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A COMPARATIVE STUDY OF THE BIOCHEMICAL ACTIVITY OF *STREPTOCOCCUS LACTIS, STREPTOCOCCUS CITROVORUS,* AND *STREPTOCOCCUS PARACITROVORUS* WHEN GROWN IN COW'S MILK AND SOYBEAN MILK

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The importance of acetylmethylcarbinol and diacetyl from the standpoint of imparting a desirable flavor and aroma to butter and other food products is widely known. The value of selected cultures of bacteria for the development of these compounds has been well established through comparative studies on butter made with and without the use of butter cultures. Hammer and Babel (6) state in their review of butter cultures that previous to 1919 it was commonly believed that butter cultures were pure cultures of lactic acid streptococci, although there had been various suggestions that the desirable flavor of butter made from ripened cream was not produced by the lactic acid bacteria growing in the cream. In that year three laboratories established the basis for an understanding of the bacteriology of butter cultures by reporting almost simultaneously that such cultures normally include two distinct types of bacteria. Boekhout and Ott de Vries (1) isolated from sour milk and cream an organism which produced the characteristic and desirable butter culture aroma when grown with an organism of the *Streptococcus lactis* type; Hammer and Bailey (7) found that butter cultures contained organisms, associated with *S. lactis,* which commonly did not curdle milk but which in combination with *S. lactis* gave high volatile acidities; and Storch (14) considered two types of organisms necessary in butter cultures, a lactic acid type and a flavor type. The latter did not coagulate milk or form much acid but produced more volatile acid than the former. The two distinct types of organisms present in the butter cultures commonly used are *S. lactis* or *Streptococcus cremoris,* which primarily attacks the lactose and forms relatively large amounts of lactic acid together with small amounts of secondary products, and *Streptococcus citrovorus* and/or *Streptococcus*

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paracitrovorus, which are characterized mainly by the fermentation of citric acid to diacetyl and related compounds, some of which add greatly to the flavor and aroma of butter cultures, certain types of butter, cultured buttermilk, various cheeses and other food products. The numbers of bacteria in active butter cultures, as determined by plate counts, commonly are in the hundreds of millions and may be over 1 billion per ml. In the cultures studied by Hammer (4), *S. lactis* often made up 90 per cent of the flora and only occasionally fell under 75 per cent; in certain cases the flavor type made up only 1 to 3 per cent of the flora.

Price *et al.* (9) found that the lactic acid organisms usually produce from 0.7 to 1.0 per cent acid in milk, with the maximum about 1.2 per cent; most of the acid is lactic. Suzuki *et al.* (15) stated that the group of bacteria represented by *S. lactis* produces from 90 to 98 per cent of the theoretic yield of lactic acid from the sugar fermented, the remainder of the sugar going to alcohols, aldehydes and esters. With 50 cultures of lactic acid streptococci, Sherman and Albus (13) found the acid produced in milk (10 days at 35° C.) ranged from 0.60 to 0.95 per cent, the average being 0.80 per cent.

The high volatile acidities of butter cultures are evident from the odor and are readily detected by chemical procedures. Hammer and Bailey (7) found that the volatile acidities of butter cultures ranged from 31.2 to 37.6 (ml. of 0.1 *N* NaOH to neutralize 1 l. steam distillate from 250 g. culture); the total acidities varied from 0.87 to 1.08 per cent. Cordes and Hammer (3) noted that the volatile acidity of a butter culture increased as the total acidity increased until, in general, it reached 10 to 15 per cent of the total acidity. With 183 butter cultures grown in pasteurized milk, Templeton and Sommer (16) found the volatile acidities averaged 15.43 per cent of the total acidities; with 28 cultures grown in pasteurized milk plus 0.2 per cent citric acid, the value was 23.10 per cent. Boekhout and Ott de Vries (2) found that symbiosis of the two types of butter culture organisms yielded not only flavor but also considerable volatile fatty acid which was acetic.

The recognition by Van Niel *et al.* (17) that acetylmethylcarbinol (AMC) and diacetyl (AC_2) either are responsible for the aroma of butter or are the principal components of the aroma material soon led to studies on the production of AMC and AC_2 in butter cultures. Schmalfuss (10), by the sense of smell, detected AC_2 in a milk culture of a rod-shaped lactic acid organism and, through analyses, confirmed the identification of this compound. Van Niel *et al.* (17) noted that certain strains of propionic acid bacteria on a special medium (yeast-dextrose-chalk-agar) produced an odor similar to that of a high quality butter. A wide variation in the production of this odor was noted among the strains of propionic acid

bacteria, and a study of the products formed indicated that AMC was related to the typical butter aroma. It also was observed that a dilute aqueous solution of AMC or AC_2 had an odor characteristic of butter. The authors concluded that AC_2 either is responsible for the aroma of butter or is the principal component of the aroma material. Michaelian *et al.* (8) determined the amounts of AMC + AC_2 in satisfactory cultures and also in cultures lacking flavor. The results showed that considerable AMC plus AC_2 was present in satisfactory cultures. The authors also found that cultures contained only small amounts of AMC + AC_2 during the early stages of ripening, while conspicuous increases occurred later. It was observed that early in the ripening pronounced changes in titratable acid or pH had little effect on the amount of AMC + AC_2 present, but later striking increases occurred with little or no change in acidity. Hammer (5) and Michaelian *et al.* (8) made an extensive study of the relationship of AMC and AC_2 to butter cultures. Hammer and Babel (6) compiled an extensive review on the bacteriology of butter cultures. Schmalfuss and Barthmeyer (11, 12) studied the presence of AMC and AC_2 in various food materials and noted that the foods examined contained much more AMC than AC_2.

Much research has been done on the study of flavor and aroma compounds in milk, butter, foods and other materials. However, to the authors' knowledge, no information has been published on the production of these compounds in soybean milk and its products. This investigation was undertaken to determine the comparative biochemical activity of the butter culture organisms, *S. lactis, S. citrovorus,* and *S. paracitrovorus,* in cow's milk and soybean milk.

Soybean or vegetable milk is used extensively throughout Japan and China for infant feeding as well as a food for adults. The introduction of soybean milk to the American people has occurred only recently. Soybean milk has been manufactured in the form of a powder. It has been used with good results in breads and cakes, in creaming vegetables, in custards, in chocolate or cocoa, and in several other food products as a substitute for cow's milk, especially in those countries that find it cheaper to use a vegetable milk. The high nutritive value of soybean milk and its many potential uses indicate that this product will continue to rise in importance as an item in the human diet. The development by butter culture organisms of AC_2 and related compounds in vegetable milk and products made from it may be desirable from a commercial standpoint.

EXPERIMENTAL PROCEDURE

The butter cultures used in this investigation were obtained from the Department of Dairy Industry, Iowa State College, Ames. The cultures

were carried in sterile skimmed milk, transferred daily, and incubated at 21° C. until coagulation occurred. The cultures were removed immediately after coagulation and held in the refrigerator at a temperature of approximately 10° C.

Seven-hundred-milliliter samples of skimmed cow's milk and of soybean milk were placed in quart milk bottles, plugged with rubber stoppers, covered with wrapping paper, and sterilized by heating to 100° C. for 20 minutes on 3 consecutive days. The soybean milk used was obtained from Harry Miller, Director of the International Nutrition Laboratory, Mt. Vernon, Ohio. The sterilized samples of milk were inoculated with butter culture organisms (3 ml.) and held at 21° C. for 0, 12, 28, 48, 72, 96, 168 and 216 hours. The hydrogen-ion concentration, titratable acidity, volatile acidity, and AMC + AC_2 were determined in duplicate on each sample at the end of each incubation period.

The pH determinations were made on 10-ml. samples of each culture of fermented milk, using a Coleman 3C glass electrode potentiometer.

The titratable acidity was determined by the titration of 10 ml. of the culture with 0.1 *N* sodium hydroxide, using phenolphthalein as the indicator. The end-point taken was that point at which a faint pink color remained for 1 minute. The acidity obtained was expressed as per cent lactic acid.

The volatile acidity was determined by the method of Michaelian *et al.* (8). Two hundred and fifty grams of the cultured milk with 250 ml. of distilled water was steam distilled after the addition of 15 ml. of *N* sulfuric acid. The first 1,000 ml. of distillate was titrated, using 0.1 *N* sodium hydroxide and phenolphthalein. The results were expressed as the ml. of 0.1 *N* sodium hydroxide required to neutralize the acidity. In determining the amounts of flavor and aroma compounds by the procedure of Michaelian *et al.* (8), a 200-g. portion of the milk was distilled with steam after adding 40 ml. of ferric chloride solution to oxidize the AMC to AC_2. Hydroxylamine hydrochloride, sodium acetate, and nickel chloride solutions were added to the distillate as a mixture. The material was allowed to stand at least 1 day in order to permit complete crystallization; the nickel salt then was filtered into a weighed crucible. The salt was washed with distilled water, dried to constant weight at 110° C., and the results were expressed as the milligrams of nickel dimethylglyoximate per 200 g. of cultured milk.

RESULTS AND DISCUSSION

The values obtained for the pH and volatile acidity were comparable in most instances for both types of milk held at the various incubation periods. After cultures of fermented soybean milk were held for 96 to 216 hours, the volatile acidities ranged from 24.2 to 32.3 (ml. of 0.1 *N* NaOH to neutralize 1 l. of steam distillate from 250 g. of culture);

TABLE 1

The relationship between certain biochemical activities of butter culture organisms when propagated in cow's and soybean milk
(Av. of two determinations. Cultures grown in 700 ml. of sterile substratum inoculated with 3 ml. of culture and incubated at 21° C.)

	Hours incubated								
	0	4	12	24	48	72	96	168	216
Cow's milk									
pH	6.6	6.1	5.7	4.3	4.3	4.3	4.4	4.4	4.3
Titratable acidity[a]	0.15	0.20	0.25	0.83	1.12	1.07	1.04	1.08	1.03
Volatile acidity[b]	4.1	4.0	4.1	9.6	20.4	23.5	23.4	33.8	27.5
Mg. Ni salt[c]	None	None	Trace	10.2	18.6	15.3	18.7	18.2	16.9
Soybean milk									
pH	6.1	5.7	5.4	4.6	4.5	4.5	4.5	4.5	4.4
Titratable acidity[a]	0.17	0.17	0.24	0.45	0.49	0.51	0.55	0.49	0.51
Volatile acidity[b]	3.8	6.4	7.1	10.5	12.5	10.9	24.2	25.0	32.3
Mg. Ni salt[c]	None	None	None	None	4.6	9.3	12.5	33.6	21.4

[a] As per cent lactic acid per 10 g. sample.
[b] Values expressed in ml. of 0.1 *N* sodium hydroxide per 1,000 ml. of distillate.
[c] Milligrams of nickel dimethylglyoximate per 200 g. of culture.

whereas, for cow's milk the values were 23.4 to 27.5 ml. (table 1). The value for the volatile acidity in cow's milk plus 0.15 per cent citric acid averaged 32.6 ml. for an incubation period of 96 to 216 hours; the corresponding value for cultured soybean milk was 33.5 ml. (table 2).

The production of AMC plus AC_2 in soybean milk by butter culture organisms was not evident until after an incubation period of 48 hours; however, upon incubation of cultured soybean milk for 168 to 216 hours, larger amounts of the flavor and aroma compounds were produced in soybean milk as compared to cow's milk (table 1). When the samples were held for 168 hours at 21° C., 33.6 mg. of Ni salt were obtained from 200 g. of cultured soybean milk. In cow's milk held under similar conditions, 18.2 mg. of Ni salt were obtained. In cow's milk a trace of AMC plus AC_2 was formed after a 12-hour incubation period. The results obtained for the production of AMC plus AC_2 in cow's milk are in agreement with those of Michaelian *et al.* (8), who found that satisfactory cultures yielded 10 mg. or more nickel dimethylglyoximate per 200 g., the maximum being 39.5 mg. The results obtained for the cultured soybean milk are comparable.

When the butter culture organisms were grown in cow's milk plus 0.15 per cent citric acid, the AMC plus AC_2 content was found to the extent of 14.2 mg., as the Ni salt, per 200 g. of culture after an incubation period of 12 hours (table 2). The AMC plus AC_2 content increased to 44.7 mg. upon incubation of the cultures for 216 hours. For cultured soybean milk plus 0.15 per cent citric acid the production of AMC plus AC_2 was retarded; 2.4 mg. of the Ni salt were obtained after an incubation period of 24 hours, which increased to 42.3 mg. after 216 hours of incubation. AMC plus AC_2 was not developed as rapidly in soybean milk as in cow's milk, but upon prolonged incubation the results obtained were in close agreement.

The AMC plus AC_2 content of samples of cultured cow's milk and soybean milk held for 72, 96, 168, and 216 hours at 21° C. averaged 17.2 and 19.2 mg. of the Ni salt, respectively (table 1). When 0.15 per cent citric acid was added to the cultures, the average values obtained were: cow's milk, 33.2 mg.; soybean milk, 32.5 mg. (table 2). This represents a 93 and 70 per cent increase in the AMC plus AC_2 content of the cultured cow's milk and soybean milk, respectively.

The values obtained for the titratable acidity in cow's milk and cow's milk to which 0.15 per cent citric acid was added were nearly twice as great as those secured for the cultured soybean milk. Early in the ripening pronounced changes in titratable acid or pH had little effect on the amount of AMC plus AC_2 present, but later significant increases occurred with little or no change in acidity. These results are in agreement with those of Michaelian *et al.* (8).

TABLE 2

The relationship between certain biochemical activities of butter culture organisms when propagated in cow's and soybean milk modified by the addition of citric acid

(Av. of two determinations. Cultures grown in 700 ml. of sterile substratum inoculated with 3 ml. of culture and incubated at 21° C.)

	Hours incubated								
	0	4	12	24	48	72	96	168	216
Cow's milk (0.15 per cent citric acid added)									
pH	6.6	6.2	4.8	4.8	4.2	4.0	4.0	4.4	4.4
Titratable acidity[a]	0.15	0.21	0.90	0.90	1.04	1.08	1.13	1.15	1.10
Volatile acidity[b]	4.3	4.4	10.8	16.1	33.6	27.5	33.5	31.0	33.5
Mg. Ni salt[c]	None	None	14.2	36.4	32.0	30.3	28.6	29.5	44.7
Soybean milk (0.15 per cent citric acid added)									
pH	6.1	5.6	4.8	4.8	4.3	4.2	4.0	4.5	4.5
Titratable acidity[a]	0.16	0.15	0.51	0.45	0.64	0.66	0.68	0.64	0.67
Volatile acidity[b]	4.0	6.6	10.8	9.1	20.2	21.2	31.3	31.5	37.7
Mg. Ni salt[c]	None	None	Trace	2.4	12.6	36.4	20.9	30.4	42.3

[a] As per cent lactic acid per 10 g. sample.

[b] Values expressed in ml. of 0.1 *N* sodium hydroxide per 1,000 ml. of distillate.

[c] Milligrams of nickel dimethylglyoximate per 200 g. of culture.

Further data are presented in table 3 for samples of cultured soybean milk to which 0.10 0.15, 0.20, and 0.30 per cent citric acid was added.

TABLE 3

The relationship between certain biochemical activities of butter culture organisms when propagated in cow's milk and soybean milk re-enforced with citric acid

(Av. of two determinations. Cultures grown in 700 ml. of sterile substratum inoculated with 3 ml. of culture and incubated at 21° C.)

Hours held at 21° C.	Added citric acid (%)	pH	Titratable acidity[a]	Volatile acidity[b]	mg. of Ni salt[c]
		Soybean milk			
0	0.10	6.1	0.17	3.8	None
48	0.10	4.6	0.68	11.5	11.9
72	0.10	4.6	0.62	23.0	23.4
120	0.10	4.3	0.64	33.5	40.1
0	0.20	6.1	0.17	3.8	None
48	0.20	4.6	0.67	12.3	15.0
72	0.20	4.7	0.70	27.0	47.0
120	0.20	4.4	0.77	40.0	44.0
72	0.15	4.4	0.65	21.1	20.3
120	0.30	4.4	0.66	40.2	35.4
		Cow's milk			
72	0.15	4.3	1.02	39.3	49.1
120	0.30	4.3	1.04	40.7	46.0

[a] As per cent lactic acid per 10 g. sample.

[b] Values expressed in ml. of 0.1 *N* sodium hydroxide per 1,000 ml. of distillate.

[c] Milligrams of nickel dimethylglyoximate per 200 g. of culture.

SUMMARY AND CONCLUSIONS

A comparative study was made of pH, titratable acidity, volatile acidity, and acetylmethylcarbinol plus diacetyl on samples of cow's milk and soybean milk inoculated with the butter culture organisms, *Streptococcus lactis, Streptococcus citrovorus* and *Streptococcus paracitrovorus.*

The values secured for the pH and volatile acidity were comparable in most instances for both types of milk held at the various incubation periods. The value for the volatile acidity in cultured cow's milk averaged 28.3 (ml. of 0.1 *N* NaOH to neutralize 1 l. of steam distillate from 250 g. of culture) for an incubation period of 96 to 216 hours; the corresponding value for cultured soybean milk was 27.2 ml. For cultured cow's milk and soybean milk plus 0.15 per cent citric acid, the values were 32.6 and 33.5 ml., respectively.

During the early stages of fermentation of cultured cow's milk and

soybean milk, only small amounts of acetylmethylcarbinol plus diacetyl were present, while after 96 hours of incubation at 21° C. appreciable increases in these substances occurred.

Samples of cultured soybean milk held 168 to 216 hours contained larger amounts of acetylmethylcarbinol plus diacetyl than cultured cow's milk; 33.6 mg. of Ni salt were obtained from 200 g. of cultured soybean milk held 168 hours at 21° C.; whereas, in cow's milk held under similar conditions, 18.2 mg. were found.

The acetylmethylcarbinol plus diacetyl content of eight samples of cultured cow's milk and eight samples of soybean milk held 72 to 216 hours at 21° C. averaged 17.2 and 19.2 mg. of the Ni salt, respectively. When 0.15 per cent citric acid was added to the cultures, the values obtained were: cow's milk, 33.2 mg.; soybean milk, 32.5 mg. The addition of citric acid resulted in a 93 and 70 per cent increase in the production by butter culture organisms of acetylmethylcarbinol plus diacetyl in cow's milk and soybean milk.

Acetylmethylcarbinol plus diacetyl was not developed as rapidly in soybean milk as in cow's milk, but upon extended holding of the cultures the results obtained were comparable.

Cultures of cow's milk held at 21° C. for 72 to 216 hours had an average lactic acid content of 1.05 per cent; whereas, cultures of soybean milk held under similar conditions had a lactic acid content of 0.51 per cent. Samples of fermented cow's milk plus 0.15 per cent citric acid held for 72 hours or longer contained nearly twice as much lactic acid as samples of fermented soybean milk.

Early in the ripening pronounced changes in titratable acid had little effect on the development of acetylmethylcarbinol plus diacetyl in cow's milk and soybean milk, but later in the incubation period significant increases occurred with little or no change in acidity.

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THE ROLE OF SURFACE-ACTIVE CONSTITUENTS INVOLVED IN THE FOAMING OF MILK AND CERTAIN MILK PRODUCTS. III. MILK LIPIDS, INCLUDING PHOSPHOLIPIDS[1]

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The role of the milk lipids in the foaming of milk is not clearly defined. Van Dam (27), by decreasing the fat content of separated milk from 0.22 to 0.08 per cent, and Mohr and Brockmann (14), by increasing it from 0.04 to 1.0 per cent, concluded that the foaming capacity and foam stability varied inversely with the fat content. Sommer and Hörrall (24) found addition of fat to a skim milk-gelatin-sugar mix greatly decreased whipping ability. El-Rafey and Richardson (5) attributed the minimum foaming of skim milk, whey, lactalbumin sols, and blood serum at approximately 27° C. to the presence of fat globules.

Leete (11), on the other hand, from studies with skim milk, milk, and cream, concluded no definite statement could be made regarding the effect of milk fat on foaming without considering temperature. Sanmann and Ruehe (22), failing to find a definite relationship between the fat or solids content of milk and its foaming ability, suggested that the foaming ability of milk from individual cows largely is dependent upon factors which are characteristic of the cow. When these latter factors were controlled, they found that increasing the fat content usually decreased the foaming ability; the reverse was true with respect to solids-not-fat. According to Holm (7), increases in the fat content of milk over that normally present result in increased foaming and great foam stability.

The effect of the physical state of the fat on its influence on foaming has been recognized. Mohr and Brockmann (14) found that milk exhibits greater foaming properties at temperatures at which the fat is liquid than where it is solidified. This appears to be contrary to the theory proposed by Leviton and Leighton (12) that the destructive action of milk fat and other lipids on foam depends upon their ability to spread on water. It has been reported that the concentration of fat in sodium caseinate solutions is of less significance on their foaming capacity than the chemical and physical condition of the fat (19).

Preliminary studies of the role of milk fat in the foaming of milks and

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creams suggested not only that the concentration, the chemical composition, and the physical state of the fat must be considered, but also that the nature of the material comprising the interface between the fat globules and the aqueous medium is of even greater significance.

EXPERIMENTAL PROCEDURE

The method of measuring foaming capacity and foam stability has been described in a previous publication (4). The milk fat used in this study was obtained by churning fresh cream from mixed herd milk, melting the butter at 50° C., and filtering the decanted fat at the same temperature. The fat was stored at 5° C.

Preliminary investigations showed that soybean phospholipids gave essentially the same results when used as emulsifiers as milk phospholipids isolated from separator slime. Soybean phospholipids,[4] which are a mixture including lecithin, cephalin, and probably inositol phosphatide, were used in these studies.

The natural emulsions were prepared by diluting cream with the appropriate volumes of its separated milk. The artificial emulsions were prepared by dispersing the fat or oil, with or without added phospholipid, in pasteurized separated milk, or other medium, using a two-cylinder hand emulsifier.[5] When stabilizers, such as gum arabic, were used, the fat first was triturated with the powdered gum and distilled water, and the resulting cream, after being diluted to the desired concentration, was passed through the emulsifier.

The size of the fat globules in the creams was determined microscopically using the technic of Cole and Smith (3). Emulsification was considered satisfactory when the globules ranged in diameter from 1 to 11 μ, with an average diameter of 3.5 μ. To attain this it sometimes was necessary to pass the mixtures through the emulsifier three to five times.

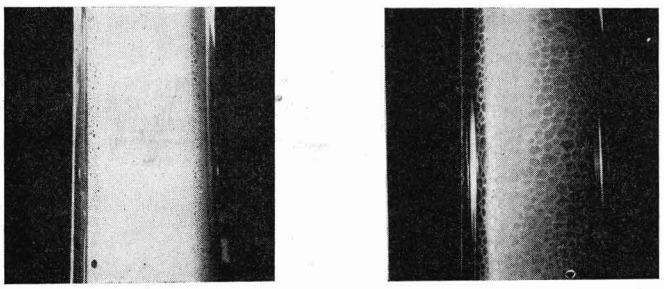
Phosphorus in the fats was determined colorimetrically (31), the fat being ashed according to the method of Horrall (8).

RESULTS

Effect of fat percentage on the foaming of milk and cream. At certain temperatures, at least, skim milk and cream yield their own distinctive type of foam, as illustrated in figure 1. In a series of milks and creams of increasing fat contents, both types of foam will be expected to be present at a certain fat content, and somewhere in the series a reversal of predominating types will take place.

[4] "Margo", 70 per cent phospholipid in soybean oil, courtesy of Dr. J. Eichberg, American Lecithin Company, Long Island City, N. Y. "Best Grade Lecithin", courtesy of Mr. D. C. Ingraham, Durkee Famous Food Company, Berkeley, California.

[5] Club Aluminum Products Co., Chicago, Illinois.



(a) (b)

FIG. 1. Types of foam from milk and cream. (a) Skim milk or protein type. (b) Cream or lipoprotein type.

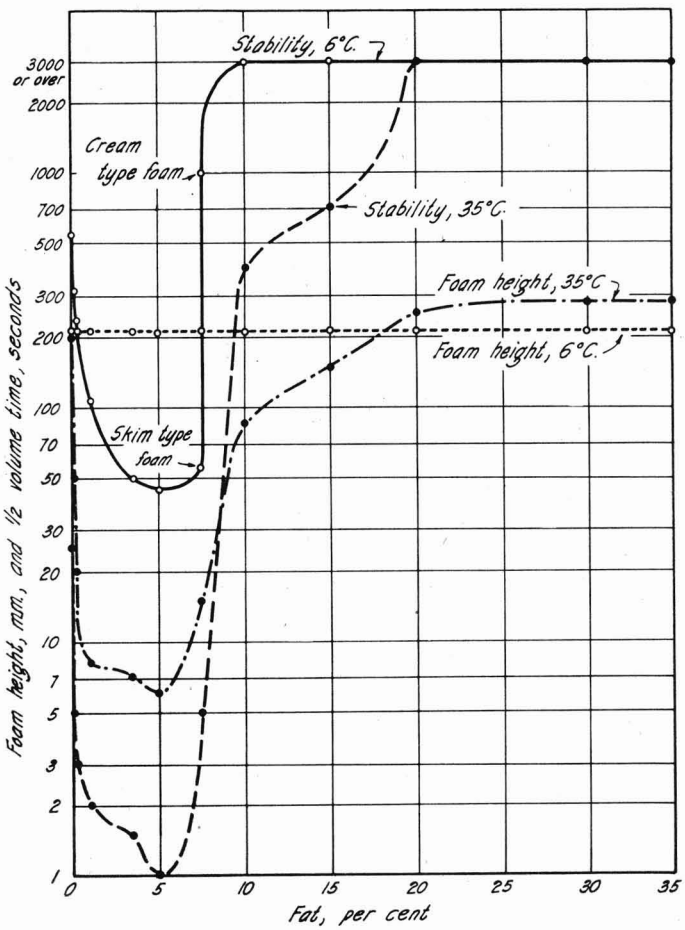


FIG. 2. The effect of increasing the fat content of skim milk from 0.01 to 35.0% on its foaming properties at 6° C. and at 35° C.

A series of samples ranging in fat contents from 0.01 to 35.0 per cent was prepared using raw separated milk and raw cream. As shown in figure 2, at both 6 and 35° C., as the fat content increases, the foam stability first decreases to a minimum value, and this is followed by a rapid increase in stability. With fat concentrations up to between 5.0 and 7.5 per cent, the foam consisted of small, compact cells with a short half-volume time. Above 7.5 per cent of fat, a cream-type or lipoprotein-type of foam predominated. This latter consisted of large cells with distorted lamellae that maintain an increasingly stable structure with increasing fat percentage.

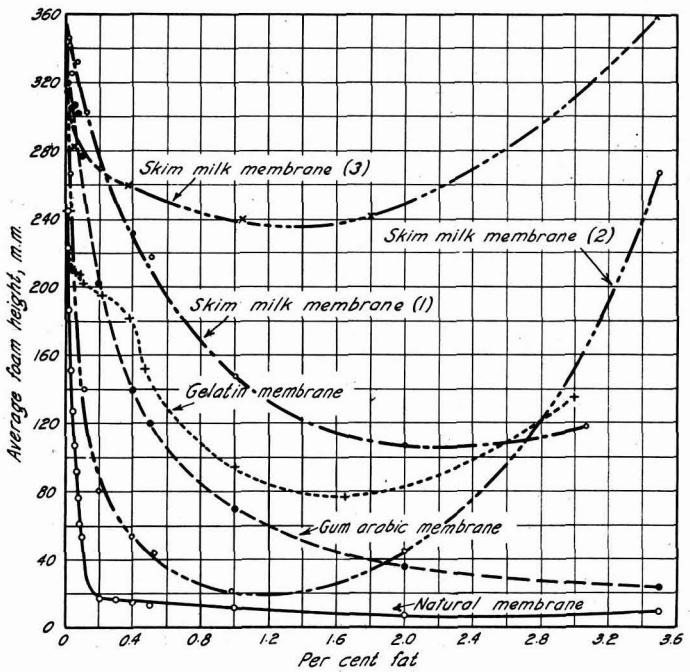


FIG. 3. The effect of the type of stabilizer on the foaming of milk fat emulsions at 35° C. (All emulsions were made by diluting creams, stabilized as indicated, with skim milk. The emulsions for curve (2) differ from those of (1) in that the fat for (2) contained 4% phospholipids added prior to emulsification. Curve (3) represents emulsions of soybean oil containing 4% added phospholipids.)

At 6° C. both types of foam were visible in the 7.5 per cent milk. At 6° C. the foam volume remained constant regardless of the fat content.

Effect of the emulsifying agent on foaming. Various emulsions were prepared as follows: (a) Natural raw cream was diluted with raw separated milk to give a series of milks ranging in fat contents up to 3.5 per cent. (b) Artificial emulsions were made by emulsifying milk fat into raw separated milk, gelatin sols, or gum arabic and diluting the resulting

creams with separated milk to give a series of milks of fat contents similar to those in (a). (c) Artificial emulsions were made by emulsifying milk fat or soybean oil, each containing 4 per cent added soybean phospholipids, into raw separated milk and diluting these with the separated milk to obtain the series of milks of varying fat contents. The foaming characteristics of these milks at 35° C. were studied.

The results are shown in figure 3. With the emulsions made with the natural cream, a rapidly progressive decrease in foaming occurs with increasing fat content up to 0.2 per cent fat, after which no marked further decreases occur. The emulsions of milk fat containing the added phospholipids showed a marked resemblance to the natural emulsions up to a fat content of 1 per cent. With increasing fat contents, however, the foaming capacity (and foam stability) increased. The type of this latter foam was distinctive. It was coarse in structure, consisting mostly of five- and six-membered rings. It exhibited a marked glistening and iridescent appearance. The lamellae became very thin and the whole structure collapsed in an explosive manner. This type of foam will be referred to as the "phospholipid" foam. As later experiments will indicate, the 4 per cent added phospholipid is excessive.

The effect of increasing fat contents is not so marked in those emulsions stabilized with skim milk or gelatin. No changes in the nature of the foams were observed with increasing fat contents. With the gum arabic-stabilized emulsions, increasing concentrations of fat exerted a slow but progressive depressing action on foaming. The depressing action of the soybean oil containing added phospholipids was found to be less marked than that of milk fat, but, in the higher concentrations, the typical phospholipid foam was observed.

Effect of increasing phospholipid content on the foaming of separated milk. A 2 per cent soybean phospholipid emulsion, prepared by dispersing it in pasteurized skim milk at 50° C. and passing it five times through the emulsifier, was diluted with the skim milk to give a series ranging from 0.0 to 2.0 per cent added phospholipid. Foaming tests were made at 21.5° C. The results are shown in figure 4. As the added phospholipid increases to 0.05 per cent, the foaming tendency decreases. This decrease is about six times that obtained by adding soybean oil in amounts equivalent to that associated with the added phospholipid.

As the concentration of phospholipid increases beyond 0.05 per cent, a typical phospholipid foam begins to appear, first as a coarse unstable foam, similar to that of buttermilk, and followed by foams of increasing height, compactness, iridescence and stability.

Effect of phospholipid concentration in the fat on foaming of milk emulsions. Four 10 per cent fat emulsions were prepared by emulsifying,

in pasteurized skim milk, milk fat containing 0.0, 0.45, 0.7, and 1.0 per cent added soybean phospholipid. The foaming properties were studied at various temperatures between 5 and 55° C. Figure 5 shows that each emulsion yielded foams of minimum stability at temperatures between 27 and 35° C. As the phospholipid content increased, the protein-type foam, characteristic

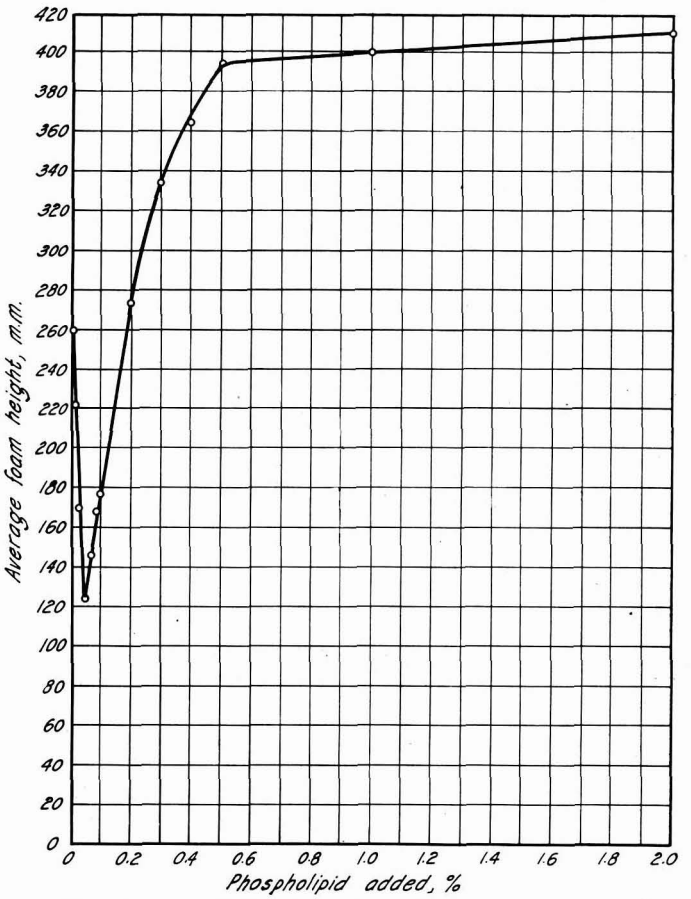


FIG. 4. The effect of added soybean phospholipids on the foaming of skim milk at 21.5° C.

of skim milk, gradually was replaced by a cream-type or lipoprotein-type foam.

No further increases in foaming capacity or foam stability occurred at temperatures above 50° C. In figure 6 it is seen that the emulsions made with fat containing about 0.8 per cent phospholipid yielded foams of minimum or low stabilities at all the temperatures below 50° C. The artificial

emulsions made with fat containing from 0.5 to 1.0 per cent phospholipid had foaming characteristics more closely resembling those of the natural emulsion made from cream and skim milk. At 23° C. the emulsions pre-

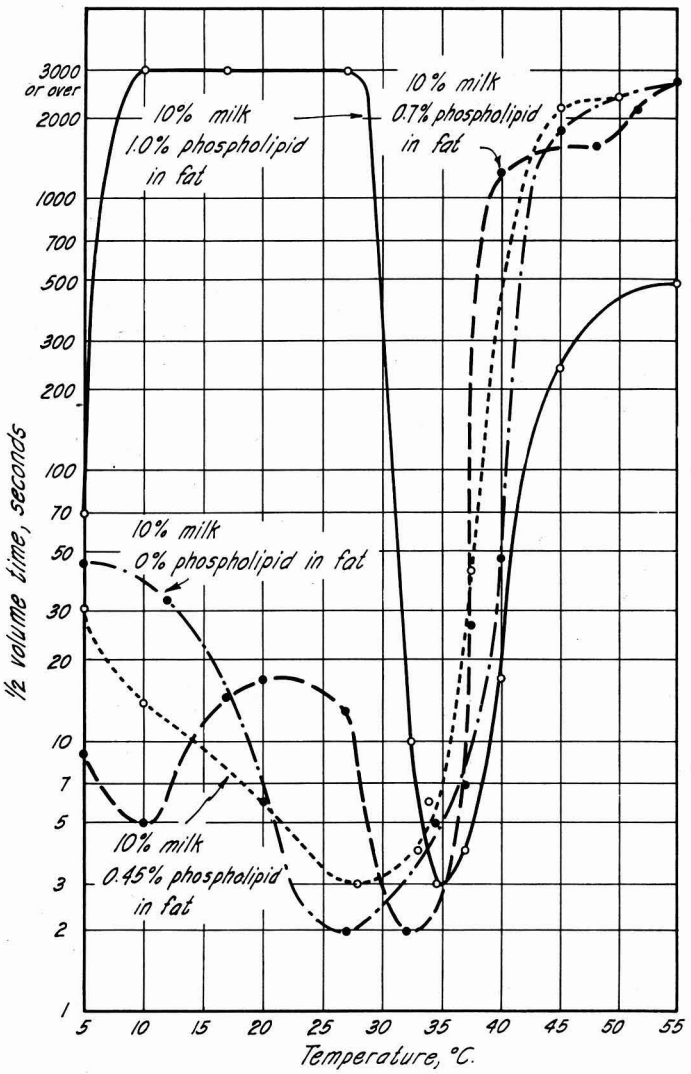


FIG. 5. The effect of temperature on the stability of foams of 10% emulsions of milk fat, containing varying concentrations of soybean phospholipids, dispersed in pasteurized skim milk.

pared with fat containing 0.8, 0.85, and 0.9 per cent phospholipid showed two types of foam, a protein foam that subsided quickly and the very stable cream-type of foam.

Effect of increasing the concentration of the fat of constant phospholipid content on the foaming of artificial creams. Artificial creams were prepared by emulsifying milk fat containing 0.5 per cent added soybean

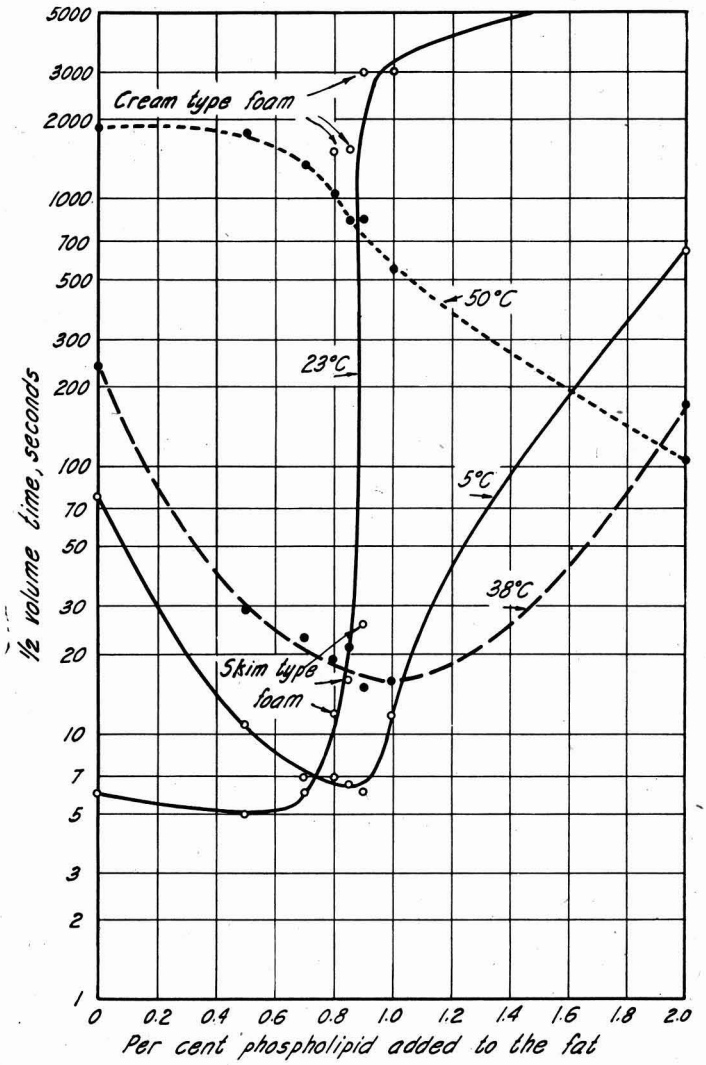


FIG. 6. The effect of increasing the phospholipid content of milk fat from 0.0 to 2.0% on the foam stabilities of a 10% emulsion in pasteurized skim milk at 5, 23, 38 and 50° C.

phospholipid into pasteurized skim milk. Natural creams were included. The results, shown in table 1, indicate that at 5 and 28° C. the foaming

tendency and the foam stability increase as the fat concentration increases from 10 to 40 per cent; the surface tension decreases with increasing fat concentration. At 50° C. the foaming capacity remains constant for increasing fat concentration, while the surface tensions and the half-volume times decrease progressively with increases in the fat. The surface tension values and the foaming properties of the 30 per cent artificial cream were very similar to those of the pasteurized natural cream of similar fat content.

Effect on foaming properties of emulsifying milk fat, with and without added phospholipids, in buttermilk. Wiese and Palmer (30) found that remade milk, made by dispersing butterfat in buttermilk, closely resembled whole milk in many respects, including the churnability of its cream. On

TABLE 1

The effect of concentration of fat of constant phospholipid content on the foaming of artificial creams

Sample	Results at 5° C.			Results at 28° C.		
	Surface tension	Foam height	½ volume time	Surface tension	Foam height	½ volume time
	(*dynes/cm.*)	(*mm.*)	(*sec.*)	(*dynes/cm.*)	(*mm.*)	(*sec.*)
10% milk	.....	115	12	43.4	40	5
20% cream	.....	162	17	45.0	100	> 3000
30% cream	.....	200	1220	42.3	240	> 3000
40% cream	.....	205	> 3500	40.0	.....	.....
31% natural cream	51.0	200	> 3000	42.7	240	> 3000

the other hand, emulsions made by dispersing milk fat in calcium caseinate, lactalbumin, lactoglobulin, or phospholipid-aqueous media behaved quite abnormally. These investigators used milk fat in which the phospholipid presumably would be absent or nearly so. They did not add phospholipid to the fat.

Two creams were made by emulsifying milk fat or milk fat containing 0.5 per cent soybean phospholipid into buttermilk from sweet, pasteurized cream. Foaming was studied at three temperatures and the results compared with those obtained with a 31 per cent pasteurized natural cream. The results (table 2) show that the addition of phospholipid to the milk fat prior to emulsification improves the foaming properties of the artificial creams to the extent that they resemble those of natural cream. The foam stability of these creams at 5° C. and 23° C. is almost eight times that of the cream with no phospholipid added to the fat.

It appears to have been established that the natural fat globule "membrane" of milk and cream is some sort of phospholipid-protein com-

plex. Palmer (15) stated, "Indeed, not only was it found that the emulsion properties and churnability of artificial emulsions of milk fat in the various colloidal sols from milk plasma are strikingly different from those of washed natural cream but also that the 'membrane' materials isolated from the washed artificial creams are also chemically distinct from the natural 'membrane' substances". To the authors' knowledge no attention has been paid to the presence or absence of phospholipid dissolved in the fat being emulsified. It is known, however, that the amount of these compounds left associated with the fat after its isolation depends upon the method of

TABLE 2

The foaming of 30% creams prepared by emulsifying butterfat, with and without 0.5% added soybean phospholipids, in buttermilk from sweet cream containing 31% fat[a]

Cream	Results at 5° C.			Results at 23° C.		
	Surface tension	Foam height	½ volume time	Surface tension	Foam height	½ volume time
	(*dynes/cm.*)	(*mm.*)	(*sec.*)	(*dynes/cm.*)	(*mm.*)	(*sec.*)
30% cream in buttermilk, no added phospholipid	54.4	200	396	48.2	220	435
30% cream in buttermilk, 0.5% phospholipid added to the fat	52.3	200	> 3000	43.5	240	> 3000
31% natural cream	51.6	200	3000	42.9	240	> 3000
Buttermilk (0.4% fat)	51.9	170	20	47.1	60	25

[a] At 50° C. all samples gave the protein-type foam.

isolation (6, 21). Jenness and Palmer (9) did have some indication that the protein as it is "eroded" during churning pulls away varying amounts of phospholipid with it. Observations in this laboratory (18) revealed striking visible differences at the interface between liquid milk fat superimposed upon a warm aqueous medium depending upon whether or not the fat contained phospholipid and whether or not the medium was pure water or contained whey proteins. The interface between the fat containing phospholipid and the pure water was cloudy, indicating solvation of the phospholipid; all the other interfaces remained clear.

The effect of incorporation of phospholipids in the fat of artificial creams on some of their physical properties. Soybean phospholipids were

dissolved in milk fat to a concentration of approximately 1.0 per cent by heating to 50° C. The solution was clear but showed a tendency to give an iridescent-type foam. A 25 per cent dispersion was prepared by emulsifying it in distilled water at 45° C. The emulsion was diluted to give creams containing 16.5 per cent butterfat, one-half being diluted with distilled water and the other half with a 0.5 per cent lactalbumin solution. The lactalbumin was isolated as described previously (4). Both creams were held overnight at 2° C.

TABLE 3

Foaming properties of creams prepared with butterfat with 1.0% soybean phospholipid added emulsified in distilled water and in 0.5% lactalbumin solution

Temp.	Surface tension	Foam height	½ volume time	Surface tension	Foam height	½ volume time
	16.5% cream (1% phospholipid in fat) in distilled water			16.5% cream (1% phospholipid in fat) in 0.5% lactalbumin		
(°C.)	(*dynes/cm.*)	(*mm.*)	(*sec.*)	(*dynes/cm.*)	(*mm.*)	(*sec.*)
5	47	190	250	48.9	210	> 3000[a]
16	40.9	40	10	46.6	185	1450[a]
25	40.5	20	6	40.5	30	10
37	29.1	100	> 3000[b]	29.6	15	5
45	29.1	105	> 3000	29.0	15	5
	Buttermilk from above cream 0.61% fat			Buttermilk from above cream 1.1% fat		
5	46.5	5	2	45.4	70	8
8	—	3	2	—	50	5
19	39.6	2	2	45.2	10	3
37	36.7	15	4	41.0	2	1
45	31.5	15	4	35.2	8	1.5

[a] Typical cream-type foam.

[b] At 37° C. the cream oiled off and an iridescent phospholipid foam originated from the oil layer on top.

Upon examination, the cream prepared in water contained large fat aggregates which stuck to the walls of the container and resembled those formed when normal cream is on the verge of "breaking" during churning. On heating to 37° C., the emulsion oiled off. Churning time at 10° C. was about 5 minutes. The butter formed a solid crumbly mass and the buttermilk contained 0.61 per cent fat (Babcock). The other cream prepared in the albumin solution had a smoother body, less tendency for fat aggregation, and a longer churning time (about 12 minutes). The butter was more

plastic, the butter granules maintained their individuality, and the buttermilk contained 1.1 per cent fat. The cream formed a stable cream-type foam between 5 and 15° C., and the foam decreased in volume and stability at increased temperatures.

Table 3 shows that the surface tensions of the creams were very similar except at the temperature of 16° C.; those of the buttermilk from the lactalbumin cream were higher than those of the other except at 5° C. The foaming characteristics of the creams and the buttermilks from them were diametrically different with respect to the effect of temperature. The lactalbumin-phospholipid stabilized cream gave the typical cream-type foam at low temperatures; the protein-free cream yielded the typical phospholipid foam at the higher temperatures.

TABLE 4

Phosphorus in the filtered butterfat from butter churned from artificial creams

Sample no.	Description	Weight of fat ashed	Total phosphorus	Phosphorus
		(*g.*)	(*g.*)	(*mg./g. fat*)
1	Original fat	4.1527	0	0
2	Original fat plus approx. 1% phospholipids	4.0591	0.855	0.211
3	Fat from butter of cream from fat no. 2 in distilled water	4.0365	0	0
4	Fat from butter of cream from fat no. 2 in 0.5% lactalbumin solution	4.017	0	0

The analyses for phosphorus in the fat from the butter of each cream (table 4) showed that protein is not necessary to "pull" the phospholipid from the fat during churning. Apparently, the mere solvation of the polar phosphoric acid-choline group of the lecithin and of the phosphoric acid-ethanolamine group of the cephalin is sufficient.

DISCUSSION

The results of this study clarify, in some measure at least, the problem of making artificial emulsions of milk fat with properties similar to the natural product, using milk solids as stabilizers. Most workers in the past have overlooked the importance of incorporating phospholipids in the fat prior to emulsification. Wiese and Palmer (30) recognized that "The butterfat-in-buttermilk dispersion resembles whole milk in every respect, in general appearance, microscopic structure, cream separation and churnability of the cream." When they prepared stable emulsions with the

proteins of milk as emulsifiers, these emulsions were abnormal in one or more of their properties. The best churning was obtained with a phospholipid stabilizer. All stabilizers were incorporated in the aqueous phase prior to emulsification. Wiese *et al.* (29), in studies on the rebodying of cream, found that only those emulsions prepared with the normal fat globule membrane present during the emulsification responded to the rebodying process. They also noted that the composition of the butterfat appears to be a factor, presumably referring to the chemical constants of the fat, rather than its purity with respect to phospholipids. As stated earlier, the phospholipid content of filtered milk fat depends upon the purification procedure (6, 21). It is known also that the phospholipids of milk, cream, and butter exist in at least three states: free, loosely-bound to protein (probably by secondary valences), and chemically-bound to protein (primary valences) (2, 17, 26). Jenness and Palmer (9) emphasized that the ratio of phospholipid to protein was greater in the serum of washed-cream butter than in its buttermilk. It would be interesting to know the ratio of cephalin to lecithin in this respect, especially in view of the work of Spiegel-Adolph (25), Chargaff (2) and Rewald (17), who showed that the phosphatides of butter consisted of 50 per cent lecithin, 36 per cent cephalin, and 14 per cent other phosphatides.

Exact duplication of the foaming or other physical properties of a milk or cream by using artifical emulsions compounded from purified milk fat and other milk solids isolated by chemical means obviously is extremely difficult. The natural membrane material from cream which has not been washed too well would be expected to be the best emulsifier.

The authors postulate the following explanation, based on thermodynamical considerations. Assuming that milk fat is elaborated separately from the plasma solids (16), that the neutral blood fat is the main precursor of milk fat, and that blood phospholipids or phospholipid-protein complexes take part in its transfer, it seems logical to assume that, initially, milk fat contains, or is associated with, phospholipids, free and/or linked to protein. These, being surface-active, would tend to migrate to the interfaces between the fat and the aqueous medium during globule formation, their relative concentration being proportional to their surface activity. The phosphoric acid-choline polar group of lecithin and the phosphoric acid-ethanolamine polar group of cephalin would orient themselves toward the aqueous phase. They would become solvated in pure water, but, in the presence of plasma proteins, functional groups of both phospholipids and proteins likely would react through primary valences to form salt-like compounds. It is known that lecithin and cephalin form complexes with bacterial cells which inhibit the action of synthetic detergents (1). Cephalin has been shown to react with serum albumin and salmine, the rate of reaction being high; lecithin appears to react more slowly (2). Other

less stable complexes are possible through secondary valences or adsorption. Macheboeuf and Sandor (13) voiced similar ideas in connection with blood.

This speculation supports the theory advanced by Rimpila and Palmer (20), *viz.*, "It appears possible that the 'membrane' may be formed before the fat globules become a part of the milk or that the fat globules may be secreted before the milk plasma is completely formed, in which case the 'membrane' materials could be considered, in part at least, as precursors of plasma materials." The speculation is not counter to the idea of a special 'membrane' protein, or to the finding that the membrane protein of cream washed four times with water, four times with rennet whey, and then four times with water, and that of an artificial whey cream washed three times with water, have a sulfur content practically identical with the natural membrane. The membrane itself was lower in lipid phosphorus when whey was not used (20). The speculation is in keeping with the lipid extraction data of Tayeau (26), who found 20 per cent of the phospholipids in milk extractable with ether, 30 per cent with ether and soap, and the remainder extractable by ether after treatment with boiling alcohol to denature the protein in combination with the phospholipids.

As an outgrowth of these studies the authors have adopted the practice of incorporating phospholipids in the fat as well as in the whey, skim milk, or other media in making remade milks or creams, providing the media do not already contain appreciable phospholipid or the lipoprotein complex; buttermilk requires none. The principle has found industrial application in making wartime substitutes for ice cream and also genuine ice cream (28). Lecithin, for example, is separately incorporated into both the aqueous and fatty phases. Josephson and Dahle (10) succeeded in imparting normal whipping properties to ice cream mixes made with butter or butter oil as the source of fat by emulsifying the fat with either dried egg yolk or the natural "membrane suspension" before incorporating the fat into the mixes. These authors considered that a protein-phospholipid complex, already formed, was essential for proper emulsification; specificity was allotted to the true "membrane" protein moiety of the complex. Sell *et al.* (23), in studies with mayonnaise, showed that the lecitho-protein complex of egg yolk was the emulsifying agent in egg yolk, whereas free lecithin and free cephalin were detrimental. The results of the present study would seem to indicate that unaltered serum proteins with functional groups available for combining with functional groups of the phospholipids are adequate for the formation of such complexes.

SUMMARY

The role of milk fat in the foaming of milk, cream, buttermilk, and their artificial counterparts has been studied at temperatures between 5 and 50° C. The results suggest the following conclusions.

1. In such emulsions two types of foam may appear separately or simultaneously, a protein type and a phospholipid-protein type. At the higher temperatures the protein type predominates.

2. Whole milk, cream, and buttermilk exhibit minimum foaming at 30–35° C.

3. At 35° C. the foam volume and the foam stability of skim milk are decreased as the fat content is increased up to about 5.0 per cent. With further increases in the fat content both the volume and stability of the foam increase up to a fat content of 20 per cent, after which no further increases occur. At 6° C. the foam volume remains constant regardless of the fat content. The stability of the foam reaches a minimum at about 5 per cent fat concentration, after which it increases rapidly until a fat content of 10 per cent is reached, above which cream-type foams of high stability are formed.

4. Artificial milks and creams were made to resemble the natural product only when phospholipids (soybean) were incorporated into the fat prior to emulsification. The medium should be a protein sol; a lactalbumin sol or a milk serum protein sol such as rennet whey was satisfactory. The most normal cream was made when the medium contained natural fat globule "membrane" material; buttermilk met this condition.

5. Emulsions of pure milk fat in skim milk, gelatin, or gum arabic sols have abnormal foaming properties.

6. For emulsions with fat dispersed to a degree comparable to that of natural milk or cream, the optimum concentration of mixed phospholipids in the fat appears to be from 0.8 to 1.0 per cent. Unbound phospholipid appears undesirable.

7. These results have been discussed as they apply to churning, to cream whipping, to cream rebodying, and, by inference, to ice cream whipping.

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A STUDY OF MULTIPLE BIRTHS IN A HOLSTEIN-FRIESIAN HERD[1]

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Dairy cows are uniparous animals. Multiple births are relatively rare and occur with variable frequency. Twin births are most frequent, and triplets, quadruplets, and quintuplets are progressively rarer. Numbers of twin births reported for individual dairy breeds range from less than 0.5 per cent to 4.5 per cent (11) and for individual herds up to 8.8 per cent (9).

There still are differences of opinion as to whether twin births are desirable or undesirable in dairy cattle breeding (4, 5). Hewitt (7) considers multiple births a sign of increased fecundity and fertility, whereas Williams (12) relates such births to unsound or even diseased conditions of the genital tract, in particular of the ovary.

A general study of fertility in dairy cows, in which twin births were recognized as one of the factors influencing reproductive performance, led to a more detailed investigation of multiple births. The results are presented herewith.

SOURCE OF DATA

The data for this study were taken from the records of the Holstein-Friesian experimental herd at the New Jersey Agricultural Experiment Station and cover a period of about 15 years. The breeding program and operations pursued in this herd are rather unique insofar as the herd is self-containing and inbreeding is practiced to a high degree. The inbreeding with rigid selection is manifested in the preservation and concentration of the young animals' relationship to the noted sire, Ormsby Sensation 45th. In publications of Bartlett and Margolin (1) and Bartlett *et al.* (2, 3) detailed accounts are given of the conduct and progress of this experimental breeding project. From these reports it is evident that the artificial selection was not specifically directed toward reproductive efficiency and twinning but toward such qualities as milk and butterfat production, butterfat percentage, and body conformation.

RESULTS

By nature of the character investigated, the results must be so evaluated

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that small changes in actual numbers could have notable effect upon some of the summaries and averages.

General occurrence of multiple births. From 1931 to 1946 there were 937 parturitions and abortions available for observation. Of these, 37 or 3.95 per cent were twin births and 2 or 0.21 per cent triplets, making a total of 39 or 4.16 per cent multiple births. In other words, the average incidence of twin births was one in every 25.3 and triplets one in every 468.5 births, or one multiple birth in every 24.0 births. The 39 multiple births were observed in 36 cows; three cows gave birth to twins twice. Since only two sets of triplets occurred and the gestation of one set ter-

TABLE 1

Multiple births in relation to age of dam

Age by parturition no.	Observed parturitions	Observed multiple births	
	(*no.*)	(*no.*)	(%)
1	269	2	0.74
2	199	10	5.03
3	142	6	4.23
4	100	5	5.00
5	77	6	7.79
6	51	5	9.80
7	41	3	7.32
8	30	0	0
9	23	1	4.35
10	4	1	25.00
11	1	0	0
Summary	937	39	4.16

minated in an abortion without sex determination of the fetuses, it was considered advisable in these investigations to include the triplets along with the twin births.

Twin births in relation to age of dam. Frequency of twinning in relation to age of dam is summarized in table 1, where age is expressed in parturition numbers. The first parturition occurred at an average age of about 29.5 months, and the average interval between parturitions was about 14.5 months. This tabulation shows that the number of twin births has been extremely low at the first parturition. From then on it increased with age at calving, first increasing abruptly at the second parturition and then gradually reaching a peak at the fifth, sixth, and seventh par-

turitions. Above these ages a decrease seemed to take place, but in view of the small number of cases not too much weight should be given to this observation.

Length of twin gestations. Confirming Hewitt's findings in British Friesian cows (7), twin gestations in this herd were of shorter duration than gestations of single calves. The highly significant difference amounted to about 7 days. Between the sexually unlike twin pairs, differences also existed, as shown in table 2. The mean length of gestation of the male pairs was greater than that of the sexually-mixed pairs and considerably greater than that of the female pairs. It appears, as in the case of single calves (10), that sex of the fetuses has an influence upon length of gestation.

Sex ratio. The sex ratio of 36 twin and one triplet births was found to be 33 males to 42 females, or 44 per cent males. The theoretical ratio

TABLE 2

Length of gestation of twins

	No. of gestations observed	Length of gestation	
		Mean days	Standard deviation days
Male pairs	6	275.33 ± 3.97	6.87 ± 1.98
Sexually-mixed pairs	11	273.64 ± 2.74	8.23 ± 1.75
Female pairs	14	270.00 ± 2.56	8.86 ± 1.68
Summary	31	272.99 ± 1.68	9.37 ± 1.19

of twin pairs, 1 ♂♂ : 2 ♂♀ : 1 ♀♀ was met by an actual one of 9 ♂♂: 13 ♂♀ : 14 ♀♀, or 1.00 : 1.44 : 1.56. Although these ratios are rather unusual, their differences from the theoretical are not statistically significant.

Vitality of multiple calves. The vitality of multiple calves was evaluated according to the number born dead or that died within 2 days after birth and by following up the individual life histories of female twin pairs.

The mortality rate at birth for each sex is presented in table 3. It was slightly higher for male calves than for females. The average for both was 22.67 per cent, in contrast with only 9.65 per cent for the whole herd.

The history of the 14 female twin pairs revealed that 6 individuals, or 21.43 per cent, were born dead or died soon after birth; 5, or 17.86 per cent, were sold when immature, mostly for reasons of selection; 6, or 21.43 per cent, freshened in the herd but showed relatively poor production and breeding records; only 3, or 10.72 per cent, remained in the herd

for the length of their natural life, averaging 8.5 years, and exhibited relatively good production and breeding records; and 8, or 28.57 per cent, still in the herd at the time of this writing were immature, ranging in age from 4 to 18 months.

The mortality rate and life history of these heifer calves strongly indicate that twins have a lower vitality and poorer prospects of productive life than single calves.

Retained placentae and twin births. The condition of retained placentae after twin births was very much aggravated when compared with births of single calves. Of 31 apparently normal multiple parturitions, 23, or 74.19 per cent, were accompanied by this condition. The percentage for the whole herd was 23.10.

Conception rate of dams after twin births. After giving birth to twins, the dam's conception rate for a succeeding pregnancy should be another

TABLE 3

Mortality of multiple calves

	Males	Females	Total
No. of calves	33	42	75
Dead and aborted calves	8	9	17
Per cent mortality	24.24	21.43	22.67

indication of the possible effect of twinning on future breeding efficiency. An analysis of the records revealed that 71 services were required for 22 safe pregnancies in as many cows after twin births, corresponding with a conception rate of 3.23. This conception efficiency was almost 50 per cent lower than that of the herd average, which amounted to 2.21.

Calving interval after twin births. After the 37 twin births, apparently normal pregnancies and parturitions were noted in 13 instances. The mean calving interval was 483.5 ± 22.3 days. This interval corresponded with a breeding efficiency of 75.41 per cent, which was considerably below the herd average of 82.59 per cent. This difference alone suggests that twin births cause a reduction in reproductive efficiency.

Influence of twin birth upon future reproductive performance. In the 39 cases of multiple parturitions, 15 cows, or 38.46 per cent, continued to produce in apparently normal fashion, except for the higher conception rate and longer calving intervals. On the average, these cows survived their twin calving age by 2.33 parturitions. Five cows, or 12.85 per cent, became sterile; 12 cows, or 30.77 per cent, were sold for various reasons shortly after giving birth to twins; and three cows, or 7.69 per cent, died after the twin parturitions, one because of hardware, another,

from a ruptured uterus, and the third from an unknown cause. Of the four remaining cows which have given birth to twins within the last 9 months and which are still in the herd, two already have exhibited breeding troubles.

This recorded information does not warrant definite conclusions in regard to the influence of twin births on the future reproductive performance of the cows. The five proved cases of sterility are not in excess of the expectation for the whole population. It must be remembered that the disposals include a number of cases which must be regarded as doubtful in this respect. On the other hand, the first group, which consists of the 15 cows with an apparently normal reproductive performance after twin births, comprises the largest proportion of the grouped twin dams. Their performance at least implies that twin births do not necessarily cause breeding troubles with sterility implications or shorten the reproductive life of the cows.

Breeding efficiency of twin dams. Previously it was shown clearly that twin births exert a depressive effect upon the dam's subsequent

TABLE 4

Breeding efficiency of twin dams compared with the herd as a whole

Group	No. of cases	Percentage of breeding efficiency			
		Mean	Standard deviation	Coefficient of variation	Skewness of distribution
Herd as a whole	144	83.99±0.94	11.32±0.66	13.48	+0.0483
Twin dams	26	83.46±1.59	7.96±1.10	9.54	—0.0302

breeding efficiency. That this will affect a cow's lifetime breeding efficiency in proportion to her life span is acknowledged for the following analysis.

The lifetime breeding efficiency was determined for 26 twin dams, the only ones with complete records available. Their mean breeding efficiency and the standard deviation and coefficient of variation, as well as the approximate measure of skewness, were compared with the respective values of the herd as a whole.

Table 4 shows that almost no difference existed in the mean breeding efficiency between these two groups. The values for the standard deviation and coefficient of variation were considerably smaller for the twin dams than for the whole herd, denoting a greater uniformity of the twin group. Moreover, the distribution of the twin group was skewed positively, that is, toward the higher values, while the distribution of the whole herd was skewed negatively. Since twinning in itself has a depressing effect

upon the breeding efficiency, as already established, and since the frequency distribution of the herd as a whole markedly is skewed toward the lower values, a strong argument is offered for a fundamentally higher breeding efficiency in favor of the twinning group.

Milk production of twin dams. A study of the relationship between twinning and milk production was another object of this investigation. For this purpose the records of the 26 twin dams already employed in the analysis for breeding efficiency were considered suitable for a comparison of the milk production between twin dams and the herd as a whole.

The milk yield in both groups was expressed in pounds of 4 per cent fat corrected milk (5) per day on a mature equivalent twice-a-day milking basis. The actual milk yield was converted to the mature equivalent by the use of conversion factors based on the production records of the herd itself. The daily average was calculated on the total adult days the individual cow stayed in the herd, starting at the age of 27 months and continuing until her last calving.

TABLE 5

Milk production of twin dams compared with the herd as a whole

Group	No. of cases	Milk yield in lb.			
		Mean	Standard deviation	Coefficient of variation	Skewness of distribution
Twin dams	26	30.08±1.06	5.32±0.74	17.70	—0.7080
Herd as a whole	144	29.10±0.59	7.11±0.42	24.42	—0.3047

The results are presented in table 5. The constants chosen for this comparison were the same as those used for the analysis of breeding efficiency. The mean milk yield was insignificantly in favor of the twin dams ($P = 0.70$). The standard deviation and the coefficient of variation of the twin dams were considerably smaller than those of the herd, indicating greater uniformity for the first group. The frequency distributions of both groups were skewed negatively. This analysis indicated that the twin dams were at least equal in milk production to the herd as a whole. Superiority on this basis alone could not be demonstrated.

Comparison of various characteristics of fertility in twin dams and in the whole herd. Although the results of some investigations (7) indicate that twinning in dairy cows is associated with high reproductive qualities, this question is far from settled. Few data are available for correct evaluation of fertility itself, much less in relation to twinning. Thus, although twinning may be an expression of female fecundity in itself, the structural form of the female reproductive tract seems to obstruct the expression.

This conflict could be noted in the records and investigational results already cited and might interfere with most comparisons.

Besides breeding efficiency, the following criteria were used in the evaluation of reproductive performance: Live and dead calves born, twin births, abortions, and retained placentae. The comparison between twin dams and a representative group of cows producing only single calves is shown in table 6.

From this tabulation it will be seen that with the exception of the per cent of live calves, these measures of fertility all were in favor of the cows giving birth to single calves. That the greater percentage of living calves

TABLE 6

Comparison of various measures of fertility between twin dams and other herd representatives

	Twin dams	Herd representatives
Total no. of cows observed	28	142
Av. coefficient of inbreeding per cow	0.09	0.09
Av. gestation number observed	3.73	3.12
Av. conception rate	2.53	2.10
Based on total no. of parturitions:		
% of live calves	100.00	83.35
% of dead calves	17.50	7.83
% of multiple births	23.33	0.0
% of abortions	9.17	6.57
% of retained placentae	30.83	18.69

for the twin dams' group was due almost exclusively to the twin births was revealed simply by adding the percentages of live and dead calves in both groups and subtracting the percentage of multiple births from the twin dams' group. Should allowance be made for the lack of vitality of twin calves, this slight superiority would vanish.

Another important point in this tabulation is the average observed gestation number, which is considerably higher for the twin dams. Since the gestation numbers stand in relation to the average age at calving of the cows, their averages indicate strongly that the twin dams were older than the cows with only single calves. Two factors might have contributed to this effect. Probably the main factor was the expression of twinning relatively late in life, making the twin dams a selected group in this respect. Many of the younger cows in the second group were potential twin dams. The other factor would be that twin dams actually were longer lived.

Twinning and inbreeding. When twinning was set in contrast with the degree of inbreeding of the cows in the herd, as illustrated in table 7, a non-uniform positive trend between these two characteristics was observed. This feature does not mean that twinning was dependently related to the degree of inbreeding as such. It indicated rather well, however, that the factors for the twinning disposition were present in some of the foundation animals. By directing the breeding operations to the inbreeding of such animals, these factors became more concentrated in some cows and expressed themselves more often than in the foundation cows.

The hereditary aspect of twinning. Heredity control of multiple births in mammals has been proved amply in sheep, goats and other animals. In

TABLE 7

Comparison of inbreeding with twinning

Group	Coefficient of inbreeding	No. of cows	No. of parturitions	No. of twin births	% of twin births
1	0.00	55	116	2	1.72
2	0.01–0.04	92	291	10	3.44
3	0.05–0.09	21	67	0	0.0
4	0.10–0.14	36	87	4	4.60
5	0.15–0.19	29	73	7	9.59
6	0.20–0.24	9	16	1	6.25
7	0.25–0.29	10	22	1	4.55
8	0.30–0.39	3	8	1	12.50
Total		255	680	26	3.82

dairy cows this proof is attained only with difficulty, because its relatively rare appearance and its dependence upon the dam's age frequently hide the presence of this character. The small number of offspring in dairy cows and, possibly, environmental influences upon twinning contribute to the difficulties. Statistical investigation of the problem would necessitate large numbers of reliable and complete records such as those accumulated in herd book organizations. Unfortunately, these generally lack completeness, because only promising offspring are reported. An alternative, used in the present study, is the investigation of individuals and family groups in large herds where complete records are kept over long periods.

In table 8 the occurrence of twin births by parturition numbers is summarized for the members of 21 cow families which make up over 90 per cent of the present herd. Cows of ten of these families never had any

twins recorded. In the remaining 11 families the rate of twin parturitions ranged from 2.63 to 18.18 per cent.

In table 9 the twin parturitions of the daughters of 19 sires are presented. These daughters are, for the most part, the same cows represented in the cow families listed in table 8. In grouping these cows according to their sires, it was found that nine sires did not have any daughters with twin births. The remaining ten sires had one or more daughters which gave birth to twins. On the basis of all parturitions of the daughters of

TABLE 8

Occurrence of twins by cow families

Cow family no.	No. of parturitions	No. of twin pairs	% of twin pairs
.3	20	0	0.0
15	17	1	5.88
20	18	0	0.0
37	23	1	4.35
61	66	6	9.09
64	22	4	18.18
66	48	2	4.17
68	20	0	0.0
69	21	1	4.76
75	14	0	0.0
78	20	1	5.00
80	26	0	0.0
82	34	2	5.88
91	7	0	0.0
92	38	1	2.63
93	24	1	4.17
95	17	0	0.0
96	14	2	14.29
97	15	0	0.0
100	13	0	0.0
103	25	0	0.0
Total	502	22	4.38

these individual sires, the ten daughter groups varied from 2.27 to 14.29 per cent in twin births.

This variation in both groups might be attributed to three sources, namely, pure chance, environment, and the genetic twinning disposition of sires and dams. The emerging combination of the daughters' germ plasm founded upon the physical basis of heredity was the determining principle of this disposition. Environmental factors such as feeding and management probably were of very minor influence. Hormonal therapy, though of considerable importance in twinning, as shown by Hammond and Bhattach-

arya (6), was of no consequence in this herd. With the generally low percentage of twin births, the expression of the disposition was very uncertain and the chance factor could not be discounted.

By means of genealogical diagrams of cow families that have had a relatively high number of twin births it can be demonstrated that the disposition for twinning is inherited.

Figure 1 is a genealogical diagram of cow family 61. It is arranged with the foundation cow on top, her female progeny following down the

TABLE 9

Occurrence of twins by sires' daughters

Sire no.	No. of daughters included	No. of parturitions	No. of twin pairs	% of twin pairs
A	6	20	0	0.0
B	4	10	0	0.0
C	13	44	1	2.27
D	4	10	0	0.0
E	19	62	3	4.84
F	8	41	1	2.44
H	3	8	1	12.50
I	18	68	3	4.41
L	6	21	0	0.0
N	15	41	2	4.88
O	27	77	7	9.09
R	4	5	0	0.0
T	32	80	2	2.50
U	18	35	2	5.71
V	7	10	0	0.0
W	5	11	0	0.0
X	6	14	2	14.29
D-1	3	8	0	0.0
F-1	3	4	0	0.0
Total	201	569	24	4.22

line, generation by generation. The individual offspring is designated by herd number. After the cow's number is given in parentheses the number of her twin parturitions, if any. For the younger offspring alive in the herd at this writing, the letter *P* for prospect was added. Animals that are twins themselves are so designated by numbers in bold-face type. Below the female's identification number is ascribed the sire's identification, generally by a letter or letter with number. Beyond that, separated by a dash, the percentage of twin births of the sire's daughters is given as far as it is known.

The figure illustrates how the transmission of the disposition for twin-

ning may work out in a relatively large cow family. Foundation cow 61, herself a twin, was sired by bull *C*, whose daughters born in the herd showed only 2.3 per cent twinning. Cow 61 had five daughters with reproduction records. They were sired by three different bulls, making three of them full sisters by sire *E*, whose daughters averaged 4.8 per cent twin births. Their records and especially those of the three full sisters suggest strongly a segregation in the Mendelian ratio. Two of the three full sisters, namely nos. 67 and 294, started progeny lines of their own. Neither of these lines exhibited any twinning. The third full sister, no. 256, produced one pair of twins in her second parturition. Unfortunately, she did not leave any progeny in the herd. Her early expression of the twinning character suggests, however, that she was highly predisposed to it.

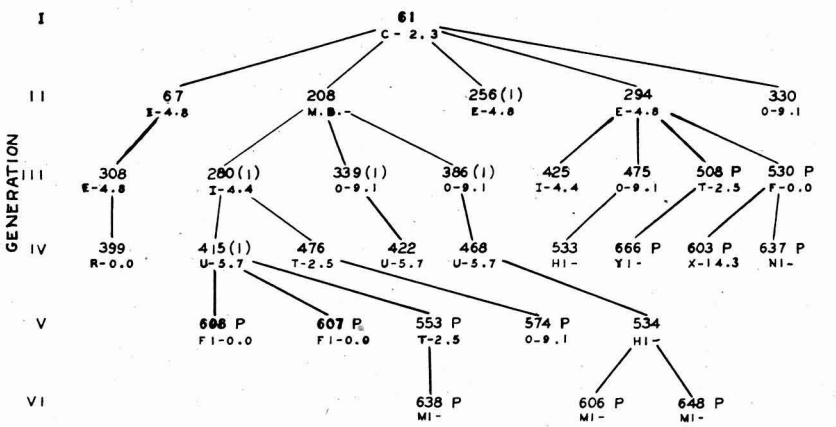

FIG. 1. Diagram of cow family 61 showing the extent and distribution of twin births, which amount to 9.09 per cent of all observed parturitions. For explanations of the diagram see text.

Daughter no. 208, which was sired by a bull designated *M. B.* and for which no twinning record could be established because of lack of daughters in the herd, started the longest line of progeny. Though no. 208 herself revealed no twinning disposition, her progeny visibly exhibited it to an extreme degree. Every one of her three daughters gave birth to one pair of twins. In turn, their progeny (generation IV) again suggest that Mendelian segregation might have been at work. The animals listed under generations V and VI are too young at present to allow any conclusion.

The fifth daughter, no. 330, of foundation cow 61 was sired by bull *O*, which was by progeny test a highly predisposed animal. Cow 330 calved only twice in the herd and produced single bull calves. Her disposition for twinning, therefore, never will be ascertained.

A similar investigation was made on cow family 64; her genealogy is diagrammed in figure 2. Family 64 was relatively small. Over a period covering five generations, this family was just about holding its own. In respect to twinning, this family's record, amounting to 18.18 per cent, was higher than that for any other cow family observed. The distribution of twin births extended over only three generations. The foundation cow did not visibly express any twinning disposition and the descendants in the fifth generation were too young to show any. A segregation into unusually predisposed and undisposed lines was hinted in this family, a feature closely resembling that of family 61.

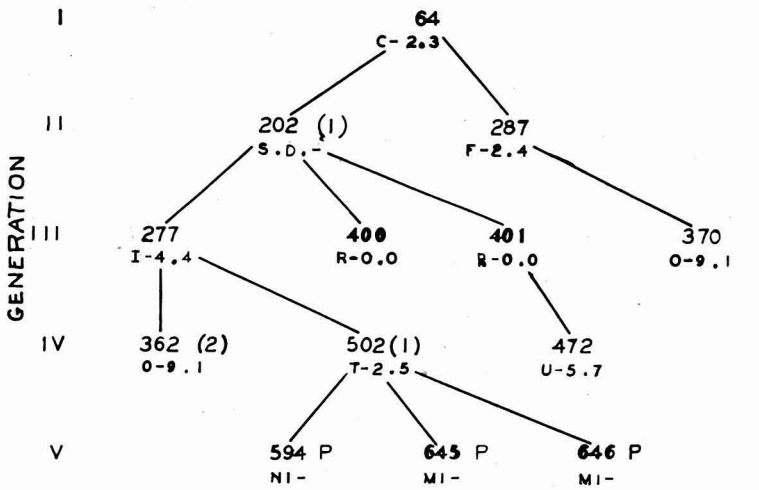

FIG. 2. Diagram of cow family 64 showing the extent and distribution of twin births, which amount to 18.18 per cent of all observed parturitions. For explanations of the diagram see text.

In both families virtually the same bulls appear as sires to the progeny. Sire *O*, with a high average twinning rate, is especially conspicuous as a sire of twin dams; so also is sire *I*, with a relatively low average rate. Both these sires are inbred to bull *C*, which is also the sire of both foundation cows. Sires *E* and *F*, both outbreds, are conspicuously present in the non-predisposed lines.

Obviously, the low frequency of twin births does not warrant definite conclusions with respect to the mode of transmission of twinning. The continued use of inbred sons and grandsons of bull *C* for several generations in succession and with it a concentration of certain genes of this bull suggest the possibility that he carried genes which foster twinning. In addition, it is observed that other inbred sons and grandsons of the same bull, as sires *T* and *R*, have very low rates for twinning. It should be

recalled that almost half of the cow families and half of the sires never showed any tendency for twinning. All of these observations point strongly to the conclusion that twinning in dairy cows is influenced chiefly by heredity yet expresses itself differently with age. The mode of transmission could well be understood by the assumption that twinning is under the control of a small number of autosomal genes which express themselves incompletely. The transmission seems to be recessive in character, with gene interactions or modifications.

DISCUSSION AND CONCLUSIONS

The observation made in this investigation that twinning is rare in the first parturition, rises to a peak in the fifth, sixth, and seventh parturitions, and then decreases with advancing age harmonizes closely with the general cycle of fecundity in most multiparous mammals. From this observation alone it is most probable that twinning is an expression of fecundity or the potential reproductive capacity of a dairy cow. However, if dairy cows are considered as strictly uniparous animals, and they should be so considered according to the structural development of their reproductive tract, twinning could be regarded as cases of reversion or atavism.

Evidence was presented that twinning is chiefly controlled by heredity. The effect of environment seems to be of very minor importance. The mode of transmission of the twinning character in dairy cattle is obscured by its relatively rare and incomplete expression, its sex-limited and age-limited appearance, and the small number of offspring inherent in cattle. The factual manifestations of transmission in two cow families over a number of generations provided impressive indications that twinning exhibits Mendelian segregation and seems to be under the control of a small number of genes. The character of twinning should be recessive with gene interactions or modifications. By making use of these findings it is feasible that in practical breeding operations twinning in dairy cattle could be influenced considerably in either direction.

The question then arises as to whether twinning is a desirable character in dairy cattle. If the available data all are accepted at their face value, twinning is associated with the increased production of calves by sheer numbers; if this fact is scrutinized from the actual reproducing value of the female line, the contrary is the case. Of the theoretical twin sex-ratio, 1 ♂♂ : 2 ♂♀ : 1 ♀♀, for all practical purposes only the female pairs are suitable for reproduction in the direct female lines. Theoretically, the number of these female twins would just be equal to the number of single females. Since twin calves have a higher mortality rate at birth and, apparently, a lower vitality throughout life than single calves, the real fact is that twinning has a harmful effect on the continuity of the female lines.

If the face value of data favoring the association of twinning with longevity is examined closely, the degree of association diminishes considerably. Twin births occur generally late in life, increase with advancing age, and are incomplete in appearance. If every animal in a group is expected to show this character, almost all the animals of this group have undergone an intense selection with respect to age. Therefore, almost any comparison of twinning in regard to age is of very questionable reliability.

Definite disadvantages of twin births include shortened gestation periods, greater parturition difficulties with subsequent increases in retained placentae, decreased conception rate, lower breeding efficiency, and increased sterility.

In summarizing all these factors, there is no doubt that twinning definitely is an undesirable character in dairy cattle, and efforts should be made to reduce its appearance by proper breeding methods and selection.

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A STUDY OF THE BROWNING REACTION IN WHOLE MILK POWDER AND ICE CREAM MIX POWDER[1]

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The development of a brown color during storage is an index of deterioration in many foods. Browning during storage usually is not observed in commercial dry milk, but some observations on milk powders prepared for other purposes led to the consideration that incipient browning might be related to the development of stale and oxidized flavors in milk powder. Consequently, it seemed advisable to investigate the factors contributing to browning and the possible relationship to other deteriorative changes in milk powder.

REVIEW OF LITERATURE

The literature on the browning reaction in foods is voluminous. Excellent reviews of literature on darkening of various foods and fundamental aspects of the browning reaction have been presented in project reports, Committee on Food Research, QMC, in the last two years. The literature on browning reaction in dairy products has been reviewed recently by Sharp and Stewart (11). The browning in dairy products, similar to other foods, is attributed to two possible reactions: the caramelization of lactose and a Maillard-type reaction between lactose and milk proteins leading to formation of amino-sugar compounds. Webb (12) believes that a lactose-amino combination may account for much of the browning of autoclaved milk, with caramelization of lactose by phosphates as a contributing factor. Regardless of the mechanism of the reaction, the intensity of browning produced in milk is known to be influenced by certain factors, namely, the pH of milk (9, 13), lactose concentration (6, 9, 11), and temperature and time of heating (9, 13). The major chemical change in milk related to the browning reaction is a partial conversion of lactose into acids (7, 14). The acids produced are mainly lactic and formic (3, 7). Kometiani (7) could not account for the total increase in acidity in browning as derived from lactose. He attributed part of the increase in acidity to an increase in free carboxyl groups in the casein molecule.

The published information on browning of dry milk products is limited to the study of Doob *et al.* (2) on browning of dried whey and skim milk. Both products were roll-dried and none of the samples studied was gas-packed. The browning of these products was affected chiefly by moisture

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[1] The subject matter of this paper has been undertaken in cooperation with the Quartermaster Corps Committee on Food Research.

content, temperature, and time of storage. According to the authors, browning is markedly accelerated at temperatures above 30° C.; however, even at 50° C. browning could be inhibited by low moisture content. An increase in titratable acidity and a decrease in pH accompanied browning of dried whey and skim milk.

EXPERIMENTAL PROCEDURE

All powders of whole milk and ice cream mix used in this study were made by the spray process under standard commercial conditions of processing. Ice cream mix powder was prepared in a commercial plant with a Rogers-type drier. Whole milk powder was prepared in a commercial experimental plant equipped with Mojonnier stainless steel processing equipment and Mojonnier drier. All samples were packed and stored in 1-lb. tin cans unless otherwise specified. Gassing of samples refers to evacuation and gassing with nitrogen. Gas analysis was made according to procedure of Peters and Van Slyke (8) with a Haldane gas apparatus. The pressure of gas in cans was taken at 22–24° C. with an attached gas apparatus manometer built for this purpose. The degree of browning (except for the first experiment) was measured by visual comparison of the sample with a set of dry powder standards, the procedure developed by Doob *et al.* (2). These standards are made up of mixtures of potassium chromate, ferric oxide, Norrit and sodium chloride to give colors ranging from white to medium brown in 14 divisions and numbered from 0 for the white to 13 for the darkest standard. Ascorbic acid and total reducing substances were determined by indophenol titration, as described by Sharp (10) and modified by Doan and Josephson (1). In one experiment the reducing substances were determined also by a modified Chapman method and expressed in terms of ferricyanide values. Moisture was determined by the toluol-distillation method, and the solubility index by the method recommended by the American Dry Milk Institute. The QMC score card of 1 to 15 was used in assigning the numerical value of the organoleptic score.

EXPERIMENTAL RESULTS

Experiment I. Effect of high humidity on browning of milk powder. In this experiment, samples of freshly prepared whole-milk powder were vacuum-packed in special laminated packages, shown by previous experiments to be water-vapor permeable but supposedly not air permeable.[2] The vacuum-packed samples were placed in a wet incubator at 95 per cent relative humidity and the thermostat set at 40° C. During the experiment the thermostat stuck and the temperature rose to 45° C. or more. This

[2] The authors are indebted to Mr. W. C. Cole and Mr. E. S. Chase, Research Laboratory, Arden Farms, Los Angeles, for their valuable contribution in securing the data of Experiments I and II.

increase in temperature undoubtedly accelerated the changes under consideration but did not destroy the value of the experiment. Samples were withdrawn at intervals from the incubator for testing. Results of these tests are presented in table 1.

Storage of whole milk powder under high relative humidity at 40 to 45° C. led to a rapid browning of samples. The browning was accompanied by a decrease in vitamin C, decrease in solubility, and increase in ferricyanide value. With the increase of moisture in powder, there was a development of mild stale flavor at first; but as browning appeared and developed, the stale flavor either decreased or was masked by a burned or caramelized flavor.

Experiment II. The effect of moisture content of powder on the rate of browning. The effect of moisture content of powder on the rate of browning also is brought out by the data of Experiment II. In this experiment, samples were prepared to contain approximately 4 per cent and 7 per cent moisture by adding water drop by drop from a pipette to milk powder as it was stirred in a Hamilton Beach Mixer. The prepared samples were packed: (a) in air, using pint mayonnaise jars, and (b) vacuum-packed (vacuum of 28 inches) in special vacuum-holding laminated packages. Samples were stored in an incubator at 45° C. Subsequent analyses showed that within the same moisture group of samples, there was a variation in the moisture up to 0.9 per cent. The vacuum-packed samples lost some moisture as the result of subjecting the samples to vacuum treatment. The data on browning and reducing groups (ferricyanide value) of the samples are presented in table 2. At 4 per cent moisture and storage at 45° C. there was no significant change in color on storage for 26 days. Samples containing 7 per cent moisture darkened significantly within the first 2 weeks of storage. Air-packed powder showed no consistent and significant difference in the degree of browning as compared with the same powder vacuum-packed at the levels of moisture tested.

The solubility index was run on the samples after 2 and 3 days of incubation. By then, the high-moisture samples were very insoluble, and continuation of the tests seemed unnecessary.

Experiment III. Gas changes during browning of whole milk powder. Early observations on browning of some ice cream mix powder samples packed under air and under 3 per cent of oxygen indicated that the browning was retarded in gas-packed samples. In one case, ungassed ice cream mix powder on storage at 45° C. for 3 months was dark brown and the same product gassed was only very slightly discolored. These samples originally were below 2 per cent in moisture but were badly contaminated with iron, which might explain why browning took place at that level of moisture in powder. Other samples of powder, free from iron contamination and with

TABLE 1

Effect of storage under high relative humidity on browning of milk powder

Length of storage at 40–45° C.	Moisture	Flavor	Color	Solubility index	Texture	Reducing substances		
						Vit. C	Dye titration	Ferricyanide value
(*hr.*)	(%)					(*mg./l.*)	(*mg./l.*)	
Control	2.25	11.5	Cream	1.20	Fluffy	13.3	12.4	10
17	3.32	11.5	Cream	1.45	Caked edges	12.9	14.3	11
24	3.73	10.5	Cream	1.55	Caked edges	13.4	14.7	11.5
41	4.61	10.5	Cream	2.00	Caked edges	12.2	12.6	12.5
48	4.97	10.5	Cream	1.85	Caked edges	11.6	12.6	13
65	5.59	9.0	Sl. brown	1.90	Hard	12.8	11.7	15
113	5.86	7.0	Brownish	7.00	Hard	9.5	11.0	25
161	7.49	6.0	Brown	7.90	Hard	7.4	11.2	33
353	7.68	.....	V. brown	7.90	Hard	4.7	35.0	> 80

TABLE 2

Effect of moisture and atmospheric pressure on browning of milk powder

Incubation time at 45° C.	Color				Ferricyanide value				Solubility index			
	4A [a]	4V	7A	7V	4A	4V	7A	7V	4A	4V	7A	7V
(*days*)												
2	1.0	0.9	1.5	0.9	18	19	28	25	0.60	0.65	8.25	6.0
3	1.0	0.9	1.5	0.9	14	14	25	25	0.95	0.90	9.20	8.00
6	1.0	0.9	1.5	1.0	15	15	34	35	.....	.....	.....	.....
15	1.0	.....	2.25	.....	30	.....	60	.....	.....	.....	.....	.....
26	1.0	.....	3.25	.....	40	.....	55	.....	.....	.....	.....	.....
33	1.75	.....	3.75	3.75	50	.....	70	100	.....	.....	.....	.....
61	2.75	3.0	4.50	.....	100	150	100	.....	.....	.....	.....	.....

[a] 4A — air-packed—4% moisture.
4V — vacuum-packed—4% moisture.
7A — air-packed—7% moisture.
7V — vacuum-packed—7% moisture.

moisture content of 2.5 per cent or lower, did not show any discoloration on storage at 44–45° C. for over 1 year. It is probable that iron accelerates browning in milk powder, as it has been shown to do in orange juice (5) and in lemonade and orangeade powders (4). This acceleration of browning does not explain the difference, however, in degree of browning in gassed and ungassed samples, unless removal of oxygen inhibits browning as in the case of orange juice (5). The data of Experiment II in this report show clearly that in samples of powder of high moisture content the degree of browning was not affected significantly by a partial removal of oxygen.

In Experiment III the samples of whole-milk powder were prepared to contain approximately 7, 4, and 2 per cent of moisture. This was accomplished by placing freshly prepared powder at 2 per cent moisture in a special stainless steel chamber of high relative humidity. The powder in this chamber was mixed frequently, and incorporation of moisture up to 7 per cent was accomplished in 44 hours. The samples at each level of moisture content were packed under three levels of oxygen, that of 21 per cent, about 10 per cent, and less than 2 per cent by packing, respectively, in air, with single gassing and double gassing. All samples were packed to contain 14 oz. of powder. The free-space gas volume, as calculated from gas pressures at 23° C., was 495±10 ml.

All samples were stored at 40° C. After 5 months of storage, the samples of 2 per cent and 4 per cent moisture failed to show a significant discoloration at 40° C. and were placed in the incubator at 60° C. for further storage. The data on browning, uptake of oxygen, production of carbon dioxide, and changes in moisture and flavor are presented in tables 3, 4, and 5 for powders of 7, 4 and 2 per cent, numbered as series 29II, 29I and 29, respectively. The partial pressure values for carbon dioxide and oxygen were calculated by converting per cent of gas on wet basis to per cent on a dry basis and multiplying this figure by total pressure on a dry basis.

Figures 1 and 2 show the rate of browning and gas changes during browning of high-moisture powder. The relationship between degree of browning and carbon dioxide production in powder of 2 per cent moisture packed under various levels of oxygen is shown in figure 3.

The data on browning and other changes of ice cream mix powder stored at 20, 37 and 45° C. are given in table 6. This powder had no added sugar and contained 53.55 per cent fat. The powder had 0.04 per cent tannic acid added as an antioxidant during the processing of the mix.

DISCUSSION

The conditions under which dry milk and ice cream mix powder undergo darkening or browning as a result of aging are apparent from the data presented. The browning of powder is a function of its moisture content

TABLE 3

Browning of whole milk powder of 7% moisture packed under various levels of oxygen (storage at 40° C.)

Oxygen level	Sample	Storage time	Moisture	Color	Partial pressure		Flavor
					CO_2	O_2	
(%)		(*days*)	(%)		(*mm.*)	(*mm.*)	
21	1	0	7.00	0.9	5.0	146.4	Good
	2	6	7.04	1.5	21.3	103.4	Burnt & stale
	3	28	7.00	2.6	39.7	1.3	Burnt & stale
	4	47	7.00	4.0	.....	.....	Caramelized & stale
	5	70	6.90	4.7	49.4	0.3	Caramelized & stale
	6	104	7.36	6.7	61.2	0.3	Caramelized & stale
10	1	0	7.22	0.9	3.5	59.0	Good
	2	6	7.34	1.7	11.1	32.4	Burnt & stale
	3	28	7.20	3.8	28.7	1.4	Caramelized & stale
	4	47	7.40	4.3	.....	.....	Caramelized & stale
	5	70	7.52	6.9	48.5	0.2	Caramelized & stale
	6	104	7.30	7.1	53.6	0.2	Caramelized & stale
2	1	0	6.74	1.0	1.1	2.8	Good
	2	6	6.70	1.8	6.1	1.0	Burnt
	3	28	7.00	5.3	22.7	0.8	Caramelized & v. sl. stale
	4	47	6.80	4.8	.....	.....	Caramelized & v. sl. stale
	5	70	7.14	7.5	42.7	0.7	Caramelized & v. sl. stale
	6	104	7.20	8.0	54.7	0.2	Caramelized & v. sl. stale

TABLE 4

Browning of whole milk powder of 4% moisture packed under various levels of oxygen

Powder	Sample no.	Storage time	Storage temperature	Moisture	Color	Partial pressure		Flavor
						CO_2	O_2	
		(*days*)	(° *C.*)	(%)		(*mm.*)	(*mm.*)	
29I	1	0	.....	3.93	0.7	2.0	156.8	Good
	2	19	40	3.73	0.9	4.7	142.8	.....
	3	61	40	3.62	1.0	13.2	84.6	.....
	4	152	40	.....	< 1.5	.....	.....	Stale
	5	11	60	5.25	>13.0	215.0	<1.0	V. caramelized
	6	15	60	5.20	>13.0	>230.0	.....	V. caramelized
29IA	1	0	.....	3.70	0.7	1.5	91.5	Good
	2	19	40	4.04	0.9	3.2	86.5	.....
	3	61	40	4.00	1.0	13.1	35.7	.....
	4	152	40	.....	< 1.5	.....	.....	Stale
	5	11	60	5.56	>13.0	277.0	<1.0	V. caramelized
	6	17	60	5.56	>13.0	>250.0	.....	V. caramelized
29IAB	1	0	.....	3.87	0.7	1.1	6.6	Good
	2	19	40	3.96	0.9	2.3	5.9	.....
	3	61	40	3.80	0.9	3.6	2.5	.....
	4	152	40	.....	< 1.5	.....	.....	Sl. stale
	5	11	60	4.84	>13.0	119.0	<1.0	V. caramelized
	6	17	60	4.80	>13.0	>260.0	0.0	V. caramelized

TABLE 5

Browning of whole milk powder of 2% moisture packed under various levels of oxygen

Powder	Sample no.	Storage time	Storage temperature	Moisture	Color	Partial pressure		Flavor
						CO_2	O_2	
		(*days*)	(° *C.*)	(*%*)		(*mm.*)	(*mm.*)	
29	1	0	.....	1.95	0.6	2.8	141.7	Good
	2	152	40	.....	0.6	.....	.....	.....
	3	17	60	2.30	1.5	16.6	<1.0	Stale
	4	43	60	2.00	1.8	23.4	<1.0	Stale
	5	108	60	2.06	4.2	80.2	<1.0	Stale & caramelized
	6	157	60	2.20	4.5	74.0	<1.0	Stale & caramelized
29A	1	0	.....	1.82	0.6	2.1	88.2	Good
	2	152	40	.....	0.6	.....	.....	.....
	3	17	60	1.70	1.5	16.2	<1.0	Stale
	4	43	60	1.80	1.8	20.8	<1.0	Stale
	5	85	60	2.10	4.5	72.6	<1.0	Stale & caramelized
	6	108	60	2.40	3.8	55.2	<1.0	Stale & caramelized
	7	157	60	2.90	7.2	123.0	<1.0	Sl. stale & caramelized
29AB	1	0	.....	1.72	0.6	1.6	3.5	Good
	2	152	40	.....	0.6	.....	.....	.....
	3	17	60	1.40	1.5	11.5	<1.0	Good
	4	43	60	2.00	1.8	15.1	<1.0	Good
	5	85	60	1.34	2.0	21.1	.....	.....
	6	108	60	1.80	2.5	27.7	.....	.....
	7	157	60	2.32	3.5	42.4	.....	Sl. caramelized

TABLE 6

Browning of ice cream mix powder

Sample no.	Packaging	Storage temperature	Storage time	Moisture	Color	CO_2	O_2	Titratable acidity	Flavor
		(° C.)	(*days*)	(%)		(%)	(%)	(*ml. 0.1 N NaOH*)	
1	No gassing	20	26	1.5	1.5	0.12	20.7	1.36	Good
2	No gassing	20	80	.....	1.5	0.18	19.77	.....	.....
3	No gassing	20	431	1.60	1.5	0.50	13.70	1.36	Stale
4	Gassed	20	26	1.50	1.5	0.03	2.28	.....	Good
5	Gassed	20	80	.....	1.5	0.08	1.49	.....	.....
6	No gassing	37	0	1.5	1.5	0.12	20.70	1.36	Good
7	No gassing	37	416	1.96	1.5	1.49	2.40	1.40	V. stale
8	Gassed	37	0	1.50	1.5	0.03	2.28	1.36	Good
9	Gassed	37	416	1.60	1.7	0.31	0.03	1.36	V. sl. stale
10	No gassing	45	0	1.50	1.5	0.12	20.70	1.36	Good
11	No gassing	45	14	1.60	1.5	0.39	19.02	.....	.....
12	No gassing	45	55	.....	1.5	0.91	13.49	.....	.....
13	No gassing	45	416	1.95	4.0	.....	.....	2.54	Oxidized & caramelized
14	Gassed	45	0	1.50	1.5	0.03	2.28	1.36	Good
15	Gassed	45	55	.....	1.5	0.44	0.84	.....	.....
16	Gassed	45	416	1.70	2.7	1.28	0.06	2.20	Sl. stale, sl. caramelized
17[a]	No gassing	60	40	2.20	10.0	.....	.....	5.10	Caramelized & stale
18[a]	Gassed	60	40	1.90	7.0	.....	.....	4.60	Caramelized

[a] Samples 17 and 18 are duplicates of Samples 7 and 9, respectively. They were stored at 37° C. for 416 days and then placed in an incubator at 60° C.

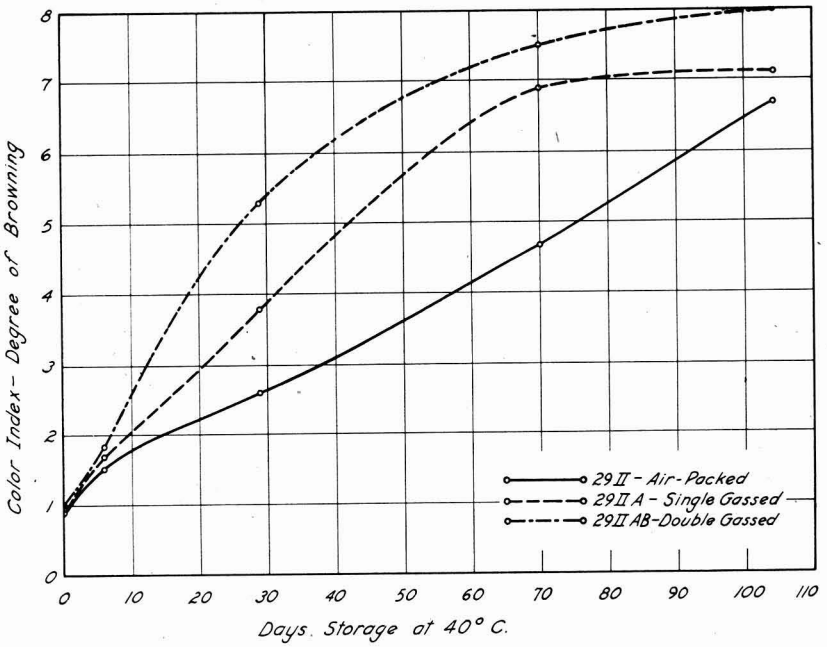

FIG. 1. Rate of browning of whole milk powder of 7% moisture (Series 29II).

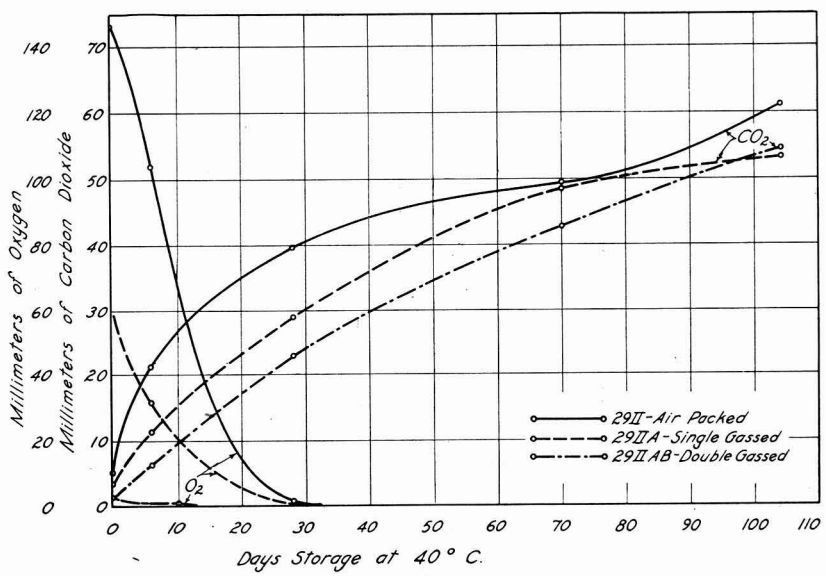

FIG. 2. Gas changes during browning of whole milk powder of 7% moisture (Series 29II).

and temperature of storage. Other factors, such as contamination with iron and possibly copper, certain added polyphenol compounds as antioxidants, and possibly vanillin in ice cream mix powder, may accelerate the rate of browning, but these factors are of comparatively minor importance in their relation to the browning of powder.

In general, the browning of powder is accompanied by: (a) production of carbon dioxide, (b) uptake of oxygen, (c) increase in reducing groups, (d) very marked decrease in solubility, (e) increase in titratable acidity,

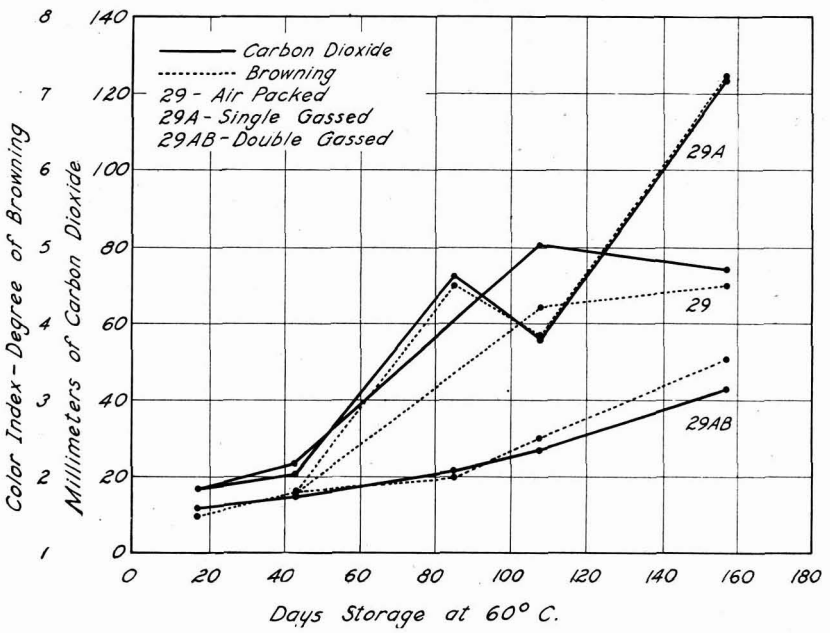

FIG. 3. Relationship between degree of browning and carbon dioxide production in whole milk powder of 2% moisture (Series 29).

and (f) development of caramelized flavor. In the advanced stages of browning there is also an increase in moisture content of powder. There was little, if any, increase in moisture in powder of 7 per cent moisture as compared to the same powder of lower moisture content.

In powder of 7 per cent moisture stored at 40° C., noticeable discoloration of powder takes place within a few days. On storage for a month, the powder becomes distinctly brown, with the appearance of caramelized flavor. At this time the oxygen practically is all gone and the partial pressure of carbon dioxide is increased to about 30 mm. under the conditions described. After the oxygen is gone, the browning and production of carbon dioxide continue but at slower rates.

In powders of 4 per cent and 7 per cent moisture content, storage at a

partial pressure of oxygen below 7 mm. (less than 1 per cent) did not retard the rate of browning. In fact, the samples packed under less than 2 per cent of oxygen have shown greater darkening than air-packed samples (see table 3 and fig. 1).

The browning of powder of 2 per cent moisture or less seemingly was retarded by packing the powder at the level of about 2 per cent of oxygen (see fig. 3 and table 6). It is possible that the retarding effect was due, at least partially, to a lower moisture content of gas-packed samples resulting from the vacuum treatment in the process of gassing.

It is evident from the data of tables 1, 4, 5 and 6 that both dry milk and ice cream mix powder of a moisture content below 4 per cent and stored at 40° C. or lower do not darken or brown in storage. Other samples of dry milk and ice cream mix powder of less than 3 per cent moisture have been stored at 40 and at 30° C. for over 2 years without showing any noticeable discoloration.

The usual deterioration in flavor of dry milk and ice cream mix powder in storage is independent of browning. Under the conditions of high available oxygen, as in the case of air-packed powder and storage at 40° C., the stale and oxidized flavor will develop with no browning at all. The same is true for a storage of powder at room temperature for a long period of time. The caramelized flavor is the only flavor that is produced by browning, and its intensity parallels the degree of browning. Caramelized flavor in gassed samples is a typical flavor of caramel. The development of stale or oxidized flavor apparently ceases when browning begins. There is no evidence that stale or oxidized flavors which have developed prior to browning disappear as browning progresses. These flavors merely are reduced or covered up when caramelized flavor appears.

SUMMARY

The conditions with respect to moisture content of powder, temperature of storage, and level of oxygen in gas-free space of container, as they may affect the browning of dry milk and ice cream mix powder as a result of aging, are given. The changes accompanying the browning, such as production of carbon dioxide, uptake of oxygen, increase in reducing groups, decrease in solubility and development of caramelized flavor, have been studied, and the extent of these changes in relation to the degree of browning is presented.

The darkening or browning of dry milk and ice cream mix powder, unlike some other dehydrated foods, is not related to the usual storage deterioration in flavor. The most common defective storage flavors of dry milk are the stale and the oxidized flavors. The development of these flavors in milk and ice cream mix powder is not the result of incipient browning.

In fact, it appears that the products of browning reaction inhibit the development of these flavors. Browning is accompanied by a development of a specific flavor, a caramelized flavor.

The browning does not take place in dry milk or ice cream mix powder when the above products are stored at 40° C. or lower if their moisture content is below 4 per cent.

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IRON AND COPPER CONTENT OF NON-MILK PRODUCTS COMMONLY USED IN ICE CREAM

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There is a lack of information on the iron and copper content of the non-dairy ingredients that commonly are used in conjunction with dairy products in the manufacture of ice cream. These materials include such products as stabilizers, emulsifying agents, sugar, cocoas, vanillas, chocolate liquors and coatings, and flavoring extracts. It is possible that one or more of these ingredients may contain enough copper or iron to accelerate the development of off-flavors of the oxidative type. Such metal contamination would be particularly important in the manufacture of dried ice cream mix that may be stored 6 to 12 months before using.

It was thought advisable, therefore, to study the iron and copper content of the previously mentioned materials. Accordingly, 74 samples of commercial non-dairy products commonly used in ice cream were analyzed. The iron content was determined by the method of Pyenson and Tracy (5) and the copper analyses were made by the method of Hetrick and Tracy (4).

EXPERIMENTAL RESULTS

The iron and copper content of stabilizers and sugars. Eighteen samples of stabilizers, gums, emulsifying agents, and sugars were analyzed for iron and copper. Magnesium nitrate (1) was added to Kragel,[1] sodium alginate, Irish moss, Vestirine and Gelox after carbonization to aid in the ashing. Two milliliters of a saturated solution of magnesium nitrate added after carbonization was found satisfactory to give a white, soluble ash. The results of the analyses are given in table 1.

All stabilizers except Gelox contained considerable amounts of iron. Irish moss contained 0.219 per cent of iron, which would be considered more than a trace amount. The emulsifying agents contained from 1 to 59 p.p.m. of iron. Egg yolk, which is sometimes used in ice cream as an emulsifying agent, contained 59 p.p.m. of iron. The sugars analyzed contained only small amounts of iron.

The copper content of the stabilizers varied from 0.92 to 10.0 p.p.m. Irish moss, locust bean gum, Kragel, and sodium alginate contained the most copper of the ten stabilizers analyzed. Glycerol monostearate did not contain any copper and Mixacoid contained practically none. Na-Pe-Co and egg yolk contained 2.85 and 3.35 p.p.m., respectively, of copper. Su-

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[1] Now modified and known as Kragelene.

crose, dextrose, and enzyme-converted corn sirup were found to contain less than 1 p.p.m.

The iron and copper content of American and Dutch process cocoas. The iron and copper contents of 17 samples of cocoa from five different manufacturers were determined (table 2). Of these samples, 13 had been treated with alkali (Dutch process) and four were untreated (American process).

The iron values for the American process cocoas varied from 73 to 119 p.p.m., with an average for the four cocoas of 92 p.p.m. In the Dutch

TABLE 1

The iron and copper content of some stabilizers, emulsifying agents and sugars

Sample no.		Iron	Copper
		(*p.p.m.*)	(*p.p.m.*)
	Stabilizers		
1	Gelatin (275 Bloom-pigskin)	14.8	2.21
2	Gelatin (125 Bloom-calfskin)	9.0	2.56
3	Gelox	1.2	2.0
4	Vestirine	28.0	1.75
5	Kragel	61.0	9.0
6	Sodium alginate	97.0	6.25
7	Irish moss	2190.0	10.0
8	Locust bean gum	16.4	9.0
9	Gum oat	47.6	5.4
10	Karaya gum	26.4	0.92
	Emulsifying agents		
11	Na-Pe-Co	19.4	2.85
12	Mixacoid	1.0	0.25
13	Glycerol monostearate	2.0	0.00
14	Egg yolk	59.0	3.35
	Sugars		
15	Sucrose, lot no. 1	0.9	0.15
16	Sucrose, lot no. 2	1.2	0.20
17	Dextrose	0.4	0.40
18	Enzyme-converted corn sirup	1.1	0.70

process cocoas the iron content averaged 117 p.p.m., with only three of them having an iron content under 100 p.p.m. Eight of the samples had an iron content between 110 and 149 p.p.m. There is some evidence that Dutch process cocoa contains more iron than American process cocoa, especially when comparisons are made between the two cocoas from the same manufacturer.

Dahlberg (2) found that a greenish-black discoloration of chocolate ice cream was caused by ferric tannate and that the cocoas that had a slightly alkaline pH value (Dutch process) were the only ones that produced the defect. The results of the present study suggest that the greater iron content of the Dutch processed product also may be a factor.

TABLE 2

The iron and copper content of American and Dutch process cocoas

Sample no.	Process	Iron	Copper
		(*p.p.m.*)	(*p.p.m.*)
	Brand A		
1	Dutch	94.5	20.6
2	American	73.0	21.0
3	Dutch	136.7	21.0
4	Dutch	142.7	21.2
5	American	84.7	20.6
6	Dutch	134.0	20.6
7	Dutch	134.0	21.0
	Brand B		
8	Dutch	149.0	22.2
9	American	119.0	23.4
10	Dutch	120.5	24.0
11	Dutch	141.0	21.6
	Brand C		
12	American	92.0	22.4
13	Dutch	110.0	23.5
14	Dutch	108.0	23.6
15	Dutch	105.0	31.2
	Brand D		
16	Dutch	69.0	27.3
	Brand E		
17	Dutch	75.0	28.3

The copper content of the samples varied from 20.6 to 31.2 p.p.m. American and Dutch process cocoas gave about the same copper values. The copper content of cocoas was more uniform than the iron content.

The iron and copper content of chocolate liquors and coating. Seven samples of chocolate liquor and one sample of milk coating were analyzed for copper and iron. The results are given in table 3.

Like cocoas, chocolate liquors and coating contain considerable quantities of iron and copper. The iron content of chocolate liquors and coating varied from 14 to 105 p.p.m., and the copper varied from 2.3 to 27 p.p.m.

TABLE 3

The iron and copper content of chocolate liquor and coating

Sample no.	Brand	Iron	Copper
		(*p.p.m.*)	(*p.p.m.*)
1	A	100.8	20.0
2	A (Milk coating)	14.0	2.3
3	B	59.0	14.9
4	B	71.0	15.5
5	C	104.5	27.0
6	D	103.0	24.8
7	E	105.0	18.9
8	F	19.4	9.8

The iron and copper content of flavoring. Twenty-two vanillas obtained from nine manufacturers were analyzed for iron and copper. The results are listed in table 4.

The three samples of powdered vanillas were uniformly low in iron and contained less than 2 p.p.m. copper. Powdered vanillas are made by grinding vanilla beans and combining the ground beans with a carrier like

TABLE 4

The iron and copper content of vanillas

Sample no.	Brand	Iron	Copper
		(*p.p.m.*)	(*p.p.m.*)
	Powdered vanillas		
1	A	4.8	1.35
2	A	4.0	1.90
3	B	1.4	0.45
	Concentrated vanillas		
4	C	59.0	9.45
5	C	58.4	4.95
6	C	43.8	36.5
7	C	32.6	4.3
8	D	2.0	9.5
9	D	4.0	10.5
	Vanilla extracts		
10	E	4.4	15.5
11	A	33.6	19.5
12	D	0.8	4.9
13	B	1.5	2.2
14	E	0.6	2.7
15	F	7.8	3.75
16	E	1.9	9.30
	Imitation or partially imitation vanillas		
17	C	58.0	3.2
18	B	46.0	7.75
19	F	4.4	3.7
20	E	5.3	3.25
21	E	3.0	1.2
22	G	4.4	15.5

sucrose or glucose. The vanilla bean as it exists in nature appears to be relatively low in iron and copper.

The concentrated vanillas studied were found to have a rather high iron and copper content. The iron content of single strength vanilla extracts varied from 0.6 to 33.6 p.p.m. Five out of the seven samples had an iron content under 4.4 p.p.m. The copper content also varied considerably; the minimum was 2.2 p.p.m. and the maximum 19.5 p.p.m.

The iron content of the imitation or partially imitation vanillas varied from 3 to 58 p.p.m. and the copper content from 1.2 to 15.5 p.p.m. Represented in this lot were six samples from five different manufacturers.

The iron and copper content of fruit flavors and extracts. Most of the samples of fruit flavors and extracts analyzed (table 5) contained less than 5 p.p.m. of iron. Six out of the nine samples contained less than 5 p.p.m. of copper. Lime flavor, black raspberry concentrate and strawberry flavor were comparatively high in copper content.

TABLE 5

The iron and copper content of some fruit flavors and extracts

Sample no.		Iron	Copper
		(*p.p.m.*)	(*p.p.m.*)
	Brand A		
1	Orange emulsion conc.	3.6	0.25
2	Lemon emulsion conc.	3.4	0.25
	Brand B		
3	Black raspberry natural flavor conc.	18.6	6.9
4	Imitation pineapple flavor	3.9	0.45
5	Strawberry flavor	5.6	5.05
6	Peach flavor conc.	1.3	2.25
7	Lime flavor	1.2	11.25
8	Pistachio imitation conc.	0.5	4.05
9	Imitation banana	1.0	0.65

DISCUSSION

The determination of copper by the direct carbamate method used in this study does not entirely eliminate the interference of nickel. Nickel exhibits maximum absorption at a wave length of 385 mμ, while copper exhibits maximum absorption at a wave length of 440 mμ. Hetrick and Tracy (4) state that when 5 γ of nickel are added to 5 γ of copper, the error is + 0.6 γ. Studies in the wave length at which maximum absorption occurs of the materials reported in this paper indicate that there was little, if any, nickel present.

The non-dairy products individually would produce only insignificant increases in the iron and copper content of ice cream. The Irish moss sample studied would be an exception, as it contained over 0.2 per cent iron. While the copper and iron content of the milk-product ingredients ordinarily would have a major bearing on the iron and copper content of the finished ice cream, the total added by non-milk products could be of such quantity as to be an important factor in the development of oxidized flavors.

Observations by Dahle and Folkers (3) and Tracy *et al.* (7) have shown that ice creams containing small amounts of fruit such as strawberries and pineapple develop a stale and/or oxidized flavor sooner than does vanilla ice cream. These authors believe that the off-flavor is due to the presence of copper and the acid of the fruit. Dahle and Folkers (3) state

that if the amount of copper in the mix equaled 1.3 p.p.m., the off-flavor always developed. Tracy *et al.* (7) state that in order to prevent the development of a stale metallic flavor in strawberry ice cream, the elimination of copper contamination is necessary. Other fruits found to accelerate the reaction responsible for the off-flavor were oranges and lemons. The copper content of any of the non-dairy products studied conceivably could be a factor in accelerating the development of the oxidized flavor, especially if used in strawberry, pineapple, orange and lemon ice creams.

The vanilla sample no. 4 in table 4 has been shown to have antioxygenic properties (6) although it contains relatively large amounts of iron and copper, indicating that a substance may be relatively high in iron and copper and still have antioxygenic properties.

CONCLUSIONS

Stabilizers, cocoas, chocolate liquors, sugars, vanillas and fruit extracts were found to contain iron and copper. The copper and iron present in some of these products is thought to be significant from the standpoint of possible cumulative effect in hastening fat oxidation and the development of off-flavor in ice cream.

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THE DEVELOPMENT OF FLAVOR IN AMERICAN CHEDDAR CHEESE MADE FROM PASTEURIZED MILK WITH *STREPTOCOCCUS FAECALIS* STARTER[1]

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This report is the first of several papers dealing with the development of a higher flavor in pasteurized-milk American Cheddar cheese. Cheddar cheese is made by a process that gives a long period for controlled lactic acid fermentation during manufacture. Most investigators have found that commercial lactic starters, chiefly *Streptococcus lactis* and *Streptococcus cremoris,* affect acidity without otherwise greatly affecting curing. As these starters in active growing condition are very important in both cheese manufacture and curing, it is obvious that proper acidity is very important in curing cheese. With the exception of some inoculations of certain lactobacilli, there have been no bacteria found that have aided in the development of good Cheddar cheese flavor. Furthermore, added enzymes, particularly lipases and proteinases, have not given very promising results.

In 1941 Wilson *et al.* (8) compared curing temperatures of 40, 50, and 60° F. They found 40° F. to be best for cheese made from poor milk, but 50° F. was preferred for cheese made from pasteurized milk of good quality. The type of curing was rather uncertain at 60° F. About this same time Dahlberg and Marquardt (2) showed that cheese made from either raw or pasteurized milk of excellent sanitary quality and ripened in vacuum in tin cans uniformly failed to develop Cheddar flavor in a year at 40° F., whereas some Cheddar flavor developed in 4 months at 50° F. and in 2 months at 60° F. In this study cheese made from raw or pasteurized milk of low bacterial count developed flavor uniformly; hence, it is evident that the effect of pasteurization of milk in slowing the curing of cheese is due chiefly to destruction of bacteria rather than the milk enzymes, and that this effect may be overcome in part by higher curing temperatures. The evidence indicates that thermoduric bacteria are a factor in cheese curing or that the pasteurized milk was recontaminated.

Consideration of the problem indicated little chance of success by the usual procedure of isolating bacteria from cheese and using them in

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its manufacture, as so much of this work already has been done. Rather, one might consider the characteristics of the bacterium desired in cheese making and curing and then ascertain if such a bacterium exists. For example, the desired bacterium should be universal in milk, as all good milk can be made into good cheese without adding this culture. Probably the organism should survive pasteurization. It should produce lactic acid from lactose rapidly and also be able to use lactates or other compounds in cheese as a source of energy; grow well at temperatures of 50° F. or less, and at temperatures as high as 106° F.; not produce gas in large amounts, although it might need to produce some carbon dioxide, as Dorn and Dahlberg (3) have shown cheese made from exeellent milk yielded limited amounts of almost pure carbon dioxide during curing; be non-proteolytic and not produce objectionable flavors and odors. It should grow anaerobically at a pH of 5.0 to 5.5 and at salt concentrations up to 6 per cent, as this concentration is about the maximum that normally occurs in the water in Cheddar cheese. The bacterium which has been described obviously may be *Streptococcus faecalis,* as this organism has the characteristics given. This organism would develop in the cheese milk and in the cheese during curing, especially if stored at 50 or 60° F. White and Sherman (6) recently have found enterococci in all raw and pasteurized milk samples which they tested.

EXPERIMENTAL METHODS

A dozen or more cultures of *S. faecalis* were obtained from several laboratories and all of them produced acid too slowly in milk to appear to be promising. The idea remained dormant for a few years and then a search was made in nature for a strain which ferments lactose rapidly, as *S. faecalis* often loses this characteristic when propagated in media which do not contain lactose. Some 15 human adults saved stools from which enterococci were isolated on the penicillin-azide agar of White and Sherman (6), and *S. faecalis* was identified by the characteristics given by Sherman (5). *S. faecalis* is the predominating streptococcus in the digestive tract of man. Approximately 40 or 50 strains of enterococci were isolated before one was found that curdled milk rapidly. A 1 per cent inoculation of this strain incubated at 85° F. curdled milk in less than 18 hours, producing a smooth curd without gas. The flavor of the starter was characteristic and definite, but quite different from ordinary lactic starter. The odor was flat without being objectionable. It was identified as *S. faecalis* and possessed all typical characteristics. It did not ferment glycerol. The tyrosine decarboxylation activity of *S. faecalis* isolated from the starter was $Q_{CO_2} = 50$ and that isolated from the ripened cheese made with the starter was $Q_{CO_2} = 60$. This indicates a moderately active strain for conversion of tyrosine into tyramine.

This *S. faecalis* strain has been carried in milk pasteurized at 200° F. for 1 hour and incubated at 88° F. The curdled starters are held at 40° F. and transferred twice weekly. The starter appears to be pure *S. faecalis* on plating, but no endeavor was made to carry it in sterile milk or other media to assure no loss in acid-producing ability while the research was in progress. Should the starter become contaminated, the *S. faecalis* bacteria could be reisolated and developed as a new starter of the same organism. Pure cultures of this organism have been prepared and are in storage.

The milk used for cheesemaking was a good quality of market milk pasteurized at 143–145° F. for 30 minutes. After cooling to 86° F., the milk was divided into three lots of 300 lb. each. To the first batch of milk was added 2 per cent of Hansen's commercial lactic acid starter; to the second batch, 1 per cent of Hansen's starter and 1 per cent of *S. faecalis* starter; and to the third batch, 2 per cent of *S. faecalis* starter. The milk then was made into cheese according to the time schedule of Wilson (7), using the 4.5-hour schedule from adding rennet to milling the curd, except that no time was allowed for the starters to develop before adding rennet. Acid development was followed by titratable acidity and pH, using a Beckman pH meter, laboratory model G, with glass electrode. After manufacture and pressing, the cheese was vacuum packed in cans and ripened at 50 and 60° F. A few samples were made into 10- or 30-lb. cheese and paraffined in accordance with the usual commercial practice.

The cheese was analyzed for moisture, salt and fat. On the day it was taken from the press, analyses were made for pH, volatile acidity by the method of Kosikowsky and Dahlberg (4), and soluble nitrogen by the method of Sharp as reported by Dahlberg and Kosikowsky (1). The samples of cheese were scored by the authors at the end of one month curing and bimonthly thereafter. The samples were analyzed bimonthly for volatile acids, soluble nitrogen and pH.

A considerable number of series of cheese were made with remarkably consistent results, and two series made on different days are presented to illustrate the results.

RESULTS

The manufacturing data (table 1) show that the rate of acid development with the *S. faecalis* starter was slower than with the commercial lactic starter, and the mixture of the two starters developed acid at a rate intermediate between those of the two cultures used singly. *S. faecalis* grows well in the salt concentration of Cheddar cheese, so the pH of all samples of cheese 1 day old was rather uniform at pH 4.9 to 5.1 (table 2), irrespective of considerable variations in acidity present in the whey when

the curd was milled. The composition of the cheese was uniform, but the salt contents were somewhat low (table 1). Most other batches of cheese in other experiments contained 1.5 to 2.0 per cent salt.

While the cheese curd was matting in the vats, it generally was possible to observe that curd containing *S. faecalis* matted slightly more rapidly and that the curd developed more of the stringy character of the meat of chicken breast. As the time approached for salting, the curd

TABLE 1

The acidity development during the manufacture of the pasteurized-milk American Cheddar cheese and the percentage composition of the cheese made with lactic, lactic plus S. faecalis, and S. faecalis starters

Manufacturing data	Series 1—10464			Series 2—10468		
	Lactic	L. F.[a]	Faecalis	Lactic	L. F.[a]	Faecalis
Fresh milk, titr. acid.	0.15	0.15	0.15	0.16	0.16	0.16
pH	6.64	6.64	6.64	6.54	6.54	6.54
Amount of starter (%)	2	1+1	2	2	1+1	2
Starter, titr. acid. (%)	0.74	.....	0.64	0.77	.....	0.61
Milk set, titr. acid. (%)	0.17	0.165	0.16	0.18	0.18	0.18
pH	6.41	6.40	6.42	6.40	6.38	6.42
Whey acid						
At cutting, titr. acid (%)	0.11	0.10	0.11	0.12	0.11	0.11
pH	6.35	6.38	6.42	6.33	6.40	6.46
Cooked, titr. acid. (%)	0.12	0.12	0.11	0.13	0.13	0.13
pH	6.22	6.28	6.35	6.21	6.32	6.32
Drawn, titr. acid. (%)	0.13	0.13	0.12	0.15	0.14	0.13
pH	6.11	6.17	6.29	6.05	6.05	6.18
Milling, titr. acid. (%)	0.52	0.45	0.38	0.50	0.38	0.25
pH	5.38	5.44	5.54	5.35	5.63	5.95
Cheese out of press						
Yield per cwt. milk (lb.)	10.4	10.4	10.4	11.0	11.5	11.9
Moisture (%)	35.9	36.9	36.3	34.9	35.8	36.9
Fat (%)	35.5	34.5	34.5	35.5	35.0	35.0
Salt (%)	1.18	1.25	1.42	1.39	1.27	1.45
Protein (%)	23.33	23.63	23.65	23.69	23.21	22.64

[a] L.F. = cheese containing 1% lactic starter and 1% *S. faecalis* starter.

containing *S. faecalis* developed a more pronounced odor of good Cheddar cheese curd. This odor of good cheese curd invariably was selected by several persons.

As the cheese cured there was a gradual increase in the pH to 5.13–5.29 for cheese held at 50° F. and to 5.19–5.33 for cheese held at 60° F. (table 2). The data are not extensive enough to show any conclusive difference in acidity due to storage temperature, but a higher pH at the

warmer curing temperature seems logical. Certainly, the acidity of the ripened cheese was not affected by the starters, for the range of pH on all samples of cheese when 6 months old was from 5.13 to 5.33.

The volatile acidity of the cheese increased more rapidly at 60° F. than at 50° F. (table 2), as would be expected. The commercial lactic starter produced more volatile acidity in the cheese than the *S. faecalis*

TABLE 2

The pH, volatile acids, and water soluble proteins during curing of pasteurized-milk American Cheddar cheese made with lactic, lactic plus S. faecalis, and S. faecalis starters

Cheese no.		pH		Volatile acids		Water-soluble proteins	
		50° F.	60° F.	50° F.	60° F.	50° F.	60° F.
				(*Ml. N acid/100 g.*)		(%)	(%)
				1 day old			
Lactic	10464	4.99	.....	12.5	.....	1.45	.....
Lactic	10468	4.88	.....	12.5	.....	2.25	.....
L.F.	10464	5.05	.....	12.5	.....	1.59	.....
L.F.	10468	5.01	.....	16.7	.....	1.74	.....
Faecalis	10464	5.15	.....	12.1	.....	1.50	.....
Faecalis	10468	5.12	.....	17.5	.....	1.54	.....
				2 months old			
Lactic	10464	5.07	5.13	19.5	27.5	5.77	6.68
Lactic	10468	5.06	5.14	14.7	22.9	6.01	7.28
L.F.	10464	5.10	5.15	21.4	32.4	5.55	6.40
L.F.	10468	5.17	5.22	13.6	20.7	5.65	6.00
Faecalis	10464	5.15	5.12	16.6	18.6	5.03	6.24
Faecalis	10468	5.19	5.20	16.9	17.2	5.00	6.05
				4 months old			
Lactic	10464	5.13	5.22	28.4	35.5	7.78	8.32
Lactic	10468	5.10	5.24	18.1	35.0	7.07	8.26
L.F.	10464	5.03	5.24	27.1	35.6	7.55	8.10
L.F.	10468	5.15	5.29	18.6	31.7	6.46	7.84
Faecalis	10464	5.07	5.24	23.9	29.6	7.23	7.99
Faecalis	10468	.....	5.14	17.2	29.1	5.70	8.16
				6 months old			
Lactic	10464	5.19	5.19	27.1	43.3	7.85	8.33
Lactic	10468	5.20	5.29	27.2	39.0	8.00	9.26
L.F.	10464	5.29	5.28	29.4	39.2	7.49	8.95
L.F.	10468	5.22	5.33	22.5	36.5	7.02	8.67
Faecalis	10464	5.13	5.22	21.0	30.6	7.61	8.89
Faecalis	10468	5.19	5.31	15.8	26.2	7.10	8.61

starter, but the difference was not great. Cheese manufactured with *S. faecalis* starter and cured for 6 months at 50° F. showed practically no increase in volatile acidity. None of the samples of cheese was high in volatile acidity for, from a beginning of 12.1 to 17.5 ml. 0.1 N acid per 100 g., the volatile acidity value increased after 6 months at 50° F. up to 15.8 to 29.4 and at 60° F. up to 26.2 to 43.3.

As anticipated, the percentage of water-soluble protein increased more rapidly at 60 than at 50° F. (table 2). The two types of starters did not affect the increase in soluble proteins, which was reasonably uniform for all samples. The soluble protein in cheese cured 6 months at 50° F. ranged from 7.02 to 8.00, and that cured at 60° F. from 8.33 to 9.26.

The significant results from the use of *S. faecalis* starter are shown in the flavor scores and comments. Numerical scores were given to the nearest half point. All samples were graded as to intensity of Cheddar cheese flavor. Other comments on flavor were not made systematically, *i.e.*, some excellent flavored samples of cheese were called excellent and others equally good were not so marked. All the comments made at scoring were entered in tables 3 and 4.

TABLE 3

Flavor development in pasteurized-milk American Cheddar cheese made with lactic, lactic plus S. faecalis, and S. faecalis starters
(Ripened at 50° F.)

Cheese no.		Total score	Flavor [a]		Body [b]	
			Score	Remarks	Score	Remarks
				1 month old		
Lactic	10464	93.0	39.0	Mild —, flat	29.0	Corky, firm
Lactic	10468	92.5	39.0	Mild —, flat	28.5	Corky, sl. crumbly
L.F.	10464	95.0	40.5	Mild +, exc., raw	29.5	Waxy
L.F.	10468	94.0	40.0	Mild +, exc., raw	29.0	Waxy, sl. crumbly
Faecalis	10464	94.5	40.0	Mild +, exc.	29.5	Waxy
Faecalis	10468	95.0	40.5	Mild, exc., raw	29.5	Waxy
				2.5 months old		
Lactic	10464	94.0	40.0	Mild —, clean	29.0	Waxy, sl. firm
Lactic	10468	93.0	39.5	Mild —, sl. curd	28.5	Sl. rubbery
L.F.	10464	95.5	41.0	Mild +, clean, raw	29.5	Waxy
L.F.	10468	95.0	41.0	Mild +, clean, exc.	29.0	Waxy
Faecalis	10464	95.5	41.0	Mild, clean	29.5	Waxy, sl. rubbery
Faecalis	10468	94.5	40.0	Mild, clean, exc.	29.0	Waxy, sl. rubbery
				4.5 months old		
Lactic	10464	94.5	40.0	Medium —, exc.	29.5	Waxy, sl. firm
Lactic	10468	93.0	39.0	Mild, past.	29.0	Sl. waxy, sl. firm
L.F.	10464	94.5	40.0	Medium +, exc.	29.5	Waxy
L.F.	10468	94.5	40.0	Medium, raw	29.5	Waxy
Faecalis	10464	94.0	39.5	Medium, exc.	29.5	Waxy
Faecalis	10468	94.0	39.5	Medium —	29.5	Waxy
				7 months old		
Lactic	10464	93.5	39.5	Medium —, sl. flat	29.0	.....
Lactic	10468	94.0	39.5	Medium	29.5	.....
L.F.	10464	95.0	40.5	Medium +	29.5	Waxy
L.F.	10468	95.0	40.5	Medium +	29.5	Waxy
Faecalis	10464	94.5	40.0	Medium	29.5	Waxy
Faecalis	10468	94.5	40.0	Medium	29.5	Waxy

[a] Flavor was scored with 45 as perfect. Intensity of flavor was rated mild—, mild, mild+, medium—, medium, medium+, sharp —, sharp.

[b] Body was scored with 30 as perfect.

Considering the cheese ripened at 50° F. (table 3), it will be noted that the cheese made with commercial lactic starter scored the lowest or possessed least flavor, whereas the cheese made with both lactic and *S. faecalis* starters scored the highest or possessed the most flavor of the

TABLE 4

Flavor development in pasteurized-milk American Cheddar cheese made with lactic, lactic plus S. faecalis, and S. faecalis starters
(Ripened at 60° F.)

Cheese no.		Total score	Flavor [a]		Body [b]	
			Score	Remarks	Score	Remarks
				1 month old		
Lactic	10464	94.5	40.0	Mild	29.5	Sl. open
Lactic	10468	93.5	39.5	Mild	29.0	Firm
L.F.	10464	95.5	41.0	Medium, raw	29.5	Waxy
L.F.	10468	96.0	41.5	Medium, raw, exc.	29.5	Waxy
Faecalis	10464	94.5	40.0	Medium, raw	29.5	Waxy
Faecalis	10468	95.5	41.0	Medium, raw, exc.	29.5	Waxy
				2.5 months old		
Lactic	10464	94.5	40.5	Mild +, past., flat	29.0	Sl. mealy
Lactic	10468	94.0	40.0	Mild, clean	29.0	Sl. crumbly
L.F.	10464	96.5	42.0	Medium +, clean, exc., raw	29.5	Waxy
L.F.	10468	96.5	42.0	Medium, clean, exc., raw	29.5	Waxy
Faecalis	10464	95.5	41.0	Medium, clean, raw	29.5	Waxy
Faecalis	10468	95.5	41.0	Medium —, exc., clean, raw	29.5	.....
				4.5 months old		
Lactic	10464	94.5	40.0	Medium +	29.5	Waxy, sl. firm
Lactic	10468	91.5	38.0	Medium, burnt	28.5	Weak, sticky
L.F.	10464	95.0	40.5	Sharp —	29.5	Waxy
L.F.	10468	95.5	41.0	Medium +, exc.	29.5	Waxy
Faecalis	10464	95.0	40.5	Sharp —	29.5	Waxy
Faecalis	10468	94.5	40.0	Medium +, exc.	29.5	Waxy
				7 months old		
Lactic	10464	93.0	39.0	Medium +, flat, burnt	29.0	.....
Lactic	10468	91.5	38.0	Medium, burnt	28.5	Sl. crumbly
L.F.	10464	94.0	39.5	Sharp —	29.5	.....
L.F.	10468	93.5	39.5	Medium +	29.0	.....
Faecalis	10464	94.0	39.5	Sharp —	29.5	.....
Faecalis	10468	93.5	39.5	Medium +	29.0	.....

[a] Flavor was scored with 45 as perfect. Intensity of flavor was rated mild—, mild, mild +, medium—, medium, medium +, sharp—, sharp.

[b] Body was scored with 30 as perfect.

three lots. The difference in the scores was obvious for cheese of all ages up to 7 months, when scoring was discontinued. Furthermore, the intensity of the Cheddar flavor was greatest for cheese made with both lactic and *S. faecalis* starters and was least for cheese made with lactic

starter only. The flavor of the cheese with lactic starter usually was slightly flat, whereas with the use of both starters the flavor was full, clean, and often said to be excellent and like good raw milk cheese. The flavor of cheese made with *S. faecalis* starter alone closely resembled that of cheese made with combination starter, but neither quality nor intensity of flavor always was as good. It should be noted that when the flavor of the cheese with lactic starter was especially good, the quality closely approached that of cheese made with lactic and *S. faecalis* starters. All lots of cheese cured well without developing any off-flavors for 7 months at 50° F.

The waxy, mellow body of cheese made with the combination commercial lactic and *S. faecalis* starters, and with *S. faecalis* starters alone, was evident (table 3). The improved body of these samples of cheese could be detected even after 7 months of curing. The difference was much more noticeable than might be supposed by observing the numerical scores on body.

Cheese cured at 60° F. developed the same character as that cured at 50° F., except that changes occurred more rapidly. At 50° F. no cheese developed a sharp flavor in 7 months, but at 60° F., cheese made with *S. faecalis* starter alone or in combination with lactic starter was sharp in flavor in 4.5 months (table 4). The intensity of Cheddar flavor at 2.5 months at 60° F. approximated the intensity of flavor after 4.5 months at 50° F. This agrees closely with the work of Dahlberg and Marquardt (2). At 60° F. cheese made with lactic starter began to show some deterioration in flavor at 4.5 months and was obviously deteriorated at 7.5 months. The defect was a burnt or caramelized off-flavor. The cheese made with *S. faecalis* starter alone or in combination with lactic starter was excellent after 7 months at 60° F., but the flavor quality was slightly less than at 4.5 months of age. Observations of other batches of cheese show that about 4 months at 60° F. should be the maximum forced curing before cold storage at 40° F.

DISCUSSION

The scientific literature on Cheddar cheese of the last 50 years contains many articles showing that the use of a certain bacterial culture or enzyme or of a specific process of manufacture has intensified good flavor. With minor exceptions, none of these promising results ever has been successfully used commercially over an extended territory. Therefore, it is with some reluctance that the authors publish these data, but the results are of scientific value irrespective of commercial usage. This study is the first to embody successfully the use of large inoculations of a special culture into pasteurized milk to produce good, high flavored

American Cheddar cheese in a short curing period. Although the flavor is like that of raw milk cheese, it never develops the very "bitey" or "snappy" flavor that stings on the upper palate of the mouth, and is so typical of old raw milk cheese. Rather, the flavor is full and pronounced without being astringent or without having any rancid or other foreign flavor. Obviously, the milk must be of good flavor before pasteurization.

Good Cheddar cheese was made in these experiments with pasteurized milk containing only a commercial lactic starter. A better Cheddar cheese was made using *S. faecalis* starter alone, so this is positive proof that ordinary lactic starter is not necessary for making good Cheddar cheese. The best Cheddar cheese was made using both starters together, indicating symbiotic action among the bacteria in the two starters in producing a maximum of flavor.

In these experiments no time was given for the starter to develop before adding rennet, and the authors actually favor an hour at 86° F. for the starter to work in the milk before setting. Under such conditions about 0.5 to 0.75 per cent of lactic starter and 1 per cent of *S. faecalis* starter gave desired acid production.

S. faecalis has characteristics of special significance in cheese. It grows at 106° F. in the cheese vat and at 50° F. in the curing room. It ferments lactose rapidly enough to be used as a starter, providing a proper strain is selected and developed. It grows anaerobically in cheese, utilizing lactates as sources of energy. It is nonproteolytic and does not produce gas or objectionable flavors. It grows at the pH and the salt concentration present in cheese.

SUMMARY

A strain of *Streptococcus faecalis* which rapidly fermented lactose was isolated. It was used as a starter for American Cheddar cheese made from pasteurized milk of excellent quality.

The *S. faecalis* starter produced acid in milk somewhat slower than a commerical lactic starter but rapidly enough for cheese making. The cheese made with *S. faecalis* developed a normal acidity, slightly lower total volatile acidity, and the same water-soluble protein level as did cheese made with a commercial lactic starter. More Cheddar flavor of better quality developed in the cheese made with *S. faecalis,* and the body of the cheese was more mellow and waxy than the cheese made with lactic starter.

American Cheddar cheese with the best flavor of highest intensity was made by using both commercial starter and *S. faecalis* starter in the same pasteurized milk. The flavor was pronounced, clean, good Cheddar but not snappy.

S. faecalis starter hastened the ripening of Cheddar cheese. A well-ripened cheese of medium flavor intensity was produced in 4.5 months at 50° F. and in 2.5 months at 60° F. when *S. faecalis* starter was used with the usual lactic starter. With commercial lactic starter, the same approximate intensity of flavor, of lower quality, was developed in 7 months at 50° F. and in 4.5 months at 60° F. The results indicate that after these curing periods, the cheese should be held at cold curing temperatures.

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THE GROWTH AND SURVIVAL OF *STREPTOCOCCUS FAECALIS* IN PASTEURIZED MILK AMERICAN CHEDDAR CHEESE[1]

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In a previous paper (1) it was shown that the addition of *Streptococcus faecalis* starter to pasteurized cheese milk increased the rate of ripening and improved the flavor of American Cheddar cheese. Such an effect naturally directed the attention of the authors to the growth characteristics of this organism in cheese.

It is well known that the bacteria normally found in commercial lactic cheese starters do not survive for any great length of time in cheese. *S. faecalis*, on the other hand, is considered to be a rugged type of organism, able to survive and grow under conditions which would soon destroy many other types. As American Cheddar cheese in many respects affords conditions unfavorable for growth of most bacteria, it would be interesting to observe the degree of adaptation that *S. faecalis* could make in such an environment. That this organism commonly is found in cheese has been noted by several investigators. Sherman and Stark (5) found *S. faecalis* in 1-day-old Swiss cheese, while Foster *et al.* (2) found large numbers of these bacteria in ripening Brick cheese. Tittsler *et al.* (6) stated that enterococci were one of the predominant bacterial types in ripening Cheddar cheese made from pasteurized milk.

Up to the present, very little study has been made of the course of growth and of the survival period of *S. faecalis* in Cheddar cheese. Data of this nature should aid in an understanding of the effect of this organism upon cheese flavor development as well as provide general information which will be required as knowledge of the relationship of *S. faecalis* to foods becomes more apparent. A study covering the foregoing phases was conducted.

EXPERIMENTAL METHODS

Methods used consisted of the total plate count using standard tryptone-glucose-extract-skim milk agar, a selective medium plate count for enterococci, and conventional physiological identification tests for enterococci. The selective medium was that developed by White and Sherman (7) for the determination of enterococci in milk. It contains 0.5 per

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cent glucose, 0.5 per cent tryptone, 0.5 per cent yeast extract, 1.5 per cent agar, 0.03 per cent sodium azide, and 325 Oxford units of penicillin per l. Tests employed for the identification of the enterococcus group were those suggested by Sherman (4) as being very important for differential purposes. They included growth at 10° C. and 45° C., rapid reduction in litmus milk, and growth in broth containing 6.5 per cent sodium chloride. In addition, microscopic obsel'vations were made. All plates were incubated at 32° C. for 4 days. Samples for plating were prepared by aseptically grinding 3 g. of cheese with 27 ml. of warm 2 per cent sodium citrate solution until the cheese was well emulsified. Dilutions then were made from this cheese solution.

RESULTS

Total and selective medium counts of milk, starters, and fresh curd. Results shown in this work were obtained on a series of three cheese. All cheese were made from milk pasteurized at 143-145° F. for 30 minutes, with the raw milk being obtained from the Cornell University herd. Twelve hundred pounds of milk were divided into three equal portions and made into cheese. The cheese obtained was the series *L 10468*, *LF* 10468 and F 10468 referred to in a previous paper (1) in which *L* was made with 2 per cellt commercial lactic starter, *LF* with 1 per cent commercial lactic starter and 1 per cent *S. faecalis* starter, and F with 2 per cent *S. faecalis* starter. The cheese were ripened at 50 and at 60° F.

Bacterial counts, using standard agar and penicillin-sodium azide medium, were made of the original raw and pasteurized milk, and starters. Although the selective medium was used to separate the enterococci, no attempt was made at this stage to identify the enterococcus colonies by additional tests. Results of these counts are shown in table 1. The two different types of starters showed relatively high total bacterial counts. The regular commercial starter showed a total bacterial count of 350 million per ml., while the *S. faecalis* starter had a total bacterial count of 950 million per ml. Results with the selective medium showed that no enterococci were present in the regular commercial starter, while approximately 94 per cent of the total count of the *S. faecalis* starter grew in the selective medium.

The original raw milk was of high quality, having a total bacterial count of 5,700, while the pasteurized milk had a low bacterial count of *500* per ml. The numbers of bacteria growing on the selective medium were very small in either milk. The total bacterial counts after the addition of the starters ranged from 5 million to 16 million, whereas the selective medium counts ranged from 70 to 12 million.

The next counts were taken on the cheese curds just before salting.

Results are listed in table 2 under 0 days. Beginning with this section and continuing through, with a few exceptions, 20 colonies from the plates of each cheese were isolated and cultured from plates containing the selective medium. These bacteria then were identified as to whether or not they were enterococci, using the tests enumerated previously.

Total and selective medium counts of cheese ripened at 50° F. Of the cheese ripened at 50° F. (table 2), cheese *L*, containing 2 per cent lactic starter, had its highest total bacterial count, 300 million per g., within the first 2 days, after which the bacterial population decreased rapidly to the

TABLE 1

Bacterial counts on starters and cheese milk containing commercial lactic starter organisms and S. faecalis organisms

Milk	Total bacterial counts per ml. on standard agar	Bacterial counts per ml. on penicillin-azide agar
Commercial starter	350,000,000	1
S. faecalis starter	950,000,000	890,000,000
Raw milk	5,700	320
Past. milk (143–145° F. for 30 min.)	500	50
L 10468—past. milk set at 86° F. containing 2% com. starter	5,000,000	70
LF 10468—past. milk set at 86° F. containing 1% com. starter and 1% *S. faecalis* starter	11,500,000	6,400,000
F 10468—past. milk set at 86° F. containing 2% *S. faecalis* starter	16,000,000	12,000,000

low total count of 1,200,000 per g. at the end of 23 days and then gradually increased to a total count of 26 million at the end of 180 days. Cheese *LF*, containing 1 per cent lactic starter and 1 per cent *S. faecalis* starter, had its highest total count, 1,150 million per g., at the time of salting the curd. The number of bacteria then decreased very slowly over the ripening period of 180 days to a low of 305 million. Cheese *F*, containing 2 per cent *S. faecalis* starter, on the other hand, had a high total count of 1,790 million at the time of salting, but this high count was maintained at the same level for 60 days, after which it slowly decreased to 855 million at the end of 180 days.

When the selective penicillin-azide medium for enterococci was used on the cheese ripened at 50° F., the following results, outlined in table 2, were obtained. The selective medium bacterial count for cheese *L* was lowest during the first 2 days. Just prior to salting of the cheese, the bacterial

count was 300, with 18 of the 20 colonies being identified as enterococci. From this low point, the bacteria in this control cheese increased to a peak of 23 million per g. However, it can be seen clearly from the summary of identification tests (table 2) that the increase in numbers was not a

TABLE 2

Bacterial counts of pasteurized-milk American Cheddar cheese (Series 10468) made from commercial starter, S. faecalis starter, and a mixture of the two, and ripened at 50° F.

Cheese [a]	Age	Total bacterial count per g. on standard agar	Bacterial count per g. on penicillin-azide agar	Positive identification of enterococci
	(days)			*(from 20 picked colonies)*
L	0	300,000,000	300	18
LF	0	1,150,000,000	520,000,000	19
F	0	1,790,000,000	1,370,000,000	20
L	2	320,000,000	100	.....
LF	2	1,070,000,000	490,000,000	.....
F	2	1,890,000,000	1,100,000,000	.....
L	11	60,000,000	5,000	4
LF	11	920,000,000	500,000,000	19
F	11	1,750,000,000	1,310,000,000	20
L	23	1,200,000	14,000	.....
LF	23	686,000,000	389,000,000	.....
F	23	1,560,000,000	1,076,000,000	.....
L	34	2,900,000	295,000	2
LF	34	740,000,000	390,000,000	19
F	34	1,810,000,000	1,065,000,000	20
L	60	11,000,000	2,700,000	6
LF	60	480,000,000	345,000,000	20
F	60	1,630,000,000	1,085,000,000	19
L	120	44,000,000	23,000,000	0
LF	120	366,000,000	290,000,000	19
F	120	970,000,000	740,000,000	18
L	180	26,000,000	11,000,000	0
LF	180	305,000,000	250,000,000	19
F	180	855,000,000	675,000,000	20

[a] L = 2% regular lactic starter in cheese milk.
LF = 1% regular lactic starter in 1% *S. faecalis* starter in cheese milk.
F = 2% *S. faecalis* starter in cheese milk.

result of an increase in enterococci but rather of another type or types of bacteria able to multiply on the selective medium. Further examination of these organisms showed them to be of the genus *Lactobacillus.*

In cheese *LF*, the highest number of enterococci, 520 million per g., was found in the curd prior to salting. The numbers of these bacteria

were maintained at almost 70 per cent of this level for 60 days, while at the end of 180 days there was a decline of about 50 per cent. Almost all the bacteria isolated were of the enterococcus group.

Cheese *F* at 50° F. had the highest enterococcus count, 1,370 million per g., just before the curd was salted, and this count was maintained at

TABLE 3

Bacterial counts of pasteurized-milk American Cheddar cheese (Series 10468) made from commercial starter, S. faecalis starter and a mixture of the two, and ripened at 60° F.

Cheese [a]	Age	Total bacterial count per g. on standard agar	Bacterial count per g. on penicillin-azide agar	Positive identification of enterococci
	(*days*)			(*from 20 picked colonies*)
L	0	300,000,000	300	18
LF	0	1,150,000,000	520,000,000	19
F	0	1,790,000,000	1,370,000,000	20
L	2	.....	.....	.....
LF	2	.....	.....	.....
F	2	.....	.....	.....
L	11	50,000,000	36,000	3
LF	11	960,000,000	450,000,000	19
F	11	1,680,000,000	1,330,000,000	20
L	23	3,200,000	2,700,000	.....
LF	23	680,000,000	420,000,000	.....
F	23	1,490,000,000	1,030,000,000	.....
L	34	14,000,000	5,500,000	1
LF	34	630,000,000	385,000,000	18
F	34	1,600,000,000	1,020,000,000	20
L	60	50,000,000	26,000,000	0
LF	60	450,000,000	305,000,000	18
F	60	875,000,000	750,000,000	20
L	120	61,000,000	28,000,000	0
LF	120	150,000,000	115,000,000	18
F	120	530,000,000	450,000,000	19
L	180	35,000,000	13,500,000	0
LF	180	61,000,000	36,000,000	13
F	180	165,000,000	90,000,000	20

[a] L = 2% regular lactic starter in cheese milk.
LF = 1% regular lactic starter in 1% *S. faecalis* starter in cheese milk.
F = 2% *S. faecalis* starter in cheese milk.

the same level for 60 days, after which it decreased to 675 million at the end of 180 days. Practically all the colonies isolated by means of the selective medium belonged to the enterococcus group, and, as only *S. faecalis* was added, presumably all or almost all of this species made up the bacterial count of cheese *F*.

Total and selective medium counts of cheeses ripened at 60° F. The bacterial counts of cheese ripened at 60° F. compared to those of the same lots of cheese ripened at 50° F. showed many similar trends (table 3). In control cheese *L*, the total bacterial count showed a rapid decrease from a high of 300 million to a low of 3 million per g. at the end of 23 days. This was followed by a steady increase until at the end of 120 days the total bacterial count was up to 61 million. Two months later the numbers of bacteria had decreased to 35 million. In cheese *LF* a high total bacterial count of 1,150 million was obtained on the curds just prior to salting. The total count then gradually decreased to 450 million at the end of 60 days, while at the end of 180 days the bacterial count had gone down to 61 million. Cheese *F* showed results in line with those exhibited by cheese *LF*, going from a high of 1,790 million in the curd to a low of 165 million at 180 days.

Selective medium counts made on these cheese ripened at 60° F. (table 3), showed control cheese *L* with a low initial count of 300, followed by a steady increase to 28 million at the end of 120 days and a drop to 13,500,000 at 180 days. This increase in cheese *L* was not due as much to enterococci as to lactobacilli. On the other hand, cheese *LF* had its highest enterococcus count just before salting, 520 million per g., and this population decreased to 36 million at 180 days. At the end of 60 days of ripening, 305 million enterococci per g. still were present. In this connection, cheese *F*, with *S. faecalis* starter only, showed counts which were maintained for long periods of time. Starting with an initial selective medium count of 1,370 million, this cheese still had a count of 750 million at the end of 60 days, and then dropped to 90 million at 180 days.

A comparison of tables 2 and 3 shows that at 50 and at 60° F. the trends of bacterial growth and survival were very similar; the difference that existed showed up as a more rapid decline in bacterial population at 60° F., a result which was expected.

A duplicate experiment made on cheese manufactured a month later produced data of strikingly similar nature. These data are not included in this paper because they would only provide repetition of the initial observations.

DISCUSSION

A study was made of the growth and survival of *Streptococcus faecalis* in pasteurized milk American Cheddar cheese over a 6-month ripening period at 50 and at 60° F. Control cured cheese made from pasteurized milks and containing only regular lactic cheese starter had the lactobacilli as their predominating organisms. These results do not agree with those of Tittsler *et al.* (6), who stated that enterococci were the predom-

inating organisms in pasteurized milk cheese. However, it is pointed out that the milk used in the present study was of very high quality, and cheese made commercially from pasteurized milk was not included. Development of lactobacilli in large numbers late in the ripening period of Cheddar cheese was noted very early by Hastings *et al.* (3) and by other investigators.

The observation that the selective medium developed by White and Sherman (7) for the separation of enterococci actually allowed bacteria of the lactobacillus group to grow confirms the earlier findings of White and Sherman (8). These investigators found that in cheese certain species of lactobacilli were able to grow in the selective medium. Where enterococci are predominant, this method of selection is very useful and surprisingly consistent in its ability to recover the organism. In the work involving cheese *F*, where there was a vast number of *S. faecalis* organisms, the selective medium was able to recover on the average about 73 per cent of the total bacterial count as enterococci. This is a good recovery when one considers the many opportunities that exist for the elimination of the less resistant bacteria in work of this nature.

Streptococcus faecalis proved able to adapt itself well to the environment provided by Cheddar cheese when added as a starter. Its best growth occurred in the milk and curd up to salting, and it was able to grow and survive in large numbers after 180 days of ripening at 50 and at 60° F. This characteristic resistance to destruction in cheese further strengthens the authors' belief that this organism is instrumental in developing increased flavor in cheese.

SUMMARY

Three lots of milk pasteurized at 143–145° F. for 30 minutes were made into American Cheddar cheese. These lots contained, respectively, 2 per cent commercial lactic starter, 1 per cent commercial lactic starter plus 1 per cent *Streptococcus faecalis* starter, and 2 per cent *S. faecalis* starter. A selective penicillin-azide medium was used to count and isolate the enterococci.

In 1-day-old cheese made with commercial lactic starter, the number of bacteria growing on the selective medium was small, 300 per ml., but these gradually increased to 23 million per ml. at the end of 120 days at 50° F., and to 28 million per ml. after 120 days at 60° F. At the end of 180 days the counts on the selective medium had decreased to 11 million and 13 million per ml. at 50 and 60° F., respectively. In this cheese most of the increase was due to lactobacilli and not to enterococci.

When *S. faecalis* was used as a starter for pasteurized milk American Cheddar cheese, the highest enterococcus count was found to exist in the

cheese curds just prior to salting, the count being 500 million per g. for cheese containing 1 per cent commercial lactic starter plus 1 per cent *S. faecalis* starter, and 1,370 million per g. for the cheese containing 2 per cent *S. faecalis* starter.

S. faecalis was able to grow and survive in Cheddar cheese in large numbers for a considerable period of time, both at 50 and 60° F. At 50° F., cheese made with both lactic and *S. faecalis* starters still gave counts of 345 million per g., and cheese made with *S. faecalis* starter gave counts of 1,085 million per g. at the end of 60 days, whereas at 60° F. the former cheese contained 305 million per g. and the latter cheese contained 750 million per g. at the end of 60 days. At the end of 180 days the numbers of bacteria in the cheese growing on the selective medium had decreased, although considerable numbers still were present.

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THE TYRAMINE CONTENT OF CHEESE[1]

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The process of cheese ripening produces a variety of nitrogenous decomposition products, some of which must exert an important influence upon the ripening process. Yet relatively little is known concerning the nature of many of these compounds, their concentration in cheese, their specific rôle in ripening, and their nutritional value. For example, information is relatively meager on the amino acids freed in cheese during ripening, and even less is known concerning the respective amines which may be formed from these amino acids.

This paper deals with a small portion of this complex problem in that a quantitative study on cheese was made of the amine derived from tyrosine. This breakdown product from tyrosine is *p*-hydroxyphenylethylamine and very commonly is referred to as tyramine. No information has been available to indicate its quantitative concentration in cheese, with the single exception of an Emmenthal cheese.

Tyramine is an alkaloid of the aromatic amine type. It can be produced by heating tyrosine with a high boiling solvent such as diphenylamine or by bacterial decomposition of tyrosine. Often it occurs in decaying protein and it also is found in ergot and mistletoe. Tyramine has a boiling point of 179–181° C. (8 mm.) and a melting point of 161° C. When injected subcutaneously or intravenously, it has the property of contracting the peripheral blood vessels, thus causing an increase in blood pressure, and for this reason it is used rather frequently in medicine. Gale (6) found the optimum production of tyramine by bacterial cells to occur at pH 4.5 to 5.5, which is in the pH range of normal American Cheddar cheese.

Tyramine was first discovered in cheese in 1903 by Van Slyke and Hart (8) in their research to show the source of carbon dioxide in cheese. They made two small batches of Cheddar cheese from fresh milk and from fresh milk to which chloroform had been added. Of specific interest in their study was the accumulation of relatively large amounts of tyrosine and no tyramine in the chloroformed cheese (a low acid cheese) after curing for 32 weeks at 15.5° C. (60° F.) as compared with lesser amounts of tyrosine and positive tests for tyramine in the normal cheese. This conversion of tyrosine to tyramine was thought to be due to bacteria. They cited the research of Emerson (5), published the previous year, which established

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that the aqueous extract of the pancreas converted tyrosine into tyramine with liberation of carbon dioxide by the following reaction:

$$HO \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2)COOH \rightarrow HO \cdot C_6H_4 \cdot CH_2 \cdot CH_2 \cdot NH_2 + CO_2$$

An abnormal Emmenthal cheese was found in 1909 by Winterstein and Küng (10) to contain tyramine. The authors did not state the reason for considering this cheese to be abnormal, but they found the tyrosine content to be abnormally low for a well-ripened cheese. They believed that bacteria probably converted the tyrosine into tyramine. Later Winterstein (9) found traces of tyramine in a skim milk Emmenthal cheese.

In 1914 Ehrlich and Lange (4) reported the presence of tyramine in samples of Roquefort, Camembert and Emmenthal cheese. The tyramine content of the Emmenthal cheese was found to be 1.08 g. in 1.8 kg. of cheese, or 0.06 per cent. When the cheese was inoculated into a bacterial culture medium containing tyrosine, tyramine was produced. A culture was isolated which produced tyramine by this method, but it did not produce tyrosol or *p*-hydroxyphenyllactic acid. This culture which produced acid in milk belonged to the colon group of bacteria. The literature includes the analysis for tyramine of only one Cheddar, one Roquefort, one Camembert and three Emmenthal cheeses. All samples tested showed the presence of tyramine, but of these six samples, only one, an Emmenthal cheese, was analyzed quantitatively.

Methods for the separation and estimation of tyramine have been known for some time, but only in recent years have newer methods been introduced which can be applied on a large scale to the estimation of this compound in such products as cheese. Henze (7) developed a quantitative method for obtaining and separating tyrosine and tyramine from cephalopods by ether extraction. With this method he isolated tyrosine and tyramine from the salivary gland of *Octopus macropus* and determined the compounds colorimetrically by the Millon reaction. Recently Bellamy and Gunsalus (1, 2), by using a continuous ether extractor and a colorimeter, adapted and applied this method to the determination of tyrosine and tyramine in bacterial cultures in their study on tyrosine decarboxylase systems. Utilizing the knowledge obtained by the foregoing investigators, an applied method for determining tyramine in cheese was evolved. With this method it was possible to test quantitatively a large number of cheeses.

EXPERIMENTAL METHODS

The principle of this method is that the phenolic hydroxyl group, which is characteristic for tyrosine and tyramine, will react to form a positive Millon test under the proper conditions. Separation of tyramine from tyrosine is based on the fact that tyramine can be extracted by ether under mildly alkaline conditions, while tyrosine is insoluble in ether. Both are

insoluble in acid ether, while phenols are soluble. The method as applied to cheese is described.

Preparation of sample. Fifteen grams of cheese were ground in a mortar with a small amount of warm (45° C.) 2 per cent sodium citrate solution. After the cheese was well emulsified, the solution was transferred quantitatively to a 250-ml. volumetric flask and enough sodium citrate solution was added to bring it to the 250-ml. mark after cooling to 25° C. The contents of the volumetric flask were transferred to a standard 300-ml.

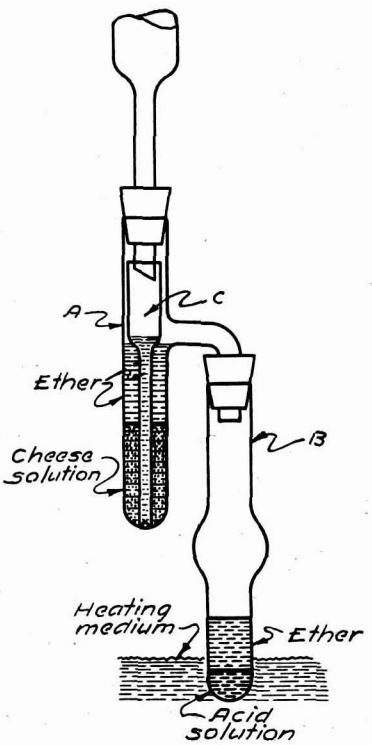

FIG. 1. A continuous extractor for separating tyramine from tyrosine. *A*, extraction tube (200 × 25 mm.); *B*, receiver tube (200 × 25 mm.); *C*, glass thimble. Condenser capacity—300 mm.

pyrex flask and heated to 80° C. for 15 minutes in a water bath to destroy the decarboxylases and then cooled to 25° C. After cooling, an 18-g. sample obtained by using a calibrated pipette was transferred to the extraction tube (A) of the continuous ether extractor shown in figure 1. This extractor, very similar to the one proposed by Wooley (11), was used by Bellamy and Gunsalus (1, 2) in their work on bacterial decarboxylases.

Acid extraction of cheese. To the receiver tube (*B*, fig. 1) of the ex-

tractor, 5 ml. of M/50 sulfuric acid were added. This acid solution is used to trap the tyramine when it comes over with the ether. The glass thimble (*C*, fig. 1) was placed in the extraction tube and then ethyl ether carefully was added to both the extractor and receiver tube. This was done until a 3-cm. layer accumulated in the receiver and until the ether just reached the side arm in the extractor tube. The two sections were attached to each other by means of cork connections and then attached to a condenser. Gentle heating of the ether in the receiver tube was accomplished by the use of an oil bath. Extraction under acid conditions was carried on for 5 hours to remove a large portion of the fat and fatty acids and any phenols that might be present. This extraction, due to the influence of the sodium citrate, was carried at slightly above the pH of most normal Cheddar cheese, or about pH 5.5–5.8.

Alkali extraction of cheese. The receiver tube was emptied, washed, and again filled to its former level with 5 ml. of M/50 sulfuric acid and sufficient ethyl ether and attached to the extraction section. The entire extraction unit then was taken from the oil bath, and enough of a solution of 10 per cent sodium carbonate was added to the thimble in the extractor tube to make the solution to be extracted slightly alkaline to phenolphthalein. The quantity required varied from 0.4 to 1.0 ml. No phenolphthalein actually was added to the extractor tube as it would be extracted by ether and would give a positive test. To find the proper amount of alkali required without adding phenolphthalein to the unit, an Erlenmeyer flask containing 18 g. of the cheese solution and phenolphthalein was used and the solution in it neutralized. The same amount of alkali required for this preliminary neutralization was added to the thimble. Extraction was resumed and continued until all the tyramine was obtained. This usually took about 42 hours, depending on the nature of the cheese. To assure complete extraction, analyses were made on the contents of the receiver tube after 30 hours and then at 12-hour intervals until the pink color no longer was produced.

After each of these extraction periods the receiver tube was disengaged from the extractor tube and the entire ether-acid mixture was cooled slightly and poured slowly into a graduated test tube. The test tube was placed into an oil bath and the ether carefully boiled off. The acid solution remaining was cooled to 25° C., and the volume noted and tyramine analyses made. The receiver tube again was filled with acid solution and ether, and the extraction resumed.

Measurement of color. One milliliter of the acid solution containing the tyramine was pipetted into a colorimeter tube (calibrated 18 × 150 mm. pyrex test tube). Three milliliters of 95 per cent acetic acid were added, followed by 2 ml. of a mercuric sulfate reagent (10 per cent mercuric sulfate in 5 per cent sulfuric acid) and the tube well agitated. This mixture

was heated for 3 minutes in boiling water, cooled and mixed. The tube then was placed in a Coleman no. 11 spectrophotometer and read at 500 γ against a reagent blank set at $G = 100$. After this turbidity reading (L_1) if any, was recorded, 1.0 ml. of fresh 0.5 per cent sodium nitrate (prepared fresh daily) was added and well mixed, and the tube read at 500 γ after 15 minutes at room temperature against a reagent blank to which sodium nitrate solution had been added. The second reading with the color-producing compound was labeled L_2.

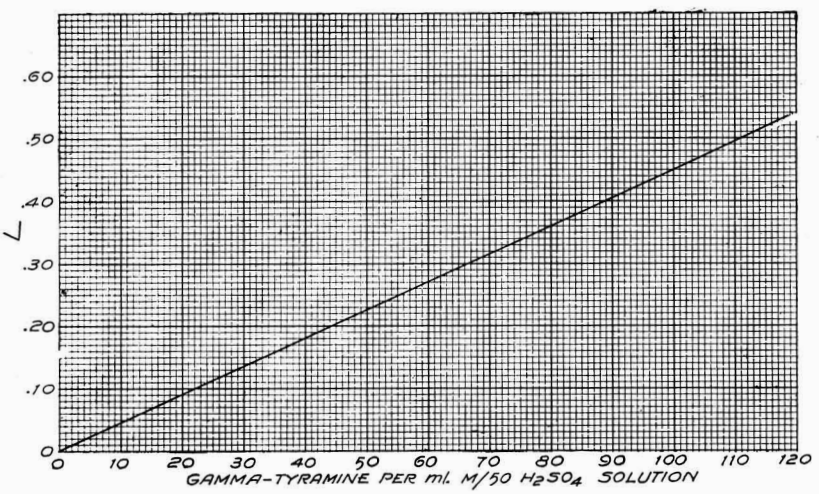

FIG. 2. Standard curve for tyramine using the Coleman spectrophotometer.

Calculations. Calculations required for this method are as follows:

$$L = 2 - \log G$$

$$L_2 - L_1 = L \text{ (proportional to tyramine concentration)}$$

G = galvanometer reading

L_1 = value of turbidity

L_2 = value for color

To find the amount of tyramine in the sample, one of two things can be done. Reference can be made to the standard curve (fig. 2), or the tyramine content can be calculated by using a constant K, which has been obtained at different levels. To use K, the standard curve must go through the origin.

$$K = \frac{\text{gamma in standard sample}}{L \text{ for same sample}}$$

$$\text{Gamma in standard sample} = KL$$

The final standard is brought to gammas of tyramine per gram of cheese, after considering all dilution factors. In this work all values are based on tyramine and not tyramine hydrochloride.

RESULTS

The recovery of added tyramine from cheese. In order to test the ability of this applied method to recover tyramine from cheese effectively and at the same time to prevent tyrosine from being extracted, a number of recovery experiments were performed. The results from several experimental trials indicated that when pure tyrosine was added to water or to a cheese solution, no amount of extraction, either at an acid or alkaline pH, would bring over the tyrosine. On the other hand, when various amounts of tyramine in the form of tyramine hydrochloride were added to a cheese solution, which then was made mildly alkaline with 10 per cent sodium carbonate, it was found (table 1) that practically all the added tyramine was recovered under the conditions of extraction and color

TABLE 1

The recovery of added tyramine from cheese (5468 Fe)
(5-hr. acid ether extraction + 40-hr. alkali ether extraction)

Tyramine added	Tyramine found in cheese	Tyramine recovered
(γ/g.)	(γ/g.)	(%)
0	1427	.....
790	2191	97.0
1580	3008	100.0

estimation previously described. In this recovery experiment 5 hours of acid ether extraction and 40 hours of alkali ether extraction were required for complete recovery. For some cheese solutions where smaller amounts of tyramine were added, complete recovery was attained in a shorter length of time.

The tyramine content of 25 Cheddar cheeses. Analyses for tyramine were conducted on 25 commercial American Cheddar cheeses. These samples were obtained from New York, Wisconsin, Illinois, and Missouri and consisted of both raw and pasteurized milk cheese. The ages of these cheese ranged from 2 months to 3 years. No attempt was made to show here the effect of such factors as treatment of milk or cheese and the effect of age upon the tyramine content, as these topics will form the basis of subsequent papers. The high, average, and low tyramine concentrations of 25 American Cheddar cheeses are shown in table 2.

The tyramine content of Cheddar cheese can be much greater than that listed in the table. One very old Cheddar cheese, not included in the group of 25 cheeses because it was not manufactured in a commercial plant, was found to contain 2,330 γ of tyramine per g., or 0.223 per cent. Data shown

here make it quite apparent that practically all commercial American Cheddar cheese must contain tyramine in a wide range of concentration.

The tyramine content of miscellaneous types of cheese. A number of cheeses representing diversified types were subjected to analyses for tyramine. The results of these analyses are shown in table 3. All samples within a type variety were purchased from different sources and at different times of the year. It was assumed from the beginning that the values shown here do not necessarily typify any particular variety of cheese, as factors tending to shift these values always are present. Data shown in table 3, however, do give some idea as to the tyramine content of an assorted group of cheeses. Here again, as with the Cheddar cheeses, a wide range exists not only between varieties but also between samples of similar types. The smallest amount of tyramine, 48 γ per g., occurred in a Roquefort, while the largest amount, 1,683 γ per g., occurred in a Liederkrantz cheese.

TABLE 2

The concentration of tyramine in twenty-five commercial Cheddar cheeses

Cheese	Tyramine	Tyramine
	(γ/g.)	(%)
Highest	1199	0.1199
Lowest	25	0.0025
Av.	384	0.0384

Two different samples of Liederkrantz both gave high concentrations of tyramine. This is undoubtedly a result of the character of this cheese, where early and extensive decomposition of proteins occurs. The Limburger sample, which is considered similar to Liederkrantz in its decomposition properties, had a relatively low value. Further examination of this sample showed it to be very atypical in that it was not broken down in body and that it resembled a Brick cheese more than a Limburger.

The isolation, purification and identification of the dibenzoyl derivative of tyramine from Cheddar cheese. A series of tyramine extractions was conducted on several Cheddar cheeses which were considered to have large concentrations of tyramine. Sufficient extractions were made from fresh samples of cheese to provide a volume of 500 ml. of N/50 sulfuric acid calculated to contain about 35 mg. tyramine. The acid solution (about pH 1) then was evaporated *in vacuo* to approximately 40 ml., washed with ethyl ether to remove any fat, and then centrifuged to remove other impurities.

The method for obtaining a dibenzoyl derivative of tyramine as described by Gale (6) was followed. Solid sodium bicarbonate was added until the pH of the solution was brought to 7.5–8.0 and the mixture was

cooled in ice to 10–12° C. Benzoyl chloride then was added, with vigorous shaking, a few drops at a time until about 3 mol. equivalents had been added, the pH being maintained in the region of 8 by the addition of solid sodium bicarbonate. This mixture was left overnight in a cold room. The following day a precipitate had formed and was removed by centrifuging. It was purified by extracting with hot absolute alcohol. The dibenzoyl

TABLE 3

The concentration of tyramine in an assorted group of cheeses

Cheese	Tyramine	Tyramine
	(γ/g.)	(%)
Edam [a]	214	0.0214
Edam	100	0.0100
Roquefort [a]	48	0.0048
Blue	49	0.0049
Blue	266	0.0266
Limburger	204	0.0204
Liederkrantz	1226	0.1226
Liederkrantz	1683	0.1683
d'Oka [a]	310	0.0310
d'Oka [a]	158	0.0158
Gouda [a]	95	0.0095
Gouda	54	0.0054
Brick	194	0.0194
Munster	110	0.0110
Swiss	50	0.0050
Swiss	434	0.0434
Romano [a]	197	0.0197
Argenti [a]	188	0.0188
Camembert	125	0.0125
Mild process [b]	164	0.0164
Cheese food	125	0.0125

[a] Imported cheeses.
[b] Cheddar.

tyramine was recrystallized once from dilute alcohol and its melting point determined on a hot-stage microscope. A sample of dibenzoyl tyramine was prepared in the same manner from highly purified Eastman Kodak tyramine hydrochloride and the melting point of the derivative taken by the hot-stage microscope. Gale (6) reported that the dibenzoyl tyramine obtained in his studies had a melting point of 171–172° C. (corr.). The derivative from the present cheese and from the known pure tyramine melted at 170–172° C. (corr.) and a melting point of the mixed samples was 169–172° C. (corr.). These data confirm that tyramine was the chemical substance being extracted from the cheese.

DISCUSSION

It has been possible to show the presence and concentration of tyramine (*p*-hydroxyphenylethylamine) in a large number of ripened types of cheese. As tyramine can be derived from tyrosine by bacterial decarboxylases, a prerequisite of tyramine production in cheese is the presence of free tyrosine. The observations of Dorn and Dahlberg (3) that the white particles in ripened Cheddar cheese actually are made up largely of tyrosine fully satisfies this condition.

The fact that tyramine was found in practically all commercial cheeses examined is not as surprising as the concentrations found. Past concepts that tyramine occurs in normal cheese only in traces, if at all, will have to be revised. A concentration of from 0.08 to 0.12 per cent cannot be considered a trace. In these analyses it was found that 5 out of 25 commercial American Cheddar cheeses fell in the above group. Because of the very unique properties of tyramine, the implications and significance of the presence of such amounts in cheese should pose some very interesting questions for future study and should prove to be a fertile field for investigation.

Compounds other than tyramine and tyrosine, but containing the same characteristic phenolic hydroxyl grouping, if present, also would give a positive Millon test. These compounds would include tyrosol, *p*-hydroxyphenyllactic acid, *p*-hydroxyphenylacetic acid, thyroxine, dopa, phenolphthalein, phenol, salicylic acid and thymol. However, practically all of these compounds can be ruled out either as being insoluble in ether or as never having been reported to be found in cheese. If free phenol were present, it would be removed by the acid ether extraction. Nevertheless, to make certain that tyramine actually was being obtained, a dibenzoyl derivative of it was isolated and purified from cheeses after standard extraction by the method.

The initial 5-hour acid extraction was introduced in the method to remove fat and fatty acids. At the end of this period most of the fat will be extracted, thus ruling out fat as an experimental factor, and no tyramine will be extracted. This was true for all Cheddar cheeses which are extracted at a pH range of 5.0 to 5.8, but in the case of well broken-down cheeses such as Liederkrantz, where the pH was higher, some tyramine was recovered. In the actual analyses these initial recoveries of tyramine were added into the total.

The method as applied to cheese usually produced results on duplicate samples with an experimental error of less than 3 per cent. Results below 50 γ per g. produced a larger experimental error. However, as the range of values was from 25 γ to more than 2,000 γ, this variation was not important.

Several precautions should be stressed. Unless the extraction rate is

very slow at the beginning, a stable sludge or emulsion will occur in the ether of the extraction tube, finally increasing in amount to such a point that it will carry over into the receiver tube. If this happens, a complete re-extraction must be begun. To overcome this emulsification effect it has been found feasible to conduct the extraction for the first 12 hours at a rate of not more than 40 drops per minute. If an emulsion has formed but has not gone through the side arm, the extraction tube may be disengaged and the thimble removed, after which the extraction tube is placed in the warm oil until the emulsion has settled. After slight cooling of the tube, the thimble is replaced, more ether is added, and the extraction process is continued. Other factors which have been observed to aid in forming this emulsion are the presence of green cheese and the extraction of greater amounts of cheese than that recommended here.

The final acid solution in the receiver tube usually is clear. If the solution contains protein particles carried over by the emulsion, usually easily visible, the results will be in error and cannot be used. Also, the ether must be completely boiled off; failure to do this will increase turbidity and will provide erroneous results. A blank should be run on reagents.

SUMMARY

A method for separating tyramine from tyrosine and for estimating the concentration of the former substance was applied to cheese. This method involved the use of a continuous ether extractor and the employment of the Millon reagent using a colorimeter.

Twenty-five samples of commercial American Cheddar cheese of different age and history were tested for tyramine. All were found to contain tyramine in varying degrees of concentration. The average for these samples was 384 γ per g. or 0.0384 per cent; the highest concentration was 1,199 γ per g. or 0.1199 per cent, and the lowest was 25 γ per g., or 0.0025 per cent.

A large number of miscellaneous varieties of ripened types of commercial cheese were tested for tyramine. Again, all samples were found to contain tyramine in varying concentrations. The largest amount was found in a Liederkrantz cheese which had a concentration of 1,683 γ per g., or 0.1683 per cent, while the smallest amount was found in a Roquefort cheese with a concentration of 48 γ per g. or 0.0048 per cent. The analyses were not sufficiently extensive to establish differences due to cheese variety.

Tyramine was isolated from a sample of Cheddar cheese by this method, purified, and identified as the dibenzoyl derivative.

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THE RELATIONSHIP OF THE AMOUNT OF TYRAMINE AND THE NUMBERS OF *STREPTOCOCCUS FAECALIS* TO THE INTENSITY OF FLAVOR IN AMERICAN CHEDDAR CHEESE[1]

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The three previous papers in this series (3, 7, 8) have shown that the flavor of experimental American Cheddar cheese was intensified by *Streptococcus faecalis* starter, that this bacterium grew and survived in large numbers in cheese, and that tyramine, a product of growth of *S. faecalis* in the presence of tyrosine, was found in all varieties of cheese that were analyzed. Problems naturally arose concerning the effect of the presence of *S. faecalis* on the production of tyramine in cheese and any relationship that might exist between the tyramine content of commercial American Cheddar cheese and the intensity of the Cheddar flavor.

EXPERIMENTAL PROCEDURE

When the cheese from earlier experiments (3) was 5 months old, it was analyzed for tyramine. The flavor intensity of this cheese had been recorded as scored, but the scoring occurred on various days and the samples were not compared together as a lot at any time. Hence, some variation in flavor ratings must be expected due to the personal factor. The obvious relationship of tyramine to flavor intensity prompted further study.

Samples of good commercial Cheddar cheese were selected by several cheese companies to give flavor of varying intensity. These samples came from Wisconsin, Illinois and Missouri. A few samples were selected by one of the authors at factories in New York State. One sample of cheese made at Cornell University from raw milk was used; this sample was 10 years old. After all of the samples were on hand, they were arranged in order of intensity of flavor by three experienced judges. In several instances, the character of the flavor was such that it was difficult to place the cheese exactly. For example, cheese S25 possessed some Swiss cheese flavor which interfered with judgment of the intensity of Cheddar flavor.

Data were obtained on the age of the cheese, and curing temperatures usually were available. The manufacturer also stated whether the cheese had been made from raw or pasteurized milk. The cheese was tested for phosphatase by the method of Sanders and Sager (9), soluble and total

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protein (2, 10), volatile acids (6), pH, moisture, salt, titratable acidity, total bacterial count by the standard plate method (1), total enterococcus count (13), and *S. faecalis* identified by characteristics given by Sherman (11). The titratable acidity was determined by weighing out 3 g. of cheese, macerating with 10 ml. warm water in a white dish, titrating with 0.1 N alkali with phenolphthalein as indicator, and calculating the results to percentage of lactic acid.

RESULTS

The experimental cheese made with lactic starter and aged 5 months showed the lowest tyramine content, averaging 38 γ per g., and the mildest Cheddar flavor (table 1). Cheese made with both commercial lactic and *S. faecalis* starters developed the highest tyramine content, 786 γ per g.,

TABLE 1

The tyramine content and intensity of flavor of experimental American Cheddar cheese made with commercial lactic and S. faecalis starters, ripened about 5 months

Cheese no.	Curing temperature	Tyramine	Flavor intensity
	(° *F*)	(γ/*g*.)	
	Commercial lactic starter		
10464	50	21	medium—
10464	60	87	medium +
10468	50	4	mild
10468	60	40	medium
	Commercial lactic and *S. faecalis* starters		
10464	50	367	medium +
10464	60	1397	sharp—
10468	50	333	medium
10468	60	1049	medium +
	S. faecalis starter		
10464	50	170	medium
10464	60	830	sharp—
10468	50	122	medium—
10468	60	302	medium +

and the most pronounced Cheddar flavor. The cheese with *S. faecalis* starter alone contained 356 γ of tyramine per g. Very little tyramine was produced in cheese made from pasteurized market milk with ordinary lactic starter, whereas *S. faecalis* starter produced large amounts of tyramine. It may be observed also that there is an associated action between the two starters, which results in the production of more tyramine and slightly more flavor than obtained by *S. faecalis* alone. The acceleration of tyramine production at 60° F. as compared with 50° F. may be noted, and will be considered further in a subsequent paper.

Some variation in the exact relationship was noted in individual samples, but the intensity of the flavor of Cheddar cheese increased as the tyramine content increased (fig. 1). The trend line, drawn empirically, not only shows the trend but also, with one exception, divides the figure so that all samples of cheese above the line were cured at 50° F. and those below the line at 60° F. This observation means that the rate of flavor

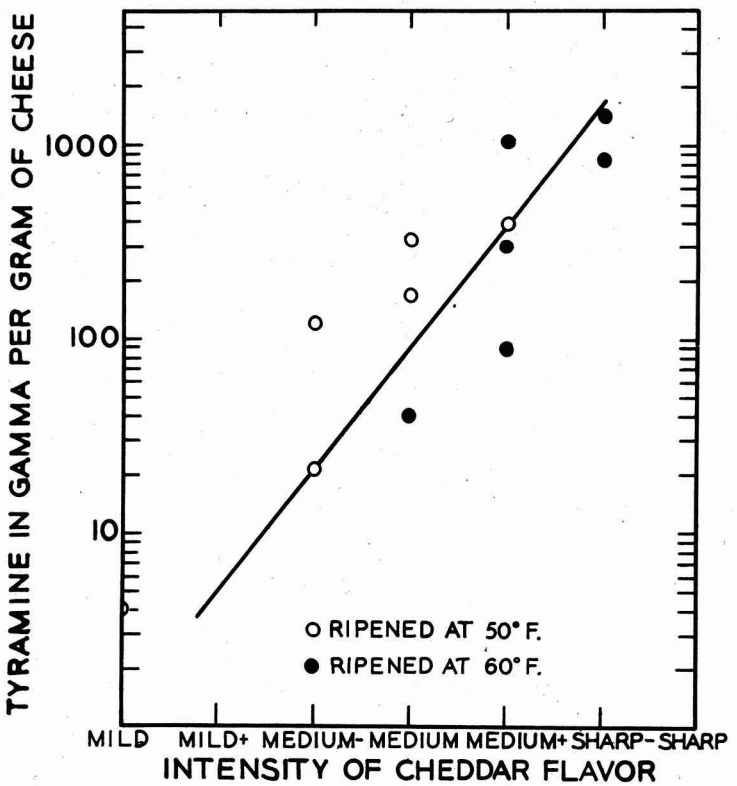

FIG. 1. The relationship between tyramine content and intensity of flavor of experimental Cheddar cheese made with commercial lactic and *S. faecalis* starters. (Ripened about 5 months.)

development at 60° F. was relatively more rapid than the increase in tyramine when compared with the rate of increases at 50° F.

The data on the 24 samples of commercial Cheddar cheese and one sample of old cheese made at Cornell University are presented in tables 2 and 3. The cheeses were grouped into units of five on the basis of flavor intensities; thus numbers 1 to 5 included the five sharpest flavored cheese in the experiment (table 4). There were fifteen raw milk cheese, six made from pasteurized milk and four were underpasteurized (table 2).

TABLE 2

The age, acidity, composition and flavor intensity of commercial American Cheddar cheese made from raw and pasteurized milk

Cheese no.	Age in months	Pasteurization		pH	Titratable acidity as % lactic	Water	Salt	Order of flavor intensity
		Mfg. report	Phosphatase test[a]					
			(*units/0.25 g.*)			(%)	(%)	
C 22	120	raw	40	5.70	4.87	37.5	2.0	1
C 21	36	raw	40	5.42	3.52	31.3	1.7	2
A 4	30	raw	40	5.49	3.36	29.8	1.7	3
M 20	24	raw	30	5.61	2.86	35.0	2.0	4
K 13	10.5	raw	40	5.35	2.87	34.5	1.4	5
K 12	9.5	raw	40	5.35	2.80	34.2	1.4	6
S 25	33	raw	30	5.35	3.42	37.5	2.0	7
St 15	8.5	raw	35	5.23	2.95	34.7	1.5	8
A 5	10.5	raw	40	5.13	2.82	34.1	2.0	9
K 3	11.5	past.	1	5.22	2.89	37.0	1.6	10
St 14	3	raw	40	5.32	2.71	35.9	1.6	11
K 11	8	raw	40	5.46	2.21	36.6	1.2	12
LM 9	12.5	past.	9	5.22	2.57	33.4	1.9	13
K 2	4.5	past.	2	5.15	2.68	36.1	1.5	14
M 18	2.5	raw	40	5.04	2.31	38.1	1.4	15
M 17	2.5	past.	3	5.15	2.26	37.3	1.5	16
CM 24	2.5	?	6	5.14	2.03	37.2	1.3	17
CM 23	2.5	?	5	5.10	2.18	37.5	2.0	18
LM 8	12.5	past.	2	5.27	2.41	34.3	1.8	19
K 19	2.5	raw	40	5.06	2.42	39.0	1.5	20
LM 7	14.5	past.	3	5.60	2.09	32.4	1.9	21
K 1	2.5	past.	3	5.03	2.16	37.0	1.5	22
K 10	1.5	raw	40	5.26	1.92	35.7	1.5	23
M 16	1.5	raw	40	5.15	1.91	39.0	1.6	24
A 6	6	past.	5	5.32	1.71	36.0	1.4	25

[a] Values of 5 or greater indicate raw milk or contamination with raw milk.

TABLE 3

The flavor intensity, volatile acidity, soluble protein, and tyramine content and Streptococcus faecalis in commercial American Cheddar cheese made from raw and pasteurized milk

Flavor		Volatile acids	Protein		Tyramine	Bacterial counts (in thousands)		
Order of intensity	Intensity grade		Soluble	Soluble % of total		Standard plate count	Enterococci	*S. faecalis*
		(*ml. 0.1 N/100 g.*)	(%)		(γ/*g.*)	(*per g.*)	(*per g.*)	(*per g.*)
1	sharp + + + +	173.3	12.6	57.3	2330	3,000	<100	<10
2	sharp + +	48.8	11.7	45.2	966	56,000	22,000	1,000
3	sharp	34.1	10.6	40.8	1199	17,000	4,000	80
4	sharp	24.8	11.7	42.9	1147	30,000	18,000	17,000
5	sharp —	37.8	8.3	31.9	847	380,000	80,000	56,000
6	sharp —	37.4	8.0	31.4	814	200,000	91,000	82,000
7	sharp —	39.5	12.0	44.0	460	3,000	900	300
8	medium +	41.3	7.7	32.1	746	129,000	28,000	3,000
9	medium +	17.5	7.4	38.5	406	125,000	39,000	8,000
10	medium +	39.3	7.5	29.7	566	285,000	53,000	3,000
11	medium	37.1	5.1	20.3	377	535,000	265,000	159,000
12	medium	34.1	6.6	25.1	233	623,000	128,000	58,000
13	medium	20.9	7.2	30.0	112	7,000	<100	<10
14	medium	40.9	6.4	25.2	177	20,000	13,000	1,000
15	medium —	35.2	4.7	20.8	230	106,000	98,000	10,000
16	mild +	32.2	5.1	20.8	147	55,000	29,000	<1,000
17	mild +	22.6	4.3	19.6	123	86,000	27,000	5,000
18	mild +	18.7	4.5	19.9	134	145,000	52,000	3,000
19	mild	11.9	7.1	29.5	55	10,000	<100	<100
20	mild	26.7	3.6	24.6	119	450,000	73,000	18,000
21	mild	15.5	7.7	32.1	58	40,000	<100	<10
22	mild	19.9	3.9	16.1	30	6,000	3,000	<100
23	mild —	24.9	3.1	18.5	59	50,000	21,000	19,000
24	mild —	17.6	3.0	13.3	99	205,000	90,000	31,000
25	mild —	16.2	4.5	18.8	25	1,000	500	<20

Although cheese must be aged to develop flavor, the relationship between age and intensity of flavor was not very exact. The cheese that was 120 months old was highest in flavor, but a 6-month-old cheese was the mildest in flavor, being less cured than some cheese only 1.5 months old (table 2). The summarized data show that the ten strongest flavored cheese were oldest, but the next 15 cheese were rather uniform in age (table 4).

It is known that the pH of cheese increases with age. Apparently variations in individual cheese exceeded the effect of aging upon pH or any possible relationship of intensity of flavor to pH (tables 2 and 4). The average pH was near 5.2, the highest flavored group averaged 5.5, and the pH of all samples varied from 5.03 to 5.70. The titratable acidity varied from 1.71 for the mildest flavored cheese to 4.87 for that with the strongest flavor.

TABLE 4

The age, acidity, soluble protein, and tyramine content of commercial American Cheddar cheese grouped in units of 5 on the basis of flavor intensity

Order of flavor intensity	Age in months	pH	Titratable acidity	Volatile acids	% soluble protein	% of total protein that is soluble	Tyramine
				(*ml. 0.1 N acid/100 g.*)			(γ/*g.*)
1– 5	44.1	5.51	3.49	63.7	11.0	43.6	1258
6–10	14.6	5.25	2.97	35.0	8.5	33.1	598
11–15	6.1	5.24	2.49	33.6	6.0	24.3	226
16–20	4.6	5.14	2.26	22.4	4.9	22.9	115
21–25	5.2	5.27	1.96	18.8	4.4	19.8	54

The higher the titratable acidity of cheese, the more intense the cheese flavor (table 4); even the results on individual samples of cheese were noticeably consistent. There was no striking exception to this general relationship (table 2).

No significance was attached to the moisture contents, which varied from 29.8 to 39.0 per cent, and to the salt contents, which varied from 1.2 to 2.0 per cent.

Although previous studies by the authors (3) had established no definite relationship between the intensity of flavor of Cheddar cheese and volatile acidity or water soluble protein, this work was repeated in this study. In a general way, in average grouped data, the volatile acidity and soluble protein values increase with increased flavor (table 4), but this relationship does not hold for individual samples (table 3). For example, the cheese wth the fourth sharpest flavor and the cheese that was twenty-third in flavor intensity both had volatile acids per 100 g. equivalent to 24.9 ml. 0.1 *N* alkali. The volatile acidity ranged from 16.2 to 173.3. The soluble

protein varied from 3.0 to 12.6 per cent, and the percentage of the total protein that was water soluble varied from 13.3 to 57.3 per cent, with noticeable exceptions to a definite correlation to flavor intensity in individual samples.

The data show a definite relationship of increased tyramine content and increased flavor. In the individual samples of cheese, the strongest flavored Cheddar cheese contained 2,330 γ of tyramine per g., and the twenty-fifth cheese in flavor intensity, the mildest of all flavors, had only

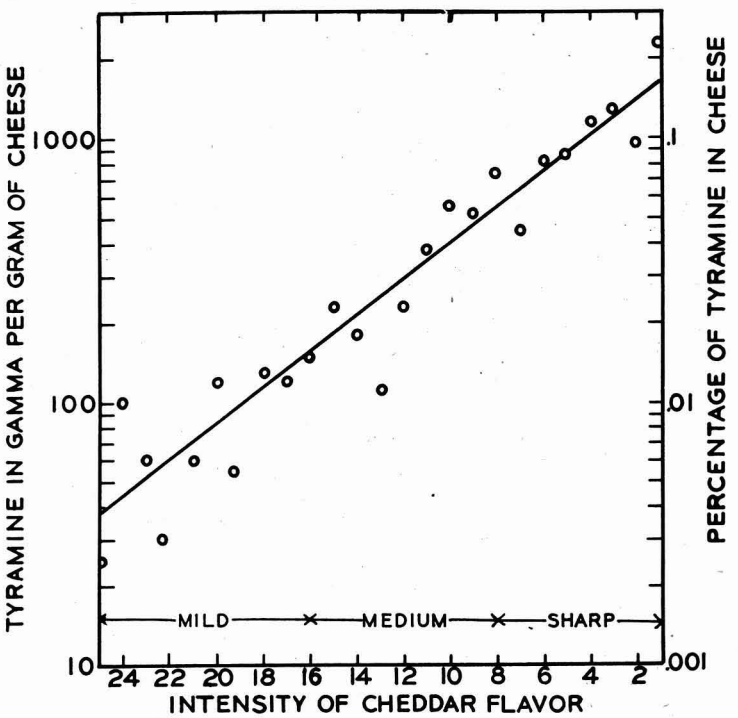

FIG. 2. The relationship between tyramine content and intensity of flavor of commercial American Cheddar cheese.

25 γ of tyramine (table 3). The tyramine ratio of these cheeses was 93 to 1. A close relationship between the tyramine content and intensity of flavor of individual samples of cheese is shown in figure 2. When one considers that there was no control over the quality of milk, method of manufacture, starter, time and temperature of ripening, and other factors of possible significance, the relationship is exceptionally good. The relationship between the tyramine content and flavor intensity was a direct semilogarithmic one. On the basis of groups of cheese as units of five in order of flavor intensity, the gamma of tyramine per gram was doubled for each group

(table 4). Tyramine added to fresh cheese curd did not give Cheddar cheese flavor and it did not aid the development of flavor during ripening.

The total bacterial counts on the cheese were made with standard tryptone-glucose-extract-milk agar, but the plates were incubated at 32° C. for 4 days to obtain maximum counts. The total bacterial counts per gram of cheese ranged from 1 million to 623 million. Counts also were made with the penicillin-azide medium of White and Sherman (13) and these counts, considered to be chiefly enterococci, ranged from less than 100,000 to 265 million. From each plate count for enterococci, 20 colonies were isolated and the number of *S. faecalis* determined, except for the possibility of confusion with *Streptococcus zymogenes,* which is not common in milk. The *S. faecalis* count was calculated from the proportion of the colonies which proved to be *S. faecalis.* The *S. faecalis* count varied from less than 20,000 to 159 million per g., and *S. faecalis* actually was isolated from 18 of the 25 samples of cheese. There appeared to be no correlation between bacterial counts and flavor intensity, and this observation was expected and in accordance with several previous publications.

DISCUSSION

It has been shown that there is a direct relationship between the tyramine content of American Cheddar cheese and the intensity of its flavor. The correlation was better for good commercial cheese selected at random than for experimental cheese made from good pasteurized market milk with special cultures and cured at different temperatures. Tyramine added to cheese curd did not give the Cheddar flavor, so tyramine is not the flavor compound. The amount of tyramine indicated the extent of activity of *S. faecalis* and probably of other bacteria, such as certain strains of the lactobacilli, which possibly may produce tyramine (4, 5). The numbers of these bacteria may increase in the early stages of cheese ripening and then decrease, so that these numbers at any one time may not be too significant.

The growth of *S. faecalis* in cheese produces Cheddar flavor, but this is only one source of flavor. That other factors are involved is indicated by the development of some Cheddar flavor in cheese with amounts of tyramine too small to indicate much growth of *S. faecalis,* and by the more rapid production of flavor than of tyramine at 60° F. when compared to 50° F. Flavor with low tyramine content always was flat, irrespective of age of the cheese.

S. faecalis and lactobacilli should be present in all raw milk Cheddar cheese, as these bacteria occur universally in raw milk. Both types of bacteria are involved in cheese ripening. *S. faecalis* is thermoduric, although survival numbers are not great, and about half of the lactobacilli

are not destroyed by pasteurization (12). Hence, pasteurized milk cheese cures slowly, as does cheese made from very low-count raw milk. The survival of *S. faecalis* and lactobacilli during pasteurization must be an important factor in present day curing of pasteurized milk cheese. The small numbers of these bacteria are increased by warmer curing temperatures.

It was reported in the first paper in this series (3) that cheese made with lactic and *S. faecalis* starters developed more flavor than cheese made with either starter alone, and that the flavor with lactic starter only was especially mild. This observation was related directly to the production of tyramine. Lactic and *S. faecalis* starters together produced more tyramine in cheese than did *S. faecalis* starter alone, even though the numbers of *S. faecalis* bacteria were greater in the cheese in which this culture alone was used, probably due to the larger inoculation in the milk. The lactic starter induced higher flavor development in cheese by its symbiotic action with *S. faecalis*.

SUMMARY

Experimental American Cheddar cheese made with commercial lactic starter from pasteurized milk developed low amounts of tyramine, 4 to 87 γ per g., and flavor of mild to medium intensity in 5 months of curing. The combination of lactic and *Streptococcus faecalis* starters in cheese produced the largest amounts of tyramine, 333 to 1,397 γ per gram, and flavor of medium to sharp intensity. *S. faecalis* starter alone in cheese produced tyramine and flavor between these two extremes.

In commercial American Cheddar cheese made from raw and pasteurized milk, cured for varying periods, there was a direct semilogarithmic relationship between tyramine content (25 to 2,330 γ per g.) and the intensity of flavor. Of the 25 cheese samples, 18 gave plate counts on the special medium of over 80,000 *S. faecalis* per g., with the high count of 159 million. The *S. faecalis* bacteria produced the tyramine, although other bacteria may contribute.

Tyramine was not the Cheddar-flavor compound, but served as a means of measuring bacterial activity that accentuated flavor production. The activity of bacteria producing tyramine did not account for all cheese flavor.

The increase in titratable acidity was related directly to cheese flavor intensity, and this relationship, even though subject to considerable variation, was too close in individual samples to be accidental. In a general way, the increase in volatile fatty acids and water soluble nitrogen was related to flavor intensity, but variations in individual samples prevented a definite correlation and also established that these changes were incidental to flavor development.

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DETERMINATION OF VITAMIN A IN MILK[1]

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Milk is an important natural source of dietary vitamin A. Recent interest in the enrichment of milk has emphasized the need for a simple and reliable method of assay for control purposes. Attempts to determine preformed vitamin A in milk in this laboratory by several methods found in the literature have been unsuccessful. These procedures, although reliable for other types of products, gave low recoveries (approximately 80 per cent of the theoretical) of the added vitamin. In these tests the milk was enriched with a vitamin A emulsion similar in composition to milk itself.[2] The low values may be attributed to a combination of causes, including destruction of the vitamin during hot saponification, inadequate cold saponification, loss due to adsorption on the milk protein, inefficient extraction, or failure to take into account the effect of inhibitors in the antimony trichloride reaction.

In the present study, vitamin A in milk is determined quantitatively by a method which involves cold saponification with potassium hydroxide, extraction of the vitamin with diethyl ether, evaporation of the solvent, and solution of the residue in chloroform.[3] The vitamin then is allowed to react with antimony trichloride and the resulting blue color measured in an Evelyn photoelectric colorimeter. The effect of compounds which inhibit the color formation is evaluated by means of an internal standard.

The importance of adequate, cold saponification in the analysis of milk for vitamin A has been recognized by other investigators (1). The use of the internal standard has been described (5) and has received considerable attention in the development of analytical procedures for the determination of vitamin A in enriched margarine.

METHOD

Reagents

Aldehyde-free alcohol. Reflux 1 l. of 95 per cent ethyl alcohol with 10 g. of potassium hydroxide on a steam bath for 5 hours and then cool. Add 0.5 g. granulated aluminum and distill on a steam bath. Prepare fresh each month.

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[1] Contribution from the Vitex Department, Vitamin Division, Nopco Chemical Company, Harrison, New Jersey.

[2] The concentrate used for enrichment consisted of a butter oil solution of vitamin A, homogenized with water and skim milk solids.

[3] The saponification, extraction, evaporation, and washing steps are essentially those employed in the laboratory of the Wisconsin Alumni Research Foundation. The details of the method were kindly supplied by Dr. Carl H. Krieger.

Sixty per cent potassium hydroxide solution. Dissolve 60 g. of potassium hydroxide in 40 ml. of distilled water.

Antimony trichloride reagent. Grind 250 g. of antimony trichloride with 250 g. of anhydrous sodium sulfate. Suspend the mixture in 1,000 ml. of freshly redistilled chloroform, stir mechanically for 15 minutes, filter through rapid paper, and store in the dark in a glass-stoppered amber bottle. The reagent is stable for at least 1 month. If turbid, filter again immediately before use.

Saturated sodium chloride solution. Suspend 800 g. of sodium chloride in 2,000 ml. of water, stir mechanically for 15 minutes and allow to stand overnight to assure saturation.

Ethyl ether. Distill U.S.P. ethyl ether freshly each day.

Chloroform. Distill C.P. grade chloroform freshly each day discarding the first 10 per cent of the distillate.[4]

Vitamin A standard. Dissolve 100 mg. of U.S.P. vitamin A Reference Standard (10,000 U.S.P. units per g.) in 100 ml. of freshly redistilled chloroform. This solution contains 10 U.S.P. units of vitamin A per ml. and is prepared fresh each day.

Acetic anhydride. Use C.P. grade.

Special Apparatus

Separatory funnels. Three 500-ml. pear-shaped separatory funnels are required.

Evelyn photoelectric colorimeter (macro) with matched colorimeter tubes. This instrument is manufactured by the Rubicon Company, Philadelphia, Pennsylvania.

Rapid delivery pipette. This may be prepared by cutting the tip from a 10-ml. volumetric pipette, leaving an opening about 2 mm. in diameter. The pipette need not be recalibrated.

Procedure

To 100 ml. of milk in an amber Erlenmeyer flask are added 50 ml. of aldehyde-free alcohol and 10 ml. of 60 per cent potassium hydroxide. The suspension is mixed and allowed to stand overnight in a dark cabinet. In the morning the sample is transferred to a 500-ml. amber separatory funnel. The flask is washed consecutively with 40-, 40-, and 20-ml. portions of distilled water and once with 100 ml. of redistilled ether. The washings are added to the separatory funnel. The latter is shaken thoroughly and then allowed to stand until a sharp separation of the phases is

[4] Occasionally samples of chloroform are obtained which are unsatisfactory as solvents for vitamin A. The vitamin standard solution in chloroform should show less than 5% decomposition when stored for 24 hours in the dark.

observed. The lower layer is drawn off into a second separatory funnel. This is shaken with 75 ml. of ether. After the phases have separated, the lower (aqueous) layer is drawn off into a third separatory funnel. The ether layer is added to the extract in the first funnel, along with a 25-ml. ether wash. The aqueous phase is re-extracted twice more with 75- and 50-ml. portions of ether, respectively. The ether phases all are combined in the first separatory funnel and are washed four times with 100-ml. portions of distilled water. To prevent the formation of emulsions, the first two washings merely are poured through the ether, without shaking, and then drawn off. The funnel is shaken gently during the third washing and vigorously during the fourth. Sufficient time for complete separation of the phases must be allowed at all times. Finally, the ether extract is washed twice with 75-ml. portions of saturated sodium chloride solution, shaking vigorously each time. The last salt solution is separated sharply from the ether phase.

The ether extract is transferred to a 500-ml. amber distilling flask containing two or three glass beads. The separatory funnel is rinsed with 25 ml. of redistilled ether and the wash added to the flask. The ether is removed by distillation on a steam bath until approximately 5 ml. remain in the flask. The remainder is evaporated off at room temperature with the aid of a stream of carbon dioxide. While still under the inert atmosphere, the vitamin A in the residue is dissolved immediately in sufficient chloroform to produce a concentration of approximately 10 U.S.P. units per ml.

Into a series of labeled Evelyn colorimeter tubes in a wooden rack are pipetted 2 ml. of chloroform solvent (tube *A*), 1 ml. of chloroform extract of sample + 1 ml. of chloroform solvent (tube *B*), and 1 ml. of chloroform extract of sample + 1 ml. of chloroform solution of vitamin A standard (tube *C*).

To tube *A*, one drop of acetic anhydride is added, followed by 10 ml. of antimony trichloride reagent, the latter from the rapid delivery pipette.[5] The colorimeter, containing a 620 mμ filter, then is set at 100 per cent transmission with the solution in tube *A*. The tube is removed and the "center setting" noted. The latter is employed to reset the instrument before each subsequent reading.

Tube *B* is placed in the instrument, one drop of acetic anhydride is added, and 10 ml. of antimony trichloride reagent are added rapidly. The galvanometer needle first fluctuates rapidly, "pauses" for a second or two, then drifts slowly as the blue color fades. The per cent transmission at the pause point is recorded. Tube *C* is measured similarly.

[5] Because antimony trichloride solution is extremely sensitive to moisture and is corrosive, it must be pipetted with a rubber bulb, not by mouth.

Because of difficulty in observing the pause point and in order to obtain reliable results, tubes B and C are set up and measured in triplicate, and the average per cent transmission of each determined. The latter (G) is converted to photometric density ($L = 2 - \log G$), employing the chart provided with the instrument. The vitamin A content of the milk sample is calculated employing the formula:

$$\frac{L_B}{L_C - L_B} \times 10 \times \frac{V}{100} \times 946 = \text{U.S.P. units of vitamin per quart.}$$

(V = cc. of chloroform solution of unsaponifiable extract)

EXPERIMENTAL RESULTS AND DISCUSSION

The colorimetric method as described by Oser *et al.* (5) has been employed successfully in these and other laboratories for the determination

TABLE 1

Recovery tests of vitamin A added to milk, employing previously published procedures (1, 5)[a]

Procedure	Milk sample	Vitamin A added	Total vitamin A found	Vitamin A recovered	
		(*USP units per qt.*)			(%)
Boyer *et al.* (1)	Jan. milk, homogenized	0	960	.....	.....
		2000	2560	1600	80
		4000	3970	3010	75
		6000	6190	5230	87
	Oct. milk, cream-line	0	1020	.....	.....
		4000	4270	3250	81
Oser *et al.* (5)	Oct. milk, homogenized	0	1510	.....	.....
		4000	4800	3290	83
	Oct. milk, homogenized	0	1420	.....	.....
		4000	4700	3280	82
	Oct. milk, cream-line	0	1320	.....	.....
		4000	4580	3260	82
	Oct. milk, homogenized	0	1200	.....	.....
		4000	4400	3200	80

[a] For each recovery test, the vitamin was added directly to the milk immediately before the analysis and the enriched sample was carried through the entire determination. The concentrate employed for enrichment consisted of a solution of vitamin A esters in butter oil, homogenized with water and skim milk solids.

of vitamin A in a wide variety of food products. Boyer *et al.* (1) have applied the antimony trichloride reaction to the analysis of milk. The recent interest in the enrichment of milk with vitamin A prompted an examination of the reliability of these procedures. The results of these tests are presented in table 1. Milk samples were enriched with vitamin A at the levels indicated, employing a concentrate similar in composition to milk itself. This concentrate consisted of a solution of vitamin A

esters in butter oil, homogenized with water and skim milk solids, and contained 30,000 U.S.P. units per ml. The blank and enriched samples were analyzed immediately according to the published procedures. Approximately 80 per cent of the added vitamin was recovered in each case. Because of the physical nature of milk, the complete extraction of its fat-soluble constituents is particularly difficult. Therefore, it is not surprising that the determination of vitamin A in milk requires a special technique.

Similar recovery tests, employing the procedure described in the present

TABLE 2

Recovery tests of vitamin A added to milk employing present procedure[a]

Milk sample	Vitamin A added	Total vitamin A found	Vitamin A recovered	
	(USP units per qt.)			(%)
	0	1310	.....	.....
A	2000	3270	1960	98
Oct. milk,	4000	5500	4190	105
homogenized	6000	6970	5660	94
B				
Oct. milk,	0	1200	.....	.....
cream-line	4000	5250	4050	101
C				
Oct. milk,	0	1070	.....	.....
cream-line	4000	4970	3900	98
D				
Jan. milk,	0	830	.....	.....
homogenized	4000	4830	4000	100
E				
Jan. milk,	0	920	.....	.....
cream-line	4000	4950	4030	101

[a] For each recovery test, the vitamin was added directly to the milk immediately before the analysis and the enriched sample was carried through the entire determination. The concentrate employed for enrichment consisted of a solution of vitamin A esters in butter oil, homogenized with water and skim milk solids.

paper, are reported in table 2. Recovery values ranged from 94 to 105 per cent, with an average of 99.6 per cent.

The use of an internal standard in the determination of vitamin A decreases the precision of the analysis. Therefore, some investigators have employed a reference curve, obtained with pure solutions, to calculate their results or, since the reaction obeys Beer's law, an external standard of 10 U.S.P. units. The data employed in calculating the results reported in table 2 demonstrate the importance of the internal standard or increment. These data are presented in table 3. In a given test extract, the photometric density has been found to be proportional to the vitamin A content. However, biological materials contain substances which inhibit

the reaction, and the constant of proportionality is not the same in all extracts. Thus in sample A, the increments due to 10 units of added vitamin A were 0.184, 0.174, 0.173 and 0.180, respectively, in the four solutions tested. Employing the same reagents at the same time, 10 units of vitamin A in pure chloroform gave a photometric density of 0.201. Thus, the use of a reference curve in calculating the vitamin A content of the above samples would give values erroneously low by approximately 13 per cent. Samples *B* and *C* likewise required the internal standard. On the other hand, either method of calculation could be employed with *D* and *E*. The

TABLE 3

Importance of the internal standard

Milk sample	Vitamin A added	Photometric density			
		Tube B	Tube C	Internal standard[a]	External standard[b]
	(*USP units per qt.*)				
	0	0.102	0.286	0.184	0.201
A	2000	0.240	0.414	0.174	0.201
	4000	0.201	0.374	0.173	0.201
	6000	0.177	0.357	0.180	0.201
B	0	0.047	0.233	0.186	0.199
	4000	0.184	0.362	0.178	0.199
C	0	0.043	0.233	0.190	0.204
	4000	0.182	0.364	0.182	0.204
D	0	0.036	0.240	0.204	0.204
	4000	0.189	0.387	0.198	0.204
E	0	0.039	0.238	0.199	0.204
	4000	0.184	0.387	0.203	0.204

[a] Photometric density due to the reaction of antimony trichloride with 10 USP units of vitamin A added to the chloroform solution of the unsaponifiable extract of the milk sample.

[b] Photometric density due to the reaction of antimony trichloride with 10 USP units of vitamin A in chloroform.

importance of the internal standard also has been established in the determination of niacin (4) and pyridoxine (3).

If the total vitamin A content of unenriched milk is of interest, β-carotene should be determined in the extract by carefully evaporating off the ethyl ether, taking up the residue in petroleum ether, and fractionating the pigments with diacetone alcohol (2). As much as one-fourth of the total vitamin A potency of unenriched milk is due to carotene, although in the case of summer milk from Guernsey cows, almost 50 per cent may be present as the provitamin (1). In milk enriched with preformed vitamin A, these proportions are considerably smaller.

Though carotene reacts with antimony trichloride to form a blue pigment, this does not interfere appreciably in the determination of the preformed vitamin because of differences in the rates and sensitivities of the two reactions. The photometric density at 620 mμ due to one U.S.P. unit of β-carotene is only one-twelfth that due to one U.S.P. unit of preformed vitamin A 4 seconds after the addition of antimony trichloride reagent (5).

SUMMARY

1. Vitamin A in milk was determined quantitatively by cold saponification with potassium hydroxide, extraction wth diethyl ether, evaporation of the solvent, and solution of the residue in chloroform. The blue color formed by reaction with antimony trichloride was measured in a photoelectric colorimeter.

2. Theoretical recoveries of added vitamin A were obtained. The concentrate employed was a solution of vitamin A in butter oil, homogenized with water and skim milk solids. Two other procedures for the determination of vitamin A gave recoveries of only 80 per cent.

3. Colorimetric evaluation of the vitamin A content of the final extract included the use of an internal standard. Calculations based upon a reference curve obtained in pure solutions gave low recovery values in some samples.

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