'HE LECITHIN CONTENT OF BUTTER AND ITS POSSIBLE RELATIONSHIP TO THE FISHY FLAVOR

A THESIS

Presented to the Faculty of the Graduate School of Cornell University for the Degree of

DOCTOR OF PHILOSOPHY

BY

GEORGE CORNELL SUPPLEE

[Reprint from the Cornell University Experiment Station Memoir 29, November, 1919]

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THE LECITHIN CONTENT OF BUTTER AND ITS POSSIBLE RELATIONSHIP TO THE FISHY FLAVOR[1]

George Cornell Supplee

The commercial value of butter is based to a great extent on its quality, which in turn is determined by its flavor. The commercial grading of butter on this basis, and the recognition of certain characteristic defects, have resulted in the establishment of certain terms more or less descriptive of the flavors found. Among the terms commonly applied to the flavors in butter are such words as *metallic, fishy, oily, rancid, tallowy.* Since the presence of any of these flavors carries with it a reduction in commercial value of the butter, considerable effort has been made to determine their causes and prevent their development. Unfortunately most of these efforts have not met with a high degree of success. This may be ascribed to several reasons, among which are the following: lack of positive identification of the same flavor by different investigators; lack of adequate chemical methods for the isolation and measurement of the small amount of substance capable of producing the flavor; and lack of cooperation between the chemist, the bacteriologist, and the experienced butter judge.

Fishiness in butter, with which this investigation is primarily concerned, is usually described as a flavor resembling that of salmon or mackerel, altho the names of other varieties of fish are occasionally used to describe the flavor more explicitly. While the typical fishy flavor in butter is readily recognized by experts, it is often accompanied by a more or less oily condition which tends to create differences of opinion as to its exact nature. But if the opinion of butter judges of long experience is to be considered as trustworthy, it may be said that the true fishy flavor is entirely distinct from the oily flavor even tho the oily condition may precede or accompany it.

PREVIOUS INVESTIGATIONS

The earlier investigations bearing on fishiness in dairy products have been largely confined to milk and butter. One instance is recorded, however, in which this condition was observed and studied in evaporated milk.

[1] Also presented to the Faculty of the Graduate School of Cornell University, December, 1918, as a major thesis in partial fulfillment of the requirements for the degree of doctor of philosophy.

Harding, Rogers, and Smith (1900)[2] report the fishy flavor in a sample of milk brought to the New York State Agricultural Experiment Station at Geneva in 1900. The source was traced to a single animal in the herd, but investigation failed to locate any pathological condition or any irregularity in the feeding which might cause the trouble. Attempts to reproduce the flavor by inoculating milk with bacteria isolated from this cow's udder also failed. The same authors mention also an instance brought to their attention by W. E. Griffith, in which a peculiar flavor developed in June butter after storage at 18° to 22° F. This flavor was described by butter experts as fishy.

Piffard (1901) discusses the fishy flavor in dairy products, and suggests the possible relationship between certain algae found in stagnant water, and fishiness in milk and butter. His theory is supported by the fact that the flavor is often produced in water by the growth of algae and diatoms, and he believes, therefore, that cows having access to such water may transmit the condition to milk. Referring to the flavor in butter, he considers the idea that salt may be responsible and mentions the ability of salt to absorb flavors and odors of materials stored near it.

Harrison (1902), discussing butter defects at about the same time, states that the characteristic off-flavors of butter — fishy butter being specifically mentioned — are caused by the growth of undesirable bacteria in the cream.

O'Callaghan (1902) published certain observations on fishiness in Australian butter. He states that he has found this condition in butter only two hours old. From his investigations he concludes that *Oïdium lactis* is the causal agent. Later (O'Callaghan, 1908) he elaborated on his former views, concluding that *Oïdium lactis* associated with the lactic-acid bacteria in cream will usually produce a fishy flavor in the butter. He mentions the presence of the defect in unsalted butter, and recommends the improvement of sanitary conditions in the creameries, and pasteurization, as a remedy. His conclusions have not been confirmed by other investigators.

Rogers (1909), after a rather exhaustive study of the occurrence and cause of fishy butter, confirms many observations commonly noted in connection with this trouble but is unable to confirm the observations of O'Callaghan. He also seemingly eliminates the theory that trimethyl-

[2] Dates in parenthesis refer to *Bibliography*, page 150.

amine is directly responsible for the flavor. After studying the effect of high-acid cream, overworking, and the consequent increased oxygen content of the butter, and by conducting bacteriological investigations, he concludes that high-acid cream is essential to bring about the condition, altho he points out that not all butter made from such cream develops the fishy flavor. In this respect he states (page 20 of reference cited) that "fishy flavor may be produced with reasonable certainty by overworking the butter made from sour cream." From his viewpoint the probability that microorganisms are the cause falls into disfavor by the advancement of the opinion that "fishy flavor is caused by a slow, spontaneous, chemical change to which acid is essential and which is favored by the presence of small amounts of oxygen" (page 20 of reference). More recently the same author (Rogers, 1914, a and b) points out that fishiness in butter may be preceded by an oily or a metallic flavor, and reiterates his views that the evidence is against the theory that the fishy condition is of a bacterial nature. He also states (1914 b) that "fishy flavor is said to occur rarely or not at all in unsalted butter and it is possible that the salt furnishes certain conditions which are essential to the development of the flavor."

Reakes, Cuddie, and Reid (1912) find no significant differences in the bacterial flora of fishy and of high-grade butter, and, in agreement with Rogers, state that "the development of fishy flavour in butter arises as a result of a chemical change inducing a splitting-up of some of the constituents into compounds possessing this peculiar character of smell and taste, the factors responsible for such change being apparently a degree of high acidity of the cream and overworking."

Hunziker (1916) states that high pasteurization temperatures (185° F.) when used on sour cream tend to produce a very poor quality of butter, which often has a disagreeable oily taste suggestive of fishiness. He points out that this is particularly true when cows are on green pasture and the butterfat contains a rather high percentage of olein, which may be oxidized with relative ease in the presence of high temperatures and high acid.

Hammer (1917) reports that he found a can of evaporated milk which possessed a marked fishy flavor and odor and from which he was able to isolate an organism heretofore undescribed. He gives to this organism the name *Bacterium ichthyosmius*, which was suggested by Dr. A. W.

Dox. The description of the organism seems to indicate that it is closely allied to the Proteus group. By inoculation experiments Hammer was able to reproduce the flavor in milk and cream under both aerobic and anaerobic conditions. He noted that the intensity of the odor was increased by the addition of alkali to the milk after the incubation period. He was unable, however, to produce fishiness in butter by direct inoculation or by inoculating the cream before churning. Bacteria counts at various intervals during the storage period showed an immediate decrease in numbers in salted butter, and an increase during the first few days in unsalted butter followed by a pronounced decrease.

Washburn and Dahlberg (1918), while studying the influence of salt on storage butter, found that salted butter was more likely to turn fishy in storage than was unsalted butter, and furthermore that there appeared to be a tendency toward a progressive development of the flavor thru metallic to oily and finally to fishy.

LECITHIN DECOMPOSITION IN BUTTER AS A POSSIBLE CAUSE OF THE FISHY FLAVOR

CHEMICAL CONSTITUTION, PROPERTIES, AND DISTRIBUTION OF LECITHIN

Lecithin, which stands in close relation to the fats, belongs to a more or less definite group of substances known as phosphatides, or phosphorized fats. These bodies appear to be a group of esters containing nitrogen, phosphoric acid, and fatty-acid radicals. Lecithin, which is the best known of the phosphatides, contains two fatty-acid radicals and the nitrogenous base choline, combined with glycerophosphoric acid. According to the kind of fatty acid present in the molecule, it is possible to have various types of lecithin, such as stearyl, palmityl, and oleyl. A number of investigators seem to agree that every true lecithin contains at least one oleic-acid radical. There seems to be uncertainty as to whether choline is the only base present in lecithin. MacLean (1909) was able to get only 42 per cent of the theoretical amount from lecithin isolated from heart muscle, and 65 per cent from lecithin of egg yolk. Other investigators have found the same to hold true of lecithin from different sources.

Lecithin has certain properties in common with the fats, particularly with respect to its solvents. It differs, however, by being less soluble in ether and more soluble in alcohol. It is precipitated from alcoholic solution by acetone; in water it swells to a colloidal mass which on microscopic examination appears as oily drops and threads. It saponifies with alkalies and baryta water, yielding the corresponding soaps, salts of glycerophosphoric acid, and choline. Hammarsten and Hedin (1915) state that it is slowly decomposed by dilute acids and enzymes (lipase). Barger (1914) states that *Bacterium prodigiosus* produces trimethylamine from choline and lecithin; he also cites references to show that lecithin is decomposed during putrefaction, yielding fatty acids, glycerophosphoric acid, choline, and ultimately trimethylamine. Hasebroek (1888) claims that methylamine, ammonia, methane, and carbon dioxide may be finally produced from choline during putrefaction. On being heated with strong caustic soda or potash, lecithin yields trimethylamine, which has a distinct fishy odor, this being one of the characteristic qualitative tests for it. Leathes (1913), in citing the work of various investigators, seems to think that lecithin is rather unstable. He thinks this property is due to the unsaturated oleic-acid radical contained, and offers this as the reason why the substance gives Pettenkofer's reaction. The work of Long (1908), however, seems to indicate that lecithin is more stable than has been generally believed. Koch (1902–03) has shown that various salts will cause lecithin to precipitate as a gelatinous mass, and that acids, if sufficiently dissociated (0.005 M sulfuric), will accomplish the same thing.

Lecithin seems to be widely found in nature, being present in many plant cells and animal fluids. It is particularly abundant in the brain, the nerve tissue, and the yolk of egg. It is also reported as existing in blood corpuscles, blood plasma, lymph, milk, and bile. Since the methods used for the quantitative estimation of lecithin depend on the determination of phosphorus in alcoholic or alcohol and ether extracts, it is doubtful whether the figures given are absolutely correct due to the fact that other phosphatides are extracted and also because the empirical formula used in the calculation may be inaccurate for the particular lecithin involved. Altho there have been conflicting statements as to whether milk contains lecithin, there seems to be sufficient evidence that it does. The results obtained by Nerking and Haensel (1908) are submitted in table 1:

TABLE 1. THE LECITHIN CONTENT OF VARIOUS MILKS
(From Nerking and Haensel)

Kind of milk	Percentage of lecithin		
	Highest	Lowest	Average
Human, 10 samples	0.080	0.024	0.050
Cow's, 17 samples	0.116	0.036	0.063
Ass's, 6 samples	0.039	0.006	0.016
Ewe's, 4 samples	0.167	0.051	0.083
Goat's, 11 samples	0.075	0.036	0.049
Mare's, 8 samples	0.017	0.007	0.011

Glikin (1909), studying the lecithin and iron content of milk, reports 0.0515 per cent lecithin in whole milk, 0.05 per cent in cream, and 0.1329 per cent in human milk. Fetzer (1911), studying the lecithin content of milk under pathological conditions, finds that it is lower in milk from cows suffering with mastitis than in milk from normal cows. He finds also that the lecithin content decreases as the fat decreases. The work of Bordas and De Raczkowski (1902) indicates that the amount of lecithin varies with the lactation period. They find that it is at the maximum at the beginning, and gradually decreases during the remainder of the period. Their observations were from seven cows.

THEORETICAL DISCUSSION

Trimethylamine as a decomposition product of lecithin was brought to the attention of the writer as a possible cause of the fishy flavor in butter about three years ago, when he was working with lecithin isolated from brain tissue. Altho there seem to be no published data concerning trimethylamine in relation to this subject, and Rogers (1909) claims that it can be worked into butter in large amounts without producing the fishy flavor, it is nevertheless believed by many that this substance is in some way responsible. On boiling lecithin isolated from brain tissue and egg yolk with strong caustic soda, the writer has been able to obtain a distinct oily and fishy odor which was asserted by many to be typical of the odor of fishy butter. The only possible source of such an odor in this case was the trimethylamine derived from the lecithin. This result, together with the fact that there seems to be good evidence that lecithin is present in milk, led to the assumption that the substance may exist also in butter and that by its decomposition it can slowly liberate trimethylamine with the consequent production of the fishy flavor and odor.

Provided that lecithin can be shown to exist in butter, the above assumption is supported by several facts. It is well known that enzymes are capable of bringing about many chemical decompositions which result from the action of acids and alkalies on organic substances. It also appears to be a fact that butter may contain enzymes derived from the udder, and from the action of bacteria in the milk or the cream before it is made into butter, and furthermore that the activity of such enzymes is not entirely stopped at the temperature at which butter is stored. Hammarsten and Hedin (1915) state that lecithin is decomposed by dilute acids and enzymes. The citations of Hasebroek (1888), Barger (1914), and others show that lecithin is decomposed by bacteria yielding choline, which finally yields trimethylamine. It is also a well-known fact that this substance in very small amounts possesses a distinct fishy odor, but in concentrated solution it has a strong ammoniacal odor. Speaking of the former property, Davis (1912), quoting Taylor, states that the " odour [referring to the peculiar fishy odor suggestive of herring brine] is gradually developed by adding lime to a solution of the base, but requires some time to reach its maximum intensity."

In addition to the foregoing facts the writer has observed certain features that may have some bearing on this problem. In inspecting butter used in the Navy, it has been noticed that certain samples of cream evolved a peculiar fishy odor on the addition of alkali used for titration. This phenomenon was first brought to the attention of the writer by A. M. Besemer, and has since been confirmed by a number of men, some of whom have wide reputation as butter judges. Since trimethylamine is a base which is liberated from its acid combinations by alkalies, it is quite possible that the odor mentioned above was due to this substance's having been liberated from its acid combination in the cream. If such were the case, it is conceivable that butter made from such cream might, during storage, give up its trimethylamine thru the action of enzymes. In this connection it has been noted that certain samples of old butter, which were not scored as fishy, when brought into contact with a warm solution of soap powder would give off a strong herring-like odor. This phenomenon might also be explained as in the case of the cream. In addition to these features it has been noted that certain samples of fishy butter may lose their characteristic flavor after a period of time. This has also been observed by other investigators. It is possible that this characteristic may be explained by the fact that, since trimethylamine is extremely volatile, it may pass off, or that the instability of the acid combination changes so that the conditions are not right for its manifestation. The writer has noted a very strong fishy odor in partially decomposed egg yolk held at refrigerator temperature, which had entirely disappeared two weeks later.

On the basis of the foregoing facts and observations and the evident lack of contradiction of most of them with what is known about fishy butter, the following experimental work was planned with the object of determining the possible relationship of trimethylamine to this flavor. In calling attention to the lecithin, it may be stated that the writer is cognizant of the fact that trimethylamine may be produced from other substances. This material has been chosen as the object of study primarily because there is exact knowledge concerning its cleavage and some of the agencies bringing this condition about.

INVESTIGATIONAL WORK

QUALITATIVE DETERMINATION OF LECITHIN IN BUTTER

The first experimental work undertaken was to demonstrate the presence of lecithin in butter, since there appeared to be no reports on this point in the literature. One hundred grams of melted butter was thoroly mixed with sufficient anhydrous calcium sulfate (about one kilogram) so that the mixture retained its dry powdered form to such an extent that it could be readily sifted between the thumb and the finger. The mixture was transferred to a specially constructed percolator and extracted for 48 hours with 95-per-cent alcohol at 60° C. The alcoholic extract was evaporated down and the residue was treated with a small amount of ether, which took up the fat, the fatty acids, and part of the lecithin. The part insoluble in ether was again taken up with warm alcohol, and what may be termed the *lecithin fraction* was precipitated by thoroly cooling the alcoholic solution. The substance thus obtained precipitated in the form of small, wart-like masses, which clung tenaciously to the sides of the beaker. On this material, which presumably contained a high proportion of lecithin, various observations were made and qualitative tests applied. The following characteristics were noted:

On drying at ordinary temperature and pressure the material appeared as a semi-amorphous and oily substance of a pale, dirty yellow color. It was entirely soluble in alcohol but was partially thrown out of solution by the addition of an excess of ether. The precipitate formed in this manner was finely granular and was white in color. In water it formed a semi-colloidal solution which on microscopic examination appeared as minute oily drops. When the watery suspension was heated, the particles would cohere to form a sticky mass which changed to a distinct brown color. Both the dry substance and the watery suspension, when heated with strong caustic soda, gave off a marked fishy odor resembling sometimes dried herring and sometimes salmon oil. This observation was in the great majority of cases confirmed by a number of colleagues. The fishy odor obtained from the material in this manner seemed to furnish positive evidence that lecithin was present. To further strengthen this belief, Pettenkofer's test with sugar and sulfuric acid was applied to the dry material with positive results. The above observations were confirmed with lecithin extracted from fresh butter, salted and unsalted, and from other miscellaneous samples of normal butter.

Altho the evidence that lecithin exists in butter in detectable quantities seemed conclusive, it was decided to determine, if possible, the presence of choline, which, as already pointed out, is one of the components of the lecithin molecule. This was accomplished by boiling the residue of the first alcoholic extract with baryta water, which removed all fat, fatty acids, and fatty-acid radicals of the lecithin in the form of barium soaps. After the barium soaps were filtered off, the excess barium was removed with carbon dioxide, the barium carbonate filtered off, and the filtrate containing choline and barium glycerophosphate evaporated to a sirupy consistency. This residue was then treated with absolute alcohol, in which choline is soluble but barium glycerophosphate is insoluble. On evaporation of the absolute alcohol a small amount of sirupy substance remained. To this material qualitative tests for choline were applied. The most characteristic of such tests is the periodide test described by Staněk (1905), which is made by adding a small amount of strong iodine solution (153 grams of iodine and 100 grams of potassium iodide in 200 grams of water) to an aqueous solution of choline. A positive test is indicated by the formation of a brown precipitate of choline periodide, which on microscopic examination in the presence of the reagent appears as dark brown refractive and notched prisms or rhomboidal leaflets. On evaporation of the reagent the crystals lose their shape and appear to liquefy, forming brown, oily droplets which again assume their crystalline structure on the addition of more reagent. On the application of this test to the choline obtained from butter lecithin, it was found that the results conformed in all respects to the descriptions of this periodide. The accompanying plate of photomicrographs (Plate VI) shows the characteristic crystals and oily droplets of the periodide formed by the choline from butter. In addition to this test it was shown that the small amount of choline obtained would give off a slight but distinct fishy odor on being heated with solid caustic soda.

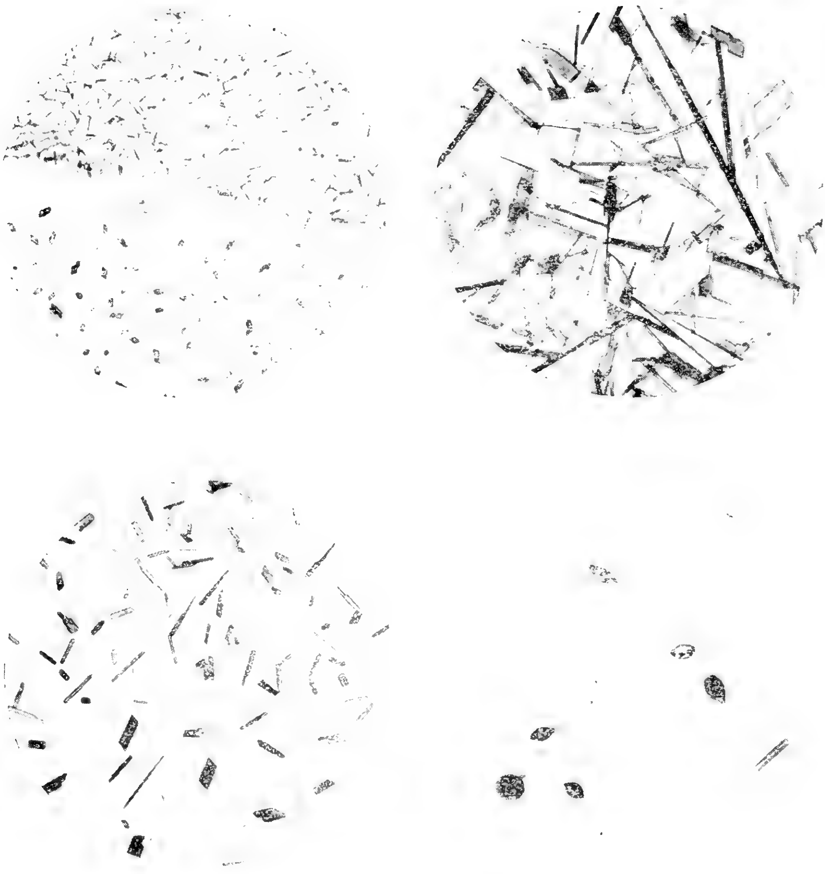
AMOUNT OF LECITHIN IN BUTTER

Since the qualitative tests seemed to leave no room for doubt as to the presence of lecithin in butter, the next step was the quantitative estimation of this substance. In view of the evident difficulty in securing an absolutely pure lecithin free from other phosphatides, the estimations were based on the phosphorus content of extracts and the amount of lecithin calculated according to the formula of the distearyl type. The results of such determinations on various types of butter made from different lots of cream are shown in table 2:

TABLE 2. LECITHIN CONTENT IN VARIOUS BUTTERS*

Sample	Type of cream from which butter was made	Age of butter (days)	P_2O_5 (per cent)	Lecithin, distearyl type (per cent)
1	Raw sweet	6	0.0127	0.0723
2	Pasteurized sweet	6	0.0127	0.0723
3	Raw ripened	6	0.0122	0.0693
4	Pasteurized ripened	6	0.0075	0.0433
5	Raw sweet	48	0.0092	0.0522
6	Pasteurized sweet	48	0.0120	0.0682
7	Raw ripened	48	0.0086	0.0488
8	Pasteurized ripened	48	0.0083	0.0471
9	Raw sweet	72	0.0111	0.0631
10	Pasteurized sweet	72	0.0089	0.0505
11	Raw ripened	72	0.0083	0.0471
12	Pasteurized ripened	72	0.0095	0.0540

* These figures were furnished by J. T. Cusick, chemist for the State Department of Agriculture, located at Cornell University.



CHOLINE PERIODIDE CRYSTALS OBTAINED FROM CHOLINE OF BUTTER LECITHIN

Photomicrographs, × 380

A study of table 2 shows a fairly constant lecithin content in butter from various lots of cream and in different types of butter made from the same lot of cream. There is one feature, however, which is worthy of note, and this is that in most instances there is a tendency toward a lower lecithin content in the ripened-cream butter than in that made from unripened cream. This may be significant in the light of the statement by Hammarsten and Hedin (1915), that lecithin is decomposed by dilute acids and enzymes. This fact applied to these results might indicate that the acidity of the cream slowly decomposed the lecithin, and its decomposition products, particularly the glycerophosphoric acid, were washed out with the buttermilk. If such were the case it would be very easy to account for the lower phosphorus content in sour-cream butter.

TRIMETHYLAMINE SALTS OF THE FATTY ACIDS

It was decided that before an attempt was made to correlate trimethylamine with the fishy flavor of butter, this substance should be prepared in a pure state and those characteristics determined which might have a bearing on this particular problem. Trimethylamine was made by heating 50 grams of ammonium chloride and 440 grams of a 40-per-cent solution of formaldehyde in the autoclave at 122° C. for thirty minutes. Any excess formaldehyde was then expelled and the trimethylamine liberated from its hydrochloride by distilling from an alkaline solution. A 10-per-cent solution was easily obtained at the ordinary temperature and pressure. The trimethylamine thus procured was combined with lactic, butyric, oleic, and stearic acids, and also with the mixed soluble and insoluble fatty acids obtained from butter according to the procedure outlined by Browne (1899). While the properties of these acid addition products seemed to offer an interesting field for study, only such of their characteristics are recorded here as might have a direct relationship to the fishy flavor in butter, namely, their stability, volatility, and behavior in the presence of sodium chloride.

The lactic-acid combination with trimethylamine proved to be a relatively stable oily liquid possessing no characteristic taste other than that shown by many common salts. The odor, especially after the liquid had been standing in a stoppered bottle, seemed to be slightly fishy. Evidence on this point is not conclusive because it is possible that this was due to excess trimethylamine added at the time of neutralization and not shown by the indicator used. Heating seemed to intensify the odor to some extent, which would indicate instability at high temperatures.

The butyric-acid combination with trimethylamine was a substance extremely volatile at ordinary temperatures. The odor greatly resembled fish oil; the taste resembling this product was manifest only when very small amounts were used, and then not regularly.

Oleic acid and trimethylamine formed a soft soap which was very unstable as evidenced by the liberation of the trimethylamine; the greater the dilution, however, the less this condition was manifested. This soap could not be obtained entirely free from water, even at the ordinary temperatures, because of the simultaneous giving-off of trimethylamine and water. This liberation was such that nothing remained but the free acid. Furthermore, on the addition of sodium chloride to its water solution, the sodium ion readily replaced the trimethylamine radical, with the consequent precipitation of the sodium soap and the formation of trimethylamine hydrochloride.

The trimethylamine stearate showed the same characteristics as the oleic soap, but to an even greater extent. Trimethylamine was constantly given off in large quantities, and the only way in which it could be handled as a soap was in a mixture of alcohol and water in a tightly stoppered bottle.

The mixed soluble and insoluble fatty acids combined with trimethylamine showed the same general characteristics as the butyric and stearic combinations, respectively.

The instability of the combinations of fatty acid and trimethylamine can undoubtedly be accounted for by the fact that they are addition products in which the trivalent nitrogen of the latter substance changes to the pentavalent condition in the presence of an acid. The fact that these are weak acids with relatively large molecules is probably also significant. It was observed that the hydrochloride was more stable than the above salts, and that the sulfate was even more stable than the hydrochloride. This instability of the fatty-acid combinations and their reaction in the presence of sodium chloride may have an important bearing on the relation of trimethylamine to fishiness in butter, and may be of particular significance in explaining why the flavor is usually found in salted butter. As yet, however, the relationship is not clear.

EFFECT OF WORKING TRIMETHYLAMINE SALTS OF THE FATTY ACIDS INTO BUTTER

The character of the trimethylamine salts of the fatty acids in pure state seemed to justify the following series of experiments, in which these salts are incorporated into various types of butter for the purpose of determining the possibility of their producing the fishy flavor in the presence of butterfat. In view of the desirability of incorporating the trimethylamine in logical amounts, the following plan was adopted:

The largest quantity of lecithin reported in cow's milk by Nerking and Haensel (1908) was used as the basis of calculation. These authors report 0.116 per cent as the largest amount found in seventeen samples. For the calculations of this experiment, this was assumed to be lecithin of the distearyl type, and it was further assumed to be pure lecithin with the empirical formula assigned to the type named. Granting these assumptions, this amount would yield on complete decomposition the equivalent of 85 parts per million of trimethylamine. This substance alone or in acid combination was therefore added to cream, wash water, or butter on this basis. It is very evident that because of the volatility of some of the materials and because of mechanical loss, none of the samples of butter when completed would contain 85 parts per million of trimethylamine. The method of arriving at the quantity to be added seemed to afford a uniform basis and to approximate in a logical manner the amount of this substance that might be produced in butter. When the fatty acids were used alone they were added in quantities equivalent to the amounts added in the corresponding trimethylamine salts. The addition of the acids was merely for the purpose of checking against the trimethylamine.

In tables 3 to 9 inclusive are shown the comments of various judges on different types of butter containing trimethylamine added as already indicated and incorporated by various means. Because of the great importance of the personal factor in judging butter, an effort was made in all cases to get a number of men familiar with the various flavor defects of the product. In all cases the samples were so labeled that the judges had no knowledge of their contents. They were instructed to comment on the flavor and to work independently of one another, and it is believed that this injunction was carried out. In presenting the results in tabular form the author has intentionally omitted comments having no direct bearing on the fishy flavor.

TABLE 3. EFFECT ON THE FLAVOR OF BUTTER, OF ADDING TRIMETHYLAMINE AND FATTY ACIDS TO RAW SWEET CREAM WITH 0.23 PER CENT ACID AT THE RATE OF 85 PARTS PER MILLION OF THE FORMER

(*S* indicates salted butter)

Sample	Material added	Comments by judges					
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
AS	Nothing	......	......	......	Oily	Oily	......
A	Nothing	......	Oily	......	......	Oily	......
1 AS	Trimethylamine	Oily	Fishy	......	Fishy	......	Fishy
1 A	Trimethylamine	Fishy	......	......	......	......	......
2 AS	Trimethylamine lactate	......	......	......	......	......	......
2 A	Trimethylamine lactate	Fishy	Oily	Fishy	Fishy	......	......
3 AS	Trimethylamine butyrate	Fishy	Fishy	Fishy	......	......	......
3 A	Trimethylamine butyrate	Fishy	Oily	......	Fishy	......	......
4 AS	Trimethylamine oleate	Fishy	......	......	Fishy	......	......
4 A	Trimethylamine oleate	......	......	......	......	......	......
5 AS	Trimethylamine stearate	Fishy	......	......	......	......	......
5 A	Trimethylamine stearate	Fishy	......	......	......	......	......
6 AS	Trimethylamine and soluble fatty acids	......	......	Fishy	Fishy	......	Fishy
6 A	Trimethylamine and soluble fatty acids	Fishy	Fishy	Oily	......	......	......
7 AS	Trimethylamine and insoluble fatty acids	......	Fishy	Fishy	Fishy	......	Fishy
7 A	Trimethylamine and insoluble fatty acids	......	Oily	......	......	......	Oily
8 AS	Lactic acid	......	......	......	Fishy	......	......
8 A	Lactic acid	Fishy	Oily	......	......	......	......
9 AS	Butyric acid	Fishy	......	......	......	......	......
9 A	Butyric acid	Fishy	......	......	Oily	......	......
10 AS	Oleic acid	......	Fishy	Fishy	Fishy	......	......
10 A	Oleic acid	......	......	......	......	......	......
11 AS	Soluble fatty acids	......	......	......	......	......	......
11 A	Soluble fatty acids	......	......	......	......	......	......
12 AS	Insoluble fatty acids		......	......	......	......	......
12 A	Insoluble fatty acids		......	......	......	......	......

TABLE 4. EFFECT ON THE FLAVOR OF BUTTER, OF ADDING TRIMETHYLAMINE AND FATTY ACIDS TO RAW SWEET CREAM WITH 0.23 PER CENT ACID AT THE RATE OF 85 PARTS PER MILLION AND THEN WASHING THE BUTTER IN WATER CONTAINING THE SAME CONCENTRATION OF THE VARIOUS SUBSTANCES

(*S* indicates salted butter)

Sample	Material added	Comments by judges					
		No. 1	No. 2	No. 3	No. 4	No. 5	No 6
BS	Nothing	......	......	......	Oily	Oily	......
B	Nothing	......	Oily	......	......	Oily	......
1 BS	Trimethylamine	......	Fishy	......	Fishy	Oily	Fishy
1 B	Trimethylamine	......	......	Fishy	......	Fishy	......
2 BS	Trimethylamine lactate	......	Oily	......	Fishy	Oily	Fishy
2 B	Trimethylamine lactate	Fishy	Oily	Fishy	Fishy	......	......
3 BS	Trimethylamine butyrate	Fishy	Oily	Fishy	......	......	Fishy
3 B	Trimethylamine butyrate	Fishy	......	......	Fishy	......	Fishy
4 BS	Trimethylamine oleate	Fishy	......	......	Fishy	......	......
4 B	Trimethylamine oleate	Fishy	......	......	......	......	Fishy
5 BS	Trimethylamine stearate	Fishy	Oily	Fishy	Fishy	......	Fishy
5 B	Trimethylamine stearate	Fishy	......	......	Fishy	......	Fishy
6 BS	Trimethylamine and soluble fatty acids	Fishy	Fishy	......	Fishy	Oily	Fishy
6 B	Trimethylamine and soluble fatty acids	Fishy	......	......	......	......	Fishy
7 BS	Trimethylamine and insoluble fatty acids	Fishy	Oily	Fishy	Fishy	......	Fishy
7 B	Trimethylamine and insoluble fatty acids	Fishy	......	......	Fishy	......	......
8 BS	Lactic acid	Fishy	......	......	......	......	......
8 B	Lactic acid	Oily	......	......	......	......	......
9 BS	Butyric acid	......	......	......	Oily	......	......
9 B	Butyric acid	......	Fishy	......	Oily	......	......
10 BS	Oleic acid	......	Oily	......	Fishy	......	......
10 B	Oleic acid	......	......	Fishy	......	......	......
11 BS	Soluble fatty acids	......	......	......	......	......	......
11 B	Soluble fatty acids	......	......	......	......	......	......
12 BS	Insoluble fatty acids	......	......	......	......	......	......
12 B	Insoluble fatty acids	......	......	......	......	......	......

TABLE 5. EFFECT ON THE FLAVOR OF BUTTER, OF ADDING TRIMETHYLAMINE AND FATTY ACIDS TO RAW SWEET CREAM WITH 0.23 PER CENT ACID AT THE RATE OF 85 PARTS PER MILLION AND THEN WORKING THE SUBSTANCES DIRECTLY INTO THE BUTTER AT THE SAME RATE

(*S* indicates salted butter)

Sample	Material added	Comments by judges					
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
CS	Nothing	......	......	......	Oily	Oily	......
C	Nothing	......	Oily	......	......	Oily	......
1 CS	Trimethylamine	Fishy	Fishy	Fishy	Fishy	Fishy	Fishy
1 C	Trimethylamine	......	Fishy	......	Fishy	Fishy	Fishy
2 CS	Trimethylamine lactate	Fishy	Fishy	Fishy	......	Fishy	Fishy
2 C	Trimethylamine lactate	Fishy	Fishy	Fishy	Fishy	Fishy	Fishy
3 CS	Trimethylamine butyrate	Fishy	Fishy	Fishy	Fishy	......	Fishy
3 C	Trimethylamine butyrate	Fishy	Fishy	Fishy	......	Fishy	Fishy
4 CS	Trimethylamine oleate	......	......	Fishy	Fishy	......	......
4 C	Trimethylamine oleate	Fishy	Fishy	Fishy	Fishy	......	......
5 CS	Trimethylamine stearate	Fishy	......	......	Fishy	Fishy	Fishy
5 C	Trimethylamine stearate	Fishy	......	......	Fishy	......	Fishy
6 CS	Trimethylamine and soluble fatty acids	Fishy	......	Fishy	......	Fishy	......
6 C	Trimethylamine and soluble fatty acids	Fishy	......	Fishy	......	Fishy	......
7 CS	Trimethylamine and insoluble fatty acids	Fishy	......	Fishy	Fishy	Fishy	Fishy
7 C	Trimethylamine and insoluble fatty acids	Fishy	......	......	Fishy	Fishy	Fishy
8 CS	Lactic acid	Oily	......	Fishy	......	......	......
8 C	Lactic acid	......	......	......	......	......	......
9 CS	Butyric acid	Fishy	Fishy	......	......	......	......
9 C	Butyric acid	Fishy	......	......	Fishy	......	......
10 CS	Oleic acid	......	......	Fishy	Fishy	......	......
10 C	Oleic acid	......	......	Fishy	Fishy	......	......
11 CS	Soluble fatty acids	......	......	......	......	......	......
11 C	Soluble fatty acids	......	......	......	......	......	......
12 CS	Insoluble fatty acids	Oily	......	......	......	Oily	......
12 C	Insoluble fatty acids	Oily	......	......	......	......	......

TABLE 6. EFFECT ON THE FLAVOR OF BUTTER MADE FROM PASTEURIZED RIPENED CREAM WITH 0.32 PER CENT ACID, WORKING TRIMETHYLAMINE AND FATTY ACIDS DIRECTLY INTO THE BUTTER AT THE RATE OF 85 PARTS PER MILLION

(*S* indicates salted butter)

Sample	Material added	Comments by judges					
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
DS	Nothing		......	......	.	......	......
D	Nothing			..		......	......
1 DS	Trimethylamine		Fishy	Fishy	Fishy	Fishy	......
1 D	Trimethylamine		......	Fishy	Fishy	Fishy	......
2 DS	Trimethylamine lactate	......	......	......	......	......	......
2 D	Trimethylamine lactate	......	......	......	......	......	......
3 DS	Trimethylamine butyrate	Oily	Fishy	......	Oily	Fishy	Fishy
3 D	Trimethylamine butyrate	......	Fishy	......	Oily	Fishy	......
4 DS	Trimethylamine oleate		.	......	......	......	......
4 D	Trimethylamine oleate	......	..	......	.	......	......
5 DS	Trimethylamine stearate		..	......	Fishy	Fishy	......
5 D	Trimethylamine stearate	Oily	......	.	Oily	Fishy	......
6 DS	Trimethylamine and soluble fatty acids		..	Fishy		......	......
6 D	Trimethylamine and soluble fatty acids	......	......	......		......	......
7 DS	Trimethylamine and insoluble fatty acids	......	Fishy	......		......	......
7 D	Trimethylamine and insoluble fatty acids	......	Fishy	..		..	......
8 DS	Lactic acid	Oily	......			..	......
8 D	Lactic acid	......	......	......		......	......
9 DS	Butyric acid	Fishy	Fishy	......		..	......
9 D	Butyric acid	......	Fishy	......	.	......	......
10 DS	Oleic acid	......	......	Fishy	......	......	......
10 D	Oleic acid	.		Fishy		......	......
11 DS	Soluble fatty acids	......	......	.		......	......
11 D	Soluble fatty acids	......	......		.	......	......
12 DS	Insoluble fatty acids	Oily				Oily	......
12 D	Insoluble fatty acids	Oily				Oily	......

TABLE 7. EFFECT ON THE FLAVOR OF BUTTER MADE FROM PASTEURIZED SWEET CREAM WITH 0.16 PER CENT ACID, WORKING TRIMETHYLAMINE AND FATTY ACIDS DIRECTLY INTO THE BUTTER AT THE RATE OF 85 PARTS PER MILLION

(*S* indicates salted butter)

Sample	Material added	Comments by judges					
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
ES	Nothing	...	...	...	...	...	...
E	Nothing	...	...	...	...	...	...
1 ES	Trimethylamine	Fishy	Fishy	Fishy	Fishy	...	Fishy
1 E	Trimethylamine	Fishy	Fishy	...	...	...	Fishy
2 ES	Trimethylamine lactate	Fishy	Fishy	Fishy	Fishy	...	Fishy
2 E	Trimethylamine lactate	...	Fishy	...	...	...	Fishy
3 ES	Trimethylamine butyrate	Fishy	Fishy	Fishy	Fishy	Fishy	Fishy
3 E	Trimethylamine butyrate	...	Fishy	...	...	...	Fishy
4 ES	Trimethylamine oleate	Fishy	Fishy	...	...	...	...
4 E	Trimethylamine oleate	...	Fishy	...	...	...	Fishy
5 ES	Trimethylamine stearate	...	Fishy	...	Fishy	...	Fishy
5 E	Trimethylamine stearate	...	Oily	Fishy	Fishy	Fishy	Fishy
6 ES	Trimethylamine and soluble fatty acids	Fishy	Fishy	Fishy	Fishy	Fishy	Fishy
6 E	Trimethylamine and soluble fatty acids	...	Fishy	Fishy	Fishy	...	...
7 ES	Trimethylamine and insoluble fatty acids	Fishy	Fishy	...	...	Fishy	...
7 E	Trimethylamine and insoluble fatty acids	...	Fishy	...	...	Fishy	...
8 ES	Lactic acid	...	Fishy	...	...	...	...
8 E	Lactic acid	...	Fishy	...	...	...	...
9 ES	Butyric acid	Fishy	...	Fishy	...	...	Fishy
9 E	Butyric acid	Fishy	...	Fishy	Fishy	Fishy	...
10 ES	Oleic acid	...	...	...	Fishy	...	...
10 E	Oleic acid	...	...	...	Fishy	...	...
11 ES	Soluble fatty acids	...	...	...	Fishy	...	...
11 E	Soluble fatty acids	...	...	...	...	Fishy	...
12 ES	Insoluble fatty acids	Oily	...	...	...	...	Oily
12 E	Insoluble fatty acids	Oily	...	...	...	...	...

TABLE 8. EFFECT ON THE FLAVOR OF BUTTER MADE FROM PASTEURIZED RIPENED CREAM WITH 0.38 PER CENT ACID, WORKING TRIMETHYLAMINE AND FATTY ACIDS DIRECTLY INTO THE BUTTER AT THE RATE OF 40 PARTS PER MILLION

(*S* indicates salted butter)

Sample	Material added	Comments by judges			
		No. 1	No. 2	No. 3	No. 4
FS	Nothing	....	....	....	....
F	Nothing	....	....	....	....
1 FS	Trimethylamine	Oily	Oily	Fishy	....
1 F	Trimethylamine	Oily	Fishy	Oily	....
2 FS	Trimethylamine lactate	Fishy	....	Fishy	....
2 F	Trimethylamine lactate	....	Fishy	....	....
3 FS	Trimethylamine butyrate	Fishy	Fishy	Fishy	Fishy
3 F	Trimethylamine butyrate	....	Fishy	Fishy	Fishy
4 FS	Trimethylamine oleate	....	....	....	....
4 F	Trimethylamine oleate	Oily	....	....	....
5 FS	Trimethylamine stearate	....	....	....	....
5 F	Trimethylamine stearate	....	....	....	....
6 FS	Trimethylamine and soluble fatty acids	....	....	Fishy	....
6 F	Trimethylamine and soluble fatty acids	Oily	....	....	Fishy
7 FS	Trimethylamine and insoluble fatty acids	....	....	....	....
7 F	Trimethylamine and insoluble fatty acids	....	....	....	Fishy
8 FS	Lactic acid	....	....	....	....
8 F	Lactic acid	....	....	....	....
9 FS	Butyric acid	....	....	....	....
9 F	Butyric acid	....	....	....	....
10 FS	Oleic acid	....	....	....	....
10 F	Oleic acid	....	....	....	....

TABLE 9. EFFECT ON THE FLAVOR OF BUTTER MADE FROM PASTEURIZED RIPENED CREAM WITH 0.28 PER CENT ACID, OF WORKING TRIMETHYLAMINE AND FATTY ACIDS DIRECTLY INTO THE BUTTER AT THE RATE OF 40 PARTS PER MILLION

(*S* indicates salted butter)

Sample	Material added	Comments by judges			
		No. 1	No. 2	No. 3	No. 4
GS	Nothing	....	....	....	....
G	Nothing	....	....	....	....
1 GS	Trimethylamine	Fishy	Fishy	Fishy	....
1 G	Trimethylamine		....	....	....
2 GS	Trimethylamine lactate	Fishy	Fishy	Fishy	....
2 G	Trimethylamine lactate	....	....	....	....
3 GS	Trimethylamine butyrate	Fishy	Fishy	Fishy	....
3 G	Trimethylamine butyrate	....	Fishy	Fishy	Fishy
4 GS	Trimethylamine oleate	Fishy	....	Oily	....
4 G	Trimethylamine oleate	....	....	....	....
5 GS	Trimethylamine stearate		....	....	....
5 G	Trimethylamine stearate	....	....	....	....
6 GS	Trimethylamine and soluble fatty acids	....	....	Fishy	....
6 G	Trimethylamine and soluble fatty acids	....	....	Fishy	Fishy
7 GS	Trimethylamine and insoluble fatty acids	....	....	....	....
7 G	Trimethylamine and insoluble fatty acids	....	....	....	....
8 GS	Lactic acid	....	....	....	....
8 G	Lactic acid	....	....	....	....
9 GS	Butyric acid	Oily	....	....	....
9 G	Butyric acid	....	....	....	....
10 GS	Oleic acid		....	....	....
10 G	Oleic acid	....	....	....	....

All of the samples of butter represented in tables 3 to 9 inclusive were scored from three to five days after making. They were then placed in storage for different lengths of time and rescored by two or more judges. The results of this examination are shown in table 10. In this table are listed only those samples showing a fishy flavor by unanimous opinion of the persons judging them.

TABLE 10. PRESENCE OF FISHY FLAVOR AFTER STORAGE, IN BUTTER TO WHICH TRIMETHYLAMINE HAD BEEN ADDED AT THE TIME OF MAKING

Sample	Material added	Age (days)	Comments by judges	
			At time of making	After storage
3 CS	Trimethylamine butyrate	40	Fishy	Fishy
3 DS	Trimethylamine butyrate	267	Fishy, oily	Fishy
3 ES	Trimethylamine butyrate	266	Fishy	Fishy
6 ES	Trimethylamine and soluble fatty acids	266	Fishy	Fishy
1 FS	Trimethylamine	243	Fishy, oily	Fishy, tallowy
3 FS	Trimethylamine butyrate	243	Fishy	Fishy
3 GS	Trimethylamine butyrate	243	Fishy	Fishy

The results obtained from these experiments bring out some very interesting facts. While there are several conflicting opinions as to the presence of the fishy flavor in any particular sample, it is nevertheless evident that the greatest number of positive comments is found in the samples containing trimethylamine in one form or another. It will also be noticed that usually the greatest uniformity of such comments is found in the samples containing trimethylamine in unstable form. This is particularly true as to the samples to which trimethylamine was added alone, in combination with butyric acid, or in combination with the mixed soluble fatty acids of butter. These results are in harmony with the volatility, the taste, and the odor of the compounds in pure state. The lack, in a few instances, of a majority opinion with regard to the samples containing trimethylamine oleate, trimethylamine stearate, and trimethylamine with the mixed insoluble acids, might be explained on the basis that, since these substances were so extremely unstable, the trimethylamine had nearly all volatilized before the time of scoring. The greater number of positive comments from the salted butter is also worthy of note, and, from what is generally known regarding the occurrence of the fishy flavor in such butter, it might tend to strengthen the trimethylamine theory of this flavor. Another feature found in this series of experiments is that a greater number of fishy-flavored samples were found where the acidity of the cream was the lowest. This condition is in harmony with the chemistry involved, for the reason that butter made from low-acid cream contains less lactic acid when fresh than is found in butter made from high-acid cream. It is therefore conceivable that trimethylamine given off by the unstable compounds which were added could not be taken up by the excess lactic acid in the butter to form the more stable trimethylamine lactate. The finding of numerous fishy-flavored samples where trimethylamine lactate had been added to low-acid butter might be explained on the basis that certain conditions were present, possibly particular enzymes, which were capable of bringing about the more rapid dissociation of the trimethylamine lactate, and that due to the absence of sufficient free lactic acid to hold the trimethylamine it passed into the free state. This explanation is borne out by the fact that in the butter from high-acid cream there were a smaller number of samples showing fishiness where the lactate was added. It might be stated further that one would naturally expect to find a greater variety of enzymes capable of bringing about the above-described decomposition in unripened than in ripened cream.

The evidence obtained from the various samples of butter to which trimethylamine butyrate was added, indicates a striking relationship between this substance and the fishy flavor. This seems to be true regardless of the type of butter, and to a certain extent regardless of the presence of sodium chloride. The extremely volatile nature of this substance and its characteristic odor in pure state easily account for the results obtained. The fact that there were more positive comments on the butters containing butyric acid alone than there were on butters containing the other acids alone, indicates that this substance may be a contributing factor in the development of the fishy flavor under natural conditions. This feature indicates also that the fishy flavor may be due to a definite balance between a decomposition yielding trimethylamine and one yielding butyric acid, with the consequent formation of trimethylamine butyrate. This associative action would be entirely possible in storage butter, judging from what is known regarding these fermentations.

In the foregoing discussion the writer has called attention to certain theoretical possibilities which might correlate the findings with what is generally known regarding the various types of normal and of fishy-flavored butter. It may be said, however, that the evidence points strongly toward trimethylamine as a contributing factor in fishy-flavored butter. The amount of this substance responsible for the flavor described as fishy in these experiments is very small. It would be a hazardous guess to assign a definite quantity, other than to say that in all cases there was less than 85 parts per million.

QUANTITATIVE ESTIMATION OF TRIMETHYLAMINE IN FISHY BUTTER

The results obtained by working trimethylamine into normal butter warranted an attempt to isolate this substance from samples of fishy-flavored butter found on the market. One of the first difficulties met with in this connection was the lack of a method which would accurately measure the small amounts of trimethylamine that would be found. The method that was finally worked out consisted of a combination and modification of the methods of Folin and Macallum (1912) for ammonia and of Budai [Bauer] (1913) for trimethylamine. The adaptation of these methods for this purpose was as follows:

The material in which trimethylamine and ammonia were to be determined was concentrated to a volume not exceeding 15 cubic centimeters. This material was then placed in the proper tube of the Folin apparatus, 10 grams of anhydrous potassium carbonate was added, and the mixture was covered with a thin layer of kerosene to prevent foaming. This mixture was aspirated for five hours. The ammonia and trimethylamine set free by the potassium carbonate was collected in N/10 hydrochloric acid. The excess acid was titrated with exactly N/100 alkali, methyl red being used as the indicator. The results of this titration gave the total amount of the mixture of ammonia and trimethylamine. To this titrated mixture 10 cubic centimeters of a neutral 40-per-cent formaldehyde solution was added. The ammonium chloride present reacted with the formaldehyde to form hydrochloric acid and hexa-methylene-tetramine according to the equation

$$6\ HCOH \text{ and } 4\ NH_4CL = (CH_2)_6N_4,\ 4\ HCL, \text{ and } 6\ H_2O.$$

The hexa-methylene-tetramine being neutral, the hydrochloric acid liberated from the ammonium chloride was titrated and the ammonia was calculated from this titration. Since the trimethylamine hydrochloride present was not affected by the formaldehyde, the trimethylamine was calculated by differences. Since this is essentially a micro method, the technique involved is of the utmost importance. In all cases the volume of the solution to be titrated was kept as nearly constant as possible, and the same amount of indicator was used for each titration. A check on the standard acid and alkali was made with each determination, and the end-points of all neutralization processes were compared colorimetrically with the standard neutral color. In table 11 are shown the results obtained by this method from mixtures of known amounts of trimethylamine hydrochloride and ammonium chloride:

TABLE 11. EFFICIENCY OF THE MODIFIED MICRO METHOD FOR ESTIMATING TRIMETHYLAMINE AND AMMONIA

Sample	Actual amount of $(CH_3)_3N$ and NH_3 as hydrochlorides (milligrams)		Amount recovered (milligrams)		Percentage	
	$(CH_3)_3N$	NH_3	$(CH_3)_3N$	NH_3	$(CH_3)_3N$	NH_3
1	6.66	6.66	6.61	6.63	99.70	99.55
2	6.66	6.66	6.51	6.63	97.75	99.55
3	2.30	2.30	2.25	2.278	97.83	99.04
4	1.84	1.84	1.79	1.820	97.28	98.91
5	0.92	0.92	0.885	0.935	96.19	101.63
6	0.92	0.92	0.914	0.935	99.35	101.63
7	0.46	0.46	0.442	0.476	96.09	103.48
8	0.46	0.46	0.476	0.476	103.48	103.48
9	0.276	0.276	0.295	0.323	106.88	117.03
10	0.276	0.276	0.295	0.297	106.88	107.61
11	0.276	0.276	0.295	0.297	106.88	107.61
12	......	0.276	....	0.289	....	104.71
13	......	1.84	....	1.820	....	98.91
14	......	0.92	....	0.918	....	99.78
15	......	0.46	....	0.459	....	99.78
16	......	0.644	....	0.646	....	100.31
17	......	0.092	....	0.105	....	114.13

The results shown in table 11 having justified the reliability of the micro method for measuring small amounts of trimethylamine, a number of fishy-flavored samples of butter were subjected to analysis. The butter was thoroly washed in a separatory funnel five times with equal volumes of water acidified with hydrochloric acid at the rate of 25 cubic centimeters of normal acid to the liter. The wash water was then evaporated to a small volume as quickly as possible, and the trimethylamine and ammonia were determined as outlined above.

In table 12 are shown the trimethylamine and ammonia results obtained from fishy butter appearing in commerce and procured from widely different localities. The ammonia results are shown as a matter of interest but they probably have no direct bearing on this particular problem. Since the experiments with artificially produced fishy flavor seemed to indicate the importance of acidity, the acid values of the samples are also included. The acidity is expressed as cubic centimeters of N/10 sodium hydroxide used to neutralize 20 grams of butter in boiling alcohol. Trimethylamine and ammonia are expressed in parts per million.

TABLE 12. TRIMETHYLAMINE AND AMMONIA CONTENT AND ACID CONTENT OF MISCELLANEOUS SAMPLES OF FISHY-FLAVORED BUTTER

Sample	Trimethylamine (parts per million)	Ammonia (parts per million)	Acid value
1	30.4	11.2	3.8
2	35.4	14.4	5.7
3	28.8	15.2	5.5
4	27.3	26.1	5.4
5	14.0	18.3	6.8
6	None	11.9	3.5
7	None	20.0	2.7
8	26.0	55.0	3.8
9	No analysis	No analysis	3.8
10	No analysis	No analysis	3.0
11	No analysis	No analysis	11.0

The data submitted in table 12 are of considerable interest in view of the history of some of the samples. On arriving at the laboratory, all of the samples, with the exception of samples 5 and 11, were scored as fishy by several judges. These two exceptions were samples of butter sent from a distance and were presumably scored as fishy when shipped but could not be so judged when received. It will be noted that in both cases there was a higher acid value than in any of the other samples, and also that the trimethylamine content of sample 5 is low. With these exceptions the acid value appears to be relatively constant, as does the trimethylamine content with the exception of samples 6 and 7, in which no trimethylamine whatever was found. The majority of these results would seem to point to a definite trimethylamine-acid relationship, as referred to elsewhere in this paper. It is to be regretted that in three instances the sample of butter submitted was too small to warrant analysis. The available data, however, point to trimethylamine as one of the causal agents in fishy-flavored butter.

DEVELOPMENT OF FISHY FLAVOR IN EXPERIMENTAL BUTTERS

In order that the development of the fishy flavor might be more carefully studied, three series of experimental butters were made with the object of determining the influence of pasteurization, of acidity developed during ripening of the cream, of adding lactic acid to the cream, of inoculating butter with lactic-acid bacteria, and of salt. The procedure followed in each of these series consisted of making nine different types of butter, salted and unsalted, from the same original lot of cream. The different series were made at intervals of from three to six weeks. The description of each of the different types of butter in each of the series, and the designation of the samples, are shown in table 13:

TABLE 13. Description of Types of Butter Made to Study the Development of Fishy Flavor

Treatment of cream or butter	Name of sample					
	Salted			Unsalted		
	A series	B series	C series	A series	B series	C series
Raw sweet cream	ASRS	BSRS	CSRS	ASR	BSR	CSR
Pasteurized sweet cream	ASHS	BSHS	CSHS	ASH	BSH	CSH
Raw cream ripened with starter	ARRS	BRRS	CRRS	ARR	BRR	CRR
Pasteurized cream ripened with starter	APRS	BPRS	CPRS	APR	BPR	CPR
Raw cream ripened naturally	ARRNS	BRRNS	CRRNS	ARRN	BRRN	CRRN
Raw sweet cream with *Bacterium lactis acidi* worked into butter	ASRBS	BSRBS	CSRBS	ASRB	BSRB	CSRB
Pasteurized cream with *Bacterium lactis acidi* worked into butter	ASHBS	BSHBS	CSHBS	ASHB	BSHB	CSHB
Raw sweet cream acidified with lactic acid	ASRLS	BSRLS	CSRLS	ASRL	BSRL	CSRL
Pasteurized cream acidified with lactic acid	ASHLS	BSHLS	CSHLS	ASHL	BSHL	CSHL

The samples indicated in table 13 were placed in storage at a temperature of 0° F. or lower, and were scored by three or four judges at various intervals. The results of these scorings are given in table 14. Noncharacteristic flavors are purposely omitted from this table.

TABLE 14. COMMENTS OF JUDGES ON THE DIFFERENT TYPES OF EXPERIMENTAL BUTTERS AFTER VARIOUS LENGTHS OF TIME IN STORAGE AT 0° F.

Sample	Acid in cream (per cent)	Days in storage	Comments by judges			
			No. 1	No. 2	No. 3	No. 4
ASR..........	0.27	45	Fishy	....	....	....
ASRS.........	0.27	45	Fishy	....	....	....
ASRS.........	0.27	130	Fishy	....	....	....
ASRS.........	0.27	285	....	Fishy	Fishy	....
BSR..........	0.18	45	Oily	....	Oily	....
BSRS.........	0.18	45	Fishy	....	Oily	....
BSRS.........	0.18	285	....	....	....	Fishy
CSRS.........	0.162	45	....	Fishy		....
CSRS.........	0.162	285	....	....	Fishy	Fishy
BSHS.........	0.16	45	Metallic	....	Metallic ..	....
CSH..........	0.144	130	....	Oily	....	....
CSHS.........	0.144	45	Metallic	....	....	....
ARR..........	0.68	45	Metallic	Metallic	....	....
ARRS.........	0.68	45	Metallic	....	Metallic	....
ARRS.........	0.68	90	....	Metallic	Oily, fishy	....
ARRS.........	0.68	130	Metallic	Oily	Metallic	....
ARRS.........	0.68	285	Metallic	Metallic	Fishy	....
BRRS.........	0.567	45	....	Metallic	....	....
CRRS.........	0.567	90	....	Metallic	Metallic	....
CRRS.........	0.567	285	Fishy	Oily	Metallic	Fishy
BPR..........	0.52	45	....	....	Oily	....
BPR..........	0.52	130	Metallic	....		....
APRS.........	0.66	130	....	Fishy	Metallic	....
APRS.........	0.66	285	....	....	....	Fishy
BPRS.........	0.52	45		Metallic		....
BPRS.........	0.52	130	Metallic	....	....	....
BPRS.........	0.52	285	....	Metallic	....	....
CPRS.........	0.562	90	Oily	....	Oily	....
ARRNS.......	0.675	130	....	Oily	Fishy	....
BRRN........	0.576	45			Oily	....
BRRNS.......	0.576	45	Oily, fishy	....	....	....
BRRNS.......	0.576	90	Fishy	Fishy	Oily, fishy	....
BRRNS.......	0.576	285	Fishy	Fishy	Fishy	Oily
CRRNS.......	0.699	90	Fishy	....	Oily, fishy	....
ASRB........	0.27	45	Fishy	....	....	....
ASRBS.......	0.27	45	Fishy	....	....	....
ASRBS.......	0.27	130	Fishy	Fishy	....	....
BSRB........	0.18	45	....	....	Oily	....
CSRBS.......	0.162	45	Oily, fishy	Fishy	....	....
CSRBS.......	0.162	90	....	....	Oily	....
CSRBS.......	0.162	285	....	Oily	Metallic	....

TABLE 14 (*concluded*)

Sample	Acid in cream (per cent)	Days in storage	Comments by judges			
			No. 1	No. 2	No. 3	No. 4
ASHBS	0.189	130	Fishy	Oily	....	....
ASHBS	0.189	285	....	....	Fishy	Metallic
CSHBS	0.144	45	Metallic	Metallic	Metallic	....
CSHBS	0.144	90	Metallic	....	....	....
ASRLS	0.638	45	Fishy	....	....	....
ASRLS	0.638	90	....	....	Fishy	....
ASRLS	0.638	130	....	Fishy	Fishy	....
ASRLS	0.638	285	Fishy	Fishy	Fishy	Fishy
BSRLS	0.665	45	....	Fishy	....	....
BSRLS	0.665	90	....	Fishy	....	....
BSRLS	0.665	285	....	Fishy	Fishy	Metallic
CSRLS	0.472	45	....	Metallic	....	....
CSRLS	0.472	285	....	Metallic	Fishy	Oily
ASHLS	0.690	130	....	Metallic	Metallic	....
ASHLS	0.690	285	....	Fishy	Fishy	....
BSHLS	0.594	45	Fishy	Oily	....	....
BSHLS	0.594	90	....	Fishy	....	....
CSHL	0.504	45	Fishy	....	....	....
CSHLS	0.504	45	Fishy	....	....	....
CSHLS	0.504	285	....	....	Fishy	Oily

In considering the results from the different types of experimental butter, it is evident that there is considerable diversity of opinion among the judges as to the presence or the absence of the fishy flavor in certain samples. It is also evident that there is some relationship between the metallic, oily, and fishy flavors, particularly when these flavors are not sufficiently pronounced to be distinctive as was the case in these samples. This would seem to indicate that there are possibly certain fundamental conditions which are common to the development of each of these flavors.

Even tho there is difference of opinion as to the presence of the characteristic flavors, certain conclusions may be drawn from these experiments. Probably one of the most significant is the presence of the fishy or the metallic flavor in the salted butters. Of a total of 105 characteristic comments, 93 are found in the samples containing salt. Another conclusion which may be drawn from the relative agreement of the judges, is that the fishy flavor appears oftener in the butter made from high-acid cream than in that from low-acid cream, there being little difference whether the acid was developed by the use of starter, by ripening naturally, or by the addition of lactic acid to raw sweet cream.

These findings, compared with the results obtained from pasteurized cream either churned sweet, ripened with starter, or acidified with lactic acid — all of which showed fewer fishy samples than did raw cream — clearly indicate that the fundamental cause of this butter defect is primarily biological, not brought about by a spontaneous chemical change in which such agencies do not play a part. While it is evident that acid plays an important rôle in the development of the fishy flavor, it is equally clear that there are other important contributing factors. Just what these factors are, is unknown. The variable results obtained from the same type of butter in the different series would indicate that the original cream or milk possessed the unknown factors which in the presence of lactic acid determined the development of the flavor. From the fact that pasteurization tends to reduce the occurrence of the fishy flavor, it is quite probable that these agencies are bacterial enzymes which are only partially inactivated by heat; or it may even be possible that certain microorganisms which are incorporated in the butter from the cream, either in a living or in a dead condition, could on autolysis liberate the enzymes capable of supplying the determining factor. It may also be added that pasteurization may kill certain enzymes and not others, the particular ones that are important being among those killed.

These contentions are further supported by the fact that in the butters made from raw sweet cream there is a suggestion of fishiness after the first storage period which is not found after the longer periods, the disappearance or lack of further development of the flavor being due to the absence of the proper acid condition. It is clear that large numbers of *Bacterium lactis acidi* added directly to butter without their usual accompanying by-products are not the cause of any characteristic change in flavor.

VARIATION IN ACID VALUE OF EXPERIMENTAL BUTTERS

The importance of acidity in the manifestation of the fishy flavor by trimethylamine, and the relatively constant acid value of the miscellaneous samples of fishy butter found on the market, emphasized the importance of studying this factor in the experimental butters described above. The variation in acid value of the different types of butter in each of the three series is shown in tables 15, 16, and 17. Results are expressed as cubic centimeters of N/10 alkali necessary to neutralize 20 grams of butter in boiling neutral alcohol.

TABLE 15. Variation in Acid Value of Experimental Butters of *A* Series after Various Storage Periods

Sample	Acid value after various storage periods			
	43 days	86 days	128 days	310 days
ASR	8.8	10.5	10.8	13.0
ASRS	7.4	8.0	8.2	10.0
ASH	5.8	8.6	10.0	12.1
ASHS	5.7	5.9	5.7	7.2
ARR	8.2	8.5	9.3	10.3
ARRS	8.7	8.4	8.6	10.3
APR	7.7	7.9	8.2	9.8
APRS	8.1	7.8	8.0	9.6
ARRN	8.9	8.6	9.2	10.5
ARRNS	8.8	8.4	8.7	10.5
ASRB	9.7	10.4	11.2	15.0
ASRBS	7.4	7.7	7.8	9.8
ASHB	5.7	8.6	9.5	14.3
ASHBS	5.8	5.9	5.8	9.7
ASRL	8.9	8.8	8.8	10.3
ASRLS	8.9	8.9	9.0	10.0
ASHL	7.0	7.1	7.2	8.0
ASHLS	7.4	6.5	7.1	8.5

The data presented in tables 15, 16, and 17 show many interesting features, some of which are worthy of discussion in connection with this preblem. It may be stated in the beginning that the variations in acid value of the different types of butter point to biological agencies as the cause of those variations. The lower acid value obtained in nearly all instances from salted butter indicates a preservative action by the salt, a function which is well known. The greatest increase in acidity is shown in the butter made from raw sweet cream. It is interesting to note that very few of the samples were scored as fishy. When such a condition was suggested, it is to be noted that it occurred after the first storage period, when the acid value was lowest.

TABLE 16. VARIATION IN ACID VALUE OF EXPERIMENTAL BUTTERS OF *B* SERIES AFTER VARIOUS STORAGE PERIODS

Sample	Acid value after various storage periods			
	20 days	88 days	126 days	286 days
BSR	13.9	14.6	15.2	17.5
BSRS	9.7	9.5	10.0	10.6
BSH	7.3	9.0	10.6	13.4
BSHS	6.3	6.5	6.8	7.6
BRR	10.1	10.2	10.8	11.9
BRRS	10.1	10.1	10.4	11.3
BPR	10.1	10.1	10.0	11.2
BPRS	9.8	10.1	9.6	11.0
BRRN	11.3	11.7	12.6	14.3
BRRNS	11.2	11.7	11.8	13.8
BSRB	13.6	13.5	14.3	15.4
BSRBS	10.6	10.8	10.8	12.0
BSHB	8.1	8.4	9.0	9.2
BSHBS	6.8	6.6	6.8	8.0
BSRL	11.5	11.5	11.6	12.9
BSRLS	11.5	11.1	11.6	12.6
BSHL	9.4	9.4	9.5	11.0
BSHLS	9.5	9.6	9.6	10.1

With reference to the samples from pasteurized cream as compared with those from raw sweet cream, it will be noticed that pasteurization has tended to cause a lowering of the acid value but has not entirely prevented its gradual increase. This would be in accord with possibilities already stated regarding bacterial enzymes. The data show also a retarding action exerted by the acid originally in the cream. This is evident in the butter made from both raw and pasteurized cream ripened with starter, from raw cream ripened naturally, and from both raw and pasteurized cream to which lactic acid has been added. In comparing the results from these samples it will be observed that cream ripened naturally shows the greatest increase in acid value, raw cream ripened

TABLE 17. Variation in Acid Value of Experimental Butters of C Series after Various Storage Periods

Sample	Acid value after various storage periods			
	6 days	48 days	90 days	272 days
CSR	7.8	10.4	11.0	13.5
CSRS	7.0	7.9	8.0	9.7
CSH	4.4	7.6	11.0	12.0
CSHS	4.4	4.7	4.4	5.2
CRR	7.6	9.3	10.4	12.8
CRRS	7.5	8.2	8.4	10.0
CPR	6.6	7.5	8.9	10.0
CPRS	6.5	7.0	7.0	8.2
CRRN	8.5	9.5	10.0	12.9
CRRNS	8.2	9.0	9.1	11.3
CSRB	7.8	10.2	11.8	13.5
CSRBS	6.9	7.4	7.9	9.0
CSHB	4.4	6.5	7.7	7.1
CSHBS	4.1	4.4	4.4	5.2
CSRL	8.3	8.1	8.0	9.8
CSRLS	8.4	8.5	8.4	9.5
CSHL	5.8	6.0	5.9	6.7
CSHLS	6.0	5.7	5.6	6.3

with starter a little less increase, pasteurized cream ripened with starter a still less increase, and cream to which lactic acid has been added the least increase of any in the group. The low acid value caused by the addition of lactic acid might possibly be explained in one of two ways: either the addition of the acid in pure form has tended to inactivate the enzymes, or the lactic acid retained in the butter has been changed to butyric acid during storage — which is entirely possible by enzymatic action. If such a change as the latter did take place, a lowering of the acid value would be manifested because of the formation of a weaker acid which probably has resulted from the splitting and condensation of two parts of the stronger lactic acid. The possibility of butyric acid being formed in this way might be supported by the fact that the other samples from high-acid cream showed a lower acid value than those from sweet cream but had a higher value than those to which pure lactic acid was added. Even tho this change did take place, however, it is improbable that it could entirely account for the low value indicated. It is more probable that the lactic acid acts as an inhibiting agent. A study of the tables will show that the increase in acid value of the sour-cream butters seems to be greater when the amount of pure lactic acid is lowest in the cream. In using the phrase "pure lactic acid," reference is made to that which was added and also to that developed by bacteria, it being logical to assume that the greatest amount so developed is found in pasteurized cream ripened with starter and the least amount in the raw cream ripened naturally. The condition mentioned above also supports the theory of devitalized enzymes, altho it is more difficult to explain why approximately the same degree of commercial lactic acid has a more marked effect than the acid produced by bacteria. It would seem that the structure of the particular lactic acids involved produced different results in this respect, or that the other acids produced by the bacteria are less inhibitive than the lactic.

Regardless of what the explanation for the variations may be, the data seem to indicate that there may be a relationship between the acid value and the fishy flavor, not so much by a constant condition as by a proper balance between the progressive development of the acid value and some other contributory cause. The most favorable condition would seem to be a very gradual increase in acid value, and one that would be in proper harmony and relationship to some other important and transient factor. If these views can to any degree serve as a basis of explanation, it is comparatively easy to see how an improper balance of any one of the conditions would determine the presence or the absence of the fishy flavor. It might also be conceivable that the intensity of the true flavor would be in inverse proportion to the degree in which these factors were out of equilibrium. Such a conception could explain the occurrence and the disappearance of the flavor in the same sample of butter at different times, why the fishy, the metallic, and the oily flavors seem to be closely related, and possibly why trimethylamine can be detected in some fishy butters and not in others.

TRIMETHYLAMINE AND AMMONIA CONTENT OF EXPERIMENTAL BUTTERS

The micro method already described was used to ascertain the trimethylamine and ammonia content in the experimental butters after different lengths of time in storage. The results of these determinations are shown in tables 18, 19, and 20. Amounts of the substances are expressed in parts per million.

TABLE 18. Trimethylamine and Ammonia Content of Experimental Butters in *A* Series after Various Storage Periods

Sample	After 128 days		After 347 days	
	$(CH_3)_3N$	NH_3	$(CH_3)_3N$	NH_3
ASR	None	36.0	None	17.6
ASRS	None	17.2	None	8.4
ASH	None	23.3	None	16.6
ASHS	None	15.0	None	6.8
ARR	None	29.2	None	30.8
ARRS	3.1	16.8	9.4	18.2
APR	None	34.0	None	24.4
APRS	None	20.7	None	24.0
ARRN	None	25.3	None	25.0
ARRNS	13.5	22.4	None	6.8
ASRB	None	18.4	None	24.4
ASRBS	None	22.1	None	12.8
ASHB	None	23.1	11.8	10.8
ASRL	None	17.3	15.2	9.4
ASRLS	None	14.2	6.0	10.7
ASHL	None	16.3	None	12.2
ASHLS	None	19.3	None	8.8

The results shown in tables 18, 19, and 20, altho erratic, are of importance as indicating the variations in decomposition in the same and in different lots of cream, and further emphasize the complexity of a problem of this nature. The same general results with respect to enzymatic activity in salted and in unsalted butter from raw, pasteurized, and ripened cream are found here as were found in connection with the acid values of the same butters. Altho the trimethylamine results are somewhat discordant, a tendency is shown for the presence of this substance to harmonize with the samples scored as fishy, metallic, or oily. Of 21 samples in which trimethylamine was found, 15 were assigned one of the characteristic flavors by one or more of the judges at some time during the storage period. In 5 of the remaining instances, it is to be noted that, while trimethylamine was found in the same type of butter of the same series, its presence did not harmonize with the characteristic flavor in the salted or the unsalted sample. On the other hand, there were 10 samples of different types of butter which were indicated as having a characteristic flavor by one or more of the judges at some time during the storage period, in which trimethylamine could not be detected.

TABLE 19. TRIMETHYLAMINE AND AMMONIA CONTENT OF EXPERIMENTAL BUTTERS IN *B* SERIES AFTER 323 DAYS IN STORAGE

Sample	$(CH_3)_3N$	NH_3
BSR	None	35.2
BSRS	None	18.0
BSH	......	......
BSHS	53.0	10.2
BRR	None	32.8
BRRS	8.2	21.6
BPR	10.6	23.8
BPRS	9.4	25.0
BRRN	4.7	27.0
BRRNS	None	32.2
BSRB	35.4	29.4
BSRBS	None	20.0
BSHB	5.9	23.0
BSHBS	None	10.4
BSRL	None	23.0
BSRLS	None	17.0
BSHL	None	7.4
BSHLS	None	11.4

While these results are not absolutely conclusive, there is nevertheless an indication that trimethylamine may be one of the contributing factors in the development of the true fishy flavor. It has been shown that this substance is capable of producing a flavor described by butter judges as fishy, this being particularly true in the presence of butyric acid. Furthermore, it has been shown that trimethylamine may be present in fishy-flavored butter. Therefore it would not appear to be beyond the realm of possibility that the results shown in this paper point to a definite trimethylamine and acid relationship as being the cause of that flavor in butter which resembles the flavor of herring or mackerel brine, and that non-typical flavors resembling other fish products, or the metallic

TABLE 20. TRIMETHYLAMINE AND AMMONIA CONTENT OF EXPERIMENTAL BUTTERS IN *C* SERIES AFTER VARIOUS STORAGE PERIODS

Sample	After 90 days		After 310 days	
	$(CH_3)_3N$	NH_3	$(CH_3)_3N$	NH_3
CSR	None	30.2	8.2	33.2
CSRS	None	20.0	None	28.2
CSH	None	32.4	......	....
CSHS	None	19.7	None	14.2
CRR	None	36.3	None	20.6
CRRS	None	31.2	None	36.2
CPR	None	34.7	None	29.2
CPRS	None	33.2	14.0	27.4
CRRN	None	37.0	18.8	31.8
CRRNS	None	27.6	8.2	29.8
CSRB	None	33.3	13.0	33.6
CSRBS	None	23.1	5.8	18.6
CSHB	None	31.6	None	31.6
CSHBS	None	34.0	17.6	15.4
CSRL	None	21.4	None	14.8
CSRLS	None	14.2	None	10.8
CSHL	None	38.7	5.8	20.6
CSHLS	None	19.1	......	....

and the oily flavor, may be due to an unbalancing of this relationship, the occurrence of these flavors being due to factors in which the presence of trimethylamine in detectable amounts is in no way contributory.

BACTERIOLOGICAL STUDIES

In an effort to correlate the preceding observations with the biological aspects of the problem, certain bacteriological studies were carried out. These included a bacterial analysis of fishy- and non-fishy-flavored butters, and inoculations with pure and mixed cultures into choline, lecithin, butterfat, and cream for the purpose of finding, if possible, an organism or a group of organisms which in some way might contribute to the development of the fishy flavor.

Bacterial analysis

Bacteriological examinations of fishy- and of normal-flavored butter from various sources seemed to show no characteristic differences in flora, neither were the quantitative results consistent. Even tho the samples examined did not appear to possess marked differences in flora, cultures of the predominating type were isolated from the fishy samples for the purpose of determining a condition under which they might contribute to the characteristic flavor. The types of bacteria found included a number of acid-producing varieties, both coccus and rod forms. Among the species commonly found were *Micrococcus lactis acidi*, *Mic. lactis albidus*, *Bacterium lactis brevis*, *Bact. aerogenes*, and *Bact. lactis flocculus*.

The bacteria content of certain samples of fishy- and of non-fishy-flavored butter is given in table 21:

TABLE 21. NUMBER OF BACTERIA FOUND IN VARIOUS SAMPLES OF FISHY- AND NON-FISHY-FLAVORED BUTTER

Sample	Character of flavor	Bacteria per gram
1	Strong	1,200,000
2	Fishy	1,400,000
3	Fishy	8,000,000
4	Fishy	21,600,000
5	Oily	30,000,000
6	Fishy	60,000
7	Fishy	135,000
8	Oily	3,900,000
9	Strong	760,000
10	Fishy	23,500
11	Fishy	8,300,000
12	Strong	1,600,000
13	Fishy	340,000
14	Storage	2,600
15	Fishy	390,000
16	Fishy	465,000

Inoculations for the purpose of developing the fishy flavor

The results of previous investigations show that little success has been attained in the attempt to develop the fishy flavor in butter by inoculating the butter itself or by inoculating the cream just prior to churning. If the enzymatic idea as already stated in this paper is to be upheld, such negative results can be explained by the fact that organisms inoculated into the cream just prior to churning, or into the butter, do not have the opportunity for growth and consequent production of by-products which they would have if allowed to grow in the milk or the cream before it is made into butter. There seems to be good evidence that there is a rapid dying-off of the bacteria in butter after the first few days of storage. Furthermore, it is well known that low storage temperatures do not entirely prevent enzymatic activity. Rogers (1909) shows that, while low temperatures delay the development of the fishy flavor, they do not entirely prevent it. In accordance with these facts it is conceivable that certain enzymes which would be produced by the growth of organisms in the cream, and carried into the butter, would there continue their activity, with the consequent manifestation of certain changes in flavor. Results obtained in the present investigation indicate also the importance of a definite acid relationship. With these factors in mind, the inoculation experiments undertaken in connection with this problem were carried out in a manner that would allow for the manifestation of the possibilities indicated.

Nine organisms, all of which were isolated from samples of fishy butter and *Bacterium ichthyosmius* — which Hammer (1917) found would produce the fishy flavor in milk — were used in these experiments. The same original lot of cream was divided into six parts, and each of these parts was further divided into ten parts, each of which was inoculated with a specific organism. Different methods of handling the six groups of ten inoculations each were carried out in such a way that the effect of acid in conjunction with the specific organism could be determined. Adequate checks were made from uninoculated cream. Pasteurized sweet cream was used as the basis for all inoculations. When the cream was neutralized the acidity was reduced to 0.18 per cent. All samples were made up both salted and unsalted, and were scored after 231 days in storage at a temperature of 0° F. or lower. The results of the experiment as regards salted butter are given in table 22.

It is characteristic of the results of this experiment that none of the samples of unsalted butter showed any of the characteristic flavors and are therefore not included in table 22. It is believed that the results shown in the table clearly confirm the opinion that biological agencies, particularly bacterial enzymes, are responsible to a great degree for the flavors indicated; also, that the fundamental condition necessary for the

TABLE 22. EFFECT ON THE FLAVOR OF SALTED BUTTER, OF INOCULATING CREAM WITH SPECIFIC ORGANISMS UNDER DIFFERENT CONDITIONS

Sample	Treatment of cream	Acidity of cream*		Comments by judges			
		1 (+)	2 (+)	No. 1	No. 2	No. 3	No. 4
1	Raw sweet	......	0.18	....	....	....	....
2	Pasteurized sweet	......	0.13	....	....	....	....
3	Pasteurized sweet, ripened	......	0.38	....	....	....	....
4	Pasteurized sweet, ripened and neutralized	0.38	0.20	....	....	....	....
5	Pasteurized sweet, acidified with lactic acid	......	0.54	Fishy	Fishy	Oily	Fishy
6	Pasteurized sweet, acidified with lactic acid and neutralized	0.54	0.18	....	....	....	....
D BI	Pasteurized sweet, inoculated with specific organisms and churned at once	......	0.13	....	....	....	....
D 100		......	0.13	....	....	....	Metallic
D 23		......	0.13	....	....	....	....
D 12		......	0.13	Tallowy	....	....	Tallowy
D 18		......	0.13	Metallic	....	....	Oily
D 19		......	0.13	....	....	....	....
D 8		......	0.13	....		....	Oily
D 11		......	0.13	....	....	....	....
D 21		......	0.13	....			....
D 10		......	......	....			....
E BI	Pasteurized sweet, inoculated with specific organisms and held 24 hours before churning	......	0.45	....	....	....	....
E 100		......	0.41	....	....	Fishy	Tallowy
E 23		......	0.41	....	Fishy	Metallic	Oily
E 12		......	0.43	....	....	....	....
E 18		......	0.47	....	....	....	....
E 19		......	0.43	....	....	....	....
E 8		......	0.46	....	....	....	....
E 11		......	0.43	....	Oily	Oily	Fishy
E 21		......	0.43	....		....	....
E 10		......	0.39	....		....	....
K BI	Pasteurized sweet, inoculated with specific organisms, held 24 hours, and neutralized before churning	0.45	0.18	Metallic	....	Fishy	Fishy
K 100		0.41	0.18	....	....	....	....
K 23		0.41	0.18	....	....	....	Oily
K 12		0.43	0.18	....	Butyric	Butyric	....
K 18		0.45	0.18	....	....	....	....
K 19		0.43	0.18	....	....	....	....
K 8		0.46	0.18	....	....	....	....
K 11		0.43	0.18	....	Tallowy	....	....
K 21		0.43	0.18	....	....	....	....
K 10		0.39	0.18	....	....	Fishy	....

*1 (+) = acidity at time of neutralizing.
2 (+) = acidity at time of churning.

TABLE 22 (*concluded*)

Sample	Treatment of cream	Acidity of cream*		Comments by judges			
		1 (+)	2 (+)	No. 1	No. 2	No. 3	No. 4
N BI	Pasteurized sweet, inoculated with specific organisms, held 24 hours, neutralized, and ripened	0.45	0.32	....	....	....	....
N 100		0.41	0.36	....	....	....	....
N 23		0.41	0.35	....	....	....	....
N 12		0.43	0.37	....	....	....	....
N 18		0.45	0.38	....	....	....	....
N 19		0.43	0.36	....	....	....	....
N 8		0.47	0.32				....
N 11		0.43	0.29	....	....	....	....
N 21		0.43	0.39	....	....	....	....
N 10		0.39	0.39	....	....	....	....
R BI	Pasteurized sweet, inoculated with specific organisms, held 24 hours, neutralized, ripened, and again neutralized	0.36	0.18	Fishy	Oily	Fishy	Fishy
R 100		0.26	0.18	....	....	....	....
R 23		0.38	0.18	....	....	....	Oily
R 12		0.31	0.18	Butyric	....	....	....
R 18		0.33	0.18	....	....	....	....
R 19		0.32	0.18	....	....	Rancid	....
R 8		0.29	0.18	....	....	....	Fishy
R 11		0.30	0.18	....	....	....	Fishy
R 21		0.32	0.18			....	....
R 10		0.29	0.18	....	....	....	....
X BI	Pasteurized sweet, inoculated with specific organisms, held 24 hours, and ripened	......	0.29	Metallic	Metallic	Tallowy	Fishy
X 100		......	0.42	....	....	....	Oily
X 23		......	0.40	Metallic	Metallic	Fishy	Metallic
X 12		......	0.39	Butyric	Butyric	....	Strong
X 18		......	0.37	....	....	....	....
X 19			0.36			....	....
X 8		......	0.34	....	....	....	....
X 11		......	0.36	....	....	....	....
X 21			[illegible]				....
X 10			[illegible]				....

*1 (+) = acidity at time of neutralizing.
2 (+) = acidity at time of churning.

manifestation of these flavors is greatly enhanced by the growth of the organisms in the cream; and furthermore, that a definite acid condition is essential for the development of these flavors, which are potentially possible from the specific bacteria or enzymes. In this experiment it is appreciated that the results are obtained by an associative action with the organisms in the starter and those surviving pasteurization; this fact, however, does not depreciate the specificity of the particular organisms that were inoculated. In reviewing the data from this experiment, it is interesting to note that the sweet pasteurized cream to which lactic acid had been added and which was uninoculated, developed the fishy flavor. The description of the flavor in the same sample of butter by different judges again calls attention to the fact that there seem to be some conditions common to the fishy, the metallic, and the oily flavor.

The most consistent comments from specific organisms seem to be from cultures BI and 23. The former is *Bact. ichthyosmius*, which was obtained from Hammer; the latter is an organism isolated from raw-ripened-cream butter which developed the fishy flavor after two months and retained it for nearly twenty months.

Butter samples E 23 and X BI were analyzed for trimethylamine and ammonia. None of the former substance was found. Sample X BI showed 35.2 parts per million of ammonia, and sample E 23 showed 17 parts per million.

LONGEVITY OF BACTERIUM ICHTHYOSMIUS IN BUTTER

Preliminary experiments with *Bacterium ichthyosmius* indicated that this organism might produce the fishy flavor in butter. It seemed desirable, therefore, to determine its longevity in butter made from the inoculated cream. Results of the quantitative determinations of the bacteria in salted butter containing this organism are shown in table 23; results are given only for those samples to which a characteristic flavor was assigned.

TABLE 23. BACTERIA CONTENT OF SALTED BUTTER MADE FROM CREAM INOCULATED WITH BACTERIUM ICHTHYOSMIUS AND STORED AT A TEMPERATURE OF 0° F. OR LOWER*

Age of sample (days)	Number of bacteria per gram		
	K BI	R BI	X BI
7	23,400,000	10,400,000	....
21	11,400,000	10,100,000	34,250,000
35	10,450,000	8,300,000	28,000,000
49	8,500,000	6,700,000	17,400,000
63	6,300,000	6,350,000	10,150,000
78	1,800,000	4,000,000	11,500,000
91	1,150,000	1,350,000	11,200,000
105	890,000	....	6,400,000
119	750,000	....	5,400,000
134	....	740,000	....
148	....	355,000	....
162	....	37,500	4,600,000

* These results were furnished by J. T. Cusick.

The decrease in bacteria content of the salted butter containing *Bact. ichthyosmius* shows that, altho this organism may contribute to the development of the fishy flavor in butter, it does not do so by active multiplication in that medium.

FURTHER STUDIES WITH BACTERIUM ICHTHYOSMIUS

The results obtained with *Bacterium ichthyosmius* seemed to warrant a further study of its relationship to the fishy flavor in butter. The following experiment was carried out with the purpose of determining the conditions in butter under which the development of the flavor could be accelerated. Pasteurized sweet cream was inoculated with this organism and held for two days at room temperature. The butter made from this cream was divided into twelve parts, and to each of these parts a different substance was added. The cream at the time of churning contained 0.23 per cent acid. The treatment of this butter, and the time of occurrence of the fishy flavor as determined by two or more judges, are shown in table 24:

TABLE 24. FISHY FLAVOR AS DEVELOPED IN BUTTER WHICH WAS MADE FROM CREAM INOCULATED WITH BACTERIUM ICHTHYOSMIUS AND TO WHICH VARIOUS SUBSTANCES WERE ADDED

Sample	Substance added to butter	Days in storage				
		52	91	136	175	328
BI 1	Nothing	...	Fishy	Fishy	Not scored	
BI 2	Berkfeldt filtrate from milk culture of *Bact. ichthyosmius*	...	...	...	...	...
BI 3	Choline, 0.0118 per cent	Oily	...	...	...	...
BI 4	Calcium caseinate	...	...	...	...	...
BI 5	Lactic acid, 0.117 per cent	Fishy	Fishy	Fishy	Not scored	
BI 6	Lecithin from butter	...	...	...	...	Fishy
BI 7	Lactic acid and choline	Fishy	...	...	...	...
BI 8	*Bact. lactis acidi* starter	...	...	...	...	Slightly fishy
BI 9	Berkfeldt filtrate and caseinate	...	...	...	...	...
BI 10	Berkfeldt filtrate and choline	...	...	...	...	...
BI 11	Berkfeldt filtrate, lactic acid, and choline	...	...	...	...	...
BI 12	Berkfeldt filtrate made alkaline	...	Oily	Fishy	Not scored	

All of the butters indicated in table 24 possessed a very disagreeable flavor and odor when fresh but they seemed to improve in quality during storage. The development of the fishy flavor in certain samples shows some very interesting features. In reviewing the results of this experiment it must be borne in mind that the cream from which they were made contained the products of two days growth of *Bact. ichthyosmius.*

The development of the fishy flavor in the sample to which nothing was added, is therefore significant. The earlier occurrence of the flavor in the sample to which lactic acid was added is significant in that it confirms certain observations already noted. The development of the flavor in the sample containing the alkaline Berkfeldt filtrate seems to be about simultaneous with its development in the sample to which nothing was added. The lack of development of the flavor in the samples to which the filtrate was added alone or in combination with other substances, might appear to be contradictory to the enzymatic idea previously expressed. It is believed, however, that this is more than offset by the other data, which point to the necessity of a definite set of conditions that must be met in order to produce the flavor. Such being the case, the absence of the flavor when the filtrate was added may be explained on the basis that the proper equilibrium had been disturbed. The final occurrence of fishiness in the sample containing lecithin is of importance as indicating that this may be the mother substance of the material causing the flavor. Other scattering results do not merit particular discussion at this time.

The trimethylamine and ammonia content of the samples shown in table 24, and their acid value, are given in table 25:

TABLE 25. TRIMETHYLAMINE AND AMMONIA CONTENT AND ACID VALUE OF BUTTER SAMPLES WHICH WERE MADE FROM CREAM INOCULATED WITH BACTERIUM ICHTHYOSMIUS AND TO WHICH OTHER SUBSTANCES WERE SUBSEQUENTLY ADDED

Sample	Age of sample (days)	$(CH_3)_3N$ (parts per million)	NH_3 (parts per million)	Acid value
BI 1	136	7.2	20.7	8.7
BI 2	328	9.4	30.2	9.5
BI 3	328	5.8	28.4	8.5
BI 4	328	4.6	26.8	7.8
BI 5	94	4.7	12.7	8.2
BI 6	328	9.4	25.4	8.0
BI 7	328	8.2	24.0	8.9
BI 8	328	1.6	22.4	8.5
BI 9	328	5.8	25.8	...
BI 10	328		...	9.2
BI 11	328		...	9.1
BI 12	136	11.8	17.8	9.3

The relatively constant trimethylamine results shown in table 25 indicate strongly that this substance has been produced in the cream by *Bact. ichthyosmius.*

TRIMETHYLAMINE AND AMMONIA PRODUCTION BY BACTERIUM ICHTHYOSMIUS IN MILK AND CREAM

In order to determine the trimethylamine production by *Bacterium ichthyosmius* in milk and cream, 50-cubic-centimeter quantities of these substances, sterilized, were inoculated with the organism alone and in combination with a lactic-acid starter. The inoculations were held for forty hours at 30° C., and the trimethylamine and ammonia were then determined in 20-cubic-centimeter quantities. The results of these determinations are shown in table 26:

TABLE 26. AMOUNT OF TRIMETHYLAMINE AND AMMONIA PRODUCED IN SKIMMILK AND IN CREAM BY BACTERIUM ICHTHYOSMIUS

Inoculation	Material inoculated	$(CH_3)_3N$ (parts per million)	NH_3 (parts per million)
Bacterium ichthyosmius and starter	Skimmilk	None	84
Bact. ichthyosmius and starter	Cream	204.0	88
Bact. ichthyosmius	Skimmilk	94.4	125
Bact. ichthyosmius	Cream	74.7	78

The results presented in table 26 are of great interest as showing beyond a doubt that the fishy flavor produced in milk and cream by *Bact. ichthyosmius* is due to trimethylamine. This being the case, it is obvious that this substance would be carried into the butter, and there, under proper conditions which have already been pointed out, be responsible for the characteristic flavor in that material. With respect to the production of trimethylamine in cream and in milk by this organism, it is desirable to again call attention to the observations of the author, in which the evolution of a fishy flavor was noted on the addition of alkali to sweet cream.

These results are of further importance in that the cream inoculated with starter and *Bact. ichthyosmius* contained a greater amount of trimethylamine than did the cream inoculated with the organism alone. This indicates that an acid condition is most favorable for this particular fermentation, which would be in harmony with the idea that lecithin furnishes the source of the trimethylamine produced by the organism. The results are supported also by the fact that lecithin is largely associated with the fat, and that according to Hammarsten and Hedin (1915) lecithin is decomposed by dilute acids. Such being the case, it is readily seen that this fermentation brought about by *Bact. ichthyosmius* would be greatly enhanced by the presence of acid. The presence of trimethylamine in skimmilk inoculated with the organism alone might be explained on the basis that the organism was able to produce this substance from proteins as well as from lecithin. Certain data not included in this paper, however, indicate that there is a certain amount of lecithin present in skimmilk. Just why there is no trimethylamine in skimmilk inoculated with the starter and the organism, is more difficult to explain. It may be that the greater acidity in the skimmilk has inhibited the particular factor responsible for trimethylamine production.

PRODUCTION OF TRIMETHYLAMINE FROM LECITHIN AND CHOLINE BY BACTERIAL ACTION

In order to determine, if possible, whether certain organisms found in milk and in butter were capable of decomposing lecithin or choline into trimethylamine, a series of inoculation experiments were carried out. Lecithin alone in 0.3 per cent concentration, and in the presence of lactic acid and salt, was inoculated with a number of organisms, some of which were obtained from milk, some from fishy butter, and some from decomposed egg yolk which had developed the fishy flavor. The following known species were also used: *Bacterium lactis acidi*, *Bact. aerogenes*, *Bacillus prodigiosus*, *B. proteus*, *Bacterium ichthyosmius*, *Pseudomonas liquefaciens fluorescens*, *Oïdium lactis*. All organisms were inoculated singly and in various combinations, and the cultures were held at 20° C. for approximately nine months. At the end of that time the cultures were tested for the presence of trimethylamine by heating with alkali. Negative results were obtained from all of the lecithin inoculations tested. Unfortunately, many of the cultures were contaminated with mold, and, since the results could not be considered trustworthy, they were discarded.

The same series of experiments was repeated by inoculating 0.1-per-cent choline alone and in the presence of lactic acid and salt. These cultures were held under the same conditions as were the lecithin inoculations. Trimethylamine was found where *Bacterium ichthyosmius* was inoculated alone, in combination with *Oïdium lactis*, and with *Bacterium lactis acidi*. The presence of salt did not seem to prevent the production of trimethylamine. Two organisms which were isolated from milk gave a pronounced test from the choline inoculation, but gave negative results in the presence of lactic acid and salt; *Bacillus prodigiosus* gave a positive reaction from the choline alone; and *Bacterium aerogenes* gave a non-typical test under the same conditions, as did *Pseudomonas liquefaciens fluorescens*. All other inoculations gave negative results.

It would appear from the results of the inoculation experiments that since trimethylamine is produced from choline by *Bacterium ichthyosmius* and certain organisms found in milk, it is quite possible that the fishy flavor and odor found in milk and in butter may be due to this substance's having been produced from the choline of the lecithin molecule. The fact that the two organisms isolated from milk gave a positive reaction and that they were selected at random, indicates that such a fermentation might be found fairly often. These results would therefore seem to point to bacterial agencies as the cause of the fishy flavor, its manifestation in butter being dependent on conditions previously mentioned.

BACTERIAL INOCULATIONS INTO BUTTERFAT

A further attempt was made to produce the fishy flavor by bacterial inoculations into a medium in which all the constituents were fairly definitely known. Pure sterile butterfat from which the phosphatides had been extracted was used as the basis of such a medium. Four series of inoculations were made, using, with a few exceptions, the species that were inoculated into lecithin and choline. These inoculations were stored at a temperature of 0° F. or lower for two hundred and nineteen days, at the end of which time they were examined for the fishy flavor by four judges. Negative results were obtained from all inoculations, not one of the judges pronouncing any of the 120 samples to be fishy in flavor. Whether any of the samples had possessed the flavor at some time during the storage period is difficult to say. All of the samples had a disagreeable tallowy flavor and odor. The composition of each of the four series, and the variation in acid value caused by the inoculations, are shown in table 27. All samples contained from 10 to 12 per cent of moisture and 2 per cent of salt.

TABLE 27. COMPOSITION OF ARTIFICIAL BUTTER AND VARIATIONS IN ACID VALUE CAUSED BY BACTERIAL INOCULATIONS

Sample	Organism or source	Composition and acid value			
		Butterfat	Butterfat and 0.15 per cent of lecithin	Butterfat and Berkfeldt filtrate from starter	Butterfat, Berkfeldt filtrate from starter, and 0.15 per cent of lecithin
1	*Bacterium lactis acidi*	5.2	6.1	6.5	8.8
2	*Oïdium lactis*	7.8	5.2	7.8	7.5
3	*Bacterium aerogenes*	7.1	6.8	5.8	7.8
4	*Bacterium ichthyosmius*	5.2	6.8	6.1	7.1
5	*Pseudomonas liquefaciens fluorescens*	6.8	7.1	7.1	7.1
6	*Bacillus prodigiosus*	8.4	6.5	7.5	7.1
7	*Bacillus proteus*	6.1	5.8	6.5	6.8
8	Fishy butter	7.1	7.1	7.5	7.8
9	Fishy butter	6.1	9.1	6.5	7.5
10	Fishy butter	6.5	6.5	7.8	7.1
11	Fishy butter	5.8	6.1	7.8	8.8
12	Fishy butter	7.1	6.1	8.8	8.1
13	Egg yolk	7.1	6.5	8.4	8.1
14	Egg yolk	7.1	6.5	8.8	9.7
15	Strong butter	9.1	6.8	8.4	7.8
16	Strong butter	8.4	8.1	8.4	8.4
17	Normal butter	7.5	6.5	8.4	8.4
18	Fishy butter	8.8	6.8	8.1	8.8
19	Fishy butter	8.1	7.8	7.8	9.7
20	Fishy butter	8.1	6.1	8.1	10.4
21	Fishy butter	6.5	7.1	8.1	11.0
22	Fishy butter	8.4	8.1	9.4	7.8
23	Fishy butter	10.4	6.5	8.4	7.8
24	Egg yolk	9.7	7.1	8.4	7.1
25	Egg yolk	8.8	7.1	6.5	7.8
26	Milk	6.8	6.1	7.5	7.8
27	Milk	7.5	6.5	7.1	9.1
28	Milk	7.8	6.8	6.1	10.0
29	Milk	6.5	8.1	8.1	5.8
30	Check	5.8	5.9	8.1	7.4

The results shown in table 27 are of interest only to the extent that they show the variation in acid value caused by different species of bacteria. Inasmuch as the samples were placed in storage immediately after being inoculated, it is probable that the changes are the result of bacterial enzymes liberated by autolysis, because it has been repeatedly shown that little or no growth takes place during storage.

SUMMARY

The data presented in this paper show beyond a doubt that there is in normal butter a sufficient amount of lecithin to yield, on decomposition, small quantities of trimethylamine, and it is shown also that small quantities of this substance are essential for the manifestation of a fishy odor. Furthermore, it is shown that when this substance is worked into butter under the proper conditions, it produces a flavor described as fishy. These results are most uniform when trimethylamine butyrate is used. An associative fermentation in butter or in cream, with the ultimate formation of this substance, is quite possible. As to whether or not this or some other volatile and unstable combination of trimethylamine is the cause of the natural fishy flavor, remains to be shown more conclusively. Certain data do indicate that trimethylamine is found in some samples of fishy-flavored butter but not in others. Altho it is possible that its presence is incidental in such samples, that is not believed to be the case. In this connection it is worth while to call attention to the confusion between the fishy, oily, and metallic flavors when they are present to only a slight degree. It seems possible that the initiation of the development of these flavors depends on a common fundamental factor. Whether or not any particular one of them develops to its typical flavor would depend on the presence of certain conditions which were specific for that flavor. With this possibility in view, it would be logical to assume that trimethylamine is responsible for the typical herring, or mackerel, flavor and odor in butter, and that the absence of this substance would result in the manifestation of similar but non-typical flavors.

There seems to be no doubt that the presence of a definite acid condition in the butter is essential for the development of the fishy flavor. This condition is best obtained when butter is made from cream containing lactic acid, regardless of whether this is developed by bacteria or added to the cream in the form of the commercial product. Furthermore, the results indicate that, while a definite acid condition is essential, it must be accompanied by some other equally important factor. The data show that this factor is determined by biological agencies. It appears that both these factors must exist in a definite and delicate relationship, and that if the proper equilibrium is disturbed, the characteristic flavor is not manifest. Numerous results and observations indicate that the unknown transient factor is trimethylamine.

The bacteriological aspects of the problem seem to involve the determination of the relationship already mentioned. It is shown that the acid value of butter is to a certain extent regulated by biological factors, probably enzymes. It is shown also that trimethylamine may be produced in milk and in cream, probably to some extent from lecithin, with the consequent production of the fishy flavor in those products. Furthermore, it is shown that *Bacterium ichthyosmius*, which produced the flavor in those substances, would produce the flavor in butter also under certain conditions. It would therefore seem possible that other species of microorganisms might bring about the same type of change. It seems highly probable that the growth of bacteria in the cream before it is made into butter determines the conditions necessary for the later manifestation of the fishy flavor.

The data dealing with lecithin as the source of trimethylamine in milk products are too meager to warrant definite conclusions at this time. However, the results presented herein, taken together with what is known regarding this substance, indicate that this is one of the most logical sources.

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