH 8.3, for 2 hrs at 2 mA per tube. The gels were then removed and half of them fixed and stained for protein. Lipolytic activity was determined on the remaining gels by immediately slicing the gel into 2 mm segments and eluting each segment with 0.5 ml of 0.1 M tris buffer, pH 8.0. A colorimetric substrate, p-nitrophenyl palmitate,[1] was used as a saturated solution in absolute methyl alcohol. An equal volume of substrate was added to the elutate and incubated at 40 C for 1 hr. Lipolytic activity is indicated by the development of a yellow color when the substrate is hydrolyzed.

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[1]California Corp. for Biochemical Research, Los Angeles.

Immunological Studies

Production of antilipase.--Mature Dutch Belt rabbits (2-4 lbs), young mice (1 month old) and mature cows were used for the production of antilipase serum. Freunds adjuvant[1] (1:1) was used as an antibody enhancing agent. For proper emulsification, the addition of adjuvant to sample was done in small increments while carefully drawing and expelling the mixture with a one ml pipette; this technique resulted in a stable emulsion which did not separate upon standing overnight at 4 C. The rabbits and cows were inoculated subcutaneously by multiple injections in the subscapular region at intervals over a several months period. These animals were bled at intervals. The mice were inoculated intraperitoneally, exsanguinated 3 weeks later, and the serum pooled and stored at -20 C.

Serological techniques.--Lipase neutralization tests were used to determine the highest dilution of antiserum which would neutralize 1 unit of lipase. One unit of lipase solution was added to each of a series of serially diluted serum. In most cases this was 1 ml of enzyme solution to 1 ml of diluted serum. The tubes were incubated in a water bath at 37 C for 1 hr. Then, either a lipase assay was done on each or the neutralization of lipase was demonstrated by the inhibition of the hydrolysis of tween on tween-agar media (Gonzalez, 1963). This was done by cutting wells into

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[1]Difco Laboratories, Detroit, Michigan.

Tween-80 agar media, filling the wells with enzyme-serum solution, and incubating at 37 C for a suitable period.

Precipitating antibodies were detected by the ring-test. The test was performed by layering the lipase solution carefully over a series of diluted antiserum in narrow test tubes. The endpoint was that dilution of antiserum which produced the smallest amount of turbidity at the interface.

The slide latex agglulation test was a slight modification of the method of Greenberg and Cooper (1960). One ml of purified lipase (2 units/ml) was added to 1 ml of polysterene latex particles[1] and left at room temperature for two hours. Ten ml of 0.1 M tris buffer, pH 8.0, was then added and the suspension was stored at refrigerator temperatures until used. This suspension was stable for at least two weeks. Two-fold dilutions of serum were made and two drops of each were placed in concavity slides. Two drops of the latex antigen were then added to each concavity and the antigen and serum mixed by gentle rocking. The results were read after two minutes.

Gel diffusion tests were made according to modifications of methods of Ouchterlony (1958). Oxoid Ionagar #2 to a final concentration of 1 per cent was added to phosphate buffered physiological saline solution (pH 7.4) and sterilized. Ten ml quantities of this melted agar were layered on standard glass lantern slides (3 1/4 x 4 in)

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[1]Difco Laboratories, Detroit, Michigan.

and allowed to harden at 4 C for 3 hr. After cutting wells to the desired pattern, antigen and serum samples were added. The glass slides were stored at 37 C under humid conditions for 4-7 days for precipitin bands to develop. The slides were then immersed in 0.85% NaCl solution for 24 hr , washed in distilled water an additional 24 hrs and the agar dried to a thin film by use of a warm air heater. The zones of precipitation were stained according to methods described by Crowle (1961). The slides containing the dried agar film were immersed into stain solution (thiazine red R, 0.1%; amidoswartz 10B 0.1%; light green SF, 0.1%; acetic acid, 2%; mercuric chloride, 0.1%) for 5-10 minutes. The slides were then washed in 5% acetic acid solution to remove excess non-specific stain.

RESULTS

Modification of Lipase Assay

Ideal measurement of enzyme activity is accomplished when the substrate concentration is sufficient to saturate the enzyme during the time when the reaction kinetics approach zero order. In general, the activity of an enzyme is measured accurately only during the initial or early period of the reaction. In nearly all procedures for the determination of lipase, a long hydrolysis period has been used because a short incubation time would not yield enough fatty acids for measurement. Therefore, conditions have to be modified to fit the specific lipolytic enzyme being studied. The method being modified is the serum lipase determination of Tietz and co-workers (1959).

Purification of substrate.--Vogel and Zieve (1963) found that alumina-treated olive oil substrate gave substantially higher results in serum lipase determinations than untreated olive oil. Since free fatty acids have an inhibitory effect, they are removed from the olive oil by the addition of 25 gm of alumina per 100 ml of olive oil. The suspension was stirred for 2-3 hrs on a magnetic stirrer, allowed to settle overnight at room temperature, and finally filtered to remove the alumina. This treatment

removes all free fatty acids as no acidity was detected when 100 ml of the purified oil was extracted with ethyl ether-hexane (1:1) and titrated with standard alkali. Untreated olive oil when extracted by this method gives varying amounts of titratable acidity. Treatment with alumina also removes an appreciable amount of the yellow pigment from the olive oil.

Emulsification of substrate.--The stock emulsion was made by dissolving 0.2 gm sodium benzoate and 7 gm Gum Arabic in 100 ml distilled water. This solution usually has to be heated as Gum Arabic is very difficult to dissolve. The warm solution is filtered to remove granular material and then placed in a Waring Blender. Best results were obtained if the purified olive oil (100 ml) was added very slowly during homogenization. The emulsion was homogenized for an additional 10 minutes by intermittent mixing, for example, 2 min mixing, 2 min off. This results in a more stable emulsion. The stock emulsion is stable for several months in a refrigerator but the pH should be checked at intervals and the reagent discarded if an appreciable deviation in pH occurs.

Enough of the working emulsion must be made up for all assays at a specific time or titration values may vary. Usually enough working emulsion was mixed for one day, kept in a Waring blender, and re-emulsified before each use.

Buffer.--One ml of 1.0 M tris buffer, pH 8.3 was needed to maintain an optimal pH of 8.2 during the 1 hr incubation

period. Lower concentrations of buffer will not adequately buffer the reaction mixture as the pH drop will retard the reaction rate.

Accelerators.--Shah and Wilson (1963) observed that calcium was required as a fatty acid acceptor for the egg yolk opacity reaction. Four calcium salts were tested for their role as fatty acid acceptors. Of these, calcium acetate (Table 1) produced the best lipolytic activity. The effect of increasing concentrations of calcium acetate on lipolysis is shown in Table 2. The optimal concentration of calcium acetate under the test conditions was 3.8×10^{-2} M. Greater concentrations than this produced precipitation of the reaction mixture during hydrolysis and breakdown of the emulsion.

Termination of reaction.--Many methods for lipase determination terminate the reaction by adding 95% ethanol. In this case, however, the addition of 95% ethanol up to a final concentration of 45%,or ethanol-acetone (1:1) up to 50%, did not stop the reaction. Heating in flowing steam or boiling water for 5 minutes inactivated the enzyme completely. Heating by flowing steam up to 20 minutes also showed no loss of free fatty acids. However, if the substrate used contains short chain free fatty acids, the test tubes containing the reaction mixture must be sealed as short chain acids are easily lost under these conditions.

TABLE 1.--Calcium salts as accelerators for lipase activity

Calcium salt[1]	% Activity
Calcium acetate	100
Calcium sulfate	89
Calcium chloride	84
Calcium phosphate	45

[1]Final concentration of 3.8×10^{-3} M.

TABLE 2.--Determining optimal concentration of calcium acetate for lipase activity

Final Concentration Molar	% Activity[1]
3.8×10^{-4}	100
3.8×10^{-3}	167
1.2×10^{-2}	210
3.8×10^{-2}	292
5.2×10^{-2}	precipitation

[1]Control with distilled water set at 100%.

Evaluation of Conditions for Optimal Production of Lipase

Media.--A variety of commercial, dehydrated as well as a few semi-synthetic, media were screened to determine which would give maximum production of lipase. The various media in 500 ml flasks were incubated on a rotary shaker at 37 C for 5 days. Lipase production in all media tested was very low, between 0.03 and 0.05 lipase units/ml/hr. O'Leary and Weld (1964) found that lipase production was much higher on agar media than in broth. Trypticase soy broth and agar were used, and it was found that the agar medium yielded approximately twice as much lipase as the broth. In spite of the fact that the yield was low, broth was elected because extraction of the enzyme was less time consuming.

Effect of incubation temperature.--When stationary cultures were incubated at various temperatures (37, 25 and 12 C) for one week, lipase was only detectable in cultures growing at 37 C.

Effect of shaking.--Lipase activity of shake versus non-shake cultures was compared over a 5 day incubation period. When S. aureus 71 was incubated on a rotary shaker at 37 C, maximal production occurred after 3 days. Shaking gave approximately twice the lipase as stationary cultures (Table 3).

Addition of Substrate.--To determine if the addition of substrate to the culture media would increase lipase production, Tween 80 and olive oil emulsions were added to

TABLE 3.--Effect of aeration by shaking upon the production of lipase by Staphylococcus aureus 71

Days	Lipase Activity (units/ml/hr)	
	Shaking	Stationary
1	0.01	0
2	0.02	0.01
3	0.06	0.03
4	0.06	0.03
5	0.06	0.04

brain-heart infusion. Incubation was at 37 C for one week with shaking. These substrates (Table 4) in general had an inhibitory effect on lipase production.

Concentration and Purification of Lipase

Production.--Initially the lipase activity of some 24 strains of phage-propagating staphylococci belonging to the International-Blair series was quantitatively measured. Figure 1 is a bar graph representing the exo-lipase produced in brain-heart infusion after 5 days of stationary incubation. The abscissa represents the various propagating strains arranged according to their major groups while the ordinate indicates lipase activity expressed as units after 12 hrs incubation. The lipase assay was incubated for 12 hrs because of the small amount of enzyme present. Three strains from group III (53, 54, 75) produced no detectable lipase.

From these results Staphylococcus aureus, P.S. 71, the highest producer, was selected for further study. Because of the low yield of lipase, large amounts of media had to be employed. Ten to twenty liter batches of either brain-heart infusion or trypticase soy broth were used.

Purification.--Diagrammatically, Figure 2 represents a flow sheet for the preparation of pure lipase. The second crude fraction of lipase was precipitated from the cell-free supernatant fluid (fraction I) at -4 C by adjusting to pH 4.1 with glacial acetic acid and enough ethanol to effect a final concentration of 10% (v/v). A final pH value of

TABLE 4.--Effect of two different substrates at various concentrations upon lipase production

Substrate	Lipase Activity (units/ml/hr)
None	0.07
Tween 80	
0.25%	0.08
1.0%	0
Olive oil emulsion	
0.1%	0.04
0.25%	0.04
0.5%	0.01
1.0%	0.01

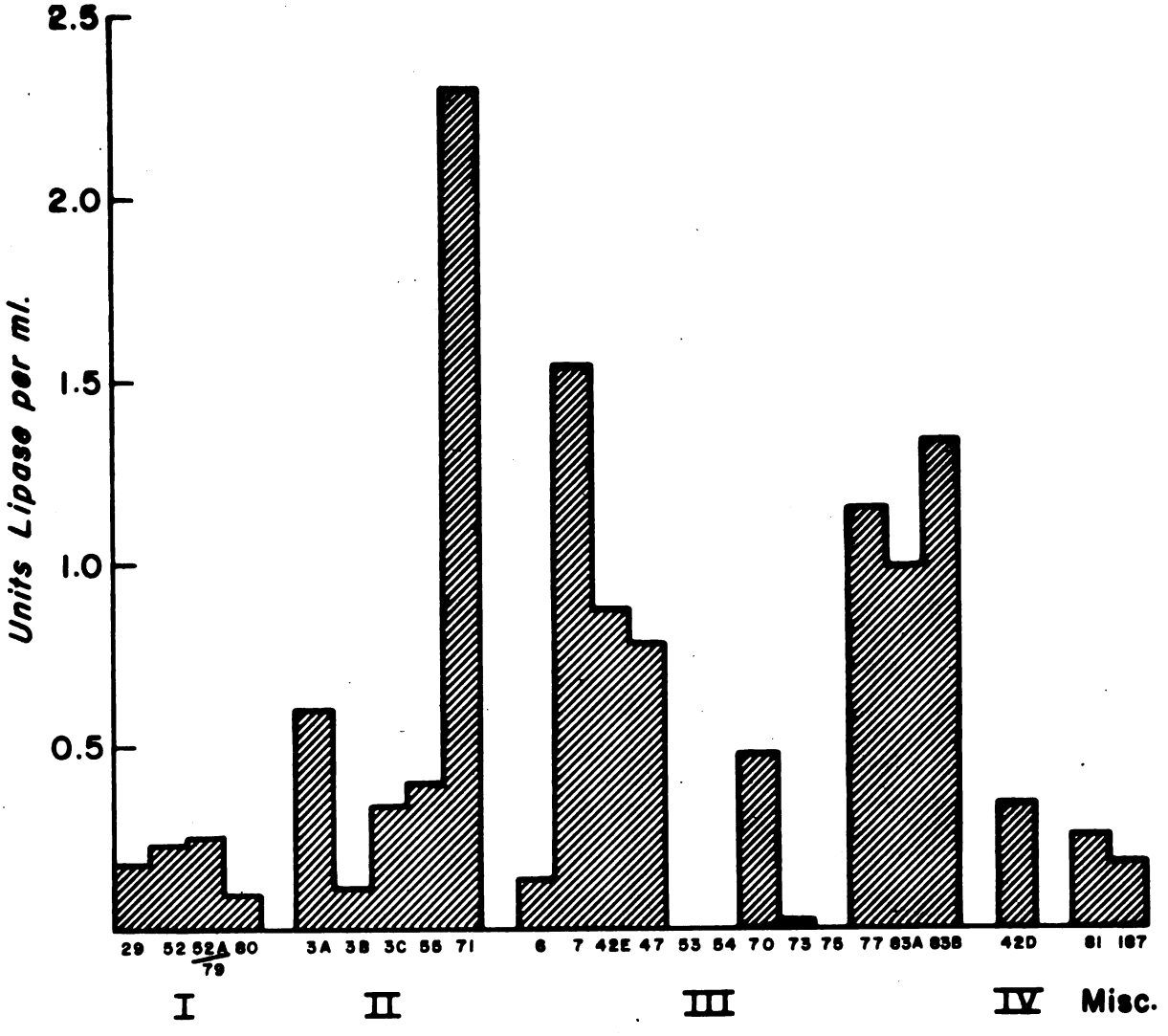

Fig. 1.--Lipase activity of the phage-propagating strains of Staphylococcus aureus after 5 days stationary incubation in brain heart infusion. Activity expressed as units/ml after 12 hrs incubation.

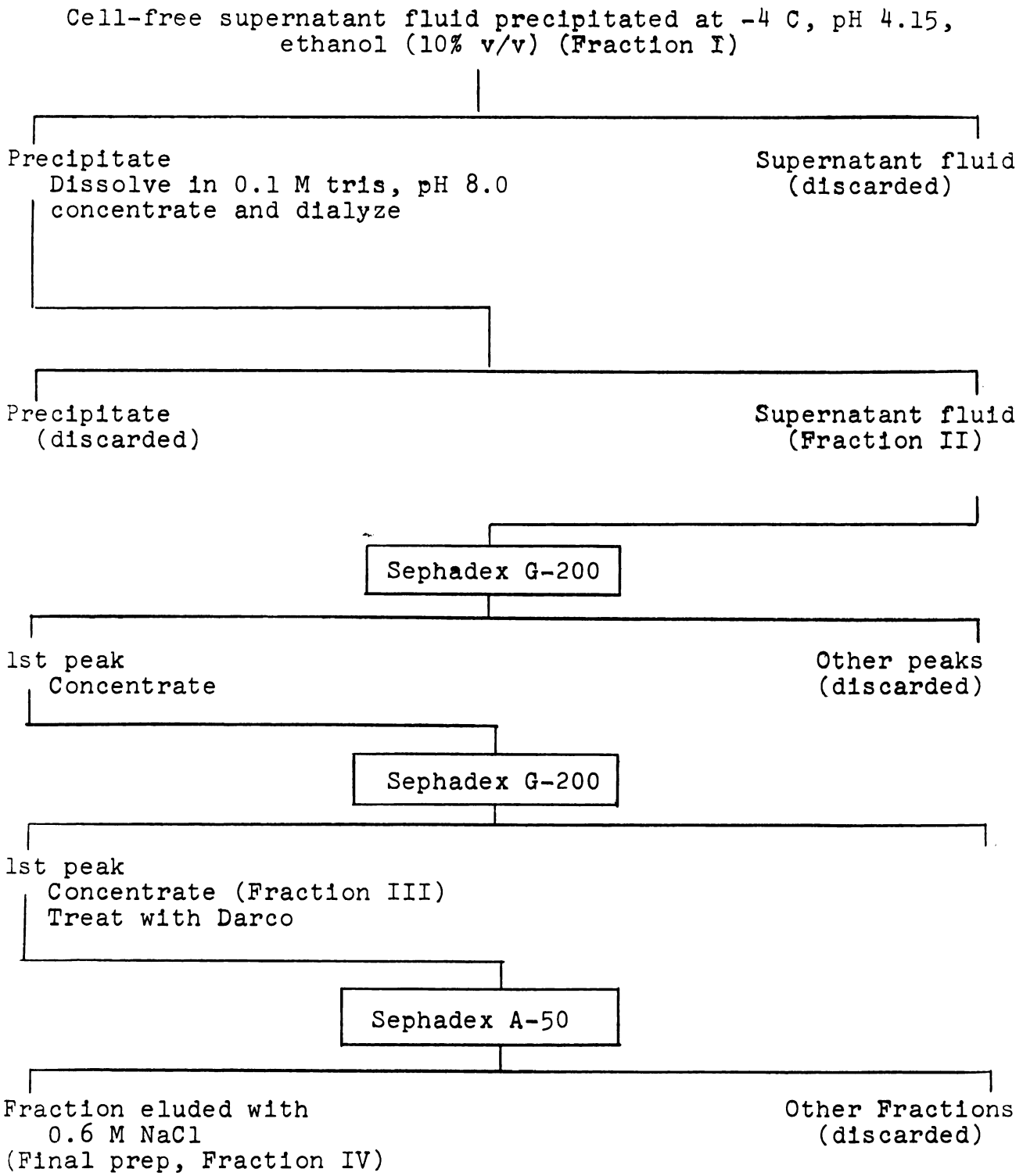

Fig. 2.--Flow-sheet diagram for preparation of purified lipase.

4.1 to 4.2 gave optimal precipitation of the product. Values below pH 4.0 caused slight inactivation of the enzyme, while values above pH 4.5 resulted in incomplete precipitation. The volume of ethanol used was not critical, concentrations between 10 and 40% gave approximately the same recovery of enzyme. However, its presence was indispensable. Complete precipitation of the enzyme required a standing period of at least 24 to 48 hrs at -4 C in the presence of 10% ethanol.

The precipitate from this step must be extracted at least four times with the tris buffer for redissolution. After dialysis and concentration, this crude material (fraction II) was passed through Sephadex G-200. The results of the active material recovered from the column are shown in Figure 3. The effluent volume between 75 and 140 ml was pooled, concentrated, and recycled through the same column. Concomitant protein and lipase peaks (Figure 4) were obtained by the recycling of this fraction through the column of Sephadex G-200. The single homogeneous peak represents a relatively pure substance. The effluent volume between 90 and 120 ml was pooled as fraction III.

This fraction is slightly opaque and usually pale yellow. Activated carbon (Darco) was added, and most of the color and opacity were removed. This step also removed a large part of the contaminating DN'ase that was present. The final purified product (fraction IV) is produced by eluting off DEAE-Sephadex A-50, concentrating, and dialyzing against 0.1 M tris buffer, pH 8.0. Characteristics of each

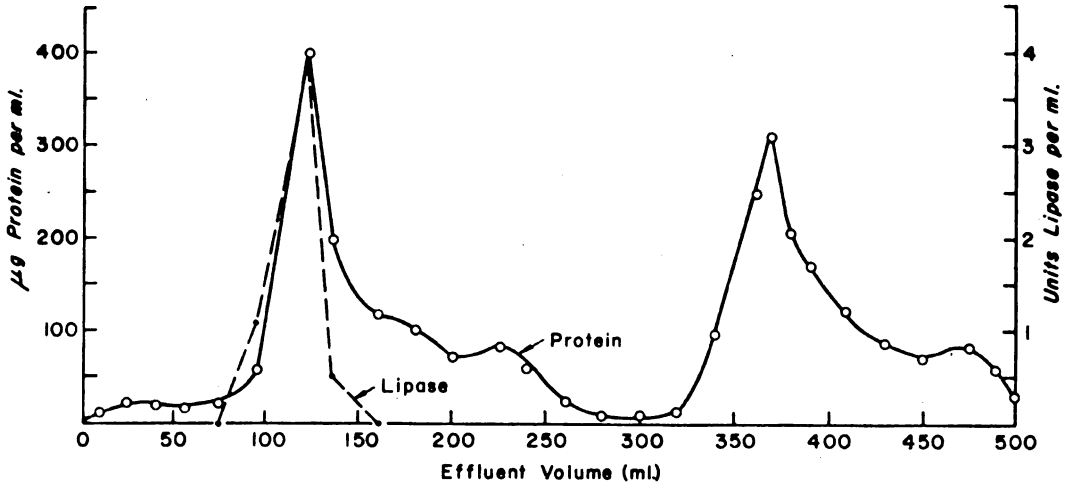

Fig. 3.--Elution pattern of crude lipase (fraction II) preparation from Sephadex G-200. The eluant was 0.1 M Tris (pH 8.0).

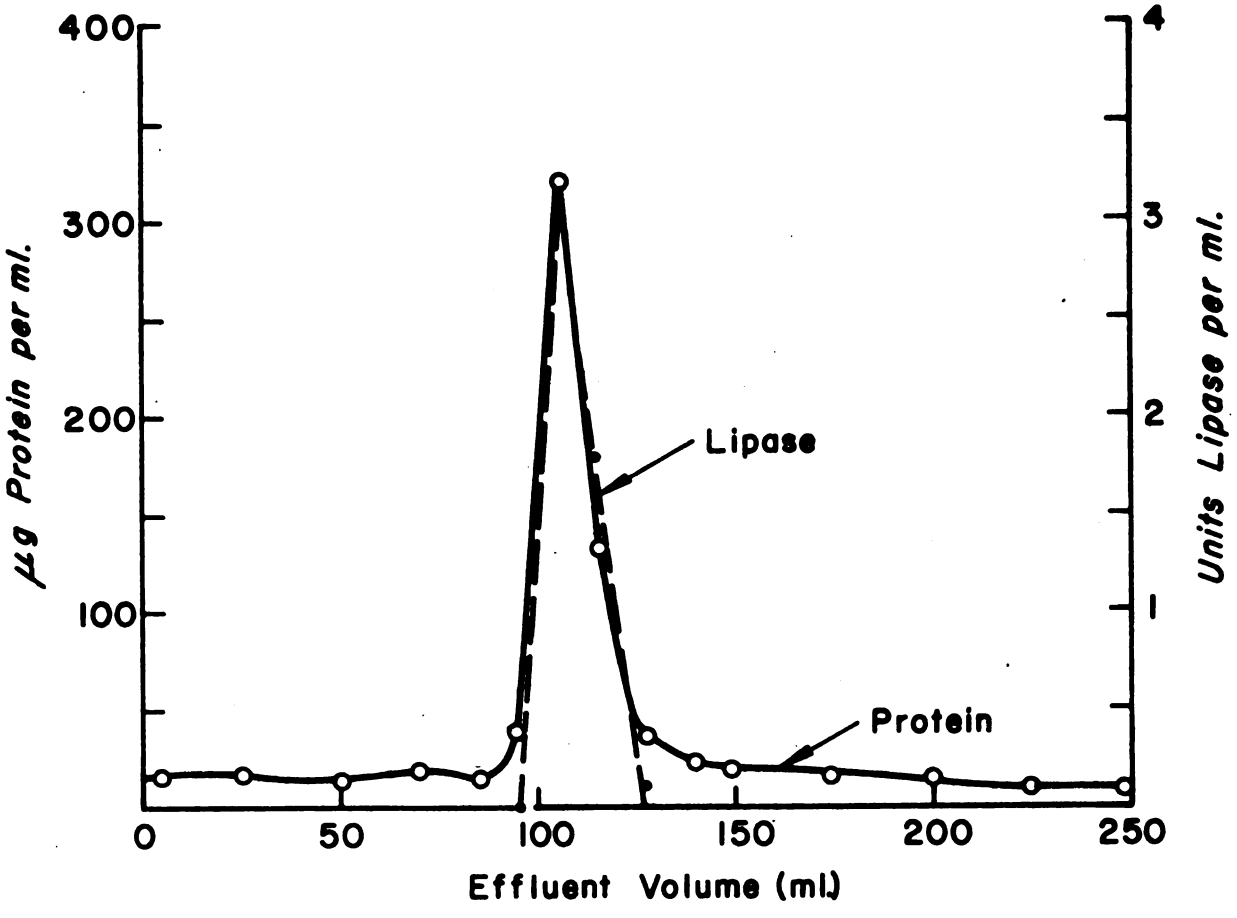

Fig. 4.--Elution pattern of recycled portion of active lipase (fraction II) through Sephadex G-200. The eluant was 0.1 M tris (pH 8.0).

fraction during a typical preparative procedure is shown in Table 5. From 20 liters of culture fluid a yield of 20-30% was obtained (100-150 mg) of a material having a purification factor exceeding 2000.

Characterization of Purified Lipase

Hydrolysis of olive oil substrate.--Figure 5 shows free fatty acid production or hydrolysis of the olive oil emulsion as a function of incubation time. Enzyme and substrate were mixed in the proper proportions and incubated at 40 C. Ten ml samples were removed at intervals and titrated. The curve deviates from linearity only slightly during the first two hours, but then deviation from linearity increases with time due to the inability of the buffer to maintain the optimal pH. There is a direct proportionality (Figure 6) between enzyme concentration and enzyme activity up to approximately 4 lipase units. Therefore, lipolysis was zero-order during the assay only until 4 lipase units were reached.

Effect of temperature on activity.--The effects of temperature on enzyme activity under these conditions are presented in Figure 7. Lipase activity was determined at various temperatures while all other variables were held constant. Optimal temperature was found to be about 40 C but measurable activity was demonstrated down to 7 C after the 1 hr incubation period.

TABLE 5.--Activities of each major fraction during purification of a lipase from Staphylococcus aureus 71

	Fraction	Specific Activity[1]	$\frac{280}{260}$ Ratio	Purification Factor
I	Cell-free supernate	0.002	0.43	1
II	Acid-alcohol precipitate	0.29	0.49	145
III	Sephadex G-200	1.56	1.27	780
IV	DEAE-Sephadex A-50	4.26	1.42	2130

[1]Expressed as lipase units/hr/mg protein.

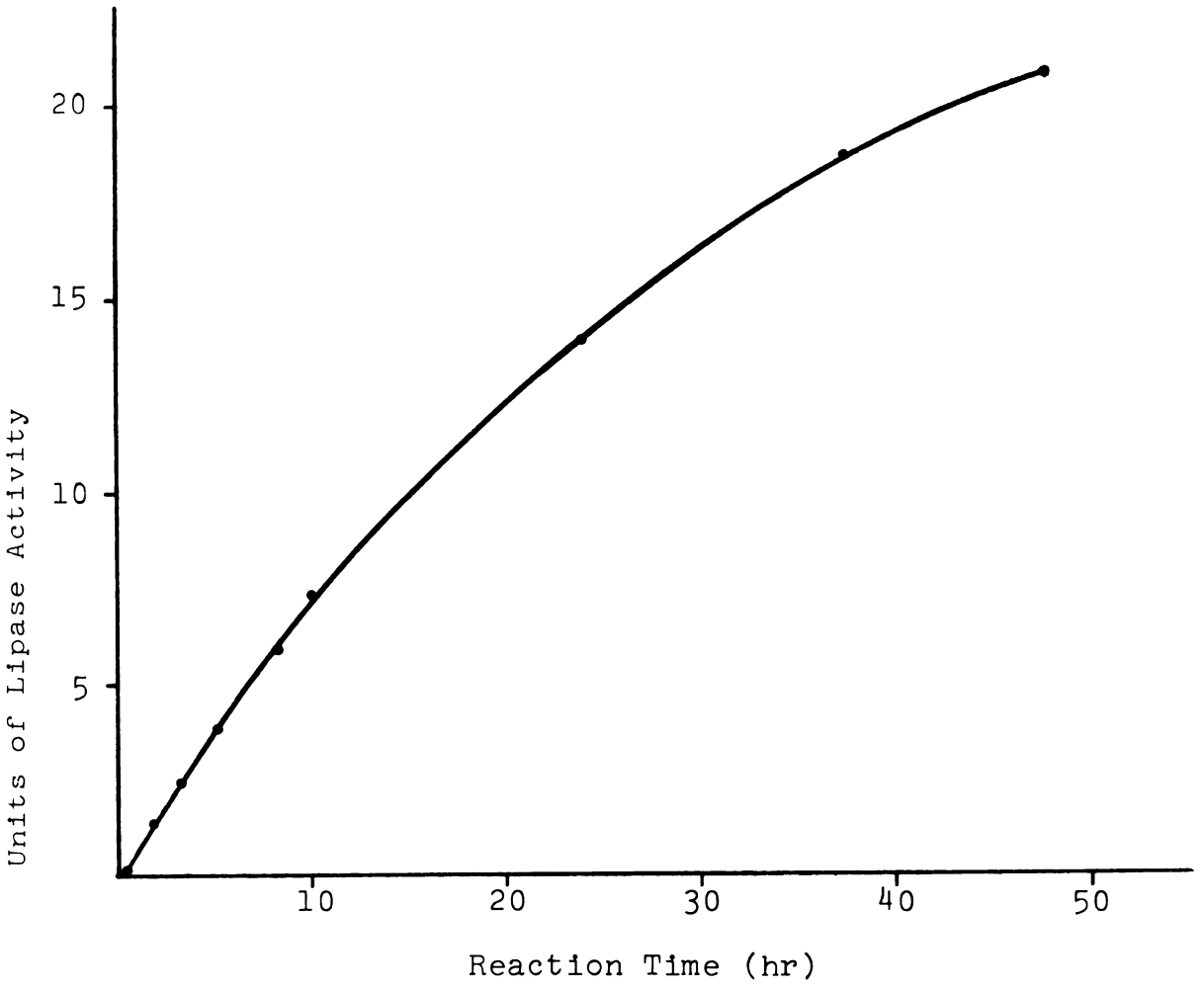

Fig. 5.--Rate of hydrolysis of the olive oil emulsion by a constant amount of purified staphylococcal lipase.

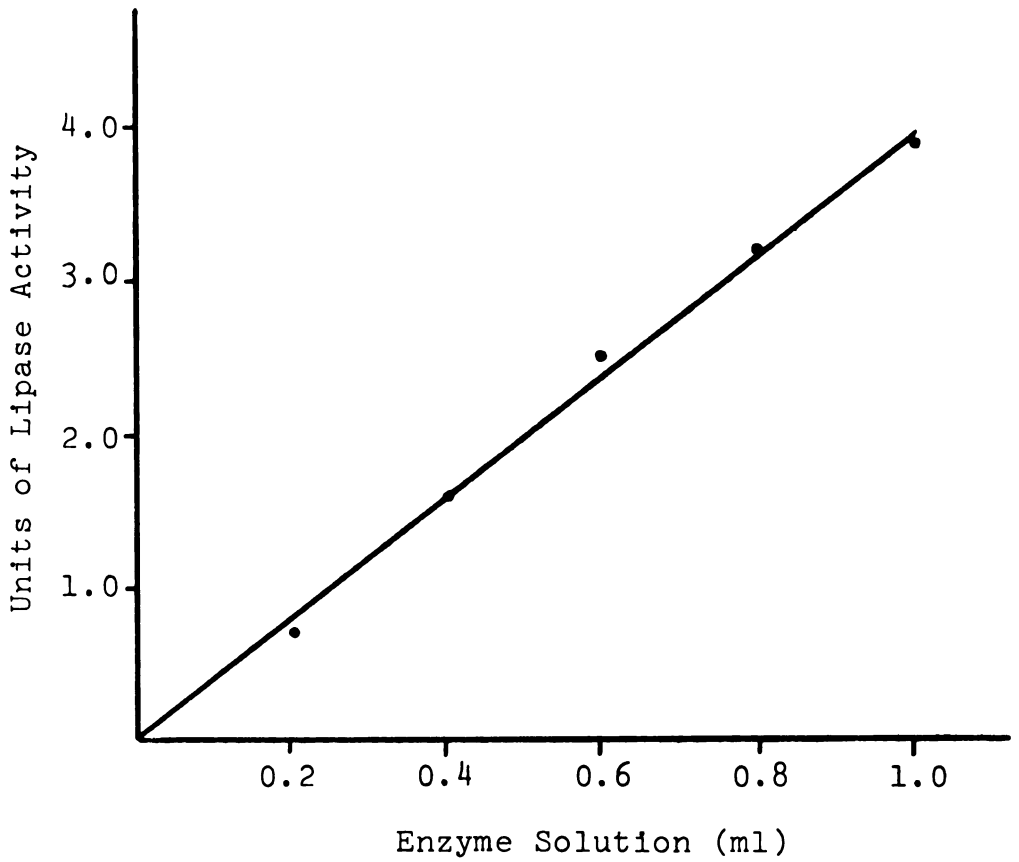

Fig. 6.--Lipase activity on olive oil emulsion as a function of enzyme concentration during a one hour period.

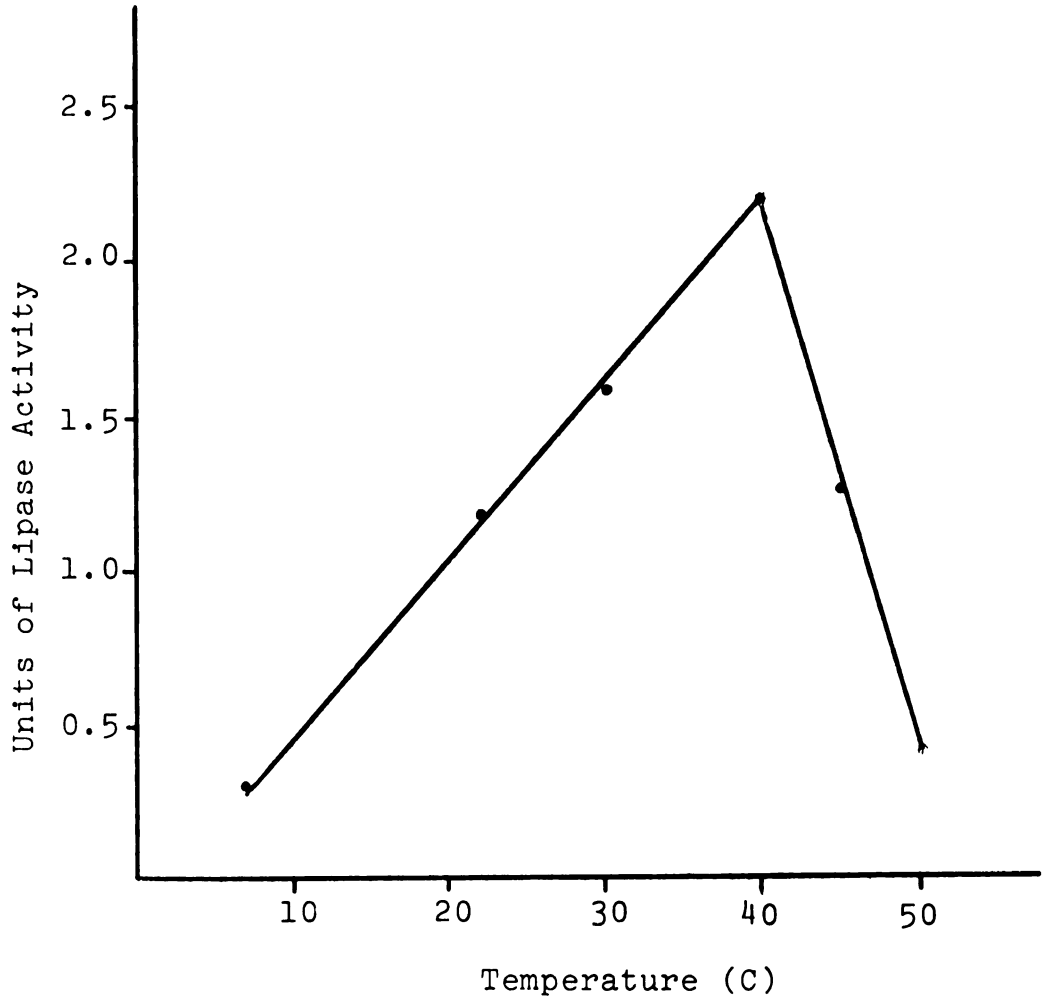

Fig. 7.--Lipase activity on olive oil emulsion as a function of temperature during a one hour period.

Effect of pH on Activity.--The effect of pH on lipolysis was determined between pH 6 and 10. Acetate buffer was used for pH 6.0, phosphate buffer for pH 7.0 and tris buffer for pH 8 to 10. Figure 8 shows the effect of pH on the lipolytic activity of the purified enzyme. The enzyme shows an optimal activity between pH 8 and 9 with maximal activity at approximately 8.2. The unexpected asymmetrical curve in Figure 8 induced a repetition of this experiment several times but the results were unchanged. The accelerated decrease in lipolysis in solutions of lower pH may be caused by the mass action effect of hydrogen ion as well as its specific inactivation of the enzyme. Other evidence (Figure 10) confirms increased enzyme stability on the alkaline side.

Effect of salts as activators.--A variety of salts and other substances have been reported to activate lipases by acting as emulsifiers, fatty acid acceptors, or cofactors. Table 6 shows the effects of various cations that were tested for their role as fatty acid acceptors. Calcium stimulated lipase activity to the greatest extent, followed by magnesium, manganese and potassium. In general, high concentrations of iron and zinc were inhibitory. Sodium desoxycholate had no effect, and 1% bovine serum albumin and ethylenediaminetetraacetic acid (EDTA) greatly decreased lipolytic activity.

Stability of the enzyme.--Staphyloccocal lipase is a relatively stable enzyme as the crude enzyme solution is

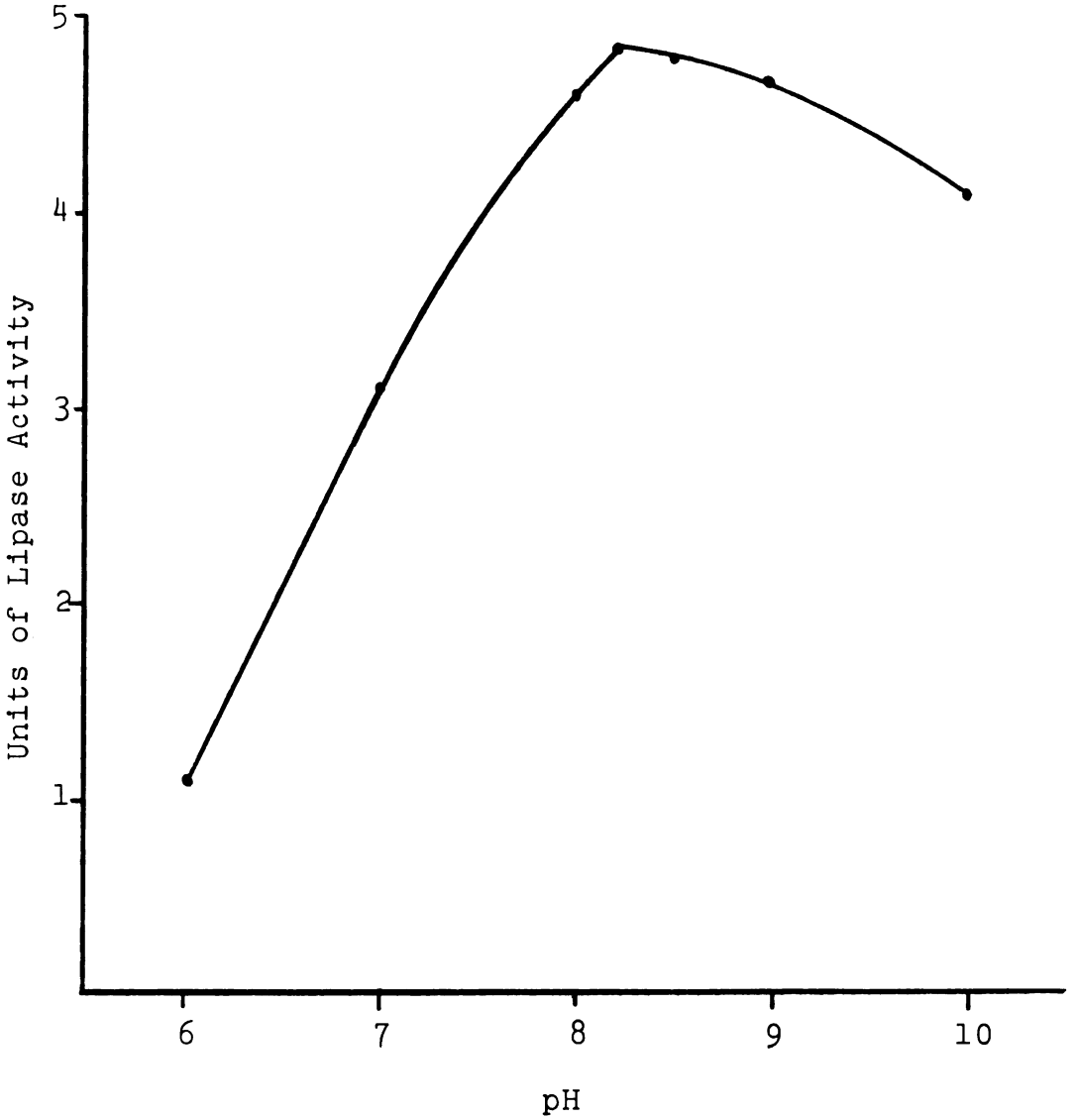

Fig. 8.--Lipase activity on olive oil emulsion as a function of pH during a one hour period.

TABLE 6.--Effect of salts as accelerators or co-factors for staphylococcal lipase

Substance	Final Conc.(M)	Relative Activity[1]
Calcium acetate	4×10^{-4}	131
	4×10^{-3}	159
Ferric sulfate	4×10^{-4}	116
	4×10^{-3}	61
Magnesium sulfate	4×10^{-4}	105
	4×10^{-3}	133
Manganous sulfate	4×10^{-4}	107
	4×10^{-3}	125
Zinc sulfate	4×10^{-4}	60
	4×10^{-3}	0
Sodium chloride	4×10^{-4}	110
	4×10^{-3}	119
Potassium chloride	4×10^{-4}	123
	4×10^{-3}	123

[1]Activity against control using distilled water arbitrarily set equal to 100.

stable for several months at room temperature. The purified enzyme was stable for over thirteen months at 7 C and over two years when stored at -20 C. Figure 9 shows the heat stability of purified lipase. One hour at 50 C had no effect, while at 56 C, over 50% of the enzyme was destroyed. One hour at 60 C completely inactivated the enzyme.

To study the effect of pH on the stability of the purified enzyme, samples were adjusted to various pH values and incubated at 56 C for 15 minutes. This time and temperature was enough to destroy approximately 25% of the activity at pH 8.0. The results are shown in Figure 10, which indicates the optimal pH for stability is approximately 9, rather than 8.2, the optimal pH for lipolysis.

Substrate specificity.--The substrate specificity of purified staphylococcal lipase was determined for several complex natural oils and a number of synthetic substrates. All natural oils were purified by alumina and lipase activity determined as previously described. The relative rates of hydrolysis of the complex oils are shown in Table 7. Most of the natural oils were either as good or better than olive oil as substrates for staphylococcal lipase. The only exception was castor bean oil which was hydrolyzed at approximately half the rate of olive oil. The effect of the purified enzyme against several synthetic substrates is shown in Table 8. Only sorbitan monolaurate showed greater hydrolysis than olive oil. More than half of the substrates showed less than one-third of the hydrolysis of olive oil.

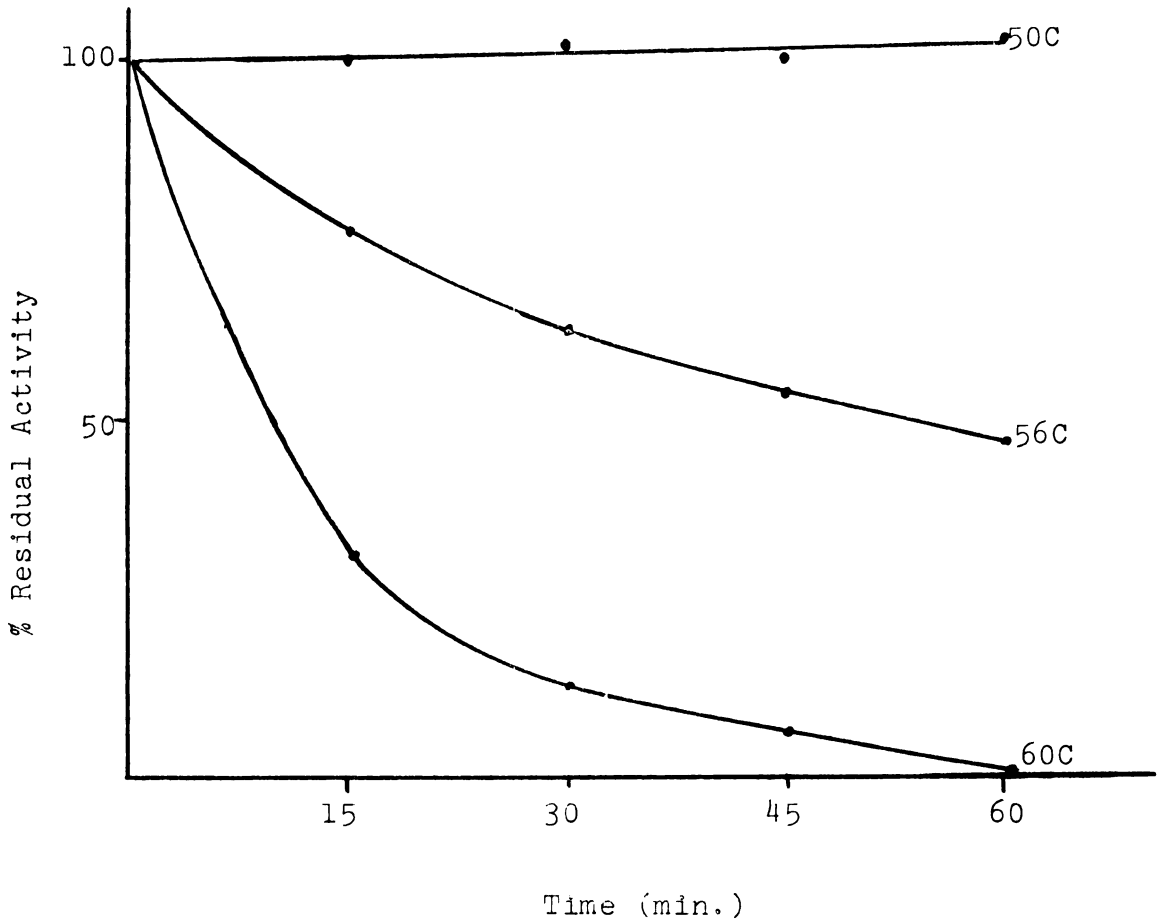

Fig. 9.--Effect of heat at three temperatures upon the stability of lipase in tris buffer (pH 8.0) as measured at 15 minute intervals.

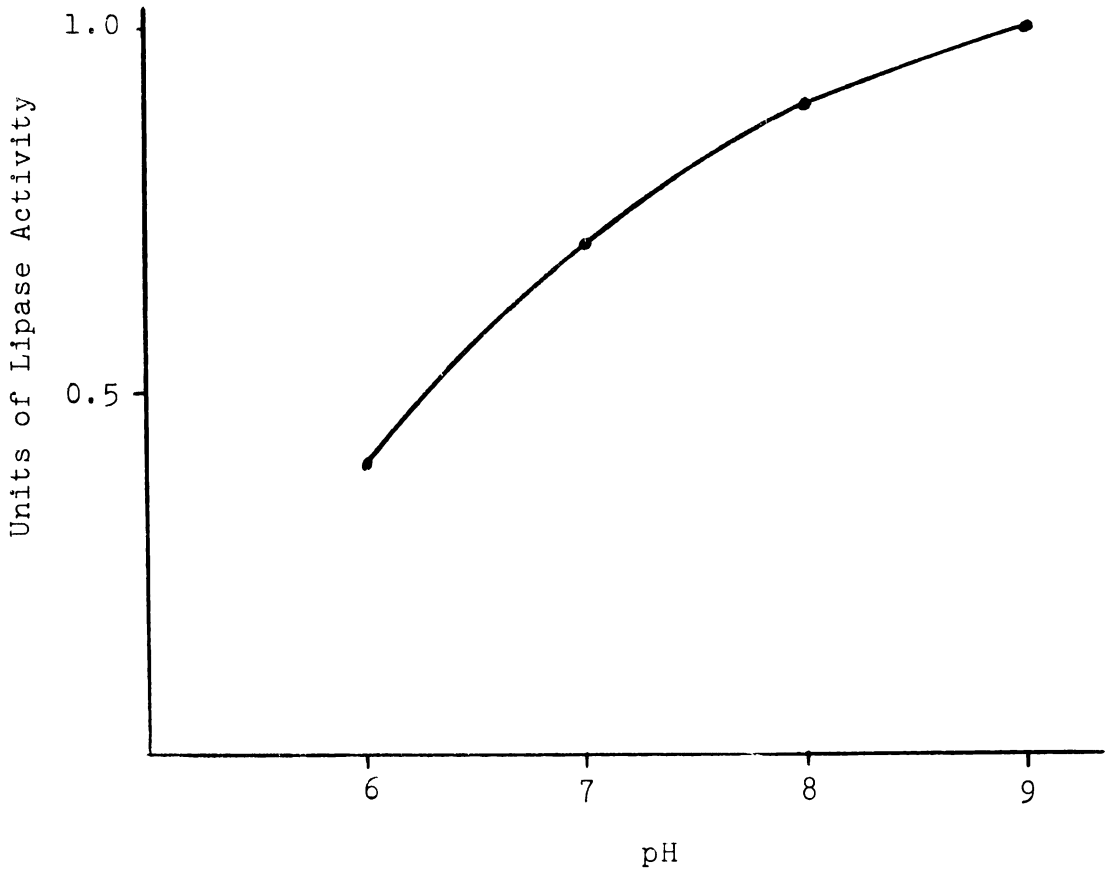

Fig. 10.--Effect of pH at four levels in tris buffer at 56 C for 15 minutes upon the stability of lipase.

TABLE 7.--Relative activity of purified lipase against natural oils as substrates

Substrate	Relative Activity[1]
Almond	143
Corn	131
Cottonseed	129
Sesame	120
Cod liver	111
Peanut	106
Safflower	106
Olive	100
Linseed	97
Castor	49

[1]Activity against olive oil arbitrarily set equal to 100.

TABLE 8.--Relative activity of purified lipase against synthetic substrates

Substrate	Relative Activity[1]
Sorbitan monolaurate	133
Glycerol monosterate	67
Glycerol monooleate	67
Sorbitan monosterate	56
Ethylene glycerol monosterate	45
Sorbitan monooleate	33
Tween 80	33
Tween 20	33
Sorbitan trioleate	22
Sorbitan tristerate	11
Tween 40	11
Sorbitan sesquioleate	0
Sorbitan monopalmitate	0

[1]Activity against olive oil arbitrarily set equal to 100.

Inhibitors.--A variety of substances have been reported to inhibit lipases. The effect of some of these substances was investigated with the following results. The two established esterase inhibitors, parathion and eserine were inhibitory only at high concentrations (Table 9). Arsenate and fluoride at 10^{-3} M inhibited staphylococcal lipase about 50% and the sulfhydryl inactivating reagent, iodoacetic acid, inhibited to a lesser extent. Isopropanol which inhibits the "hormone-sensitive" lipase of animals (Strand et al., 1964), also inhibits staphylococcal lipase. Triton WR-1339, an effective inhibitor of pancreatic lipase (Traxel and LeQuire, 1964), had no effect on staphylococcal lipase. The effect of metallic compounds was dependent upon the specific cation and its concentration (Table 10). High concentrations (10^{-3} M) of mercury, copper, silver and iron were inhibitory. High concentrations of magnesium and manganese, however, produced increased activity.

Electrophoresis.--Evaluation of protein homogeneity was obtained by use of cellulose acetate as well as disc electrophoresis. Cellulose acetate strips were divided in half lengthwise. One half was stained while the other half was placed on a tween agar plate to locate lipolytic activity. For comparative purposes, electrophoretic patterns of lipase fractions II and IV are indicated in Figure 11. The purified preparation (fraction IV) showed one main peak with a very small amount of inactive trailing material while the crude fraction (II) showed multiple protein components.

TABLE 9.--Effect of various inhibitors upon lipase activity

Inhibitor	Concentration	% Residual Activity
Parathion	10^{-3}M	4
	10^{-5}M	92
	10^{-7}M	100
Eserine	10^{-3}M	44
	10^{-5}M	88
Sodium fluoride	10^{-3}M	48
	10^{-5}M	92
Iodoacetic acid	10^{-3}M	64
	10^{-5}M	96
Sodium arsenate	10^{-3}M	52
	10^{-5}M	100
Isopropanol	7×10^{-2}M	52
	7×10^{-3}M	96
Triton WR-1339	10 mg/ml	100

TABLE 10.--Effects of certain metallic compounds on lipase activity

Compound	Concentration	% Residual Activity
$MgSO_4$	$10^{-3}M$	130
	$10^{-5}M$	100
$MnSO_4$	$10^{-3}M$	108
	$10^{-5}M$	80
$FeSO_4$	$10^{-3}M$	40
	$10^{-5}M$	104
$AgNO_3$	$10^{-3}M$	4
	$10^{-5}M$	44
$CuSO_4$	$10^{-3}M$	10
	$10^{-5}M$	36
$HgCl_2$	$10^{-3}M$	0
	$10^{-5}M$	20

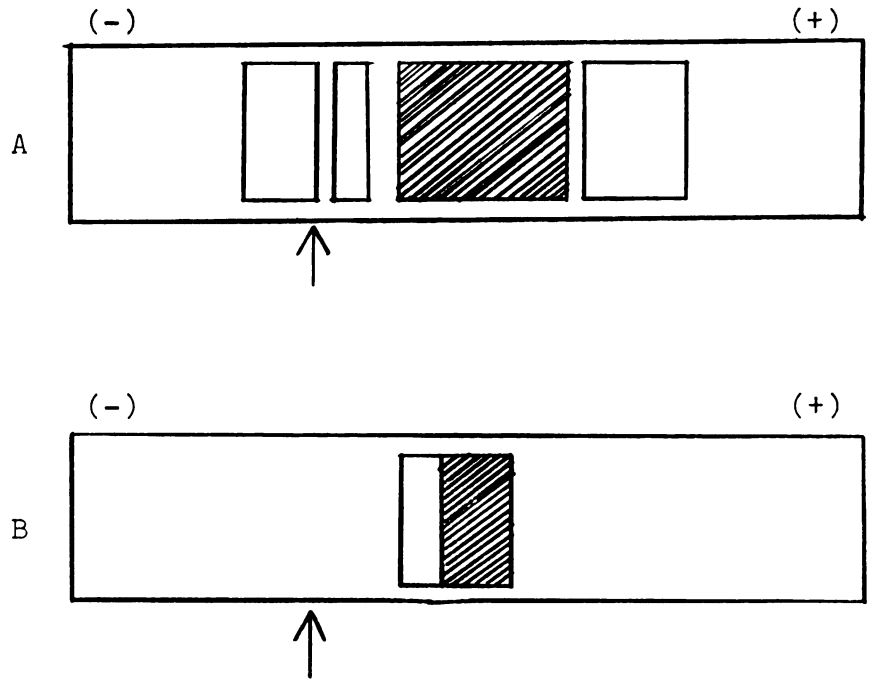

Fig. 11.--The complex electrophoretic pattern (Fig. 11A) on cellulose acetate of the crude lipase fraction II as contrasted with the simple pattern (Fig. 11B) of the final purified fraction IV. The diagonal lines indicate areas of lipase activity.

Disc electrophoresis of the purified preparation (Figure 12) shows two large bands with lipolytic activity and one small inactive band.

Effect of proteolytic enzymes.--To determine what effect proteolytic enzymes would have on purified lipase, 2% solutions of four enzymes were incubated for 30 minutes at 40 C with equal volumes of lipase solution. A lipase assay was performed on each and the per cent residual activity calculated (Table 11). Only the endopeptidase, trypsin, completely inactivated staphylococcal lipase.

Miscellaneous examinations.--To determine if this staphylococcal lipase might be identical to the "egg yolk factor" or the lipase producing free fatty acids from plasma lipids, qualitative plate assays were performed. Egg yolk agar was prepared by adding one part fresh egg yolk to 99 parts of 1% agar, pH 8.0; while plasma agar plates were prepared by adding 10% pooled human plasma to agar. Wells were cut into the plates, 0.25 ml of each fraction from the purification procedure added, and the plates incubated at 37 C. All fractions hydrolyzed both egg yolk and plasma, with fraction IV showing positive results within the first hour. In each case there was a progressive increase of hydrolysis as the lipase fractions became more pure.

As a criterion of homogeneity of the lipase preparation, specific enzymes known to be elaborated by S. aureus were qualitatively assayed for in each fraction. Five enzymes were present in S. aureus, P.S. 71. Coagulase was

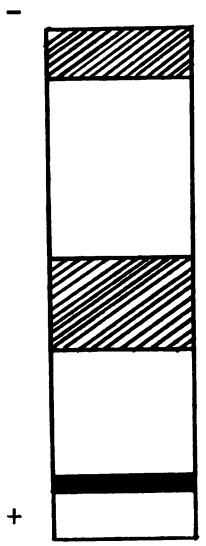

Fig. 12.--A diagrammatic representation of the protein pattern of purified staphylococcal lipase obtained by disc electrophoresis. The diagonal lines indicate areas of lipolytic activity.

TABLE 11.--Effect of certain enzymes on staphylococcal lipase

Enzyme	% Residual Activity
Rennin	100
Diastase	49
Papain	40
Trypsin	0

assayed for by adding 1 ml of diluted human plasma (1:4 with saline solution) to an equal amount of each lipase fraction, followed by incubation at 37 C for 24 hours. A visible clot formation constituted a positive test. Phosphatase activity was detected by the procedure of Barnes and Morris (1957) using p-nitrophenylphosphate as the substrate, and a pH of 10.2. Alpha-hemolysin was measured by incubating 0.5 ml of a 1% suspension of washed rabbit erythrocytes with 0.5 ml of each lipase fraction. Any hemolysis after 3 hrs incubation at 37 C constituted a positive test. Fibrinolysin was detected on fibrin plates made by adding 1% bovine fibrinogen to very hot agar. Wells were cut into the agar, the various fractions added and the plates incubated at 37 C for 24 hours. A positive test was the appearance of a clear zone around the well. DNase Test Agar (Difco) was used to detect deoxyribonuclease activity. Wells were cut into the agar, samples applied and incubation was at 37 C for 12 hours. After flooding the plates with 1N HCl, positive reactions were recognized by clear zones around the wells. These enzymes (Table 12) were progressively eliminated during the purification process. Coagulase, phosphatase, and alpha-hemolysinwere separated by molecular sieving through Sephadex G-200. Fibrinolysin was apparently concentrated during the acid-alcohol precipitation step, but eliminated by passage through Sephadex G-200. DNase, on the other hand, was not eliminated until the final step of purification.

TABLE 12.--Elimination of associated staphylococcal products during purification of lipase

Staphylococcal Product	Purification Fraction			
	Cell-free Supernate	Acid-alcohol Precipitate	Sephadex G-200	DEAE-Sephadex A-50
Coagulase	+	+	-	-
Phosphatase	+	+	-	-
Alpha-hemolysin	+	+	-	-
Fibrinolysin	-	+	-	-
DNase	+	+	+	-

A micro-Kjeldahl nitrogen determination as well as the determination of total phosphorus by the Fiske-Subbarow method (Kabat and Mayer, 1961) were done on the purified enzyme. Total nitrogen found was 15.7%, while there was less than 2% phosphorus present, indicating that the enzyme is probably a protein of high purity.

Immunology

Antilipase production.--Five rabbits were used for the production of antilipase serum. Three of the rabbits were given a total of 10 mg each of a 50-50 mixture of fractions II and IV for use in the gel diffusion studies. The other two rabbits received 1 and 2 mg of purified lipase (fraction IV), also given over a three months period in three equal injections. A series of eight mice received 1 mg each of the purified lipase; and two cows were given 7 and 19 mg respectively, over a period of four months. The rabbits and mice received complete Freund's while the cows received incomplete Freund's as the adjuvant. Two of the eight mice died within two days, probably indicating that this amount of enzyme is close to the toxic level. Five of the six surviving mice also developed some morbidity for approximately six days. The injections, however, had no noticeable effect on either the rabbits or cows.

Because Freund's adjuvant contains oils, the effect of purified lipase on the complete adjuvant was studied to determine if appreciable hydrolysis takes place. A lipase assay

using the adjuvant as the emulsion was set up and aliquots were titrated at intervals. No detectable hydrolysis of the emulsion occurred during the first six hours, and by 24 hours only a small amount of titratable acidity was detected.

Serological studies.--All three of the animal species produced detectable antibody to lipase. Both rabbits and cows also produced antibody to lipase without use of the adjuvant, showing that the enzyme is fully antigenic. The antibody titers of the three species as determined by the various serological tests are shown in Table 13. Agglutinating, precipitating, and neutralizing antibodies were produced in all animals injected with purified lipase. No antibodies were detected in the preinjection sera of any of the animals.

Gel diffusion studies were used as one of the several criteria for the determination of protein homogeneity. By injection of a mixture of crude (fraction II) and purified (fraction IV) lipase into rabbits and testing the antisera against each of the increasingly pure antigenic fractions, a progressively decreasing number of precipitin bands has occurred. Results (Figure 13) show this to be true. Many precipitin bands are formed with fraction II, three bands occur with fraction III, and with the purified lipase (fraction IV), two bands are present, a heavy band, probably indicating the lipase-antilipase reaction, and a weak component indicating the presence of a trace impurity.

TABLE 13.--Antibody titers of various serological tests on animals injected with purified staphylococcal lipase

Animal	Antibody Titer			
	Latex-slide Agglutination	Ring Test	Tween Neutralization	Lipase Neutralization
Rabbit	1:80	1:40	1:32	1:4
Mouse	1:160	1:20	1:8	1:8
Cow	1:20	1:10	1:8	1:4

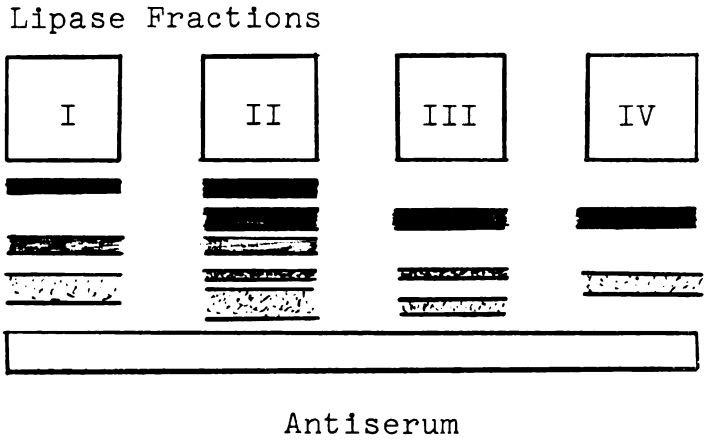

Fig. 13.--Reproduction of the precipitation bands prepared from the gel diffusion technique for each of the four fractions of lipase obtained during the purification procedure.

Survey of Staphylococcal Strains

Some 160 strains of coagulase-positive and coagulase-negative staphylococci were checked for lipolytic activity. These included clinical isolates of both human and animal origin, as well as the propagating strains. Lipolysis was detected by streaking on plates of tweens 20, 40 and 80 (Sierra, 1957). The results of this survey are shown in Table 14. Tween 20 was easily hydrolyzed by all strains. While all human strains degraded tween 40, some of the animal strains did not. On the other hand tween 80 was the most resistant of the group. In the latter case, the human strains, coagulase-positive, were the most active. It should also be observed that tween 80 was not hydrolyzed by phage-propagating strains 53, 54 and 75. From Figure 1 it should be noted that neither did the cell-free filtrates of the same strains hydrolyze the emulsified olive oil.

TABLE 14.--Hydrolysis of tweens by human and animal staphylococcal strains

Species	Percent of Strains Hydrolyzing		
	Tween 20	Tween 40	Tween 80
Human coag-pos. (27)*	100	100	89
coag- neg. (81)	99	98	76
Animal coag-pos. (41)	100	85	75
coag-neg. (12)	100	83	83

*Number of strains.

DISCUSSION

In the investigation of enzymes it is very important to have a sensitive and specific assay method as well as one which is relatively simple and not too time consuming. For most lipolytic procedures a long incubation period is essential because the rate of hydrolysis of an oil emulsion is very slow. The colorimetric procedures (Nachlas and Seligman, 1949; Kramer et al., 1964) using chromogenic substrates were first investigated as these have short incubation periods as well as great sensitivity. However, the use of these procedures was rejected because of the instability of the chromogenic substrates as well as the possibility of measuring esterase instead of true lipase activity.

The various procedures using oil emulsion as the substrate were investigated and the olive oil procedure of Tietz et al. (1959) was modified for use with this particular staphylococcal enzyme. The modifications included an increase in buffering capacity and the addition of calcium acetate to accelerate the reaction. A strong buffering capacity is important in this system because of the large amounts of fatty acids being released during the relatively short incubation period. Shah and Wilson (1963, 1965) found that calcium was essential as a fatty acid acceptor

for the staphylococcal lipase which produced the egg yolk reaction. They found $CaCl_2$ in a concentration of 10^{-3} to 10^{-4} M to be optimal in their test system. In the present investigation calcium acetate proved to be the best fatty acid acceptor of the various salts tested, but the actual optimal concentration was indeterminate because of the adverse effect upon the stability of the oil emulsion. These results appear to indicate that calcium ions act as a fatty acid acceptor for lipase activity and not as a co-factor or coenzyme.

In this assay the addition of calcium acetate, as well as a strong buffer, greatly enhances the reaction rate and reduces the time of incubation from a fraction of a day to one hour. Because of this increased rate it is important to terminate the reaction rapidly. With other lipases this precaution is unnecessary because of the slow reaction rates. Most methods use either ethanol or acetone to inhibit the enzymatic reaction, but these inhibitors did not work with staphylococcal lipase as concentrations up to 50% failed to stop the lipolysis. Therefore, a boiling water bath or flowing steam were adequate as enzyme inactivators providing certain substrates such as olive oil were used.

Staphylococcal lipase is believed to be an extra-cellular enzyme which is produced in very low quantities. Most of the commercial dehydrated media which supports staphylococcal growth, as well as some semi-synthetic media, were screened for lipase production, but no medium was

superior to any other. Conditions for cultivation were studied and in no instance could lipase production be increased greater than two or three fold. It therefore appears that staphylococcal lipase is a constitutive enzyme and is not adaptive or inducible. Gonzalez (1963) found lipase to be a constitutive enzyme for Aeromonas and Vibrio, while in Clostridium it was both constitutive and adaptive.

Because of the low yield of staphylococcal lipase, several different purification methods were attempted. Initially, a procedure using acid and alcohol precipitation under controlled conditions (Renshaw and San Clemente, 1964) yielded a highly purified enzyme preparation. Later it was found that recycling through Sephadex G-200 produced essentially the same degree of purification with a much greater yield of enzyme. An additional step involving DEAE-Sephadex A-50 completed the purification process to an extremely high degree. Final preparations showed an approximate 2000-fold increase in activity per mg protein. O'Leary and Weld (1964) purified a staphylococcal lipase by a somewhat similar procedure with a final purification factor of approximately 225. Lipases from other sources have been purified by various workers with purification factors ranging from 50 to approximately 450 fold. It was therefore concluded that our method gave an additional 5 to 10-fold increase in purity over that achieved by other investigators.

Staphylococcal lipase, like pancreatic lipase, is an enzyme of high molecular weight. On Sephadex G-200, the

lipase occurs in the first peak immediately after the void volume of the column, and represents a particle with a molecular weight exceeding 200,000. Hallander (1963), who fractionated staphylococcal toxins by gel-filtration, found that lipase, alkaline phosphatase and delta-hemolysin all had molecular weights of greater than 200,000, as these all passed out in the void volume of Sephadex G-200. Gelotte (1964) as well as Sarda and co-workers (1964), studied the behavior of hog pancreatic lipase on Sephadex. They found pancreatic lipase to be separated into two fractions, probably consisting of polymer and monomer forms. The molecular weight of the monomer was estimated to be approximately 35,000 to 38,000. Sarda et al. (1964) suggest that the enzyme might be a lipoprotein in its higher polymeric form because a unit of smaller molecular weight was produced when the enzyme was subjected to treatment known to rupture lipoproteins, i.e., Na deoxycholate treatment. In the present investigation, however, no low molecular weight forms were detected, even after treatment with Na deoxycholate or ethanol-ether, so it appears that staphylococcal lipase is probably neither a lipoprotein nor a polymer made up of these distinct units, at least under the conditions studied here.

Immuno-diffusion studies were conducted to determine serological distinction. Kabat and Mayer (1961) stated that gel diffusion methods are among the most powerful tools which the immunochemist has available for establishing the presence

of mixtures of antigens and antibodies; however, these procedures also possess certain limitations. For instance, the quantity of antibody may be insufficient to produce discernible precipitin bands. Also, the presence of non-precipitating antibody may be responsible for similar results. On the other hand, the presence of two bands does not always indicate a mixture of antigenic or antibody components since an excess of antigen may diffuse beyond the original zone of specific precipitate to form a broad or second band. By using a mixture of relatively crude and highly purified lipase preparations, the antisera should contain antibodies against all of the contaminating proteins. This antiserum, when tested against each of the series of pure fractions, should show a decreasing number of precipitin bands. Two such precipitation zones were present with the highly purified lipase (fraction IV), a very heavy sharp zone, probably indicating the lipase-antilipase system and a weak, diffuse zone. This weak precipitation zone was only present with high concentrations of the purified enzyme, indicating that it was a trace impurity.

Besides immuno-diffusion, other studies were made to determine the homogeneity of this enzyme. Cellulose acetate electrophoresis showed one main band with a very small amount of inactive trailing material.

Disc electrophoresis, which is much more sensitive than cellulose acetate electrophoresis, showed two main bands with lipolytic activity and a small band of inactive

material. The band nearest the cathode showed approximately three times as much lipolytic activity as the middle band, which appears to contain more protein. However, as a water soluble substrate was used, it is not possible to say that these two bands are true lipases or isozymes. Another possibility is that the band nearest the cathode is the polymeric form while the middle band is the monomer. Disc electrophoresis also separates by molecular sieving and large proteins (19S globulins) will not penetrate the gel to any extent.

As a further criterion for the homogeneity of the lipase preparation, detection of specific enzymes known to be elaborated by this strain of S. aureus was effected in each fraction. Five such enzymes (coagulase, phosphatase, alpha-hemolysin, fibrinolysin and DNase) were eliminated during the purification process.

Staphylococcal lipase is a very stable enzyme, with broad tolerances of both pH and temperature; it survives storage in .01 M calcium acetate at 7 C for over one year and probably indefinitely at -20 C. The staphylococcal lipase purified by O'Leary and Weld (1964) was sensitive to repeated freezing and thawing while our enzyme was not. The optimal pH for lipolysis was found to be 8.2 which agrees with that found by O'Leary and Weld (1964), but differs slightly from that found by Shah and Wilson (1965) which was 7.8. These latter workers also found greatest enzyme stability to be at pH 7, while the optimal pH for

stability found here was 9.0 during incubation at 56 C for 15 minutes.

Several of the characteristics of this enzyme are similar or at least comparable to the enzyme responsible for producing free fatty acids from plasma lipids, reported in the recent paper of O'Leary and Weld (1964). It is also somewhat comparable to the enzyme responsible for the "egg yolk factor" (Shah and Wilson, 1964); it is possible, indeed likely, that these three are the same enzymatic entity and differ only by degree of purity and methods of study. The enzyme isolated here showed activity against both egg yolk and human plasma, showing increased activity with each purification step. However, it may be that staphylococcal lipase contains a family of enzymes or isozymes. Unfortunately our attempts to detect lipase isozymes was a failure because the emulsified oil substrate precluded observable lipolysis prior to extensive diffusion of the lipase preparation. Until a suitable substrate for true lipase activity is devised to permit a rapid reaction, characteristic of most colorimetric reactions, the obstacle to the elucidation of a potential lipase isozyme pattern will remain.

The term "lipase" usually covers a large series of enzymes which hydrolyze various soluble and insoluble aromatic or aliphatic esters. This term has also been used in a synonymous manner with the term "esterase." Sarda and Desnuelle (1958) showed that the true substrate of

pancreatic lipase is an insoluble ester emulsion rather than a soluble ester. Substrate specificity shows this to be true; all natural oil emulsions proved to be very good substrates for this staphylococcal enzyme. The one exception is castor bean oil which is hydrolyzed at approximately half the rate of the other oils. Castor bean lipase, however, is an acid reacting lipase, with an optimal pH of 4.0-4.2. With the exception of sorbitan monolaurate, all the soluble synthetic substrates were hydrolyzed at low rates, if at all. These observations suggest that staphylococcal lipase is able to hydrolyze emulsions rapidly, but also acts very slowly on substrates in true solution.

That the staphylococcal enzyme is a lipase rather than an esterase is also supported by inhibition studies. True lipases are resistant to most enzyme inhibitors, only sulfhydryl inactivating agents and heavy metals are effective inhibitors. These two groups showed active inhibition of the purified enzyme, while two established esterase inhibitors (parathion and eserine) were operative only at high concentrations. These esterase inhibitors are very potent and will inhibit esterases at very low concentrations (10^{-5} to 10^{-7} M), and lipases only at high concentrations.

Davies (1954) suggested that the lipases of staphylococci are antigenically homogeneous and found no evidence of naturally occurring antilipase antibody in normal rabbits. Gonzalez (1963 a,b), using purified lipases of Aeromonas and Vibrio, found both precipitating and neutralizing antibodies

in rabbits. He also found the enzymes to be fully antigenic and that their capacity was enhanced with Freund's adjuvant. In the present investigation, staphylococcal lipase was fully antigenic, producing agglutinating, precipitating and neutralizing antibodies in rabbits, mice and cows, and no naturally occurring antilipase was detected in any of the animals.

SUMMARY

An extracellular lipase of Staphylococcus aureus, P.S. 71 was purified some 2000-fold by acid-ethanol precipitation, followed by two passages through Sephadex G-200, treatment with activated carbon and a final passage through DEAE Sephadex A-50. This enzyme, of high molecular weight, was free of coagulase, phosphatase, alpha-hemolysin, fibrinolysin and DNase, but electrophoresis and gel diffusion indicated that it was not completely homogeneous.

Enzymatic activity was assayed by electrometric titration of the liberated free fatty acids from an emulsified olive oil substrate. By use of a strong buffering capacity as well as the addition of calcium acetate the time of incubation was greatly reduced over that of standard lipase procedures.

Staphylococcal lipase is a very stable enzyme with broad tolerances of both pH and temperature. It survives storage at 7 C for over one year and probably indefinitely at -20 C. It is active over a range of 7 to 50 C, with an optimum at 40 C. It is, however, completely inactivated in one hour at 60 C. Its optimal pH is 8.2 with a range of 6 to 10, while the optimal pH for stability is 9.0. It requires calcium salts as an accelerator, and is easily

inactivated by trypsin. Substrate specificity and inhibitor studies show it to be a true lipase. It is fully antigenic and produces agglutinating, precipitating and neutralizing antibodies in rabbits, mice and cows.

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