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The action of lipolytic bacteria on some simple tri-glycerides and some natural fats

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THE ACTION OF LIPOLYTIC BACTERIA ON SOME SIMPLE TRI-GLYCERIDES AND SOME NATURAL FATS

By

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TABLE OF CONTENTS

GENERAL INTRODUCTION

In each branch of bacteriology a systematic classification of the organisms which are of special importance is essential. The criterion of the value of any such classification is primarily its usefulness, which is determined by the adaptability of the classification to the needs of the investigators in the field.

Medical bacteriologists are able to classify microorganisms which are pathogenic for man through a knowledge of their relationship to changes in certain tissues or organs of the body; soil bacteriologists are aided in their classification work by the chemical changes brought about in certain soil constituents; while the dairy bacteriologist may base a very useful classification upon the action on the important constituents of dairy products.

Classifications of the bacteria of interest in dairying have included primarily their action on the milk sugar and on the milk proteins, with little consideration of their effect on the milk fat. While a great deal of work has been done on the chemical deterioration of butter fat and while some authors have attributed these changes to the action of microorganisms, there has been very little work done on the

specificity of the lipolytic microorganisms for the various tri-glycerides, and no previous studies have utilized the lipolytic powers of microorganisms as a criterion for their classification.

It should be recognized that butter fat is rather unique among natural fats and oils because of its relatively high content of the tri-glycerides of the volatile fatty acids. This being true, one might expect to find distinct differences between the flavors and odors produced by lipolytic bacteria in butter fat and those produced in other natural fats and oils. The importance of these differences will assume more significance, from the standpoint of certain deteriorations in natural fats and oils, if it is found that the action of bacteria involves primarily the tri-glycerides which contain some of the lower saturated fatty acids.

GENERAL STATEMENT OF THE PROBLEM

The work herein reported was undertaken to obtain information concerning the characteristics and importance of the lipolytic bacteria, with special attention to the following:

1. Some considerations of the Nile-blue sulfate technique and its application to the study of bacterial action on simple tri-glycerides and natural fats.

2. The isolation, identification and classification of lipolytic bacteria.

3. The numbers of lipolytic bacteria in certain dairy products and the relationship of these organisms to rancidity.

4. The ability of lipolytic bacteria to produce rancidity in butter fat, in some other natural fats and oils and in some simple tri-glycerides.

SECTION I

SOME CONSIDERATIONS OF THE NILE-BLUE SULFATE TECHNIQUE AND ITS APPLICATION TO THE STUDY OF BACTERIAL ACTION ON SIMPLE TRI-GLYCERIDES AND NATURAL FATS

GENERAL CONSIDERATIONS

Various investigators, particularly in the field of histology, but in other branches as well, have studied the staining reactions of the fats in the tissues. Of the various dyes investigated Nile-blue sulfate has been applied extensively by different workers to the detection of the action of bacteria on fats. In this connection the staining reactions of Nile-blue sulfate have been considered with special reference to (a) the specificity of Nile-blue sulfate in its staining reaction for oleic acid, (b) the value of Nile-blue sulfate as an indicator for the detection of fat hydrolysis and (c) the application of the Nile-blue sulfate test to the detection of the action of bacteria on various fats dispersed in solid media.

STATEMENT OF THE PROBLEM

In view of the conflicting opinions regarding some of the points mentioned in connection with the detection of lipolysis by bacteria, the work herein reported was undertaken to obtain information on the following points:

1. The staining reaction of Nile-blue sulfate with saturated fatty acids, oleic acid and the simple tri-glycerides of these.

2. The Nile-blue sulfate test and the application of this technique to the detection of lipolysis by bacteria.

3. The factors influencing the accuracy of the Nile-blue sulfate test in the detection of lipolysis by bacteria.

4. The value of the action of bacteria on some simple tri-glycerides and some natural fats (indicated by the Nile-blue sulfate technique) as a criterion for the identification and the classification of lipolytic bacteria.

REVIEW OF THE LITERATURE

Methods for the detection of fat hydrolysis by microorganisms or by lipase produced by them have been employed for many years.

As early as 1894 Sommaragua (70) studied the lipolytic ability of microorganisms by growing them in a solid medium in which was dispersed 2.0 percent of pure olive oil or other fat. The medium which he used was composed of 1000 cc. of fresh meat juice, 1.0 percent peptone, 0.5 percent NaCl and 1.5 percent agar or 10.0 percent gelatin. No indicator was used. The fat was added to the agar or gelatin before sterilizing, and after sterilization it was emulsified with the medium by shaking vigorously and cooling until the fat would not rise to the surface when the tubes were slanted. The prepared slants were either streaked or stabbed with the cultures, and the hydrolysis of the fat was shown by the disappearance of the globules from the region of the growth.

A somewhat different method for the determination of fat hydrolysis by microorganisms was reported by Eijkman (23) in 1901. This investigator inoculated a melted agar medium with an organism and poured it onto a thin layer of tallow which covered the bottom of a petri dish. A lipolytic organism

produced colonies which caused clear zones in the tallow beneath them. Eijkman stated that bacteria which produce alkali may hydrolyze fat without the production of a lipase.

Smith (68) in 1908 studied the staining of neutral fats and fatty acids by oxazine dyes and showed that fat globules present in tissues could be stained by basic aniline dyes if they were first changed into fatty acids. He pointed out that, in the presence of Nile-blue sulfate, new methylene blue or Mendola's blue, globules of olein or of other neutral fats were red, while those of oleic acid were blue, the blue color being due to the formation of a blue soap between the fatty acid and the oxazine base. He found that the oxazine base is also soluble in palmitin and stearin. From his work Smith concluded that Nile-blue sulfate is a specific stain for fatty acids in general and not alone for oleic acid.

In an investigation upon the keeping quality of butter, Sayer and others (62) determined the presence of lipolytic bacteria in a litmus agar, prepared from sugar-free broth. They put a small amount of pure butter fat in each tube of agar before sterilizing. After sterilization and partial cooling they emulsified the fat by shaking, and then slanted the tubes and inoculated them. The hydrolysis of the fat was indicated by the disappearance of the fat from beneath the growth on the streak.

Boeminghaus (11) studied the effect of Nile-blue sulfate on the color of palmitin, stearin and olein globules and their corresponding acids. His results indicated that,

1. Nile-blue sulfate is a specific dye for oleic acid and its ester and that palmitic and stearic acids are little affected by it.

2. Globules of olein are colored a bright red by Nile-blue sulfate while globules of palmitin or stearin are little affected by it.

3. Olein or oleic acid when added to the glycerides of the higher fatty acids or the fatty acids themselves, increased the ability of these substances to absorb Nile-blue sulfate.

Buchanan (15) showed that lipolytic organisms, when grown on a solid medium in which was dispersed an emulsion of a suitable fat or oil, produced a lipase which caused the disappearance of the fat from the immediate vicinity of the growth. Similar results were secured by Waksman and Davison (76). They detected fat hydrolysis by the color changes of litmus or other indicators as a result of the freeing of fatty acids from the fat in the medium.

In the first of a series of papers dealing with a medium for the detection of lipase producing bacteria, Turner (74) in 1928 outlined the following procedure. The inoculum was mixed in a petri dish with a small quantity of a medium composed

of 1000 cc. of sugar-free meat digest fluid, 5 grams of dibasic sodium phosphate and 30 grams of agar, the reaction of the medium being adjusted to a pH of 7.6 before sterilizing. When the medium was cooling 0.125 grams of Nile-blue sulfate (made up in 100 cc. of 25.0 percent ethyl alcohol) and about 10.0 percent of a sterile emulsion of fat were added to it. Turner stated that the dye was absorbed by the fatty acids or by the soaps. No organisms were found which produced a blue zone about the colonies that were not fat hydrolyzers. Some organisms produced a transient blue zone with fat but did not produce lipase in pure culture when fat was not present. Certain anaerobic bacteria were found to decolorize aniline dyes while some bacterial colonies were shown to absorb the color. In a later paper Turner (73) compared the relative merits of the various methods which had been employed for the determination of fat hydrolysis by microorganisms. These methods involved the use of various combinations of fats or oils and dyes. He concluded that the method he had outlined (74) gave the quickest and most satisfactory results. Turner prepared the fat emulsion by heating together 100 cc. of cottonseed oil, 3 grams of India gum and 200 cc. of hot distilled water. This emulsion was sterilized alone and was added to the agar containing Nile-blue sulfate just before pouring in the proportion of 0.75 cc. of emulsion to 5.5 cc. of agar. The plates were incubated anaerobically at 37° C.

for 48 hours. The growth of fat splitting organisms was surrounded by a deep blue zone full of blue globules. When he used the tri-glycerides of saturated fatty acids Turner found that the clear zones never developed with tri-stearin and that they developed in two days, six days and ten days with tri-butyrin or tri-caprylin, tri-myristin and tri-palmitin respectively. He observed that tri-butyrin greatly inhibited bacterial growth while the other tri-glycerides did not. He also found that Nile-blue sulfate in a concentration of 1:8000 inhibited the growth of some organisms. His experiments showed that a good differential plating medium should result from either an emulsion of fat made up of short chained tri-glycerides or the use of bile in the medium along with an oil like cottonseed oil. Regarding the use of bile, Neill (55) found that the addition of bile to oleic or stearic acids hastened their diffusions through a collodion membrane.

In 1929 Turner (73) made a photomicrographic study of the changes taking place in the fat globules during hydrolysis by bacteria. He was convinced that the changes occurred far removed from the growth. He concluded that the unsaturated fatty acids, oleic and linoleic, stain deeply with Nile-blue sulfate and also that an alkaline reaction in the medium facilitates fat hydrolysis through the production of more readily soluble soaps.

Kaufmann and Lehmann (43), 1926, worked with pure substrates in the presence of the oxazine dye, Nile-blue sulfate. They found that it colored fatty acids dark blue and fatty esters red. They also thought that the color was absorbed by the unsaturated fatty acids and that this absorption was greatly influenced by the number of double bonds. Theyfound intense blue color in mixtures which did not contain any unsaturated acids, however, and concluded that a tri-glyceride having one oleic acid radical would react the same as pure tri-olein. They considered that for this reason all natural fats give the red color. They did not consider that Nile-blue is a specific dye for the fatty acids. These investigators did not agree with the conclusion of Boeminghaus (11) that Nile-blue sulfate is a specific reagent for oleic acid and its esters.

In 1931 Rettie (60) investigated the use of Nile-blue sulfate as a fat stain. He showed that the oxazone of Nile-blue sulfate is pink and that, since it is soluble in fats and fat solvents, it is the substance which is responsible for the color in the fat stain; he called this substance Nile-pink.

Turner's technique for the determination of the lipolytic ability of bacteria was somewhat modified by Hussong (42), 1932. He used an ordinary beef infusion agar with a pH of 6.8 to 7. To this agar he added Nile-blue sulfate (as an alcoholic solution) in the ratio of 1:10,000, and fat in the proportion of 1:200. The fat was emulsified with a 0.5 percent agar; it

was sterilized alone, and was added to the beef infusion agar at the time of pouring. Cultures were streaked on the surface of the medium. A change in the color of the fat globules, as well as a deep blue zone about the colony growth, was accepted as a positive test for hydrolysis.

Berry (9), 1933, recently reported some work, on the hydrolysis of fat by microbial lipase, on which he used the technique first employed by Carnot and Mauban (18). The organisms were grown on the surface of nutrient agar containing a suitable fat. Following incubation period, at the optimum temperature of the organisms used, the plates were flooded with a saturated copper sulfate solution for 10 minutes and then rinsed with distilled water. If the fat had been hydrolyzed, the fatty acids united with the copper sulfate to form an insoluble, blue, copper soap which was easily recognized in the medium.

METHODS USED

Methods Used in the Determination of Lipolytic Action by Bacteria

The method used for the determination of lipolysis by bacteria was based on the modification by Hussong (42) of the method of Turner (74). It depends on the differential staining, by Nile-blue sulfate, of fats and fatty acids dispersed in solid media. In the case of the tri-glycerides of the soluble fatty acids, a positive hydrolysis is indicated by the disappearance of the fat globules from the region of the growth.

Regular method used in the study of lipolysis by bacteria. The medium used was a beef infusion agar adjusted to a pH of 6.8 to 7.0. There was then added to the agar, in the proportion of 10 cc. of the solution to 100 cc. of the medium, a 0.1 percent aqueous solution of Nile-blue sulfate. The agar was sterilized at 15 pounds pressure for 25 minutes. This medium will be referred to as Nile-blue sulfate agar when it does not contain fat and as Nile-blue sulfate medium when it does contain fat.

The fat emulsion which was added to the agar was prepared in the following manner: The natural fat which was to be used

was added to a melted 0.5 percent agar solution in the proportion of 10 cc. of fat to 90 cc. of the solution. This was then sterilized at 15 pounds for 25 minutes. After the sterilized solution had been allowed to cool until it was partly solidified, it was vigorously shaken to secure an emulsion of the liquid fat. The fat emulsion was stored in this condition and just before use was heated to a temperature that would give a soft jelly-like mass which could be easily transferred with a pipette.

When plates were to be poured the Nile-blue sulfate agar was melted and to this hot medium the fat emulsion was added in the proportion of 1 cc. of the emulsion to 20 cc. of the agar. After the dye had been added to the agar the medium was allowed to remain hot for a few minutes before it was poured; this was done since the fat globules became much redder if they were in contact with the very hot Nile-blue sulfate agar for a short time.

Method for the simple tri-glycerides and the fatty acids. The liquid tri-glycerides and fatty acids were dispersed in the Nile-blue sulfate agar in the same general manner as the natural fats excepting that the smallest possible quantities were used. The tri-glycerides and the fatty acids which are solid at room temperature had to be dispersed in a special manner, as follows: The Nile-blue sulfate was added to the

plates and kept very hot over a low Bunsen flame while a small amount of the solid tri-glyceride or fatty acid was added and vigorously stirred into it. The agitation was continued until the fat or fatty acid had solidified in small globules or masses.

Method for studying the action of bacteria on the simple tri-glycerides and the natural fats. In studying the effect of bacteria on the simple tri-glycerides and the natural fats the procedure was as follows: The plates, having been poured as described above, were left at room temperature until the surface of the medium was dry (at least 12 hours) to prevent an abnormal spreading of the bacterial colonies which would form upon inoculation. The bottoms of the petri dishes were marked off (on the outside) into 8 to 12 subdivisions, and the plates were then inoculated with small loop-fulls of 48 hour litmus milk cultures. The plates were inverted and incubated for 7 days at 21° C. During incubation they were examined frequently for the purpose of recording the rates of lipolysis and enzyme diffusion.

The hydrolysis of the fats was indicated by a change in the color of the dispersed globules from red to blue. The theory of the test has been treated in the "Review of the Literature" and is dependent on the different color reactions of the natural fats and of the fatty acids in the presence of

the Nile-blue sulfate. When the simple tri-glycerides of the lower fatty acids were used it was necessary to accept the disappearance of the globules from the region of the growth as a positive test, since the fatty acids were soluble in the medium.

The absorption of the Nile-blue sulfate by the simple tri-glycerides and the fatty acids and the color changes resulting in the globules or masses of these materials were studied with non-nutrient Nile-blue sulfate agar (containing no peptone or beef extract) as well as with the regular Nile-blue sulfate agar. In both cases the simple tri-glycerides and the fatty acids were dispersed in the agar in the manner already mentioned and the general procedure was the same excepting that the media were not inoculated.

Method for making examinations of lipolytic action. All the examinations in the study of the action of bacteria on the various simple tri-glycerides and natural fats and in the study of the absorption of the Nile-blue sulfate by the simple tri-glycerides and the fatty acids were made by the aid of a hand lens or a wide field binocular.

Sources of the simple tri-glycerides, fatty acids and natural fats. The simple tri-glycerides with the exception of tri-olein were obtained from the Research Laboratories of the Eastman Kodak Co., Rochester, N. Y. The tri-olein was

secured from Doctors Fraenkel and Landau, Berlin. Some of the fatty acids were secured from the Eastman Kodak Co., while the others were obtained from Merck, N. Y. The natural fats and oils were obtained from local stores.

RESULTS OBTAINED

The Absorption of Nile-blue Sulfate by Some Simple Tri-glycerides When Dispersed in Solid Media

The absorption of Nile-blue sulfate by simple tri-glycerides was studied with tri-acetin, tri-propionin, tri-butyrin, tri-caproin, tri-caprylin, tri-caprin, tri-laurin, tri-myristin, tri-palmitin, tri-olein and tri-stearin. Four trials, in each of which the tri-glycerides were dispersed in both non-nutrient and nutrient Nile-blue sulfate agar, were carried out, and similar results were secured in all the trials. The data secured are given in Table I and may be summarized as follows:

1. Tri-acetin in the concentrations used was completely soluble in the media.

2. The dispersed globules of tri-butyrin, tri-caproin and tri-caprylin were quite insoluble and bright red in color in the media.

3. The dispersed masses of tri-caprin, tri-laurin, tri-myristin, tri-palmitin and tri-stearin were solid and became red to a degree which decreased rapidly with the increase in the melting points. It was possible to obtain a uniform distribution of fairly round solid globules with tri-caprin and tri-laurin while with tri-myristin, tri-palmitin and tri-stearin a disper-

TABLE I

Absorption of Nile-blue Sulfate by Some Simple Tri-glycerides
When Dispersed in Solid Media

(summary of four trials with non-nutrient and nutrient agars)

Tri-glyceride	Size, distribution, state and color of the dispersed globules Non-nutrient and nutrient nile-blue sulfate agar
Tri-acetin	Completely soluble, no apparent change in the color of the medium.
Tri-propionin	Small, uniformly dispersed, bright red globules.
Tri-butyrin	Small, uniformly dispersed, red globules.
Tri-caproin	Small, uniformly dispersed, bright red globules.
Tri-caprylin	Small, uniformly dispersed, bright red globules.
Tri-caprin	Small, uniformly dispersed, regular and irregular solid globules which are a duller red than those above.
Tri-laurin	Small, uniformly dispersed regular and irregular flaky, solid globules which vary in color from dull red to faintly purplish red.
Tri-myristin	Small, not uniformly dispersed, regular and irregular, flaky, solid masses which vary in color from dull red to purplish red.
Tri-palmitin	Small, not uniformly dispersed, regular and irregular, solid masses which vary in color from purplish red to reddish purple or to a yellowish whitish blue.
Tri-olein	Small, uniformly dispersed, regular, bright red globules.
Tri-stearin	Small, not uniformly dispersed, regular and irregular solid masses which vary in color from reddish purple to bluish white.

sion of only somewhat flaky, irregularly shaped masses could be obtained; the color of these globules or masses ranged from a faintly purplish red to a faintly reddish purple and sometimes to a bluish white.

4. The dispersed globules of tri-olein were very uniformly bright red and quite regular in size.

The results obtained show that Nile-blue sulfate is not a specific coloring reagent for the unsaturated tri-olein but that it becomes red when absorbed by the lower fatty esters also; this is in agreement with some of the literature reported. It would also seem that the absorption by the 3 higher saturated tri-glycerides is not sufficiently definite to permit the use of these for an accurate and practical differential test in the detection of lipolysis by bacteria. On the other hand the liquid or solid globules respectively of the lower saturated tri-glycerides or of tri-olein, when dispersed in the presence of Nile-blue sulfate, provide a very excellent differentiation.

The Absorption of Nile-blue Sulfate by Free Fatty Acids Dispersed in Solid Media

The absorption of Nile-blue sulfate by some fatty acids was studied with acetic, propionic, butyric, caproic, caprylic, capric, lauric, myristic, palmitic, oleic and stearic acids. Four trials, in each of which the fatty acids were

dispersed in both non-nutrient and nutrient agar were carried out, and similar results were secured with all the trials. The data are presented in Table II.

The results may be summarized as follows:

1. Acetic, propionic and butyric acids were soluble in the media in the concentrations used and, with the occasional exception of butyric acid, they did not affect the color; the butyric acid sometimes caused a slight turbidity in the media.

2. The dispersed globules of caproic and of caprylic acids were distinctly blue and seemed to uniformly absorb the dye from the surrounding medium. With the caprylic acid there were sometimes outlines or partial outlines of the globules left in the media.

3. The dispersed, solid, disc-like globules of capric and of lauric acids ranged in color from a much deeper blue to a much paler blue than the surrounding media.

4. The dispersed, solid, disc-like masses of myristic, palmitic and stearic acids generally assumed the color of the media although the small particles were sometimes more blue while the larger, flatter masses were often less blue than the surrounding media.

5. The dispersed globules of oleic acid were always a clear, much deeper blue than the surrounding media.

These results indicate that Nile-blue sulfate is not a specific color reagent for unsaturated oleic acid but that it

TABLE II

Absorption of Nile-blue Sulfate by Free Fatty Acids
Dispersed in Solid Media

(summary of four trials with non-nutrient and nutrient agars)

Acid	Solubility of acid in media	Size, distribution, state and color of the dispersed globules in Non-nutrient and nutrient Nile-blue sulfate agar
Acetic	Soluble	Completely soluble, no apparent change in the color of the medium.
Propionic	Soluble	Completely soluble, no apparent change in the color of the medium.
Butyric	Soluble	Completely soluble, apparent change in the color of the medium resulting in slight turbidity.
Caproic	Slightly soluble	Small to medium, well distributed, oily, clear, blue globules. The globules are bluer than the medium. Only the outlines of some globules remain.
Caprylic	Insoluble	Small to medium, well distributed, oily, clear, blue globules. The globules are much bluer than the medium which is faintly blue and slightly opaque.
Capric	Insoluble	Small to medium, well distributed, solid masses which are generally bluer than the surrounding medium. Some of the disc-like globules are quite blue.
Lauric	Insoluble	Small to medium, well distributed, solid masses which are sometimes bluer than but usually the same color as the surrounding medium.
Myristic	Insoluble	Small to large, poorly distributed, flat, solid masses which vary in color. The small particles are bluer than the medium; the large ones are whitish blue.
Palmitic	Insoluble	Difficult to prevent a film of solid acid on surface. When well distributed the smaller particles are bluer than the medium while the larger ones are yellowish blue.

(continued on following page)

TABLE II (continued)

Acid	Solubility of acid in media	Size, distribution, state and color of the dispersed globules
		Non-nutrient and nutrient Nile-blue sulfate agar
Oleic	Insoluble	Small, well distributed, oily, clear, deep blue globules which are much bluer than the surrounding medium.
Stearic	Insoluble	Small, well distributed, solid particles which are usually deeper blue than the surrounding medium but are sometimes whitish blue.

is absorbed in much the same manner by the saturated fatty acids which are insoluble and liquid in the medium; this is in agreement with some of the literature reported. The results also indicate that some of the solid saturated acids, if finely and uniformly dispersed, will show an appreciable dye absorption.

A Comparison of Nile-blue Sulfate and Litmus as Indicators for the Detection of Lipolysis by Bacteria

The comparative usefulness of Nile-blue sulfate and litmus for the detection of lipolysis by bacteria was determined with 16 lipolytic and 2 non-lipolytic organisms on beef infusion agar (without added sugar) in which some of the simple tri-glycerides were dispersed. The same batch of beef infusion agar was used with both indicators. Comparisons were made both by culturing on the surface and by stabbing slants of the media. Positive hydrolysis was indicated, with the Nile-blue sulfate, by the disappearance of the fat globules or by a definite change from a red to a blue color of the dispersed globules and, with the litmus, by the disappearance of the globules or by the reddening of the litmus through the production of free fatty acids from the tri-glycerides. A summary of the data obtained is given in Table III. Positive and negative signs are used to indicate lipolysis as judged by one or more criteria.

The recorded and unrecorded results show that:

1. Tri-propionin was hydrolyzed by all the cultures as shown by both indicators.

2. With tri-butyrin, tri-caproin and tri-caprylin, partial or complete agreements of lipolysis were shown by both indicators in 94.4 percent of the trials.

3. With tri-caproin, tri-caprylin, tri-caprin and trilaurin there were more definite and more positive results shown with the Nile-blue sulfate than with the litmus, the latter giving only indefinite and slight suggestions of lipolysis.

4. With tri-myristin and tri-palmitin or tri-stearin (not recorded in Table III) there was practically no lipolysis shown by either of the criteria and hence there was a very high agreement between the two indicators.

5. With tri-olein there was a 94.4 percent agreement between the two dyes. This agreement was definite and complete since there was a very definite color change with Nile-blue sulfate and a rapid color change in the litmus.

It is evident from the foregoing results that Nile-blue sulfate or litmus would be equally useful as indicators for the detection of lipolysis in the presence of tri-propionin, tri-butyrin or tri-olein, and that Nile-blue sulfate gives a more consistently definite differentiation with more tri-glycerides than does litmus. It also seems logical from the foregoing results that Nile-blue sulfate would be the better

TABLE III

A Comparison of Nile-blue Sulfate and Litmus as Indicators for the Detection of Lipolysis by Bacteria

Culture	Hydrolysis of some simple tri-glycerides by lipolytic bacteria																			
	Tri-propionin		Tri-butyrin		Tri-caproin		Tri-caprylin		Tri-caprin		Tri-laurin		Tri-myristin		Tri-olein					
	* Lit.	** N.S.	Lit.	N.S.	Lit.	N.S.	Lit.	N.S.	Lit.	N.S.	Lit.	N.S.	Lit.	N.S.	Lit.	N.S.				
6	+	+	+	+	+	+	+	sl.+	+	sl.+	+	sl.+	+	-	-	+	+			
7	sl.+	+	+	sl.+	+	sl.+	+	sl.+	+	-	-	-	-	-	-	+	+			
8	+	+	+	sl.+	+	sl.+	+	sl.+	+	sl.+	+	sl.+	+	-	-	+	+			
12	+	+	+	+	+	+	+	sl.+	+	sl.+	+	-	+	-	-	+	+			
13	+	+	+	+	+	sl.+	+	sl.+	+	sl.+	+	-	-	-	-	+	+			
17	+	+	+	+	+	sl.+	+	sl.+	+	sl.+	+	sl.+	-	-	-	+	+			
18	+	+	+	+	+	+	+	sl.+	+	sl.+	+	-	-	-	-	+	+			
28	+	+	+	+	+	sl.+	+	sl.+	+	sl.+	+	-	-	-	-	+	+			
32	sl.+	+	+	sl.+	+	-	-	-	-	-	-	-	-	-	-	sl.+	?			
33	sl.+	+	+	sl.+	+	-	sl.+	-	sl.+	-	sl.+	-	-	-	-	+	sl.+			
34	+	+	+	+	+	+	+	+	+	sl.+	+	sl.+	?	sl.+	-	+	+			
35	+	+	+	sl.+	+	sl.+	+	sl.+	+	sl.+	+	sl.+	-	-	-	+	+			
37	+	+	+	+	+	sl.+	+	sl.+	+	sl.+	+	sl.+	+	-	-	+	+			
38	+	+	+	sl.+	+	sl.+	+	sl.+	+	sl.+	-	sl.+	-	-	-	+	+			
39	+	+	+	sl.+	+	sl.+	+	sl.+	+	sl.+	-	-	-	-	-	+	+			
41	+	+	+	+	+	+	+	+	+	sl.+	+	sl.+	+	-	-	+	+			
42	+	+	+	+	+	+	+	+	+	sl.+	+	sl.+	+	sl.+	?	+	+			
43	sl.+	+	+	sl.+	-	-	-	-	-	sl.+	-	-	-	-	-	-	-			
A	18	18	18	17	15	16	15	16	15	13	10	7	2	0	17	16				
B	100.0	100.0	100.0																	
C	18		17		17		17		14		13		16		17					
D	100.0		94.4		94.4		94.4		77.7		72.2		88.8		94.4					
E	0		1		1		1		4		5		2		31					
F	0		5.6		5.6		5.6		22.3		27.8		11.2		5.6					

* Litmus.
** Nile-blue sulfate.
A Number of cultures hydrolyzing.
B Percent of cultures hydrolyzing.
C Number of partial or complete agreements.
D Percent of partial or complete agreements.
E Number of questionable or complete disagreements.
F Percent of questionable or complete disagreements.

indicator of the two to use when common natural fats are dispersed in a solid medium.

A Comparison of the Disappearance and the Color Change of Fat Globules Dispersed in Nile-blue Sulfate Agar for the Detection of Lipolysis by Bacteria

The relative value of the disappearance and the color change of the fat globules in the region of the growth was studied with 100 cultures, most of which were lipolytic, grown on Nile-blue sulfate medium in which various simple tri-glycerides were dispersed. The examination for both changes was made at the same time, on the growths resulting from the inoculation of plates, with pure culture of bacteria.

The data presented in Table IV are representative results obtained with 50 of the 100 cultures; the results secured with tri-myristin, tri-palmitin and tri-stearin are not included but are discussed with those recorded.

The recorded and unrecorded results show that:

1. When tri-propionin and tri-butyrin were hydrolyzed their acids were completely miscible with the medium.

2. It was not possible to make a comparison of the two methods with tri-propionin, tri-butyrin or tri-olein since the fatty acids formed by the hydrolysis of the first two were soluble and oleic acid was insoluble in the media. With tri-caproin, tri-caprylin, tri-caprin and tri-laurin there were complete or partial agreements between the two methods in

TABLE IV

A Comparison of the Disappearance and the Color Change of Fat Globules Dispersed in Nile-blue Sulfate Agar for the Detection of Lipolysis by Bacteria

Culture	Tri-propionin Hydrolysis * sol.	Tri-propionin Hydrolysis ** c.c.	Tri-butyrin Hydrolysis sol.	Tri-butyrin Hydrolysis c.c.	Tri-caproin Hydrolysis sol.	Tri-caproin Hydrolysis c.c.	Tri-caprylin Hydrolysis sol.	Tri-caprylin Hydrolysis c.c.	Tri-caprin Hydrolysis sol.	Tri-caprin Hydrolysis c.c.	Tri-laurin Hydrolysis sol.	Tri-laurin Hydrolysis c.c.	Tri-olein Hydrolysis sol.	Tri-olein Hydrolysis c.c.
6	+	No color change due to the solubility of the acid	+	No color change due to the solubility of the acid	+	-	+	+	+	+	-	+	?	+
7	+		+		+	-	+	+	+	+	-	sl.+	-	+
8	+		+		+	-	+	+	?	+	?	sl.+	-	+
9	+		+		-	-	-	-	-	-	-	-	-	+
10	+		sl.+		-	-	-	-	-	-	-	-	-	-
12	+		+		+	-	+	?	sl.+	-	-	-	-	+
13	+		+		+	-	+	?	sl.+	-	-	-	-	+
16	+		sl.+		+	-	+	+	-	-	-	-	-	+
17	+		+		+	+	+	+	-	-	+	-	-	+
18	+		+		+	-	+	?	-	-	+	-	-	+
19	+		-		-	-	-	-	-	-	-	-	-	-
20	+		+		+	+	+	+	+	+	+	+	+	+
21	+		+		+	?	+	+	-	-	+	-	?	+
22	+		-		-	-	-	-	-	-	-	-	-	-
23	+		+		+	+	+	+	-	-	+	-	?	+
24	+		-		-	-	-	-	-	-	-	-	-	-
26	+		+		+	+	+	+	+	-	-	-	?	+
27	+		+		+	+	+	+	-	-	-	-	+	+
28	+		+		+	+	+	+	sl.+	sl.+	+	+	?	+
29	+		-		-	-	-	-	-	-	-	-	-	-
30	+		-		-	-	-	-	-	-	-	-	-	-
31	+		sl.+		-	-	-	-	-	-	-	-	-	+
32	+		+		+	?	-	-	-	-	-	-	-	-
33	+		sl.+		-	?	sl.+	+	-	?	-	?	-	-

(continued on following page)

TABLE IV (continued)

Culture	Tri-propionin Hydrolysis * sol.	Tri-propionin Hydrolysis ** c.c.	Tri-butyrin Hydrolysis sol.	Tri-butyrin Hydrolysis c.c.	Tri-caproin Hydrolysis sol.	Tri-caproin Hydrolysis c.c.	Tri-caprylin Hydrolysis sol.	Tri-caprylin Hydrolysis c.c.	Tri-caprin Hydrolysis sol.	Tri-caprin Hydrolysis c.c.	Tri-laurin Hydrolysis sol.	Tri-laurin Hydrolysis c.c.	Tri-olein Hydrolysis sol.	Tri-olein Hydrolysis c.c.
34	+	+	+	+	+	+	+	+	+	+	+	-	?	+
35	+	+	+	+	+	+	-	+	?	-	-	-	-	+
36	+	+	+	+	+	+	-	+	?			-	-	+
37	+	+	+	+	+	+	+	+	?			-	+	+
38	+	+	+	+	+	+	?	+	?			-	+	+
39	+	+	+	+	-	-	-	+	?			-	+	+
40	+	+	-	-	-	-	-	-	-	-	-	-	-	-
41	+	+	+	+	+	+	-	+	?	-	-	-	+	+
42	+	+	+	+	+	+	?	+	-	-	-	-	+	+
43	+	+	-	-	-	-	-	-	-	-	-	-	-	-
45	+	+	+	+	-	-	-	+	?	-	-	-	-	+
46	+	+	sl.	+	-	-	-	-	-	-	-	-	-	+
47	+	+	+	+	+	+	+	-	+	+	?	-	-	+
48	+	+	+	+	sl.	+	-	+	?	+	?	-	-	+
49	+	+	+	+	+	+	-	+	-	-	-	-	-	+
50	+	+	+	+	+	+	-	+	?	+	?	-	-	+
F54	+	+	sl.	+	-	-	-	-	-	-	-	-	-	+
F56	+	+	sl.	+	-	-	-	-	-	-	-	-	-	+
56	+	+	sl.	+	-	-	-	-	-	-	-	-	sl.	+
57	+	+	+	+	+	+	-	-	-	sl.	-	-	-	+
82	+	+	+	+	-	-	-	-	-	-	-	-	-	
83	+	+	-	-	+	+	-	+	?	-	-	-	-	-
90	+	+	+	+	+	+	-	+	+	-	-	+	+	+
91	+	+	+	+	+	+	+	+	+	sl.	-	-	+	+
92	+	+	+	+	+	-	+	-	-	-	-	-	-	+
93	+	+	+	+	-	-	-	+	-	-	-	+	-	+
A	50		42		31	14	31	15	14	4	11	9	36	

(continued on following page)

TABLE IV (continued)

Culture	Tri-propionin Hydrolysis * sol.	Tri-propionin Hydrolysis ** c.c.	Tri-butyrin Hydrolysis sol.	Tri-butyrin Hydrolysis c.c.	Tri-caproin Hydrolysis sol.	Tri-caproin Hydrolysis c.c.	Tri-caprylin Hydrolysis sol.	Tri-caprylin Hydrolysis c.c.	Tri-caprin Hydrolysis sol.	Tri-caprin Hydrolysis c.c.	Tri-laurin Hydrolysis sol.	Tri-laurin Hydrolysis c.c.	Tri-olein Hydrolysis sol.	Tri-olein Hydrolysis c.c.
B	100.0		84.0		62.0	28.0	62.0	30.0	31.7	8.7	22.0	18.0		72.0
C					32		32		35		31			
D					64.0		64.0		76.0		62.0			
E					18		18		11		19			
F					36.0		36.0		24.0		38.0			

* Disappearance of globules.

**Color change of globules.

A Number cultures hydrolyzing.
B Percent cultures hydrolyzing.
C Number cultures showing complete or partial agreement.
D Percent cultures showing complete or partial agreement.
E Number cultures showing questionable or complete disagreement.
F Percent cultures showing questionable or complete disagreement.

64.01, 64.0, 76.0 and 62.0 percent of the trials respectively. The disappearance of the fat globules was more sensitive to lipolytic action with these tri-glycerides than the color change of the globules.

3. With the tri-glycerides of the intermediate fatty acids, capric, lauric and myristic, it was easier to detect a color change than a disappearance of the fat globules.

4. There was no solubility noted with tri-myristin, tri-palmitin or tri-stearin, and there was no color change in the globules of tri-palmitin or tri-stearin. There were three questionable color changes noted for tri-myristin; these indefinite results were attributed to the presence of insufficient free acid to bring about a definite color change and the rather doubtful red color of the original globules.

The above summary suggests that the method adopted for the detection of lipolytic action by bacteria is largely dependent on the simple tri-glyceride dispersed in the Nile-blue sulfate medium.

The Effect of the pH of the Medium on the Hydrolysis by Bacteria of Some Simple Tri-glycerides and Cottonseed Oil

The effect of the pH of the medium on the hydrolysis by bacteria of simple tri-glycerides and cottonseed oil was determined by dispersing tri-butyrin, tri-olein and cottonseed

oil, respectively, in Nile-blue sulfate agar. Each medium was divided into 3 parts and the 3 parts standardized to hydrogen ion concentrations of 5.3, 6.7 and 7.8, respectively. The study was carried out with 26 cultures of lipolytic bacteria with which the prepared media were inoculated. The data secured on the comparison of the hydrolysis of the fats at the different pH concentrations are presented in Table V.

The results may be summarized as follows:

1. With tri-butyrin there were 5 cultures, which did not show good growth or any lipolysis at a pH of 5.3; these cultures grew well and hydrolyzed the fat at a pH of 6.7 or 7.8. This indicates that (a) either the tri-butyrin at the pH of 5.3 retarded the growth of the bacteria or (b) the small amount of butyric acid produced inhibited further growth and also lipolysis.

2. There was a 100 percent agreement of the hydrolysis of tri-olein and of cottonseed oil at the three hydrogen ion concentrations.

3. The most luxuriant growth and the most positive lipolysis occurred at the highest hydrogen ion concentration in each series.

From the above results it may be concluded that (a) reasonable variations in the hydrogen ion concentrations of the media have very little effect on the hydrolysis, by bacteria, of

TABLE V

The Effect of the pH of the Medium on the Hydrolysis by Bacteria of Some Simple Tri-glycerides and Cottonseed Oil

Culture	Tri-butyrin pH value			Tri-olein pH value			Cottonseed oil pH value		
	5.3	6.7	7.8	5.3	6.7	7.8	5.3	6.7	7.8
6	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+
41	+	+	+	+	+	+	+	+	+
42	+	+	+	+	+	+	+	+	+
67	+	+	+	+	+	incomplete	+	+	incomplete
82	sl. +	sl. +	sl. +	+	+	+	incomp.	+	incomp.
86	sl. +	+	+	+	+	+	+	+	+
87	+	+	+	+	+	+	+	+	+
89	+	+	+	+	+	+	incomp.	incomp.	incomp.
91	+	+	+	+	+	+	+	+	+
92	-	-	-	incomp.	sl. +	+	-	-	-
93	incomp.	+	incomp.	incomp.	+	+	+	incomp.	+
94	+	+	+	+	+	+	+	+	+
95	incomp.	+	+	+	incomp.	incomp.			
97	+	+	+	+	+	+	+	+	+
108	-	+	+	+	+	+	+	+	+
109	-	+	incomp.	+	incomp.	incomp.	+	+	+
111	-	incomp.	-	incomp.	incomp.	incomp.	+	+	incomp.
113	-	incomp.	sl. +	incomp.	incomp.	incomp.	-	sl. +	incomp.
F114	-	-	-	-	-	-	-	-	-
118	sl. +	+	+	+	+	+	incomp.	incomp.	incomp.
119	-	-	incomp.	+	+	+	+	+	+
120	sl. +	incomp.	incomp.	+	+	+	+	+	+
123	incomp.	+	+	+	+	+	+	+	+
124	incomp.	incomp.	incomp.	+	incomp.	+	+	+	+
125	-	-	incomp.	+	incomp.	+	sl. +	sl. +	sl. +

(continued on following page)

TABLE V (continued)

Culture	Tri-butyrin pH value			Tri-olein pH value			Cottonseed oil pH value		
	5.3	6.7	7.8	5.3	6.7	7.8	5.3	6.7	7.8
A		20			26			24	
B		76.9			100.0			96.0	
C		6			0			1	
D		23.1			0			4.0	

A Number of partial or complete agreements.
B Percent of partial or complete agreements.
C Number of disagreements.
D Percent of disagreements.

either tri-olein or cottonseed oil and (b) the more alkaline reaction of the medium favors the hydrolysis, by bacteria, of either tri-olein or cottonseed oil.

A Comparison of the Hydrolysis by Bacteria of Some Simple Tri-glycerides When Dispersed In and Out of an Agar Emulsion

The effect of the method of dispersing the fat on its hydrolysis by bacteria was studied with 20 definitely lipolytic cultures and 6 cultures of doubtful lipolytic ability. Reasonable good dispersions were obtained with the emulsified tri-glycerides while relatively uneven dispersions were obtained when the tri-glycerides were added to the Nile-blue sulfate agar without previous emulsification. In the latter case a considerable amount of fat remained as a liquid or solid film over the surface of the solid medium. The media were inoculated from fresh litmus milk cultures in the usual manner. The data secured are recorded in Table VI.

The results show that:

1. The percentages of partial or complete agreements between the two methods of dispersion ranged from 38.5 for tri-myristin to 100.0 for tri-olein and tri-stearin, tri-myristin being the only tri-glyceride to show an agreement of less than 80.8 percent with the two methods.

2. More than 80 percent of the cultures showed partial

TABLE VI

A Comparison of the Hydrolysis by Bacteria of Some Simple Tri-glycerides

Culture	Tri-butyrin Hydrolysis * in E	Tri-butyrin Hydrolysis ** out E	Tri-caproin Hydrolysis in E	Tri-caproin Hydrolysis out E	Tri-caprylin Hydrolysis in E	Tri-caprylin Hydrolysis out E	Tri-caprin Hydrolysis in E	Tri-caprin Hydrolysis out E	Tri-laurin Hydrolysis in E	Tri-laurin Hydrolysis out E
6	+	+	+	+	+	+	+	+	+	?
20	+	+	+	+	+	+	+	+	-	-
41	+	+	+	+	+	+	+	+	+	+
42	+	+	+	+	+	+	+	+	+	+
67	+	+	+	+	+	+	sl.+	sl.+	+	?
82	+	sl.+	+	+	+	+	sl.+	sl.+	-	+
86	sl.+	sl.+	+	+	+	+	+	+	+	+
87	+	+	+	+	+	+	+	+	+	+
89	sl.+	sl.+	-	-	?	-	-	-	-	-
90	+	+	+	+	+	+	+	sl.+	+	+
92	sl.+	sl.+	sl.+	sl.+	-	-	-	-	-	-
93	sl.+	sl.+	sl.+	sl.+	incomp.	incomp.	-	-	+	+
94	+	+	+	+	+	+	+	+	+	+
95	+	+	+	+	+	+	+	+	+	+
105	+	+	+	+	+	+	sl.+	sl.+	-	-
108	+	+	+	+	+	+	+	+	+	sl
109	+	+	sl.+	+	+	+	sl.+	sl.+	sl.+	+
111	-	-	-	-	?	-	-	-	-	-
113	sl.+	sl.+	-	-	?	-	-	-	-	-
F114	-	-	-	-	sl.+	+	-	-	-	-
118	sl.+	sl.+	-	+	+	+	-	+	sl.+	+
119	+	+	+	+	+	sl.+	sl.+	+	?	?
120	+	+	+	+	+	+	+	+	+	+
123	+	+	+	+	+	+	+	+	sl.+	+
124	+	+	+	+	+	+	+	+	+	+
125	+	+	sl.+	+	+	+	+	sl.+	sl.+	sl.+
A	24	24	21	22	22	22	19	20	17	16
B	92.3	92.3	80.1	84.6	84.6	84.6	73.1	76.9	65.4	61.5
C	26		25		23		25		21	
D	100.0		96.2		88.5		96.2		80.8	
E	0		1		3		1		5	
F	0		3.8		11.5		3.8		19.2	

* In emulsion.
** Out of emulsion.
A Number of cultures hydrolyzing.
B Percent of cultures hydrolyzing.
C Number of partial or complete agreements.
D Percent of partial or complete agreements.
E Number of questionable or complete disagreements.
F Percent of questionable or complete disagreements.

TABLE VI

of Some Simple Tri-glycerides When Dispersed In and Out of an Agar Emulsion

Tri-caprin		Tri-laurin		Tri-myristin		Tri-palmitin		Tri-olein		Tri-stearin	
Hydrolysis		Hydrolysis		Hydrolysis		Hydrolysis		Hydrolysis		Hydrolysis	
in E	out E	in E	out E	in E	out E	in E	out E	in E	out E	in E	out E
+	+	+	?	-	?	-	-	+	+	-	-
+	+	-	-	-	?	-	-	+	+	-	-
+	+	+	+	-	?	-	-	+	+	-	-
+	+	+	+	-	?	-	-	+	+	-	-
sl.+	sl.+	+	?	-	?	-	-	sl.+	sl.+	-	-
sl.+	sl.+	-	+	-	?	-	-	sl.+	sl.+	-	-
+	+	+	+	-	?	-	-	+	+	-	-
+	+	+	+	-	?	-	-	+	+	-	-
-	-	-	-	?	?	-	-	-	-	-	-
+	sl.+	+	+	?	+	-	-	+	+	-	-
-	-	-	-	-	-	-	-	incom-plete incomp.	incom-plete incomp.	-	-
-	-	-	+	-	-	-	-	incomp.	incomp.	-	-
+	+	+	+	?	+	-	-	+	+	-	-
+	+	+	+	+	+	-	-	sl.+	+	-	-
sl.+	sl.+	-	-	-	-	-	-	sl.+	+	-	-
+	+	+	+	sl.+	sl.+	-	-	+	+	-	-
sl.+	sl.+	sl.+	+	+	sl.+	-	-	incomp.	incomp.	-	-
-	-	-	-	-	-	-	-	"	"	-	-
-	-	-	-	?	+	-	-	sl.+	sl.+	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	+	sl.+	+	-	?	-	-	sl.+	sl.+	-	-
sl.+	+	+	?	+	+	-	-	+	+	-	-
+	+	+	+	-	?	-	-	+	+	-	-
+	+	sl.+	+	?	+	incomp.		+	+	-	-
+	+	+	+	-	sl.+	-	-	+	+	-	-
+	sl.+	sl.+	sl.+	sl.+	sl.+	-	-	incomp.	incomp.	-	-
19	20	17	16	5	10	1	0	24	24	0	0
73.1	76.9	65.4	61.5	19.2	38.5	3.8	0	92.3	92.3	0	0
25		21		10		25		26		26	
96.2		80.8		38,5		96.2		100.0		100.0	
1		5		16		1		0		0	
3.8		19.2		61.5		3.8		0		0	

ents.
ments.

or complete agreements in their reactions with 8 of the 9 tri-glycerides.

3. Tri-myristin was hydrolyzed more easily when not emulsified with agar than when emulsified with it, but there was no regular difference with the other tri-glycerides.

4. The percentage of questionable or complete disagreements ranged from 0.0 for several tri-glycerides to 61.5 percent for tri-myristin; with 3 tri-glycerides there were no questionable or complete disagreements and with the remaining five there were less than 20.0 percent complete disagreements with the two methods.

5. Observations which are not included in Table VI show that the emulsified fat was more uniformly dispersed throughout the medium than the non-emulsified fat.

From the above summary it is evident that the method of dispersing a fat, which would ordinarily be used for the detection of fat hydrolysis by bacteria, has little effect on the result. Very indefinite results and a small percentage of agreement were shown with the 2 methods when tri-myristin was used, but this is not significant as this tri-glyceride would not commonly be employed in the Nile-blue sulfate test.

The explanation for the greater ease of hydrolysis of tri-myristin when not in emulsion than when in an emulsion may be explained on the basis of the air relationship; the non-emulsified tri-myristin spread out in a thin film over the surface

of the medium and presented more surface to the bacterial colonies.

The Hydrolysis of Some Simple Tri-glycerides and Butter Fat by 119 Cultures of Bacteria Isolated from Various Sources

The hydrolysis of the simple tri-glycerides of the fatty acids occurring normally in butter fat, as well as the hydrolysis of tri-propionin, tri-normal valerin, tri-iso valerin and tri-heptylin which are not normally found in butter fat, was studied with 119 cultures of bacteria, most of which were lipolytic. The data recorded represent the typical reaction observed in 3 or 4 trials with each tri-glyceride, and butter fat, and also gives a comparison between (a) the hydrolysis of tri-butyrin and tri-olein, (b) the hydrolysis of tri-butyrin and butter fat and (c) the hydrolysis of tri-olein and butter fat. Table VII presents the data secured.

The results may be summarized as follows:

1. The percentages of the cultures showing lipolysis ranged from 100.0 for tri-propionin to 0.0 for tri-stearin.

2. With a slight exception in the case of tri-caprylin there was a regular decrease in the percentages of the cultures showing lipolysis as the molecular weights of the acids in the tri-glycerides increased.

3. Tri-normal valerin, tri-iso valerin and tri-heptylin

TABLE VII

The Hydrolysis of Some Simple Tri- lycerides and Butter Fat by Il

Hydrolysis determined by color change or disappearance o

Culture #	Hydrolysis of Tri-propionin	Tri-butyrin	Tri-n-valerin	Tri-iso-valerin	Tri-caproin	Tri-heptylin	Tri-caprylin	Tri-caprin	Tri-laurir
6	plus	plus	plus	plus	plus	plus	plus	plus	plus
7	"	"	"	"	"	neg.	"	neg.	neg.
8	"	"	"	"	"	"	"	plus	plus
9	"	"	"	neg.	neg.	"	neg.	neg.	neg.
F10	"	neg.	neg.	"	"	"	"	"	"
M10	"	plus			"	**	"	"	"
12	"	"	plus	plus	plus	quest.	plus	plus	plus
13	"	"	"	"	"	"	"	"	neg.
F15	"	"			"		neg.	neg.	"
16	"	"	"	"	"	"	plus	plus	"
17	"	"	"	quest.	"	neg.	"	"	"
18	"	"	"	"	"	"	"	"	"
F19	"	neg.	neg.	neg.	neg.	"	neg.	neg.	"
20	"	plus	plus	plus	plus	plus	plus	plus	plus
21	"	"	"	"	"	"	"	"	"
F22	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
23	plus	plus	plus	plus	plus	plus	plus	plus	plus
F24	"	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
26	"	plus	plus	plus	plus	plus	plus	plus	plus
27	"	"	"	neg.	neg.	"	"	neg.	"
28	"	"	"	plus	plus	"	"	plus	neg.
F29	"	neg.	neg.	neg.	neg.	neg.	neg.	neg.	"
F30	"	"	"	"	"	"	"	"	"
31	"	"	quest.	"	"	plus	"	"	"
F32	"	plus			"		"	"	"
32	"	"	plus	"	"	neg.	"	"	"
33	"	"	neg.	incomp.	plus	quest.	plus	plus	"
34	"	"	plus	plus	"	plus	"	"	ques
35	"	"	"	quest.	"	quest.	"	"	neg
36	"	"	"	plus	neg.	neg.	"	neg.	ques
37	"	"	"	"	plus	plus	plus	plus	plu
38	"	plus	"	"	"	"	"	neg.	"
39	"	"	"	"	"	"	"	"	"
F40	neg.	neg.	neg.	neg.	neg.	neg.	neg.	"	neg
41	plus	plus	plus	plus	plus	plus	plus	plus	plu
42	"	"	"	"	"	"	"	"	"

TABLE VII

ter Fat by 119 Cultures of Bacteria Isolated from Various Sources

appearance of fat globules in Nile-blue sulfate medium

sis of								Agreement between the hydrolysis of		
Tri- aprin	Tri- laurin	Tri- myris- tin	Tri- palmi- tin	Tri- olein	Tri- stearin	Butter fat	Tri- butyrin and tri- olein	Tri- butyrin and butter fat	Tri- olein and butter fat
plus	plus	neg.	neg.	plus	neg.	plus	plus	plus	plus
neg.	neg.	"	"	"	"	" slow	"	"	incomp.
plus	plus	"	"	"	"	plus	"	"	plus
neg.	neg.	"	"	"	"	"	"	"	"
"	"	"	"	neg.	"	neg.	"	"	"
"	"	"	"	plus	"	plus	"	"	"
plus	plus	"	"	"	"	"	"	"	"
"	neg.	"	"	"	"	"	"	"	"
neg.	"	"	"	"	"	"	"	"	"
plus	"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"	"
neg.	"	"	"	neg.	"	neg.	"	"	"
plus	plus	incomp.	"	plus	"	plus	"	"	"
"	"	neg.	"	"	"	"	"	"	"
neg.	neg.	"	"	neg.	"	neg.	"	"	"
plus	plus	"	"	plus	"	plus	"	"	"
neg.	neg.	"	"	neg.	"	neg.	"	"	"
plus	plus	"	"	plus	"	plus	"	"	"
neg.	"	"	"	"	"	"	"	"	"
plus	neg.	"	"	"	"	"	"	"	"
neg.	"	"	"	neg.	"	neg.	"	"	"
"	"	"	"	"	"	"	"	"	"
"	"	"	"	plus	"	plus	neg.	neg.	"
"	"	"	"	incomp.	"	"	incomp.	plus	incomp.
"	"	"	"	quest.	"	"	"	"	"
plus	"	"	"	neg.	"	incomp.	neg.	incomp.	"
"	quest.	"	"	plus	"	plus	plus	plus	plus
"	neg.	"	"	"	"	"	"	"	"
neg.	quest.	"	"	"	"	"	"	"	"
plus	plus	"	"	"	"	"	"	"	"
neg.	"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"	"
"	neg.	"	"	neg.	"	neg.	"	"	"
plus	plus	quest.	"	plus	"	plus	"	"	"
"	"	"	"	"	"	"	"	"	"

(continued on following page)

TABLE VI
(continue

43	plus	neg.	neg.	neg.	neg.	neg.	neg.	neg.
45	"	plus	plus	quest.	"	quest.	plus	"
46	"	quest.	neg.	neg.	"	neg.	neg.	"
47	"	plus	plus	plus	plus	plus	plus	plus
48	"	"	"	"	"	"	"	neg.
49	"	"	"	incomp.	neg.	quest.	quest.	"
50	"	"	"	plus	plus	plus	plus	plus
F51	"	"	"	"	"	"	"	"
F52	"	neg.	neg.	neg.	neg.	neg.	neg.	neg.
52	"	plus			plus		plus	plus
F53	"	neg.	"	"	neg.	"	neg.	neg.
53	"	"			"		"	"
53a	"	plus			"		plus	"
F54	"	quest.			"		neg.	"
55	"	plus	plus	quest.	"	"	"	"
F56	"	neg.			"		"	"
56	"	plus	incomp.	neg.	"	"	"	"
57	"	"	quest.	"	"	"	plus	"
F58	"	" slow			"		neg.	"
58	"	neg.	plus	"	"	quest.	"	"
59	"	plus			slow		slow	"
F60	"	" slow			neg.		neg.	"
60	"	plus			slow		slow	quest.
61	"	" slow			"		"	neg.
62	"	"			"		"	quest.
63	"	neg.	neg.	"	neg.	neg.	neg.	neg.
F64	"	plus			"		"	"
66	"	"	"	"	"	"	"	"
67	"	"	plus	plus	plus	"	plus	"
68	"	"			"		"	"
69	"	"			"		"	"
70	"	"			"		"	quest.
71	"	neg.			neg.		neg.	neg.
72	"	"			"		"	"
73	"	plus			plus		plus	"
74	"	quest.			neg.		neg.	"
75	"	plus			plus		plus	plus
76	"	"			"		"	"
77	"	"			neg.		neg.	neg.
78	"	"			"		"	"
79	"	neg.			"		quest.	"
80	"	plus			plus		plus	quest.
81	"	incomp.			neg.		neg.	neg.
82	"	"			quest.		"	"
83	"	"			neg.		"	"
84	"	plus			plus		plus	"

TABLE VII
continued)

neg.	neg.	neg.	neg.	neg.	neg.	neg.	plus	plus	plus
"	"	"	"	plus	"	plus	"	"	"
"	"	"	"	"	"	neg.	incomp.	incomp.	neg.
plus	"	"	"	"	"	plus	plus	plus	plus
neg.	"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"	"
plus	"	"	"	"	"	"	"	"	"
"	plus	"	"	"	"	"	"	"	"
neg.	neg.	"	"	neg.	"	neg.	"	"	"
plus	"	"	"	plus	"	plus	"	"	"
neg.	"	"	"	neg.	"	neg.	"	"	"
"	"	"	"	plus	"	plus	neg.	neg.	plus
"	"	"	"	neg.	"	incomp.	"	incomp.	incomp.
"	"	"	"	"	"	"	incomp.	"	"
"	"	"	"	quest.	"	plus	incomp.	plus	"
"	"	"	"	neg.	"	neg.	plus	"	plus
"	"	"	"	plus	"	slow	"	incomp.	incomp.
"	plus	"	"	"	"	plus	"	plus	plus
"	neg.	"	"	quest.	"	"	incomp.	incomp.	incomp.
"	"	"	"	plus	"	neg.	neg.	plus	neg.
"	"	"	"	"	"	plus	plus	"	plus
"	"	"	"	quest.	"	"	incomp.	incomp.	incomp.
uest.	"	"	"	plus	"	"	plus	plus	plus
neg.	"	"	"	"	"	"	incomp.	incomp.	"
uest.	"	"	"	incomp.	"	"	plus	"	incomp.
neg.	"	"	"	neg.	"	neg.	"	plus	plus
"	"	"	"	"	"		neg.		
"	"	"	"	plus	"	plus	plus	"	"
"	"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"	"
uest.	"	"	"	"	"	"	"	"	"
neg.	"	"	"	neg.	"	"	"	neg.	neg.
"	"	"	"	"	"	neg.	"	"	plus
"	"	"	"	plus	"	plus	"	plus	"
"	"	"	"	neg.	"		"		
plus	"	"	"	plus	"		"		
"	"	"	"	"	"		"		
neg.	"	"	"	"	"		"		
"	"	"	"	"	"		"		
"	"	"	"	"	"		neg.		
uest.	"	"	"	"	"		plus		
neg.	"	"	"	quest.	"		incomp.		
"	"	"	"	plus	"		"		
"	"	"	"	neg.	"	quest.	"	incomp.	incomp.
"	"	quest.	"	plus	"	plus	plus	plus	plus

(continued on following page)

TABLE VII
(continued)

85	plus	plus			neg.		neg.	neg.	ne
86	"	"			plus		plus	plus	
F87	"	quest.			neg.		quest.	neg.	
87	"	plus			plus		plus	plus	
F88	"	neg.			neg.		neg.	neg.	
88	"	plus			plus		plus	"	
89	"	"			"		neg.	"	
90	"	"			neg.		plus	"	pl
91	"	"			plus		"	quest.	
92	"	"			"		neg.	neg.	ne
93	"	"			neg.		quest.	"	
94	"	"			plus		plus	"	pl
95	"	"			"		"	plus	
F96	"	"			neg.		neg.	neg.	ne
97	"	"			plus		plus	quest.	pl
98	"	"			" slow		" slow	slow	ne
F102	"	neg.			neg.		neg.	neg.	
105	"	plus			plus		plus		
106	"	"			"		"	"	
108	"	"			"		"	plus	pl
109	"	"			"		"	"	
111	"	quest.			neg.		neg.	neg.	que
112	"	plus			plus		plus	quest.	
113	"	incomp.			neg.		neg.	incomp.	pl
F114	"	neg.			"		"	neg.	ne
115	"	plus			plus		plus	"	
116	"	incomp.			"		neg.	"	
117	"	plus			incomp.		plus	"	que
118	"	"			"		"	"	
119	"	"			neg.		quest.	"	pl
120	"	"			plus		plus	plus	
121	"	"			"		"	quest.	
122	"	"			"		"	plus	
123	"	"			"		"	"	
124	"	"			"		"	quest.	
125	"	"			"		"	neg.	

Cultures									
plus	83.3%	78.2%	68.0%	50.0%	53.8%	36.0%	56.2%	27.7%	26.
neg.	1.7%	17.6%	28.0%	40.0%	44.5%	48.0%	39.5%	63.0%	70.
quest.	0.0%	4.2%	4.0%	10.0%	1.7%	16.0%	4.3%	9.3%	3.

* F cultures were used to show the ease of hydrolysis of tri-propionin; th
** Questionable hydrolysis.
*** Incomplete hydrolysis.

TABLE VII
(continued)

neg.	neg.	neg.	neg.	plus	neg.	plus	plus	plus	plus	
plus	"	"	"	"	"	"	"	"	"	
neg.	"	"	"	"	"	neg.	incomp.	incomp.	neg.	
plus	"	quest.	"	"	"	plus	plus	plus	plus	
neg.	"	"	"	neg.	"	neg.	"	"	"	
"	"	neg.	"	plus	"	plus	"	"	"	
"	"	"	"	"	"	"	"	"	"	
"	plus	"	"	"	"	"	"	"	"	
quest.	"	plus	incomp.	"	"	"	"	"	"	
neg.	neg.	neg.	neg.	"	"	"	"	"	"	
"	"	"	"	"	"	incomp.	"	incomp.	incomp.	
"	plus	plus	"	"	"	plus	"	plus	plus	
plus	"	neg.	"	"	"	"	"	"	"	
neg.	neg.	"	"	"	"		"			
quest.	plus	plus	"	"	"		"			
slow	neg.	neg.	"	incomp.	"		incomp.			
neg.	"	"	"	neg.	"		plus			
"	"	"	"	plus	"		"			
"	"	quest.	"	"	"		"			
plus	plus	plus	"	"	"		"			
"	"	"	"	"	"	plus	"	plus	plus	
neg.	quest.	neg.	"	neg.	"	"	incomp.	incomp.	neg.	
quest.	"	"	"	plus	"	"	plus	plus	plus	
incomp.	plus	plus	"	"	"	"	incomp.	incomp.	"	
neg.	neg.	neg.	"	neg.	"	"	plus	neg.	neg.	
"	"	"	"	plus	"	"	"	plus	plus	
"	"	"	"	"	"	"	incomp.	incomp.	"	
"	quest.	"	"	"	"	"	plus	plus	"	
"	"	"	"	"	"	"	"	"	"	
"	plus	plus	"	"	"	"	"	"	"	
plus	"	neg.	"	"	"	"	"	"	"	
quest.	"	"	"	"	"	"	"	"	"	
plus	"	plus	"	"	"	"	"	"	"	
"	"	"	incomp.	"	"	"	"	"	"	
quest.	"	neg.	neg.	"	"	"	"	"	"	
neg.	"	incomp.	"	"	"	"	"	"	"	
27.7%	26.5%	9.2%	1.7%	74.8%	0.0%	82.3%	94.1%	95.1%	94.1%	
63.0%	70.0%	87.6%	98.3%	19.3%	100.0%	16.6%				
9.3%	3.5%	3.2%	0.0%	5.9%	0.0%	1.1%				

propionin; they are not included in the regular series.

were about as easily hydrolyzed as simple tri-glycerides of the lower fatty acid which normally occur in butter fat.

4. Tri-butyrin, tri-olein and butter fat were hydrolyzed by 78.2 percent, 74.8 percent and 82.3 percent, respectively, of the cultures studied.

5. Only 8 cultures, 4 of which were Pseudomonas acidicon-coquens, hydrolyzed tri-butyrin and tri-olein without hydrolyzing some of the intermediate tri-glycerides.

6. Three of the 6 cultures which definitely hydrolyzed tri-myristin were identified as Achromobacter lipolyticum; 2 others were identified as Pseudomonas fragi while the other was identified as Bacterium viscosum.

7. A large percentage of the cultures were not consistent in their hydrolysis of the intermediate tri-glycerides.

8. There was a 94.1 percent agreement between the hydrolysis of tri-butyrin and tri-olein; some cultures which hydrolyzed tri-butyrin did not hydrolyze tri-olein, and the opposite was also true.

9. There was a 95.1 percent agreement between the hydrolysis of tri-butyrin and butter fat and a 94.1 percent agreement between the hydrolysis of tri-olein and butter fat. Butter fat was hydrolyzed by more cultures than either tri-butyrin or tri-olein.

From the above summary it may be concluded that (a) tri-propionin is too easily hydrolyzed, in comparison with tri-

butyrin, tri-olein or butter fat, to be used for the detection of definitely lipolytic bacteria, (b) tri-butyrin, tri-olein or butter fat are excellent fats to be used in the separation of lipolytic and non-lipolytic cultures and (c) the hydrolysis, by bacteria, of the members of a series of tri-glycerides comprising tri-butyrin, tri-caprylin, tri-laurin, tri-myristin and tri-olein would be a very useful criterion for the identification of organisms since it would provide three distinct groups as follows:

Group 1. Those organisms which can hydrolyze only tri-butyrin and tri-olein.

Group 2. Those bacteria which can hydrolyze tri-butyrin, tri-olein and also the slightly soluble tri-glycerides including tri- caprylin.

Group 3. Those bacteria which can hydrolyze all the members of the series.

The Hydrolysis of Some Natural and Hydrogenated Fats by 92 Cultures of Bacteria Isolated from Various Sources

The hydrolysis of butter fat, lard, beef fat, corn oil, olive oil, linseed oil, cocoanut oil and cottonseed oil, as well as of the 2 hydrogenated fats, Crisco and Clix, was studied with 92 cultures, most of which were lipolytic. Table VIII

presents the data which represent reactions given in 2 trials with each culture.

The results may be summarized as follows:

1. Twelve of the cultures showed no lipolytic action on any of the fats.

2. Two of the cultures (24 and 58) hydrolyzed olive oil somewhat but did not affect the other fats.

3. Three of the cultures (38, 56 and F54) did not hydrolyze cocoanut oil but did hydrolyze the other fats.

4. The percentages of the cultures showing lipolysis ranged from 76.3 for cocoanut oil to 84.4 for lard.

5. Eighty and five-tenths of the cultures agreed in their actions upon all the fats used.

From the above summary it is evident that (a) the hydrogenation of the natural fats resulting in more stable fats with higher melting points did not appreciably influence their susceptibility to lipolysis, (b) olive oil which contains a relatively high percentage of unsaturated tri-glycerides showed a tendency to be more quickly hydrolyzed than the other fats, (c) cocoanut oil which contains a relatively high percentage of tri-laurin and tri-myristin and a relatively low percentage of tri-olein was somewhat more difficultly hydrolyzed than the other fats and (d) there were such inappreciable differences in the lipolysis of the natural fats that a differentiation of bacteria through their action on natural fats would seem infeasible.

TABLE VIII

The Hydrolysis of Some Natural and Hydrogenated Fats by 92 Culture of Bacteria Isolated from Various Sources

Hydrolysis determined by color change or disappearance of fat globules in Nile-blue sulfate medium

Culture*	Hydrolysis of fats								
	Natural								Hy
	Butter fat	Lard	Beef fat	Corn oil	Olive oil	Linseed oil	Cocoa-nut oil	Cotton-seed oil	Cri
6	plus	plus	plus	plus	plus	plus	incomp.	plus	p
7	slow	slow	slow	slow	slow	slow	slow	slow	
8	plus	plus	plus	plus	plus	plus	plus	plus	
9	"	"	"	"	"	"	"	"	inc
F10	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	n
M10	plus	plus	plus	plus	plus	plus	plus	plus	
12	"	"	"	"	"	"	"	"	p
13	"	"	"	"	"	"	"	"	
16	"	"	"	"	"	"	"	"	
17	"	"	"	"	"	"	"	"	
18	"	"	"	"	"	"	"	"	
F19	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	n
20	plus	plus	plus	plus	plus	plus	plus	plus	p
21	"	"	"	"	"	"	"	"	
F22	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	n
23	plus	plus	plus	plus	plus	plus	plus	plus	p
F24	neg.	neg.	neg.	neg.	incomp.	neg.	neg.	neg.	n
26	plus	plus	plus	plus	plus	plus	plus	plus	p
27	"	"	"	"	"	"	"	"	
28	"	"	"	"	"	"	"	"	
F29	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	n
F30	"	"	"	"	"	"	"	"	
31	plus	plus	plus	plus	plus	plus	plus	plus	p
F32	"	"	"	"	"	"	"	neg.	
32	"	"	"	"	"	"	"	plus	inc
33	slow	slow	slow	slow	slow	slow	slow	slow	s
34	plus	plus	plus	plus	incomp.	plus	plus	plus	p
35	"	"	"	"	plus	"	"	"	
36	"	"	"	"	"	"	"	"	
37	"	"	"	"	"	"	"	"	
38	"	"	"	"	"	"	neg.	"	
39	"	"	"	"	"	"	plus	"	
F40	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	n
41	plus	plus	plus	plus	plus	plus	plus	plus	p
42	"	"	"	"	"	"	"	"	
43	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	n

(continued on following

TABLE VIII

ydrolysis of Some Natural and Hydrogenated Fats by 92 Cultures of Bacteria Isolated from Various Sources

ydrolysis determined by color change or disappearance of fat globules in Nile-blue sulfate medium

Hydrolysis of fats								
Natural							Hydrogenated	
Lard	Beef fat	Corn oil	Olive oil	Linseed oil	Cocoa-nut oil	Cotton-seed oil	Crisco	Clix
plus	plus	plus	plus	plus	incomp.	plus	plus	plus
slow	slow	slow	slow	slow	slow	slow	"	"
plus	plus	plus	plus	plus	plus	plus	"	"
"	"	"	"	"	"	"	incomp.	"
neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
plus	plus	plus	plus	plus	plus	plus		
"	"	"	"	"	"	"	plus	plus
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
plus	plus	plus	plus	plus	plus	plus	plus	plus
"	"	"	"	"	"	"	"	"
neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
plus	plus	plus	plus	plus	plus	plus	plus	plus
neg.	neg.	neg.	incomp.	neg.	neg.	neg.	neg.	neg.
plus	plus	plus	plus	plus	plus	plus	plus	plus
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
"	"	"	"	"	"	"	"	"
plus	plus	plus	plus	plus	plus	plus	plus	plus
"	"	"	"	"	"	neg.		
"	"	"	"	"	"	plus	incomp.	"
slow	slow	slow	slow	slow	slow	slow	slow	"
plus	plus	plus	incomp.	plus	plus	plus	plus	"
"	"	"	plus	"	"	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	neg.	"	"	"
"	"	"	"	"	plus	"	"	"
neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
plus	plus	plus	plus	plus	plus	plus	plus	plus
neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.

(continued on following page)

TABLE VIII
(continued)

45	plus	plus	plus	plus	plus	plus	plus	plus	pl
46	slow	"	"	"	"	"	"	"	"
47	plus	"	"	"	"	"	"	"	"
48	"	"	"	"	"	"	"	"	"
49	"	"	"	"	"	"	"	"	"
50	"	"	"	"	"	"	"	"	"
F51	"	"	"	"	"	"	"	"	"
F52	neg.	incomp.	incomp.	neg.	neg.	neg.	neg.	neg.	ne
F53	"	neg.	neg.	"	"	"	"	"	"
53	incomp***	plus	plus	plus	plus	plus	plus	"	
F54	"	"	"	"	"	"	neg.	plus	
55	plus	"	"	"	"	"	plus	"	
F56	neg.	"	"	"	neg.	"	neg.	neg.	
56	slow	"	slow	"	incomp.	"	"	plus	
57	plus	"	plus	"	plus	slow	plus	"	
58	neg.	neg.	neg.	neg.	"	neg.	neg.	neg.	
63	"	"	"	"	neg.	"	"	quest.	
66	plus	plus	plus	plus	plus	plus	plus	plus	
68	"	"	"	"	"	"	"	slow	
69	"	"	"	"	"	"	"	plus	
70	"	"	"	"	"	"	"	"	
71	"	"	"	"	neg.	"	neg.	neg.	
72	neg.	neg.	neg.	neg.	"	neg.	"	"	
73	plus	plus	plus	plus	plus	plus	plus	plus	
83	quest.**		quest.	"	quest.	"	neg.	neg.	
83a	neg.		plus	"	neg.	"	quest.	plus	
84	plus		"	"	plus	"	plus	"	
85	"		"	"	"	"	"	"	
86	"		"	"	"	"	"	"	
F87	neg.		neg.	neg.	neg.	neg.	neg.	neg.	
87	plus		plus	plus	plus	plus	plus	plus	
F88	neg.		neg.	neg.	neg.	neg.	neg.	neg.	
88	plus		plus	plus	plus	plus	plus	plus	
89	"		"	"	"	"	incomp.	"	
90	"		"	"	"	"	"	"	
91	"		"	"	"	"	plus	"	pl
92	"		"	"	"	"	"	"	inco
93	incomp.	plus	incomp.	incomp.	"	slow	incomp.	"	pl
94	plus	"	plus	plus	"	plus	plus	"	"
95	"	"	"	"	"	"	"	"	"
109	"	"	"	"	"	"	"	"	"
111	"	"	"	"	"	"	"	incomp.	
112	"	"	"	"	"	"	"	plus	

(continued on following page)

TABLE VIII
(continued)

plus	plus	plus	plus	plus	plus	plus	plus	plus
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
comp.	incomp.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
neg.	neg.	"	"	"	"	"	"	"
plus	plus	plus	plus	plus	plus	"		
"	"	"	"	"	neg.	plus		
"	"	"	"	"	plus	"		
"	"	"	neg.	"	neg.	neg.		
"	slow	"	incomp.	"	"	plus		
"	plus	"	plus	slow	plus	"		
neg.	neg.	neg.	"	neg.	neg.	neg.		
"	"	"	neg.	"	"	quest.		
plus	plus	plus	plus	plus	plus	plus		
"	"	"	"	"	"	slow		
"	"	"	"	"	"	plus		
"	"	"	"	"	"	"		
"	"	"	neg.	"	neg.	neg.		
neg.	neg.	neg.	"	neg.	"	"		
plus	plus	plus	plus	plus	plus	plus		
	quest.	"	quest.	"	neg.	neg.		
	plus	"	neg.	"	quest.	plus		
	"	"	plus	"	plus	"		
	"	"	"	"	"	"		
	"	"	"	"	"	"		
	neg.	neg.	neg.	neg.	neg.	neg.		
	plus	plus	plus	plus	plus	plus		
	neg.	neg.	neg.	neg.	neg.	neg.		
	plus	plus	plus	plus	plus	plus		
	"	"	"	"	incomp.	"		
	"	"	"	"	"	"		
	"	"	"	"	plus	"	plus	plus
	"	"	"	"	"	"	incomp.	"
plus	incomp.	incomp.	"	slow	incomp.	"	plus	"
"	plus	plus	"	plus	plus	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"	"
"	"	"	"	"	"	incomp.		
"	"	"	"	"	"	plus		

(continued on following page)

TABLE VIII
(continued)

113	plus	incomp.	incomp.	incomp.	plus	plus	plus	incomp.		
F114	"	"	"	"	"	"	"	neg.	neg.	
115	"	plus	plus	plus	"	"	"	plus		
116	"	incomp.	neg.	"	"	"	"	incomp.		
117	"	plus	plus	"	"	"	"	plus		
118	"	"	"	"	"	"	"	"	plus	
119	"	"	"	"	"	"	"	"		
120	"	"	"	"	"	"	"	"		
121	"	"	"	"	"	"	"	"		
122	"	"	"	"	"	"	"	"		
123	"	"	"	"	"	"	"	"		
124	"	"	"	"	incomp.	"	"	"	"	
125	"	"	"	"	plus	neg.	"	"	"	
Cultures plus	80.4%	86.0%	83.7%	84.8%	82.6%	85.7%	77.2%	78.3%	79.2%	
Cultures neg.	18.5%	14.0%	15.4%	15.2%	16.3%	15.2%	21.7%	20.6%	20.8%	
Cultures quest.	1.1%	0.0%	1.1%	0.0%	1.1%	1.1%	1.1%	1.1%	0.0%	

* F cultures not in regular series.
** Questionable hydrolysis.
*** Incomplete hydrolysis.

TABLE VIII
(continued)

ıcomp.	incomp.	incomp.	plus	plus	plus	incomp.		
"	"	"	"	"	"	neg.	neg.	neg.
plus	plus	plus	"	"	"	plus		
ıcomp.	neg.	"	"	"	"	incomp.		
plus	plus	"	"	"	"	plus		
"	"	"	"	"	"	"	plus	plus
"	"	"	"	"	"	"		
"	"	"	"	"	"	"		
"	"	"	"	"	"	"		
"	"	"	"	"	"	"		
"	"	"	"	"	"	"		
"	"	"	"	"	"	"		
"	"	"	incomp.	"	"	"	"	"
"	"	"	plus	neg.	"	"	"	"
36.0%	83.7%	84.8%	82.6%	83.7%	77.2%	78.3%	79.2%	79.2%
14.0%	15.4%	15.2%	16.3%	15.2%	21.7%	20.6%	20.8%	20.8%
0.0%	1.1%	0.0%	1.1%	1.1%	1.1%	1.1%	0.0%	0.0%

t in regular series.
hydrolysis.
drolysis.

SUMMARY

1. Nile-blue sulfate was not specific in its staining reaction for either tri-olein or oleic acid. With this indicator the simple tri-glycerides of all the volatile fatty acids from butyric to capric, inclusive, were colored red or reddish pink, and caproic, caprylic and capric acids were colored blue.

2. Nile-blue sulfate and litmus were equally useful as indicators for the detection of the hydrolysis of some of the lower tri-glycerides.

3. The disappearance of fat globules and the color change of fat globules, in the presence of Nile-blue sulfate, were equally accurate tests for the detection of the hydrolysis by bacteria of the tri-glycerides of the lower fatty acids.

4. Reasonable variations in the pH of the medium did not appreciably affect the hydrolysis by bacteria of triolein or cottonseed oil; it was quite evident, however, that a rather alkaline reaction favored the lipolytic action of bacteria.

5. The method of dispersing the fats which would ordinarily be dispersed in Nile-blue sulfate agar, for the detection of fat hydrolysis by bacteria, had little influence

on the results.

6. Tri-butyrin, tri-olein and butter fat were generally hydrolyzed by the same culture of bacteria and were therefore equally useful in the Nile-blue sulfate test for the detection of lipolysis.

7. There seemed to be very little difference between the action of lipolytic bacteria upon butter fat and upon certain other natural fats and oils.

SECTION II

THE ISOLATION, IDENTIFICATION AND CLASSIFICATION OF LIPOLYTIC BACTERIA

GENERAL CONSIDERATIONS

The isolation, identification and classification of lipolytic bacteria were undertaken with the following considerations in mind:

1. The application of the Nile-blue sulfate technique (see Section I) to the isolation of lipolytic bacteria.

2. The application of the information contained in Section I, concerning the action of lipolytic bacteria on some simple tri-glycerides and some natural fats, to the identification of lipolytic bacteria.

3. The classification of the lipolytic bacteria which are of very great interest, commercially, because of their action on fats and because of their prevalence in dairy products.

PART I

THE ISOLATION AND GENERAL CHARACTERISTICS OF LIPOLYTIC BACTERIA

STATEMENT OF THE PROBLEM

The isolation of lipolytic bacteria was undertaken to determine the numbers and types of lipolytic bacteria in various materials and to study some of the general characteristics of these organisms so that the group can be readily identified.

REVIEW OF THE LITERATURE

Various investigators have reported the lipolytic action of certain microorganisms while some of these workers have also shown the relationship of microorganisms to the rancidity of butter.

In 1886, Escherich (25) studied the relative lipolytic ability of a number of bacteria and found the percentages of fat hydrolyzed by these organisms as follows: Bact. fluorescens non-liquefaciens, 8.0; Bact. fluorescens liquefaciens, 56.7; streptococcus, 24.0; Micrococcus ovalis, 31.0; Bact. aerogenes, 43.0; Bact. coli, 41.0 percent.

Sommaragua (70), 1894, by means of his method (see Section I) found that B. pyocyaneus and M. tetragenus hydrolyzed fat.

From a study of the hydrolysis of tallow under a solid medium, Eijkman (23) showed that some organisms produced a diffusible lipolytic enzyme (A-lipase) while others produced an enzyme (B-lipase) which diffuses less readily and is more sensitive to the pH of the medium. In the group of rapid hydrolyzers he placed Bact. pyocyaneus, Staph. pyogenes aureus, B. prodigiosus and B. fluorescens, while in the group of slow hydrolyzers he placed B. indicus and B. ruber.

Orla-Jensen (57), 1902, credited the following organisms with the ability to produce hydrolytic rancidity: Oidium lactis, Cladosporium butyri, B. fluorescens and B. prodigiosus.

According to Laxa (48) the splitting of fats occurred principally with oidia, penicillia and mucor moulds as well as with Bacillus fluorescens liquefaciens and to a lesser degree with some saccharomyces, Bacillus 2 and Bacillus 3. Barthel (7) found that Oidium lactis, Bacillus fluorescens liquefaciens and Bacterium prodigiosum can split butter fat to cause rancidity but are incapable of splitting glycerol and that Cladosporium butyri and Penicillium glaucum can split glycerol.

The lipase of Bacillus tuberculosis and other bacteria was investigated by Wells and Corper (77), 1912, who used olive oil, ethyl butyrate and triacetin for the detection of hydrolysis. They found that B. pyocyaneus and S. pyogenes aureus were the most hydrolytic while B. dysentariae and B. coli split the fats to a lesser extent. Each organism produced a corresponding effect on all the esters used which suggests the non-specificity of lipases.

Evans (27) in 1917 reported fat-splitting by Bact. abortus var. lipolyticus which is commonly found in freshly drawn milk. Further studies by this author (26) in 1918 showed that some strains of bacteria which seemed to be closely related to B. lipolyticus were able to decompose the fat in the cream layer of litmus milk.

In 1922, Seliber (67) investigated the fat-splitting ability of B. pyocyaneus and some moulds using olive oil. He showed that B. pyocyaneus was lipolytic especially in the presence of a good supply of peptone. According to Haag (33), 1928, Bact. pyocyaneum is the only bacterium that can attack palmitic and stearic acids, but even this organism cannot split the tri-glycerides of these acids. He showed that palmitic acid could be readily attacked by Mucor mucedo, P. glaucum, an Aspergillus and an Oidium from the air. Most of the cultures which he studied could hydrolyze oleic acid and tri-olein. B. fluorescens liquefaciens, Mycobacterium lacticola, O. lactis and other moulds showed good growth in these substances, while streptococci, micrococci, B. coli, B. subtilis and Torula grew rather poorly.

Hussong (42), in 1932, showed the relationship of an organism, which he identified as Ps. fragi, to rancidity in butter.

According to Berry (9), 1933, who used the copper sulfate soap method for the detection of microbial lipase, fat-splitting was observed with Staph. aureus, Staph. albus, Sarcina lutea, Ps. fluorescens and moulds, but it was not observed with B. subtilis, B. mycoides, and yeasts in general.

METHODS USED

Isolation

Most of the lipolytic bacteria were isolated without the use of enrichment methods by one of the following procedures:

Method I. Nile-blue sulfate agar (Section I) was used with the regular plating technique for the isolation of lipolytic bacteria from dairy products and other materials. In this method the inoculum was placed in the plates and the agar thoroughly mixed with it. The dilutions were sufficiently high to limit the number of colonies of lipolytic organisms to less than 50 per plate and thus to prevent the fusing of the hydrolyzed zones. Emulsions of any one of several natural fats or oils were used with equally good results. The plates were incubated at room temperature for 3 days unless the presence on the plates of rapidly diffusing lipase made it necessary to count them earlier.

Method 2. Proteolytic cultures were picked from milk powder agar* into litmus milk and were then incubated at room temperature for 10 days. The cultures were observed closely and at the end of the incubation period the various types were tested for lipolytic ability.

*Ayers and Mudge (5).

Method III. Some cultures, especially of fluorescent types, were secured from various workers in the laboratory; most of them had been isolated from beef infusion agar plates, and their sources were air, water or factory equipment.

Purification

All the cultures were purified by plating or by making a series of dilutions before they were studied further.

Establishment of the Lipolytic and Proteolytic Abilities of the Bacteria Isolated

Lipolysis. The amount and the type of lipolysis brought about by the purified cultures were determined by culturing the organisms on the surface of Nile-blue sulfate agar containing dispersions of butter fat. The procedure was carried out according to the method outlined in Section I.

The lipolytic action of the bacteria studied was described as follows: Lipolytic action on the fat under the colony growth was recorded as "complete" when all the globules had changed color or had disappeared, and was recorded as "incomplete" when all the fat globules had not changed color or disappeared. Production of a diffusible lipolytic lipase was recorded by the use of one or several positive signs; a negative sign was used to indicate the absence of a lipase.

Proteolytic action. The proteolytic ability of the bacteria studied was recorded from observations of litmus milk

cultures which were held at temperatures that previously had been found suitable. Proteolysis was considered negative, i.e., the cultures were considered non-proteolytic in litmus milk, when no digestion was evident in three weeks. Definite proteolysis was recorded by one positive sign when the litmus milk was completely or almost completely digested in 10 days, while it was recorded by two positive signs when the litmus milk was completely digested in 3 or 4 days. Chemical determinations to detect proteolysis not visible to the eye were not made.

Action of Lipolytic Bacteria in Butter

The effects of the lipolytic bacteria in butter were determined by inoculating small amounts of cream, with such quantities of young litmus milk cultures, that the finished butter would contain from 50 to 500,000 bacteria per ml. The butter was churned in quart jars from one pound quantities of medium rich, sterile cream; it was unsalted, packed in sterile, glass jars and stored at room temperature. The lengths of the holding periods depended on the development of definite defects in the butter. When no defect was detected, by means of the senses of taste and smell within three weeks, it was considered that the culture used was not injurious to butter, since the storage conditions were optimum for bacterial growth.

The detection of the inoculated organism as well as the

initial numbers and the development of these in the fresh and stored butter were determined by means of the regular plating technique, with either beef infusion agar or with the Nile-blue sulfate medium.

RESULTS OBTAINED

The Lipolytic Bacteria Studied - the Sources, the Lipolytic and Proteolytic Actions and the Defects Produced in Butter

The identification, the sources, the lipolytic and proteolytic actions and the defects produced in butter of 159 lipolytic organisms studied are presented in Table IX and summaries are given in Tables IXa and IXb. The type of lipolytic action and the defect produced in butter are considered general characteristics since they facilitate a rapid classification of lipolytic bacteria into general types.

The sources of the organisms may be summarized as follows:

1. Fifteen cultures, consisting chiefly of Ps. fluorescens or related types, were isolated from creamery air, tap water and various pieces of creamery equipment.

2. Fifty-five cultures, including several species, were isolated from raw cream or raw skimmilk aged at low temperatures, while three cultures were isolated from fresh raw milk.

3. Thirty-five cultures, including several species, were isolated from raw cream butter which had developed rancidity, cheesiness or other defects when stored for a short time at 6° C.

4. Thirty-four cultures, including several species, were isolated from raw cream or raw milk which developed rancidity when rather fresh. Some of the cows in the herd from which the milk was obtained were well advanced in lactation.

5. Six cultures, including 3 cultures of Ps. fragi, were isolated from butter which had been made in poorly washed churns and which had developed rancidity during a 7 month storage period at 0° C.

6. Two cultures were isolated from cream which had been pasteurized at 145° F. for 30 minutes.

7. Seven cultures were isolated from cream of unknown origin.

8. Ps. fragi types were commonly isolated while Ps. fluorescens types were seldom isolated from stored butter.

Table IXa summarizes the data on the relationship between the lipolytic and proteolytic actions of the bacteria studied. An analysis of the results presented leads to the following conclusions:

1. All the lipolytic bacteria were not evidently proteolytic but with the lipolytic bacteria that were proteolytic there was a fairly close agreement between the rates of lipolysis and proteolysis.

2. Many lipolytic bacteria rapidly hydrolyzed the fat under the colony growth but did not produce a rapidly diffusible

lipase while some organisms which did not completely hydrolyze the fat beneath the colony growth produced a rapidly diffusible lipase. These characteristics seemed to be constant for the various groups.

3. With the organisms not showing evident proteolysis, incomplete lipolysis was more common than complete lipolysis. This observation suggests that the ability of lipolytic bacteria to bring about proteolysis was more closely correlated with their direct action on the fat than to their indirect action through the production of rapidly diffusible lipolytic enzymes.

Table IXb gives a summary of the data on the relationship between the lipolytic and proteolytic actions of the lipolytic bacteria and their production of defects in butter.

An analysis of the results shows that:

1. Of the 80 cultures investigated from the standpoint of their action on butter, 58 (72.5 percent) produced definite rancidity, while almost half of this number produced very strong rancidity. The species of lipolytic organisms most effective in the production of rancidity were A. lipolyticum, Ps. fragi, Ps. fluorescens and Ps. mucidolens.

2. Twenty-five (31.3 percent) of the cultures produced strong rancidity in butter and also showed complete lipolysis, with a rapidly diffusible lipase, and rapid proteolysis.

3. There was an almost complete agreement between the lipolytic action and the production of rancidity with 47 (55.7 percent) of the cultures and between the proteolytic action and the production of rancidity with 33 (41.3 percent) of the cultures.

4. The organisms that rapidly hydrolyzed the fat under the bacterial growth and were actively proteolytic produced rancidity in butter very rapidly.

5. Of the 28 cultures (18.8 percent) which did not produce rancidity in butter 5 produced cheesiness, 4 produced a "May Apple" flavor and odor, 4 brought about slightly putrefactive conditions and the remainder produced undefined or no defects.

TABLE IX

The Lipolytic Bacteria Studied-the Sources, the Lipolytic and Pr

*Culture	Species or morphologic type	Description of sources of organisms		
		Dairy product or other source	Age of product	Storage temp. of product
1	gram neg. rod	raw cream	2 days	15° C.
2	" " "	" "	" "	"
3	" " "	" "	" "	"
4	" " "	" "	" "	6° C.
5	Ps. fluorescens	raw skim milk	fresh	
6	Ps. synxantha	creamery air		
7	Ps. schuylkilliensis	" "		
8	Ps. fluorescens	raw skim milk	6 days	"
9	Ps. fluorescens var. patula	" " "	" "	"
12	Ps. fluorescens	churn skim milk		
13	Ps. fluorescens	" " "		
14	Ps. myxogenes	" " "		
15	gram neg. rod	raw cream	15 days	0° C.
16	Ps. fluorescens	" "	12 "	6° C.
17	Ps. fluorescens	" "	20 "	0° C.
18	Ps. fluorescens	" "	21 "	"
20	Ps. fluorescens var. zymogenes	" "	20 "	"
21	Ps. fluorescens	" "	" "	"
23	Ps. fluorescens	" "	10 "	6° C.
26	Ps. fluorescens	" "	5 "	"
27	Ps. fluorescens var. zymogenes	" "	21 "	"
28	Ps. fluorescens var. patula	" "	21 "	"
31	gram neg. rod	" "	14 "	0° C.
32	Ps. synxantha	" "	" "	"
33	Ps. schuylkilliensis	tap water		
34	Ps. fluorescens var. patula	raw skim milk		
35	Ps. schuylkilliensis	tap water		
36	Ps. synxantha	air		
37	gram neg. rod	raw cream	15 "	15° C.
38	Ps. fluorescens var. radians	" "	10 "	0° C.
39	" " " "	" "	" "	"
41	Ps. mucidolens	" "	" "	15° C.
42	Ps. fluorescens	tap water		
43	gram neg. rod	" "		
45	Ps. fluorescens	raw cream butter		21° C.
46	gram neg. rod	" " "		"
47	Ps. fluorescens var. patula	" " "		6° C.
48	Ps. fluorescens	" " "		21° C.
49	" "	raw cream	6 days	0° C.
50	Ps. fluorescens var. patula	" "	20 "	15° C.
51	Ps. fluorescens var. radians	" "	15 "	"

TABLE IX

ytic and Proteolytic Actions and the Defects Produced in Butter

of organisms** of duct	Storage temp. of product	Under bacterial growth	A Lipolytic action by diffusible enzyme	B Proteolytic action in litmus milk	#Agreement between A and B	Defect produced in butter
days	15° C.	incomp.	-	+	-	
"	"	"	-	+	-	
"	"	"	-	+	-	
"	6° C.	"	-	+	-	
esh		complete	++	++	++	rancidity +
		"	-	++	++	slow rancidity
		incomp.	+	+	+	very slow rancidity
days	"	complete	+	++	+	rancidity +
"	"	incomp.	-	slow	+	slow rancidity
		complete	+	++	+	rancidity +
		"	+	++	+	rancidity +
		"	++	++	++	rancidity +
days	0° C.	incomp.	-	+	-	
"	6° C.	complete	+	++	+	slow rancidity
"	0° C.	"	+	++	+	rancidity +
"	"	"	+	++	+	cheesiness and rancidity
"	"	"	+++	+	+	rancidity
"	"	"	+++	++	++	cheesiness and rancidity
"	6° C.	"	++	++	++	" " "
"	"	"	+++	++	++	
"	"	"	+++	+	+	rancidity
"	"	incomp.	-	slow	+	"
"	0° C.	"	++	+	+	slow rancidity
"	"	"	-	+	-	
		"	+	+	+	very slow rancidity
		complete	+	+	+	
		"	+	+	+	very slow rancidity
		"	+	++	+	objectionable, not rancid
"	15° C.	"	+	slow	-	slow rancidity
"	0° C.	"	++	++	++	
"	"	"	++	++	++	
"	15° C.	"	++	++	++	rancidity ++
		"	++	++	++	" +
		incomp.	-	slow	+	
	21° C.	complete	++	++	++	rancidity +
	"	incomp.	-	+	-	no rancidity
	6° C.	"	++	slow	-	
	21° C.	complete	+	++	+	
days	0° C.	"	+	++	+	rancidity +
"	15° C.	incomp.	++	slow	-	
"	"	complete	+	"	+	

(continued on following page)

TABLE IX (c

*Culture	Species or morphologic type	Description of sources of organisms: Dairy product or other source	Age of product	Storage temp. of product
52	A. lipolyticum	raw cream	1 day	15° C.
53	A. connii	" "	8 days	6° C.
54	Ps. acidiconcoquens	" "	15 "	0° C.
55	gram neg. rod	" "	" "	"
56	Ps. acidiconcoquens	" "	8 "	6° C.
57	A. connii	" "	20 "	0° C.
58	micrococcus	" "	" "	"
59	Ps. acidiconcoquens	" "	15 "	"
60	A. connii	" "	10 "	"
61	Ps. acidiconcoquens	" "	9 "	6° C.
62	" "	" "	" "	0° C.
63	Ps. fragi	" "	10 "	"
64	gram neg. rod	" "	12 "	6° C.
65	" " "	" "	15 "	0° C.
66	Ps. acidiconcoquens	" "	10 "	6° C.
67	Ps. fluorescens var. patula	" "	5 "	"
68	A. connii	" "	fresh	
69	" "	" "	"	
70	Ps. fluorescens var. patula	" "	14 days	"
73	Ps. fluorescens	" "	" "	0° C.
74	reducing, fluorescent gram neg.rod	" "	20 "	"
75	Ps. fluorescens	" "	" "	"
76	" "	" "	" "	15° C.
77	gram neg. rod	" "		
78	" " "	" "	15 "	6° C.
79	reducing, fluorescent gram neg.rod	" "	" "	0° C.
80	Ps. fluorescens	" "	" "	"
81	gram neg. rod	" "	6 "	6° C.
82	Ps. fragi	" "	" "	0° C.
83	S. marcescens (probable)	agar		
84	A. connii	raw cream butter		6° C.
85	Ps. fluorescens var. patula			
86	Ps. fluorescens var. glycerolytica	raw cream		
87	" " " "	" "		
88	micrococcus	churn		
89	"	"		
90	A. lipolyticum	rancid butter		
91	" "	" "		
93	Ps. fragi	old cream		
94	A. lipolyticum	rancid butter		
95	Ps. fluorescens var.patula	air		
97	A. lipolyticum	"		
98	Ps. schuylkilliensis			

TABLE IX (continued)

of organisms of product	Storage temp. of product	**Lipolytic action (A): Under bacterial growth	A: by diffusible enzyme	Proteolytic action (B) in litmus milk	#Agreement between A and B	Defect produced in butter
day	15° C.	incomp.	-	+	-	cheesiness ++, rancidity +
days	6° C.	complete	-	-	-	
"	0° C.	"	-	++	+	slow rancidity, putrefaction
"	"	incomp.	-	+	-	
"	6° C.	"	-	++	-	very slight change
"	0° C.	complete	-	++	+	very slow change
"	"	very slow	-	+	-	slight cheesiness
"	"	complete	-	++	+	slow rancidity
"	"	"	-	-	+	off - not rancid
"	6° C.	"	-	++	+	slow rancidity
"	0° C.	"	-	++	+	" "
"	"	incomp.	-	very slight	+	"May Apple"
"	6° C.	"	-	very slow	+	very object'ble, not rancid
"	0° C.			" "		very slow rancidity
"	6° C.	complete	-	++	+	off - not rancid
"	"	incomp.	+	+	-	
ash		very slow	-	-	+	
"		" "	-	-	+	rancidity
days	"	incomp.	+	+	+	rancidity
"	0° C.	"	+	+	+	
"	"	"	+	+	+	no definite defect
"	"	complete	+	+	+	rancidity +
"	15° C.	"	+	+	+	rancidity +
		incomp.	+	slow	+	
"	6° C.	complete	+	+	+	
"	0° C.	"	+	+	+	no definite defect
"	"	"	+	+	+	
"	6° C.	"	-	slow	+	
"	0° C.	"	-	very slight	-	"May Apple", rancidity
		incomp.	-	+	-	
	6° C.	complete	+	-	-	
		"	++	slow	-	
		"	+	+	+	rancidity +
		"	+	+	+	
		"	+	-	-	
		incomp.	++	slow	+	
		complete	++	+	+	rancidity ++
		"	+	+	+	rancidity ++
		incomp.	++	slow	+	rancidity
		complete	+	+	+	rancidity ++
		incomp.	+	+	+	rancidity
		complete	+	+	+	rancidity ++
		slow	+	+	+	slow rancidity

(continued on following page)

TABLE IX (co

*Culture	Species or morphologic type	Description of sources of organisms: Dairy product or other source	Age of product	Storage temp. of product
105	gram neg. rod	raw cream	7 days	6° C.
108	A. lipolyticum	rancid butter	" "	variable
109	Ps. fragi	raw cream	" "	6° C.
111	" "	rancid butter	" mos.	0° C.
112	gram neg. rod	" "	" "	"
113	Ps. fragi	" "	" "	"
115	gram neg. rod	" "	" "	"
116	" " "	" "	" "	"
117	Ps. fragi	" "	" "	"
118	" "	" "	" days	"
119	Bact. viscosum	" "	" "	6° C.
120	Ps. fragi	" "	" "	"
121	gram neg. rod	rancid dirty butter	" "	"
122	" " "	rancid butter	" "	"
123	A. lipolyticum	cheesy ranc. butter	" "	"
124	Ps. fragi	cheesy butter	" "	"
125	" "	" "	" "	"
126	Ps. fluorescens var. radians	market milk	fresh	
127	Ps. fragi	rancid cream	3 days	10° C.
128	A. connii	raw cream	" "	"
129	" "	" "	" "	"
130	Ps. fluorescens var. glycerolytica	" "	" "	"
131	Ps. fragi	" "	" "	"
133	A. lipolyticum	cheesy butter	7 "	6° C.
134	Ps. fragi	"May Apple" butter	" "	"
135	" "	cheesy ranc. butter	" "	"
136	gram neg. rod	cheesy butter	" "	"
137	A. connii	cheesy ranc. butter	" "	"
138	gram neg. rod	rancid milk	4 "	5° C.
139	micrococcus	" "	" "	"
141	"	putrefactive butter	30 "	various
142	"	" "	" "	"
143	"	" "	" "	"
145	"	sour cream butter	2 wks.	
146	"	sweet cream butter	" "	
147	"	" " "	10 days	
148	"	sour cream butter	" "	
149	"	" " "	" "	
150	"	rancid butter		10° C.
152	Ps. fluorescens var. zymogenes	rancid milk	3 "	
153	gram neg. rod	" "	2 "	6° C.
154	Ps. fragi	" "	2 "	"
156	" "	" butter	7 "	"

ABLE IX (continued)

organisms	Storage temp. of product	**Lipolytic action by bacterial growth (A)	Lipolytic action by diffusible enzyme (A)	Proteolytic action in litmus milk (B)	#Agreement between A and B	Defect produced in butter
ys	6° C.	incomp.	++	slow	+	
	ariable	complete	+	+	+	rancidity ++
	6° C.	incomp.	-	-	+	
s.	0° C.	"	-	very slight	+	no definite defect
	"	complete	+	+	+	
	"	incomp.	+	very slight	+	rancidity
	"	"	+	slow	+	putrefaction
	"	"	-	-	+	
	"	"	+	slow	+	
ys	"	"	-	very slight	-	
	6° C.	complete	+	-	-	rancidity
	"	incomp.	++	slow	+	
	"	"	-	slow	+	
	"	complete	+	very slight	-	
	"	"	+	+	+	rancidity ++
	"	incomp.	+	very slight	-	rancidity
	"	"	+	" "	-	rancidity +
h		complete	+	+	+	rancidity
ys	10° C.	"	+	-	-	"May Apple", rancidity
	"	"	+	-	-	
	"	"	+	-	-	
	"	incomp.	+	+	+	rancidity
	"	"	+	very slight	-	"May Apple", rancidity
	6° C.	complete	++	+	+	rancidity ++
	"	"	++	-	-	
	"	"	++	-	-	rancidity ++
	"	"	++	-	-	
	"	"	-	-	-	
	5° C.	"	+	-	-	
	"	"	+	-	-	
	various	incomp.	-	-	+	
	"	"	-	-	+	
	"	"	-	-	+	
s.		complete	-	-	-	no definite defect
		"	-	-	-	
ys		"	+	-	-	
		incomp.	-	-	+	
		"	-	-	+	
	10° C.	"	-	-	+	" " "
		complete	+	++	+	moldy flavor
	6° C.	"	+	-	-	
	"	"	+	-	-	
	"	"	++	-	-	

(continued on following page)

TABLE IX

*Culture	Species or morphologic type	Description of sources of organis		
		Dairy product or other source	Age of product	Stora temp. produ
157	Ps. fluorescens var. glycerolytica	rancid butter	7 days	6°
158	" " " "	" "	" "	"
159	" fragi	" "	" "	"
160	closely related to A. connii	contest butter	" "	"
161	Ps. fragi	rancid milk	2 "	"
162	gram neg. rod	" butter	" "	"
163	Ps. fluorescens	" "	7 "	"
164	A. connii	market milk	fresh	
165	gram neg. rod	" "	"	
166	Ps. fluorescens var. glycerolytica	raw cream	"	
167	" " " "	" "	"	
168	" " " "	" "	"	
169	Bact. viscosum	" "	"	
170	Ps. fluorescens var. glycerolytica	" "	"	
171	closely related to A. connii	past. cream (145°F.)	"	
172	Ps. fluorescens var. zymogenes	rancid cream	2 days	6°
173	gram neg. rod	" "	" "	"
174	closely related to A. connii	" "	fresh	"
175	Ps. fluorescens var. zymogenes	" "	2 days	"
176	" " " "	" "	" "	"
177	Ps. fluorescens var. glycerolytica	" "	1 day	"
178	A. lipolyticum	" "	7 days	"
179	" "	" "	" "	"
180	closely related to A. connii	" "	1 day	"
181	" " " "	" "	" "	"
182	A. lipolyticum	" "	" "	"
183	closely related to Ps. acidiconco-quens	" "	2 days	"
184	closely related to A. connii	" "	1 day	"
185	Ps. fluorescens var. zymogenes	" "	" "	"
186	closely related to A. connii	control butter	1 week	"
187	Bact. viscosum	sweet cream	fresh	"
188	gram neg. rod	pasteurized cream	7 days	"

* Because of inadequate information some cultures were not included in the co culture numbers and the number of cultures (159).

**Nile-blue sulfate medium.

Positive or negative agreement is decided by a fairly close correlation bet

TABLE IX (continued)

f organisms of uct	Storage temp. of product	A **Lipolytic action under bacterial growth	A by diffusible enzyme	B Proteolytic action in litmus milk	#Agreement between A and B	Defect produced in butter
ays	6° C.	incomp.	+	++	+	rancidity ++
"	"	"	+	++	+	
"	"	complete	+	-	-	
"	"	"	+	-	-	
"	"	"	+	-	-	
"	"	"	+	-	-	
"	"	"	+	-	-	putrefaction
sh		"	+	-	-	
		"	+	-	-	off - not rancid
		"	++	++	++	
		"	++	++	++	
		"	+++	++	++	
		"	+	-	-	" " "
		"	+	++	+	
		"	+	-	-	
ays	6° C.	"	+	++	+	
"	"	"	+	-	-	
sh	"	"	-	-	-	
ays	"	"	-	++	+	
"	"	"	+	++	+	
ay	"	"	+	++	+	
ays	"	"	+	+	+	rancidity ++
"	"	"	+	+	+	
ay	"	"	+	-	-	not rancid
"	"	"	+	-	-	
"	"	"	+	+	+	
ays	"	"	slight	++	+	slight putrefaction
ay	"	"	+	-	-	
"	"	"	+	++	+	
eek	"	"	+	-	-	
sh	"	"	+	-	-	slow rancidity
ays	"	incomp.	-	+	-	no definite defect

in the compilation of this table; this accounts for the discrepancy between the

ation between the lipolytic and proteolytic actions recorded.

TABLE IXa

The Relationship Between the Lipolytic and Proteolytic Actions of 159 Lipolytic Bacteria

	Lipolytic action by bacterial growth	Lipolytic action by diffusible enzyme	Proteolytic action	Cultures number	Cultures percent
1. Cultures showing	complete	+,++ or +++		85	59.7
2. " "			+ or ++	85	59.7
3. Agreement between 1 and 2				52	32.7
4. Cultures showing	complete	++ or +++		22	13.8
5. " "			++	40	25.1
6. Agreement between 4 and 5				14	8.8
7. Cultures showing	incomplete	+ or ++		20	12.5
8. " "	complete	negative		16	10.0
9. " "	incomplete	"		26	16.3
10. " "			negative or very slight	55	34.5
11. Agreement between 8 and 10				8	5.0
12. Agreement between 9 and 10				14	8.8
13. Cultures described as			non-proteol.	46	28.9
14. Cultures showing practically complete agreement in their lipolytic and proteolytic actions				98	61.6
15. Cultures showing practically incomplete agreement in their lipolytic and proteolytic actions				61	38.3

TABLE IXb

The Relationship Between the Lipolytic and Proteolytic Actions and the Production of Defects in Butter by 80 Lipolytic Bacteria

Relationships summarized	Cultures: number	Cultures: percent
1. Cultures producing complete lipolysis, a diffusible enzyme and strong rancidity in butter	22	27.5
2. Cultures producing rapid proteolysis and strong rancidity in butter	23	28.7
3. Cultures producing complete lipolysis, a diffusible enzyme, rapid proteolysis and strong rancidity in butter (agreement between 1 and 2)	21	26.2
4. Cultures producing definite rancidity in butter	58	72.5
5. Cultures producing rancidity + or ++ in butter	25	31.3
6. Cultures producing lipolysis in Nile-blue sulfate and rancidity in butter to about the same extent	47	55.7
7. Cultures producing proteolysis in litmus milk and rancidity in butter to about the same extent	33	41.3
8. Cultures producing cheesiness in butter	5	6.2
9. Cultures producing "May Apple" flavor in butter	4	5.0
10. Cultures producing slight putrefaction in butter	4	5.0
11. Cultures producing undefined or no defects in butter	15	18.8

SUMMARY

1. A variety of lipolytic bacteria were isolated from various sources about a dairy plant and from dairy products.

2. Pseudomonas types, in general, with the exception of Ps. fragi, were most commonly isolated from sources other than dairy products.

3. A. lipolyticum, Ps. mucidolens, Ps. fragi and Ps. fluorescens were most effective in the production of rancidity in butter; these species, excepting Ps. mucidolens, were very commonly isolated from certain dairy products, in stored butter the Ps. fragi being especially common and Ps. fluorescens rather uncommon.

4. All lipolytic bacteria were not evidently proteolytic, but with the lipolytic organisms that were proteolytic there seemed to be a fairly close relationship between the rates of lipolysis and proteolysis.

5. It appears that the ability of lipolytic bacteria to bring about rancidity in butter was more dependent on the direct action of the organism on the fat than on the indirect action upon the fat of a rapidly diffusing lipase.

6. Approximately 75 percent of the lipolytic organisms inoculated into sterile cream produced rancidity in butter made from it, this being by far the most common defect produced in

butter by lipolytic organisms.

7. With the exception of Ps. fragi, the organisms which were most effective in the production of rancidity in butter rapidly hydrolyzed the fat beneath the bacterial growth in Nile-blue sulfate medium and also were actively proteolytic.

PART II

THE IDENTIFICATION AND CLASSIFICATION OF LIPOLYTIC BACTERIA

STATEMENT OF THE PROBLEM

The work herein reported was undertaken in an attempt to identify and classify the organisms isolated.

The lipolytic bacteria have been identified insofar as possible according to the information available in other sources of classification. Occasionally, however, previous descriptions have been insufficient to warrant their uses, and in such instances these descriptions have been expanded from information secured in this investigation. Several organisms have been identified according to these supplemented descriptions since it seems unethical to create new species when the descriptions available, although inadequate, probably refer to the same organisms. Previous descriptions have not been supplemented and new species have not been described with information obtained on less than four cultures.

A large number of fluorescent organisms have been studied, and many of these differ enough to justify the separation of the organisms described on a varietal basis. For this reason, four organisms have been named as varieties of Ps. fluorescens, and a large number of cultures which show slight variations have been identified as Ps. fluorescens. These cultures probably could have been identified as other Pseudomonas species

if the former descriptions had been complete.

The key outlined for the classification of lipolytic bacteria includes some cultures which have been studied but which have not been identified in this investigation.

METHODS USED

Characters Considered in the Study of Lipolytic Bacteria

The lipolytic bacteria were studied on the basis of the following characters:

MORPHOLOGY:

Form and size, arrangement, staining reaction, motility, flagellation, spore formation and presence of capsules.

CULTURAL CHARACTERISTICS:

Beef infusion agar colony, beef infusion agar slant, growth on and liquefaction of gelatin, growth in bouillon, utilization of amino acids and of ammonia as the sole sources of nitrogen, growth on potato, reaction in litmus skimmilk and in whole milk.

BIOCHEMICAL FEATURES:

Fat hydrolysis, production of hydrogen sulfide from proteose-peptone, hydrolysis of starch, gas production, reduction of nitrates to nitrites or to free nitrogen, production of indol, production of ammonia, production of acetyl methyl carbinol from glucose, methyl red reaction and the fermentation of carbohydrates.

GROWTH CONDITIONS:

Oxygen relationship, range of temperature over which growth

was observed and the changes produced in butter.

Detailed Descriptions of the Characters Considered

MORPHOLOGY:

Size. The size was determined by means of a Filar micrometer eye piece and a stage micrometer.

Staining. The Gram stain was used throughout, on smears obtained from fresh agar slopes, 48 hour litmus milk cultures or 24 hour nutrient bouillon cultures.

Motility. The motility was determined with a hanging drop.

Flagellation. The flagellation was determined according to the method of Gray outlined in the "Manual of Methods for the Pure Culture Study of Bacteria" (69).

Spore formation. The spore forming ability of the organisms was determined by heating week old cultures of litmus milk or of nutrient bouillon to 80° C. for 10 minutes and also by staining with methylene blue or with ammonium oxalate violet.

Capsule formation. The formation of capsules was determined according to the method of Huntoon, outlined in the Manual (69).

CULTURAL CHARACTERISTICS:

Growth on beef infusion agar. The descriptions of the colonies were recorded from purification plates on which the colonies were thinly distributed; the observations were

recorded from plates which had been incubated for 3 days at room temperature. Agar slant growths were recorded from pre-dried slants in which there was no water of condensation. In the detection of fluorescence a special agar was also used, Levine and Anderson (50).

Gelatin growth and liquefaction. A 10 percent nutrient gelatin was used for the study of these characters; both descriptions were obtained from stabs made in tubes of gelatin which were a few days old.

Utilization of amino acids as the sole source of nitrogen. This was determined with Frankel's modification of Uschinsky's medium (69).

Utilization of ammonia as the sole source of nitrogen. This was determined with the medium described in the Manual (69).

Potato. The growth was determined on potato slants which were kept moist by means of some wet absorbent cotton in the bottoms of the tubes; the slants were submerged in water during sterilization, after which the water was decanted and the slants dried at 98° C. before they were inoculated.

BIOCHEMICAL FEATURES:

Fat hydrolysis. This was determined by the method outlined (see Section II, Part I).

Production of hydrogen sulfide. This was determined according to the method of Levine et al (51) by stabbing tubes of medium of the following composition:

Ferric citrate	0.5 g.
Proteose-peptone	20.0 g.
Di-potassium phosphate	1.0 g.
Agar agar	15.0 g.
Distilled water	1000.0 cc.

This medium was sterilized in tubes at 15 pounds pressure for 15 minutes. Definite tests for H_2S were indicated by a blacking of the medium along the line of inoculation and eventually, perhaps, of the whole medium.

Starch hydrolysis, production of nitrates and the production of indol. These criteria were determined according to the methods outlined in the Manual (69).

Production of ammonia. This was determined with the nitrate broth after the tests for nitrate reduction had been made, according to the method outlined by Buchanan (16). The tubes were heated and the fumes brought in contact with pieces of filter paper which had been moistened with Nessler's solution. Definite tests were indicated by the brown coloration of the paper.

Production of nitrogen. This was also determined with the nitrate broth. The production of gas from nitrate broth was best detected by using a small inverted tube within the larger one, although it was found that the reduction of the nitrates to the free nitrogen was always readily observed at the surface of the medium. Cultures which

showed a lot of gas, even in a short time, were usually negative for nitrites as the reaction had already gone past that point.

Production of acetyl methyl carbinol from glucose (Voges-Proskauer) and methyl red. These tests were carried out by growing cultures for four days at room temperature in a medium of the following composition:

Glucose	0.5 percent
Proteose-peptone (Difco)	0.5 percent
Dibasic potassium phosphate	0.5 percent

The Voges-Proskauer reaction was carried out according to the modification of O'Meara (56); the medium described above was not used by him, but he suggested that the creatine test worked equally well with different media. A very small pinch of creatine was added to 5 cc. of the medium, followed by 5 cc. of 40 percent NaOH. A definite red coloration above the zone of contact of the two solutions was accepted as a positive test. Tests were also carried out with the method of Werkman (78).

The methyl red test was run on the remainder of the original medium which had been divided into two lots. Five drops of methyl red solution were added to the medium, and a positive test was indicated by a distinct red color; a negative test was indicated by a yellow color. The

methyl red solution was prepared by dissolving 0.1 grams of methyl red in 300 cc. of 95 percent alcohol and diluting to 500 cc. with distilled water.

Fermentation of carbohydrates. This was determined by the addition of 0.5 percent of the sugar to regular nutrient bouillon in which the change of reaction was indicated by means of a 1.6 percent alcoholic solution of brom cresol purple, using 1 part of indicator to 1000 parts of the medium. The inoculated sugar solutions were incubated at room temperature and observed for two weeks.

GROWTH CONDITIONS:

Oxygen relationship. Conclusions regarding this criterion were drawn from close observations of the reactions of the organisms in various media.

The most favorable temperature for growth was determined by incubating litmus milk cultures at 6° C., 15° C., 22° C., 30° C. and 37° C.

The effect of the organisms in butter was determined as was previously described under Section II, Part I.

RESULTS OBTAINED

Identification of Lipolytic Bacteria

A. Cultures identified according to previous descriptions.

1. Pseudomonas fragi. Nineteen cultures were identified as Ps. fragi (Eichholz), Hussong (42), 1932.

2. Pseudomonas fluorescens. Nineteen cultures were identified, on the basis of the very inadequate descriptions available, as Ps. fluorescens (Flugge), Migula (53). A complete description of the characteristics of the cultures identified in this investigation as Ps. fluorescens is found under "Descriptions of lipolytic bacteria".

3. Achromobacter lipolyticum. Eleven cultures were identified as A. lipolyticum (Huss) (41), Bergey et al. A further description of this species is found under "Descriptions of lipolytic bacteria".

4. Achromobacter connii. Ten cultures were identified according to the brief descriptions of Chester (19) and Bergey et al. as A. connii (Chester), Bergey et al. Seven other cultures were considered to be closely related to this species. Additional information about the group is found under "Descriptions of lipolytic bacteria".

5. Pseudomonas schuylkilliensis. Two cultures have

been identified as Ps. schuylkilliensis (Wright), Chester (19). Two other cultures were considered closely related to these. None of the four cultures agree in every detail with the short descriptions available.

6. Pseudomonas synxantha. Three cultures were identified as Ps. synxantha (Ehrenberg), Hammer (35) according to the description previously published by Hammer (36).

7. Bacterium viscosum. Three cultures were identified as Bact. viscosum (Adametz) (1), Buchanan and Hammer (17), the species being called A. viscosum by Bergey et al. A further description of this species is found under "Descriptions of lipolytic bacteria".

8. Pseudomonas mucidolens. One culture was identified according to the description of Anderson (4) and later of Levine and Anderson (50) as Ps. mucidolens.

9. Pseudomonas myxogenes. One culture was identified as Ps. myxogenes, Fuhrman (29).

10. Serratia type. One culture was identified as a Serratia type, according to Bergey's Manual (8).

11. Thirty cultures of gram negative rods were studied and described but were not identified because there were not sufficient cultures of any one type to justify their identification.

B. Descriptions of lipolytic bacteria.

Complete descriptions of a new species, Pseudomonas acidiconcoquens, of the species Ps. fluorescens, of four new varieties of Ps. fluorescens; Ps. fluorescens var. zymogenes, Ps. fluorescens var. glycerolytica, Ps. fluorescens var. radians and Ps. fluorescens var. patula, and supplementary information to descriptions already available, and which have been used in this work, are presented here.

1. Pseudomonas acidiconcoquens (nov. sp.)

The description given here applies primarily to 4 of the 6 cultures isolated. Two of the cultures showed a few minor variations which are mentioned at the close of the description.

SOURCES:

All the cultures were isolated from raw cream held at temperatures above 15° C.

MORPHOLOGY:

Form and size. Small oval rods; cells from a 12 hour beef infusion agar culture or from a fresh litmus milk culture varied from .4 to 0.9 by 1.0 to 1.8 microns and averaged about 0.6 by 1.2 microns.

Arrangement. Cells were arranged singly and in pairs from agar or milk cultures.

Staining reaction. Gram negative; stained unevenly, usually

showed granules with Gram stain.

Motility. Very actively motile; flagellation was generally one polar but very occasionally two polar flagella were seen; may be considered monotrichous.

Spore formation. Spores were not detected.

Capsule formation. Capsules were not observed.

CULTURAL CHARACTERISTICS:

Agar colony. Colonies were evident on beef infusion agar plates at 21° C. in 24 to 48 hours. Three day old surface colonies were from 2 to 6 mm. in diameter, round in general outline, margin entire to irregular. The colonies were medium profuse, medium convex, opaque to white, smooth, glistening, not granular and not viscous. Old colonies remained smooth, glistening and butyrous.

Agar slope on both beef infusion and standard agars. Growth on the slopes was filiform to echinulate, medium profuse, convex, opaque white, smooth, glistening, butyrous consistency; margin was entire to serrate.

Gelatin stab. Growth and liquefaction were evident in 24 hours at 21° C. The liquefaction was fairly rapid, slightly infundibliform to saccate, liquefied portion was turbid; the first surface growth was white but the later growth was slightly yellowish white and settled to the bottom of the liquefied area.

Nutrient bouillon. Bouillon became turbid with a thin, filmy, easily broken pellicle; a definite white precipitate soon settled to the bottom of the tube.

Uschinsky's solution. There was scarcely any evidence of growth in this medium, although the organisms survived for a number of days.

Utilization of ammonia nitrogen. Acid was fairly quickly produced in the medium by a smooth white surface growth.

Potato slope. Growth on potato was medium profuse, convex, shiny, buff to citrous yellow in color.

Litmus milk. Litmus milk first became slightly acid followed soon by a reduction, soft coagulation and a fairly rapid digestion. The milk was completely digested in less than a week at room temperature and in 3 to 4 days at 30° C., with a pinkish yellow serum which turned to a pinkish red and finally to a yellow with age. Following complete digestion a flaky white residue was left in the bottom of the tube. The odor was pungent, objectionable and very characteristic.

Whole milk. Whole milk was less rapidly digested than skim-milk; the serum and coagulum became slightly pink in color. No color change was produced in the surface layer of fat.

BIOCHEMICAL FEATURES:

Fat hydrolysis. Butter fat and several other common fats and oils were slowly but completely hydrolyzed beneath the

bacterial growth. The diffusion of a lipolytic enzyme was not observed. The simple tri-glycerides of butyric and oleic acids were definitely hydrolyzed while those of caproic and caprylic acids were slightly or indefinitely hydrolyzed. The colony growth on the surface of Nile-blue sulfate beef infusion agar was distinctly raised to convex, white and glistening.

Production of H_2S from proteose-peptone. Hydrogen sulfide was produced definitely along the line of the stab in one to two weeks at 21° to 30° C.

Hydrolysis of starch. Starch was quickly hydrolyzed.

Gas production. Gas was never noticed in any liquid medium.

Nitrates. Nitrates were reduced to nitrites in 24 hours but were never reduced to free nitrogen.

Indol. Indol was formed; traces were present in 24 hours.

Ammonia. Was rapidly produced.

Acetyl methyl carbinol from glucose. Acetyl methyl carbinol was produced; i. e., cultures were V. P. positive.

Methyl red. Cultures were M. R. positive.

Fermentation of carbohydrates. Acid was rapidly produced from glucose, galactose, maltose, sucrose, levulose, inulin, salicin, mannitol, glycerol and was slowly produced from lactose. Acid was not formed from raffinose.

GROWTH CONDITIONS:

Oxygen relationship. Facultative; grew well aerobically.

Temperature range for growth. There was no growth at 6° C., good growth at 21° C. and rapid growth at 30° C.; there was a fairly rapid coagulation but a slower digestion at 37° C. than at 30° C. The most rapid growth occurred from 30° to 35° C.

One of the 6 cultures studied showed minor variations from the above description. This culture produced indol faster, produced acetyl methyl carbinol more slowly, hydrolyzed natural fats more slowly, produced acid more slowly in some of the sugar broths and, in general, grew less profusely on the surface of solid media. The organism was smaller, having an average size of 0.4 to 1.0 microns in length.

The sixth culture varied somewhat from the other cultures in its action on litmus milk, digesting more rapidly without the definite coagulation and the occurrence of a pink to red serum; the serum remained a brownish yellow from the start. This culture produced hydrogen sulfide from proteose-peptone in 2 days at room temperature.

All the cultures produced rancidity very slowly in butter.

2. Pseudomonas fluorescens (typical).

The following description of Ps. fluorescens (Flugge), Migula (53) applies primarily to 14 of the 19 cultures included as typical Ps. fluorescens. Five of the 19 cultures

showed variations which are mentioned at the close of this description. It also should be recognized that there are minor variations in the characteristics of the 14 cultures and that other authors might not completely agree on such an inclusive grouping.

Description

SOURCES:

All the cultures were isolated from air or water contamination or from dairy products held at low temperatures.

MORPHOLOGY:

Form and size. Small to medium sized oval to linear rods; the cells from 12 hour beef infusion cultures varied in length from 0.4 to 2 μ. and averaged about 1.0 to 1.4 μ.

Arrangement. Cells were arranged singly and in pairs, from cultures of agar or litmus milk.

Staining reaction. Gram negative; often stained unevenly. The regular, linear shaped rods generally stained more uniformly and more deeply than the thicker oval cells with Gram stain.

Motility. Actively motile; flagellation was generally one polar, but sometimes two, three or four polar flagella were present

Spores. Were never observed.

Capsules. Were not observed in any medium.

CULTURAL CHARACTERISTICS:

Agar colony. Colonies were quite evident on beef infusion agar plates at 21° C. in 24 to 36 hours and varied a great deal in size and appearance. Generally they were circular, raised to convex, smooth to granular, glistening; margin entire to fimbriate, the central portion of the colony often becoming yellowish when the periphery was thinner and appeared pale blue through the medium. The colony was butyrous and gave a decided yellowish green or bluish green tinge to beef infusion or special agar.

Agar slope. Growth on the beef infusion agar slope was medium profuse, filiform to echinulate, raised to convex; surface was generally smooth and glistening but occasionally granular. Growth was butyrous and in old cultures sometimes had a reddish tinge; the medium itself was always tinged yellowish or bluish green.

Standard agar colony and slope. The colony and slope growths were similar to those with beef infusion agar, but the fluorescence noted above was either very slight or absent.

(Note:) Rough and smooth colonies were often observed from these cultures on both beef infusion and standard agar.

Gelatin stab. Growth and liquefaction were evident in 24 hours at 21° C. Liquefaction was infundibuliform to stratiform, normally rather rapid. The liquefied area was turbid,

having a light whitish pellicle. The mass of growth which was somewhat yellow, accumulated at the solid-liquid interface. The liquefaction was distinctly aerobic.

Nutrient bouillon. Bouillon became turbid to opaque with a light filmy pellicle and a white precipitate.

Uschinsky's solution. The ability of the organism to utilize amino acids as the sole source of nitrogen was indicated by good growth in this medium; the solution became slightly fluorescent and turbid.

Utilization of ammonia nitrogen. Acid was slowly produced in this medium by a scanty, yellow, smooth growth on the surface at the point of inoculation; the medium was completely yellow in 10 days or less.

Potato slope. Growth in potato varied from a yellow brown, with a tinge of red, to a citrous yellow; it was smooth, glistening and medium profuse.

Litmus milk. The reactions in litmus milk varied considerably with the batch of milk. The most common reaction was one which involved: First a deepening of the blue color at the surface and as this change continued downwards in strata formation the surface was digested and became greenish yellow to bluish green. The digestion continued fairly rapidly from the top down, and if coagulation was ever evident it was difficult to observe. When digestion was complete the lower portion of the serum was more of a purplish

color while the upper layer of green became deeper. At this time there was a fairly heavy, yellowish green pellicle which adhered to the wall of the tube. A fairly heavy, yellowish white to pinkish white precipitate was also formed. As the cultures increased in age, the greenish serum usually became light yellowish green or practically yellow. The tubes produced a putrefactive odor.

Whole milk without litmus. The digestion was slower than in skimmilk; the fat layer often became slightly yellow.

BIOCHEMICAL FEATURES:

Fat hydrolysis. Butter fat and several other common fats and oils were rapidly and completely hydrolyzed beneath the bacterial growth. A diffusible lipolytic enzyme was produced in small amounts. The simple tri-glycerides of butyric, caproic, caprylic, capric and oleic acids were always hydrolyzed while tri-laurin was occasionally and tri-myristin never showed definite hydrolysis.

Production of H_2S. H_2S was not produced, but the brownish white, smooth, surface growth produced a faint browning of the medium from the top down.

Hydrolysis of starch. Starch was not hydrolyzed.

Nitrates. Nitrates were rapidly reduced to nitrites and very often to free nitrogen within 2 days.

Indol. Indol was not produced.

Ammonia production. Ammonia was produced from nitrate broth.

Acetyl methyl carbinol from glucose. Acetyl methyl carbinol was not produced from glucose, i. e., cultures were V. P. negative.

Methyl red. The cultures varied in their reactions to methyl red, ranging from positive to negative.

Fermentation of carbohydrates. Acid was quickly produced from glucose. Acid was not formed in galactose, maltose, lactose, sucrose, levulose, salicin, inulin, raffinose, mannitol and glycerol. Gas production was not observed in any sugar solution.

GROWTH CONDITIONS:

Oxygen relationship. Culture grew well aerobically.

Temperature range for growth. There was slow growth and digestion at 6° C. and fairly rapid growth at 15° C.; the most rapid growth occurred between 20 and 25° C.; a medium growth occurred at 30° C. and usually there was no growth at 37° C.

Effect in butter. Cultures rapidly produced a strong rancidity in butter when made from sterile cream inoculated with a specific lipolytic culture.

Five of the 19 cultures produced a rapidly diffusible lipolytic enzyme in Nile-blue sulfate medium. Three of the 5 cultures produced rancidity in butter rather slowly.

3. Varieties of Pseudomonas fluorescens.

Among the fluorescent cultures studied there were several groups that showed rather distinct variations from the typical Ps. fluorescens, but they were considered as closely related types and were therefore named as varieties of Ps. fluorescens; small variations have also been recognized within the varieties. The names and the descriptions of the characteristics on which the varietal designations were based are given here.

Group I (7 cultures) Ps. fluorescens var. zymogenes.

Cultures produced acid in galactose broth. They did not reduce nitrates. Two cultures produced large amounts of lipolytic enzyme which rapidly diffused through Nile-blue sulfate medium while 4 of them produced only small amounts of a diffusible lipolytic enzyme.

Group II (10 cultures) Ps. fluorescens var. glycerolytica.

Cultures produced acid in glycerol. They formed large, flat colonies which were only slightly fluorescent on beef infusion agar. They rapidly digested litmus milk with a purplish serum which often did not turn fluorescent but frequently became yellowish grey. If pellicles were produced they were very slight. Six cultures reduced nitrates; four cultures did not reduce nitrates.

Group III (4 cultures) Ps. fluorescens var. radians.

Cultures produced acid in glucose only. They formed flat, spreading, iridescent colonies which were surrounded by medium large luminous areas and which produced a smothering odor, characteristic of a sewage disposal plant. Three cultures reduced nitrates while 1 culture did not reduce nitrates.

Group IV (9 cultures) Ps. fluorescens var. patula.

Cultures produced very flat, rapidly spreading colonies which did not bring about complete hydrolysis of the fat in Nile-blue sulfate medium. They proteolyzed milk slowly.

4. Achromobacter lipolyticum (Huss), Bergey et al.

A careful study of 11 cultures in this group showed that 7 of them were very similar while 4 varied from the others in some characteristics. The following information is supplementary to the description given by Huss (41) and applies to 10 cultures (exceptions noted later).

CULTURAL CHARACTERISTICS:

Nutrient bouillon. Bouillon became turbid to opaque; pellicle was usually lacking but if present was light and membranous; a slight white precipitate was formed and the liquid was slightly viscous.

BIOCHEMICAL FEATURES:

Fat hydrolysis. The fat beneath the colony growth in Nile-blue sulfate medium was rapidly and completely hydrolyzed

and a slowly diffusible lipolytic enzyme was produced. The simple tri-glycerides from tri-propionin to tri-myristin, inclusive, were hydrolyzed.

Reduction of nitrates. Nitrates were not reduced to free nitrogen.

Production of indol. Indol, if produced, was present in very slight amounts.

Acetyl methyl carbinol from glucose. Cultures did not produce acetyl methyl carbinol from glucose, i. e., they were V. P. negative.

Methyl red. Reaction with methyl red was positive.

Uschinsky's solution. Cultures utilized amino acids as the sole source of nitrogen.

Utilization of ammonia nitrogen. Cultures rapidly acidified the medium, indicating rapid utilization of the ammonia nitrogen.

Ammonia production. Cultures produced ammonia from nitrate broth.

Production of H_2S. Proteose-peptone agar was turned brown but positive H_2S production was never observed.

Fermentation of carbohydrates. No acid or gas was ever produced from lactose or inulin by any of the cultures; acid and gas were produced from glucose, maltose, raffinose, salicin, mannitol, levulose and galactose; acid alone was produced from sucrose and glycerol.

Effect in butter. All the cultures rapidly produced a very strong rancidity in butter.

The following 4 cultures varied from the above description in the following details:

Three cultures produced a very slimy consistency in litmus milk and bouillons; one of these cultures showed long chains of organisms contained in a capsular sheath. Another of these organisms produced a very fishy odor in litmus milk or nutrient bouillon. The remaining culture produced no acid or gas from raffinose. Three of the 4 cultures formed a more purplish yellow serum in litmus milk than the others. One of these cultures slightly hydrolyzed tri-palmitin. The fourth culture, which was considered quite different from the other ten, formed a very rapidly spreading lobular colony on beef infusion agar.

CULTURAL CHARACTERISTICS:

Litmus milk. Litmus milk turned a dark blue from the top down, with the color soon fading at the bottom; eventually there was a yellowish white portion and a yellowish white precipitate at the bottom. Some cultures produced a slight pellicle while others did not.

BIOCHEMICAL FEATURES:

Fat hydrolysis. Cultures brought about a slow complete hydrolysis of the fat globules beneath the colony growth and produced very little diffusible lipolytic enzyme. They hydrolyzed the lower simple tri-glycerides including tri-

caproin or tri-caprylin.

Production of H_2S. Cultures did not produce H_2S from proteose-peptone medium.

Production of ammonia. They produced ammonia from nitrate broth.

Utilization of Uschinsky's solution. Cultures utilized amino acids as the sole source of nitrogen.

Acetyl methyl carbinol from glucose. Cultures did not produce acetyl methyl carbinol from glucose, i. e., they were V. P. negative.

Hydrolysis of starch. They did not hydrolyze starch.

Effect in butter. No cultures produced strong rancidity in butter although some of them produced slight rancidity. The remainder of the cultures produced in butter either no defects or defects which were not defined.

5. Bact. viscosum.

The three cultures which were identified as Bact. viscosum (Adametz) (1) Buchanan and Hammer (17), were found to completely hydrolyze the fat globules under the colony growths in Nile-blue sulfate medium and to produce small quantities of a diffusible lipolytic enzyme. They hydrolyzed the simple tri-glycerides, inconsistently, up to tri-laurin. The production of rancidity by these cultures was variable, one having produced pronounced rancidity and the other two having produced off flavors or suggestions of rancidity.

6. Achromobacter connii.

Seventeen cultures were studied in this group and 10 of these were identified as A. connii (Chester) (19), Bergey et al. The description applies to the 17 cultures studied. The differences which will be noted at the end were considered sufficient to keep 7 cultures out of this species.

The additions to the previous description are:

Five of the 10 cultures just described produced a strong, stale urine odor in litmus milk and also a light, white, granular pellicle. These organisms were less active in butter than the others.

Seven cultures were considered closely related to A. connii; these cultures varied in the following respects:

Morphologically they were longer slimmer gram negative rods and showed distinct variations in morphology. Litmus milk was changed much more slowly; an alkaline reaction usually was not shown, if ever, for at least 2 weeks. These cultures produced different off flavors in butter but were not important in the production of rancidity.

Classification of Lipolytic Bacteria

Proposed key for the classification of lipolytic bacteria - including a key for the Pseudomonas types studied.

A. Cells spherical.

A.A. Cells not spherical - cells rod shaped.

B. Cells motile.

C. Cells motile by peritrichous flagella.

11 cultures: Achromobacter lipolyticum.

D. Normally produce a marked greenish yellow or greenish blue fluorescence or pigment in beef infusion agar.

E. Cells motile by six or more polar flagella.

1 culture: Pseudomonas myxogenes.

E.E. Cells motile by less than six polar flagella.

F. Optimum temperature about 37° C.

G. Produce a chloroform soluble, bluish green pigment.

H. Produce H_2S from proteose-peptone medium.

1 culture: closely related to Ps. schuylkilliensis.

H.H. Do not produce H_2S from proteose-peptone medium.

2 cultures: Ps. schuylkilliensis.

G.G. Do not produce a chloroform soluble, bluish green pigment.

1 culture: closely related to Ps. schuylkilliensis.

F.F. Optimum temperature below 37° C.

I. Produce acid in glycerol broth in less than three days.

10 cultures: Ps. fluorescens var. glycerolytica.

II. Do not produce acid in glycerol broth in less than three days

J. Produce acid in galactose broth.

7 cultures: Ps. fluorescens var. zymogenes.

J.J. Do not produce acid in galactose broth.

K. Produce a deep, dandelion yellow color on the fat of whole milk and also a very putrefactive odor.

3 cultures: Ps. synxantha.

K.K. Do not produce a deep, dandelion yellow color on the fat of whole milk nor a putrefactive odor.

L. Proteolyze litmus skimmilk rapidly.

M. Produce a musty, old potato odor in common media.

1 culture: Ps. mucidolens.

M.M. Do not produce a musty, old potato odor in common media.

N. Produce a flat, iridescent, spreading colony on beef infusion or

standard agar which is surrounded by a clear zone in the medium.

4 cultures: Ps. fluorescens var. radians.

N.N. Do not produce a flat, iridescent, spreading colony on beef infusion or standard agar which is surrounded by a clear zone in the medium.

O. Litmus skimmilk serum becomes rapidly green to greenish yellow.

19 cultures: Ps. fluorescens.

O.O. Litmus skimmilk serum does not become rapidly green to greenish yellow - remains blue.

1 culture: closely related to Ps. fluorescens.

L.L. Do not proteolyze litmus skimmilk rapidly.

P. Produce a rapidly spreading colony with incomplete hydrolysis of the fat globules beneath the growth in Nile-blue sulfate medium.

9 cultures: Ps. fluorescens var. patula.

P.P. Do not produce a rapidly spreading colony with

incomplete hydrolysis of the fat beneath the growth in Nile-blue sulfate medium.

Q. Completely reduce litmus milk.

2 cultures (74 and 79): not identified.

Q.Q. Do not completely reduce litmus milk.

2 cultures (81 and 115): not identified.

D.D. Do not normally produce a marked greenish yellow or greenish blue fluorescence or pigment in beef infusion agar.

R. Produce red pigment on beef infusion and standard nutrient agars.

1 culture: Serratia type.

R.R. Do not produce a red pigment on beef infusion and standard agars.

S. Produce from litmus skimmilk or from growth on agar the sweet ester like odor of the "May Apple" blossom.

19 cultures: Ps. fragi.

S.S. Do not produce from litmus skimmilk or from growth on agar the sweet, ester like odor of the "May Apple" blossom.

6 cultures: Ps. acidiconcoquens (Nov. Sp.)

B.B. Cells not motile.

T. Produce a deep blue color and an alkaline reaction in litmus skimmilk with the subsequent yellowish whitening of the bottom part of the medium.

U. Produce a very ropy condition in litmus milk and bouillon. Capsules were observed.

3 cultures: Bact. viscosum.

U.U. Do not produce a ropy condition in

litmus milk and bouillon. Capsules were not observed.

V. Produce a strong odor of stale urine and form a light, white, granular pellicle.

5 cultures: A. connii (variants)

V.V. Do not produce a strong odor of stale urine and do not form a light, white, granular pellicle.

5 cultures: A. connii (typical)

T.T. Do not produce a deep blue color in litmus milk and produce an alkaline reaction very slowly if at all.

7 cultures: closely related to A. connii.

SECTION III

THE NUMBERS OF LIPOLYTIC BACTERIA IN CERTAIN DAIRY PRODUCTS AND THE RELATIONSHIP OF THESE ORGANISMS TO RANCIDITY

GENERAL CONSIDERATIONS AND STATEMENT OF THE PROBLEM

The numbers of lipolytic bacteria in milk, cream and butter, and the relationship of these types to the flavor defects of butter, were studied with special consideration to the following:

1. The relative numbers of lipolytic bacteria in fresh milk and cream, in fresh butter of widely varying qualities, and in these products after they had been subjected to varying storage conditions.

2. The growth of lipolytic bacteria in butter.

3. The influence of the air on the growth of lipolytic bacteria in butter as shown by the differences in the numbers of organisms found in surface and sub-surface portions.

4. The numbers of bacteria present in butter when rancidity was first detected.

5. The influence of the development of rancidity on the growth of bacteria in butter, particular interest being attached to the inhibitory effect on bacterial growth of the products formed.

REVIEW OF THE LITERATURE

In reviewing the literature dealing with the relationship of microorganisms to the development of rancidity in butter, it seems advisable to include some of the more important literature on the conceptions of rancidity. This material is presented first, in order to show the fairly close agreement among authors concerning the type of chemical change brought about by bacteria in fats, especially butter fat. The literature reported has therefore been divided into two parts.

Conceptions of Rancidity in Relation to the Types of Chemical Changes Involved

One of the first clearly defined explanations of rancidity in butter was given in 1882 by Hagemann (34), who said that rancidity is due to the presence in the butter of fatty acids, particularly butyric acid. He supposed that the lactic acid from lactose caused a splitting of the glycerides of butyric acid.

Schaedler (64), 1883, spoke of oxidative rancidity which is accelerated by a primary formation of free fatty acids, especially oleic acid, the latter acid subsequently being oxidized to cause rancidity. The idea of oxidative rancidity was also favored by Duclaux (22), 1887, who thought that the defect was characterized by an increase in the lower fatty acids as a

result of an oxidation of the higher ones.

Gröger (31) showed that the defect which he called rancidity was characterized by a lowering of the mean molecular weights of the fats concerned. This indicates the increase of the lower molecular weight fatty acids at the expense of the higher ones.

According to Fermi (28), 1890, there is an enzyme secreted by the protoplasm which splits the fat into glycerol and the free fatty acids, thus causing rancidity. Thumm (70) studied the development of rancidity in palm and olive oil and found that during the development of rancidity the liquid and solid fatty acids of these two oils are freed in exactly the same proportion as that in which they are found in the neutral fats.

Amthor (3) said that tallowiness follows rancidity and that rancid butter contains esters of the free volatile fatty acids, principally the ethyl ester of butyric acid. The glyceride of butyric acid being the least stable, it is the most quickly affected.

Schmidt (65) did not define the defect rancidity but considered that it developed most rapidly in the sunlight and quite rapidly in the incubator. This would indicate that he was talking of oxidative rancidity. On the other hand, he concluded that low temperatures, pasteurization and heavy salting

retarded rancidity, and these precautions, in the light of present knowledge, would be taken to prevent hydrolytic rancidity.

Browne (13), in 1899, defined rancidity as "a defect resulting from any chemical or physical change in the character of a fat from the normal". He obtained all the fatty acids of the homologous series from formic to capric, as well as certain di-basic and oxy-acids, by the oxidation of oleic acid. He also expressed the opinion that there is a breaking down of all the tri-glycerides of butter during the development of rancidity because a sample of tri-palmitin which he had prepared from butter fat became rancid during a long storage period. Since oleic acid is the most easily oxidized, he felt that the changes which so soon affect the oleic acid would no doubt hasten the disintegration of the entire molecule.

Scala (63), 1897, showed that, during long holding periods, oleic acid markedly increased in weight while palmitic and stearic acids did not. From his observations he concluded that the increase in weight, which occurred with different fats during the development of rancidity, was in direct proportion to their content of oleic acid.

The conception of hydrolytic rancidity must have been held by Reinmann (59) who showed that sterile butter in the presence of light and air did not become rancid and that the complete

absence of oxygen did prevent rancidity. He later suggested that rancidity must be caused by enzymes. He concluded that there was no relationship between the amount of free acids and the rancid taste of butter, that a high content of casein and milk sugar accelerated the development of rancidity, and that light played no role in the development of this defect.

Orla-Jensen (57) favored the concept of hydrolytic rancidity since he stated that oxygen merely aids the development of the organisms which cause the hydrolysis of the fats. The fatty acids which are thus freed unite with the alcohol to form the butyric ester. Sayer and others (62) expressed a similar opinion, that oxygen does not play the direct role in the production of rancidity.

Lewkowitsch (52), 1913, attributed rancidity to the direct oxidation of the fatty acids by the oxygen of the air, assisted and intensified by the exposure to light; he concluded that rancidity was not due to the presence of free fatty acids alone. Rancidity is defined by Vintilesco and Popesco (75), 1915, as a defect which is characterized by a disagreeable odor and flavor resulting from an alteration of fatty matter causing a disagreeable odor and a disagreeable taste characteristic of rancid butter, the degree of acidity not being proportional to the intensity of rancidity.

The following discussion of rancidity was given by Barthel

(7), in 1910: "The normal spontaneous decomposition of butter is seen in every day life when butter turns rancid. This process consists in the continued splitting up of the glycerides of the butter into glycerine and free fatty acids. These free fatty acids, particularly butyric acid, impart the objectionable rancid taste and smell."

Kerr (44) expressed the opinion that rancidity is a chemical change in the fat, due to the action of oxygen. Its development is accelerated by light, heat, the presence of moisture and contact with certain metals; these conditions are helpful, but oxygen is absolutely essential. He also found that aldehydes, ketones and fatty acids of lower molecular weights than those found in the original butter are always present in rancid butter.

Emery and Henley (24), 1922, discussed oxidative rancidity and concluded that oxygen rather than air accelerated the development of rancidity. They were unable to produce this type of rancidity at summer temperatures when fats were kept in contact with metals and exposed to light in the absence of air.

An exhaustive study of the rancidity of fats was conducted by Powick (58) in 1923. This author stated that azelaic acid and most of the saturated acids and aldehydes from acetic to nonylic had been recovered and identified from certain specimens of rancid fats. He further concluded that rancidity is

due to the oxidation of the oleic radicle since neither stearic acid nor glycerol do not, and since oleic acid does develop a rancid taste upon exposure to air and light. This reasoning indicates that Powick favors the concept of oxidative rancidity, which view is confirmed by his conclusions that butyric acid and butyric aldehyde do not contribute to the rancid odor of butter fat. Powick's work confirmed the view of Scala (63) that heptylic aldehyde may be responsible for this "so called" rancidity.

In a paper on the analytical detection of rancidity, Kerr and Sorber (45) defined the term for their purpose as follows: "Rancidity will be used only in reference to changes in the fat itself and not at any time to alterations in any substance with which the fat is mingled or to hydrolysis". They attribute rancidity to spontaneous oxidation and indicate that rancid fats contain a variety of substances of lower molecular weight than the original compounds. Holm and Greenbank (38), in 1923, drew the following conclusions regarding oxidative changes of fats: "The various oxidations show that the oleic acid radicle as the main constituent concerned in the production of tallowiness or rancidity and confirm the previous conclusion that the oldfactory sense gives no true criterion of how highly oxidized a fat or oil may be".

Browne (14) analyzed butter that had been stored at room

temperature for four years and noted the following: The fats which showed the greatest loss in iodine absorption exhibited the greatest increase in free and volatile acids. The free insoluble acids of the decomposed fats contained oleic acid in nearly the same proportion as in the neutral fat. These findings confirm the previously mentioned opinions which point out that the lower molecular weight compounds formed during oxidation may be an indication of either hydrolytic or oxidative rancidity. Browne concluded that, in agreement with the view of spontaneous oxidation, the saturated glycerides, such as stearin or palmitin, should show a much higher stability in the pure condition than when exposed to air in contact with an unsaturated glyceride such as olein.

The relationship of the hydrolysis of the fat to the rancidity of butter is recognized by the authors of many leading textbooks.

Mojonnier and Troy (54), 1922, expressed the opinion that butter becomes rancid as a result of the splitting of the molecule, since the fatty acids when freed from the glyceride radicle have very characteristic and pungent odors and flavors.

Holm and Greenbank (38), 1928, recognized the importance of enzymes or of free acids, aided by water, in the hydrolysis of fats. They were of the opinion that the lower fatty acids, butyric and caproic, are produced by hydrolysis and that these acids are responsible for the rancid odors and flavors.

Hammer (35) expressed the opinion that rancidity is characterized by a flavor and an aroma suggestive of butyric acid. A similar conception of hydrolytic rancidity was expressed by Gortner (30), in 1929. This author recognized that the hydrolysis of glycerides with the liberation of free fatty acids constitutes the defect rancidity, as known by the dairy industry. The oxidation of fats and oils containing unsaturated acids, resulting in the formation of aldehydes, ketones and acids having lower molecular weights than the original acids, is known as "rancidity" by the oil and fat industry but is known as "tallowiness" in the dairy industry. He stated further that the strong odor characteristic of the lower fatty acids, especially of butyric acid, is readily produced in milk fat upon slight hydrolysis.

A classification of rancidity, which is much in line with that of Gortner, was used by Triebold (72), 1931, who was interested in the decomposition of the fat in flour. He divided rancidity into (a) oxidative, (b) hydrolytic and (c) ketonic. Triebold defined hydrolytic rancidity as the ordinary splitting of glycerides with the liberation of free fatty acids as end products. This type of deterioration is very important in dairy products due to the production of butyric acid. In reference to the oxidative process Triebold suggested that active oxygen and ozone produced during the oxidation of oleic acid might bring about the glyceride decomposition which could

not be effected by molecular oxygen.

Davies (21) in 1928 limited the primary cause of rancidity to auto-oxidation, which idea he considered was generally accepted at that time. A similar opinion has been expressed by Barnicoat (6), 1931. He considered that (a) there is no absolute correlation between the free fatty acid content and the rancidity in fats and (b) when fats or fat containing materials are stored in the presence of air they sooner or later become rancid. Hood (39) described a rancid odor in cheese as resembling most the odor of butyric acid.

Briggs (12) concluded that hydrolysis of the fat does not occur in the earlier stages of the breakdown of butter and that substances such as acrolein which are found in rancid fats result from the oxidation of some unsaturated molecules.

The effect of adding oleic acid, glycerol and white of egg upon the rate of hydrolysis of beef fat was studied by Bevis (10). He concluded that the most rapid increase in free fatty acid occurred in the presence of the greatest amount of albuminous material and that this hydrolysis did not affect the Kreis test for rancidity; the addition of oleic acid to the fat, however, hastened the development of a positive Kreis test.

Briefly summarizing the literature reviewed above on the conceptions of rancidity, it is evident that:

1. Some workers restrict the use of the word rancidity to a defect resulting from the hydrolysis of butter or other mixed

fat. These investigators associate a rancid condition with the presence of lower free fatty acids, especially butyric acid.

2. Others restrict the use of the word rancid to a defect which results from an oxidation of the unsaturated fatty acids.

3. A few authors wish to extend the meaning of the word rancid to include hydrolytic, oxidative and ketonic deterioration of fats.

Relationship of the Growth of Bacteria to the Development of Rancidity in Butter

In 1886 Escherich (25) studied the intestinal bacteria of infants and reported that the "colon" bacteria have the ability to split fats and produce free fatty acids and glycerol in the intestines. He thought that the saturated fatty acids were split by moulds chiefly but was doubtful whether or not organisms played a direct part in the production of rancidity. He also recognized the inhibitory effect of rancidity on the growth of organisms and concluded that pure, water-free fat and oil do not serve as nutrients for bacteria.

Klecki (46), 1894, was of the opinion that the acidity of butter is due chiefly to the action of bacteria and very little to direct oxidation and that the growth of these bacteria in butter is eventually retarded by the acid which they

produce. The latter idea was also supported by Duclaux (22) who said that butyric acid is toxic and antiseptic for microbes.

In 1891, Lafar (47) named two new species of bacteria, Bacterium butyri colloidium and Bacillus butyri fluorescens. He stated that these organisms, when inoculated into the cream, produced rancidity in the butter.

Rancidity was attributed by Amthor (3) to the esters of the fatty acids, particularly of butyric acid, which are formed by combination with the alcohol which the microorganisms produce from the milk sugar. Browne (13), in 1899, substantiated the opinion of previous authors that rancidity could be brought about in butter by microorganisms but he added that this was not possible in pure butter fat.

Reinmann (59) reported that it was not possible to make sterile cream butter rancid by pure cultures or by mixtures of cultures of bacteria by inoculating them into the butter. He was of the opinion that enough information was not available at that time to justify a conclusion concerning the cause of rancidity but thought that it was due to either fermentative (enzymatic) or to bacterial action.

Fat splitting by microorganisms was thoroughly studied by Schreiber (66), 1902, who drew the following conclusions:

1. Pure fat, by itself, is not a food for microorganisms.

2. A number of bacteria which occur normally in the body and also in nature have the ability to split fat in the presence

of food and oxygen.

3. The fat splitting ability of microorganisms is inappreciable under anaerobic conditions.

The ability of some moulds, such as Penicillium, to split higher tri-glycerides was attributed by Laxa (48) to their production of an enzyme which was also capable of splitting monobutyrin and butter fat. Orla-Jensen (57), in 1902, concluded that the fatty acids are first formed by bacteria and later by the associative action of moulds; from this associative action arises the acid which results in the butyric ester.

Rubner (61) concluded that fat splitting occurs in the presence of certain bacteria. These bacteria grow better when salts are present to bind the fatty acids. The latter statement indicates that Rubner probably recognized the inhibitory influence of the lower free fatty acids on bacteria. Barthel (7) was of the opinion that the splitting of glycerol could be accomplished by moulds but not by bacteria; he made the rather indefinite statement that esters could not be formed by bacteria.

The lipolytic ability of bacteria, in the presence of a suitable food supply, was recognized by Lewkowitsch (52), who admitted that whether these changes were due to the direct action of living organisms or to the action of an enzyme produced by them was extremely questionable.

Haag (33) showed that members of the aliphatic acid series, especially those of double bond combinations, are decomposed by microorganisms. He concluded that the ease of hydrolysis of a fat by bacteria was dependent on its chemical rather than on its physical properties. He also recognized that the higher fungi have greater lipolytic action on the higher saturated fatty acids than the bacteria while almost all the organisms with which he worked were able to attack oleic acid and triolein. The decomposition of oleic acid by microorganisms is brought about without the formation of intermediate products and therefore is entirely different from the chemical decomposition brought about by oxidation. Haag concluded that, of the three most important fatty acids - palmitic, stearic and oleic - only oleic is important as a source of carbon for microorganisms. Furthermore, he was of the opinion that microorganisms were not responsible for the formation of rancidity in fats and oils and in any event that an analysis of the decomposition products would not prove that the microorganisms were responsible for the changes.

Hammer (35), 1928, recognized that microorganisms play an important part in the production of rancidity through fat hydrolysis but that lipase and chemical action may also aid in this change. The influence of microorganisms upon the production of rancidity in butter was also mentioned by Buchanan (16),

in 1921. The relationship of microorganisms to rancidity in butter was shown by Collins (20), 1931, who concluded that although there was at first a rapid increase in the numbers of bacteria as rancidity developed there was later a progressive and a rapid development of rancidity concurrent with a rapid decrease in the numbers of bacteria. This would seem to indicate the importance of enzymatic or chemical action in the later stages of this defect.

Hood and White (40), 1931, attributed the objectionable rancid flavor of certain lots of cheese to the presence of butyric acid freed through the action of microorganisms. Lea (49) thought that the free acid content of butter could be taken as an index to the degree of hydrolysis by microorganisms.

The relationship of a lipolytic microorganism to the development of rancidity in butter was investigated by Hussong (42), in 1932. His results indicated that at 21° C. organisms increased rapidly in butter and brought about a rancid condition in about four days. The development of the rancid condition was accompanied by a rapid increase in the amount of total acid in the butter.

METHODS USED

Samples of dairy products. The samples of milk and cream examined were secured from the Iowa State College dairy industry plants and dairy farm, and from one other Iowa dairy farm. The samples of butter were secured either from experimental churnings made in the laboratory or from commercial plants in Iowa and a few other states.

Numbers of bacteria. The total numbers of bacteria were determined by the plate method, using beef infusion agar or Nile-blue sulfate medium (Section I) with an incubation period of 3 days at 21 - 23° C.

The numbers of lipolytic bacteria were always determined with Nile-blue sulfate medium.

Predominant species or morphologic types. The species names were recorded when the types which were most prevalent on the plates were identified. The morphologic types of bacteria were recorded on the basis of microscopic studies only.

RESULTS OBTAINED

Numbers of Lipolytic Bacteria in Raw, Fresh Milk of Good Quality

A study of the total numbers of bacteria, the numbers of lipolytic bacteria and the percentages of the total bacteria that were lipolytic was made with 20 samples of good quality milk obtained from the Iowa State College market milk plant. The data obtained are presented in Table X.

The results show that the total numbers of bacteria varied from 5,000 to 1,520,000 per ml., all but 2 of the samples having less than 100,000 per ml. The numbers of lipolytic bacteria ranged from less than 100 to 30,000 per ml., 13 of the samples having less than 1,000 bacteria per ml. The percentages of the total bacteria that were lipolytic ranged from less than 0.4 to 66.6 percent, 17 of the samples having less than 5.0 and only 1 sample having over 10.0 percent.

The results show that there were lipolytic bacteria in much of the raw milk examined but these organisms made up only a small percentage of the total number of bacteria.

Numbers of Lipolytic Bacteria in Raw, Sweet Cream

The total numbers of bacteria, the numbers and types of

TABLE X

Numbers of Lipolytic Bacteria in Raw, Fresh Milk of Good Quality

Nile-blue sulfate medium

Sample	Numbers of bacteria per ml.		Percent lipolytic bacteria
	Total	Lipolytic	
1	100,000	< 100	< 1.0
2	46,000	< 100	< 2.2
3	5,000	< 100	< 2.0
4	48,000	2,000	4.1
5	3,000	< 100	< 3.3
6	7,900	300	3.8
7	45,000	30,000	66.6
8	276,000	< 100	< 0.4
9	45,000	1,600	3.55
10	11,200	< 100	< 0.9
11	1,520,000	< 100	< 0.65
12	6,900	200	2.9
13	27,000	300	1.1
14	140,000	11,000	7.85
15	40,000	400	1.0
16	45,000	< 1,000	< 2.2
17	51,000	1,000	2.0
18	62,000	2,000	3.2
19	8,000	< 100	< 1.25
20	32,000	3,100	9.7

lipolytic bacteria and the percentages of the total bacteria that were lipolytic were studied with 20 samples of cream delivered to the Iowa State College butter plant. The results obtained are given in Table XI.

The data show that the total numbers of bacteria ranged from 20,000 to 120,000,000 per ml., with 16 of the samples having less than 20,000,000 per ml. The numbers of lipolytic bacteria varied from less than 1,000 to 3,000,000 per ml, 14 samples having less than 500,000 bacteria per ml. The percentages of the total bacteria that were lipolytic ranged from less than 1.0 to 19.6 percent, 10 of the samples having 5.0 percent or less and none of the samples having more than 20.0 percent. When determined, the predominant species were Ps. fragi, Ps. fluorescens, Bact. viscosum and A. lipolyticum.

The above summary indicates that there were considerable numbers of lipolytic bacteria in raw, fresh cream and that the percentages of the total bacteria that were lipolytic were slightly higher in the raw fresh cream than in the raw fresh milk.

Numbers of Lipolytic Bacteria in Rancid, Raw Milk from Individual Cows

At the time the studies on lipolytic bacteria in milk and cream were being carried out, an Iowa dairy farm was having trouble with the rather rapid development of rancidity in table

TABLE XI

Numbers of Lipolytic Bacteria in Raw, Sweet Cream

Nile-blue sulfate medium

Sample	Flavor of cream	Numbers of bacteria per ml.		Percent lipolytic bacteria	*Predominant species
		Total	Lipolytic		
1	clean	6,040,000	250,000	4.1	Ps. fragi
2	slightly unclean	1,950,000	210,000	10.7	" "
3	slightly old	15,000,000	3,000,000	20.0	
4	fairly clean	1,600,000	60,000	3.75	Ps. fluorescens
5	slightly rancid	61,000	< 1,000	< 1.6	
6	clean	219,000	3,000	1.7	Ps. fragi
7	"	1,590,000	100,000	6.3	
8	frozen cream	4,380,000	370,000	8.4	
9	clean	210,000	< 10,000	< 4.8	
10	fairly clean	14,060,000	210,000	1.5	
11	slightly stale	1,600,000	130,000	8.1	Ps. fluorescens
12	" "	4,060,000	800,000	19.6	
13	suggests rancidity	27,000,000	2,800,000	1.0	Bact. viscosum
14	clean	20,000	< 1,000	< 5.0	
15	fairly clean	37,000,000	1,870,000	5.05	
16	suggests rancidity	120,000	< 10,000	< 8.3	
17	clean	360,000	20,000	5.5	
18	slightly stale	16,100,000	1,560,000	9.7	Bact. viscosum
19	malty, sweet	120,000,000	300,000	0.2	A. lipolyticum
20	clean, sweet	80,000,000	1,300,000	1.6	

*The predominant type was isolated from some samples and identified.

cream. Samples of milk from individual cows in this herd were taken and analyzed for numbers of lipolytic bacteria. Samples were secured from 5 cows that had been yielding milk which developed an off flavor rather quickly. The samples were obtained with the usual amount of care and were held below 8° C. until they were analyzed.

The data secured are presented in Table XII.

The results show that, at the approximate age of one week, the first 18 samples were rancid and that they contained from 3,000 to 1,240,000,000 bacteria per ml., only 6 of the samples having total counts below 10,000,000. The ages of the last 5 samples were variable and the total counts were about normal for such milk.

The lipolytic bacteria ranged from less than 100 to 48,000,000 per ml.; only 4 of the first 18 samples contained less than 1,000,000 bacteria per ml., while the last 5 all contained 500,000 or less.

The percentages of the total bacteria that were lipolytic ranged from 1.1 to 33.8 percent among the first 18 samples and from 1.7 to 73.7 percent among the last 5 samples.

The organisms which, when determined, were found to be predominant were Ps. fragi and Ps. fluorescens species, and micrococcus types.

From the above summary of results it is evident that

TABLE XII

Numbers of Lipolytic Bacteria in Rancid, Raw Milk from Individual Cows

Nile-blue sulfate medium

Sample	Age of sample in days	Flavor of sample	Numbers of bacteria per ml.		Percent lipolytic bacteria	*Predominant species or morphologic type
			Total	Lipolytic		
1	6	rancid	18,560,000	1,050,000	5.7	
2	"	very rancid	4,980,000	1,220,000	24.3	
3	"	rancid	219,000	30,000	13.7	
4	"	"	54,400,000	8,800,000	16.2	
5	"	slightly rancid	910,000	10,000	1.1	
6	"	rancid	150,000,000	10,000,000	6.7	
7	"	"	1,320,000	124,000	9.4	
8	"	"	11,000,000	2,400,000	21.8	
9	"	rancid, "May Apple"	35,500,000	6,000,000	16.9	
10	"	very rancid	42,000,000	7,056,000	16.8	
11	"	"	9,400,000	2,800,000	29.7	micrococcus
12	"	sl. rancid, "May Apple"	27,200,000	6,500,000	23.9	"

(continued on following page)

TABLE XII (continued)

Numbers of Lipolytic Bacteria in Rancid, Raw Milk from Individual Cows

Nile-blue sulfate medium

Sample	Age of sample in days	Flavor of sample	Numbers of bacteria per ml.		Percent lipolytic bacteria	*Predominant species or morphologic type
			Total	Lipolytic		
13	6	sl. rancid, "May Apple"	3,000	< 100	< 3.3	
14	7	rancid	35,400,000	8,900,000	25.1	Ps. fragi
15	"	"	35,000,000	8,000,000	22.8	" "
16	"	"	1,240,000,000	17,000,000	1.4	non-proteolytic gram neg. rods
17	"	"	165,000,000	19,000,000	11.5	"
18	"	"	142,000,000	48,000,000	33.8	Ps. fragi
**19	2	"	9,550,000	160,000	1.7	non-proteolytic gram neg. rods
**20	3	"	5,440,000	500,000	9.2	Ps. fluorescens
**21	2	very rancid	76,000	56,000	73.7	" "
**22	fresh	slightly rancid	767,000	55,000	7.2	Bact. viscosum
**23	"	suggests rancidity	547,000	41,000	7.5	Ps. fluorescens

*The predominant type was isolated from some samples and identified if possible.
**These samples were selected from cows yielding milk which soon became rancid.

(a) many of the high counts must have been due to the increases in the numbers of bacteria during the storage of the cream, (b) the percentages of the total bacteria that were lipolytic were much higher than those for raw milk and (c) the species and types of organisms found in some of the fresh samples indicated that water contamination may have been a factor in the high numbers of lipolytic bacteria.

Numbers of Lipolytic Bacteria in Aseptically Drawn Milk

The total numbers of bacteria, the numbers of lipolytic bacteria and the percentages of the total bacteria that were lipolytic were determined in 9 samples of aseptically drawn milk: These samples were secured for analysis because the results given, particularly in Tables X and XI, indicate that there are considerable numbers of these types of bacteria in fairly fresh milk and cream. It was considered important to obtain the aseptically drawn milk from the dairy farm where the outbreak of rancidity occurred. The data secured are presented in Table XIII.

The results show that the total numbers of bacteria ranged from less than 10 to 61,400 per ml., 7 samples having less than 100 per ml. and 3 of the samples having less than 10 bacteria per ml. None of the 9 samples contained any lipolytic bacteria in dilutions of 1:10.

The results obtained from such a small number of samples

TABLE XIII

Numbers of Lipolytic Bacteria
in Aseptically Drawn Milk
Nile-blue sulfate medium

Sample	Numbers of bacteria per ml.		Percent lipolytic bacteria
	Total	Lipolytic	
1	30	0	0
2	< 10	0	0
3	70	0	0
4	20	0	0
5	61,400	0	0
6	< 10	0	0
7	< 10	0	0
8	< 10	0	0
9	7,150	0	0

do not justify a too definite conclusion, but they at least show that lipolytic bacteria were not obtained from the udders of some cows which were yielding milk of a rather poor keeping quality.

Numbers of Lipolytic Bacteria in Unsalted, Raw Cream Butter Held at 6° C. for Different Periods

The total numbers of bacteria, the numbers of lipolytic bacteria and the percentages of the total bacteria that were lipolytic were studied with 18 samples of unsalted butter churned from raw cream obtained from the butter laboratory. Following churning the butter was stored at 6° C. and analyzed after 7 days and after 30 or 42 days, respectively, to determine the relative rates of growth of the total and of the lipolytic bacteria at a practical, short-time, storage temperature. The butter was unsalted since it has already been shown that the lipolytic organisms studied are not very salt tolerant. The data obtained are presented in Table XIV.

The results show that 9 samples developed some rancidity during the first 7 days while the number had increased to 13 by the ends of the storage periods. The total numbers of bacteria after 7 days ranged from 216,000 to 119,000,000 per ml., 4 of the samples having less than 1,000,000 and 14 having more than 10,000,000 per ml. At the ends of the storage periods

TABLE XIV

Number of Lipolytic Bacteria in Unsalted, Raw Cream Butte

Nile-blue sulfate medi

Sample	First Examination							
	Age of sample in days	Flavor of sample	Numbers of bacteria per ml.		Percent lipolytic bacteria	Age of sample in days	Fl	
			Total	Lipolytic				s
1	7	slightly rancid	66,000,000	2,500,000	3.8	42	bare	ranc
2	"	slightly rancid	78,000,000	1,600,000	2,05	"	not	ranc
3	"	not rancid	13,000,000	2,600,000	12.3	"	slig	ranc
4	"	bitter, rancid	33,000,000	1,100,000	3.3	"		
5	"	rancid	19,000,000	400,000	2.1	"	ranc	
6	"	slightly rancid	46,000,000	500,000	1.1	"		
7	"	malty, sl. rancid	18,000,000	100,000	0.55	"		
8	"	not rancid	4,000,000	200,000	5.0	"	chee	
9	"	rancid	13,000,000	500,000	3.8	"	ranc	
10	"	clean cheesy	119,000,000	3,700,000	3.1	"	chee	"Ma
11	"	unclean cheesy	63,000,000	6,000,000	9.5	"	slig	ranc
12	"	rancid	928,000	208,000	22.4	30	ranc	
13	"	slightly rancid	216,000	56,000	25.9	"		
14	"	barely rancid	750,000	90,000	12.0	"		
15	"	"	20,600,000	8,600,000	41.7	"		
16	"	not rancid	8,000,000			"		
17	"	slightly rancid	366,000	64,000	17.5	"	not	ranc
18	"	suggests rancidity	91,000,000	7,000,000	7.7	42	slig	ranc

TABLE XIV

ted, Raw Cream Butter Held at 6° C. for Different Periods

le-blue sulfate medium

ercent polytic cteria	Age of sample in days	Flavor of sample	Second Examination: Numbers of bacteria per ml. Total	Lipolytic	Percent lipolytic bacteria	Change in percent of lipolytic bacteria
3.8	42	barely rancid	610,000	< 10,000	< 1.6	decrease
2,05	"	not rancid	410,000	< 10,000	< 2.4	
12.3	"	slightly rancid	400,000	< 10,000	< 2.5	large decrease
3.3	"	"	1,140,000	< 10,000	< 0.8	"
2.1	"	rancid	740,000	< 10,000	< 1.35	decrease
1.1	"	"	1,780,000	10,000	0.6	decrease
0.55	"	"	2,260,000	< 10,000	< 0.4	small decrease
5.0	"	cheesy	2,700,000	< 10,000	< 0.4	large decrease
3.8	"	rancid	600,000	< 10,000	< 1.7	decrease
3.1	"	cheesy, "May Apple"	6,000,000	< 500,000	8.3	medium increase
9.5	"	slightly rancid	11,700,000	4,600,000	39.0	large increase
22.4	30	rancid	1,250,000	20,000	1.6	large decrease
25.9	"	"	6,800,000	1,500,000	22.0	small decrease
12.0	"	"	9,800,000	1,700,000	17.3	small increase
41.7	"	"	5,100,000	1,000,000	19.6	decrease
	"	"	10,000	< 10,000		
17.5	"	not rancid	350,000	10,000	2.85	large decrease
7.7	42	slightly rancid	20,000,000	5,500,000	27.5	large increase

7 samples had less than 1,000,000 and none of the samples had more than 10,000,000 bacteria per ml.

The numbers of lipolytic bacteria ranged from 56,000 to 8,600,000 per ml. after 7 days and from less than 10,000 to 5,500,000 per ml. at the ends of the storage periods, at which time 11 of the samples had counts of 10,000 or less. The percentages of the total bacteria that were lipolytic ranged from 0.55 to 41.7 percent after 7 days and from less than 0.4 to 39.0 percent at the ends of the storage periods. Twelve of 16 samples with which comparisons were possible showed smaller percentages of lipolytic bacteria after the long storage periods than after 7 days.

The results indicate that although there were large numbers of lipolytic bacteria in unsalted butter stored for 1 week at 6° C., the numbers decreased appreciably during continued storage at this temperature while there was an increase in rancidity. There seemed to be very little correlation between rancidity and large numbers of total bacteria; however, the rapid decrease of bacteria in some of the samples during the time that rancidity was decreasing suggests an inhibitory influence on bacteria of the products formed in butter by them.

Numbers of Lipolytic Bacteria in Unsalted,
Pasteurized Cream Butter Held at 0° C.
for Seven Months

The total numbers of bacteria, the numbers and types of lipolytic bacteria and the percentages of the total bacteria that were lipolytic, in unsalted, storage butter were studied with 12 samples made in a churn which had been carelessly cleaned. The butter was studied because it was considered important to determine the numbers and types of lipolytic bacteria in butter after it had been stored at 0° C. for 7 months, this being a comparatively long storage period for commercial conditions. Table XV presents the data obtained.

The results clearly indicate that the butter was of very poor quality and that all the samples were rancid to some degree. The total numbers of bacteria ranged from 90,000 to 135,000,000 per ml., 7 of the samples having more than 10,000,000 and 3 having less than 1,000,000 per ml. The numbers of lipolytic bacteria varied from 6,000 to 12,000,000 per ml., only 1 sample having more than 10,000,000 and 8 samples having less than 1,000,000 per ml. The percentages of the total bacteria that were lipolytic ranged from 0.1 to 16.7 percent, 8 of them being below 5.0 percent.

Ps. fragi types predominated in 9 samples, Ps. fluorescens types in 1 sample and in another sample these two types were found together in large numbers.

The results indicate that there were large numbers of lipolytic bacteria in unsalted butter after it had been stored at

TABLE XV

Numbers of Lipolytic Bacteria in Unsalted, Pasteurized Cream Butter Held at 0° C. for Seven Months

Nile-blue sulfate medium

Sample	Total score of butter	Flavor of butter	Numbers of bacteria per ml.		Percent lipolytic bacteria	*Predominant species
			Total	Lipolytic		
1	85.0	definitely rancid	600,000	10,000	1.7	Ps. fragi
2	85.0	strongly rancid	45,000,000	1,000,000	2.2	" "
3	85.0	definitely rancid	2,900,000	110,000	3.8	" "
4	85.0	"	12,000,000	430,000	3.6	
5	85.0	"	1,800,000	60,000	3.3	" "
6	86.0	strongly rancid	135,000,000	12,000,000	8.9	" "
7	85.0	definitely rancid	26,000,000	270,000	1.0	Ps. fluorescens
8	85.5	cheesy and rancid	34,000,000	8,000,000	2.3	Ps. fluorescens Ps. fragi
9	86.0	strongly rancid	17,000,000	1,100,000	6.5	Ps. fragi
10	85.0	cheesy and rancid	90,000	6,000	6.7	" "
11	85.0	definitely rancid	22,000,000	30,000	0.1	" "
12	85.0	"	240,000	40,000	16.7	" "

*The predominant type was isolated from some samples and identified.

0° C. for 7 months, that Ps. fragi was the most common species identified from these samples and that rancidity was the common defect which occurred in the samples under such storage conditions.

Numbers of Lipolytic Bacteria in Good Quality, Lightly Salted Butter

The total numbers of bacteria, the numbers of lipolytic bacteria and the percentages of the total bacteria that were lipolytic, in high quality, unsalted butter were studied with 23 samples of butter secured from widely separated sources. The samples were chosen since it was thought that the more careful selection of the cream for this butter might result in a low total bacterial count and a low percentage of lipolytic bacteria. The data secured are presented in Table XVI.

The results show that the total numbers of bacteria ranged from 11,000 to 1,456,000 per ml., 13 of the samples having less than 100,000 and only 4 having more than 1,000,000 per ml. The numbers of lipolytic bacteria ranged from less than 1,000 to 40,000 per ml., 17 of the samples having counts of 1,000 or less while only 1 sample had more than 10,000 bacteria per ml.

The percentages of the total bacteria that were lipolytic ranged from less than 0.1 to 18.5 percent with 9 of the samples having less than 1.0 and only 1 sample having more than 10.0 percent.

The results show that there were some lipolytic bacteria

TABLE XVI

Numbers of Lipolytic Bacteria in Good Quality, Lightly Salted Butter

Nile-blue sulfate medium

Sample	Total score of butter	Flavor of butter	Numbers of bacteria per ml.		Percent lipolytic bacteria
			Total	Lipolytic	
1	94.0	mild and clean	102,000	< 1,000	< 0.9
2	93.0	coarse	130,000	< 1,000	< 0.8
3	92.0	poor raw product	23,000	1,000	4.3
4	93.0	coarse	580,000	< 1,000	< 0.2
5	94.25	good	1,500,000	4,000	0.3
6	94.0	"	1,328,000	< 1,000	< 0.1
7	92.5	"	340,000	< 1,000	< 0.3
8	92.0	"	25,000	2,000	8.0
9	92.0	flat	158,000	4,000	2.5
10	93.5	wintry	11,000	1,000	9.1
11	93.0	burnt	12,000	< 1,000	< 8.3
12	93.5	clean, mild	12,000	< 1,000	< 8.3
13	91.0	wintry	20,000	< 1,000	< 5.0
14	93.0	good	10,000	1,000	10.0
15	93.0	slightly rancid	3,000	< 1,000	
16	93.5	good	54,000	10,000	< 18.5
17	93.0	"	77,000	2,000	2.6
18	93.5	"	976,000	< 1,000	< 0.1
19	93.0	flat	1,456,000	< 1,000	< 0.1
20	92.0	feed	17,000	< 1,000	< 5.9
I.S. 1		good (medium salt)	1,040,000	40,000	0.4
I.S. 2		" " "	42,000	< 1,000	< 2.4
I.S. 3		" " "	11,000	1,000	9.1

in good quality butter but that the numbers were generally much smaller than those obtained with the raw milk, raw cream and butter that had been previously examined.

Numbers of Lipolytic Bacteria in Defective Butter from Various Sources

The total numbers of bacteria, the numbers and types of lipolytic bacteria and the percentages of the total bacteria that were lipolytic were studied with 26 samples of defective butter obtained from different commercial plants in Iowa and a few other states. It appeared that selecting samples in this manner might result in finding additional types of lipolytic bacteria which might be related to the development of rancidity in butter. Most of the samples were salted but none of them contained more than 1.5 percent salt. The data obtained are given in Table XVII. Samples 1, 2, 3, 4, 5, 14, 15, 25 and 26 were rather old samples of Iowa butter which had been stored for a few weeks at 0° C. Little is known about the actual ages and the storage conditions of the other samples.

It is evident from the data given that the samples showed many defects although only 5 of them were described as rancid.

The total numbers of bacteria ranged from 3000 to 42,800,000 per ml., 7 of the samples having more than 1,000,000 per ml. The numbers of lipolytic bacteria varied from less than 100 to 900,000 per ml., with 14 of the samples having less than 10,000

TABLE XVII

Numbers of Lipolytic Bacteria in Defective Butter from Various Sources-Salting Variable

Nile-blue sulfate medium

Sample	Source of butter	Flavor of butter	Numbers of bacteria per ml.		Percent lipolytic bacteria	*Predominant species or morphologic type
			Total	Lipolytic		
1	Ia.	suggests rancidity	300,000	1,000	0.3	
2	"	slightly rancid	22,500	< 1,000	< 0.4	
3	"	oily	3,000	100	3.3	micrococcus
4	"	"	60,000	< 1,000	< 1.7	"
5	"	rancid	150,000	< 1,000	< 0.7	
6	Ill.	roquefort	20,000	< 1,000	< 5.0	
7	Ia.	rancid	5,000,000	mostly moulds		lipolytic moulds
8	"	roquefort	42,800,000	500,000	1.2	micrococcus
9	Wash.	rancid	300,000	< 1,000	< 0.3	
10	Mont.	stale, putrefactive	96,000	4,000	4.2	"
11	Tex.	tallowy, stale	420,000	3,000	0.7	"
12	"	stale, cheesy	2,700,000	300,000	11.1	"

(continued on following page)

TABLE XVII (continued)

Numbers of Lipolytic Bacteria in Defective Butter from Various Sources-Salting Variable

Nile-blue sulfate medium

Sample	Source of butter	Flavor of butter	Numbers of bacteria per ml.		Percent lipolytic bacteria	*Predominant species or morphologic type
			Total	Lipolytic		
13	Ia.	good	1,040,000	40,000	3.85	Ps. fragi
14	"	"	42,000	< 1,000	< 2.4	
15	"	"	10,000	< 1,000	<10.0	
16	"	objectionable	2,000,000	30,000	1.5	" "
17	"	storage	520,000	60,000	11.5	Ps. fluorescens
18	"	yeasty	950,000	40,000	4.2	Ps. fragi
19	"	slightly cheesy	5,000,000	40,000	0.8	
20	"	storage	36,000	3,000	8.3	
21	Ill.	slightly rancid	650,000	150,000	23.0	Ps. fragi Ps. fluorescens
22	Ore.	bitter, cheesy	10,900,000	900,000	8.3	micrococcus
23	Ill.	rancid	360,000	53,000	14.7	lipolytic yeasts
24	Ia.	"	180,000	90,000	50.0	A. lipolyticum Ps. fragi
25	"	slightly rancid	3,000	< 100	< 3.3	
26	"	"	77,000	2,000	2.6	

*The predominant type was isolated from some samples and identified if possible.

per ml. The percentages of the total bacteria that were lipolytic ranged from less than 0.3 to 50.0 percent, 8 of these being less than 1.0 percent.

The lipolytic bacteria isolated and identified were similar to the organisms generally obtained from the dairy products examined. There was a predominance of micrococci in the samples obtained from Montana, Texas, Oregon and some Iowa plants; these samples were not rancid. A very actively proteolytic yeast was isolated from a sample obtained from Illinois.

The results indicate that there were appreciable numbers of lipolytic bacteria in ordinary defective butter from widely distributed sources but that there seemed to be little correlation between the bacterial counts and the types of defects present in the butter. The micrococci which were isolated proved to be only weakly lipolytic and were not identified.

Growth of Lipolytic Bacteria in Unsalted Butter Churned from Inoculated Sterile Cream and Held at 21° C.

The growth of lipolytic bacteria in unsalted butter churned from inoculated sterile cream was determined with 19 samples which were examined when fresh and at least once after storage at 21° C. The data secured from two and usually from three examinations of these samples are given in Table XVIII.

The results show that the initial numbers of lipolytic

TABLE XVIII

Growth of Lipolytic Bacteria in Unsalted Butter Churned from Inoculated, Sterile Cream and Held at 21° C.

Beef infusion agar

Culture used	Numbers of bacteria in fresh butter	Second Examination			Third Examination		
		Age of sample in days	Flavor of butter	Numbers of bacteria per ml.	Age of sample in days	Flavor of butter	Numbers of bacteria per ml.
6	70,000	7	good	3,000,000	10	dirty	24,000,000
8	10,000	7	"	300,000	10	slightly rancid	4,000,000
8	160,000	12	off	38,000,000			
16	12,400	7	good	1,600,000	10	good	6,400,000
17	7,400	7	"	300,000	10	slightly rancid	2,300,000
20	490,000	4	slightly rancid	8,000,000	7	rancid	50,200,000
27	870,000	7	off	9,000,000			
36	220,000	2	"	80,000,000	14	very objec-tionable	343,000,000
37	520,000	2	slightly rancid	55,520,000	3	very rancid	36,800,000
41	1,000,000	4	rancid	20,000,000	7	"	11,600,000
41	7,720,000	1	slightly rancid	10,060,000	4	"	50,000,000

(continued on following page)

TABLE XVIII (continued)

Growth of Lipolytic Bacteria in Unsalted Butter Churned from Inoculated, Sterile Cream and Held at 21° C.

Beef infusion agar

Culture used	Numbers of bacteria in fresh butter	Second Examination			Third Examination		
		Age of sample in days	Flavor of butter	Numbers of bacteria per ml.	Age of sample in days	Flavor of butter	Numbers of bacteria per ml.
41	3,150,000	3	very rancid	27,720,000	7	very rancid	120,000,000
42	9,000,000	4	rancid	159,000,000	17	"	1,050,000,000 135,000,000
42	400,000	2	good	32,000,000	7	rancid	55,000,000
45	1,000,000	2	"	107,000,000			
49	900,000	10	slightly rancid	97,000,000	21	"	130,000,000
76 (surface)	10,000	4	"May Apple"	42,000,000	7	"May Apple", rancid	22,300,000
97 (surface)	200,000	4	rancid	96,000,000	7	very rancid	17,600,000
108 (surface)	330,000	4	"	82,000,000	7	very rancid	9,500,000

bacteria in 14 of 19 samples were less than 1,000,000 per ml. while in the remaining 5 samples they were over 1,000,000 per ml. The counts of lipolytic bacteria at the second examination ranged from 300,000 to 159,000,000 per ml., 13 of the samples having counts above 10,000,000 per ml.

The numbers of lipolytic bacteria at the third examination ranged from 2,300,000 to 1,050,000,000, with 3 samples having more than 10,000,000 per ml. and 1 of these having more than 1,000,000,000 bacteria per ml. All the samples showed definite increases at the second examination over the first. At the third examination, 5 of the samples showed definite decreases in numbers concurrent with the development of strong rancidity. One sample which was examined 4 times showed an extremely large increase in the numbers of lipolytic bacteria per ml. at the third examination and a very large decrease at the fourth examination.

From the above considerations it is evident that there was a rapid increase for a short while in the numbers of lipolytic bacteria in unsalted butter held at 21° C.; this increase was soon followed by a rather rapid destruction of the organisms.

Growth of Lipolytic Bacteria in Surface and Sub-surface Portions of Unsalted Butter

The growth of lipolytic bacteria in surface and sub-surfac

portions of unsalted butter was studied to determine the effect of the air supply on the growth of these organisms. The study was made with 18 samples of butter, churned from samples of sterilized cream, each of which had been inoculated with a pure culture of a lipolytic organism. The data obtained (from one or two examinations, in addition to the one made on the fresh butter) are included in Table XIX.

The results show that the numbers of inoculated organisms in the fresh samples were, on the average, quite uniform, ranging from less than 10,000 for 2 samples, to 490,000 per ml. At the second examination the numbers of bacteria in the surface portions ranged from 70,000 to 138,000,000 per ml., while in the sub-surface portions they varied from less than 10,000 to 80,000,000 per ml. higher than the sub-surface counts in 13 of 15 samples and slightly lower in 2 samples.

At the final examination the numbers of bacteria in the surface portions ranged from 8,400,000 to 50,200,000 per ml, while in the sub-surface portions they varied from 140,000 to 54,600,000 per ml. The surface counts were higher than the sub-surface counts in 6 of the samples and lower in the remaining 3 samples. In 2 of the 3 samples which had higher sub-surface than surface counts strong rancidity had already developed.

The results indicate that there were large differences

TABLE XIX

Growth of Lipolytic Bacteria in Surface and Sub-surface Portions of Unsalted Butter Held at 21° C.

Beef infusion agar

Culture used	Numbers of bacteria in fresh butter	Second Examination (4-7 days) Flavor of butter	Numbers of bacteria per ml. Surface	Sub-surface	Third Examination (10-20 days) Flavor of butter	Numbers of bacteria per ml. Surface	Sub-surface
20	490,000	rancid	8,000,000	1,500,000	medium rancid	50,200,000	6,000,000
33	70,000	good	23,100,000	15,000,000	off		
56	30,000	"	520,000	50,000	good		
61	10,000	off	100,000	10,000	"		
67	10,000	"	101,300,000	13,500,000	sl. rancid	10,050,000	600,000
76	10,000	"May Apple"	42,000,000	2,600,000	very rancid	22,300,000	55,000,000
86	23,000	rancid	70,000	10,000	rancid	8,400,000	140,000
97	200,000	"	96,000,000	500,000	very rancid	17,600,000	19,800,000
98	80,000	good			unclean	14,000,000	3,750,000
108	330,000	rancid	82,000,000	2,800,000	very rancid	9,500,000	5,400,000
129	170,000	mutty	12,900,000	22,900,000	not rancid		
131	23,000	bitter "May Apple"	54,000,000	56,000,000	"May Apple"		
133	456,000	very rancid	2,670,000	370,000	very rancid		
135	154,000	"May Apple" rancid	138,000,000	80,000,000	"		
145	338,000	good			good	47,600,000	54,600,000

TABLE XIX (continued)

Growth of Lipolytic Bacteria in Surface and Sub-surface Portions of Unsalted Butter Held at 21° C.

Beef infusion agar

Culture used	Numbers of bacteria in fresh butter	Second Examination (4-7 days) Flavor of butter	Second Examination (4-7 days) Numbers of bacteria per ml. Surface	Second Examination (4-7 days) Numbers of bacteria per ml. Sub-surface	Third Examination (10-20 days) Flavor of butter	Third Examination (10-20 days) Numbers of bacteria per ml. Surface	Third Examination (10-20 days) Numbers of bacteria per ml. Sub-surface
157	78,000	rancid	2,800,000	330,000			
166	230,000	good			sug-gests ran-cidity	32,224,000	11,720,000
168	113,000	unclean	2,600,000	60,000	sl. rancid		

in the numbers of lipolytic bacteria in surface and sub-surface portions of unsalted butter held at room temperature. During the first part of the storage period the most rapid and greatest increases were shown in the surface samples, but after 7 or 10 days the numbers of bacteria in the sub-surface portions exceeded those in the surface portions. When the numbers were decreasing in the surface portions they were still increasing in the sub-surface portions. Eventually the inhibitory effect on bacterial growth which was first evident in the surface portions also became effective in the sub-surface portions and this resulted in a decrease in the numbers of bacteria in representative samples of the butter.

Numbers of Bacteria in Unsalted Butter When Rancidity is First Evident

The numbers of bacteria in unsalted butter when rancidity is first evident were studied with 15 samples of unsalted butter held at 21° C. Some of the samples were churned from sterile cream and the remainder from sweet raw cream. The data on the ages and the bacterial counts of the samples are recorded in Table XX.

The results show that when rancidity was first evident in the samples, at ages ranging from 4 to 16 days, the numbers of bacteria varied from 1,050,000 to 78,000,000 per ml. Six of the 9 samples containing only lipolytic bacteria had less than

TABLE XX

Numbers of Bacteria in Unsalted Butter when Rancidity is First Evident*

Beef infusion agar

Sample	Treatment of cream before churning	Age of sample in days	Numbers of bacteria per ml.
1	inoculated with pure culture	15	36,800,000
2	"	14	4,000,000
3	"	4	10,060,000
4	"	7	32,000,000
5	"	10	97,000,000
6	"	4	8,000,000
7	"	16	1,050,000
8	"	14	11,720,000
9	"	14	56,000,000
10	"	15	2,300,000
11	sweet raw cream	8	78,000,000
12	"	8	33,000,000
13	"	8	46,000,000
14	"	8	18,000,000
15	"	8	20,600,000

*Butter held at 21° C.

15,000,000 per ml., and none of the 5 samples churned from raw cream contained less than 18,000,000 bacteria per ml. when rancidity was first noted.

The results indicate that there were rather large numbers of bacteria present in unsalted butter held at 21° C. when rancidity was first evident but that these numbers were smaller when pure cultures of lipolytic bacteria were used than when mixed cultures of organisms were used.

Relationship Between the Development of Rancidity and the Decrease in the Numbers of Lipolytic Bacteria in Unsalted Butter

The relationship between the development of rancidity and the decrease in the numbers of lipolytic bacteria in unsalted butter was studied with 11 samples of unsalted butter, each of which was churned from sterile cream that had been inoculated with a pure culture of a lipolytic organism. The study was made because the results recorded in previous tables suggested that rancidity has an inhibitory effect on bacterial growth.

Table XXI presents the data obtained. The results show that the numbers of bacteria in the fresh samples ranged from less than 10,000 to 9,000,000 per ml. At the second examination all the samples were rancid and showed much higher counts than originally. The numbers of bacteria per ml. then ranged from 70,000 to 159,000,000 per ml., only 1 sample having less

TABLE XXI

Relationship between the Development of Rancidity and the Decrease in the Numbers of Lipolytic Bacteria in Unsalted Butter*

Beef infusion agar

Culture used	Numbers of bacteria in fresh butter	Second Examination (4 days) Flavor of butter	Second Examination (4 days) Numbers of bacteria per ml.	Third Examination (7-10 days) Flavor of butter	Third Examination (7-10 days) Numbers of bacteria per ml.	Fourth Examination (15-20 days) Flavor of butter	Fourth Examination (15-20 days) Numbers of bacteria per ml.
20	490,000	rancid	8,000,000	rancid	50,800,000	rancid	2,510,000.
41	1,000,000	"	20,000,000	"	11,600,000		
42	9,000,000	"	159,000,000	very rancid	1,059,000,000	very rancid	135,000,000
76	10,000	"May Apple"	42,000,000	"	22,300,000	"	11,400,000
86	23,000	rancid	70,000	rancid	3,400,000	"	910,000
97	200,000	"	96,000,000	very rancid	17,600,000	"	2,400,000
108	330,000	"	82,000,000	"	9,500,000	"	4,200,000
133	456,000	very rancid	2,670,000	"	33,400,000	"	5,500,000
135	154,000	"May Apple", rancid	138,000,000			"	23,000,000
157	78,000	rancid	2,800,000			rancid	1,360,000
41	7,800,000	"	35,840,000	very rancid	50,000,000	**very rancid	10,000

* Butter stored at 21° C.

**Held 3 months.

than 2,000,000 per ml.

At the third examination all the samples examined were rancid or very rancid and the numbers of bacteria ranged from 8,400,000 to 1,059,000,000 per ml. When the samples were between 7 and 10 days old, 4 of the 9 showed large decreases in counts over the previous examination. At the fourth examination the rancid flavors were more pronounced than previously in all the samples and the numbers of bacteria had decreased in every instance, the final counts ranging from less than 10,000 to 135,000,000 bacteria per ml.

From the above considerations it is quite evident that there were rapid decreases in the numbers of bacteria simultaneously with the development of rancidity in butter and that these decreases continued with age.

Salt Tolerance of Some Lipolytic Bacteria

The salt tolerance of lipolytic bacteria was studied with 1 or more cultures of 8 species or morphologic types of lipolytic bacteria. The ability of the organisms to grow in brine concentrations simulating those in commercial butter was determined in nutrient bouillon brine at 21° C.: Cultures were examined every day for 7 days to detect turbidity or other changes which would indicate growth. The concentrations of sodium chloride in nutrient bouillon were equivalent to brine concentrations in butter of 0.8 to 2.5 percent salt in 15 percent moisture. The abilities of the organisms to survive

these concentrations of salt without growth were not determined. The data obtained are presented in Table XXII.

The results show that 20 of the 24 organisms definitely grew in 5.0 percent bouillon brine, 13 definitely grew in 6.25 percent, 2 grew in 7.5 percent and only 1 culture showed growth in brine concentrations above these. The last mentioned organism, which was a micrococcus, grew well in 12.0 percent bouillon brine. Three cultures showed no evident growth and 1 culture gave questionable growth in 5.0 percent bouillon brine while 10 cultures showed no evident growth and 1 culture showed questionable growth in 6.25 percent bouillon brine. None of the identified species gave any evidence of growth in bouillon brine concentrations above 6.25 percent (equivalent to 1.0 percent NaCl in 15.0 percent moisture).

The results indicate that a very large percentage of lipolytic cultures studied did not tolerate brine concentrations higher than 6.25 percent (equivalent to 1.0 percent NaCl in 15.0 percent moisture). The 2 cultures which grew in 7.5 percent bouillon brine, one of which also grew in a 12.0 percent concentration, were micrococcus types. The fact that the micrococcus types were more salt tolerant than the gram negative non-spore forming rods possibly accounted for the isolation of large numbers of these organisms from salted butter.

TABLE XXII

SALT TOLERANCE OF SOME LIPOLYTIC BACTERIA

*Growth determined in bouillon brine

Culture	Species or morphologic type	Concentration of NaCl in nutrient bouillon						
		5.0%	6.25%	7.5%	8.75%	10%	12%	16%
		Growth	Growth	Growth	Growth	Growth	Growth	Growth
6	Ps. synxantha	+	-	-	-	-	-	-
7	Ps. schuylkilliensis	+	+	-	-	-	-	-
12	Ps. fluorescens	+	-	-	-	-	-	-
17	Ps. fluorescens	+	-	-	-	-	-	-
20	Ps. fluorescens-var. zymogenes	+	+	-	-	-	-	-
28	Ps. fluorescens-var. patula	+	-	-	-	-	-	-
33	Ps. schuylkilliensis	+	+	-	-	-	-	-
38	Ps. fluorescens-var. radians	+	+	-	-	-	-	-
41	Ps. mucidolens	+	-	-	-	-	-	-
56	Ps. acidiconcoquens	-	-	-	-	-	-	-
M10	Ps. fragi	+	-	-	-	-	-	-
57	micrococcus	+	+	+	-	-	-	-
59	Ps. acidiconcoquens	?	-	-	-	-	-	-
73	Ps. fluorescens	+	+	-	-	-	-	-
75	Ps. fluorescens	+	+	-	-	-	-	-
76	Ps. fluorescens	+	+	-	-	-	-	-
80	closely related to Ps. fluorescens	+	+	-	-	-	-	-
82	Ps. fragi	+	?	-	-	-	-	-
90	A. lipolyticum	+	+	-	-	-	-	-
94	A. lipolyticum	+	+	-	-	-	-	-
108	A. lipolyticum	+	+	-	-	-	-	-
128	A. connii	-	-	-	-	-	-	-
148	micrococcus	+	+	+	+	+	+	-
164	Ps. fluorescens	-	-	-	-	-	-	-

*Cultures incubated for 1 week at 21° C.

DISCUSSION OF RESULTS

The results obtained indicate that there were considerable numbers of lipolytic bacteria in fresh milk and cream, that there were few, if any, in aseptically drawn milk and that there were considerable numbers in butter. Fresh unsalted and lightly salted butter, of average quality, contained more lipolytic bacteria than good quality, lightly salted butter or than butter of unknown salt content from various sources.

There seemed to be a fairly close correlation between the numbers of lipolytic organisms and the incidence of rancidity in milk, cream and unsalted butter when the rancidity developed during extended holding periods of these products: With the samples that developed rancidity when fairly fresh, however, this correlation was less evident. These observations suggest the importance of an inherent lipolytic enzyme in the production of rancidity in some samples of milk. It is also noteworthy that the milk and cream which developed rancidity when fairly fresh did not become appreciably worse without rapid increases in the numbers of lipolytic bacteria.

Unrecorded data obtained in this investigation have shown that rancidity can be produced through the action of enzymes

elaborated by bacteria. Enzyme preparations were made by growing pure cultures of lipolytic organisms in sterilized skim-milk. When the milk was completely digested the organisms were all destroyed, by the addition of "Formalin" (1 part to 800 parts of the digested milk), before the preparations were used.

These preparations were inoculated into small quantities of sterile cream which were churned and held at 21° C. Of the enzyme preparations from 15 organisms only 2 produced the same degree of rancidity in butter as was brought about by the organisms themselves.

Concerning the inhibition of bacterial growth by rancidity, the results show that there was a rapid decrease in the numbers of bacteria when strong rancidity developed. This was attributed to the inhibitory influence of butyric or of other lower fatty acids produced by the hydrolysis of the tri-glycerides. The inhibitory effect of butyric and caproic acids upon bacterial growth was confirmed by unrecorded data obtained in this work. Concentrations of these two fatty acids as low as 1 part of acid in 1000 parts of nutrient bouillon prevented bacterial growth, while 1 part of oleic acid to 10 parts of nutrient bouillon did not prevent growth. The hydrogen ion concentrations of the bouillons were reduced as low as 4.7 to 5.0 with the concentrations of the lower fatty acids mentioned above, and it is not known whether the inhibitory effect upon the

growth of the bacteria was exerted by the toxicity of the acids or by the low pH induced in the media.

The work herein reported and the unrecorded general observations concerning the inhibition of bacterial growth by a strongly rancid condition in butter are in agreement with much of the literature.

The influence of the air supply on the increase in the numbers of bacteria in butter appears to be important. Bacterial examinations of surface and sub-surface portions of unsalted butter showed that there was a more rapid and extensive increase of organisms in the surface than in the sub-surface portions.

The results on the salt tolerance of a number of lipolytic organisms indicates that, of the lipolytic cultures used, only the micrococcus forms grew well in a bouillon brine containing an amount of salt equivalent to 1.0 percent salt in butter containing 15.0 percent moisture. The Ps. fluorescens types were a little less salt tolerant than some of the other types. This confirms an observation which was often made throughout the work that greenish fluorescent colonies were much less commonly found on beef infusion agar from samples of commercial, salted butter than were some other types. The data considered as well as general observations have shown that Ps. fragi, A. lipolyticum, micrococcus types and non-proteolytic gram negative rods which produced an alkaline reaction in litmus milk,

were most commonly isolated from butter of different salt concentrations.

No data have been reported in this study on the effect of the moisture supply on the growth of bacteria and the development of rancidity in butter. Constant observations, however, indicated that both the growth of bacteria and the development of rancidity in unsalted butter were greatly favored by the presence of free moisture resulting from weak bodied, poorly worked butter.

SUMMARY

1. There were considerable numbers of lipolytic bacteria in average fresh milk and cream but not in aseptically drawn milk.

2. Rancidity which developed in fairly fresh milk and cream probably was not due to any great extent to the lipolytic organisms present.

3. There were rapid increases in the numbers of lipolytic bacteria in unsalted butter held at 0°, 6° or 21° C.

4. The development of rancidity in milk, cream and unsalted butter during extended holding periods was always accompanied by large increases in the numbers of lipolytic bacteria.

5. Lipolytic bacteria produced strong rancidity in butter, and when this defect was well initiated it developed simultaneously, after a certain point, with decreases in the numbers of lipolytic organisms.

6. The growth of bacteria was favored more by conditions in surface than in sub-surface portions of unsalted butter.

7. Normal dairy products did not become rancid unless fairly large numbers of lipolytic bacteria were present.

8. Rancidity inhibited bacterial growth in butter.

9. The important lipolytic bacteria appear to be rather

intolerant of salt and therefore grow poorly in salted butter.

10. Lipases elaborated by bacteria produced rancidity in butter made from sterilized cream inoculated with sterile enzyme preparations.

SECTION IV

THE ABILITY OF LIPOLYTIC BACTERIA TO PRODUCE RANCIDITY IN BUTTER FAT, IN SOME OTHER NATURAL FATS AND OILS AND IN SOME SIMPLE TRI-GLYCERIDES

GENERAL CONSIDERATIONS

A consideration of some of the literature reviewed in Section III on the conceptions of rancidity indicates that the term "rancidity" has been used rather indiscriminately by investigators in different industries to define distinctly different defects or chemical changes of fats and oils.

The word "rancidity" is used in the dairy industry to indicate a defect which is characterized by the hydrolysis of the tri-glycerides with the formation of chemical substances having the characteristic odor of butyric acid. Since the most probable source of these substances is the tri-glycerides, it seems logical that they would occur most readily in the hydrolytic products of a natural fat or oil which contains a considerable amount of the lower tri-glycerides.

In view of the fact that such fats and oils as lard, beef fat, corn oil, wheat kernel oil, linseed oil, etc. contain none or only small amounts of the lower fatty acids (as tri-glycerides), while butter fat contains relatively large amounts, it seems that hydrolytic rancidity should not be encountered in other fat and oil industries as it is in the dairy industry. On the other hand, the defects caused by an oxidation of the unsaturated molecules should be encountered frequently with

various fats including butter fat.

A consideration of the results presented in Section I shows that tri-laurin and the higher simple tri-glycerides, with the exception of tri-olein, are very difficultly hydrolyzed by bacteria and, since this is true, it appears that the mixed tri-glycerides containing large amounts of the higher saturated fatty acids must also be hydrolyzed with great difficulty. The tri-glycerides of certain natural fats and oils other than butter fat are composed almost entirely of the higher fatty acids and therefore when these fats and oils undergo a chemical change the molecules primarily involved are no doubt those which contain large amounts of oleic or other unsaturated acids.

The changes resulting when the unsaturated fatty acids in the molecules are saturated through the addition of oxygen have been defined as oxidative or autoxidative by many investigators, and these changes result in a defect which is known in the dairy industry as "tallowiness".

It should be recognized that uniformity in the use and interpretation of the terms "rancidity" and "tallowiness" would result in much less confusion in the literature and would clarify the whole general understanding of the deterioration of fats and oils.

Rancidity Defined

In the work herein reported the term "rancidity" has been used to designate a condition of fats which is characterized by the odor and flavor of the lower fatty acids, especially of butyric acid.

The defect rancidity has been detected by the senses of smell and taste since it is recognized that certain chemical compounds can be more accurately detected by the senses than by chemical means, and it is thought that this is especially true of butyric acid. Grossfeld and Battay (31) found that 1 part of butyric acid in 12,500 parts of a medium could be detected by the sense of smell.

STATEMENT OF THE PROBLEM

The work herein reported was undertaken to determine the relative susceptibilities of some simple tri-glycerides and some natural fats and oils to the development of rancidity through the action of lipolytic bacteria. The fats employed were dispersed by the use of an agar emulsion, fine sawdust or shredded filter paper to make them more accessible to the action of the inoculated bacteria.

The study was carried out because the work reported in Section I indicated that the tri-glycerides of the lower fatty acids (contained in butter fat) are much more susceptible to lipolysis by bacteria than those of the higher acids which constitute the bulk of the saturated acids in olive oil, corn oil, etc. It appeared that if "rancidity", as interpreted in the dairy industry, were caused by the breakdown of the unsaturated molecules, corn oil and olive oil, which have large percentages of oleic acid, should readily become rancid.

METHODS USED

In the trials involving an agar emulsion, the fat and the peptone solution were emulsified with a 0.5 percent agar solution. The peptone solution used in some of the trials contained 5 grams of peptone in 95 cc. of distilled water.

The sawdust used was sifted through cheesecloth, washed, drained and dried in petri dishes.

The filter paper used was prepared by shredding or grinding moist filter paper with sand in a mortar. The paper was separated from the sand by floating on water, divided into small lots and dried in petri dishes.

The butter fat, corn oil and olive oil used were of the finest quality and were filtered by means of a hot water funnel. The tri-butyrin and tri-olein were commercial preparations (see Section I) and were not filtered.

The ingredients were either mixed together and then sterilized or were sterilized separately and then mixed. Regardless of the form in which the constituents were sterilized, they were autoclaved at 15 pounds pressure for 25 minutes and were emulsified when cooling.

Each medium in a series was inoculated when partly cooled, with a small quantity of growth from an agar slant, and was then

treated in the manner which would give the greatest homogeneity in the emulsion. An inoculated check was kept for each medium in a series. The samples were incubated at room temperature and were examined often by several persons over a long period of time, the taste and smell being used entirely for the detection of defects.

RESULTS OBTAINED

The Susceptibilities of Some Simple Tri-glycerides and Some Natural Fats to Rancidity Through the Action of Lipolytic Bacteria

Six series of trials were carried out, in each of which various fats were used, and each fat preparation was divided into a number of parts so that various organisms could be tested.

The detailed description of the medium or media employed for each series is as follows:

Series 1. Five cc. of a 5.0 percent stock peptone solution were added to 15 cc. of distilled water and 80 cc. of filtered butter fat. This mixture was divided into four parts and each part placed in a four ounce, glass-stoppered bottle and sterilized.

Series 2. To 16 cc. quantities of the respective fats was added 1 cc. of a sterile 5.0 percent peptone solution, and the mixtures were emulsified with 3 cc. of a 0.5 percent agar solution. Each medium was divided equally among four test tubes before sterilizing.

Series 3. Small volumes of the prepared sawdust were sterilized in each of 10 petri dishes. The sawdust was then moistened with sterile 5.0 percent peptone and each lot mixed

with a sufficient quantity of one of the fats to make it distinctly oily. The preparations were inoculated and mixed as well as possible by means of sterile glass rods.

Series 4. The procedure was exactly the same as for Series 3 excepting that 1.5 percent peptone solution replaced the 5.0 percent peptone solution.

Series 5. To 16 cc. quantities of the respective fats was added 0.1 cc. of sterile 5.0 percent peptone solution, and the mixtures were emulsified with 4.9 cc. of a 0.5 percent agar solution. Each medium was divided equally between two test tubes before sterilizing.

Series 6. To small volumes of the dried filter paper in petri dishes was added enough 1.5 percent peptone to make them distinctly moist. The moist masses of filter paper were then mixed thoroughly with small quantities of the respective fats and sterilized in the petri dishes.

Nine lipolytic organisms from three genera were used. The identified organisms are Ps. fluorescens, Ps. mucidolens, Ps. fluorescens var. zymogenes and A. lipolyticum. The unidentified cultures belong to the genus Serratia.

The data obtained and a summary of them are presented in Tables XXIII and XXIIIa, respectively.

The results, considered from the standpoint of the individual fats, show:

TABLE

The Susceptibilities of Some Simple Tri-glycerides and Some Natu

Series number	Fat used	Artificial medium	Trial I		C
			Culture	Defect	
1	butter fat	Emulsion of 0.25% peptone in aqueous solution and 80.0% butter fat.	42	very rancid	
	checks		none	normal	
2	butter fat	Emulsion of 0.5% agar solution containing 1.25% peptone and 80.0% fat; in test tubes.	41	very rancid	
	corn oil		41	musty potato	
	olive oil		41	musty potato	
	checks		none	normal	
3	butter fat	Mixture of fine sawdust, with enough 5.0% peptone solution to moisten and enough fat to make distinctly oily; in sterile petri dishes.	123	very rancid	
	corn oil		123	slightly rancid	
	olive oil		123	change but not rancid	
	tri-butyrin		123	no change	
	tri-olein		123	change but not rancid	
	checks		none	normal	
4	butter fat	Mixture of fine sawdust, with enough 1.5% peptone solution to make 16% of the amount of fat used, and enough fat to make distinctly oily; in test tubes.	20	rancid	
	corn oil		20	change but not rancid	
	olive oil		20	"	
	tri-butyrin		20	no change	
	tri-olein		20	slightly putrefactive	
	checks		none	normal	
5	butter fat	Emulsion of 0.5% agar solution, 0.2% peptone and 80.0% fat; in test tubes.	200	very rancid	
	corn oil		200	change but not rancid	
	olive oil		200	smells like lemon	
	tri-butyrin		200	odor is fainter	
	tri-olein		200	change but not rancid	
	checks		none	butter fat tallowy, others normal	
6	butter fat	Mixture of shredded filter paper dampened with 1.5% peptone solution and enough fat to make distinctly oily.	123	very rancid	
	corn oil		123	very tallowy	
	olive oil		123	slightly tallowy	
	tri-butyrin		123	no change	
	tri-olein		123	tallowy	
	checks		none	normal	

TABLE XXIII

ne Natural Fats to Rancidity Through the Action of Lipolytic Bacteria

	Trial II		Trial III		Trial IV	
	Culture	Defect	Culture	Defect	Culture	Defect
	41	very rancid	91	very rancid	21	rancid, cheesy
	none	normal	none	normal	none	normal
	91	very rancid	83	very rancid	42	very rancid
)	91	slightly putrid	83	change but not rancid	42	change but not rancid
)	91	change but not rancid	83	change but not rancid	42	change but not rancid
	none	normal	none	normal	none	normal
	83	very rancid				
ncid	83	tallowy				
not	83	change but not rancid				
	83	no change				
not	83	change but not rancid				
	none	normal				
	41	very rancid	91	very rancid	133	very rancid
not	41	musty potato	91	change but not rancid	133	change but not rancid
	41	"	91	"	133	"
	41	no change	91	no change		
	41	objectionable, not rancid	91	change but not rancid		
	none	normal	none	normal	none	normal
	83	very rancid				
not	83	change but not rancid				
	83	smells like lemon				
nter	83	odor is fainter				
not	83	change but not rancid				
	none	normal				
ers						
	41	very rancid				
	41	musty potato				
		slightly tallowy				
llowy	41	musty potato				
	41	no change				
	41	slightly tallowy				
	none	normal				

Cultures Used

20. Ps. fluorescens-var. zymogenes
21. Ps. fluorescens
41. Ps. mucidolens
42. Ps. fluorescens
83. Serratia type
91. A. lipolyticum
123. A. lipolyticum
133. A. lipolyticum
200. Serratia type

TABLE XXIIIa

Summary of Table XXIII

Fat used	Times each fat was used	Times each culture was used	Trials with each fat	Trials in which fat became rancid: Number	Trials in which fat became rancid: Percent	Trials in which fat became tallowy: Number	Trials in which fat became tallowy: Percent
butter fat	6	9	18	18	100.0	0	0.0
corn oil	5	8	14	1	7.2	3	21.7
olive oil	5	8	14	0	0.0	1	7.2
tri-butyrin	4	5	9	0	0.0	0	0.0
tri-olein	4	5	9	0	0.0	2	15.5
checks on all fats	6	0	24	0	0.0	1	4.2

1. Butter fat. With butter fat rancidity was produced by each of the nine lipolytic bacteria in every instance. The rancidity developed more slowly in one of the series with sawdust and with the filter paper series than with the liquid emulsions. The rate of the development of the rancid condition was about the same for all the organisms excepting culture 20, Ps. fluorescens var. zymogenes, which was less active than the other cultures.

2. Corn oil. Rancidity was produced in only one instance in corn oil, and this occurred in the solid mixture of sawdust in Series 3, through the action of culture 123, A. lipolyticum. The same culture produced tallowiness in 2 other trials with corn oil. Culture 41, Ps. mucidolens, produced slight tallowiness in 1 trial and a characteristic musty potato flavor and odor in 3 trials. Culture 83, a Serratia type, caused tallowiness in the sawdust mixture in Series 3. The other 4 cultures produced changes in corn oil but, like the other cultures with the exception of 123, did not cause rancidity in any trial.

3. Olive oil. Rancidity was not produced in olive oil by any of the 8 cultures in the 14 instances. Culture 123, A. lipolyticum, produced tallowiness in 1 trial and culture 41, Ps. mucidolens, produced a musty potato flavor and odor in every trial. The Serratia types, cultures 200 and 83, produced a distinct odor of lemon extract in olive oil in Series 5 while

the remaining 4 cultures brought about slight changes but no rancidity.

4. Tri-butyrin. Of the 5 cultures inoculated into media containing tri-butyrin, only the Serratia types, cultures 200 and 83, produced any change, and this occurred only in the fat-agar emulsion (Series 5). The 2 cultures caused a marked decrease in the characteristic odor of this simple tri-glyceride. There was no evidence of growth with the other organisms in any of the trials. The odor of butyric acid was never detected in the trials with tri-butyrin.

5. Tri-olein. Rancidity was not produced in tri-olein by any of the 5 cultures in the 9 instances. Cultures 123, A. lipolyticum, and 41, Ps. mucidolens, brought about tallowiness in the tri-olein - filter paper medium. Changes were produced in the other trials by the remaining 3 cultures used but rancidity was never detected.

6. Checks. The check sample on butter fat in Series 5 developed a tallowy flavor while the other 23 checks remained normal during the periods of observation.

The above summary indicates that rancidity developed regularly in all the artificial media containing butter fat but that it seldom, if ever, developed when the sources of fat were not butter fat. Tallowiness developed frequently with corn oil as the source of fat and occasionally with olive oil and tri-olein.

In general, tri-butyrin was least affected by the organisms used. The amount of protein (in the form of peptone) present apparently did not greatly influence the results but the looser media, which presumably were more aerated, appeared to favor the development of tallowiness.

Talloviness which developed in the butter fat check in Series 5 was not evident in the samples which were inoculated and therefore did not influence the results obtained.

DISCUSSION OF RESULTS

The study of the susceptibilities of some simple tri-glycerides and some natural fats and oils to the development of rancidity has brought out some very interesting facts which correlate with the results obtained in the other sections.

The cultures used were chosen because they had rapidly produced rancidity in butter when inoculated into the cream; it was therefore assumed, when they produced rancidity in all the artificial media containing butter fat, that the media were capable of supporting growth of the organisms.

In the 1 trial in which slight rancidity was produced in corn oil, culture 123, A. lipolyticum, was used; this is the only one of the cultures studied in Section I which hydrolyzed tri-palmitin. This culture also appeared to be the most effective in the production of tallowiness in corn oil, olive oil and tri-olein, especially in the presence of a large air supply. Since these fats contain entirely or very largely unsaturated acids (as tri-glycerides) they probably would have become rancid were tri-olein of any direct importance in the production of this defect. It is quite conceivable, however, that very actively lipolytic organisms under the most favorable conditions for growth could break down the mixed tri-

glycerides of the solid fatty acids or even the acids themselves to form substances which might be responsible for a slightly rancid condition.

Ps. mucidolens rapidly produced rancidity in the artificial media containing butter fat and also in butter and medium rich cream but not in skimmilk or in artificial media containing corn oil or olive oil. This indicates that in corn oil and olive oil, as also in skimmilk, the constituents which are responsible for rancidity in butter fat are either absent or present in very small amounts, and that rancidity is not likely to occur ordinarily in these substances as the result of the action of lipolytic bacteria. This culture also grew well in tri-olein but did not cause it to become rancid.

Two Serratia types were used which produced rancidity in butter fat; these cultures produced the unusual characteristic odor of lemon extract in olive oil. They were also the only cultures that brought about changes in tri-butyrin. The fact that they caused a decrease in the odor of media containing tri-butyrin apparently cannot be explained on the basis of hydrolysis unless all the butyric acid volatilized.

The results of the trials with tri-butyrin, regarding its inhibitory influence on the growth of bacteria, confirm previous observations in this investigation and are in agreement with the literature reported.

A final analysis of the results indicates clearly that (a) the artificial media used were suitable for the growth of the organisms providing the dispersed fat was not toxic to them, (b) lipolytic bacteria, in general, produce rancidity in butter fat and produce tallowiness or other defects in corn oil, olive oil and tri-olein under identical environmental conditions and (c) the media which permitted the most accessible air supply became tallowy most readily and most often.

SUMMARY

1. Lipolytic bacteria inoculated into artificial media produced rancidity in every instance with butter fat, once with corn oil and in no trials with olive oil, tri-olein or tri-butyrin.

2. The lipolytic organism which produced slight rancidity in corn oil also hydrolyzed tri-myristin and tri-palmitin. Tallowiness was the defect which most frequently accompanied the growth of lipolytic bacteria in corn oil and tri-olein.

3. The lipolytic cultures produced various defects in olive oil but rancidity was never evident.

4. The constituents of butter fat which are responsible for the development of rancidity with it are either not present in tri-olein and in the oils studied, or their decomposition products are present in quantities too small to be noticed in the presence of the oxidation products of oleic and closely related unsaturated acids.

GENERAL DISCUSSION

The work done with the Nile-blue sulfate technique indicates that it has an important application in the study of lipolytic bacteria. A study of the absorption of the Nile-blue sulfate dye by the fatty acids showed that caproic and caprylic acids became blue in the presence of the dye in much the same manner as oleic acid did. This indicates that Nile-blue sulfate is not specific for oleic acid as has been previously reported by some investigators.

A study of the effects, on lipolysis by bacteria, of the nutrients in the medium, the method of dispersing the fat and the pH of the medium showed that the test gave satisfactory results within a fairly wide range of conditions. It was evident, however, in agreement with some of the literature cited, that a rather alkaline reaction favors lipolysis by bacteria.

The application of the Nile-blue sulfate technique in determining the effect of lipolytic bacteria on some simple tri-glycerides and some natural fats showed that:

1. The ease of hydrolysis of the simple tri-glycerides of the saturated fatty acids decreased as the series was ascended.

2. Tri-olein was easily hydrolyzed by lipolytic bacteria.

3. The hydrolysis of simple tri-glycerides has an application in the differentiation of lipolytic bacteria since certain

species hydrolyzed more of the simple tri-glycerides, in the ascent of the series, than did other species.

4. The natural fat or oil dispersed in the medium did not appreciably affect the results of the test since practically all of the lipolytic organisms that hydrolyzed butter fat also hydrolyzed the other fats and oils used.

The isolation of lipolytic bacteria was easily carried out by the use of the Nile-blue sulfate technique while the type of reaction given by the test was a useful criterion for the identification and classification of these organisms. This was definitely shown by an examination of more than 5,000 colony growths of lipolytic cultures on Nile-blue sulfate, beef infusion agar, in which was dispersed one of many simple tri-glycerides or natural fats or oils.

The Nile-blue sulfate test was also used in the determination of the numbers of lipolytic bacteria in certain dairy products. Comparisons of the bacterial counts obtained on regular beef infusion agar and on Nile-blue sulfate medium indicated that the Nile-blue dye was often responsible for a lower count and inhibited particularly streptococcus and micrococcus types, and spore forming rods; these types, however, were only slightly, or definitely not, lipolytic. The Nile-blue sulfate dye appeared to have very little inhibitory effect on the gram negative non-spore forming rods which were most

actively lipolytic, and therefore it was considered suitable for use in a medium intended for the detection of the numbers of lipolytic bacteria which are of the greatest significance in dairy products.

The organisms which occurred most frequently in sources about a dairy plant, other than the dairy products, were types which produced greenish fluorescent colonies on beef infusion agar and were considered to belong to the genus Pseudomonas. The organisms most frequently isolated from certain dairy products were identified as Ps. fragi, A. lipolyticum, Ps. fluorescens with closely related varieties, A. connii with closely related types, Ps. acidiconcoquens, and micrococcus types.

Following the determination of the types of lipolytic bacteria and the relationship of these to rancidity in butter, an attempt was made to produce rancidity by lipolytic bacteria in artificial media containing one of the following fats: butter fat, corn oil, olive oil, tri-butyrin and tri-olein. The results of these trials indicated that lipolytic bacteria normally produced rancidity in butter fat, very seldom in corn oil and never in olive oil or tri-olein. The results obtained with tri-butyrin indicated that the growth of most organisms was inhibited by the concentrations used. It is important to note, however, that a slight odor of butyric acid

was observed from the growth of A. lipolyticum cultures on Nile-blue sulfate agar in which small quantities of tri-butyrin were dispersed. The hydrolysis of the tri-butyrin was proved definitely in the above trial by the disappearance of the fat globules and by the detection of a volatile fatty acid with moistened litmus paper.

In conclusion it should be appreciated that a considerable amount of work remains to be done on the detection and identification of the specific substance which is responsible for the characteristic odor and flavor of rancidity.

GENERAL CONCLUSIONS

1. The Nile-blue sulfate technique is very useful and has many applications in the study of lipolytic bacteria.

2. The hydrolysis of simple tri-glycerides, dispersed in Nile-blue sulfate agar, may be used as a criterion in the identification and the classification of lipolytic bacteria.

3. The type of colony and the type of lipolytic action produced on Nile-blue sulfate medium are very useful criteria in the grouping of lipolytic bacteria.

4. The numbers of actively lipolytic bacteria in dairy products are conveniently determined by the Nile-blue sulfate technique.

5. The numbers of weakly lipolytic bacteria, including micrococcus and streptococcus types, and spore forming rods are not very accurately determined by this method since the Nile-blue sulfate dye has an inhibitory effect on these organisms.

6. There are considerable numbers of lipolytic bacteria in average fresh milk and cream but not in aseptically drawn milk.

7. Larger numbers of lipolytic bacteria are found in

unsalted than in salted butter.

8. The lipolytic bacteria most commonly isolated from dairy equipment, wash water and other materials, and from certain dairy products, are Pseudomonas fluorescens, Pseudomonas fragi, Achromobacter lipolyticum, Achromobacter Conni and micrococcus types.

9. The actively lipolytic bacteria studied do not tolerate more than 1.0 percent salt in butter of average composition.

10. Micrococci are more salt tolerant than the more actively lipolytic types of bacteria.

11. More rapid increases of lipolytic bacteria are shown in surface than in sub-surface portions of butter.

12. A strongly rancid condition in butter causes decreases in the numbers of lipolytic bacteria.

13. Rancidity is a defect which is specific for butter fat and which is rarely produced by lipolytic bacteria in other fats and oils and never in tri-olein.

14. Tri-butyrin and butyric acid in very small concentrations inhibit bacterial growth.

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