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The lipase of Pseudomonas fragi

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NOTE TO USERS

THE LIPASE OF PSEUDOMONAS FRAGI

by

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INTRODUCTION

Many microorganisms are capable of attacking fats and causing objectionable flavors in dairy products. In most cases, the microorganisms isolated from defective dairy products have been largely gram-negative bacteria. Among these, Pseudomonas fragi has been found to occur frequently. The organism is widely distributed in nature and commonly is encountered in water, soil and farm surroundings.

The systematic study of Ps. fragi, regarding its physiological and morphological characteristics, has been adequately covered. The ability of the growing cells of Ps. fragi to produce lipase and cause defects in dairy products, especially butter and cream, has been the object of much study. However, little information has been reported concerning the factors that affect lipase production by this organism, and still less is known regarding the characteristics of the liberated lipase.

In determining whether a certain organism is lipolytic or not, much will depend upon the suitability of the medium for growth and for lipase production. Even though the organisms might find the conditions conducive to growth, yet these same conditions might not necessarily favor lipase production. Lipase, like other extracellular enzymes, usually is liberated

in the growth medium and begins to act on its substrate independent of the parent cells. Therefore, the study of lipolytic systems independent of the living cells offers a better insight into the behavior of lipase action.

Recently, the Food and Drug Administration has been condemning butter and cream in Interstate Commerce on the basis of their content of water-insoluble fatty acids. These fatty acids are primarily the result of the hydrolysis of butterfat by the different lipases. It has been shown that under some circumstances microbial lipases were more active in this respect than milk lipase. Also, the lipase produced by Ps. fragi during growth in cream and butter attacks butterfat and consequently causes an excessively high content of water-insoluble fatty acids, rendering the product unfit for marketing.

This study has been undertaken in order to supply information regarding the characteristics of the lipase of Ps. fragi and the conditions under which it is produced, so that the difficulties caused by its presence in dairy products might be minimized.

STATEMENT OF PROBLEM

The object of this work was to obtain information on the lipolytic system of Ps. fragi. For that purpose, the work was subdivided into the following phases:

Establishment of a reliable method for the measurement of lipase activity.

Characterization of the lipase produced by Ps. fragi.

Investigation of the factors that influence lipase production by this organism.

Application of some of the results to cream and butter.

REVIEW OF LITERATURE

Description and Distribution of *Pseudomonas fragi*

The organism has been encountered by several workers and has been known under different names. This early work has been reviewed by Hussong *et al.* (1937) and will not be repeated here. Hussong (1932) studied this organism in detail and proposed the name *Pseudomonas fragi*. Hussong *et al.* (1937) were able to isolate the organism from a considerable number of normal and abnormal samples of various dairy products. *Ps. fragi* produced three distinct types of colonies -- S (smooth), O (intermediate), and R (rough) -- when grown on the usual agar media. The smooth type regularly hydrolyzed butterfat and olive oil, the intermediate type usually did not hydrolyze fat, and the rough type had a variable action on fat, some cultures hydrolyzing it and others having no action. The organism was found to be gram-negative non-sporeforming rod, motile by means of polar flagellum. It grew well at 20° C. but not at 37° C., and grew slowly at 3-5° C. It was found to be sensitive to heat, only one of five cultures tested surviving heating at 68.2° C. for 10 minutes and most cultures were killed by very much shorter exposures than this. The organism invariably produced a small ring of acid curd at the

surface of litmus milk; this acid curd did not extend to the bottom of the test tube until after long incubation. Another consistent characteristic was the production of an odor resembling that produced by the flower of the may apple. Variant types which were unable to hydrolyze fat could be secured from parent cultures with a marked fat-splitting action.

Long (1936) found that the production of an acid ring in litmus milk was not always observed except with most of the freshly isolated cultures. Out of 44 cultures examined on agar plates containing cottonseed oil, 28 cultures hydrolyzed only a part of the fat globules below the growth area, while 16 hydrolyzed all of the globules. All the cultures were relatively consistent in the production of a diffusible lipolytic enzyme, only one culture failing to hydrolyze the fat within a considerable area around the growth.

Collins (1933) found that the lipolytic bacteria most commonly isolated from dairy equipment, wash water, and from certain dairy products were Ps. fragi, Pseudomonas fluorescens, Achromobacter lipolyticum, and Micrococcus sp. From the studies of Morrison and Hammer (1941) on barnyard soil from many states, it was evident that Ps. fragi was widely distributed. They isolated it from 29 out of 176 samples of milk delivered to an Iowa plant during the cool seasons.

A full description of Ps. fragi is given by Breed et al. (1948).

Determination of Lipase Activity

Numerous procedures have been used in determining the activities of various lipases during the last half century.

In his investigation of the lipase of Penicillium glaucum Gerard (1897) titrated a substrate containing two per cent monobutyrin with 0.1 N sodium hydroxide after incubation for various intervals. Camus (1897) and Garnier (1903) used the same titration procedure in their work on the lipases of aspergilli.

Wells and Corper (1912) used olive oil, ethyl butyrate and triacetin emulsions as substrates for testing the activities of the lipases of various microorganisms. They extracted the free fatty acids from the substrates with ether and then titrated the ether extract with alcoholic sodium hydroxide. These investigators stated that the fatty acids liberated by the action of the lipases on the substrate are held in the emulsion in such a way that the aqueous alkaline solution does not readily reach them, with the result that titrations in aqueous suspensions are not at all accurate. Kendal et al. (1914) employed 0.25 per cent triacetin or ethyl butyrate as substrate, adjusted the reaction to the phenolphthalein endpoint, and titrated the medium after a reaction period of 24 hours at 37° C. Naylor and Smith (1930) also used ethyl butyrate as a substrate, allowing it to react

with the lipase of Penicillium roqueforti for 10 days at 30° C., and then titrating it with standard alkali.

Willstätter et al. (1923), in their work on pancreatic lipase, used olive oil as a substrate. At the end of the reaction period, they added 95 per cent ethanol and ether to their substrate and titrated it with 0.2 N alcoholic sodium hydroxide, using thymolphthalein as an indicator.

Fleming and Neill (1927) measured the activity of the lipase of the Welch bacillus (Clostridium perfringens) by observing the change in pH of the tributyrin substrate at the end of the reaction period. Tammisto (1933) also used tributyrin as a substrate for measuring the activity of the lipases of B. fluorescens liquefaciens (Pseudomonas fluorescens) and B. prodigiosum (Serratia marcescens).

Milk was used for emulsification of the different fats and oils on which the action of pancreatic lipase was studied by Hartwell (1938). The emulsions were titrated with 0.1 N sodium hydroxide. Lubert et al. (1949a) devised an extraction-titration procedure for estimating microbial lipases. They used tributyrin emulsified in skim milk as a substrate, and a reaction period of 30 minutes at 37° C. Butyric acid was extracted with ether after acidification with phosphoric acid.

The action of the lipase of Penicillium roqueforti was tested in buffered three per cent butterfat emulsion

containing 0.5 per cent arabic gum as an emulsifier by Thibodeau and Macy (1942). Fifty ml. of a 1:1 mixture of alcohol and ether were added to a 10 ml. sample of the emulsion and the mixture then titrated with 0.1 N alcoholic potassium hydroxide, using phenolphthalein as an indicator. Peters and Nelson (1948a) adopted a buffered five per cent butterfat emulsion with agar as an emulsifier in their determination of the activity of the lipase of Mycotorula lipolytica (Candida lipolytica). At the end of the reaction period they added 50 ml. of a 50:50 mixture of ether and neutral 95 per cent ethanol to 10 g. of the emulsion and titrated it with 0.05 N methanolic potassium hydroxide, using phenolphthalein as an indicator.

In order to measure the activity of milk lipase more accurately, Johnson and Gould (1949b) developed an extraction-titration procedure. Cream or milk was acidified to pH 2 with sulfuric acid, ethanol was added and shaken and then extracted with a mixture of petroleum ether and diethyl ether. The ether extract was recovered after centrifugation, vaporized and the residue titrated according to the alcoholic potassium hydroxide method proposed by Breazeale and Bird (1938).

Characterization of Lipases

The lipases of various microorganisms have been reported to possess widely different pH optima. Avery and Cullen (1920), in their studies on the esterase of Pneumococcus observed that it had a maximum activity at pH 7.8. Stevens and West (1922) found that the lipase of a hemolytic streptococcus was active above pH 5.6, and had a maximum activity at pH 7.9. Gorbach and Guentner (1932) reported that the lipase of brewer's yeast had an optimum reaction on olive oil at pH 6.6 to 6.8. Tammisto (1933) found that the lipase of B. prodigiosum had an optimum action on tributyrin at pH 8.3. Kirsh (1935) observed that the water-soluble lipases of Penicillium oxalicum and Aspergillus flavus were most active in the hydrolysis of olive oil at pH 5.0. Thibodeau and Macy (1942) observed that the activity of the lipase of Penicillium roqueforti was favored between pH 5.3 and 7.5, using a butterfat emulsion as a substrate. Peters and Nelson (1948b) found that the optimum pH for the lipase of Mycotorula lipolytica on butterfat was 6.2 to 6.5. Fodor and Chari (1949) observed that optimum pH for the activity of lipases secreted into the culture medium by Aspergillus niger and Penicillium roqueforti was about 8.0. Lubert et al. (1949c) found that the lipase of Pseudomonas fluorescens exhibited maximum activity at pH 8.0 to 9.0.

Sumner and Somers (1947) stated that the type of buffer employed may have considerable effect on the optimum pH of enzymes. Platt and Dawson (1925) found that the optimum pH for the splitting of ethyl butyrate by pancreatic lipase differed with the different buffers used. In phosphate-borax buffer the optimum pH was 7.0, but in borax-boric acid buffer solution it was 8.4. Rabinowitch and Wynne (1936) also observed that with phosphate buffer the hydrolysis of tripropionin and methyl butyrate by pancreatic lipase was greatest at 7.2, whereas with glycine buffer the optimum pH was 9.3.

Platt and Dawson (1925) reported that formaldehyde in concentrations less than 0.2 per cent had no appreciable effect on pancreatic lipase action. Fouts (1940) observed that none of the organisms tested showed appreciable activity in cream containing one part of formaldehyde in 1600 parts of cream. Peters and Nelson (1948b) used a concentration of 1:1500 of formaldehyde to prevent microbial growth and permit strong enzyme activity during a reaction period of 48 hours at 37° C.

Oppenheimer (1925) observed that bile salts inhibited the action of lipases having an optimum pH on the acid side and activated those with an optimum pH on the alkaline side of neutrality. Fodor and Chari (1949) reported that a concentration of 0.18 per cent sodium taurocholate inhibited

the lipase activities of both Aspergillus niger and Penicillium roqueforti.

Lipases generally are considered to be of low specificity in their substrate requirements. Kirsh (1935) indicated that the lipase of Penicillium oxalicum was highly non-specific, bringing almost the same degree of hydrolysis of esters, triglycerides and a variety of oils. However, Hartwell (1938) found cocomut oil was digested by pancreatic lipase more rapidly than was any other fat. Collins and Hammer (1934) observed that tripropionin was more easily hydrolyzed by bacteria than were various other simple triglycerides or natural and hydrogenated fats. They also noticed that the hydrolysis of simple triglycerides of saturated fatty acids became more difficult as their molecular weights increased; triolein was easily hydrolyzed. Long (1936) reported that tripropionin and tributyrin were hydrolyzed by all the cultures of Ps. fragi that grew on the medium. Trivalerin and triolein were hydrolyzed by a majority of the cultures studied, whereas the hydrolysis of triisovalerin, tricaproin, tricaprylin and tricaprin was variable. Triheptylin, trilaurin, trimyristin, tripalmitin and tristearin were not hydrolyzed.

Most lipases are susceptible to heat treatment. Avery and Cullen (1920) inactivated the lipase of Pneumococcus by heating it for 10 minutes at 70° C. Stevens and West (1922)

were able to destroy the lipase activity of a hemolytic streptococcus after heating the lipase preparation at 55° C. for 10 minutes. Earlier Sohngen (1911) observed the production by B. fluorescens liquefaciens of a thermoduric lipase that could withstand heating at 100° C. for five minutes. He also reported that the lipases of Oidium lactis (Geotrichum candidum) and Penicillium glaucum were inactivated at 80° C. Tammisto (1933) found the lipases of fresh bacterial cells of B. fluorescens liquefaciens were relatively insensitive to the effect of higher temperatures. The lipase of the cells was diminished by about 30 per cent after heating at 95° C. for 10 minutes.

The temperature at which the enzymatic reaction takes place has a marked influence upon lipase activity. Kirsh (1935) reported that the lipase of Penicillium oxalicum showed maximum activity between 37 and 40° C. Peters and Nelson (1948b) found that the lipase of Mycotorula lipolytica had its maximum activity at 37° C. if the reaction period was 48 hours or less. Lubert et al. (1949c) observed that 42° C. was optimum for the activity of the lipase of Pseudomonas fluorescens.

Gyotoku and Tarashima (1930) succeeded in precipitating the lipases of blood, pancreas and stomach by the use of a 55-60 per cent concentration of ammonium sulfate. Glick and King (1933) used, with varying success, magnesium sulfate,

half and fully saturated ammonium sulfate and 10 per cent sodium chloride for the precipitation of pancreatic lipase. Peters and Nelson (1948b) stated that the only procedure they found for the salting out of the lipase of Mycotorula lipolytica was by saturation with ammonium sulfate.

Kirsh (1935) noticed that the lipases of both Aspergillus flavus and Penicillium oxalicum were extremely unstable in aqueous solutions preserved at 3° and at 20-25° C. at pH 5.0. Peters and Nelson (1948b) observed that the activity of the lipase of Mycotorula lipolytica tended to decrease upon storage. This decrease was more pronounced at 23-25° C. than at 3-5° C.

Lipase Production by Microorganisms

It has long been known that lipases diffuse out of the cells into the medium in which the microorganisms are growing. Camus (1897) found filtrates of the growth medium of Penicillium glaucum possessed evident but weak action on monobutyrin. Gerard (1897) found that extracts of the same mold had a marked action on monobutyrin as a substratum. Using the same technique, Garnier (1903) noticed that filtrates of Aspergillus glaucus had appreciable lipase activity. Neill and Fleming (1927) found that the botulinus bacillus (Clostridium botulinum) yielded an extracellular lipase that retained its hydrolytic activity independent of the presence

of formed cells. Tammisto (1933) studied lipase production by B. fluorescens liquefaciens and B. prodigiosum and found that the growth medium to which toluol was added had a marked ability to split tributyrin. Lubert et al. (1949b), after studying the lipolytic activity of several organisms, observed that Pseudomonas fluorescens had the greatest lipase activity among the organisms studied.

Hussong (1932) found that Ps. fragi had pronounced lipolytic activity on butterfat. The hydrolytic activity of 40 pure cultures of bacteria on triolein has been studied by Castell and Garrard (1941). They found that Ps. fragi produced over 0.1 per cent acidity in a triolein emulsion containing one per cent peptone after incubation at 25° C. for 11-17 days.

Factors Affecting Lipase Production by Microorganisms

Weisbrodt (1927) was able to increase lipase production by Penicillium roqueforti by substituting ammonium chloride for sodium nitrate in Czapek's medium. Naylor, Smith and Collins (1930) confirmed the above results. Thibodeau and Macy (1942), who investigated the lipase production by Penicillium roqueforti, noticed that when a sugar was present in the culture medium it tended to retard lipase production. They also found that organic nitrogenous compounds increased the production of lipase as compared with that obtained on

plain Czapek's medium. The lipase production of Aspergillus niger and Penicillium roqueforti was found by Fodor and Chari (1949) to be highest in a culture medium containing Bacto-peptone, marmite and glucose. However, the growth and lipolytic activity of the molds were very poor when glucose was the only source of carbon.

Peters and Nelson (1951) reported that nutrient broth was by far the best medium for lipase production by Candida lipolytica. A basal medium containing 0.1 per cent glucose, 0.03 per cent ammonium sulfate, phosphate, Fe^{++} and Mg^{++} did not yield detectable lipase after three days at 30° C. Addition of thiamin or its pyridine component resulted in considerable lipase production but still not as much as from a control in nutrient broth.

The concentration of phosphate buffer in the growth medium affects lipase production. Jezeski and Halvorson (1950) observed that an M/6 phosphate buffer was inhibitory to the organisms tested. Peters and Nelson (1948a) found that a concentration of 0.225 M disodium phosphate and citric acid in nutrient broth at pH 4.5 to 5.5 gave the highest yield of lipase by Mycotorula lipolytica. Similar concentration of disodium phosphate and monosodium phosphate also gave a high yield of lipase by the same organism, while sodium acetate-acetic acid buffer was unsatisfactory. Weisbrodt (1927) and Naylor et al. (1930) reported lipase production by

Penicillium roqueforti was highest at pH 4.5 in a modified Czapek's medium.

According to Peters and Nelson (1948a), highest lipase production by Mycotorula lipolytica occurred at 30° C. during incubation periods varying from 48 to 144 hours. Fouts (1940) reported that lipolysis by a mixed culture in cream was greater at 5° C., due to inhibition by acid formation at the higher temperatures.

Hussong (1932) found that salt markedly restricted the growth of Ps. fragi. Collins (1933) observed that the actively lipolytic bacteria studied by him did not tolerate more than one per cent salt in butter of average composition. He also found that Ps. fragi grew in five per cent but not 6.25 per cent salt.

Hussong et al. (1937) observed that the addition of one to 2.5 per cent salt to butter obtained from cream inoculated with Ps. fragi delayed the appearance of rancidity, the delay being greater with the higher salt content than with the lower. Kester (1950) found that two per cent salt inhibited microbial liberation of water-insoluble fatty acids in the inoculated experimental butter.

Hussong (1932) reported that butter cultures did not seem to have a very marked action in restraining the growth of Ps. fragi. Hussong et al. (1937) found that when 10 per cent butter culture was added to the cream, the resultant

butter developed rancid flavor somewhat less rapidly than when the butter was made without culture. Fouts (1940) observed that there was a considerable decrease in the acid numbers of the butterfat of cream inoculated with Achromobacter lipolyticum, Alcaligenes lipolyticus and Pseudomonas fluorescens when butter cultures were included.

Action of Lipase in Cream and Butter

Reinmann (1900) found that Oidium lactis, B. fluorescens liquefaciens and Mucor sp. did not produce rancidity in butter but increased its acid number. Hussong (1932) inoculated sterile butter with Ps. fragi and observed that on storage the total acid value increased considerably over the control of unsalted butter. There also was a greater increase in volatile acidity in the inoculated butter over the control. With salted butter, these values were much lower. Hussong et al. (1937) reported that when cream was inoculated with Ps. fragi the resultant butter showed a conspicuous increase in total acids in all the cases except when the intermediate type was used.

Fouts (1940) showed that 25 per cent of commercial unsalted butter samples became rancid when held at 21° C. for six days, while comparatively few samples of salted butter became rancid under the same conditions. He also observed that microorganisms were more active in fat hydrolysis in

cream than was milk lipase. All the organisms studied were more actively lipolytic in cream than in butter, especially at 5° C.

Richards et al. (1949) reported that molds produced much higher amounts of total acids when grown in butterfat emulsions than did bacteria.

Kester (1950) in his studies on the water-insoluble fatty acids in cream and butter, found that more fatty acids appeared in cream held at 38° F. for a considerable length of time than at higher temperatures for the same length of time. He also observed that butter obtained from sterile cream which was inoculated with Ps. fragi showed appreciable fat decomposition.

EXPERIMENTAL PROCEDURES

Cultural Methods

Source of cultures

Cultures O-1, K-1, E-1 and E-3 were class isolations for course D.I. 656 in 1949 and 1950. The rest of the cultures (C, 10, P, I, II, 11 and W2) were obtained from the stock culture collection of the Department of Dairy Industry, Iowa State College.

Media for carrying cultures

Stock cultures were carried on tryptone-beef-extract agar slants. The cultures were transferred at monthly intervals by first inoculating them into nutrient broth, peptone broth or vitamin-free casamino acids medium, and after incubating them at 21° C. for 24 hours they were streaked on agar slants, incubated at 21° C. for 24 hours, and then kept at 2-3° C.

Inoculation of media and growth of cultures

Before any experimental medium was inoculated, the cultures were transferred twice, usually into vitamin-free

casamino acid medium, and earlier in the work into nutrient broth or peptone broth. These successive transfers were incubated at 21° C. for 24 hours. However, when the growth medium was a chemically-defined one, glucose broth with the following composition was used in the two transfers: 0.5 g. glucose c.p., 0.2 g. ammonium chloride, 0.05 g. magnesium sulfate heptahydrate, 10 ml. 0.5 M potassium dihydrogen phosphate, 41 ml. 0.1 N sodium hydroxide and 48 ml. distilled water. The inoculum consisted of one drop of the second 24-hour transfer of the culture into 100 ml. of the medium. All media, unless otherwise stated, were incubated at 15° C. for three days. The flasks containing the media were set at the desired temperature overnight before inoculation so that the media would be at the required temperature. All liquid growth media were dispensed in 100 ml. amounts into 300 ml. Erlenmeyer flasks before sterilization, unless stated otherwise.

Determination of bacterial counts

Plate counts were made according to Standard Methods for the Examination of Dairy Products (American Public Health Association, 1948), with the exception that no milk was included in the agar. All plates were counted after an incubation period of 48 hours at 21° C. Exceptions to this procedure will be noted in the various studies.

In order to help disperse the clumps of bacteria, the growth medium was poured into sterile 6 oz. bottles containing glass beads and then shaken 25 times before a sample was taken. Despite this attempt, the colony counts frequently varied between duplicate plates by 10 per cent or more. All counts reported were averages of counts on duplicate plates.

Sterilization of culture media

Unless otherwise stated, culture media were sterilized by autoclaving at 15 lb. for 15 minutes.

Measurement of Lipase Activity

Lipase preparations

At the end of the incubation period, the growth medium was poured into 100 ml. centrifuge tubes and whirled in an International angle centrifuge at 4000 r.p.m. for 30 minutes, using a head 14 inches in diameter. The clear supernatant was decanted into 125 ml. Erlenemeyer flasks and then 0.1 per cent of 36 per cent formaldehyde was added to prevent bacterial growth. These flasks were kept at 2-3° C. for further use.

To be used as a blank the lipase preparation was heated in boiling water in 125 x 16 mm. pyrex screw-cap test tubes for 15 minutes. In the latter part of this study the holding

time was increased to 20 minutes.

Test substrate for lipase activity

The routine test substrate consisted of the following ingredients: 10 g. coconut oil, 10 ml. 0.5 M potassium dihydrogen phosphate, 35 ml. 0.1 N sodium hydroxide, 0.1 ml. formaldehyde (36%), 0.4 g. sodium taurocholate and enough distilled water to make 75 or 100 ml.

When more than 1 ml. of lipase preparation was used, the stock emulsion was made up to a total volume of 75 ml. by the addition of water. Coconut oil was heated to get it in liquid form. The rest of the ingredients, after dissolving the sodium taurocholate, were added to the melted coconut oil and then homogenized twice with a hand homogenizer. If the above volume was 75 ml., 30 ml. portions of the homogenized emulsion were dispensed into 6 oz. medicinal bottles and enough distilled water added to each bottle so that the final volume totaled 40 ml. when the lipase preparation was added. Otherwise, 40 ml. portions of the coconut oil emulsion were dispensed into the bottles when the volume of the emulsion was 100 ml. instead of 75 ml. The bottles were set overnight in the incubator at 36° C. before the lipase preparations were added. The pH of the resulting emulsion was 7.2 ± 0.05.

Coconut oil was refined before being used. The refining procedure consisted of treating the warm liquid oil twice with a warm 0.2 per cent sodium carbonate solution, recovering the oil after each treatment with a separatory funnel, and finally washing it three times with warm distilled water, removing the water after each washing in a separatory funnel. The oil was freed from the remaining water by centrifuging it at 1500 r.p.m. for five to 10 minutes, and drawing the water from the bottom of the centrifuge tubes. The refined oil was stored at 2-3° C. until used.

After addition of the lipase preparation, the amount used depending upon expected lipase activity (ranged from 0.1 ml. to 2 ml.), to the test substrate it then was left to react at 36° C. for 24 hours.

Quantitative determination of lipase activity

A modification of the extraction-titration procedure proposed by Johnson and Gould (1949b) was used. At the end of the reaction period, one or more 10 g. samples were weighed on a torsion balance into regular Mojonnier fat extraction flasks. After addition of five drops of thymol blue indicator, the contents of the flasks were acidified with sufficient 25 per cent sulfuric acid that a definite pink color persisted after subsequent extractions (usually 0.25 to 0.3 ml. would suffice), and then the contents were mixed by tilting the

flasks back and forth several times with moderate shaking. This was followed by adding 10 ml. of 95 per cent ethanol, stoppering the flasks, vigorously shaking them up and down for 15 seconds, and then letting them stand for five minutes (five or less flasks could be so treated at the same time by setting them in a suitable container). Two successive extractions with 10 ml. portions of an ether mixture (two volumes of ethyl ether and three volumes of petroleum ether) were then made by delivering the ether from a buret, and after stoppering the flasks, they were shaken vigorously for 30 seconds, and allowed to stand until the ethereal layer became clear (0.5 - 1 minute). After adjusting the level of the aqueous layer with distilled water using an eye dropper, the ethereal layer was cautiously poured off into dry 125 ml. Erlenmeyer flasks. Care was taken not to pour any of the aqueous contents of the flasks with the ethereal portion because that would introduce significant errors in titrations. The ether extract then was titrated with 0.05 N potassium hydroxide in absolute methanol, using ten drops of one per cent phenolphthalein in absolute ethanol as an indicator. A faint pink color that persisted after shaking for 10 to 15 seconds was used as the endpoint of titration. In this work a semimicro buret which delivers about 75 drops per milliliter was used for titration. Readings to the third decimal place were made and then recorded to the nearest second decimal

place. The titration values given after deduction of the blanks represent hydrolysis in approximately 1 g. of coconut oil.

The above procedure was modified when cream was being extracted. This change involved whirling the Mojonnier flasks in a Mojonnier hand centrifuge for 1-1.5 minutes after each addition and shaking of the ether portions. The precipitation of the proteinaceous materials in cream was facilitated by the addition of 2-3 ml. of saturated sodium chloride solution before centrifugation (Bird, 1951).

Another modification was introduced in the case of butter. The sample was melted at 50-55° C., mixed well, and weighed as usual. Due to the high fat content of butter, 20 ml. portions of ether mixture were used instead of the usual 10 ml. portions. This was followed by whirling in a Mojonnier hand centrifuge for 30 seconds after each extraction.

Having made the trials on cream in the summer months, the butterfat was intensely colored, and consequently the resulting ether extracts were highly colored, somewhat masking the phenolphthalein endpoint.

Properties of the Lipase

Heat inactivation studies

These studies have been made on lipase produced in phosphate buffered peptone broth. The original lipase preparation was diluted to twice its volume with potassium dihydrogen phosphate-sodium hydroxide buffer and 0.1 N hydrochloric acid so that the pH of the resulting mixture, after bringing it up to volume with distilled water, was 7.0±0.05. The final concentration of phosphate in the diluted lipase preparation was 0.05 M. This preparation was pipetted into 125 x 16 mm. pyrex screw-cap test tubes in 10 ml. amounts. The test tubes were then immersed in constant-temperature water baths at 61.6 or 71.6 ± 0.1° C. so that the tops of the caps were at least one inch below the surface of the water, and the level of the lipase preparation in the test tubes was about two inches below the surface of the water in the baths. In order to obtain temperatures of 98-99° C., the test tubes were immersed in a bath of boiling water. The temperature of the lipase preparation in the test tubes was followed by a thermometer in a control test tube, containing the same lipase preparation, fitted with a rubber stopper that kept it firmly closed. When the desired temperature was reached, timing would begin, and as soon as the time interval

ended the test tube immediately was placed in an ice water bath and cooled to below 20° C. Aliquots of the lipase preparations before heating, after the desired temperature was reached, and at the end of the heating period, were pipetted into the lipase test substrate and treated as usual.

Salting out of lipase

Salting out of the lipase of Ps. fragi was accomplished by bringing 50 ml. of peptone broth lipase preparation to nearly full saturation with ammonium sulfate (about 26 g.) in 100 ml. centrifuge tubes. After dissolving the sulfate, the tubes were allowed to stand at 5-7° C. for 16-18 hours. At the end of this period the centrifuge tubes were whirled in an angle centrifuge at 4000 r.p.m. for 30 minutes, using an angle head 14 inches in diameter, and then the supernatant was decanted. In order to prevent any loss of precipitate, the last portion of the supernatant was run through Whatman no. 30 filter paper. The precipitate caught on the filter paper was washed with cold distilled water and added to the washings of the precipitate from the centrifuge tube into 50 ml. volumetric flask and made up to volume with cold distilled water. The activity of this solution was checked against that of the original lipase preparation. The filtrate was combined with the decanted supernatant and the mixture was referred to in the table as the supernatant. It is to be

noted that all glassware and equipment were kept in the cold room and all the steps made at the temperature of 5-7° C.

It was noticed that a fluffy white material immediately formed at the surface of the lipase preparation on saturation with ammonium sulfate. In order to achieve the separation of this precipitate from the brownish precipitate that formed mostly at the bottom of the centrifuge tube after standing for 16-18 hours, 50 ml. of the lipase preparation was saturated with ammonium sulfate in a 500 ml. separatory funnel. As soon as the fluffy material had risen to the surface, the aqueous solution beneath it was drawn into a 100 ml. centrifuge tube which was set at 5-7° C. for 16-18 hours, centrifuged and the brownish precipitate dissolved by adding 50 ml. of cold distilled water to the centrifuge tube. The fluffy material that was left in separatory funnel was dissolved by the addition of 50 ml. cold distilled water. These two solutions were tested separately for lipase activity.

Preparation of Buffer Solutions and Determination of pH

Preparation of buffer solutions

McIlvaine's citric acid-disodium hydrogen phosphate buffer solution was prepared according to Hodgman (1949), except that the final molarity of the phosphate in the media

was fixed at 0.056. Clark and Lubs potassium dihydrogen phosphate-sodium hydroxide and boric acid-sodium hydroxide were prepared according to Lang (1944), except that 0.5 M boric acid or phosphate solutions were used.

Measurement of pH

A Leeds and Northrop glass electrode potentiometer was used in the early part of this study for the measurement of pH in growth media and in test substrates. However, most of the pH determinations reported in this work have been obtained by the use of a Leeds and Northrop quinhydrone-calomel potentiometer.

Treatment of Cream and Butter

Inoculation and incubation of cream

For studies on lipase production by Ps. fragi in cream, 100 ml. portions of sterile cream in 300 ml. Erlenmeyer flasks were inoculated with one drop of a 24-hour culture in vitamin-free casamino acids medium and then incubated at 15° C. for three days. When S. lactis was used one drop of 14-16 hour culture grown at 32° C. in litmus milk constituted the inoculum. However, in the studies on residual lipase in butter, 500 ml. portions of sterile cream in 2-liter flasks were inoculated with five drops of the usual inoculum and

incubated at 15° C. for three days.

Pasteurization and buttermaking

In trial 1, two 500 ml. portions of inoculated cream at the end of the incubation period were combined aseptically in one 2-liter flask which then was set in a constant-temperature water bath at 71.6° C. ± 0.1°. It took 17 minutes for the cream to reach the temperature of 70° C., after which timing began. The temperature of the cream continued to rise until it reached 71.5° C. after 20 minutes. The cream was stirred frequently with a sterile rod throughout the holding time. After pasteurization, the inoculated cream was cooled in ice water to below 10° C., split into 500 ml. portions and churned in sterile quart jars simultaneously with the uninoculated cream. It took 42 minutes for the inoculated cream to churn as compared to 25 minutes for the control cream, the difference in churning time being probably due to lack of crystallization of the fat globules in the inoculated cream. The butters while still in the jars were washed with cold sterile distilled water. The test butters in the two jars were combined and the same procedure was followed with control butters from the other two jars, then worked thoroughly with sterile paddles in sterile bowls, and finally dispensed into sterile 2 oz. sample jars which were stored at the various temperatures.

The same procedures were followed in trial 2 except that the cream was sterilized by steaming on two successive days in an Arnold sterilizer for a period of one hour, instead of sterilizing in the autoclave at 15 lb. for 15 minutes as in the case of trial 1. The modification of the pasteurization procedure consisted of heating the inoculated cream in a water bath at 80-85° C. bringing the cream up to the temperature of 71.5° C. in eight minutes, after which period the cream was transferred to the constant-temperature water bath at 71.6° C. and held for 30 minutes. Also, the inoculated cream, after pasteurization and cooling to below 10° C. in an ice bath, was held overnight at 4-6° C. together with the control cream before being churned. The churning times in trial 2 were 35-39 minutes for the control and 38-44 minutes for the inoculated cream.

Tests for composition and flavor

Butterfat in cream was determined by the Babcock test (Hunziker, 1940).

Testing butter for moisture content, butterfat, and curd was carried out according to the modified Kohman test (Hunziker, 1940).

The butter samples were tested organoleptically for flavor defects at the beginning and at the end of the holding periods.

RESULTS

Measurement of Lipase Activity

Many of the methods employed in the determination of lipase activity consist of titrating the test substrate in toto after a given reaction period. Such methods have inherent errors due to the interference of buffers, the existence of the fat and aqueous phases, and the formation of soaps by the liberated fatty acids, especially when the pH of the test substrate is on the alkaline side of neutrality.

In the course of preliminary work, it was found that whenever the whole test substrate was titrated, the titration values invariably were extremely low even after prolonged reaction periods. It was decided to shift to an extraction-titration procedure that would permit of acidification of the test substrate to a pH at which all the fatty acids would be in the free form before starting the extraction. Such a procedure obviously was much superior to the previous unsuccessful method. Although the extraction procedure is by no means perfect, since some of the lower fatty acids are only partially recovered, yet the coconut oil used contained such low percentages of these fatty acids (Hilditch, 1941) as to render this discrepancy rather insignificant.

The extraction procedure

The extraction procedure, which is a modification of the method reported by Johnson and Gould (1949b) was adapted to the use of Mojonnier butterfat extraction flasks. Volumes of ethanol greater than 10 ml. did not improve extraction efficiency but rendered pouring of the ethereal layer difficult. It remained to be seen what ratio of ethyl ether to petroleum ether (Skelly-solve B) in the ether mixture would give maximum extraction of fat and free fatty acids.

The mixture of two volumes of ethyl ether and three volumes of petroleum ether suggested by Johnson and Gould (1949a) gave net activities within the optimal range, as shown in Table 1. The greater the quantity of ethyl ether in the mixture the greater were the blank titration values. These values gradually decreased from 1.30, when three volumes of ethyl ether to one volume of petroleum ether were present in the mixture, to a low value of 0.32 when this ratio was reversed. Moreover, the ether extract became turbid during titration with methanolic potassium hydroxide whenever a ratio of three volumes of ethyl ether to two volumes of petroleum ether was used, or any other ratio in which the ethyl ether was increased. However, the net activities were practically the same when the ratio of ethyl ether to petroleum ether was within 1:2 to 2:1. Use of petroleum ether

alone caused marked fading of the endpoint of titration.

The results presented in Table 2 indicate that the efficiency of extraction was significantly increased whenever two extractions were made. Preliminary trials demonstrated there was nothing to be gained by carrying the number of extractions any further. Using 10 ml. portions of ether mixture, the net activity for the first extraction was 1.55, for the second extraction 0.22, and only 0.01 for the third extraction. Doubling the portions of ether mixture to 20 ml. did not result in the same total net activity obtained with two 10 ml. portions used in two extractions, but a second extraction was necessary to get a net activity of 1.76 which is practically identical to the value of 1.77 obtained by the first procedure. It seems that the value of blank titrations is directly proportional to the quantity of ether mixture used. The blank titrations for the two 10 ml. portions of ether mixture totaled 0.41, which was exactly the same as that obtained with one 20 ml. portion of ether mixture.

Recovery of added fatty acids by the extraction procedure used was reasonably satisfactory when the length of the carbon chain of the fatty acid was eight carbons or more (Table 3). In these trials the fatty acid solutions were prepared by dissolving the individual fatty acids in absolute ethanol and determining the normality of the solutions against

Table 1

Effect of the ratio of ethyl ether to petroleum ether in the extraction mixture upon the efficiency of extraction (0.4 ml. lipase preparation per 40 ml. substrate)

Volumes petroleum ether	Volumes ethyl ether	Active		Blank		Net* activity
		Titration*	Ether extract	Titration*	Ether extract	
1	3	3.66	Turbid	1.30	Turbid	2.36
1	2	3.38	"	0.93	"	2.45
2	3	3.16	Sl. "	0.73	Sl. "	2.43
1	1	2.94	Clear	0.51	Clear	2.43
3	2	2.83	"	0.40	"	2.43
2	1	2.78	"	0.36	"	2.42
3	1	2.71	"	0.32	"	2.39

* Ml. 0.05 N methanolic potassium hydroxide

Table 2

Effect of quantity of ether mixture used and number of extractions made upon the efficiency of extraction (0.1 ml. lipase preparation per 40 ml. substrate)

Extraction no.	10 ml. portions of ether			20 ml. portions of ether		
	Active	Blank	Net activity	Active	Blank	Net activity
1	1.85	0.30	1.55	2.07	0.41	1.66
2	0.33	0.11	0.22	0.26	0.16	0.10
3	0.10	0.09	0.01	-	-	-
			1.78			1.76

Table 3

Recovery of added fatty acids

Trial no.	Per cent recovery of fatty acids				
	Butyric	Caproic	Caprylic	Lauric	Stearic
1	44.3	76.1	91.5	97.2	96.2
2	42.8	75.6	91.5	96.6	97.3
3	45.3	-	91.5	98.2	-
4	44.8	-	91.0	97.2	-
Average	44.3	75.9	91.4	97.3	96.8

standard alkali. These solutions were added in 0.5 ml. portions per 10 g. of test substrate and extracted as usual. The figures in Table 3 show that only 44.3 per cent of the added butyric acid was recovered, denoting that the partition of the acid between the water and ether phases was in favor of the former. With caproic acid the percentage recovered rose to 75.9. Recoveries of 91.4 per cent for caprylic acid, and about 97 per cent for lauric and stearic acids were obtained. Recoveries of the fatty acids tried checked fairly well between the different trials.

Choice of emulsifier

Several compounds were tried in order to find an emulsifier that would give a stable coconut oil emulsion, would not interfere with the extraction procedure, and would not inhibit lipase activity. Among the emulsifiers tried were: carboxy-methyl cellulose, asolectin, glyceryl monococate, tragacanth gum, ghatti gum, carob bean gum, arabic gum, locust gum, gelatin, oxgall, sodium ricinoleate, mixifier, sodium glycocholate, and sodium taurocholate. The gums were unsatisfactory because they interfered with the extraction procedure by clogging the necks of the Mojonnier flasks after shaking with the ether mixture. Others such as sodium ricinoleate, oxgall, sodium glycocholate, and glyceryl monococate inhibited lipase activity very markedly, while the

rest gave an unstable emulsion or else were difficult to handle.

Tragacanth gum was found to be the most satisfactory emulsifier among the gums that were tried and was surpassed only by sodium taurocholate. In Table 4 these two emulsifiers are compared. The use of sodium taurocholate as an emulsifier resulted in a net activity of 1.48 as compared to 0.83 with tragacanth gum. Not only did sodium taurocholate give a higher lipase activity, but it also was easy to handle, requiring no heating or centrifugation after extraction with ether, because the emulsion immediately broke down after acidification. The only obvious drawback for the use of sodium taurocholate was that of giving a higher blank titration than did tragacanth gum.

The data presented in Table 5 show that the closely related compound, sodium glycocholate, was much inferior to sodium taurocholate. Apparently it inhibited lipase activity, as with 0.3 per cent sodium glycocholate the net activity was 0.55, but the value dropped to 0.15 when 0.5 per cent was used. In comparison, sodium taurocholate gave the same net activity values whether 0.3 or 0.5 per cent of the emulsifier was used. Both emulsifiers gave higher blanks with the use of 0.5 than with 0.3 per cent of these materials.

Further data on the effect of sodium taurocholate concentration upon lipase activity are shown in Table 6. This

Table 4

Comparison between sodium taurocholate and tragacanth gum as emulsifiers

Coconut oil emulsion with	Active 1	Active 2	Active Average titration	Blank 1	Blank 2	Blank Average titration	Net activity
Tragacanth gum	1.14	1.15	1.15	0.31	0.33	0.32	0.83
Sodium taurocholate	1.96	1.93	1.95	0.48	0.46	0.47	1.48

Table 5

Comparison between sodium taurocholate* and sodium glycocholate as emulsifiers

Emulsifier Kind	Per cent	Active Titration**	Blank Titration**	Net activity
Sodium taurocholate	0.3	1.78	0.36	1.42
" "	0.5	1.85	0.43	1.42
Sodium glycocholate	0.3	0.85	0.30	0.55
" "	0.5	0.56	0.41	0.15

* 42 per cent preparation of sodium taurocholate
** Average of two titrations

product was described by the manufacturer as pure, whereas the emulsifier used in the previous table contained 42-43 per cent sodium taurocholate. Net activity increased with increase in sodium taurocholate concentration from 0.1 to 0.4 per cent. The blank titrations also increased with the increase in the concentration of sodium taurocholate. A concentration of 0.4 per cent sodium taurochate, the minimum effective amount, was used throughout the entire course of this study.

Prevention of bacterial growth during the reaction period

In order to determine whether formaldehyde had an adverse effect upon lipase activity, concentrations from zero to 0.4 per cent by volume of a 36 per cent formaldehyde reagent were examined. Table 7 shows that the emulsions devoid of formaldehyde supported bacterial growth and resulted in oiling off of the cocomut oil. A slight but definite inhibition of lipase activity appeared when the test substrate contained over 0.036 per cent formaldehyde. Apparently, the concentration of formaldehyde did not affect the blank titration values, these values being about 0.39 with formaldehyde and 0.43 for the test substrate devoid of formaldehyde. A concentration of 0.036 per cent formaldehyde was used during the major part of this work, but it was increased to 0.072 per cent toward the end of the work in order to

Table 6

Effect of concentration of sodium taurocholate* upon lipase activity
(2 ml. lipase preparation per 40 ml. substrate)

% Sodium taurocholate	Active			Blank			Net activity
	1	2	Average titration	1	2	Average titration	
0.1	3.21	3.19	3.20	0.13	0.15	0.14	3.06
0.2	4.19	4.19	4.19	0.21	0.21	0.21	3.98
0.4	4.98	4.97	4.98	0.31	0.32	0.32	4.66
0.6	5.10	5.08	5.09	0.41	0.41	0.41	4.68
0.8	5.09	5.09	5.09	0.50	0.54	0.52	4.57

* Described by manufacturers as pure.

Table 7

Effect of concentration of formaldehyde upon lipase activity
(2 ml. lipase preparation per 40 ml. substrate)

% Formaldehyde	Active			Blank			Net activity
	1	2	Average titration	1	2	Average titration	
0.000*	3.14	3.15	3.15	0.42	0.44	0.43	2.72
0.036	3.73	3.74	3.74	0.38	0.38	0.38	3.36
0.072	3.47	3.47	3.47	0.39	0.39	0.39	3.08
0.108	3.45	3.45	3.45	0.38	0.39	0.39	3.06
0.144	3.16	3.14	3.15	0.38	0.38	0.38	2.77

* Bacterial growth occurred

correct the oiling off trouble.

Choice of buffer system

The nature of the buffer used was observed to have an effect on lipase activity. A comparison of the effect of three buffer systems used in this study upon lipase activity is given in Table 8. With boric acid buffer the final pH of the substrate with active lipase dropped appreciably, from pH 7.88 for the blank to 7.30 for the active preparation. Although a high net activity was obtained with the boric acid buffer, yet its poor buffering capacity at the pH level employed resulted in a greater drop in pH than with the other two buffers, thus coming closer to the optimum pH for lipase activity. McIlvaine's citric acid-phosphate buffer system gave a slightly lower net activity within the range of 7.8 to 7.56 than did Clark and Lubs phosphate buffer system. The same is true at the lower pH level of 7.25 to 7.0.

In an attempt to reduce the shift in pH of the substrate during the reaction period, phosphate concentrations varying from 0.028 to 0.112 M were tried. The results in Table 9 show that concentrations of monopotassium dihydrogen phosphate above 0.056 M had no effect on lipase activity but slightly decreased the shift in pH during the reaction period. With a phosphate concentration of 0.028 M, the net activity was somewhat lower than with 0.056 M, and the drift in pH amounted

Table 8

Effect of some buffer systems in test substrate upon lipase activity
(2.0 ml. lipase preparation per 40 ml. substrate)

Buffer system	Active		Blank		Net activity
	Final pH	Titration	Final pH	Titration	
KH_2PO_4 + NaOH	7.58	2.08	7.81	0.30	1.78
Boric acid + NaOH	7.30	2.61	7.88	0.48	2.13
Na_2HPO_4 + citric acid	7.56	1.90	7.87	0.30	1.60
KH_2PO_4 + NaOH	7.03	3.03	7.22	0.35	2.68
Na_2HPO_4 + citric acid	7.03	2.86	7.25	0.32	2.54

Table 9

Effect of concentration of potassium di-hydrogen phosphate in test substrate upon lipase activity
(2.0 ml. lipase preparation per 40 ml. substrate)

Buffer concentration	Final pH	1	2	Average titration	Blank Final pH	Blank Titration	Net activity
0.028 M	6.97	3.11	3.13	3.12	7.26	0.34	2.78
0.056 M	7.01	3.43	3.45	3.44	7.22	0.37	3.07
0.084 M	7.01	3.28	3.31	3.30	7.17	0.35	2.95
0.112 M	7.05	3.40	3.39	3.40	7.17	0.33	3.07

to 0.29 unit. Duplicate titrations of the active samples were given in this table in order to demonstrate the satisfactory checks obtained with the extraction-titration procedure. A concentration of 0.056 M phosphate was selected for use throughout this work.

Characterization of the Lipase of Ps. fragi

The lipase preparations used in this study represent crude enzyme preparations undoubtedly containing other enzymes, notably proteolytic. However, the characteristics determined probably would apply in a considerable measure to a system which had undergone some purification. Also the enzyme as encountered in dairy products would not be acting in a purified system.

The effect of the presence of cells of Ps. fragi in lipase preparations upon lipase activity

Attempts were made during the preliminary studies to obtain cell-free lipase preparations by using Selas microporous porcelain filters. It soon was observed that considerable lipase activity was lost during filtration, probably due to adsorption of the lipase onto the filter, or possibly due to inactivation by foaming which took place during filtration. In order to conserve the activity of

lipase produced in the growth media, centrifugal removal of cells was employed. Even though over 98 per cent of the cells were centrifuged out, yet a relatively high number of bacteria per milliliter of the medium was left, as shown in Table 10. It was desirable then to determine whether the remaining bacterial cells would contribute any appreciable amount of lipase activity during the reaction period. The net lipase activity in 2 ml. of the preparation before centrifugation amounted to 1.09, whereas it dropped to 0.98 in the preparation after centrifugation. Assuming that the difference of 0.11 was due to the cells removed during centrifugation, the remaining cells would contribute about one-hundredth of 0.11 or 0.001, a value not measurable by the method used. It is to be noted that the blank titrations were unaffected in the presence or absence of cells. Consequently, it is safe to assume that the lipase activity measured is due predominantly to extracellular lipase.

The influence of reaction time and temperature upon lipase activity

In the early trials, the lipase activities obtained were relatively low, even after prolonged reaction periods. Preliminary trials were run at 32°, 36° and 40° C. for the periods of 24, 48 and 72 hours. The results are presented in Figure 1. At 32 C. the lipase activity seems to be

Table 10

Effect of the presence of cells of *Pseudomonas fragi* in lipase preparation upon its activity
(2 ml. lipase preparation per 40 ml. substrate)

Lipase preparation with	Count per ml. ($\times 10^6$)	Active			Blank			Net activity
		1	2	Average titration	1	2	Average titration	
Cells present	870	1.32	1.30	1.31	0.22	0.22	0.22	1.09
Cells* removed	8.6	1.21	1.19	1.20	0.23	0.21	0.22	0.98

* Cells removed by centrifugation

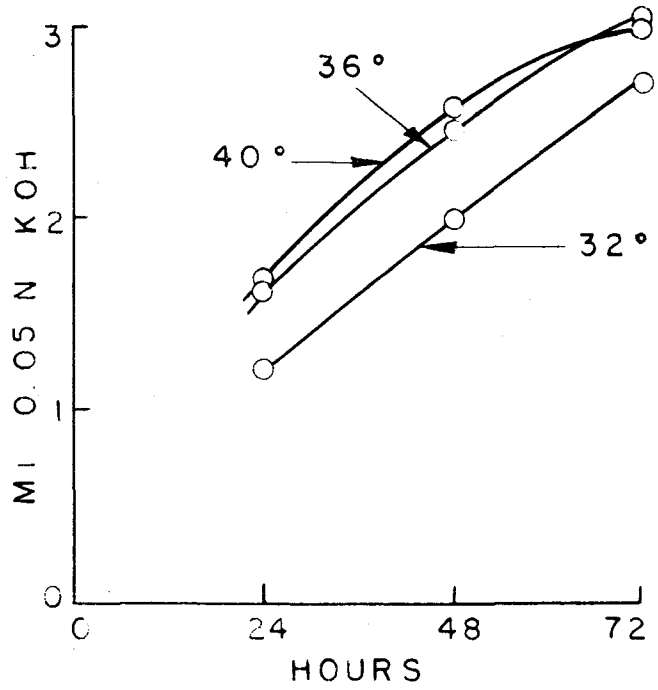


Figure 1. Relation of lipase activity to time.

essentially linear between 24 and 72 hours. That the rate of lipase activity is different during the early activity is readily seen if the curve is projected to the left, as such a projection passes above the zero point, indicating the rate of lipase activity during the first 24 hours is probably higher than during the rest of the reaction period. Unfortunately, values for this early portion of the curve were not obtained in this run and interpretation is based upon the fact that the value for lipase activity during the first 24-hour period was 1.22 as compared to 0.78 and 0.70 during the second and third 24-hour periods, respectively. The lipase activities at 36° C. are appreciably higher than those at 32° C.; the relationship between lipase activity and time is not linear and there is a slow but constant lowering of the rate of lipase activity. At 40° C. the net activity values are slightly higher than those at 36° C., and the curve deviates more from the straight line, possibly due to a faster rate of inactivation of the lipase at the higher temperature.

It was hoped that conditions might be found under which the relationship between lipase activity and time would be linear, perhaps with shorter reaction periods at 36° C. Figure 2 presents a rather smooth curve of the relationship between lipase activity and time during the first 24 hours, but it definitely deviates from a straight line in much the

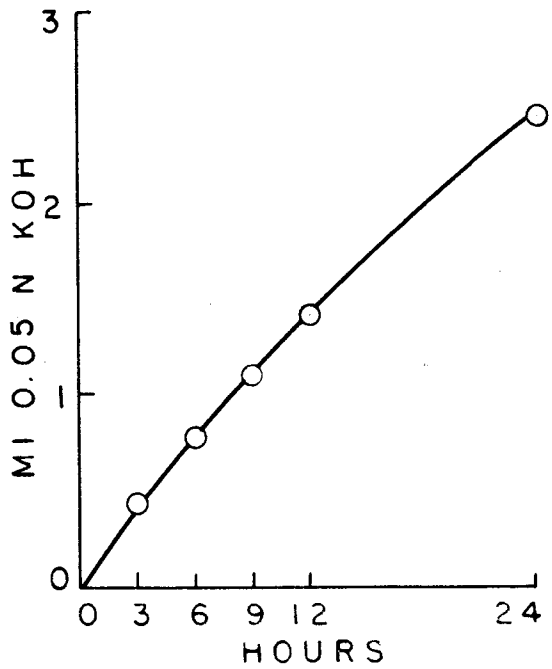


Figure 2. Relation of lipase activity at 36° C. to time.

same manner one would expect from extrapolation of the 36° C. curve in Figure 1. This deviation probably is due to a gradual inactivation of the lipase as was the case with the longer reaction periods. However, it is obvious that the relationship between lipase activity and time is regular. The increases in net activity were 0.42, 0.36, 0.33 and 0.31 for the successive three-hour periods. After 24 hours the net activity was 2.45, representing a six-fold increase of the value after the first three hours, instead of the calculated eight-fold increase. A reaction period of 24 hours at 36° C. was chosen in order to allow better comparison between different lipase preparations, especially those possessing low lipase activities, even though this portion of the curve deviated considerably from a straight line function.

The influence of the temperature of the enzyme reaction upon lipase activity is shown in Figure 3, data from two series being included. These studies were made by addition of 0.3 ml. lipase preparation to the usual test substrate set at the different temperatures in constant-temperature water baths with deviations not to exceed ± 0.1 to 0.2° C. The test substrate was dispensed in the usual 6 oz. bottles which were immersed in the water baths so that the level of the contents was at least two inches below the surface of the water in the baths. The optimum temperature for lipase activity in 24 hours was at 40° C. Although 36° C. was not

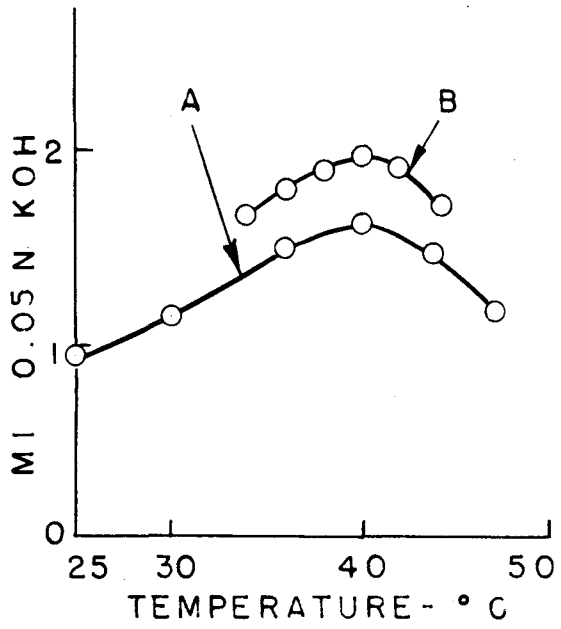


Figure 3. Effect of temperature of test substrate upon lipase activity.

optimum for enzyme action, this temperature was employed in the other studies because of its convenience and because it gave results not greatly less than those obtained with holding at 40° C.

Action of lipase on various substrates

The variations in action of the lipase of Ps. fragi upon some natural oils and fats are shown by representative data given in Table 11. The emulsions of the different oils and fats were prepared by substituting 10 g. of the test material for coconut oil in the usual test substrate. However, butterfat and the solution of other ingredients both were kept warm enough to prevent fat solidification during homogenization in the preparation of the butterfat emulsion. It is evident from the net activities presented that the lipase attacked the various oils and fats to approximately the same extent. Coconut oil was hydrolyzed most easily, and it was followed by corn oil and cottonseed oil, while olive oil, butterfat and soybean oil were least attacked. In addition to being hydrolyzed more rapidly, coconut oil does not have any ether-soluble pigments and thus yields a colorless ether extract, whereas corn oil, olive oil and particularly soybean oil give colored ether extracts, thus masking the pink endpoint of phenolphthalein in the final titration for measuring lipase activity.

In testing the action of lipase on pure triglycerides, one per cent of tributyrin was used as a substitute for coconut oil in the usual test substrate. Amounts of the other triglycerides equivalent upon the basis of molecular weights to one per cent tributyrin were used. Emulsions were prepared in the usual manner except in the case of trilaurin and trimyristin where the medium was heated and homogenized while hot, and then dispensed into 6 oz. medicinal bottles. However, some solidification of trilaurin occurred on cooling, this solidification becoming more extensive with trimyristin. With tripalmitin and tristearin no satisfactory emulsion could be obtained. Triolein gave a good emulsion, as did all the other triglycerides which are liquid at room temperature. In Table 12 data on the extent of hydrolysis of six pure triglycerides by lipase are presented. The figures in the last column are averages of the three trials corrected to 100 per cent recovery on the basis of Table 3. In the case of oleic acid, a percentage recovery of 97 was assumed. Obviously the triglycerides were not hydrolyzed at the same rate. The extent to which the lipase hydrolyzed these triglycerides increased from tributyrin to tricaprylin. The net activity and amount of hydrolysis dropped with triolein, while tricaproin and trilaurin yielded intermediate values. Somewhat surprisingly tricaprylin underwent the greatest degree of hydrolysis, with some of the triglycerides both above and

Table 11

Action of lipase on some natural fats and oils
(2 ml. lipase preparation per 40 ml. substrate)

Fat or oil	Active*	Blank*	Net activity	Color of ether extract
Butterfat	1.84	0.46	1.38	Amber
Coconut oil	2.21	0.41	1.80	Colorless
Corn oil	1.83	0.31	1.52	Faint yellow
Cottonseed oil	1.78	0.27	1.51	Colorless
Olive oil	1.89	0.59	1.30	Light green
Soybean oil	1.67	0.28	1.39	Greenish yellow

* Average of duplicate titrations

Table 12

Action of lipase on some pure triglycerides

Tri-glyceride	Net activity			Average* corrected values	Per cent** hydrolysis
	Trial 1	Trial 2	Trial 3		
Tributyrin	0.77	0.49	0.46	1.28	5.28
Tricaproin	1.81	1.34	1.35	1.97	7.63
Tricaprylin	3.56	2.95	2.85	3.41	15.85
Trilaurin	2.00	1.71	1.67	1.85	7.98
Trimyristin	1.26	-	-	-	-
Triolein	1.48	0.92	0.80	1.10	4.98

* Corrected to 100 per cent recovery (See Table 3)
** Calculated on the basis of corrected values

below tricaprylin being affected less the further removed they were in respect to molecule size. Triacetin and tripropionin were tried, but no demonstrable lipase activity could be obtained with the former, while the latter gave a net activity of 0.13. This possibly was due in part to poor recovery of the respective acids. However, in the case of triacetin there was no detectable change in pH during the reaction period, while the pH dropped 0.1 unit with tripropionin, indicating that there was little or no activity with triacetin, and a net activity appreciably lower than that with tributyrin was obtained with tripropionin. Tricaprin was not available at the time these trials were carried out.

The effect of substrate concentration upon lipase activity

The influence of concentration of coconut oil as substrate on lipase activity is presented in Figure 4. Coconut oil concentrations as low as one per cent gave fairly good lipase activities. The net activity with one per cent coconut oil was 0.88 which is about a third of that obtained with 40 per cent coconut oil. There was a tendency toward leveling off of lipase activity after a concentration of five per cent coconut oil was reached, although there was a slow increase in activity with the increase in coconut oil concentration up to 40 per cent. With 10 per cent coconut oil the net activity amounted to 1.56 as compared to 2.61 with

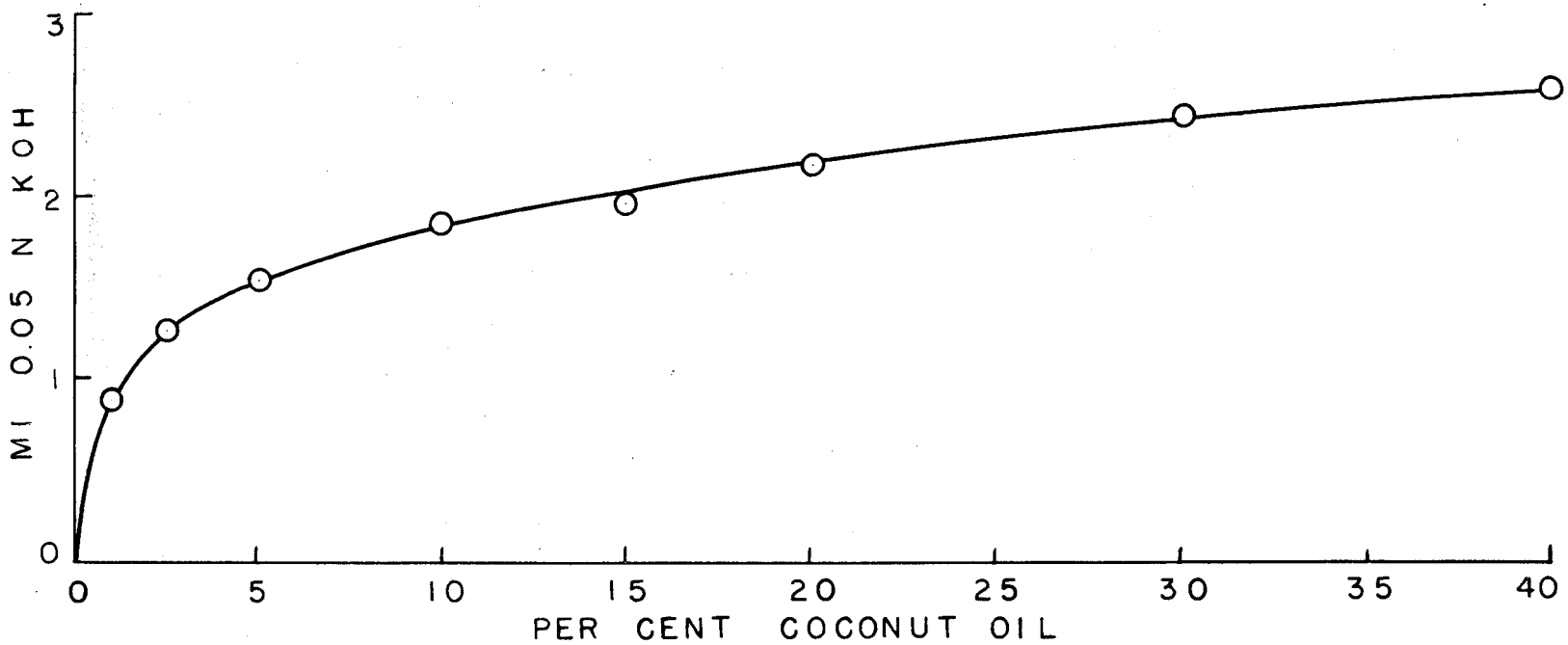


Figure 4. Effect of concentration of coconut oil in test substrate upon lipase activity.

40 per cent. A 10 per cent coconut oil emulsion was used throughout this work because satisfactory lipase activities were obtained with that concentration.

The effect of concentration of lipase upon its activity

The relationship between the concentration of lipase, measured in milliliters of crude lipase preparation, and its activity expressed in milliliters of standard alkali is not linear, indicating some sort of inhibition of enzyme activity. The shape of the curve in Figure 5 reveals that it approximates a straight line at values below a net activity of 3.0. As the amount of lipase preparation is increased the curve deviates from a straight line. However, all the points fall on a smooth curve. With 0.1 ml. lipase preparation the activity is 1.12, while it is 5.99 with 1.0 ml., or only about half the theoretical value obtained by multiplying the activity of 0.1 ml. lipase preparation by 10. Using 2 ml. lipase preparation the net activity was 8.40, a little over one-third of the calculated value arrived at by multiplying 1.12 by 20.

Even though there was no linear relationship between the concentration of lipase and its measured activity, yet it is clear from the curve presented that the relationship is regular. The curve is reproducible, so that the net activity of other values can be used to find the relative

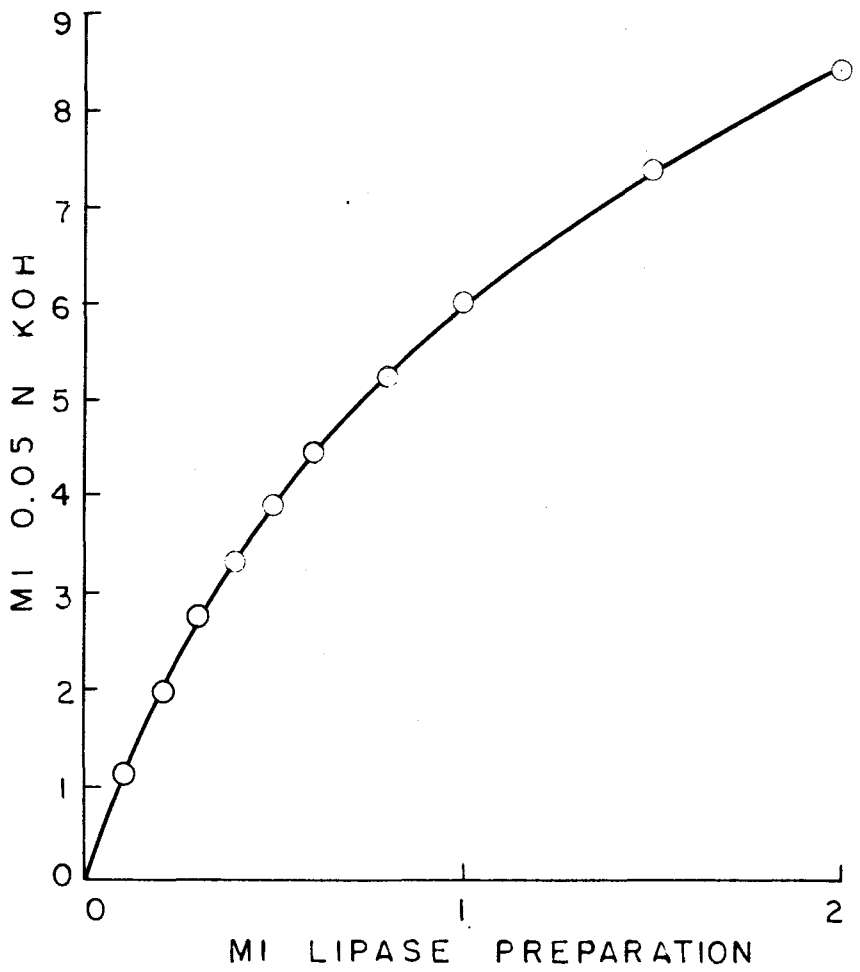


Figure 5. Effect of lipase concentration upon its activity.

concentration of the lipase by insertion on this curve. It was attempted in most trials to use such amounts of lipase preparation as to obtain net activities below 3.0, especially with studies on the characterization of the enzyme, because these values would fall on the part of the curve which approaches a straight line. However, this curve was not employed for correcting lipase activities reported in this study, because it seemed desirable to use actual experimental data.

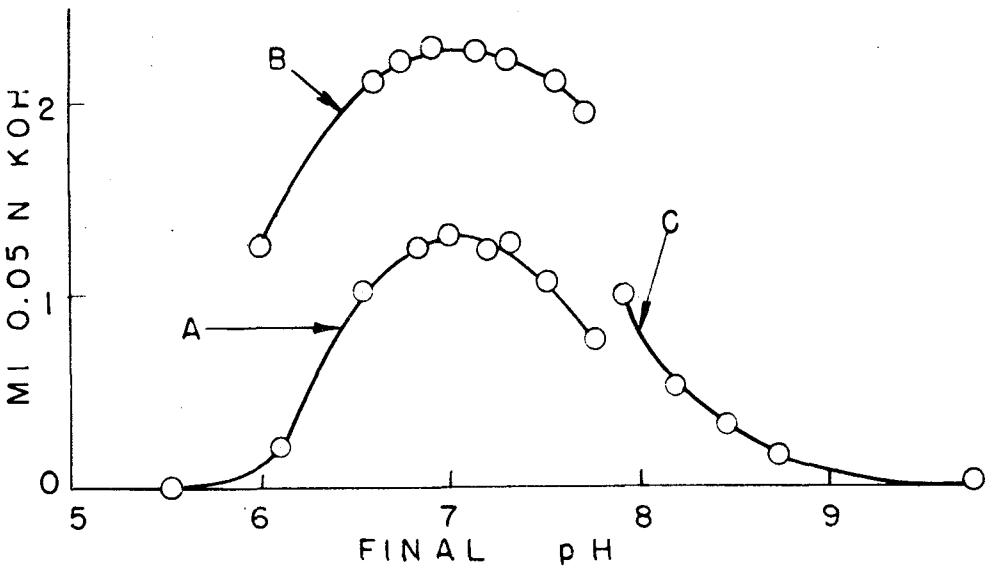
The effect of pH and buffer system of the test substrate upon lipase activity

In these trials the concentration of phosphate or borate in the final substrate after addition of the lipase preparation was fixed at 0.056 M, and the reaction of the test substrate was brought to the desired pH by addition of acid or alkali, depending upon the particular buffer system used.

The lipase of Ps. fragi was found to be active over a wide range of pH extending from slightly below 6.0 to a little higher than 8.7. Such a range of pH cannot be covered efficiently by any one buffer system. Therefore, McIlvaine's citric acid-disodium hydrogen phosphate or Clark and Lubs' potassium dihydrogen phosphate-sodium hydroxide and boric acid-sodium hydroxide buffer systems were used.

McIlvaine's system showed very good buffering capacity between pH 6.5 to 7.2, allowing hardly any shift in pH during the reaction period. During the reaction period a drop amounting to 0.1 to 0.25 pH unit was observed between pH 7.4 and 8.0. With Clark and Lubs' potassium dihydrogen phosphate-sodium hydroxide buffer, a drop of only 0.1 pH unit during reaction was detected between pH 6.7 and 7.2, but between pH 7.4 and 8.0 it increased to 0.17 to 0.3 unit. The boric acid-sodium hydroxide buffer showed a drop in pH of 0.1 to 0.2 units within the range of pH 8.0 to 8.5, the drop decreasing at the high pH levels. In order to make the curves clearer only the final pH values (measured at the end of the reaction period) are given. Figure 6 shows representative data taken from 11 trials, using several different enzyme preparations, the levels of the three curves thus not being directly comparable. Curve A depicts the effect of pH upon lipase activity using McIlvaine's citric acid-phosphate buffer. There was no detectable lipase activity at pH 5.6, in the neighborhood of which the coconut oil emulsion frequently was unstable. Slight net lipase activity was apparent at pH 6.1 and very considerable activity at pH 6.5. The lipase activity was highest between the final pH values of 6.8 and 7.3.

Using Clark and Lubs phosphate buffer (Curve B), the optimum pH for lipase activity was between 6.9 and 7.2 as in



A — Citric acid - Na_2HPO_4 buffer
B — KH_2PO_4 - NaOH buffer
C — Boric acid - NaOH buffer

Figure 6. Effect of pH of test substrate upon lipase activity.

the case of McIlvaine's buffer, except that the activity declined slightly more slowly above pH 7.0 and the level of lipase activity was invariably much higher at pH 6.0 with this buffer system than with McIlvaine's system. Although there was hardly any buffering action with Clark and Lubs phosphate buffer below pH 6.0, lower pH levels were obtained by the addition of 0.1 N hydrochloric acid in order to investigate the region below pH 5.8. In these trials the coconut oil emulsion was unstable below pH 5.8, but there was a sharp drop in enzyme activity between pH 6.0 and 5.8 and little or no activity was detectable at pH 5.4 to 5.75.

The pH range between 8.0 and 9.75 was investigated by the use of Clark and Lubs boric acid buffer system. Curve C shows that there was a rapid drop in lipase activity between pH 8.0 and 8.7 with little activity above pH 8.7.

Apparently lipase preparations from different cultures may vary in their response to pH. Lipase preparations obtained after growth of culture O-1 in nutrient broth invariably showed a steeper drop in activity above pH 7.2 than did preparations from the same culture grown in peptone broth. Culture E-1 showed more rapid drop in lipase activity than did culture O-1 above pH 7.2, whether nutrient broth or peptone broth was used as lipase preparations. The optimum pH, however, always was within the same range with the two cultures grown in the two media mentioned above.

Inactivation of lipase by heat

The temperatures of 61.6°, 71.6° C. and that of boiling water (about 99° C.) were selected, because the first two temperatures are used in the pasteurization of dairy products, and the last was employed for the inactivation of lipase in the preparation of blanks. These studies were attempted in order to elucidate some of the aspects of the susceptibility of this lipase to the heat treatments commonly used in commercial dairy plants.

The three curves shown in Figure 7 are representative data taken from a family of 10 curves obtained in the course of this study. In every case, considerable lipase activity was destroyed during the time required to bring the enzyme preparations up to the desired temperatures. This time interval will be referred to hereafter as the "coming up" time. In the curve labelled 61.6° C., the original lipase activity was 3.26 and it dropped to 1.77 during a "coming up" time of 3 min. 37 sec., 45.7 per cent of the activity being destroyed. It is obvious from the dotted portion of the curve that the drop in lipase activity during the "coming up" time was rather rapid. After reaching the temperature of 61.6° C., the decline in lipase activity with time became very gradual, and the relationship appears to be logarithmic. This slow inactivation of the lipase left after the "coming

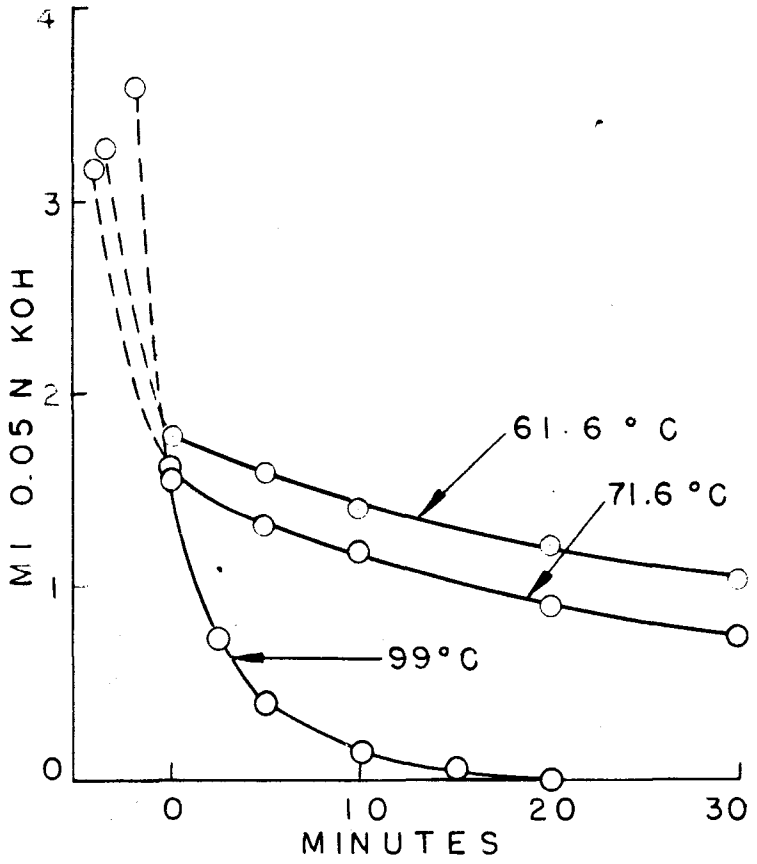


Figure 7. Representative data on the effect of heat upon lipase activity.

up" time was evident in all trials. At zero time the remaining lipase activity was 1.77, and it dropped to 1.03 after holding at 61.6° C. for 30 minutes. From this and data from other trials a higher percentage of the lipase activity was destroyed in preparations having a low activity than those showing higher lipase activities. Even after heating a lipase preparation at 61.6° C. for 60 minutes an activity of 0.29 remained out of an initial activity of 2.14, the corresponding lipase activity after 30 minutes being 0.37.

Inactivation of the lipase heated to 71.6° C. followed much the same pattern as that of the lipase heated at 61.6° C., except that the extent of inactivation was slightly greater. A notable difference between the two curves is the dotted portion of the curve, representing the rate of inactivation of lipase during the "coming up" time, which seemed to be more in continuity with the solid portion, representing the rate of inactivation of the lipase after the lipase preparation had reached the temperature of 71.6° C., than the corresponding portions of the curve at 61.6° C. Out of an initial net activity of 3.17, the residual activity of 1.56 remained after the "coming up" time, representing 50.8 per cent inactivation during that period. Despite heating the lipase preparation at 71.6° C. for 30 minutes, the residual activity was 0.76.

With most other trials on different lipase preparations, a higher percentage was inactivated during the "coming up" time when using 61.6° C. than when using 71.6° C. In a trial where portions of the same lipase preparation were subjected to heat treatments at 61.6° and 71.6° C. for the "coming up" time, the inactivation percentages were 67.3 and 46, respectively. This was the case in spite of the fact that the "coming up" time at 61.6° C. was three minutes while at 71.6° C. it was 3.5 minutes. Such a seemingly anomalous result may be due to the destruction of some inactivating agent at the higher temperature.

Before this heat resistance of the lipase of Ps. fragi was found, the enzyme preparations were subjected to heating in boiling water for 15 minutes in order to get inactivation of the lipase in preparations used for blanks. Consequently, a number of trials were run at the temperature of boiling water. The curve labelled 99° C. shows that there was a rapid drop in lipase activity during the "coming up" time. This decline continued at that rate up to a period of 2.5 minutes when it began to level off. Complete inactivation was reached after heating the lipase preparation at 99° C. for 20 minutes beyond the "coming up" time. The percentage inactivation at this temperature during the coming up time varied from trial to trial, ranging from 56 to 83; the higher percentage was obtained with the lipase preparation

having the lowest initial activity.

Stability of lipase at various temperatures

For the purpose of these studies, lipase preparations obtained after growth of culture 0-1 in peptone broth were used to determine the stability of lipase at the different temperatures. The final pH of the lipase preparations ranged from 7.6 to 7.7, which range is usual in this type of medium. In all this work, lipase preparations were used as such without adjustment of pH. Lipase preparations were dispensed in 50 ml. portions into 250 ml. Erlenmeyer flasks, and after the addition of 0.05 ml. of 36 per cent formaldehyde reagent to each flask in order to prevent bacterial growth during incubation, the contents of the flasks were adjusted to the desired temperatures and set at 3-5°, 15° and 36° C. The lipase activity was tested before incubation and after one, three and seven days, using 0.3 ml. lipase preparation per 40 ml. of test substrate. These three temperatures were chosen because they were regularly used throughout the work. The temperatures of 3 to 5° C. were employed for storing lipase preparations for further use; 15° C. was chosen as an incubation temperature of all growth media for lipase production, while lipase activity determinations were made at 36° C.

Table 13 shows representative data on the effect of storage temperature of lipase preparations upon their lipase

Table 13

Stability of lipase in peptone broth at various temperatures (0.3 ml. lipase preparation per 40 ml. substrate)

Holding (days)	Net* activity at		
	3-5° C.	15° C.	36° C.
0	5.08	5.08	5.08
1	4.66	4.41	1.04
3	4.46	3.55	0.26
7	4.33	2.55	0.12

* After deduction of blanks (0.40 - 0.42)

activity. A relatively small loss of lipase activity occurred during a period of seven days at 3-5° C., the net activity decreasing from 5.08 to 4.33. A more pronounced loss of activity was observed in lipase preparations kept at 15° C., the lipase activity dropping to 2.55 after a period of seven days. Even after holding the lipase preparations at 15° C. for three days, the net activity dropped appreciably. Very marked inactivation of lipase was observed within 24 hours at 36° C., the remaining activity being only about 20 per cent of the original. Little activity was detectable when the lipase preparation was held at 36° for three to seven days.

Salting out of lipase

Lipase could be salted out from a peptone broth preparation by means of half to full saturation with ammonium sulfate. Greater recovery of lipase activity was accomplished the closer the lipase preparation was to full saturation with ammonium sulfate. It was imperative to keep the preparation as cold as possible at all times to prevent lipase losses during manipulation. Considerable loss of lipase activity occurred when the preliminary salting out procedure was carried out at room temperature, although the saturated enzyme preparation was later allowed to stand at 3-5° C. No lipase activity was detectable in solutions of the precipitate

obtained by the use of ethanol in conjunction with saturation with ammonium sulfate, as suggested by Peters and Nelson (1948b) for salting out of Mycotorula lipolytica lipase. Also it was not possible to recover any lipase activity by substituting acetone for ethanol.

Data from three trials on the salting out of lipase are presented in Table 14. Taking into consideration the unavoidable losses in the various manipulations, it is apparent that almost all the lipase activity in the original preparation could be accounted for by the precipitate obtained in trial 1, and to a lesser degree in trial 2. In both trials the lipase activity left in the supernatant was negligible.

In all trials, a fluffy white material formed on the surface of the lipase preparation immediately after saturation with ammonium sulfate, while a brownish precipitate formed and sank to the bottom of the centrifuge tubes after standing the saturated solution at 5-7° C. for 16 to 18 hours. These two materials were combined after centrifugation and filtration and tested for lipase activity in trials 1 and 2 in the table. In trial 3 these two materials were collected separately and then tested for lipase activity. The figures under trial 3 in Table 14 show that the lipase activity retained by each precipitate was identical, and the total of both net activities of the two materials was equal to the net activity of the original lipase preparation from which

Table 14

Salting out of lipase from peptone broth by saturation with ammonium sulfate

No.	Material	Net activity* Trial 1**	Trial 2**	Trial 3**
1	Original lipase preparation	4.73	3.66	3.38
2	Solution of precipitates recovered***	4.59	3.24	--
3	Solution of fluffy precipitate on surface	--	--	1.69
4	Solution of precipitate on standing	--	--	1.69
5	Supernatant from material No. 2	0.09	0.04	--
6	Supernatant from material No. 4	--	--	0.04

* After deduction of blanks
** In trial 1, 0.3 ml. of lipase preparation per 40 ml. substrate was used, and 0.2 ml. in the other trials.
*** Lipase preparations stood at 5-8° C. for 16-18 hr. after saturation with ammonium sulfate.

they were prepared.

Effect of various divalent cations, sodium thioglycollate and cysteine upon lipase activity

Solutions of cobaltous sulfate heptahydrate, magnesium sulfate heptahydrate, anhydrous manganous sulfate, calcium chloride dihydrate, cupric sulfate pentahydrate, ferrous sulfate heptahydrate, sodium thioglycollate and cysteine hydrochloride were made with a molarity of 0.036. To each 30 ml. of four-thirds strength test substrate, 10, 1 and 0.1 ml. of these solutions were added with enough distilled water to make a final substrate volume of 40 ml. having molar concentrations of 0.01, 0.001 and 0.0001, respectively. This addition was made just before pipetting 0.1 ml. of lipase preparation into the test substrate. Cysteine hydrochloride was adjusted to pH 7.15 in the process of making the stock solution.

Cupric ion showed marked inhibition of lipase action when present in 0.01 M concentration, some inhibition at 0.001 M and no inhibition at 0.0001 M concentration, the lipase activity values being 0.06, 1.04 and 1.35 respectively as compared to a control value of 1.38. Cobaltous, manganous, calcium and ferrous ions resulted in copious precipitates when they were present in 0.01 M concentrations. Calcium, ferrous and manganous ions showed limited inhibition of

lipase activity when the final concentrations in the test substrates were 0.01 or 0.001 M, while cobaltous ion resulted in a low activity of 1.00 in concentrations of 0.01 M, but had no effect in lesser concentrations, the activities being 1.47 and 1.46 as compared to the control value of 1.47. In the case of these ions, the lowering of lipase activity probably was associated with the formation of precipitates in the test substrate. Cysteine hydrochloride and sodium thioglycollate had no appreciable effect on lipase activity.

Physical and Physico-Chemical Factors Affecting Lipase Production by *Ps. fragi*

In the past limited studies have been made on the physical factors which influence the production of lipase by *Ps. fragi*. This work is an attempt to supply additional information.

The influence of temperature of incubation on lipase production

A number of trials were made using two cultures of *Ps. fragi* in order to determine the effect of temperature of incubation upon lipase production. In all trials the incubation time at the various temperatures was three days. The results presented in Table 15 are representative data

covering four trials with culture 0-1, except that in one of the four trials lipase production was higher per unit volume of the medium when incubated at 12° C. than at 8° C. However, lipase production per unit count always was higher at 8° C. than at 12° C. With an incubation temperature of 6-6.5° C. the production of lipase per unit count of cells was at a maximum, although the lipase production, as judged by net activities per unit volume, was lower than that observed at temperatures of 12° or 16° C. Even though 16° C. was the optimum for cell proliferation of culture 0-1, yet the lipase production per unit volume was lower than that at 12° and 8.5° C. Furthermore, there was a rapid decline in lipase production by this culture at temperatures above 16° C. In other trials not shown in the table, there was little or no demonstrable lipase production by culture 0-1 after three days of incubation at temperatures of 30° C. or above. The shift in pH became greater as the temperature of incubation was raised, the final pH ranging from 7.5 to 8.12.

Similar trends were observed with culture E-1, except that the optimum temperature for lipase production under these conditions was 15-15.5° C. Table 16 shows that very little detectable lipase production was obtained in three days at 8.5° C. or lower. As in the case of culture 0-1, the lipase production declined markedly at temperatures above 15.5° C., there being virtually no demonstrable lipase activity in

Table 15

Effect of incubation* temperature of nutrient broth upon lipase production by culture O-1
(2 ml. lipase preparation per 40 ml. substrate)

Temperature of incubation (° C.)	Initial pH	Final pH	Count/ml. (x 10^6)	Net** activity
3 - 3.5	7.53	7.50	31	0.00
6 - 6.5	"	7.72	570	2.29
8 - 8.5	"	7.83	1,700	4.12
11.5 - 12.5	"	7.92	3,400	3.34
15.5 - 16.5	"	7.99	3,700	2.74
17 - 18	"	8.00	1,900	1.38
21 - 22	"	8.12	1,000	1.03

* Incubated for 3 days.
** Average blank deducted = 0.25

Table 16

Effect of incubation* temperature of nutrient broth upon lipase production by culture E-1
(1 ml. lipase preparation per 40 ml. substrate)

Temperature of incubation (° C.)	Initial pH	Final pH	Count/ml. (x 10^6)	Net** activity
3 - 3.5	7.48	7.45	14	0.02
6 - 6.5	"	7.49	15	0.00
8 - 8.5	"	7.65	190	0.06
12 - 12.5	"	7.95	3,300	1.35
15 - 15.5	"	7.93	3,200	2.30
21 - 22	"	7.93	1,700	0.87
29 - 30	"	7.98	1,100	0.03
35 - 35.5	"	7.53	0.01	0.00

* Incubated for 3 days
** Average blank deducted = 0.23

1 ml. portions of the lipase preparation from culture incubated at 30° C., despite a build-up in population of 1 billion per milliliter. The optimum temperature for both lipase production and cell proliferation after an incubation period of three days coincided at 15-15.5° C. These results are of practical importance, since lipase production by Ps. fragi is at its highest at the temperatures frequently used for holding cream on the farms. Again, the shift in pH somewhat paralleled the increase in temperature, and to a lesser degree the bacterial count.

The previous findings suggested a more detailed study of the production of lipase at several low temperatures at daily intervals of incubation. Incubation temperatures of 6, 8, 12 and 15° C. were used in this study. In Figure 8 the log of the count of bacteria is represented by solid lines, while lipase activities are indicated by broken lines. Naturally, lipase activities lagged behind cell proliferation. Maximum lipase production is relative and should be qualified by stating the temperature and incubation time. Thus after incubation for one day, lipase production was highest at 15° C., at which temperature the colony count reached 430 million per milliliter. The maximum lipase activity after a two-day incubation period occurred at 12° C. After the third day of incubation, maximum lipase activity was found at a temperature of 8° C., and at the end of seven days the highest activity

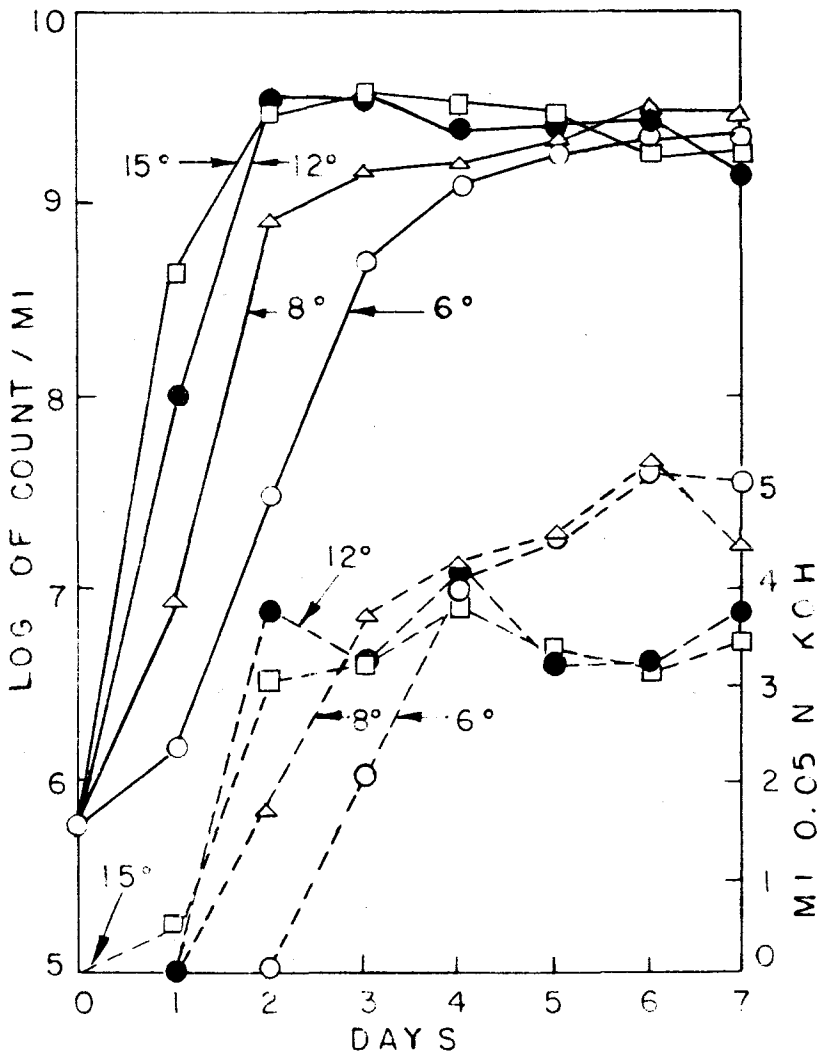


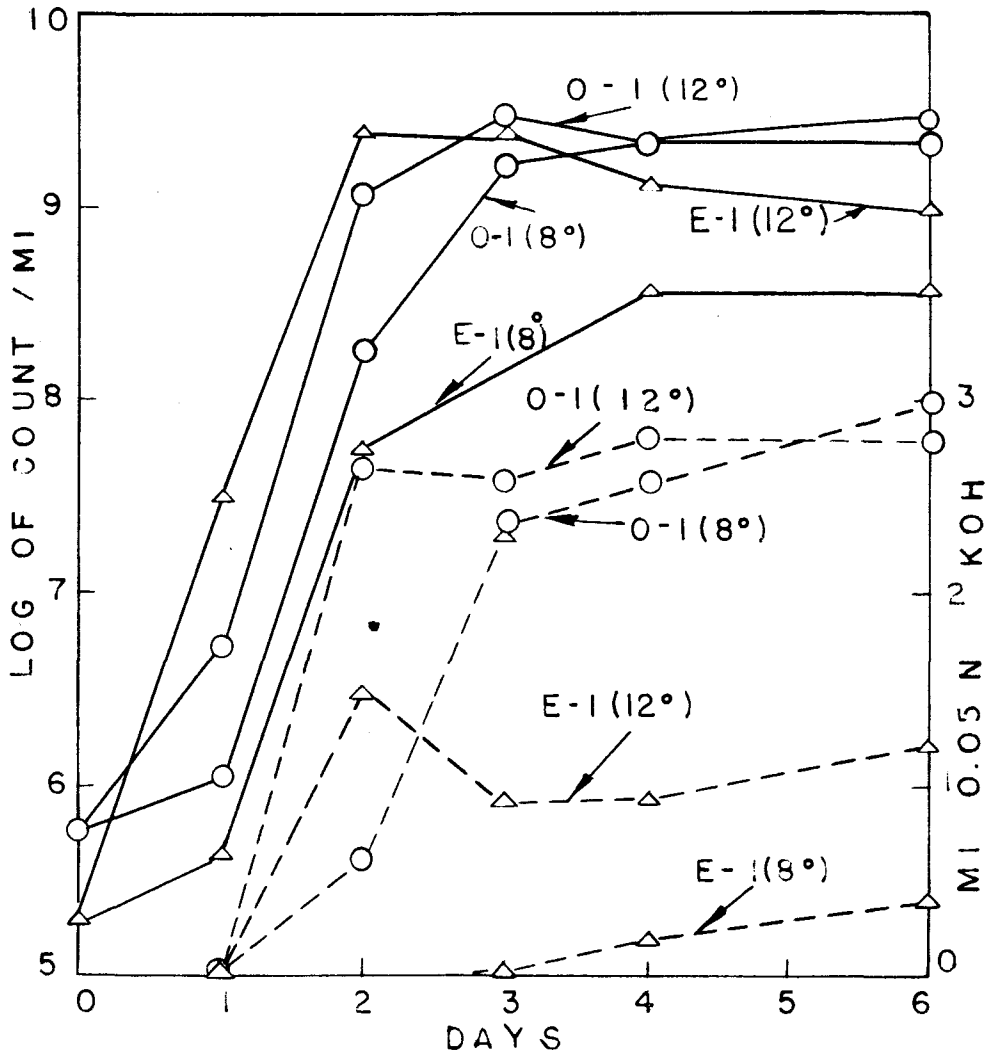
Figure 8. Relation of count of bacteria to lipase production by culture 0-1 at low temperatures.

was found at 6° C. With incubation at 12° and 15° C., the bacterial count reached its peak after incubation for two days, with a count of three billion per milliliter; the respective net activities were 3.76 and 3.05. Lipase activity per unit count was greatest at an incubation temperature of 6° C. from the third day until the end of the incubation period.

Comparing the growth curves with the lipase activity curves it is obvious that lipase production was related to the log. of bacterial counts during the latter part of the phase of logarithmic growth, when the temperatures of incubation were 6° and 8° C. Furthermore, the lipase activity of the growth medium continued to increase, though at a lower rate, even after the period of maximum stationary growth had been reached. The lipase activity produced at 12° and 15° C. rose sharply after one day of incubation, reaching a peak after two days and then fluctuated throughout the rest of the incubation period. This leveling off of lipase activity produced at 12° and 15° C. coincided with the decline in bacterial counts. There was a drop in the lipase activity of the growth medium incubated at 15° C. and sometimes at 12° C. after the third day of incubation, the drop persisting in all trials made with cultures O-1 or E-1.

The differences in the optimum temperature for lipase production by the two cultures used seemed so interesting as

to deserve the extension of the studies presented in Figure 8 to a comparison of the behavior of two cultures at 8° and 12° C. during an incubation time of six days. Figure 9 was made along the same lines as was Figure 8. The initial count on culture 0-1 was 580,000 per milliliter as compared to 190,000 on culture E-1. From a study of the population of culture E-1 at 8° C., it is clear that the count leveled off after the fourth day of incubation at 400 million per milliliter, and then was more or less constant up to the sixth day of incubation. As a consequence, the lipase production, which was hardly measurable after the fourth day, increased at a very slow rate up to the end of the incubation period. On the contrary, the count on culture 0-1 in the medium incubated at 8° C. increased rapidly after the first day until the third day of incubation, and then continued to increase slowly up to the end of the incubation period. Lipase production by culture E-1 was not detectable until after the third day of incubation period. There was appreciable lipase production by culture 0-1 after two days incubation, and this production increased rapidly up to the third day, and continued to increase up to the sixth day of incubation. This increase in lipase activity by culture 0-1 at 8° C. almost ran parallel to the increase in the log. of counts. The difference in the abilities of these two cultures to produce lipase at low temperatures was great.



——— Log of bacterial counts/ml.
- - - Ml. 0.05 N. Potassium hydroxide.

Figure 9. Relation of count of bacteria to lipase production by two cultures at 8 and 12° C.

The count on culture E-1 reached a peak on the second day at 12° C., but culture 0-1 reached its maximum count on the third day. In this and two other trials, a drop in lipase activity was observed on the third day of incubation, though it was less marked in this trial with culture 0-1 than with culture E-1, and was followed by a slow rise in lipase activity up to the end of the incubation period. Lipase activity curves of both cultures showed an abrupt jump during the second day of incubation, coinciding with the phase of logarithmic growth of the two cultures. In both cases, the lipase activity leveled off at the end of the second day of incubation. The level of lipase activity was much higher with culture 0-1 than with culture E-1 throughout the period of incubation.

The effect of pH of growth media upon lipase production

It was observed from the outset that there was a considerable shift in pH to the alkaline side of neutrality with media such as nutrient, peptone, casamino acids, citrate and lactate broths, and to the acid side when glucose was present in the medium. This shift made the study of the effect of pH upon lipase production rather complicated. When the buffer concentration was raised above 0.05 M potassium dihydrogen phosphate, the lipase production declined. As shaking of the growth medium during incubation

was found to affect lipase production, no recourse to periodic neutralization of the medium was made.

The influence of the pH of nutrient broth on lipase production by culture 0-1 is presented in Table 17. The buffer concentration was kept constant at 0.05 M, and the desired pH was obtained by addition of varying volumes of 0.1 N sodium hydroxide. There was a shift of 0.3 to 0.4 pH during the three-day incubation period, except with an initial pH of 7.8, where the shift was 0.16 unit. Apparently there are two maxima of lipase production, one at an initial pH of 6.5 and the other at 7.8, the former showing a higher lipase activity than the latter. The lipase activity per unit count was highest with an initial pH of 6.5. The plate count was highest at initial pH's of 6 and 7.4.

Representative data on culture E-1 are given in Table 18. The shift in pH ranged from practically nothing at pH 8.39 to 1.20 pH units with an initial pH of 5.14. The pH values of nutrient broth below 6.0 were obtained by addition of 0.1 N hydrochloric acid, although the phosphate buffer used had essentially no buffering capacity at that lower range of pH. Even though a count of two billion colonies per milliliter was reached at an initial pH of 8.39, there was no detectable lipase activity in 1 ml. portions of broth. Lipase activity in the medium with an initial pH of 5.6 was relatively low. Here again, the rise in lipase activity

Table 17

Effect of pH of nutrient broth upon lipase production by culture O-1
(2 ml. lipase preparation per 40 ml. substrate)

Initial pH	Final pH	Count/ml. ($\times 10^6$)	Net* activity
6.02	6.48	2,100	1.03
6.48	6.78	520	1.84
6.96	7.25	840	1.10
7.40	7.70	2,100	1.47
7.82	7.98	1,600	1.69

* Average blank deducted = 0.31

Table 18

Effect of pH of nutrient broth upon lipase production by culture E-1
(1 ml. lipase preparation per 40 ml. substrate)

Initial pH	Final pH	Count/ml. ($\times 10^6$)	Net* activity
5.14	6.34	1,800	0.17
5.59	6.47	1,600	0.16
6.08	6.68	2,100	0.27
6.48	6.99	1,200	0.82
6.97	7.47	1,900	0.74
7.47	7.99	2,500	1.97
8.03	8.34	2,200	0.76
8.14	8.35	1,800	0.17
8.39	8.40	2,000	0.00

* Average blank deducted = 0.26

with an initial pH of 6.5 is noticeable, although it is less marked than in the case of culture 0-1. The maximum lipase activity per unit volume of nutrient broth and per unit count, occurred with an initial pH of about 7.5. These two peaks of lipase production were noticeable in two trials with culture 0-1 and in two other trials with culture E-1.

Peptone broth rather than nutrient broth commonly was used in the latter part of this study because it was found to be much superior to the latter as a medium for lipase production by Ps. fragi. Table 19 shows the effect of pH of peptone broth upon lipase production within a narrow range of pH. Although the count was almost the same at the various pH values, the lipase activity per unit volume of the medium increased with the decrease in initial pH of the medium.

The data presented in Table 20 show the effect of pH on lipase production by culture 0-1 growing in casamino acids medium. The lipase activity in the medium with initial pH 7.0 or 7.2 was similar, and was appreciably greater than with a pH of 7.44. This trend was somewhat similar to that with peptone broth in the previous table. However, the count was higher the higher the initial pH. As already noticed in the case of nutrient broth and peptone broth, there was a shift of about 0.6 pH unit in medium reaction during the incubation period.

Table 21 presents results with two cultures grown in a

Table 19

Effect of pH of peptone broth upon lipase production by culture 0-1
(0.3 ml. lipase preparation per 40 ml. substrate)

Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
7.17	7.62	4,700	4.88
7.29	7.77	4,300	4.35
7.50	7.85	4,500	3.78

* Blank deducted = 0.41

Table 20

Effect of pH of vitamin-free casamino acids medium upon lipase production by culture 0-1
(0.3 ml. lipase preparation per 40 ml. substrate)

Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
7.02	7.58	3,800	2.37
7.20	7.81	4,100	2.37
7.44	7.99	5,300	1.61

* Blank deducted = 0.41

glucose-containing defined medium to demonstrate the drop in pH of the medium after growth of Ps. fragi and the effect of this drop on enzyme production. Lipase production per unit volume was highest with an initial pH 7.4 in the case of culture O-1, but the optimum was not clear-cut with culture E-1. A drop in pH ranging from 0.5 to 0.6 pH unit was observed.

A defined medium with L-leucine as the sole source of both nitrogen and carbon was observed to have a fairly constant pH throughout the incubation period. Table 22 gives the results obtained with culture O-1 growing in this medium at different pH levels. The shift in pH was not greater than 0.1 pH unit. Although the net activities obtained were rather low, the maximum lipase activity per unit volume and also per unit count was found at pH 7.56. However, the lipase activity in the medium at pH 7.37 was practically the same as that at pH 7.56, the difference being within the experimental error of the method. Counts were usually low as compared to those obtained with the other media such as nutrient, peptone, vitamin-free casamino acids and glucose broths.

In dairy products Ps. fragi usually is competing and growing with acid producing microorganisms. Therefore, the determination of the lower limits of pH for the production of lipase by this organism is of great importance. The

Table 21

Effect of pH of glucose defined medium upon lipase production by cultures O-1 and E-1
(2 ml. lipase preparation per 40 ml. substrate)

Culture	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
O-1	6.97	6.48	270	1.30
O-1	7.18	6.77	2,100	2.40
O-1	7.40	6.93	2,000	3.22
E-1	6.97	6.48	2,000	2.35
E-1	7.18	6.82	1,200	1.75
E-1	7.40	6.93	1,500	2.37

* Average blank deducted = 0.35

Table 22

Effect of pH of L-leucine defined medium upon lipase production by culture O-1
(1 ml. lipase preparation per 40 ml. substrate)

Initial pH	pH* after 24 hr.	pH after 48 hr.	pH after 72 hr.	Count/ml. at 72 hr. (x 10^6)	Net** activity (72 hr.)
7.37	7.35	7.33	7.33	650	0.46
7.56	7.52	7.45	7.48	500	0.49
7.82	7.75	7.72	7.74	860	0.37
7.95	7.95	7.86	7.87	630	0.34

* The flasks were shaken before taking pH.
** Average blank deducted = 0.38

adjustment of the pH of peptone broth below 5.8 was accomplished by omission of sodium hydroxide or addition of 0.1 N hydrochloric acid to the medium, while maintaining the usual 0.05 M concentration of potassium dihydrogen phosphate. It was attempted to study the growth and lipase production by culture 0-1 between pH 4.0 and 4.70 (Table 23). Media with initial pH values of 4.3 or below showed no visible turbidity after incubation at 15° C. for three days. Also, the counts after incubation were less than the usual initial count, which ranges between one to two million per milliliter. Turbidity was visible in the medium with an initial pH of 4.5, accompanied by a shift of 0.5 pH unit, whereas fairly good growth occurred in the medium adjusted to pH 4.69 with considerable shift in pH. Practically no lipase activity was detectable at or below initial pH values of 4.5. A fair lipase activity was demonstrable in the medium with an initial pH of about 4.7. This run really establishes no true lower level of lipase production because of the considerable rise in pH during incubation in the one case where considerable enzyme production occurred.

As a continuation, Table 24 gives some of the lipase activities produced by culture 0-1 in peptone broth with an initial pH range of 4.83 to 7.08. As the initial pH increased up to 5.5 there was an increase in the amount of demonstrable

Table 23

Effect of pH of peptone broth upon lipase production by culture 0-1
(0.3 ml. lipase preparation per 40 ml. substrate)

Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
4.00	4.05	0.02	0.01
4.17	4.19	0.28	0.04
4.30	4.44	0.26	0.02
4.53	5.04	8	0.05
4.69	6.14	1,500	0.95

* Blank deducted = 0.40

Table 24

Effect of pH of peptone broth upon lipase production by culture 0-1
(0.3 ml. lipase preparation per 40 ml. substrate)

Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
4.83	6.19	2,400	1.44
5.03	6.27	3,100	2.98
5.49	6.41	3,200	4.40
5.82	6.55	5,000	4.06
5.98	6.59	1,600	3.97
6.08	6.65	3,100	5.20

* Average blank deducted = 0.43

lipase activity. However, the final pH values in the various cultures are so much higher than the original values that about the only thing demonstrated is the ability of the organism to initiate fairly rapid growth at a given pH level, thus permitting the organism to adjust the medium reaction to a point where better growth and greater lipase production may occur.

The effect of phosphate buffer concentration in peptone broth upon lipase production

It was desirable to determine whether the increase of the concentration of potassium dihydrogen phosphate in peptone broth would result in higher lipase production and a lesser shift in pH by culture 0-1. Concentrations of phosphate from zero to 0.1 M were obtained by addition of 0-20 ml. of 0.5 M phosphate per 100 ml. of final medium and enough 0.1 N sodium hydroxide to get the desired pH levels in peptone broth. It appears from the results in Table 25, which are representative data of three trials, that concentrations of 0.025 to 0.05 M phosphate were optimal for lipase production by this culture and by culture E-1 in another trial. At the higher concentrations lipase production invariably was inhibited slightly. A concentration of 0.05 M phosphate was adopted in the growth media in this work because there was less shift in pH than at the lower

Table 25

Effect of concentration of monobasic potassium phosphate in peptone broth upon lipase production by culture 0-1 (0.3 ml. lipase preparation per 40 ml. substrate)

Concentration of KH_2PO_4	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
0.000	7.09	7.74	1,900	1.83
0.025M	7.10	7.67	4,100	4.75
0.050M	7.16	7.53	3,400	4.76
0.075M	7.16	7.52	3,700	4.31
0.100M	7.16	7.43	4,200	4.43

* Average blank deducted = 0.40

concentrations, while keeping lipase production at an optimum.

Effect of sodium chloride concentration upon lipase production

Ps. fragi is reportedly quite sensitive to low concentrations of sodium chloride. Therefore, it was desirable to obtain data on the effect of the salt in buffered peptone broth upon lipase production by this organism.

Culture 0-1 showed hardly any detectable lipase activity in 0.3 ml. portions of lipase preparation when the peptone broth contained three per cent or more of sodium chloride (Table 26). Even one per cent salt had a considerable effect in reducing both the enzyme activity and population level. In another trial, the count with three per cent salt was one billion per milliliter and the corresponding lipase activity was 0.05, while a count of 140 million per milliliter was found with a four per cent salt concentration. Also, the plate count with five per cent salt was 90 million per milliliter, whereas no visible turbidity developed when the medium contained six per cent salt and the plate count was two million per milliliter. The effect of sodium chloride concentration on lipase production is appreciably greater than the effect upon cell population, especially with the higher salt concentrations.

What was obtained in Table 27 with cultures E-1, C and 10 confirms some of the results presented in the preceding table with culture 0-1. In this trial, a comparison was made between lipase production in buffered peptone broth and that in similar broth containing four per cent sodium chloride. The three cultures liberated no demonstrable lipase in 0.3 ml. of the medium containing four per cent salt. Furthermore, the counts dropped appreciably in broths in which the salt was present. The differences in the abilities of the three cultures to produce lipase in peptone broth is also demonstrated.

Influence of aerobic relationships in the growth medium on lipase production

Ps. fragi is notably aerobic, and usually forms a pellicle on the surface of a liquid growth medium. Therefore, any shaking of the medium during growth probably would disturb the aerobic conditions of the growing organisms.

A chemically-defined medium with 0.3 per cent L-leucine, 0.1 per cent DL-isoleucine and 0.3 per cent DL-valine as sole sources of carbon and nitrogen, with the usual phosphate buffer and 0.05 per cent magnesium sulfate heptahydrate, was used for this study, because of the negligible shift in pH during incubation. The medium was adjusted to the different pH levels by addition of 0.1 N sodium hydroxide. Two flasks

Table 26

Effect of concentration of sodium chloride in peptone broth upon lipase production by culture 0-1
(0.3 ml. lipase preparation per 40 ml. substrate)

Per cent NaCl	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
0	7.30	7.80	5,500	5.28
1	7.17	7.74	2,900	3.12
2	7.19	7.68	1,800	0.70
3	7.16	7.78	1,200	0.07
4	7.16	7.75	330	0.00

* Average blank deducted = 0.40

Table 27

Effect of concentration of sodium chloride in peptone broth upon lipase production by 3 cultures
(0.3 ml. lipase preparation per 40 ml. substrate)

Per cent NaCl	Culture	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
0	E-1	7.28	7.67	4,400	4.96
4	E-1	7.27	7.32	300	0.00
0	C	7.28	7.74	6,600	1.52
4	C	7.27	7.46	330	0.01
0	10	7.28	7.73	3,800	0.47
4	10	7.27	7.48	330	0.01

* Average blank deducted = 0.48

at each pH level were inoculated with culture 0-1 and one of each pair of flasks was shaken twice daily, while the other was left undisturbed during the period of incubation. In all cases, the lipase production per unit volume of the shaken culture was about half as much as in the undisturbed medium (Table 28). However, the net lipase activity per unit count was somewhat higher in the media adjusted to pH 7.53 and 7.65 when they were shaken, indicating that at least a considerable portion of the reduction in lipase production as the result of shaking was attributable to decreased numbers of cells. Except in the medium with an initial pH of 8.06, there was very little shift in pH during the incubation period. Obviously, counts were lower in the shaken flasks than in those left undisturbed.

In Table 29 representing one trial, cultures 0-1 and E-1 reacted somewhat differently to the effect of shaking when grown in peptone broth, E-1 showing a higher lipase activity in the undisturbed medium than when shaken, while 0-1 gave similar lipase activities with the two treatments. The richer character of this medium might account for the differences between these results and those of Table 28.

With aerobic microorganisms, the relationship between the surface area of the growth medium and its depth may affect the physiological activity of the growing organisms. In order to permit considerable difference in the proportion

Table 28

Effect of twice-a-day shaking of growth medium* upon lipase production by culture O-1
(1 ml. lipase preparation per 40 ml. substrate)

Treatment	Initial pH	Final pH	Count/ml. (x 10^6)	Net** activity
None	7.53	7.54	1,300	0.94
Shaken	7.53	7.52	620	0.50
None	7.65	7.65	1,500	1.07
Shaken	7.65	7.65	530	0.50
None	7.89	7.82	540	0.54
Shaken	7.89	7.83	510	0.26
None	8.06	7.90	780	0.45
Shaken	8.06	7.90	550	0.27

* L-leucine, DL-isoleucine and DL-valine as sources of carbon and nitrogen.
** Average blank deducted = 0.38

of surface area to depth, 100 ml. of peptone broth were dispensed in 34 x 300 mm. test tubes and one-liter flasks, then after sterilization they were inoculated with cultures 0-1, E-1 and 10. In all instances, the counts per milliliter and the net lipase activities per unit volume were much higher in the containers exposing a large surface than in the test tubes (Table 30). The net lipase activity per unit count was higher in the test tubes than in the flasks. It is to be noticed that greater shifts in the final pH values occurred in the media dispensed into the flasks than in those in the test tubes, this probably being associated with the increased growth.

Nutritional Factors Affecting Lipase Production

Production of lipase by some microorganisms has been reported to be poor in chemically-defined media, and to be influenced markedly by certain medium constituents. This study was planned to determine some of the nutritional factors that influence the production of lipase by Ps. fragi.

Lipase production in some protein digests and hydrolyzates

Phosphate-buffered nutrient broth containing 0.5 per cent sodium chloride was used in the early part of this study as a medium for lipase production by Ps. fragi. An inorganic

Table 29

Effect of twice-a-day shaking of peptone broth cultures upon lipase production by cultures 0-1 and E-1 (0.5 ml. lipase preparation per 40 ml. substrate)

Culture	Treatment	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
0-1	None	7.45	7.75	4,200	4.63
0-1	Shaken	"	7.76	3,900	4.68
E-1	None	"	7.85	4,000	6.27
E-1	Shaken	"	7.87	4,800	4.66

* Average blank deducted = 0.37

Table 30

Effect of ratio of surface area to volume of peptone broth upon lipase production by three cultures (0.3 ml. lipase preparation per 40 ml. substrate)

Culture	Container	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
0-1	Test tube**	7.18	7.32	1,500	3.65
0-1	Flask***	"	7.68	3,600	5.82
E-1	Test tube	"	7.34	1,200	2.64
E-1	Flask	"	7.72	4,600	4.83
10	Test tube	"	7.33	1,000	0.78
10	Flask	"	7.77	3,900	1.06

* Average blank deducted = 0.40
** Depth of medium = 10.5 cm.; surface area = 9 $cm.^2$
*** Depth of medium = 1 cm.; surface area = 114 $cm.^2$

buffer, consisting of potassium dihydrogen phosphate and sodium hydroxide, was used. This choice of an inorganic buffer was made in order to avoid use of compounds that might be utilized by Ps. fragi as additional sources of energy.

Lipase production in the modified nutrient broth by cultures of Ps. fragi used in this study was satisfactory. It remained to be seen which components of the modified nutrient broth were necessary for lipase production. Table 31 gives data representative of two trials. In these trials the beef extract was increased from 0.3 to 0.5 per cent. The modified nutrient broth consisted of 0.5 g. Bacto-peptone, 0.5 g. beef-extract, 0.5 g. sodium chloride, 10 ml. of 0.5 M potassium dihydrogen phosphate solution, 47 ml. sodium hydroxide and 41.5 ml. distilled water. Other media were obtained by the omission of one ingredient at a time. In the first trial with culture O-1 alone, a slightly higher lipase production was obtained when sodium chloride was omitted, so it was not included in the second trial. Both cultures O-1 and E-1 gave maximum lipase production per unit volume and per unit count when buffered peptone broth was the medium of growth, that is, when beef extract was omitted from the nutrient broth. Beef extract, although supporting good growth when peptone was omitted, apparently did not

Table 31

Effect of omission of individual components of nutrient broth upon lipase production by two cultures of Pseudomonas fragi (1 ml. lipase preparation per 40 ml. substrate)

Culture	Medium	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
0-1	Nutrient broth	7.56	7.93	3,700	3.89
0-1	Peptone omitted	7.63	8.05	640	0.38
0-1	Beef extr. "	7.63	7.82	2,400	6.74
E-1	Nutrient broth	7.56	8.11	3,500	2.69
E-1	Peptone omitted	7.63	8.13	2,200	0.09
E-1	Beef extr. "	7.63	7.83	2,600	4.55

* Average blank deducted = 0.35

contribute much to lipase production. Presence of beef extract in nutrient broth increased the bacterial count but appreciably reduced the net lipase activity resulting from growth of the organisms in that medium. Upon the basis of these studies, a growth medium consisting of buffered peptone without beef extract was adopted.

Different strains of Ps. fragi have different abilities to produce lipase in various media. In Table 32 the variations between nine strains of Ps. fragi as to their abilities to produce lipase in 0.5 per cent peptone broth are demonstrated. The net lipase activities ranged from zero with culture II to 2.27 with culture E-1, using 0.5 ml. of enzyme preparation for testing. Colony counts also varied from 500 million to 3.7 billion per milliliter. In addition, the shift in pH in the medium during incubation varied in magnitude between the different strains, but there was no correlation between that shift and the plate count or the lipase activity.

After establishing that peptone served as a good substratum for lipase production by several cultures of Ps. fragi, it was desirable to determine whether the concentration of peptone had any effect upon lipase production. Concentrations of peptone ranging from 0.1 to 2.0 per cent in a medium buffered with 0.05 M potassium dihydrogen phosphate were tried, using culture O-1 as a test organism. As shown

Table 32

Lipase production in 0.5 per cent peptone broth by nine cultures of Pseudomonas fragi
(0.5 ml. lipase preparation per 40 ml. substrate)

Culture	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
E-1	7.53	7.75	3,300	2.27
K-1	7.53	7.75	1,100	1.44
P	7.53	7.85	1,100	0.28
C	7.53	7.88	3,000	1.07
I	7.53	7.96	700	0.02
II	7.53	7.78	500	0.00
E-3	7.53	7.80	1,000	1.25
10	7.53	7.94	1,700	0.76
11	7.53	7.89	3,700	0.04

* Average blank deducted = 0.38

Table 33

Effect of concentration of peptone upon lipase production by culture O-1
(1 ml. lipase preparation per 40 ml. substrate)

Per cent peptone	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
0.10	7.45	7.54	500	1.82
0.25	7.51	7.62	1,500	4.57
0.50	7.52	7.75	2,400	6.92
1.00	7.54	7.78	3,600	8.03
1.50	7.50	7.78	5,300	7.27
2.00	7.46	7.76	5,300	7.37

* Average blank deducted = 0.41

in Table 33, the plate count continued to increase with increase in concentration of peptone in the medium up to the level of 1.5 per cent. Lipase production per unit volume increased with the build-up in population up to a count of 3.6 billion in the medium with one per cent peptone then leveled off. However, maximum lipase per unit count occurred with 0.1 per cent peptone concentration. The shift in the pH of the medium increased with increase in concentration of peptone up to about 0.5 per cent. In the subsequent trials a concentration of one per cent peptone was adopted.

The next step was to determine which of the components of peptone was responsible for lipase production by Ps. fragi. As peptone, which is a peptic digest of protein, seemed to give a good yield of lipase, a purified protein hydrolyzate offered a good starting material. Lipase production by two cultures in buffered Bacto-vitamin-free casamino acids broth was found to be fairly high, indicating that probably amino acids were required for maximum production of the enzyme. A comparison of lipase production by four cultures in peptone broth versus vitamin-free casamino acids broth is given in Table 34. The peptone broth contained 1 g. Bacto-peptone, 0.4 g. sodium chloride, 10 ml. 0.5 M potassium dihydrogen phosphate solution, 42 ml. 0.1 N sodium hydroxide and 47 ml. distilled water, while the casamino acids medium was composed of 1 g. Bacto-vitamin-free casamino acids, 10 ml. of 0.5 M

potassium dihydrogen phosphate solution, 44 ml. 0.1 N sodium hydroxide and 45 ml. distilled water. As casamino acids powder, which is an acid hydrolyzate of casein, contains some sodium chloride, an equivalent amount of the salt was added to peptone broth in order to offset the effect of the salt in the former medium. Cultures O-1 and E-1 gave considerably higher lipase activities in 0.5 ml. portions of lipase preparation when peptone broth was used as the growth medium than in vitamin-free casamino acids medium, while culture C showed a smaller difference in ability to produce lipase in the two media. However, culture 10 gave a higher lipase activity with casamino acids medium than with peptone broth, being the only culture of the group acting that way. Except in the case of culture C, where the count on the casamino acids medium is questionable, the net lipase per unit count follows the same trend as did the lipase per unit volume of growth medium. The pH change was consistently higher after the incubation period in the vitamin-free casamino acids than in the peptone medium.

Having found that vitamin-free casamino acids medium gave promising results with several cultures of Ps. fragi, it was plausible to determine the optimum concentration to use in further studies. Concentrations of vitamin-free casamino acids ranging from 0.5 to 1.5 per cent were used. The effect of these concentrations upon lipase production by

cultures O-1 and E-1 is shown in Table 35. Lipase production, as determined by lipase activity in 0.5 ml. portions of the medium, was at a maximum when the concentration of vitamin-free casamino acids reached one per cent. Also, culture E-1 gave higher counts and higher lipase activities per unit volume of the medium than did culture O-1. There was a greater shift in the pH of the medium with the increase of casamino acids concentration.

Lipase production in vitamin-free casamino acids medium by culture O-1 and most of the cultures used was consistently lower than in peptone broth. Numerous attempts were made to increase the production of lipase by culture O-1 and sometimes culture E-1 in vitamin-free casamino acids medium by supplementing it with various compounds, but with little success. The addition of peptone ash (obtained by ashing Bacto-peptone at 650-700° C. for 12 hours) to the casamino acids medium decreased rather than increased lipase production by cultures O-1 and E-1. The same was true when magnesium and calcium ions were added. Addition of thiamin, niacin, pantothenic acid, and pyridoxine singly and together also was tried. Thiamin and niacin, when added in quantities of 200 γ per 100 ml. of medium, slightly increased lipase production by culture O-1 over that of the unsupplemented medium, whereas pantothenic acid and pyridoxine had no effect. Moreover, supplementing the casamino acids medium with uracil,

Table 34

Lipase production in vitamin-free casamino acids medium and in peptone broth by four cultures
(0.5 ml. lipase preparation per 40 ml. substrate)

Culture	Medium	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
O-1	Peptone	7.38	7.71	5,700	4.65
O-1	Cas. acids	7.41	7.97	4,700	2.31
E-1	Peptone	7.38	7.71	3,600	6.03
E-1	Cas. acids	7.41	8.11	5,100	3.66
C	Peptone	7.38	7.87	5,200	1.21
C	Cas. acids	7.41	8.09	(?) 500	1.06
10	Peptone	7.38	7.87	4,100	1.14
10	Cas. acids	7.41	8.07	3,000	1.88

* Average blank deducted = 0.32

Table 35

Effect of concentration of vitamin-free casamino acids upon lipase production by two cultures
(0.5 ml. lipase preparation per 40 ml. substrate)

Culture	Per cent casamino acids	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
O-1	0.5	7.39	7.87	4,800	2.26
O-1	1.0	7.38	7.93	5,000	2.34
O-1	1.5	7.37	8.11	4,300	1.83
E-1	0.5	7.39	7.88	5,600	2.69
E-1	1.0	7.38	8.11	6,000	3.61
E-1	1.5	7.37	8.10	6,400	3.17

* Average blank deducted = 0.31

thymine, guanine and adenine resulted in no appreciable increase in lipase production by culture 0-1. Neither the addition of cystine and tryptophan nor that of the ten "indispensable" amino acids, in groups or together was instrumental in raising the level of lipase production by culture 0-1 over that of the control vitamin-free casamino acids medium.

The suitability of the various commercial protein digests as media for lipase production by culture 0-1 was tested. A phosphate-buffered medium containing one per cent of the particular digest was used in this trial. The results presented in Table 36 show that peptone gave the highest net activity per unit volume of growth medium, whereas proteose-peptone showed the highest activity per unit count. Vitamin-free casamino acids yielded higher lipase activities than trypticase or tryptose media. Proteose-peptone medium exhibited the least shift in pH among the materials tried.

Lipase production in chemically-defined media

A citrate medium was among the first chemically-defined media to be tested for suitability for lipase production. It was composed of 0.4 g. sodium citrate, 0.2 g. ammonium chloride, 0.1 g. potassium dihydrogen phosphate, 0.05 g. magnesium sulfate heptahydrate, 3.5 ml. 0.1 N sodium

Table 36

Lipase production in some protein digests and hydrolyzates by culture 0-1
(0.3 ml. lipase preparation per 40 ml. substrate)

Medium	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
Casamino acids**	7.39	7.93	3,600	2.00
Tryptone	7.46	7.82	2,800	1.96
Proteose-peptone	7.44	7.65	3,900	3.43
Tryptose	7.51	7.76	1,900	0.24
Peptone	7.49	7.84	4,500	3.60
Trypticase	7.48	7.93	3,200	0.71

* Average blank deducted = 0.43
** Vitamin-free casamino acids (Difco)

hydroxide and distilled water to make 100 ml. The pH of the medium was adjusted to 6.45. There was considerable shift in pH amounting to 1.5 pH units during the three-day incubation period. Although the above medium supported a relatively large population of two billion per milliliter, yet the lipase production by the cultures used was negligible.

The citrate medium was supplemented with some "indispensable" amino acids in 20 mg. quantities of amino acid per 100 ml. of medium. The figures presented in Table 37 show that the addition of L-leucine, DL-isoleucine and DL-valine resulted in a lipase activity per unit volume of the medium as high as that when the nine amino acids were added. The citrate medium supplemented with threonine, methionine and phenylalanine showed a count of 4.4 billion colonies per milliliter with little lipase production. Considerable shifts in pH of the media during incubation were noticeable.

With culture O-1 it was questionable whether L-leucine or DL-valine was the amino acid responsible for the increase in lipase production. In Table 38 the effect of addition of 0.2 per cent of each of the two amino acids to the regular citrate medium upon lipase production by culture E-1 is demonstrated. Obviously, L-leucine caused a much greater increase in lipase production than did DL-valine. The lipase activity of the various cultures was not proportional to the small shifts in population observed with the various

Table 37

Effect of addition of indispensable amino acids to citrate medium upon lipase production by culture O-1 (1 ml. lipase preparation per 40 ml. substrate)

Citrate medium supplemented with	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
Threonine, methionine and phenylalanine	6.47	8.13	4,400	0.06
Lysine, arginine, and histidine	6.42	8.08	2,000	0.11
Leucine, isoleucine and valine	6.48	8.08	2,500	0.45
All above amino acids	6.40	8.02	2,500	0.45

* Average blank deducted = 0.38

Table 38

Effect of addition of L-leucine and DL-valine to citrate medium upon lipase production by culture E-1 (1 ml. lipase preparation per 40 ml. substrate)

Citrate medium supplemented with	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
-	6.05	7.88	3,300	0.14
L-leucine	6.04	7.95	5,800	2.34
DL-valine	6.04	7.96	4,400	0.21

* Average blank deducted = 0.34

addenda. Here again the considerable shift in pH is observed.

It was interesting to determine whether the addition of 0.2 per cent L-leucine to the defined citrate medium would cause similar increases in lipase production by other cultures of Ps. fragi. As shown in Table 39, appreciable increases in lipase production by cultures 10 and C in the supplemented over those of the control medium were evident, but the increase was less marked in the case of culture K-1, which gave very low lipase production on both the control and supplemented media. The plate count on culture C was much higher in the presence of L-leucine than on the control medium, while this was not the case with the other two cultures. As in the case of the previous tables, there were marked shifts in pH of the media during the incubation period with the three cultures studied.

Since the combination of L-leucine, DL-isoleucine and DL-valine was found to increase lipase production when used as a supplement to the citrate medium, it remained to be seen whether lipase production was affected if these amino acids were used as the sole source of nitrogen and reduced carbon. In this trial the medium consisted of 0.34 per cent of the amino acid, 0.05 per cent magnesium sulfate heptahydrate, and a final concentration of 0.05 M potassium dihydrogen phosphate with enough 0.1 N sodium hydroxide to get

Table 39

Effect of addition of L-leucine to citrate medium upon lipase production by three cultures
(1 ml. lipase preparation per 40 ml. substrate)

Citrate medium with	Culture	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
-	10	6.04	8.00	3,400	0.22
L-leucine	10	6.07	7.79	3,400	0.97
-	C	6.04	7.81	3,900	0.09
L-leucine	C	6.07	8.02	6,300	1.74
-	K-1	6.04	7.83	1,500	0.09
L-leucine	K-1	6.07	7.53	1,200	0.24

* Average blank deducted = 0.39

Table 40

Lipase production by culture O-1 in a medium in which amino acids formed the sole source of nitrogen and carbon
(1 ml. lipase preparation per 40 ml. substrate)

Medium	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
L, Il, V**	7.48	7.38	2,500	3.40
L-leucine	7.48	7.44	1,700	1.29
DL-isoleucine	7.47	7.49	42	0.00
DL-valine	7.48	7.48	46	0.00

* Average blank deducted = 0.43
** L = L-leucine; Il = DL-isoleucine; V = DL-valine

the desired pH. Table 40 shows that in the simultaneous presence of the three amino acids, a high net activity coupled with an appreciable count was observed. However, when these amino acids were used singly, only L-leucine supported good growth and gave a fairly good lipase production with culture 0-1. The media containing DL-isoleucine or DL-valine yielded no demonstrable lipase activity in 1 ml. portions of these media, probably due in some degree to the low level of growth. Apparently there was very little shift in the pH of these media during the incubation period.

Due to the low counts obtained, especially when DL-isoleucine or DL-valine were used as a sole source of reduced carbon, it was thought that the addition of glucose as a source of carbon might help to increase the count. These media differed from those shown in the preceding table just by containing 0.5 per cent glucose. The L-leucine medium continued to give higher lipase activities than did either of the two amino acids, even when the counts on the three media were almost identical (Table 41). Because of the presence of glucose, an appreciable decrease in the pH of the three media was observed.

Lipase production in glucose media was invariably lower than with media containing hydrolyzed protein. A modified glucose medium (Baker, 1950) was composed of 0.5 per cent glucose, 0.2 per cent ammonium chloride, 0.05 per

cent magnesium sulfate heptahydrate, and 0.1 N sodium hydroxide to get the desired pH. In order to determine the optimum concentration of this component, the glucose concentration was varied from 0.25 to 1.5 per cent. Using culture 0-1 as the test organism, a concentration of 0.5 per cent glucose resulted in the highest lipase activity per unit volume of the medium. The plate counts were more or less constant except in the medium containing 1.5 per cent glucose, where the count appears to be out of line (Table 42). Again there was a drop in the pH of the media during the incubation period, this drop becoming more pronounced with the increase in glucose concentration up to one per cent. Quite probably this low pH attained was a factor in reducing the lipase activity of the preparations from the media with higher glucose concentration.

In attempts to increase lipase production in the glucose medium by culture 0-1, additions of 0.34 per cent of L-leucine, DL-isoleucine and DL-valine were made. Table 43 shows that lipase production in glucose medium was increased appreciably whenever it was supplemented with the three a amino acids or with L-leucine alone. The unexpectedly low count on the unsupplemented glucose medium probably was due to an experimental error. Here again, the drop in the pH of the media is obvious and may be a factor in low lipase production in the presence of readily fermentable carbohydrate.

Table 41

Lipase production in some amino acid media supplemented with glucose by culture 0-1
(1 ml. lipase preparation per 40 ml. substrate)

Medium	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
L-leucine	7.36	6.92	2,000	1.42
DL-isoleucine	7.39	7.00	2,100	0.77
DL-valine	7.37	6.93	2,100	0.44

* Average blank deducted = 0.45

Table 42

Effect of concentration of glucose in a defined inorganic medium upon lipase production by culture 0-1
(2 ml. lipase preparation per 40 ml. substrate)

Per cent glucose	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
0.25	7.09	6.80	2,700	2.07
0.50	7.10	6.74	2,500	2.19
1.00	7.10	6.49	2,300	1.02
1.50	6.91	6.60	260	0.44

* Average blank deducted = 0.35

Lactates usually are present in dairy products showing developed acidity, especially sour cream. In one trial several cultures of Ps. fragi were tested for lipase production in a lactate medium (Table 44). The medium was composed of 0.8 per cent sodium lactate (50 per cent), 0.2 per cent ammonium chloride, 0.05 per cent magnesium sulfate heptahydrate, 10 per cent by volume of 0.5 M potassium dihydrogen phosphate solution and 0.1 N sodium hydroxide to get the required pH. Lipase production in this medium was comparable to what was obtained in the glucose medium. The net activities detected with the five cultures studied varied from one culture to another, as was the case with other media. This serves to demonstrate again the differences in the abilities of the various cultures of Ps. fragi to produce lipase under similar nutritional conditions. It is to be noted that culture C showed greater lipase production in lactate broth than did cultures O-1 and E-1, which usually produced more lipase in other media than all the other cultures including culture C. In the lactate medium as with the citrate medium, considerable rise in pH was observed during the incubation period.

Table 43

Effect of addition of some amino acids to glucose medium upon lipase production by culture 0-1
(1 ml. lipase preparation per 40 ml. substrate)

Glucose medium with	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
-	7.32	6.74	200	0.91
L, Il, V **	7.18	6.55	2,300	1.80
L-leucine	7.22	6.58	1,600	1.52
DL-isoleucine	7.21	6.56	2,100	0.96
DL-valine	7.22	6.61	2,200	0.46

* Average blank deducted = 0.38
** L = L-leucine; Il = DL-isoleucine; V = DL-valine

Table 44

Lipase production in lactate medium by five cultures of *Pseudomonas fragi*
(1 ml. lipase preparation per 40 ml. substrate)

Culture	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
0 - 1	6.27	7.58	3,600	1.04
E - 1	6.27	7.63	3,600	0.65
10	6.27	7.68	5,400	0.80
C	6.27	7.63	3,200	1.10
K - 1	6.27	7.44	1,400	0.55

* Average blank deducted = 0.37

Effect of some pure triglycerides, fatty acids and butterfat upon lipase production

Ps. fragi elaborates lipase in media devoid of substrates upon which the enzyme acts. It seemed logical that the presence of lipid materials might induce greater lipase production. To the usual one per cent vitamin-free casamino acids medium several pure triglycerides and butterfat were added at the rate of 0.1 per cent and then sterilized as usual by autoclaving at 15 lb. for 15 minutes. The results presented in Tables 45 and 46 show that tricaprylin greatly increased lipase activity when added to the casamino acids medium. Phenomenal lipase activities of 8.56 and 9.76 were detected with 0.3 ml. portions of the medium supplemented with tricaprylin. Tricaprylin usually increased the count on the supplemented medium as compared to the control medium. Triacetin, tricaproin and tricaprin slightly increased the net lipase activities in the vitamin-free casamino acids medium when they were added. The presence of trilaurin in the medium had a variable effect, appreciably increasing lipase production as judged by net activities in two out of four trials, and suppressing it in the other two trials (see Table 46). Whenever reasonable growth occurred in the presence of trilaurin, there was an increase in lipase activity of the supplemented medium over that of the control.

Table 45

Effect of addition of some pure triglycerides and butterfat to vitamin-free casamino acids medium upon lipase production by culture 0-1
(0.3 ml. lipase preparation per 40 ml. substrate)

Casamino acid medium with	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
-	7.14	7.90	2,800	3.36
Triacetin	6.82	7.54	1,700	3.95
Tributyrin	7.14	7.72	4,200	3.44
Tricaproin	7.19	7.70	6,400	3.86
Tricaprylin	7.17	7.62	5,700	8.56
Tricaprin	7.16	7.32	700	4.20
Trilaurin	7.17	7.52	< 100	0.03
Trimyristin	7.16	7.91	5,400	0.20
Triolein	7.16	7.55	2,900	0.22
Butterfat	7.17	7.68	4,600	0.04

* Average blank deducted = 0.42

Table 46

Effect of addition of some pure triglycerides and butterfat to vitamin-free casamino acids medium upon lipase production by culture 0-1
(0.3 ml. lipase preparation per 40 ml. substrate)

Casamino acid medium with	Net activity Trial 1	Trial 2	Trial 3
-	3.84	1.74	3.36
Tributyrin	2.31	2.95	2.87
Tricaprylin	-	9.61	9.76
Trilaurin	9.33	0.74	6.62
Tripalmitin	-	0.09	0.73
Butterfat	-	0.03	0.26

The effect of tributyrin also might be considered somewhat inconsistent, sometimes being slightly stimulatory and sometimes being slightly inhibitory to lipase production. The addition of butterfat to the casamino acids medium at the rate of 0.1 per cent invariably decreased the lipase activity of the supplemented medium almost to the vanishing point as compared to the control medium. This marked inhibition of lipase production was not accompanied by any inhibition of cell proliferation, but instead usually gave slight stimulation of growth. Trimyristin, tripalmitin and triolein showed behavior similar to that of butterfat. With the exception of trimyristin and tripalmitin, addition of triglyceride to the casamino acids medium caused the final pH of the medium at the end of the incubation period to be lower than that of the control medium, presumably because of partial hydrolysis of the triglycerides.

In Table 47 data on the effect of addition of 0.1 per cent tricaprylin to the vitamin-free casamino acids medium upon lipase production by cultures E-1, C and 10 are presented. The three cultures tried gave greatly increased lipase production in presence of tricaprylin, especially in the case of culture C, where the increase in lipase activity per unit volume of the medium was over six-fold. The plate counts were variable, two cultures showing increased counts in the presence of tricaprylin, while the third showed a

decrease. Tricaprylin addition resulted in a final pH lower than that of the control, presumably due to the presence of neutralized free fatty acids.

Since the addition of tricaprylin to vitamin-free casamino acids resulted in such extraordinary increases in lipase production by several cultures of Ps. fragi, it was interesting to determine whether tricaprylin as such or one of its breakdown products was responsible. Final concentrations in the casamino acid medium amounting to 0.0022 M tricaprylin and glycerol and 0.0066 M caprylic acid were used (equivalent to 0.1 per cent tricaprylin). The results in Table 48 show that caprylic acid is the component largely responsible for the increase in lipase production when added to vitamin-free casamino acids medium. The discrepancy between the net activities obtained by the addition of tricaprylin and caprylic acid may be due to the concentration of the added caprylic acid being higher than that of the fatty acid resulting from hydrolysis of tricaprylin, but the counts indicate that this may not have been the responsible factor. Addition of glycerol caused no appreciable increase in lipase activity in the supplemented medium over that of the control. The final pH of the medium to which tricaprylin was added was lower than that of the other three media.

The experiment with culture O-1 was repeated on cultures E-1 and C with regard to the effect of addition of tricaprylin

Table 47

Effect of addition of tricaprylin to vitamin-free casamino acids medium upon lipase production by three cultures (0.3 ml. lipase preparation per 40 ml. substrate)

Culture	Casamino acids with	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
E-1	-	7.26	7.69	3,500	2.07
E-1	Tricaprylin	"	7.50	5,400	7.55
C	-	"	7.85	5,100	1.06
C	Tricaprylin	"	7.54	5,800	6.88
10	-	"	7.92	4,200	1.03
10	Tricaprylin	"	7.51	3,000	3.38

* Average blank deducted = 0.39

Table 48

Effect of addition of tricaprylin, caprylic acid, and glycerol to vitamin-free casamino acids medium upon lipase production by culture O-1 (0.3 ml. lipase preparation per 40 ml. substrate)

Casamino acids with	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
-	7.16	7.77	2,800	2.32
Tricaprylin	7.16	7.43	3,800	8.95
Caprylic acid	7.12	7.69	5,300	7.79
glycerol	7.17	7.70	3,300	2.45

* Average blank deducted = 0.40

and caprylic acid to vitamin-free casamino acids medium upon lipase production. Apparently a picture similar to that obtained with culture 0-1 can be seen in Table 49 in the case of cultures E-1 and C. The counts on culture E-1 were somewhat higher in the presence of caprylic acid than in the other two media, whereas the situation was reversed in the case of culture C; possibly the differences are not significant. The medium supplemented with caprylic acid showed slightly less lipase activity than when the triglyceride was used, as was the case in the preceding table. Again the final pH of the medium containing tricaprylin was lower than that of the other two media. This drop probably is due to the effect of free caprylic acid liberated by lipase during the incubation period in the absence of suitable neutralizing agents.

In Table 50 the effect of addition of caprylic acid or tricaprylin to one per cent peptone broth upon lipase production by cultures 0-1 and E-1 is shown. The two cultures responded in the same manner to the presence of tricaprylin or caprylic acid in peptone broth as they did when they were included in the vitamin-free casamino acids medium. However, the actual enzyme activity per unit volume continued to be much higher in the peptone broth than in the casamino acids medium, despite the supplementation of both. The count was appreciably higher on the two cultures in the supplemented

Table 49

Effect of addition of tricaprylin and caprylic acid to vitamin-free casamino acids upon lipase production by cultures E-1 and C
(0.3 ml. lipase preparation per 40 ml. substrate)

Culture	Casamino acids with	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
E-1	-	7.17	7.75	4,200	3.36
E-1	Tricaprylin	7.19	7.22	4,000	9.95
E-1	Caprylic acid	7.17	7.54	6,000	8.62
C	-	7.17	7.79	5,400	1.16
C	Tricaprylin	7.19	7.39	5,300	6.49
C	Caprylic acid	7.17	7.65	4,500	5.96

* Average blank deducted = 0.42

Table 50

Effect of addition of tricaprylin and caprylic acid to peptone broth upon lipase production by cultures O-1 and E-1
(0.1 ml. lipase preparation per 40 ml. substrate*)

Culture	Peptone broth with	Initial pH	Final pH	Count/ml. (x 10^6)	Net** activity
O-1	-	7.19	7.67	4,200	2.40
O-1	Tricaprylin	7.21	7.22	6,800	7.72
O-1	Caprylic acid	7.23	7.50	6,300	7.26
E-1	-	7.19	7.66	5,200	2.27
E-1	Tricaprylin	7.21	7.38	8,300	6.17
E-1	Caprylic acid	7.23	7.57	8,600	5.21

* 0.2 per cent formaldehyde was used.
** After deduction of blanks.

media than on the control medium. Medium containing tricaprylin exhibited lower final pH than did the other media, conforming with the previous results on the casamino acids medium.

Having established that the addition of caprylic acid to vitamin-free casamino acids or peptone media results in extraordinary increases in lipase production, the question arose regarding the effect of other fatty acids upon lipase production by Ps. fragi.

It was decided to determine the concentration of caprylic acid that would give the highest lipase activity per unit volume of the supplemented vitamin-free casamino acids medium, and to use equivalent molar concentrations with the other fatty acids. Therefore, concentrations ranging from 0.001 M to 0.008 M caprylic acid in the final vitamin-free casamino acids medium were used. A concentration of 0.0035 M caprylic acid gave slightly the highest lipase activity per unit volume of the medium, using culture 0-1 as the test organism (Table 51). Even at the lowest concentration of caprylic acid tested, a substantial increase in lipase production was noted. Although the differences were not large, the plate count was slightly lower at the higher concentrations than at the lower concentrations of caprylic acid, but still higher than in the control medium.

A trial to determine the effect of some of the saturated fatty acids upon lipase production when added to vitamin-free casamino acids medium was made. The final concentrations of the fatty acids in the medium were 0.0035 M. The medium was heated in order to melt and incorporate capric and lauric acids. Addition of capric acid to the growth medium resulted in a very marked increase in lipase production by culture O-1, being slightly higher than that obtained with caprylic acid (Table 52). Butyric acid caused a small decrease in lipase activity of the medium in which it was included, although it did not affect the plate count. Caproic acid addition resulted in possibly a slight increase of lipase production in the supplemented medium over that of the control. Lauric acid was inhibitory to bacterial growth and net enzyme activity per unit volume dropped appreciably when this acid was present in the growth medium. The greater the molecular weight of the acid the less the pH of the medium changed during organism growth, although the count was essentially the same in all cases except when lauric acid was present.

Since capric acid greatly stimulated lipase production by culture O-1, it was necessary to determine its effect on other cultures of Ps. fragi. Capric acid was thus compared with caprylic acid by addition to the regular vitamin-free casamino acids medium so as to get a final concentration of

Table 51

Effect of addition of different concentrations of caprylic acid to vitamin-free casamino acids upon lipase production by culture 0-1
(0.1 ml. lipase preparation per 40 ml. substrate*)

Caprylic acid (molarity)	Initial pH	Final pH	Count/ml. (x 10^6)	Net** activity
-	7.09	7.75	3,800	1.47
0.001	7.14	7.89	5,400	3.58
0.002	7.13	7.69	5,800	5.37
0.0035	7.09	7.56	5,200	5.57
0.004	7.13	7.59	4,700	4.94
0.008	7.08	7.61	4,500	5.15

* 0.2 per cent formaldehyde was used.
** Average blank deducted = 0.44

Table 52

Effect of addition of some saturated fatty acids to vitamin-free casamino acids medium upon lipase production by culture 0-1
(0.2 ml. lipase preparation per 40 ml. substrate*)

Casamino acids with	Initial pH	Final pH	Count/ml. (x 10^6)	Net** activity
-	7.15	7.86	5,100	2.90
Butyric	7.16	7.81	5,200	2.45
Caproic	7.14	7.78	5,300	3.08
Caprylic	7.13	7.57	6,200	7.95
Capric	7.12	7.43	5,200	8.62
Lauric	7.11	7.11	260	0.85

* 0.2 per cent formaldehyde was used.
** After deduction of blanks (0.32 - 0.36)

0.0035 M. In Table 53 the effect of the two fatty acids upon lipase production by cultures E-1 and C is presented. Both cultures showed a great increase in lipase production in the growth media to which either of the fatty acids was added. Addition of capric acid resulted in slightly more lipase activity than did caprylic acid. The level of lipase production was appreciably lower with culture C than with culture E-1. This observation is in keeping with the individual abilities of these cultures to produce lipase in other growth media.

Results of addition of some long-chain fatty acids upon lipase production in vitamin-free casamino acids medium by two cultures of Ps. fragi are presented in Table 54. In the preparation of these media they were heated to above 80° C. in order to melt and incorporate the fatty acids before sterilization. Final concentrations of these fatty acids in the medium were kept at 0.0035 M. Myristic acid showed some stimulation of lipase production by the two cultures used. Stearic acid exhibited marked inhibition of lipase production. Palmitic and oleic acids also were inhibitory, though to a lesser degree than stearic acid. In all cases, addition of the fatty acids to the growth medium apparently was somewhat stimulatory to cell proliferation of the two cultures tried, particularly in the case of palmitic acid.

Table 53

Effect of addition of caprylic and capric acids to vitamin-free casamino acids medium upon lipase production by cultures E-1 and C
(0.1 ml. lipase preparation per 40 ml. substrate)

Casamino acids with	Culture	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
-	E-1	7.15	7.85	4,200	1.51
Caprylic	E-1	7.14	7.56	6,300	3.35
Capric	E-1	7.13	7.52	4,600	4.00
-	C	7.15	7.58	3,400	0.30
Caprylic	C	7.14	7.53	4,000	1.89
Capric	C	7.13	7.57	4,300	1.99

* Average blank deducted = 0.33

Table 54

Effect of addition of some long-chain fatty acids to vitamin-free casamino acids medium upon lipase production by cultures E-1 and O-1
(0.2 ml. lipase preparation per 40 ml. substrate)

Casamino acids with	Culture	Initial pH	Final pH	Count/ml. (x 10^6)	Net* activity
-	E-1	7.26	7.78	2,700	2.23
Myristic	"	7.24	7.63	3,400	3.08
Palmitic	"	7.24	7.78	5,100	0.90
Stearic	"	7.24	7.58	3,100	0.03
Oleic	"	7.23	7.69	4,800	1.42
-	O-1	7.26	7.79	2,300	2.68
Myristic	"	7.24	7.75	3,900	3.91
Palmitic	"	7.24	7.63	5,300	1.32
Stearic	"	7.24	7.71	3,800	0.10
Oleic	"	7.23	7.70	2,800	1.09

* Average blank deducted = 0.32

Lipase from Ps. fragi in Cream and Butter

Presence of Ps. fragi lipase in cream and butter leads to the development of objectionable flavors, which would render the resulting butter less marketable. It is the common belief that lipase would be inactivated during the heat treatment the cream usually undergoes during the commercial process of buttermaking.

Heat-resistance studies reported earlier in this work suggested additional work with cream in order to investigate the extent of lipase production under laboratory conditions somewhat comparable to the established practices of holding cream on the farm before delivery to the creamery, and the susceptibility of the resulting lipase to heat treatments.

Lipase production in cream by Ps. fragi

For these studies whipping cream obtained from the College creamery was standardized to about 30 per cent butterfat, sterilized and inoculated with the various cultures of Ps. fragi. The cream was incubated at 15° C. for three days, after which 12 ml. portions were pasteurized by heating in 125 x 16 mm. screw-cap test tubes at 71.5° C. for 30 minutes and then cooled to below 20° C. in ice water. The lipase activity was tested as usual before and after pasteurization.

Data representative of two trials on lipase production in cream by six cultures of Ps. fragi are given in Table 55. The cultures varied widely in their abilities to produce lipase under the conditions of the experiment, cultures O-1 and E-1 giving the highest net activities per unit volume of cream. Undoubtedly the pH of the cream, although normal, was too acid for maximum lipase production. Bacterial counts did not correlate with the lipase activities. While culture P showed the highest count, yet it produced the least lipase activity per milliliter of cream. The final pH of the inoculated cream was slightly more acid than the initial pH, the shift varying in magnitude between the different cultures. In the last column on the right hand side of the table, the residual net activities per unit volume of cream after pasteurization are given. In all cases, over 50 per cent of the original lipase activities remained after undergoing the rigorous heat treatment. The extent of the loss of activity during heating varied somewhat between the six cultures.

Effect of S. lactis on lipase production by Ps. fragi in cream

In these trials a concentration of 1:120,000 of crystal violet in tryptone-glucose-extract agar was used in order to get differential plate counts on Ps. fragi when grown with

S. lactis. Tryptone-glucose-extract agar fortified with one per cent sterile litmus milk was used in obtaining plate counts whenever S. lactis was included. Plates with crystal violet were incubated at 21° C. for three days in order to get easily countable colonies.

The results presented in Table 56 are representative of three trials. Whenever S. lactis (culture W2) was grown with Ps. fragi (culture 0-1 and two other cultures in the other trials), lipase production of the latter organism was suppressed markedly. This reduction in lipase activity occurred despite the fact that there was no appreciable decrease in the count on Ps. fragi. No detectable lipase production was observed in the uninoculated cream or in cream inoculated with S. lactis alone. From comparative data on counts by the two procedures, the concentration of crystal violet used in plating apparently was somewhat inhibitory to Ps. fragi. The low pH of the combinating cultures would not be conducive to any considerable stability or activity of the lipase, even had it been produced in some quantity.

Residual lipase in butter

It has been shown that an appreciable portion of the lipase in cream was not inactivated by heating to 71.5° C. for 30 minutes. The role of this residual lipase in the deterioration of the quality of butter during storage or

Table 55

Lipase production in cream by six cultures of Ps. fragi and the residual lipase left in cream after pasteurization at 71.5° C. for 30 minutes
(1.0 ml. cream per 40 ml. substrate)

Culture	Initial pH	Final pH	Count/ml. (x 10^6)	Net activity*	
				Before pasteurization	After pasteurization
0-1	6.42	6.29	380	1.05	0.64
E-1	"	6.32	250	1.22	0.67
C	"	6.37	430	0.52	0.32
10	"	6.41	370	0.73	0.49
K-1	"	6.36	270	0.82	0.64
P	"	6.40	910	0.36	0.26

* Average blank deducted = 0.39

Table 56

Lipase production in cream by Ps. fragi 0-1 in association with S. lactis W2
(1 ml. cream per 40 ml. substrate)

Cream inoculated with	Initial pH	Final pH	Count/ml. (x 10^6)				Net* activity
			Initial		Final		
			T.G. E.M.	T.G.E.** with cr. violet	T.G. E.M.	T.G.E.** with cr. violet	
0-1	6.45	6.03	1.20	0.20	480	380	1.07
0-1,W2	"	4.51	1.60	0.87	2,000	360	0.19
W2	"	4.39	0.88	0	1,300	0	0.01
Uninoculated	"	6.32	0	0	0	0	0.00

* Average blank deducted = 0.33
** Tryptone-glucose-extract agar with 1:120,000 crystal violet incubated at 21° C. for 3 days.

quality tests thus was investigated. Two trials on sterile sweet cream were made. The growth of culture 0-1 in cream for a period of three days at 15° C. resulted in net lipase activities of 0.52 and 0.63 per milliliter in trials 1 and 2, respectively. The corresponding residual activities after pasteurization were 0.20 and 0.41. During the incubation period, appreciable fat degradation occurred due to the action of the lipase. For instance, extraction and titration of fat and fatty acids from 10 g. portions of cream showed increases of 1.13 and 1.65 ml. 0.05 N potassium hydroxide in the inoculated cream over those of the controls in trials 1 and 2, respectively. These fat degradation products are carried over to the butter churned from these two batches of cream and are responsible for the differences between the titration of the butters from the inoculated and control creams at zero time (see Table 58).

There were greater variations between the composition of butters obtained from inoculated and the control cream in trial 1 than in trial 2. This was accomplished in trial 2 by holding the creams overnight at 4-6° C., after pasteurization of the inoculated cream and before churning, so that the fat globules would have crystallized to the same extent in both lots of cream. The composition of experimental butters is given in Table 57. Butter churned from inoculated cream in trial 1 had a relatively high moisture and curd content

Table 57

Composition of experimental butter

	Percentage composition of butter		
	Moisture	Fat	Curd
		Trial 1	
Inoculated	22.1	75.3	2.6
Uninoculated	17.4	81.0	1.6
		Trial 2	
Inoculated	18.4	80.0	1.6
Uninoculated	17.8	80.5	1.7

Table 58

Effect of the residual lipase upon the flavor and titration of the butter after holding at different temperatures for various intervals

Storage temp. (° C.)	Period of holding (days)	Trial 1				Trial 2			
		Inoculated		Uninoculated		Inoculated		Uninoculated	
		Titration*	Flavor	Titration*	Flavor	Titration*	Flavor	Titration	Flavor
-	0	4.27	Cooked	0.87	Sl. tallowy	8.48	Rancid	1.51	Cooked, feed.
36	2	9.17	Rancid	1.12	" ", cooked	22.50	Very rancid, peppery	1.20	"
21	7	13.66	Very rancid	1.37	" "	38.16	Extremely rancid and peppery	1.30	"
2-5	30	10.59	" "	1.29	" "	29.12	Very rancid and peppery	1.36	" , oxidised
-10	60	6.26	Sl. rancid	1.13	Oxidised	15.28	Very rancid and peppery	1.43	Oxidized, tallowy

* Average of duplicate titrations of 10 g. samples, expressed in ml. 0.05 N methanolic potassium hydroxide.

and low fat test, whereas the butter made from control cream had a normal composition. However, in trial 2 the compositions of the butters of inoculated and control creams were both within the normal range.

In running quality tests on butter, the samples usually are held at 20° C. for five to seven days, and then they are tested organoleptically for off-flavors. Ordinarily, butter is stored at -17.8° C. (0° C.) or lower in the course of marketing the product. In two trials, the results of which are given in Table 58, portions of the churned butters from the inoculated and control creams were held at the various temperatures shown in the table for different periods of time. Samples of the butter, amounting to 10 g., were taken just after the butter was made and at the end of the respective holding periods, and carried through the extraction-titration procedure as modified for butter. Also, the samples were tested organoleptically prior to the chemical test. Butter tested in 1 ml. portions showed no growth on T.G.E. agar after incubation at 21° C. for two to three days. In trial 1 the titration values of the butter from inoculated cream averaged between about one-half to one-third of those obtained in trial 2 from the corresponding butter, apparently due to the higher residual lipase in the respective cream, which was retained to an appreciable extent in the butter. Considerable increases in the titrations

of butter churned from inoculated cream occurred after holding at all the temperatures used, whereas there was no significant change in the titration values of the control butter. The butter containing residual lipase showed the greatest increase in titration when held at 21° C. for seven days. Butter held at 36° C. melted during the holding period, a condition that probably lowered the demonstrable lipase activity. Organoleptically all the butter samples containing residual lipase became rancid or the rancidity increased markedly after holding at the temperatures used. However, the control butter samples were criticized only for being oxidized or having a cooked flavor, but in no case were these butter samples detectably rancid.

DISCUSSION

Characterization of the Lipase of Ps. fragi

The choice of an extraction-titration procedure for the measurement of lipase activity was made because low titration values were obtained when the test substrate, as such or with the addition of a mixture of acetone and ether, was titrated. With the test substrate adjusted to pH 7.2, the fatty acids liberated by the action of lipase would be largely in the form of soaps, which would not contribute appreciably to the titration values. Actually, the titration of the medium would represent the alkali required to neutralize the unbound fatty acids between the final pH of the medium (6.9 - 7.2) to the phenolphthalein endpoint (slightly above 8.0), instead of the complete neutralization of all the fatty acids freed by the action of lipase. Use of an extraction-titration procedure permits the acidification of the lipase test substrate to a pH of 2 or below, rendering all the released fatty acids in the medium in the free state prior to the extraction of the fat and fatty acids and subsequent titration. In addition, the extraction-titration procedure permits the titration with potassium hydroxide in absolute methanol of a homogeneous ether solution of fats and

fatty acids, thus minimizing fading of the titration end-point. Also, since buffers are not extracted by this procedure, the blank titrations are lowered. Although trials on added fatty acids resulted in poor recoveries of caproic and butyric acids, yet the fact that the coconut oil contains only traces of these two fatty acids (Hilditch, 1941) makes the titration values representative of hydrolysis of the oil, since the recoveries on caprylic and the fatty acids above it in the homologous series were appreciably over 90 per cent.

Coconut oil was reported to be hydrolyzed by pancreatic lipase more rapidly than are other natural fats and oils (Hartwell, 1938). The lipase of Ps. fragi hydrolyzed coconut oil to a greater extent than it did the other natural fats and oils tried. The high lipase activity with coconut oil probably is due to its high content of esterified caprylic acid. Lipases ordinarily are thought of as possessing a low order of specificity regarding their ester substrates. Due to the fact that the triglycerides beginning with trilaurin and those higher in the series, except triolein, did not give stable emulsions, an uncontrolled physical factor which may be of greater importance than the chemical nature of the substrate, the hydrolysis of these higher triglycerides by the lipase has not been investigated. However, tricaprylin underwent hydrolysis by the lipase of

Ps. fragi to a degree two to three times as extensive as did the triglycerides lower in the series or those above it that have been tried. This would indicate that the lipase of Ps. fragi apparently has a preference for tricaprylin as a substrate. These findings appear contradictory to those of Collins and Hammer (1934) and Long (1936), who showed that tripropionin and tributyrin were more easily hydrolyzed by Ps. fragi and other lipolytic bacteria than the higher triglycerides, but the difference may lie in the fact that these workers were determining qualitative lipolysis by growing cells of the organisms rather than the action of a lipolytic enzyme as such. A study of that type naturally would involve the problem of lipase production in addition to lipase action.

Reports on the effect of bile salts on various animal and microbial lipases are not in agreement, some workers observing inhibition and others claiming activation (Fodor, 1949; Oppenheimer, 1925; Mallenby and Wolley, 1914; and Weinstein and Wynne, 1936). The lipase of Ps. fragi was activated by sodium taurocholate in concentrations ranging from 0.1 to 0.6 per cent in the coconut oil emulsion used as a lipase test substrate. The possibility exists that the increase in lipase activity results from a more efficient emulsification of the oil. However, since sodium glycocholate

which apparently gave as stable an emulsion as sodium taurocholate, showed marked inhibition of lipase activity, the indication is that the taurocholate may have an activating effect upon the enzyme as such.

The relationship between demonstrable lipase activity and reaction time was found to be curvilinear. Smith et al. (1949) showed that the curve representing the relation between reaction time and milk lipase activity deviated from a straight line function after 20 minutes reaction time. They assumed that the deviation was due to heat inactivation. Willstätter et al. (1923) found that the relationship between quantity of pancreatic lipase and acid produced in a given time was not linear. Bullock (1947) obtained similar results in his studies on pancreatic lipase. He theorized that this deviation from a linear relationship may be due partly to the fall in pH during digestion and partly to the fact that digestion is occurring in an oil-water interface and not in a homogenous phase, thus the rate of digestion per unit of enzyme falls off with increasing quantities of enzyme. In the present study, the relationship between the quantity of lipase preparation and lipase activity was curvilinear. Apart from the reasons given above, inhibition by the end-products of the enzymatic reaction and inactivation by co-existing proteases may be named as other possible factors. Evidence of appreciable proteolytic activity in the present

lipase preparation was found by van der Zant (1951), using a modification of the method proposed by Anson (1938), but no study was made to determine whether the inactivation rate of lipase paralleled proteolytic activity of the preparation.

Stability studies of the lipase of Ps. fragi in peptone broth at pH 7.6 shed some light on the foregoing problem. It was observed that about 80 per cent of the original lipase activity was lost when the lipase preparation was kept at 36° C. for 24 hours. This loss was comparatively small at 15° C. and negligible at 3-5° C. during the same period. It is unlikely that such marked inactivation of the lipase is due to the effect of heat alone, since lipase preparations retained appreciable lipase activities after heating to 71.6° C. for 30 minutes. Heating the lipase preparations in boiling water for over 20 minutes was required to accomplish complete inactivation of the enzyme. The inactivation of lipase in oil emulsions at 36° C. apparently was less marked than in aqueous solutions, since the lipase added to coconut oil emulsion was still appreciably active during the third day of incubation, whereas very little lipase activity was demonstrable in lipase preparations in peptone broth after being kept at 36° C. for three days. Bullock (1947) found that lipase powder in oily suspensions was more stable to heat inactivation the lower the moisture content of the emulsion. Other possible factors are the formation of an

enzyme-substrate complex or the presence of materials that offer some protection of the enzyme against inactivating agents. The lipase of Ps. fragi in cream withstood heating to 71.5° C. for over 30 minutes without excessive loss of lipase activity. In most instances, over 50 per cent of the lipase activity was demonstrable in the cream after that heat treatment, while in buffered peptone lipase preparations less than 25 per cent of the lipase activity was detected after a similar heat treatment. Whether this protective action in cream is due to the butterfat or to other components is not known.

It was observed that the rate of loss of lipase activity during the time required to heat the lipase preparations to 61.6°, 71.6° and 99° C. was disproportionately greater than that during the subsequent heating period, even though the heating period was several times longer than the "coming up" time. The fact that the percentage destruction of lipase activity during this "coming up" time varied between the three temperatures tried, and even between different runs involving lipase preparations of varying lipase activities, makes the explanation of this behavior rather difficult. In most trials on different lipase preparations there was apparently more destruction of lipase during the time required to reach the temperature of 61.6° C. than during a slightly longer interval required to bring the lipase

preparations up to the temperature of 71.6° C. The results of one trial at both temperatures on the same lipase preparation agreed with the preceding observation. Possibly some inactivating agent is destroyed to a greater extent at 71.6° C. than at 61.6° C. The heat inactivation of the lipase in phosphate-buffered peptone broth proceeds at a slow rate after the temperatures of 61.6° and 71.6° C. are reached. This behavior of the lipase of Ps. fragi is not strange, since Söhngen (1911) and Tammisto (1933) reported the unusual heat resistance of the lipase of B. fluorescens liquefaciens. However, the fact remains that this extraordinary heat resistance of lipase is unexpected because most enzymes usually are inactivated more easily, presumably due to the denaturation of the proteins.

As for all enzymes, the activity of lipase increases with increase in temperature until an optimum is reached, where the increase in activity is offset by the inactivation of the enzyme, so that further increase in temperature will result in decreased enzyme activity. In the case of the lipase of Ps. fragi, this optimum temperature was found to be 40° C. when a reaction time of 24 hours was employed. As was shown in the butter storage experiments, the lipase was active to a limited extent even at -10° C. Obviously, the lipase of Ps. fragi is active over a wide temperature range, including the low temperatures used in holding dairy products.

The lipase of Ps. fragi had optimum activity at pH 7.0 to 7.2. This contrasts with the results of Lubert et al. (1949c) who reported that the lipase of Pseudomonas fluorescens exhibited maximum activity at pH 8.0 to 9.0. In comparison of pH optima, the buffer system, the length of reaction period, reaction temperature and type of substrate have to be taken into consideration. These workers employed tributyrin and the extremely short reaction period of 30 minutes at 37° C., whereas in the present work the reaction period was 24 hours at 36° C. with coconut oil as a substrate. In the present study, the optimum pH for maximum lipase activity was the same whether McIlvaine's citrate-phosphate or Clark's phosphate buffer was used.

The lipase activity present in peptone broth could be salted out without much loss when the lipase preparation was saturated with ammonium sulfate in the cold. As in the salting out of pancreatic lipase by Glick and King (1933), the recovery of lipase activity from Ps. fragi preparations increased with the increase in degree of saturation with ammonium sulfate. Part of the lipase appears at the surface of the saturated preparation immediately after the solution of ammonium sulfate, and the rest appears near the bottom after standing. The part that floated at the surface showed about half the original activity of the lipase preparation and was readily soluble in water. Whether there are any

basic differences between these two fractions needs further investigation.

Factors Influencing Lipase Production

Microorganisms produce extracellular enzymes conceivably to attack compounds that are not readily diffusible through the cell wall, so as to break them down to simpler compounds capable of penetrating the cellular barrier. Although it is not known how large protein molecules, such as the enzymes, can be liberated from the cell, there is unmistakable evidence that such enzymes are found in the medium in which the microorganisms are growing. The findings of Peters (1947), who showed that the lipase activity in the supernatant was much greater than that in the cells of Ps. fragi which were centrifuged out, were confirmed in this work where it was found that the removal of the cells of Ps. fragi by centrifugation did not appreciably lower the lipase activity of the supernatant.

Physical and nutritional factors materially affect lipase production by Ps. fragi. Also of considerable importance is the fact that different strains exhibit unequal abilities to produce lipase under the same conditions of growth. This is not unexpected, since different strains of the same organism commonly vary considerably in extent of biochemical activity.

Among the most important physical factors that influence lipase production by Ps. fragi is the temperature of incubation. One cannot speak of the effect of the temperature of incubation apart from the length of incubation period. The optimum temperature for lipase production depends upon the incubation time; the longer the incubation period the lower the optimum temperature. Growth is restricted at lower temperatures (10° C. or below), and the build up of population requires longer incubation. This is coupled with the fact that the cells remain physiologically active for longer periods at these lower temperatures. Maximum lipase production in three days occurred at a temperature of 15° C. or below, the exact optimum depending upon the culture strain. Lipase production at higher temperatures was appreciably less. Possibly the reason for lower lipase production at the higher temperatures (above 15° C.) is an increased instability of the lipase, as brought out in the stability studies at different temperatures, so that the balance between lipase production and lipase inactivation is more favorable at the lower temperatures. At any rate, the fact that optimum lipase production falls within the range of 10 to 15° C. during an incubation period of three days is very significant from the standpoint of the dairy industry. Ordinarily, cream is held at about this temperature range in the cooling tanks on the farms before being delivered to the

creameries. Even with twice-a-week delivery, appreciable lipase production could occur if considerable numbers of the organism gained access to the product. This temperature range also would favor growth of Ps. fragi in competition with S. lactis.

The pH shift during incubation depends upon the composition of the growth medium. In nutrient broth, peptone and casamino acids media, the rise in pH during incubation may be due to liberation of ammonia, decarboxylation of amino acids or both. With citrate or lactate media the utilization of the citrate and lactate radicals resulted in an increase of uncombined sodium ion which increases the alkalinity of the medium. When glucose is present in the medium, it is utilized by the organism, resulting in acid endproducts that lower the pH of the medium. Due to these widely different reaction changes in the various media, the optimum initial pH for lipase production differed from one medium to another. The effect of pH of nutrient broth was studied in detail on two cultures of Ps. fragi. Two pH optima were evident, one at an initial pH of 6.5 and the other at about 7.5, lipase production at the latter pH being higher with one culture and slightly lower with the other. Sweet cream has a pH close to 6.5, which coincides with one optimum for lipase production. Lower limits of pH for lipase production were not very clear due to the enormous shift in pH during

incubation. However, little or no lipase was produced at pH 4.5 or below, because little growth occurred. If the organism could initiate growth, the pH of the medium was raised up to the level at which lipase production is detectable when the pure culture of Ps. fragi was employed in a medium buffered poorly in an acid pH range.

Peters and Nelson (1948a) found that lipase production by Mycotorula lipolytica increased with the increase in buffer concentration up to 0.225 M disodium phosphate-citric acid buffer. In the present work, monopotassium phosphate concentrations above 0.05 M did not increase lipase production by Ps. fragi, but were slightly inhibitory. Buffers containing potentially metabolizable compounds were not tried, because they might complicate the picture in nutritional studies by supplying additional sources of energy.

Aeration of the growth medium definitely restricted lipase production by Mycotorula lipolytica (Peters and Nelson, 1948a). The increase in surface area to volume relationship in cream was found to increase the extent of fat hydrolysis by Geotrichum candidum (Purko and Nelson, 1951). Similar results were obtained in the present study in the case of Ps. fragi growing in peptone broth. More lipase was produced when the same amount of medium was placed in flasks giving a large surface area than in test tubes with a small surface area. This increase in lipase production was

associated with the proportionately higher plate count in peptone broth in the flasks. Such a result is not strange, since Ps. fragi, which is markedly aerobic, usually shows vigorous growth on the surface of the medium, commonly forming a pellicle, while there is little growth in the depths of the medium.

Growth and fat hydrolysis by Ps. fragi had been demonstrated earlier to be depressed in the presence of sodium chloride in the growth medium (Hussong, 1932; Collins, 1933; Hussong et al., 1937). In the present study, peptone broth containing four per cent sodium chloride showed no detectable lipase activity in 0.3 ml. portions of the medium with four cultures of Ps. fragi. The effect of sodium chloride at low concentrations is manifest in low counts, with accompanying decline in lipase production. In concentrations of sodium chloride above two per cent, lipase production declines more rapidly than does the count. With a three per cent concentration of sodium chloride in the medium, lipase production was insignificant although the count was still over one billion per milliliter.

Ps. fragi is a non-fastidious organism and can grow fairly well in simple, vitamin-free chemically-defined media in which inorganic compounds form the only source of nitrogen. Apart from the variations in the abilities of the different organism strains to produce lipase under optimum physical

and physico-chemical conditions, the presence of the proper sources of nitrogen and reduced carbon, and the occurrence of stimulatory and inhibitory factors may affect the level of lipase activity in the different growth media. In many cases, the stimulatory or inhibitory effects were appreciably greater upon lipase production than upon growth of the organism. Good growth is essential for high lipase production but good growth is not always accompanied by high enzyme production. In a simple citrate medium with ammonium chloride as a source of nitrogen, lipase production was negligible, even though appreciable growth occurred, whereas a glucose or a lactate medium with ammonium chloride as a source of nitrogen supported fairly good lipase production. However, higher levels of lipase production could be obtained by supplementing citrate or glucose media with L-leucine alone or in combination with DL-isoleucine and DL-valine. Also it was possible to obtain good lipase production in a medium consisting of a combination of these three amino acids as sources of both nitrogen and reduced carbon, but the level of lipase activity was higher if glucose also was present in the medium to permit better growth. L-leucine was the only amino acids tested which could act as the sole source of carbon and nitrogen and support appreciable growth and lipase production, although it was inferior in these respects to the combination of the above three amino acids.

High levels of lipase production could be obtained only in media containing protein digests or hydrolyzates, although considerable differences in the yields of lipase on media containing the various protein breakdown products were observed. However, maximum lipase production during these studies were attained by supplementing some of these complex nitrogenous compounds with certain pure triglycerides or their component fatty acids, indicating that the character of the nitrogen source was not necessarily the only important factor.

A semi-defined medium containing a complex source of amino nitrogen in the form of Bacto-vitamin-free casamino acids was shown to support activity levels much higher than those of the simple media. These data and those on peptone could be interpreted as showing that amino nitrogen in the form of certain amino acids or peptides apparently is essential for high lipase production. However, the basis for the quite specific stimulatory effect of L-leucine on lipase production is not clear.

Although considerable lipase production was obtained in media containing a number of complex protein breakdown products, yet Bacto-peptone and Bacto-proteose-peptone yielded the highest levels of lipase activity. A Bacto-vitamin-free casamino acids medium, which supported high lipase activities, could not be made to equal Bacto-peptone

for lipase production, despite supplementation with some vitamins, purine and pyrimidine bases, various ions and "indispensable" amino acids. It is possible that the differences between the various protein digests and hydrolyzates may lie in specific stimulatory substances or groupings found in some and not in others, or that some of these preparations contain inhibitory materials. An example of the second possibility seems to be the demonstrable inhibitory action on lipase production of beef extract when added to peptone broth. Of course the possibility exists that the correct combination or balance of addenda was not employed or that some compound or complex not tested would have shown the desired stimulatory effect.

The presence of the specific substrate in the growth medium frequently stimulates the production of the enzyme which acts upon that substrate. Although there are many examples of such stimulatory action, few apply to enzymes concerned with fat degradation. Baker (1950) found that the addition of some fatty acids to a synthetic basal medium caused appreciable increase in the ability of Ps. fragi cells to oxidize the salts of these fatty acids, using the Warburg technique. Although the lipase of Ps. fragi is not an adaptive enzyme in the strict sense of the term, as the enzyme may be produced in media devoid of any quantity of material that ordinarily serve as a substrate for lipase

action, yet the enzyme production has been stimulated by the presence of some triglycerides, particularly tricaprylin. However, the ineffectiveness of the triglycerides containing fatty acids of carbon-chain lengths shorter than that of tricaprylin and the marked inhibition of lipase production by the triglycerides of long-chain fatty acids and by butterfat does not support the explanation that the triglyceride structure of tricaprylin is largely responsible for the extraordinary increase in lipase production in the casamino acids medium supplemented with that triglyceride. Also, the marked stimulatory effects of caprylic and capric acids, which resulted in increases in lipase production comparable to that obtained by supplementation with tricaprylin, lends additional strength to the interpretation that the triglyceride structure is not responsible for the observed stimulation. Instead, the effect of tricaprylin in increasing lipase production probably is due to the action of the component fatty acid which is liberated by the lipase elaborated during the incubation period. That this may be the case, is demonstrable by the similarity of effect on lipase production by most triglycerides and of their component fatty acids. The data available give no clue as to why caprylic or capric acids have marked stimulatory action, while most other fatty acids have no consistent stimulatory action or are inhibitory to lipase production, although nearly all seem somewhat

stimulatory to organism growth. It would be desirable to know if caprylic and capric acids are necessary as components of the enzyme system, merely stimulate the synthetic activity of the bacterium or affect the permeability of the cellular membrane, but there is nothing in the data presented to offer an answer to this question.

Lipase in Cream and Butter

The growth of several cultures of Ps. fragi in cream at 15° C. invariably was slow, and the counts in cream after an incubation of three days were below one billion per milliliter, whereas in artificial media the counts reached several billions per millliliter under the same conditions. Possibly, the physical or physico-chemical conditions in cream are such that extensive proliferation of the organism is not favored. Similarly, lipase production in cream was relatively low. However, the residual lipase in cream after pasteurization at 71.5° C. for 30 minutes was appreciable and was capable of causing considerable fat degradation.

Hussong (1932) and Hussong et al. (1937) reported that butter cultures had no marked action in inhibiting the growth of Ps. fragi in cream, but the resultant butter developed rancid flavor somewhat less rapidly than butter made without butter culture. In the present study, the growth of Ps.

fragi in cream was not decreased appreciably by the presence of *S. lactis*, although the lipase activity was markedly reduced. The growth of *S. lactis* in association with *Ps. fragi* resulted in lowering the pH of the cream to 4.5, at which reaction *Ps. fragi* was unable to grow in peptone broth; thus the observed growth of the organism and the limited lipase production probably occurred before that low pH was reached. Since the pH would have been below that optimum for lipase production during much of the period when enough bacteria were present to produce significant amount of lipase under more favorable conditions, it is not strange that lipase production was at a low level. Apparently, the growth of acid-producing organisms in association with *Ps. fragi* in cream would exert a restraining effect on lipase production by this organism and would depress lipase action on cream during incubation.

Growth of pure cultures of *Ps. fragi* in cream involves appreciable fat degradation during the period of growth. Hussong *et al.* (1937) reported that sterile cream inoculated with *Ps. fragi* resulted in butter with appreciable content of total acids. Kester (1950) also found that butter obtained from sterile cream in which *Ps. fragi* has grown showed high water-insoluble fatty acids. In the present study, lipase attacked the butterfat in cream during the incubation period and most of the free fatty acids liberated were

retained in the resulting butter. Worse still, an appreciable portion of the lipase, which is not completely inactivated by the pasteurization procedures normally employed in the manufacture of butter, apparently also remained in the resultant butter as indicated by the considerable fat degradation subsequent to churning. In fact, in trials conducted with six cultures of Ps. fragi, considerable lipase activity was demonstrable in the cream after heating to 71.5° C. for 30 minutes not including the time required to reach the holding temperature. Storage experiments on butter made from cream in which Ps. fragi was allowed to grow at 15° C. for three days brought out the alarming fact that the residual lipase, which was retained in butter after the heat treatment of cream, churning and washing of butter, was active to some degree in butter stored at -10° C. for 60 days, the butter developing rancidity even when the defect was not organoleptically detectable at the beginning of the storage period. Extensive fat degradation took place in the test butter samples stored at 21° C. for seven days, 2-5° C. for 30 days and at 36° C. for two days, in that order of decreasing fat deterioration, whereas the control samples exhibited no appreciable change. Neutralization as now commonly practiced for storage butter returns the serum to a pH at which any residual lipase activity would be favored considerably more than in butter from unneutralized cream.

It follows that it is undesirable to allow any appreciable build-up of population of Ps. fragi in cream before churning, because the lipase produced will inevitably lead to the deterioration of the product during storage because of incomplete inactivation during pasteurization.

SUMMARY AND CONCLUSIONS

1. A modified extraction-titration method was developed for measurement of lipase activity. The titration values represent quantitative determination of coconut oil hydrolysis by the lipase of Ps. fragi, since over 90 per cent of the released fatty acids would contribute to the titration values.

2. Sodium taurocholate proved to be a good emulsifier of coconut oil from pH 5.8 to pH 8.0. It also showed some activation of the lipase when used in 0.1 to 0.6 per cent concentration in the lipase test substrate, whereas sodium glycocholate was inhibitory in similar concentrations.

3. Formaldehyde was slightly inhibitory when present in the lipase test substrate in concentrations above 0.036 per cent.

4. The relatively small number of cells of Ps. fragi left in the lipase preparation after centrifugation contributed no detectable lipase activity.

5. The relationship between lipase activity and reaction time was not a straight-line function. This deviation from linearity was more marked at 36° and 40° than at 32° C. Also, the relationship between lipase concentration and

enzyme activity during a reaction period of 24 hours at 36° C. was not linear, the deviation from a straight line function becoming more pronounced with the increase in lipase activity.

6. Maximum lipase activity in coconut oil was observed at 40° C. when a reaction period of 24 hours was employed.

7. Coconut oil was hydrolyzed at a more rapid rate than were some other natural fats and oils, presumably due to its higher content of caprylic acid esters. Tricaprylin was hydrolyzed to a greater extent than the other triglycerides tried, indicating probable substrate preference by the lipase of Ps. fragi.

8. The lipase was active between pH 6.0 and pH 8.7 with an optimum at pH 7.0 to 7.2, using 10 per cent coconut oil emulsion buffered with monopotassium phosphate during a reaction period of 24 hours at 36° C.

9. Considerable lipase activity was lost during the time required to bring the lipase preparations up to temperatures of 61.6°, 71.6° and 99° C. The decline in lipase activity after the holding temperatures were reached occurred at a much slower rate. Lipase preparations were heated in boiling water for 20 minutes before complete inactivation of the enzyme was accomplished. A greater percentage of lipase was inactivated during the time required to bring the lipase preparation up to 61.6° C. than in the corresponding

interval at 71.6° C.

10. Lipase preparations were more stable when allowed to stand at 3-5° C. than at 15° or 36° C., considerable loss in lipase activity occurring in 24 hours at 36° C. In coconut oil emulsions this loss in enzyme activity at 36° C. was less marked than in peptone broth.

11. The salting out of the lipase could be accomplished almost quantitatively by fully saturating the preparations with ammonium sulfate below 7° C. About half the lipase activity of the enzyme preparation could be accounted for in a fluffy material that appears at the surface of the saturated preparation immediately after solution of ammonium sulfate. The remainder of the activity was recovered in the precipitate that formed near the bottom of the receptacle after the saturated solution stood at 5 to 8° C. for 16 to 18 hours.

12. Lipase production in the various growth media varied widely between the different strains of Ps. fragi.

13. Maximum lipase production by Ps. fragi in three days occurred at 15° C. or below, depending upon the different strains. Lipase production at higher temperatures was appreciably less, little or no detectable lipase production being observed at 30° C. or above. This optimum temperature for lipase production coincides with one common range of holding temperatures for cream on the farm. With

longer incubation periods the lower temperatures were more favorable for lipase production than were the higher temperatures.

14. Two pH optima in nutrient broth were evident, one at an initial pH of 6.5 and the other at about pH 7.5, lipase production at the latter figure being higher with one test culture and slightly lower with another culture.

15. Ps. fragi produced considerably more lipase in shallow layers of peptone broth with a large surface area than in deep layers with a small surface area. The greater lipase production was associated with increased counts.

16. There was no detectable lipase activity after the cultures had grown in peptone broth containing four per cent sodium chloride. The effect of sodium chloride at low concentration is to reduce lipase production somewhat in proportion to lowering of the counts, while at concentrations above two per cent salt lipase production declines more rapidly than does the count.

17. Appreciable lipase was produced by Ps. fragi in glucose and lactate defined media containing ammonium chloride as the only source of nitrogen. Little or no lipase production occurred in a similar medium with sodium citrate as the source of reduced carbon, although extensive growth was observed. Lipase production in citrate and

glucose defined media was increased materially when the medium was supplemented with L-leucine alone, or even more with a combination of L-leucine, DL-isoleucine and DL-valine. These three amino acids supported appreciable lipase production when used collectively as the sole source of nitrogen and reduced carbon, but L-leucine was the only single amino acid tested capable of supporting appreciable growth and lipase production.

18. Lipase production in peptone, proteose-peptone, tryptone and vitamin-free casamino acids media by Ps. fragi was much higher than in chemically-defined media. Peptone was the ingredient in nutrient broth that was responsible for lipase production, since it resulted in higher lipase activity, when used alone, than did the complete nutrient broth.

19. Addition of tricaprylin to vitamin-free casamino acids and peptone media resulted in pronounced increase in lipase production by Ps. fragi. Lipase production was increased somewhat in vitamin-free casamino acids medium supplemented with tricaprin. Addition of triacetin, tributyrin, and tricaproin caused no appreciable effect on lipase production, whereas trimyristin, tripalmitin, triolein and butterfat markedly inhibited lipase production even though the counts often were increased. The effect of trilaurin was variable, sometimes stimulating and sometimes inhibiting

lipase production.

20. The effect on lipase production of supplementing the vitamin-free casamino acids medium with tricaprylin was due largely to caprylic acid, since the fatty acid caused stimulation of lipase production comparable to that obtained with the triglyceride, whereas glycerol had no effect. Capric acid caused somewhat more increased lipase production than did caprylic acid in the same medium. Myristic acid caused appreciable increase in lipase production when supplemented to the vitamin-free casamino acids medium, whereas lauric acid inhibited growth and lipase production in the concentration tried. Supplementation with caproic, or butyric acids was without effect on lipase production, but palmitic, stearic and oleic acids markedly inhibited lipase production, even though they stimulated growth somewhat.

21. Growth and lipase production in cream by Ps. fragi invariably were rather poor. The low level of lipase in cream was offset by the greater stability of the enzyme to heat inactivation. However, a low level of lipase in cream caused an appreciable fat degradation during incubation and, after being subjected to pasteurization at 71.5° C. for 30 minutes and later churned, enough lipase was retained by the resultant butter to cause considerable fat degradation during storage.

22. The growth of S. lactis in association with Ps. fragi considerably lowered the pH of cream and caused marked reduction in lipase production, but the count of Ps. fragi was practically unaffected.

23. Butter containing residual lipase exhibited appreciable fat degradation even when held at -10° C. for 60 days, developing rancidity even when the defect was not organoleptically detectable at the beginning of the holding period. Similar butter held at 36° C. for two days, 2 to 5° C. for 30 days or 21° C. for seven days showed extensive fat hydrolysis, peppery taste and pronounced rancidity. Thus neither the usual pasteurization procedures nor the low temperatures commonly employed in commercial storage of butter can completely prevent the action of the lipase of Ps. fragi once it has been produced in cream, and the keeping qualities of such butter consequently are lowered.

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