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THE EFFECT OF AGITATION UPON THE LIVABILITY OF BOVINE SPERMATOZOA[1]

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In the routine operation of most artificial breeding associations, diluted semen samples are transported from the central bull stud to the outlying inseminators, and often considerable distances are involved. Various means of transportation are employed and it is obvious that the semen samples are subjected to different amounts of agitation enroute.

Bretschneider (2) reported that the vigorous shaking of bull semen for 3 minutes destroyed motility. Spermatozoa from the normal ejaculate showed more resistance to shaking than did those secured from the testicle or epididymis. Smirnov-Ugrjumov (14) observed that the transportation of undiluted bull semen in thermos flasks at 15–20° C. for distances of 0.6 to 5.9 miles brought about a reduction in spermatozoan activity. Hronopulo (6) reported that transportation of undiluted bull semen did not affect the fertility of the semen during the first 4 hours of storage. After a storage period of 4 hours, however, the fertility of semen transported distances greater than 21.7 miles was markedly reduced. Ayyar (1) noted that hand shaking during transport killed bull spermatozoa.

Several workers have noted effects of agitation in connection with studies of semen physiology. Gunn (4) reported that periodic shaking of ram semen contained in rubber-stoppered test tubes was effective in providing the aeration which he considered necessary for maintenance of spermatozoan motility. Motility was maintained even when shaking was vigorous. On the other hand, Nagornyi and Smirnov (11) found that the resistance of ram spermatozoa to sodium chloride was decreased by continuous agitation. During the course of metabolism studies with ram and bull semen, Mann (10) observed that when a suspension of spermatozoa was shaken vigorously in the presence of air, the cytochrome enzyme within the cell was oxidized rapidly.

In the process of examining samples of diluted semen shipped to this laboratory from the several artificial breeding cooperatives in Pennsylvania, it was

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แผนกห้องสมุด กรมวิทยาศาสตร์
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noted that the motility of the spermatozoa usually was lower in tubes of diluted semen shipped partially filled than in completely filled tubes. Poor livability often was evident, particularly in samples shipped in 14-ml. capacity test tubes which contained only 3-4 ml. of diluted semen. The decrease in semen quality observed may have been due in part to harmful effects of agitation in transit. Thus, the present study was proposed to determine the effect of mechanical agitation of diluted bull semen upon the livability of the spermatozoa.

EXPERIMENTAL PROCEDURE

Fifteen semen samples were obtained by means of an artificial vagina from three fertile bulls of the College dairy herd representing the Ayrshire, Brown Swiss and Holstein breeds. The 15 ejaculates were diluted at a constant rate of one part of fresh semen to 24 parts of egg yolk-citrate diluter. The diluter was composed of one part of fresh egg yolk and one part of citrate buffer prepared by dissolving 3.6 g. of sodium citrate dihydrate in 100 ml. of water distilled over glass.

The effect of agitation upon spermatozoan livability was studied by subjecting diluted semen in three series of test tubes to mechanical agitation for 6, 12 and 24 hours. A fourth series received no mechanical agitation and served as the controls. Each of the series consisted of four test tubes (15×125 mm.), which contained 3.5 ml., 7.0 ml., 10.5 ml. and approximately 14.0 ml. of diluted semen. With these amounts of material the tubes were approximately one-quarter filled, half filled, three-quarters filled and filled to the bottom of the cork stopper. This experimental design made it possible to determine not only the effect of varying amounts of agitation but also the effect of agitation upon the livability of spermatozoa in test tubes containing different volumes of semen.

The test tubes were prepared by placing the desired volume of diluted semen in each sterile tube by means of a sterile pipette. The tubes were stoppered with sterile corks, and melted paraffin was applied to the juncture of cork and glass to complete the closure. The tubes of diluted semen were placed in a water bath at room temperature and gradually cooled to about 7° C. in a mechanical refrigerator. In order to maintain a temperature of from 5 to 10° C. during agitation and at the same time simulate field shipping conditions, each series of tubes to be agitated was packaged in refrigerated cardboard cartons. Methods and materials as described by Perry (12) were used in packaging the samples. Refrigeration was provided by 800 g. of ice contained in rubber balloons. A test tube containing water at 5° C. was packaged next to the tubes of diluted semen. When the cartons were opened, the temperature was determined by inserting a cooled thermometer (5° C.) into the tube of water. The control tubes of diluted semen were not packaged and were stored in a refrigerator at 5° C.

Agitation was provided by placing the shipping cartons on a mechanical agitator which operated at the rate of 76 oscillations per minute through a horizontal distance of 4 inches. The cartons were placed on the agitator so that the longitudinal axis of the test tubes was parallel to the horizontal axis of the

agitator frame. Following the prescribed period of agitation, the cartons were opened and the temperatures of the contents of those cartons subjected to 24 hours of agitation were determined. The samples then were placed in a 5° C. water bath and stored in a refrigerator maintained at that temperature.

In addition to motility estimations made before and after agitation, estimations were made every 2 days during the 20-day storage period. In order to minimize bias on the part of the observer making the motility estimations, randomized numbers were placed on the test tubes prior to agitation.

RESULTS

The 15 semen samples studied had a mean concentration of 1,141,000 spermatozoa per cubic millimeter, a mean initial motility of 63 per cent active spermatozoa and a mean methylene-blue reduction time of 12 minutes. The mean temperature to which the diluted samples were cooled prior to packaging was 7.1° C. and ranged from 5.5 to 8.9° C. The mean temperature of the samples after 24 hours agitation was 6.8° C., with a range of from 5.5 to 8.9° C.

The mean percentages of motile spermatozoa during 20 days of storage are presented in table 1. Each figure represents a mean of 15 ejaculates. Mechanical agitation of the partially filled tubes brought about a significant reduction in spermatozoan livability. The decrease in livability was related directly to the length of the agitation period. In addition, it was noted that the effect of agitation was related to the volume of semen contained in the tubes. Thus, the ability of the spermatozoa to remain motile during storage following agitation for 6, 12 and 24 hours was less in the one-quarter filled tubes than in the half filled tubes and less in the half filled than in the three-quarters filled tubes. When the tubes were completely filled, spermatozoan livability was not affected as

TABLE 1

Effect of mechanical agitation upon the livability of bull spermatozoa

Fullness of test tube	Length of agitation	Per cent motile spermatozoa (15 ejaculates)					
		Before storage	After storage at 5° C. for				
			4 days	8 days	12 days	16 days	20 days
	(*hr.*)						
Filled	0	63	49	43	32	17	5
	6	63	52	42	31	16	7
	12	63	50	41	29	16	7
	24	63	51	42	30	15	5
Three-quarters filled	0	63	51	39	28	17	7
	6	63	47	32	23	9	3
	12	63	43	31	16	6	3
	24	63	41	27	13	6	1
Half filled	0	63	51	42	26	13	7
	6	63	41	29	19	6	1
	12	63	38	27	13	4	0
	24	63	36	19	11	4	1
One-quarter filled	0	63	49	26	13	5	3
	6	63	39	21	9	3	0
	12	63	31	21	11	3	1
	24	63	27	10	5	2	1

markedly by agitation. Differences in livability of the spermatozoa also were obtained in the controls which received no mechanical agitation. Spermatozoa in the completely filled tubes maintained a significantly higher level of motility during storage than spermatozoa in one-quarter filled tubes.

Analysis of variance (table 2) involving 2,400 motility observations showed highly significant differences ($P = < 0.01$) between the two treatments, length of agitation (L) and fullness of tube (F), as well as between ejaculates and storage intervals. The interaction of the two treatments ($L \times F$) also was found to be highly significant, as were all other first and second order interactions.

TABLE 2

Analysis of variance of per cent motile spermatozoa during 20 days of storage following agitation for 0, 6, 12 and 24 hours

Source of variation	Degrees of freedom	Sum of squares	Mean square
Total	2,399	9,724	
Length of agitation (L)	3	304	101.33[a]
Fullness of tube (F)	3	708	236.00[a]
Storage intervals (S)	9	5,371	596.78[a]
Ejaculates (E)	14	1,588	113.43[a]
Interactions:			
$L \times F$	9	63	7.00[a]
$S \times F$	27	100	3.70[a]
$S \times L$	27	40	1.48[a]
$L \times E$	42	42	1.00[a]
$F \times E$	42	75	1.79[a]
$S \times E$	126	437	3.47[a]
$L \times F \times E$	126	138	1.10[a]
$S \times L \times E$	378	171	0.45[a]
$S \times L \times F$	81	62	0.77[a]
$S \times F \times E$	378	225	0.60[a]
Remainder	1,134	400	0.35

[a] = Significant at the 1 per cent level.

According to the least mean differences required for significance, differences in livability between the partially filled tubes and the filled tubes were highly significant after 6, 12 and 24 hours of agitation. The differences between the filled tubes of semen which received no agitation and those agitated for 6 and 12 hours were not statistically significant. However, after agitation for 24 hours, the differences barely reached significance at the 5 per cent level. Highly significant differences were found between the one-quarter filled and the completely filled tubes which were not subjected to mechanical agitation, while the differences between the filled tubes and the half and three-quarters filled tubes were not statistically significant.

DISCUSSION

The present study was designed to determine the effect of agitation upon the livability and metabolism of bovine spermatozoa. It was hoped that the latter information would be useful in explaining results obtained in the livability phase. Because of difficulties in obtaining reliable results with the methods employed

in the metabolism study, this phase of the problem was not completed. However, it is possible that the detrimental effect of mechanical agitation upon spermatozoan livability is related to the amount of atmospheric oxygen in the test tubes.

The results of the livability study showed that irrespective of the length of agitation an inverse relationship existed between the amount of air in the test tubes and the livability of the spermatozoa. However, decreases in livability were greatest in those tubes which contained the largest volumes of air and which were agitated for the longest periods of time. When a minimum of air was present (filled tubes) agitation did not affect markedly the livability of the spermatozoa. These observations of the detrimental effects of aeration are supported by the statistical analysis of these data. As shown in table 2, all of the sources of variation were found to be highly significant. However, the table shows that a greater mean square was obtained for fullness of tube than for length of agitation. The mean square for the interaction, length of agitation × fullness of tube, also was larger than the mean square for any of the other first order interactions.

It has been shown (3, 8, 13, 16 and others) that although a certain volume of oxygen normally is utilized in the metabolism of bovine spermatozoa, respiration is not essential for motility (7). While the exact role of oxygen in the metabolic processes is not clear, there is evidence that an excess of oxygen, in certain instances, may be detrimental to spermatozoan livability. Walton (15) concluded that protection of semen against exposure to air may be beneficial to livability and recommended that the semen be covered with a layer of medicinal paraffin oil. Willett and Salisbury (17) also found that motility was maintained longer during storage when semen was covered with a layer of mineral oil. MacLeod (9) found that oxygen was detrimental to the motility of human spermatozoa. Recently Salisbury (13) reported that bovine spermatozoa in low concentration were harmed by oxygen. Based on these findings, it is possible in the present study that excess aeration of the diluted semen was responsible, in part, for the decreases in livability obtained.

CONCLUSIONS

1. In the routine shipment of partially filled tubes of diluted bull semen a decrease in spermatozoan livability may be encountered due, in part, to the effect of agitation.

2. The harmful effect of agitation may be minimized by completely filling the test tubes with diluted semen.

3. On the basis of the data obtained in this experiment, it seems advisable to ship diluted semen in completely filled test tubes of different capacities to meet the individual requirements of the inseminators.

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HEAT INACTIVATION OF MILK PHOSPHATASE IN DAIRY PRODUCTS

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A description of the phosphatase test and the modifications that make it applicable to various dairy products to determine the adequacy of pasteurization was published in 1947 (12). In a later publication (13), it was pointed out that negative results with the phosphatase test indicate that the pathogenic organisms that may have been present were destroyed.

With a phosphatase test available that is quantitative over a wide range (from no heating to complete inactivation) and that is relatively sensitive and precise, and with considerable data available in the literature on the thermal death points of various microorganisms, it seemed desirable to determine experimentally the heating conditions necessary to produce various degrees of inactivation, including complete inactivation, of the phosphatase enzyme in milk and in some other dairy products. Such results would be useful in formulating pasteurization standards for various dairy products besides milk.

This report describes a laboratory pasteurizer that was used in these studies for controlling the temperature and duration of heating with a high degree of precision, and presents the results of phosphatase-inactivation experiments on whole milk, skim milk, cream (20 and 40 per cent fat content), ice cream and sherbet mixes, Cheddar cheese and cheese mixtures with emulsifiers or various other substances added.

Precise control of the temperature and duration of heating is necessary for reliable results in studying experimentally the thermal destruction of bacteria and phosphatase. North and Park (10), determining thermal death points of tubercle bacilli, used a laboratory pasteurizer fitted with a tubular metal coil immersed in a bath at a controlled temperature. The milk, heated to the desired temperature, was inoculated and was allowed to flow by gravity into the tubular coil where it was held at the experimental temperature. This method offered relatively more precise results than older methods, in which samples, at room temperature, were placed in glass tubes, inoculated and the tubes then placed in the heated bath. It reduced the heat lag, allowed more precise control of the heating time, and eliminated surface cooling.

Some investigators, heating samples in glass tubes (11) or in metal containers (6, 8), used a series of three water baths—the first one at a temperature below the desired holding temperature in order to preheat the samples uniformly, the second one at a temperature somewhat higher than the holding temperature in order to decrease the time lag by heating the samples rapidly, and the third one at the desired holding temperature. Despite the need for increasing the temperature rapidly, there appears to be considerable possibility of over-heating some particles

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of the sample when any part of the equipment containing the sample is heated at a temperature higher than the specified experimental temperature.

Gilcreas and O'Brien (4) recently have described a laboratory pasteurizer, used in bacteriological experiments, fitted with an automatic inoculating and sampling device actuated by an electric timer. With this equipment, inocula can be injected into a medium that is held constantly at the desired temperature, and samples likewise can be withdrawn with time intervals controlled very accurately. Although the heating-time lag is thus greatly reduced, this equipment could not be used for obtaining phosphatase-test data, because in phosphatase experiments it is necessary to control the heating conditions of the entire sample rather than only the portion used as an inoculum in bacteriological experiments.

EXPERIMENTAL METHODS

Attempts were made at first to make use of a Mallory-type heater, with the heating tube surrounded by steam, but it was difficult to determine and control the temperatures of small samples with the desired precision without installing a highly sensitive thermocouple and without other modifications of the control of the temperature. The results of the phosphatase tests on the samples heated in this manner were not sufficiently consistent for this purpose.

A laboratory pasteurizer, illustrated in figure 1, was assembled and used for heating fluid samples. The pasteurizer comprised a thin-wall tubular metal coil 30 feet long and one-eighth inch internal diameter, with a metal holding chamber connected at the lower end. This assembly was immersed in a water bath fitted with heaters, stirrer and thermoregulator, which controlled the temperature of the bath with a variation not greater than $\pm$ 0.2° F. The pasteurizer coil as used at first was fitted at the midway point with a T-tube connection (not shown in diagram) in which a four-junction thermocouple in thin-wall glass tubing was installed. A similar thermocouple was installed permanently through the stopper in the holding chamber. Temperatures were determined by means of a Leeds and Northrup type K potentiometer. Six mercury thermoregulators set at approximately 5° intervals between 142 and 168° F. were used. A special thermometer, reading 140–180° F. in 0.1° intervals, calibrated against one that had been checked at the National Bureau of Standards, was used in the bath and for calibrating the thermocouples. Heating-time periods were regulated with an electric stopclock calibrated in seconds.

To reduce the heating-time lag uniformly, the samples first were warmed in the phosphatase-test bath to 99–100° F., and then forced into the pasteurizer coil at high speed by means of air pressure. A suitable initial pressure, controlled by pressure regulator *A* (fig. 1) set at between 9 and 12 inches of mercury for milk and 12 to 15 inches for cream, was built up by means of a slow flow of air into flask *B*. Twenty to thirty-five ml. of warmed sample was put into sample chamber S_1, which was stoppered, and stopcock *SC* was turned to build up the pressure on the sample. Then rubber inlet tube S_2 was opened and the stopclock was started. The pressure forced the sample into and through pasteurizer coil *C* and into the bottom of holding chamber *HC*. The lower clamp on the inlet tube immediately

was placed below the water line in the bath and the inlet tube was closed. Under these conditions, the largest decrease of temperature that could be detected by means of the thermocouple at the midway point in the pasteurizer coil during flow was 0.2° F., and such decrease was only momentary. No change of temperature in the holding chamber could be detected.

At the ends of specified time periods, 2- to 3-ml. test samples were withdrawn quickly from the holding chamber through narrow-bore glass tubes *D*, by means of suction, into test tubes immersed in ice water. To allow for lag, a correction of 3 seconds for the smaller samples and 4 seconds for the larger ones was subtracted from the total heating time from the beginning of flow. The heating times thus corrected and recorded in the graphs are believed to be the averages of the periods during which the entire sample of each product was held at the experimental temperature. This seems a more feasible time to record than the over-all heating time.

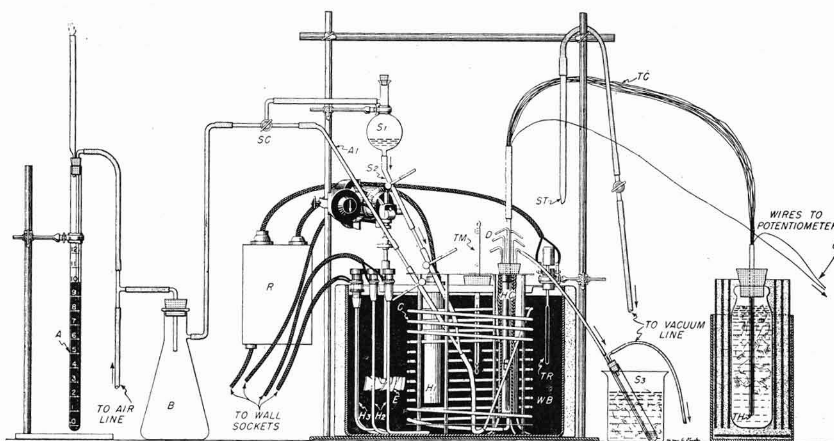

FIG. 1. Laboratory pasteurizer. *A*—adjustable air-pressure regulator. *B*—air-pressure reservoir flask, 1 liter. *SC*—three-way stopcock. S_1—sample chamber. S_2—sample inlet tube. A_1—air inlet tube for agitating sample. *C*—pasteurizer coil, 30 ft. long, ⅛ in. internal diameter (tin alloy). *HC*—metal holding chamber, insulated above water line. *D*—glass tubes for removing samples. S_3—samples collected in tubes in ice water. *WB*—water bath. *H*—heaters, 300-watt: H_1 operated by thermoregulator, H_2 on constantly, H_3 for increasing bath temperature rapidly. *E*—stirrer. *TM*—thermometer. *TR*—thermoregulator. *R*—relay. *TC*—thermocouple, copper-constantan, 4-junction in series. *TH*—thermocouple reference junction in insulated ice bath. *ST*—suction tube for cleaning.

For the fluid samples that were heated at the higher temperatures and for the shorter periods, the time required to heat the entire sample was decreased by using only 20 ml. of sample and increasing the initial pressure to as high as 12 inches of mercury for milk and 15 inches for cream. Under these conditions it required not more than 4 seconds for the entire sample to pass into the pasteurizer coil and 6 seconds from the beginning of flow for the front end of the sample to reach the holding chamber.

To avoid the difficulty caused by the cream rising or a protective pellicle form-

ing on the samples, mentioned by Smith (14) and by North and Park (10), the samples were agitated by means of air. As soon as the sample had passed in and the clamp on the sample inlet tube S_2 had been closed, stopcock SC was turned and the clamp on air inlet tube A_1 was released, permitting air to flow slowly through the pasteurizer coil and to agitate the sample with two or three bubbles per second. The temperature of the air above the sample was found to be the same as that of the sample. The temperatures in the holding chamber and in the sample did not fluctuate as much as those in the water bath.

At least six test samples, each heated for a different period of time, were obtained of each product heated at each temperature. Sufficient test samples were obtained so that at least one was negative (zero value) and at least four were positive. If the results did not meet these conditions, the experimental heating was repeated at the same temperature but under a modified set of time conditions. For example (fig. 2), a 20-ml. sample of whole milk was heated at 158.2° F. and test samples were obtained first that were heated for 42, 50, 60, 75, 90, and 110 seconds, respectively; the first two yielded phosphatase values less than 5 units per ml. and the last four were negative. The heating was repeated and test samples obtained that had been treated for 24, 27, 30, 35, and 42 seconds; the first yielded more than 40 units and the last yielded less than 2 units per ml.

The milks tested were fresh and were taken from the composite milks obtained from a large herd. The tests on creams and skim milks were run on samples prepared by separating portions of the same whole milks that were tested. The ice cream mixes contained 15 per cent fat, 8.5 per cent milk serum solids, 14 per cent sugar, and 0.3 per cent stabilizer, and had an average pH of 6.22. The sherbet mix contained 4 per cent fat, 3.5 per cent serum solids, 25 per cent sugar, and 0.3 per cent stabilizer, and had a pH of 6.30 before pasteurization and the addition of acid. The fat and phosphatase present in the mixes were from the raw cream, additional serum solids being furnished by condensed skim milk. The Cheddar cheese, which was made from raw milk, was of normal composition and between 1 and 2 months old when tested. It had a pH value of 5.29 and a phosphatase value of 3,450 units per g. The original phosphatase values of the other products were normal and within the ranges stated earlier (13).

In addition to tests on fluid products, 3-g. samples of ground cheese were placed in heat-sealing metal foil envelopes, and these were pressed to a uniform thickness of 1 mm., warmed in the phosphatase-test bath and immersed in the pasteurizing bath. With a thermocouple coated with shellac and Miracle adhesive and sealed in with such cheese samples, the time required for the temperatures of the samples to reach within 0.25° F. of that of the bath at 150° F. was found to be approximately 18 seconds. A correction of 18 seconds was subtracted from the total heating time.

To determine the effects of pH on the inactivation of the enzyme by heat, 5-ml. portions of a sample of milk (a mixture of 10 per cent of raw milk with 90 per cent of pasteurized milk) were placed in a series of 12 test tubes, various quantities of normal acid or alkali were added—*i.e.*, from 0.35 ml. of acid to 0.1 ml. of alkali, yielding a step-wise pH range of 4.09 to 8.42—the pH values were

determined, the samples were funneled into clean tubes so that there was no liquid on the inner surfaces of the tubes above the samples, and they were placed in a rack, warmed to 99–100° F., and then heated with agitation in a water bath at 140° F. for 5 minutes. Two and one-fourth minutes additional time was allowed for the temperatures of the samples to reach 139° F. The samples were cooled in

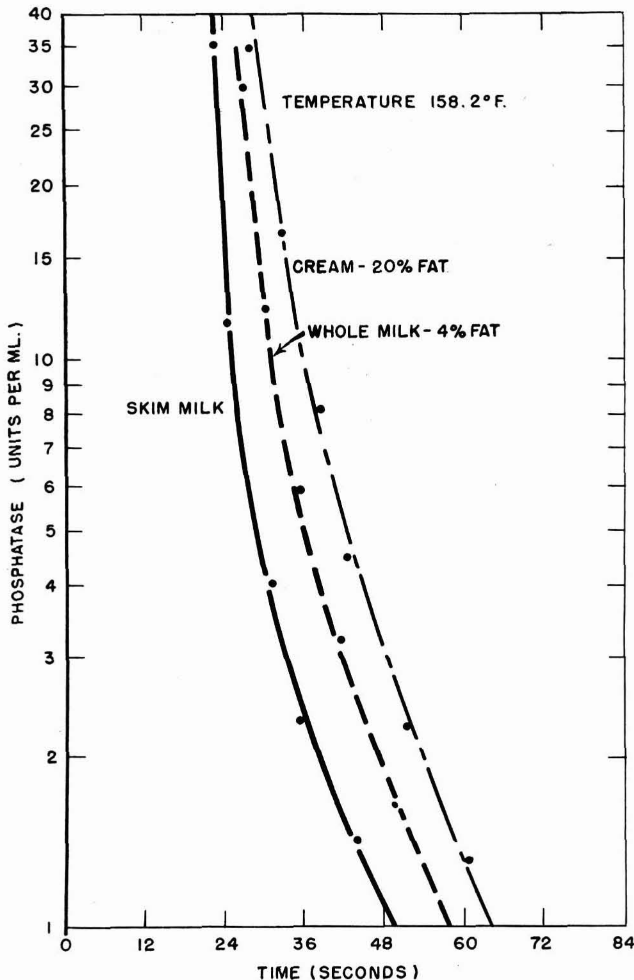

FIG. 2. Effects of duration of heating, at one temperature, on inactivation of phosphatase in skim milk, whole milk, and cream.

ice water, titrated back to the original pH with alkali or acid and tested for phosphatase activity. Corrections were made for the volumes of added acid and alkali.

Phosphatase tests were made by the method described earlier (12), and determinations of the intensity of the color were made with a Klett-Summerson photoelectric colorimeter with round matched tubes. Samples yielding less than 1 unit

per 1 ml. or per 0.5 g. of product in the test were considered negative, since such small values are difficult to determine accurately, even with a colorimeter, because of slight possible variations in the readings made on the controls.

RESULTS

The time-temperature inactivation results are summarized graphically. Figure 2 shows test data obtained on skim milk, whole milk, and 20 per cent cream, all were heated at 158.2° F., and illustrates the method of plotting the results. By conducting a series of tests on samples heated at a sufficient number of dif-

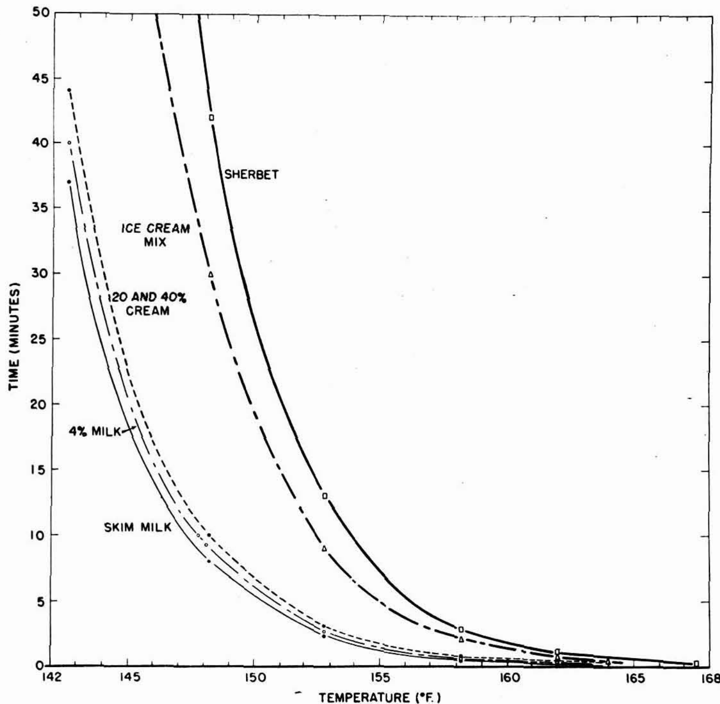

FIG. 3. Times and temperatures of heating required to reduce phosphatase activity to 2 units per 0.5 ml. (4 units per ml.) in different dairy products—test data plotted on an arithmetic scale.

ferent time periods, and thus plotting the data, it was possible to determine the heating times required at this temperature to produce zero values and also different degrees of inactivation.

With the phosphatase values plotted on a logarithmic scale and the duration of heating on an arithmetic scale (fig. 2), the curves in all experiments deviated from a straight-line course in a direction that indicates a marked decrease in the rate of inactivation as the time of heating is prolonged—*i.e.*, the rate of destruction of the enzyme by heat is most rapid at first and diminishes greatly with time. The curves prepared in this manner from data obtained at the lower temperatures

intersect the horizontal axis at a narrow angle, and the curves from data at the higher temperatures intersect the horizontal axis at a wide angle, showing that complete destruction is approached more slowly at low temperatures than at high temperatures.

By means of a similar series of experiments at each different temperature, data were obtained and plotted to determine the heating conditions just sufficient to reduce phosphatase activity to a value of four units per ml. in skim milk, whole milk, 20 and 40 per cent cream, ice cream mix, and sherbet mix. With data plotted arithmetically, as shown in figure 3, it is difficult to evaluate accurately the

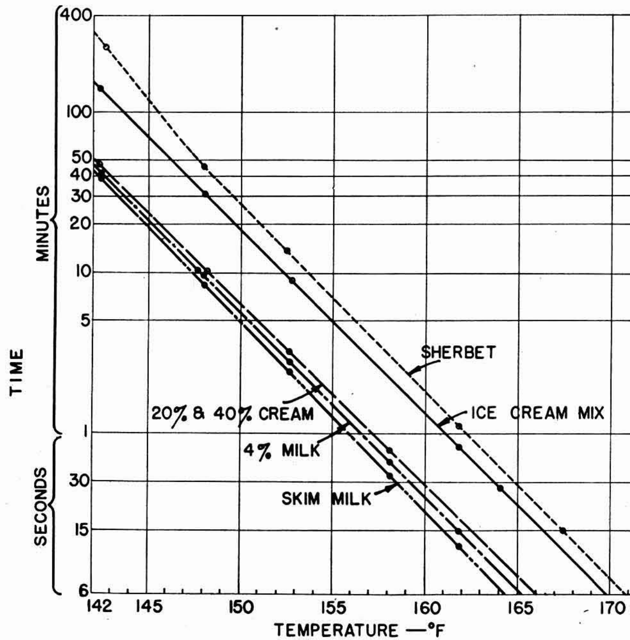

FIG. 4. Times and temperatures of heating required to reduce phosphatase activity to 2 units per 0.5 ml. (4 units per ml.) in different dairy products—data the same as in figure 3, but plotted on a semi-logarithmic scale.

heating times required in the high-temperature range. With the same data plotted on semi-logarithmic graph paper, however, as shown in figure 4, the points for each product form a straight line and the plot is readily usable.

The straight-line course of the time-temperature inactivation data conforms with results obtained earlier by Holland and Dahlberg (8) and Marquardt and Dahlberg (9) in studies on the effect of heat on cream layer volumes; with North and Park's results, as interpreted by Dahlberg (3), on the killing of tubercle bacilli; and with Hening and Dahlberg's (6) and Holland and Dahlberg's (8) results on the killing of *Escherichia coli,* the inactivation of phosphatase, and the effects on other properties. Mathematical equations pertaining to the straight-line pattern of time-temperature effects on a semi-logarithmic scale, produced in heat-

ing milk, were presented by Marquardt and Dahlberg (9) for reduction of cream layer volumes; by Sommer (15) for thermal death points, present pasteurization standards, and reduction of cream layer volumes; by Van Bever (16) for destruction of tubercle bacilli, reduction of cream layer volumes, decrease in solubility of milk proteins, and inactivation of peroxidase and of phosphatase; and by Hetrick and Tracy (7) for inactivation of phosphatase. The data in figure 4 show that the equation for phosphatase destruction is necessarily different for different products, since different time-temperature conditions are required to inactivate phosphatase in each product. As pointed out by Van Bever, all these effects induced by heat are correlated, and, furthermore, may be attributed to heat dena-

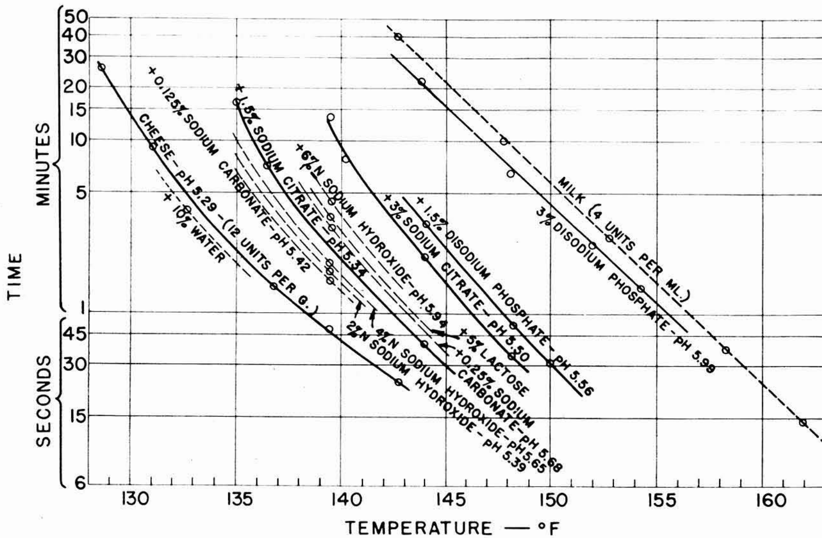

FIG. 5. Times and temperatures of heating required to reduce phosphatase activity to 3 units per 0.25 g. (12 units per g.) in cheese, and in cheese with various substances added.

turation of proteins. These relationships strengthen the belief that the phosphatase test is a reliable criterion of pasteurization.

Pasteurization holding periods required in connection with this test to reduce phosphatase activity to four units per ml. in whole milk were: 37.5 minutes at 143° F., 30 minutes at 143.7° F., 24 seconds at 160° F. and 15 seconds at 161.8° F., respectively. It will be noted that there is a slight difference between these conditions and the minimal heating conditions for pasteurization recommended in the U.S. Public Health Service milk ordinance and code. Since the straight-line semilogarithmic graph of North and Park's (10) data, as interpreted by Dahlberg (3), shows that the Public Health Service standards allow considerably less margin of safety at 160° F. than at 143° F., some increase in the margin of safety at 160° F. seems desirable.

The temperature required to reduce the activity to four units per ml. in any given time generally was found to be about 0.7° F. lower for skim milk than for

whole milk, about 0.7° F. higher for 20 and 40 per cent cream than for whole milk, about 4.5° F. higher for ice cream mix than for whole milk and about 5.7° F. higher for sherbet than for whole milk. The time required, at 143° F., was about three times as long for ice cream mix as for whole milk.

Results obtained in tests on Cheddar cheese and on mixtures of cheese with emulsifying salts, alkalies, lactose or water, are shown in figure 5. Phosphatase was inactivated at considerably lower temperatures and shorter holding times in Cheddar cheese than in milk, *e.g.*, to a value of 12 units per g. at 130° F. for 13 minutes and at 140° F. for slightly less than three-fourths minute in cheese having a pH of 5.29. Mixing sodium carbonate or sodium hydroxide with the cheese to increase the pH had some effect in stabilizing the enzyme against heat inactiva-

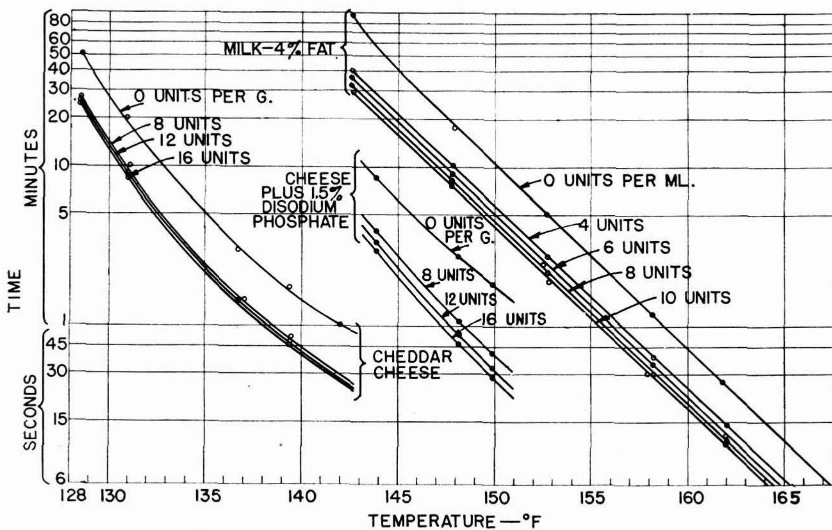

FIG. 6. Times and temperatures of heating required to cause various degrees of inactivation of phosphatase in milk, in Cheddar cheese, and in Cheddar cheese with 1.5% of *anhydrous* disodium phosphate added.

tion. Adding emulsifiers, such as sodium citrate or disodium phosphate, in quantities that affected the pH less, had a greater effect in stabilizing the enzyme. For example, in cheese with 1.5 per cent anhydrous disodium phosphate added (pH 5.56), a temperature of 150° F. for approximately 0.5 minute was needed to reduce the activity to 12 units per g. The addition of lactose increased the stability of the enzyme. The addition of water decreased its stability slightly.

Data showing the temperatures and times found necessary to produce various degrees of inactivation in milk and in cheese are shown in figure 6. As pointed out above, the last few remaining units were found to be the most difficult to inactivate. Differences between zero and four units were found to indicate considerably more heating than differences between four and eight units per ml. A pasteurization criterion of zero for milk, with this test, apparently would be too

severe, because it would require heating at temperatures several degrees higher than the temperatures specified in present pasteurization standards.

Figure 7 shows the effects of the pH values, at the time of heating, on the inactivation of the enzyme in milk. The heat stability was greatest when the pH was within a range of 6.5 to 7.4. In samples in which the pH was decreased progressively below 6.5, heating at 140° F. for 5 minutes decreased the activity markedly, until at pH 5.1 this heating reduced the activity to zero. There was a similar but less marked decrease in heat stability of the enzyme with increases in alkalinity beyond approximately pH 7.4.

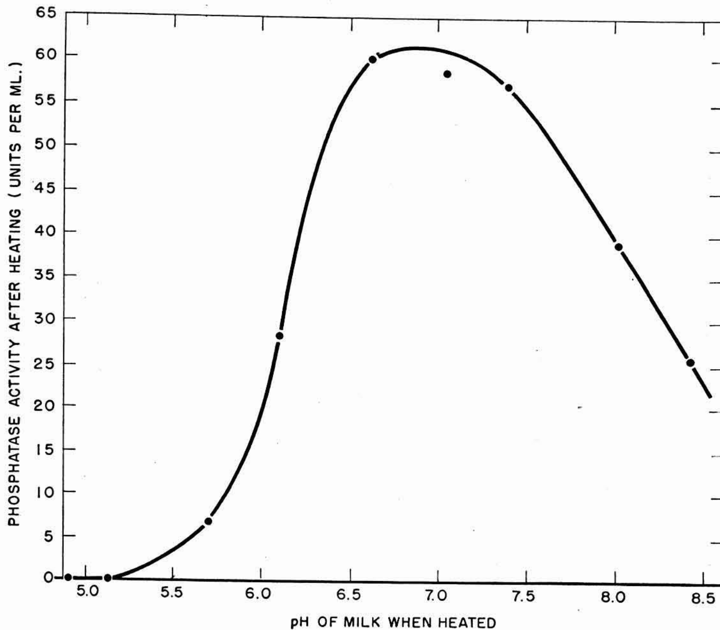

FIG. 7. Effects of pH on heat inactivation of milk phosphatase—mixture of 10% raw and 90% pasteurized milk; original phosphatase activity of mixture 195 units per ml.; heated for 5 minutes at 140° F.

DISCUSSION

Smith (14) showed that at 140° F. a considerably longer time is required to destroy tubercle bacilli in the pellicle that forms on the surface of milk than in the milk itself. Brown and Peiser (1) demonstrated that the temperature required, for a given period of time, to kill certain strains of *Streptococcus lactis* and of *E. coli* in cream is higher than that required to kill them in milk. Hening and Dahlberg (6), experimenting on the destruction of *E. coli*, inactivation of phosphatase, effect on flavor and effects on certain other properties, concluded that the present standards for pasteurization of milk are not adequate for cream. In addition to the protective effect present in cream, others (2) have shown that sugar also increases the stability of the enzyme against heat and present results

(fig. 4) corroborate this conclusion. Caulfield and Martin (2) reported that a temperature of 150° F. for 15 to 25 minutes was required to produce negative tests in ice cream mixes, and Hahn and Tracy (5) obtained similar results. It is evident that neither the present pasteurization standards for milk nor higher standards proposed by Hening and Dahlberg (6) for cream are adequate for ice cream and sherbet.

The heat stability of the enzyme is not as great in cheese as in milk. Although the stability is increased when the concentration of fat is increased (see data for cream, fig. 4), it will be noted that it is decreased much more by acidity when the pH is reduced (fig. 7) to values found normally in cheese. The acidity apparently has a predominant effect on the heat stability.

A large number of samples of process cheese, cheese foods and cheese spreads have been tested for phosphatase in these laboratories, and all of them have yielded zero values. The heating conditions used commercially in processing these products should be, and evidently are, adequate to accomplish the purposes of pasteurization.

In manufacturing cottage cheese curd from raw skim milk, a large proportion of the enzymic activity—frequently more than 80 per cent and sometimes nearly 100 per cent—is lost during manufacture. This decrease is attributed to the fact that the curd is heated, usually for a considerable period of time, after acidity has developed.

Experimental results have shown that the heat-inactivation reaction which phosphatase undergoes at the pH of normal milk is irreversible. On the other hand, when partial inactivation occurs only because of the development of acidity, the activity can be largely restored by adding sufficient alkali—*e.g.*, mixing 1 ml. of a 1.25 per cent aqueous solution of anhydrous sodium bicarbonate with 0.5 g. of cottage cheese—to increase the pH to approximately 7, and allowing the mixture to stand for several hours before testing. In the case of cottage cheese curd that has been washed thoroughly during manufacture, the presence of traces of added magnesium stimulates this reactivation, and the presence of magnesium and zinc stimulates it more.

SUMMARY

A laboratory pasteurizer is described, for controlling the heating temperatures and time accurately in pasteurization experiments.

Phosphatase test data for samples heated at any specific temperature for various periods of time show that the rate of destruction of the enzyme by heat is very rapid at first and diminishes to a relatively very slow rate with time. The experimental data for phosphatase destruction show that, in tests on milk and other fluid dairy products, a straight line results when the logarithms of the times of heating are plotted against the corresponding temperatures.

Holding periods required in this test to reduce phosphatase activity to four units per ml. of whole milk were: 37.5 minutes at 143° F., 30 minutes at 143.7° F., 24 seconds at 160° F., and 15 seconds at 161.8° F., respectively. The temperature required to produce a negative phosphatase test in any given time generally was

found to be about 0.7° F. lower for skim milk than for whole milk, about 0.7° F. higher for 20 and 40 per cent cream than for whole milk, about 4.5° F. higher for ice cream mix than for whole milk, and about 5.7° F. higher for sherbet than for whole milk. The time required, at 143° F., was about three times as long for ice cream mix as for whole milk.

Phosphatase was inactivated at considerably lower temperatures and shorter holding times in Cheddar cheese than in milk—*e.g.*, at 130° F. in 13 minutes and at 140° F. in slightly less than three-fourths minute in cheese at pH 5.29. Mixing alkalies with the cheese to increase the pH had some effect in stabilizing the enzyme against heat. Adding emulsifiers had a greater effect, as the temperature required to produce a negative test in approximatly 0.5 minute was 150° F. when 1.5 per cent anhydrous disodium phosphate was added and the pH of the mixture was 5.56. The addition of lactose to cheese increased the stability of the enzyme; the addition of water decreased its stability slightly. Experiments on milks adjusted to different pH levels and heated showed that the milk phosphatase was most stable towards heat when the reaction was within a range of pH 6.5–7.4. Heating at lower or higher pH levels produced more rapid inactivation.

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THE ADAPTABILITY OF TWO STRAINS OF LACTIC STREPTOCOCCI TO GROWTH IN THE PRESENCE OF HOMOLOGOUS BACTERIOPHAGE[1]

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Several investigators have reported on the adaptation of cultures of lactic streptococci to growth in the presence of bacteriophage. In all cases, the resistant cultures developed proved to be susceptible to other races of bacteriophage. In the present study, an attempt was made to determine more specifically how long it takes to adapt cultures of lactic streptococci to homologous bacteriophage, and after a strain of organisms has been made resistant, how long this characteristic will persist. Data from single trials, using two cultures of lactic streptococci and their homologous races of bacteriophage, are presented in this paper.

REVIEW OF LITERATURE

Whitehead and Hunter (5) found that a resistant culture of lactic streptococci developed by the action of bacteriophage on a sensitive strain was susceptible to attack by a new race of bacteriophage. They suggested that this type of action lends support to the theory that the bacteriophage is a product of the organism. Nelson and Hammer (3) isolated bacteriophage-resistant strains of *Streptococcus lactis* from the secondary growth of a culture upon which an inhibitory principle obtained from "slow" butter cultures had acted. Later work by Nelson *et al.* (4) showed that the secondary-growth organisms, which were not sensitive to the strain of inhibitory principle used, still were sensitive to other races of bacteriophage. Experiments by Anderson and Meanwell (1) indicate that bacteriophage-resistant strains of lactic streptococci can be developed, but on reintroduction into factory use these cultures become susceptible to secondary races of bacteriophage. Hunter and Whitehead (2) state that resistant strains of lactic streptococci usually develop sometime between 24 and 48 hours after bacteriophage has caused the lysis of a sensitive strain of organisms.

MATERIALS AND METHODS

Origin of cultures. The culture designated as H. P. is a strain of *Streptococcus cremoris* secured from the Dairy Research Institute in New Zealand Culture no. 4 is a strain of *S. lactis* from the culture collection at Iowa State

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College. Homologous strains of bacteriophage for each were received with the cultures.

Preparation of cultures. The cultures were propagated in autoclaved reconstituted commercial dry milk solids not fat used at the rate of 90 g. per 1,000 ml. of distilled water. Transfers were made either daily or on alternate days. The cultures were incubated at 25° C. until coagulation occurred, after which they were refrigerated at 0° C. Cultures for use on any particular day were prepared by using a 1 per cent inoculum into milk late in the afternoon of the previous day.

Preparation of the bacteriophage filtrate. Sterile milk was inoculated with a milk culture of the test organism using a 1 per cent inoculum. At the same time, a few drops of whey filtrate containing the homologous bacteriophage were added. After incubation at 25° C. until coagulation occurred, usually 48 to 72 hours, the whey was filtered through a sterile Seitz filter. The resulting bacteria-free filtrate was transferred aseptically to a sterile container and stored at 0° C. until used. Several filtrates of the bacteriophage under study were prepared during the course of the experiment in order to have on hand a filtrate of maximum titer for use with each series.

Preparation of serial dilutions of bacteriophage. Serial dilutions of the whey filtrate containing bacteriophage were made directly into sterile milk. Dilutions ranging from 10^{-2} to 10^{-10} were used. The final volume, in all cases, was 100 ml. contained in a screw-capped 6-ounce prescription flask.

Titratable acidity determination. Titratable acidities were determined on weighed 9 g. samples, using 0.1 N NaOH and phenolphthalein as the indicator. The results were expressed as per cent of lactic acid.

EXPERIMENTAL

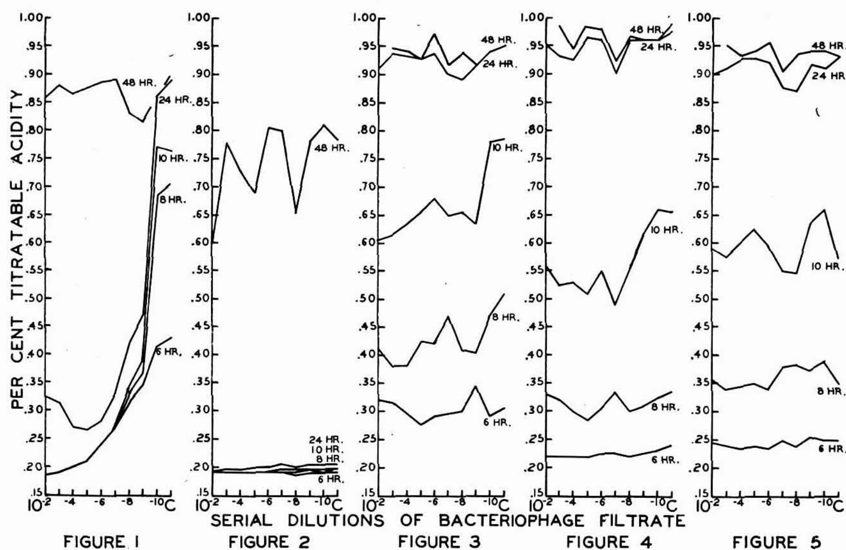
Adaptability of strain H. P. to the homologous bacteriophage. This experiment included five successive culture series. The first series consisted of ten flasks each containing 100 g. of autoclaved skim milk inoculated with a 1 g. amount of coagulated milk culture. These aliquots were dispensed aseptically from a stock flask in which the milk and the inoculum had been mixed thoroughly by vigorous agitation for a period of 1 minute. To the first nine of these flasks were added serial dilutions of the homologous bacteriophage whey filtrate ranging from 10^{-2} to 10^{-10}, inclusive. The tenth flask received no bacteriophage and served as a control to show acid production of the bacteriophage-free culture. The whole series was warmed to 30° C. in a water bath, after which the flasks were placed in an incubator adjusted to that temperature.

In the general plan of the experiment, the culture containing the lowest dilution of bacteriophage filtrate permitting coagulation of the milk after 24 hours of incubation was used to inoculate the flasks of the succeeding series. However, with series 2 it was necessary to wait 48 hours to secure a coagulated inoculum for use in the next series, as all cultures of this series, including the control, still were inhibited markedly after 24 hours by the residual bacteriophage carried over with the inoculum from series 1. Serial dilutions of the

homologous bacteriophage filtrate also were added to the flasks in all of the series by the method used in preparing series 1.

A 9 g. portion for acidity titration was withdrawn with a sterile pipette from each of the flasks after 2, 4, 6, 8, 10, 24 and 48 hours of incubation. The portions from the flasks in each series always were weighed and titrated in the same order, so as to maintain as closely as possible the desired time interval between each set of titrations.

Two series a week were started in this experiment, on Tuesdays and Saturdays, thus making one 3-day and one 4-day interval between the series of each week. The culture selected from each series for use in the succeeding series



FIGS. 1 through 5. Adaptability of strain H. P. to the homologous bacteriophage. Amount of acidity developed at 30° C.

was stored in the refrigerator at 0° C. until it was needed to make the necessary inoculation.

The data from the titrations after 6, 8, 10, 24 and 48 hours of incubation of the milk cultures are shown in figures 1 through 5. The data from the 2- and 4-hour titrations were not graphed because they did not show any significant change or differences between the various cultures containing serial dilutions of bacteriophage.

The data from series 1 (fig. 1) show that the titer of the bacteriophage filtrate used was at least as high as 10^{-9} and that the higher the serial dilution of bacteriophage filtrate added, the less was the degree of inhibition of the organisms in the culture.

The culture used to inoculate the flasks of milk of series 2 was from the 10^{-10} serial dilution of series 1. It is apparent from the data of series 2 (fig. 2) that considerable residual bacteriophage had been carried over in the inoculum from

the culture of series 1. The fact that the control culture, which had received no additional bacteriophage filtrate, was inhibited to the same extent as the others in the experimental series would seem to justify this conclusion.

The data in figure 2 also indicate that bacteriophage-resistant strains of this culture were not developed when the organisms were growing actively in the presence of a dilute concentration of bacteriophage. If any resistance to the bacteriophage had been built up in the culture used to inoculate the flasks of milk for series 2, these organisms would not have been inhibited so markedly after they had been transferred to new milk. The recovery of the organisms from the inhibiting effects of a high concentration of bacteriophage, sometime between the 24- and 48-hour titration intervals, indicates that bacteriophage-resistant strains are developed when the organisms are prevented from growing by the presence of a sufficient concentration of a homologous bacteriophage. No satisfactory explanation has been found for the rather wide and inconsistent variations in the titratable acidities after 48 hours of incubation of the cultures in series 2.

The data from series 3 (fig. 3) show that strains with some degree of bacteriophage resistance were developed sometime near the end of series 2, because the rate of acid production of nearly all cultures containing added bacteriophage was much more rapid at the 6-, 8- and 10-hour titration intervals in series 3 than was the case in either series 1 or 2.

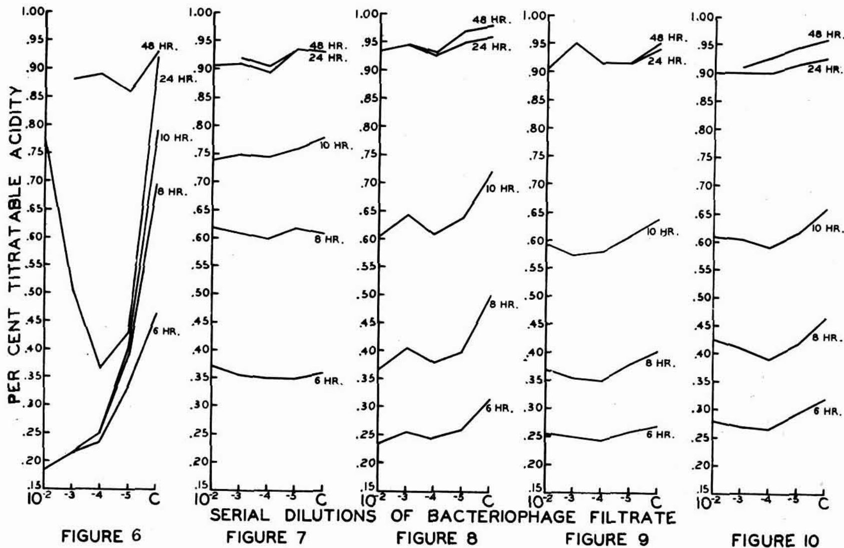
The steadily increasing resistance to bacteriophage of the newly developed strains of lactic acid organism became noticeable soon after the start of series 3. In series 3 and 4 (figs. 3 and 4) the culture which received the greatest concentrations of added bacteriophage filtrate still showed some inhibition in acid production as compared to the controls, especially at the 10-hour titration period, but in series 5 (fig. 5) the acidities followed very nearly a straight line across the graph, except for slight variations which could well be expected. The fact that the culture of series 5, which contained a serial dilution of 10^{-2} of bacteriophage filtrate, showed practically the same titratable acidity at each titration interval as the control indicates that the lactic streptococci of this strain had become well adapted to growth in the presence of active bacteriophage after only a relatively few transfers in milk to which bacteriophage filtrate had been added.

The somewhat lower acidities developed by each culture in series 3, 4 and 5 at the 6-, 8- and 10-hour titration periods as compared to the control of series 1, which was inoculated from an actively growing culture, can be attributed, in part at least, to the general "slowing down" effect on organisms in cultures that are not transferred frequently enough. The magnitude of this effect could have been determined in the present experiment had a control culture, with no added bacteriophage filtrate, been included with each experimental series. However, at the 24- and 48-hour titration intervals the titratable acidity of practically every culture in series 3, 4 and 5 was higher than the control culture of series 1.

Adaptability of strain no. 4 to the homologous bacteriophage. This experiment included five successive culture series. The general experimental procedure

was the same as that used in the experiments with strain H. P. In the studies with the no. 4 strain, only five flasks were used in each series because the titer of the bacteriophage filtrate active against this organism, as shown by preliminary observations, was not very high. To four of these flasks were added serial dilutions of the homologous bacteriophage filtrate ranging from 10^{-2} to 10^{-5}, inclusive, and the fifth served as a control.

The data from the titrations after 6, 8, 10, 24 and 48 hours of incubation of the milk cultures are presented in figures 6 through 10. The data from the 2- and 4-hour titrations were not graphed because they did not show any significant change in acidity.



FIGS. 6 through 10. Adaptability of strain no. 4 to the homologous bacteriophage. Amount of acidity developed at 30° C.

The data from series 1 (fig. 6) indicate that the titer of the bacteriophage filtrate used in this experiment was at least as high as 10^{-5}. It cannot be stated conclusively whether or not it was higher than this, because a culture containing a serial dilution of 10^{-6} of filtrate was not included in this experimental series. It is very evident, however, that the bacteriophage filtrate active against culture no. 4 was not nearly as potent as the one used in the studies with culture H. P. It will be noted further that the organisms in two of the cultures in series 1 had become fairly well adapted, sometime between the 10- and 24-hour titration periods, to growing in the presence of the bacteriophage to which they were initially susceptible. The titratable acidity readings of series 1 at the 24-hour interval are very significant, because they show that the organisms of this particular culture developed a resistance to the bacteriophage more quickly in the presence of a heavy inoculation of the bacteriophage filtrate than they did when

a lesser quantity was added. The earlier recovery was particularly noticeable in the cultures to which serial dilutions of 10^{-2} and 10^{-3} of bacteriophage filtrate were added.

The results show that culture no. 4 had attained its maximum adaptability for growth in the presence of the homologous bacteriophage by the end of series 2 (fig. 7). The somewhat lower acidities developed by each culture in series 3, 4 and 5 (figs. 8, 9 and 10) at the 6-, 8- and 10-hour titration periods as compared to the control of series 1 and all cultures in series 2 can be attributed, at least partially, to the general "slowing down" effect on organisms in cultures that are not transferred frequently enough. It will be noted, however, that there were no great differences in the titratable acidities of any of the cultures in series 2, 3, 4 and 5 at the 24- or 48-hour titration intervals.

TABLE 1

Comparison of adapted and non-adapted cultures after storage at 0° C.

Hr. incubated at 30° C.	Titratable acidity (% lactic acid)					
	Culture—H. P.			Culture—no. 4		
	Adapted	Non-adapted	Control	Adapted	Non-adapted	Control
			After 1 month			
2	0.190	0.185	0.180	0.180	0.185	0.180
4	0.255	0.185	0.220	0.240	0.220	0.225
6	0.390	0.185	0.330	0.400	0.235	0.345
8	0.585	0.185	0.535	0.620	0.235	0.555
10	0.705	0.185	0.670	0.725	0.250	0.695
24	0.820	0.375	0.800	0.815	0.445	0.825
48	0.820	0.730	0.805	0.830	0.830	0.860
			After 4.5 months			
2	0.175	0.180	0.180	0.180	0.180	0.180
4	0.195	0.195	0.230	0.245	0.250	0.250
6	0.195	0.195	0.345	0.410	0.370	0.420
8	0.195	0.195	0.600	0.590	0.390	0.590
10	0.200	0.195	0.685	0.685	0.405	0.685
24	0.520	0.250	0.740	0.795	0.430	0.810
48	0.760	0.720	0.760	0.815	0.610	0.830

The ability of adapted strains of organisms to retain their adaptability to a specific race of bacteriophage. Transfers were made of the adapted cultures at intervals of 10 to 14 days. Except for the time during which newly-inoculated cultures were being incubated, the cultures were stored in the refrigerator at 0° C. Approximately one month after the completion of the original adaptation studies, each of the "adapted" cultures was compared with a normal culture of the same strain to determine if the acquired characteristic was temporary, or whether it persisted after repeated transfers to new milk. The procedure for this test was as follows: One milliliter of the adapted culture was added to a flask containing 100 ml. of autoclaved milk. Also, 1 ml. of a normal culture of the same strain of organism was added to each of two similar flasks of milk. One milliliter of bacteriophage filtrate active against the strain of or-

ganism then was added to the flask containing the adapted inoculum, and the same amount of filtrate was added to one of the other flasks. The third flask of milk served as a control. The cultures were incubated at 30° C. Titratable acidity determinations were made after 2, 4, 6, 8, 10, 24 and 48 hours of incubation. The results of these determinations are presented in table 1. The data indicate that both the adapted H. P. culture and the adapted no. 4 culture retained their ability to resist attack by the bacteriophage active against the original cultures of the same strains of organisms. The same type of determination was made on these cultures after 4.5 months of storage at 0° C. The results of this experiment also are presented in table 1. The results of this trial

TABLE 2

Comparison of adapted and non-adapted cultures after approximately 4.5 months of storage at 0° C.

Hr. incubated at 30° C	Titratable acidity (% lactic acid)					
	Culture—H. P.			Culture—no. 4		
	Adapted	Non-adapted	Control	Adapted	Non-adapted	Control
2	0.175	0.180	0.180	0.180	0.180	0.180
4	0.195	0.195	0.230	0.245	0.250	0.250
6	0.195	0.195	0.345	0.410	0.370	0.420
8	0.195	0.195	0.600	0.590	0.390	0.590
10	0.200	0.195	0.685	0.685	0.405	0.685
24	0.520	0.250	0.740	0.795	0.430	0.810
48	0.760	0.720	0.760	0.815	0.610	0.830

show that the adapted H. P. culture had almost completely lost its previously-acquired resistance to the homologous bacteriophage. The only indication that it had retained a part of its acquired resistance was the fact that the titratable acidity of the adapted culture was markedly above the acidity of the normal culture at the 24-hour titration interval. The adapted no. 4 culture developed acid just as rapidly as the control culture, which contained no added bacteriophage; therefore, it must be concluded that it had retained its acquired resistance to the bacteriophage active against it even after 4.5 months.

These data indicate that one cannot predict accurately how long adapted cultures of lactic acid streptococci will retain the resistance that they acquire when grown in the presence of a homologous bacteriophage. The limited data available show that the degree of retention of this acquired characteristic will vary between different strains of organisms.

SUMMARY

In the present study, experiments were conducted to determine if single-strain cultures of lactic-acid streptococci could become adapted to grow in the presence of homologous bacteriophage, and if so, how long the acquired characteristic would persist.

The data show that single-strain cultures of lactic streptococci will acquire

an adaptation for growth in the presence of homologous bacteriophage. The length of time during which this acquired characteristic will persist varies with the strain of organism.

ACKNOWLEDGMENTS

Thanks are tendered to Dr. H. R. Whitehead of the Dairy Research Institute, Palmerston North, New Zealand, for the H. P. strain of *S. cremoris* and its homologous bacteriophage, and to Dr. F. J. Babel, formerly of the Department of Dairy Industry, Iowa State College, for the no. 4 strain of *S. lactis* and its homologous bacteriophage.

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EFFECT OF HIGH-TEMPERATURE SHORT-TIME HEAT TREATMENTS ON SOME PROPERTIES OF MILK. I. INACTIVATION OF THE PHOSPHATASE ENZYME[1]

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Since the development of the phosphatase test by Kay and Graham (8) and its subsequent modifications, several studies have been made to determine the suitability of the test for detecting irregularities in the pastuerization of milk and other dairy products. The literature on this phase of the phosphatase test up to 1939 has been thoroughly reviewed by Burgwald (2). The reported studies showed that all raw milk contains phosphatase, that the thermal resistance of phosphatase is greater than that of pathogens, and that the test is sufficiently sensitive to detect important variations in pasteurizing conditions on the basis of the residual phosphatase activity. Other work has been directed toward improvement of the phosphatase test to make it more sensitive, more rapid, and more quantitative (3, 5, 13, 15).

Several investigators (6, 7, 11, 14, 17) have reported on the time-temperature relationships necessary to inactivate the phosphatase enzyme in milk. The results obtained differ considerably, probably because of the variety of methods used for heating the milk and for testing the phosphatase activity, as well as differences in accepted standards of what constitutes satisfactory destruction of the enzyme. Holland and Dahlberg (6) stated that most of the discrepancies probably could be accounted for by the variations in the length of time required to heat to and cool from the temperatures at which they are holding. They did not, however, present any data to show the effect of various rates of heating. Later Lythgoe (9) commented that if milk is heated very quickly the time of inactivation of phosphatase necessarily may be longer than if milk is heated more slowly, but no data were given.

Previous experimental work has shown that at temperatures above 140° F. phosphatase destruction proceeds with sufficient rapidity to make time of heating to temperature extremely important and, as the temperature to which milk is heated becomes higher, the cumulative effects of heat in reaching the temperature become progressively more important. Using a heating time of 35–40 seconds, Prucha and Corbett (11) found the phosphatase to be inactivated by an instantaneous exposure at 160° F. They used Scharer's (15) test, measuring the indophenol color with the Hahn and Tracy (5) photoelectric cell set-up, and placed the standard for satisfactory destruction of phosphatase at 0.8 p.p.m. phenol equivalent. Holland and Dahlberg (6) secured a negative phosphatase

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test by heating to 170° F. using a 10-second heating time above 140° F. and the Kay and Graham (8) test with the standard of 0.04 mg. phenol equivalent.

In June, 1946, an experimental unit built on Mallory (10) principles by the Illinois Creamery Supply Company, Chicago, Illinois, was installed in the research laboratory of the Dean Milk Company, Rockford, Illinois. With this equipment, it was possible to heat cold milk to any desired temperature well above the boiling point in 0.83 second.

Since the trend in design of continuous milk heating equipment is toward faster, more efficient heating with more precise control of flow rate and temperature, it would seem that higher temperatures and shorter time processing will result and more information will be necessary to set time-temperature relationships. It also was apparent that information should be secured on the effects of various rates of heating on the temperatures required to inactivate the phosphatase to provide a better basis for evaluating the results of other investigators in this field. The results of such a study could be used to form a basis for formulating a practical mathematical time-temperature solution for the inactivation of the phosphatase enzyme which would explain the results secured by any heating method. It also was decided to study seasonal variations in phosphatase content of milk and the distribution of the phosphatase enzyme in various milk fractions.

EXPERIMENTAL METHODS

All of the experimental work was done in the research laboratory of the Dean Milk Company. The milk used for this study was fresh Grade A raw milk as received at the plant from the company's patrons. The Mallory small-tube heat exchanger was used to process the milk when various times and temperatures required to inactivate the phosphatase enzyme were studied. This unit had a capacity of 80 gallons per hour. It was composed of five independently operating heating sections and two cooling sections. Milk was forced through the unit with an 80-gallon-per-hour Manton-Gaulin homogenizer used as a high pressure pump. A pressure of 800 to 1,000 lb. per square inch was required to force milk through the unit. Each heating section was composed of four 58.5-inch lengths of ¼-inch O. D. stainless steel tubing, through which the milk flowed, surrounded by a larger pipe containing dry steam as the heating medium. The time required for the milk to flow through one heating or cooling section was calculated to be 0.83 second and the milk flowed through the unit at a calculated velocity of 23.6 feet per second. After the milk was heated to the desired temperature, it flowed through a copper coil of ⅜-inch O. D. immersed in a water bath held at the desired holding temperature. The coil was so constructed that milk could be removed after any desired length of time directly into test tubes which previously had been immersed in ice water. The temperatures were measured with a mercury-in-glass thermometer inserted in a mercury well which was placed in the line between the Mallory unit and the copper holding coil. In this study, the time required to heat to all maximum temperatures was 0.83 second.

The phosphatase activity in terms of phenol equivalent was measured by the

method of Sanders and Sager (13) with one modification; *i.e.*, the color development buffer used was the one proposed by them (12) in a previous publication. One-hour incubation time at 37° C. and half-hour color development at room temperature were used. The indophenol was extracted with 10 ml. of buffered butyl alcohol and the transmission measured at 650 mμ, using a Coleman model 11 spectrophotometer and a 1.3-cm. square cuvette. Boiled milk controls were run with each series of determinations. The quantities of phenol, after consideration of the boiled controls, were read directly from a standard transmission-concentration curve prepared with known amounts of phenol. The results are expressed as micrograms of phenol per milliliter of milk or parts per million on a milk basis (not on the basis of the parts per million phenol in the butyl alcohol extract). This method was found to be sensitive and reproducible quantitatively; 0.05 per cent raw milk in boiled milk could be detected readily. Phenol added to pasteurized milk could be recovered satisfactorily.

The phosphatase tests were run approximately 24 hours after treatment of the milk.

RESULTS

Phosphatase activity in raw milk and distribution of the phosphatase enzyme in various milk fractions

Variations in the initial phosphatase concentration, if large, possibly would make some difference in the time and temperature required to inactivate the enzyme. Unfortunately, the phosphatase contents of the raw milk in the initial stages of the study were not accurately determined and only those values determined since February, 1947, will be given. The initial phosphatase content was so high that insufficient di-sodium phenyl phosphate was used and this was not recognized until the values were checked by diluting the raw milk samples with boiled milk to bring the phenol concentrations within the range of the standard curve concentration. Since the kinetics of the reaction between the enzyme and the substrate in the test itself is reported to be first order, the initial concentration will have some effect on the rate of the reaction when a standard length of time of incubation is used. For this reason all raw samples were diluted with boiled milk to make the final phenol concentration fall within a rather narrow range (range of standard curve, 0–20 γ phenol), and the concentrations then were calculated on the raw milk basis.

The phosphatase values of ten lots of raw milk from February 20 to July 9, 1947, ranged from 1,920–3,000 p.p.m. with an average of 2,230 p.p.m. These values are of the same order of magnitude as the value reported by Sanders and Sager (13) for whole milk and are considerably higher than those secured by previous investigators.

To secure some idea of the distribution of phosphatase in milk, raw milk was separated into cream and skim milk. The raw cream was churned into butter and the raw buttermilk drained off. The butter was melted at 110° F. and centrifuged to secure butter oil devoid of phospholipid material. The pH of the raw skim milk was adjusted to pH 4.6 with 0.1 *N* hydrochloric acid and the casein

was filtered off. The filtrate was neutralized to pH 6.7 with 0.1 N sodium hydroxide and the phosphatase activities of all fractions were determined. Each sample was diluted with boiled milk before running the phosphatase test to bring the final phenol concentrations in range of the standard curve. The results are calculated on the product basis by weight and reported as p.p.m. phenol equivalent. The results (fig. 1) indicate that the enzyme probably is concentrated at the fat-serum interface, perhaps in a manner similar to agglutinin, since butter oil showed no phosphatase activity and the buttermilk showed an activity approximately ten times that of skim milk. This is in agreement with the observations of Kay and Graham (7). It should be observed, too, that the phosphatase activity of the raw milk was recovered quantitatively in the skim milk and cream.

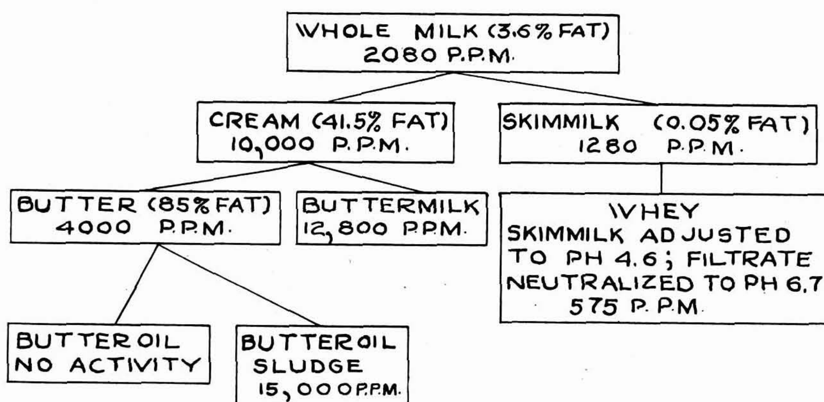

FIG. 1. Distribution of phosphatase in the various milk fractions. Phenol equivalent on the product basis by weight (p.p.m.).

The separation temperature in this case was 86° F. Figure 1 also shows that there is sufficient phosphatase activity in the various milk fractions to permit use of the phosphatase test to determine whether or not most dairy products have been properly pasteurized. The only exception appears to be butter oil.

Since the phosphatase enzyme apparently was concentrated at the fat-serum interface, a study of the effect of separating temperature on the distribution of the enzyme between cream and skim milk was thought desirable. If held in a similar manner as agglutinin, it was thought that an increase in the separating temperature would result in release of the enzyme from fat and the skim milk upon separation would show more enzyme activity. Consequently three lots of raw milk from the same milk source were separated, one at 80° F., another at 100° F., and a third at 120° F. Each lot of cream was standardized to 39.5 ± 0.5 per cent fat with skim milk from the same separation, and all samples (milk, cream and skim milk) were tested for phosphatase activity. The results are recorded in table 1.

Although some reduction in phosphatase activity of the cream resulted from increasing the separating temperature, a corresponding increase in the skim milk

fraction was not observed, which suggests the possibility that some phosphatase activity may be lost in the separator slime as the separating temperature is increased. It commonly is observed that as the temperature of separation and centrifugal clarification increases, the quantity of slime also increases, which may account for the losses in phosphatase activity observed in the cream (table 1). Actual test of the separator slime in another experiment showed it to contain considerable phosphatase activity. The reduced phosphatase activity also may be due to slight inactivation by the higher temperatures, but this is not borne out by data to be presented later. At any rate, the phosphatase apparently is held to fat more tenaciously than is agglutinin. It should not be inferred, however, that the phosphatase is entirely on the fat.

TABLE 1

Effect of separating temperature on the distribution of phosphatase in the cream and skim milk fractions

Separating temp. (°F.)	Phosphatase activity (p.p.m. phenol)			
	Milk	Cream	Skim milk	Reconstituted milk
80	2,130	10,400	1,370	2,190
100	2,100	9,400	1,360	2,090
120	2,090	8,600	1,340	2,000

Selection of phenol concentration standard for satisfactory inactivation of the phosphatase enzyme

The distribution of phosphatase activity in cream and skim milk brought up an interesting thought with respect to phenol standards for satisfactory destruction of the enzyme. If one were to pasteurize milk and select the 4 p.p.m. phenol equivalent standard as proposed by Sanders and Sager (13), cream separated from this milk would, it was thought, show a positive phosphatase test. Positive phosphatase tests on cream separated from pasteurized milk have been reported by Scharer (16), using his rapid method.

Milk was heated in the steam jacketed hot well in such a way as to give phenol equivalents greater than 4 p.p.m. and less than 4 p.p.m. and the resulting milk cooled and held two hours. The lots of milk then were heated to 80° F. and separated into 32 per cent cream and skim milk, and the phosphatase activities of the milk, cream, and skim were determined to see if the distribution of the enzyme would be essentially the same as when unheated milk was separated. When milk with a phosphatase activity of 7.5 p.p.m. was separated, the cream and skim milk showed phosphatase activities of 12.8 and 5.4 p.p.m., respectively. When milk with a phosphatase activity of 3.3 p.p.m. was separated, the phosphatase of the cream and skim milk separated form this milk were found to be 4.9 and 2.2 p.p.m., respectively.

While the cream showed much higher phosphatase activity than the milk, the differences were less than when cream and skim milk were separated from raw milk. The cream showed a positive phosphatase test even when the milk was

properly pasteurized according to the Sanders and Sager (13) standard. In another experiment in which milk was heated to give a phosphatase activity of 1.9 p.p.m. phenol equivalent and the resulting milk separated into 25 per cent cream, the cream showed a phosphatase activity of 2.8 p.p.m. phenol. Apparently this relationship would hold no matter what phenol concentration is selected unless the phosphatase were completely inactivated. However, the lower the phosphatase activity of the milk the smaller the spread between the phosphatase activity of milk and cream seems to be. Then too, values of less than 1.0 p.p.m. are not readily distinguishable by the test. To minimize the discrepancy between properly pasteurized milk and cream separated therefrom, keeping in mind the accuracy of the test, a value of 1.0 p.p.m. was adopted as the standard for satisfactory inactivation in this study. This standard requires temperatures higher, approximately 1–3° F., depending upon the corresponding holding time, than the 4 p.p.m. standard of Sanders and Sager (13). Gilcreas and O'Brien (4) indicated that higher temperatures than present pasteurization standards are necessary to kill *E. coli,* which may justify elevating the standards for the phosphatase test.

From a practical standpoint, it should be pointed out as a conclusion to this phase of the study that either the phenol standards must be varied to suit the various dairy products being pasteurized at constant time-temperature conditions or, if a single phenol standard is selected, more severe heat treatments will have to be administered to some products than others to call them properly pasteurized. The data of Sanders and Sager (14) verify this conclusion.

Kinetics of inactivation of phosphatase by heat treatment and time-temperature relationships

If the reaction is first order, as indicated by Van Bever and Straub (17), a plot of the log of the concentration against the time should yield a straight line. Milk was heated in the hot well to 143° F. and the relationship of time at constant temperature (143° F.) to the concentration is given in figure 2 for skim milk and whole milk. The reaction is not strictly first order but possibly a pseudo-first order. Much the same results were secured at higher temperatures using the Mallory unit to heat and the holding accomplished with the coil described previously. These data are given in table 2.

Van Bever and Straub (17) have derived a mathematical expression from the first order reaction rate formula and Arrhenius equation with which they should be able to predict the residual phenol concentration for any time-temperature cycle if these relationships hold. From the results shown in figure 2, this did not seem to be readily possible because the reaction did not appear to be strictly first order. As the destruction of phosphatase proceeded, the rate of destruction at constant temperature became slower with time than would be indicated by straight semi-log relationship. This difference possibly could be explained by differences in the method of determination of the phosphatase used in this investigation and that used by Van Bever and Straub (17), and differences in the range

of concentrations studied. They found the relationship to hold during up to 96 per cent destruction of the original phosphatase content.

The thermal processing work of Ball (1) suggested an approach to the problem through mathematical solution. Several investigators (6, 8, 14, 17) have

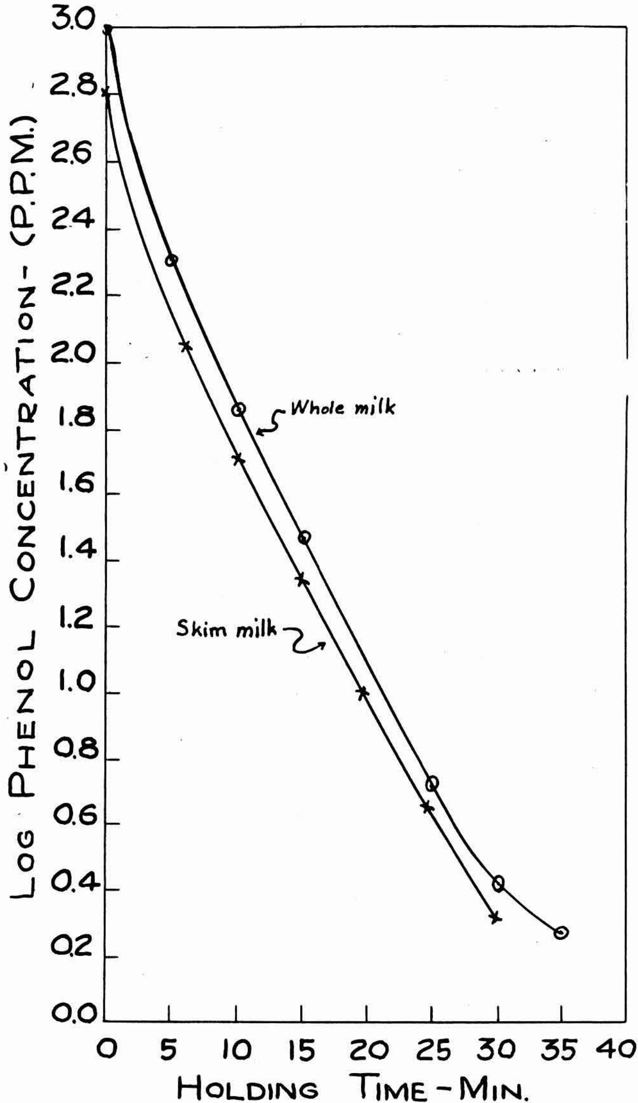

FIG. 2. Change in phosphatase concentration with time at 143° F.

shown that a plot of the temperature vs. the log time for inactivation at that temperature yields a straight line. Using 0.8-second heating time and holding in the coil described previously, the times required at various temperatures from 143 to

185° F. to reduce the phosphatase concentration to 1 p.p.m. phenol equivalent were determined. The results plotted in figure 3 show the same relationship secured by previous investigators. The formula for the curve shown in figure 3 is: $T = 174 - 9 \log t$, where T is the temperature in °F. and t is the time in seconds required at temperature T to inactivate the phosphatase. From this relationship it would seem that a practical solution for time-temperature cycles could

TABLE 2

Effect of time and temperature on the phosphatase activity in whole milk (0.8 sec. required to heat to all temperatures)

Temperature	Time	Phenol
(°F.)	*(sec.)*	*(p.p.m.)*
Raw milk	....	2,180
150	306	9.3
	361	**2.0**
	420	1.0
155	2	1,265
	32	67
	64	15
	92	1.6
	112	0.9
Raw milk	....	2,020
160	2	340
	13	6.0
	22	1.4
	32	0.6
165	2	55
	7	....
	10	1.0
	15	0.6
Raw milk	....	3,000
159	.03	2,480
165	.03	1,580
170	.03	855
175	.03	137
180	.03	4.8
185	.03	1.0
171	1	11.8
172	1	6.0
173	1	1.6
174	1	1.0
175	1	0.4

be developed if one considered a single final phenol concentration (1 p.p.m.). The destructive effect (hereafter called the D value) of any temperature for each second hold in inactivation of the phosphatase (1 p.p.m.) can be given as: $D = \log^{-1}_{10} \frac{(T - 174)}{9}$ and the summation of the D values times the time at the corresponding temperatures must be 1 or greater to insure satisfactory inactivation of the phosphatase.

Using this formula it can be shown that it would require 6 hours to inactivate the enzyme at 135° F. This was verified experimentally and this temperature was selected as a starting point for calculating the D values listed in table 3. The third column in table 3 shows the cumulative D values at any temperature as-

suming straight line heating and 1-second per degree F. heating rate. It should be stated that this was arranged for convenience in calculating cumulative D values for experimental data secured later using constant heating rates. But, according to this method, one could calculate a cumulative D value by plotting the individual D values corresponding to the temperatures used against the time in seconds. The area under the resulting curve would be the cumulative D value and this should be 1 or greater for any time-temperature cycle if the phosphatase is inactivated.

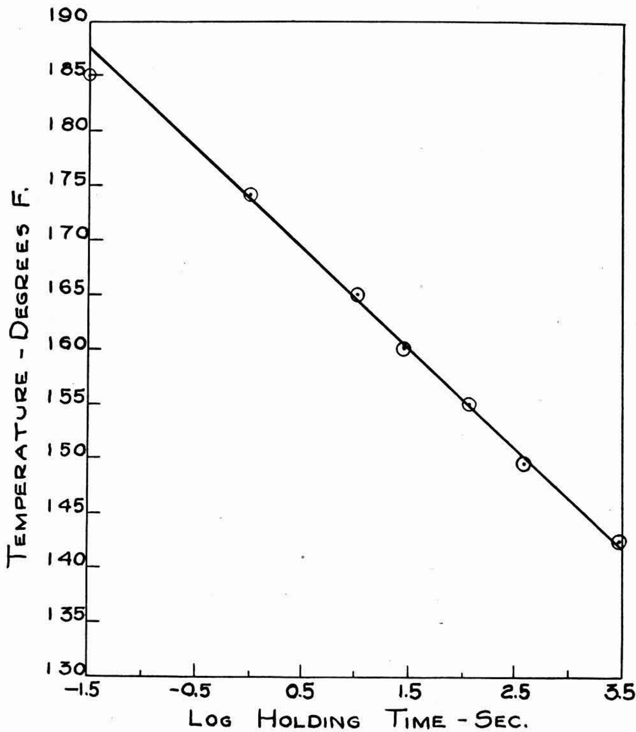

FIG. 3. Time-temperature relationship for the inactivation of the phosphatase enzyme in milk.

Effect of heating rate on temperature necessary to inactivate the phosphatase enzyme in milk

To secure some evidence on whether or not the mathematical solution has practical and proved application and to find the relationship between hot well heating at various rates and Mallory heating, the following experiments were performed.

Raw whole milk was heated in the hot wells by varying the quantities of milk and steam so that various line (constant change in temperature with time) heating and various heating rates from 2 to 20° F. per minute were secured. Samples were obtained at various temperatures and cooled rapidly in ice water as the

milk was being heated; the temperature at which the phenol concentration became 1 p.p.m. was determined. The results are listed in table 4, along with

TABLE 3

Comparative destructive effect of heat on the phosphatase enzyme activity in milk

Temperature	D value/sec. hold	Cumulative D value (1 sec./°F.) (Straight line heating)
(°*F.*)		
135	0.000046	0.000046
136	0.000059	0.000105
137	0.000077	0.000182
138	0.0001000	0.000282
139	0.000129	0.000411
140	0.000167	0.000578
141	0.000215	0.000793
142	0.000277	0.001070
143	0.000358	0.001428
144	0.000462	0.001890
145	0.000597	0.002487
146	0.000772	0.003259
147	0.00100	0.00426
148	0.00129	0.00555
149	0.00167	0.00722
150	0.00215	0.00937
151	0.00277	0.01214
152	0.00358	0.01572
153	0.00462	0.02034
154	0.00597	0.02631
155	0.00772	0.03403
156	0.0100	0.0441
157	0.0129	0.0570
158	0.0167	0.0737
159	0.0215	0.0952
160	0.0277	0.1229
161	0.0358	0.1587
162	0.0462	0.2049
163	0.0597	0.2646
164	0.0772	0.3418
165	0.100	0.442
166	0.129	0.571
167	0.167	0.738
168	0.215	0.953
169	0.277	1.230
170	0.358	1.588
171	0.462	2.050
172	0.597	2.647
173	0.772	3.419
174	1.00	4.42
175	1.29	5.71
176	1.67	7.38
177	2.15	9.53
178	2.77	12.30
179	3.58	15.88
180	4.62	20.50
181	5.97	26.47
182	7.72	34.19
183	10.0	44.2
184	12.9	57.1
185	16.7	73.8

data secured by Mallory heating and holding. The corresponding calculated cumulative D values also are shown. The dates on which the individual experi-

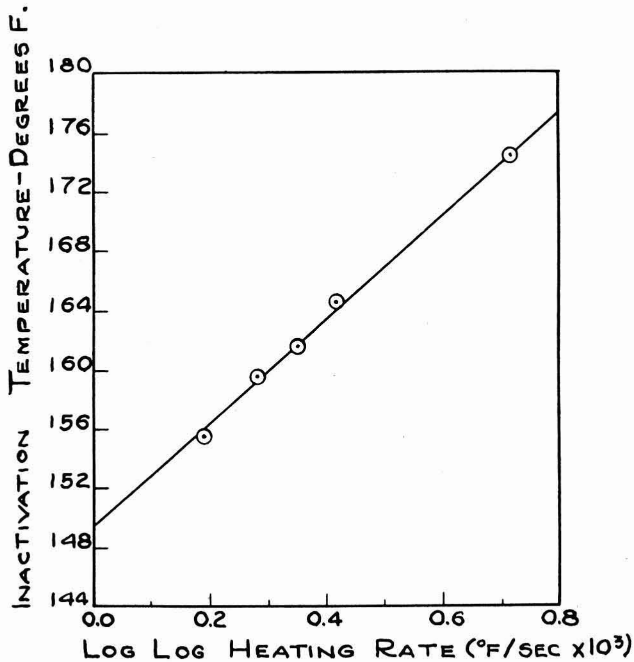

FIG. 4. Effect of heating rate on the temperature required to inactivate the phosphatase enzyme in milk.

ments were performed are given to indicate any effect season may have had on time-temperature relationships.

As a sample calculation of the cumulative *D* values, consider the first set of values given in table 4. The heating rate was 0.035° F. per second or the milk was held 28.6 seconds per °F. as it was heated from 135 to 155° F. The cumulative *D* value at 155° F. is 0.03403 if held only 1 second per °F. Therefore, by

TABLE 4

Effect of heating rate on the temperatures required to inactivate the phosphatase enzyme in milk (1.0 p.p.m. phenol equivalent)

Type of heating	Date	Heating rate	Temp.	Holding time	*D* value
		(°F./sec.)	*(°F.)*	*(sec.)*	
Hot well	10/ 1/46	0.035	155	1	0.98
	10/21/46	0.085	159	1	1.14
	9/17/46	0.167	161	1	0.99
	9/17/46	0.390	164	1	0.95
	10/ 1/46	0.178	143	3,000	1.08
	11/27/46	0.177	152	270	1.05
Mallory	8/ 9/46	137	174	1	1.03
	3/28/47	150	185	0.03	0.99
	5/14/47	120	165	10	1.00
	5/14/47	108	150	420	0.91

the time the milk reached 155° F., the D value equals 28.6×0.03403 or 0.97. To this must be added the D value × time at 155° F. = $0.0077 \times 1 = 0.0077$. Thus, the cumulative D value would be 0.98.

Figure 4 shows the relationship between heating rate and temperature necessary to inactivate phosphatase with one second hold at the top temperature. The temperature of inactivation is a log log function of the heating rate. Extending the curve to 0 log log heating rate (°F./sec. $\times 10^3$) indicates a temperature of 149° F. to be sufficient to inactivate the phosphatase. Theoretical calculations from the D values indicate a temperature of 150° F., which is fairly good agreement.

Apparently the mathematical solution is essentially satisfactory considering the constancy of the cumulative D values. If one considers an error of 1° F. in top temperature as being possible, the cumulative D values may vary approximately 0.2 and all values listed in table 4 are within this limit.

SUMMARY

Information was secured on the phosphatase activity of raw milk and the distribution of phosphatase in various milk fractions. The phosphatase is believed to be located to a large extent but not entirely at the fat-serum interface. Separation temperatures up to 120° F. did not appreciably change the distribution of the phosphatase in the cream and skim milk fractions. Separation of heated milks possessing various phosphatase activities showed that the lower the phosphatase activity in the milk the less the difference in the phosphatase activity in the cream over that in the milk. A standard of 1 p.p.m. phenol was selected as satisfactory destruction considering the limits of accuracy of the test and the smallest increase from a practical standpoint in phosphatase activity in cream over that in milk from which this cream was separated.

A study was made of the kinetics of phosphatase inactivation by heat, and time-temperature relationships for the inactivation of phosphatase are given over the temperature range of 143 to 185° F.

A mathematical solution for time-temperature cycles is given which takes into consideration accumulative effects of heating to the holding temperature in reducing the phosphatase activity to 1 p.p.m. phenol. Data secured with various rates of heating indicated that the mathematical solution is satisfactory for practical use in determining time and temperature necessary to give a negative phosphatase test in milk with various heating methods. It follows that, if the phosphatase test is used as the standard for adequate pasteurizaion, this mathematical solution can be applied to determine the proper time-temperature conditions.

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EFFECT OF HIGH–TEMPERATURE SHORT–TIME HEAT TREATMENTS ON SOME PROPERTIES OF MILK. II. INACTIVATION OF THE LIPASE ENZYME[1]

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Although there was considerable disagreement in the early literature concerning the presence of lipase enzyme in milk, the work of Rice and Markley (13) and Palmer (11) in 1922 showed lipase to be a normal constituent of cow's milk.

This enzyme is capable of splitting the glyceride structure of milk fat, liberating free fatty acids. The low molecular weight fatty acids thus formed, such as butyric and caproic acids, give the milk its characteristic rancid odor and flavor. It appears as though there may be more than one lipase present in milk (6). In general, two types of lipolysis now are recognized; one is the spontaneous and the other induced lipolysis. This study deals with the induced type of lipolysis. In induced lipolysis, the system must be "activated", possibly by changing the normal adsorbed layer surrounding the fat globules, thus making the fat more susceptible to lipase action. This can be accomplished by shaking (9), by homogenization at temperatures below 130° F. (2), and by temperature treatment between 40–80° F. (8). In this study, lipolysis was induced by homogenization at 105° F.

The lipase enzyme is heat labile. While it is not known whether both types of lipolysis respond similarly to heat treatment, the indications are that both types are destroyed under standard pasteurization conditions using the holder method. Tarassuk (14) found that 130° F. for 30 minutes would prevent the spontaneous type of lipolysis. Dorner and Widmer (2) and Halloran and Trout (5) found that pasteurization before homogenization stopped this type of induced lipolysis. Doan and Minster (1) have shown that rancidity invariably was present 24 hours after homogenization unless the milk had been previously heated to 150° F. without holding. Gould and Trout (4) found no change in fat constants or acid degree of milk fat when milk was pasteurized at 145° F. for 30 minutes and homogenized at a pressure of 1,500 lb. per square inch. In their experiments, homogenization of raw milk caused an average increase of 1,625 per cent in fat acidity during the first 24 hours of storage at 35–40° F. In an experiment where milk was heated to 140, 150, 160 and 170° F. without holding and the milk subsequently made into sweetened condensed milk, Rice (12) found that only the milk preheated to 140° F. developed rancid flavor after 8 months of storage at room temperature. When milk was heated in the

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presence of sugar, a temperature of 180° F. was necessary to prevent this defect. Palmer (11) stated that heating milk to 75° C. (167° F.) for a few minutes would stop rancid and bitter flavor development. In these studies the heating times were not given.

The present study was designed to secure more information on the holding time of temperatures from 145 to 185° F. which is necessary to prevent the lipolysis induced by homogenization of raw milk, with extremely short heating and cooling times.

EXPERIMENTAL METHODS

The small-tube Mallory heat exchanger and the copper holding coil described previously (7) were used to process the milk. Raw whole milk was heated to 105° F. in the steam-jacketed hot well. The milk then was homogenized at a pressure of 1,500 lb. per square inch and pumped through the heat exchanger by means of the homogenizer acting also as a high pressure pump. Within 5 seconds after homogenization, the milk was heated to the desired holding temperature. After the desired holding times had elapsed, samples were drawn out of the holding coil into test tubes immersed in ice water. All samples were stored 44–48 hours at 40° F. before examination for rancid flavor and other properties.

Preliminary experiments were run to ascertain the most applicable methods for determining the extent of lipase action. Gould and Trout (4) stated that fat acidity was the most reliable criterion, but difficulty was experienced by the present authors in churning the homogenized milk and extraction methods were not considered reliable. Titratable acidity and pH measurements of the milk did not prove as sensitive as organoleptic examination. Doan and Minster (1), Halloran and Trout (5), and Tarassuk and Henderson (15) have used surface tension changes as an index of extent of rancidity. The usual surface tension of the milk used in this study was about 44 dynes/cm. at 20° C. and the surface tension of butyric acid is about 26 dynes/cm. at 20° C. Preliminary experiments indicated that surface tension changes were sufficient when rancidity developed to use this as a measure of lipase activity. These measurements always were supplemented with organoleptic examination.

Surface tension was measured with a du Nouy tensiometer at 20–21° C. Measurements were made in a constant temperature (21° C.) room after the samples had been brought to a temperature of 20° C. in a water bath. The tensiometer was standardized to absolute units with materials of known surface tension and the results are reported in dynes per centimeter. Each reported result is an average of at least three determinations whose maximum variations were within ± 0.5 dynes/cm. of the average. Organoleptic examinations were made on the same samples. The samples were scored by at least three competent judges for rancid flavor, and the results reported on the basis of 0 for no rancid flavor, ? for questionable, 1 for definite, 2 for pronounced, and 3 for very pronounced rancidity.

EXPERIMENTAL RESULTS

Raw milk was heated in 0.83 second to various temperatures within 5 seconds after homogenization, held for various lengths of time and then rapidly cooled. The changes in flavor and surface tension of the milk after 44–48 hours of storage at 40° F. are shown in table 1. Four different lots of milk were used to secure the data listed. No rancid flavor developed in any of the lots of raw unhomogenized milk in the 44 to 48-hour storage period at 40° F., and the surface tension of these lots of milk varied from 44.0 dynes/cm. to 44.7 dynes.

TABLE 1

Effect of heating milk on lipolytic action induced by homogenization of raw milk at 105° F.

Lot no.	Sample no.	Temperature	Time held	Rancid flavor[a]	Surface tension[a]
		(°F.)	*(sec.)*		*(dynes/cm. 20° C.)*
1	1a	177	0.03	2	42.0
	b	180	0.03	2	41.7
	c	184	0.03	?	42.4
	d	186	0.03	0	45.4
1	3a	160	2	2	39.9
	b	163.5	2	2	41.9
	c	167	2	0	44.0
	d	169	2	0	44.8
2	2a	164	1	3	39.5
	b	167	1	2	39.5
	c	170	1	?	43.5
	d	173	1	0	44.5
3	4a	160	2	2	40.6
	b	160	6	1	42.6
	c	160	10	0	45.4
	d	160	14	0	45.6
3	5a	155	2	3	39.3
	b	155	12	1	42.1
	c	155	22	0	44.7
	d	155	32	0	45.0
4	6a	150	33	2	40.8
	c	150	60	?	43.2
	d	150	120	0	44.0
2	7a	145	200	1	41.5
	b	145	300	?	43.2
	c	145	400	?	43.8
	d	145	500	0	44.0

[a] After storage at 40° F. for 44–48 hours.

Very pronounced rancid flavor was present in each of the lots of milk which were homogenized raw at 105° F. and stored 44–48 hours at 40° F. The surface tension of the raw homogenized milk after storage ranged from 35.0 dynes/cm. to 37.1 dynes/cm.

When milk was heated sufficiently to prevent lipolytic activity, as indicated by the organoleptic examination, the surface tension of the heated milk was essentially the same as that of raw unhomogenized milk. Decreases in surface tension accompanied the rancid flavor development on those samples which were

not heated sufficiently to stop lipase activity. This is in agreement with the work of others (1, 5, 15).

From the data in table 1 and consideration of other trials in which flavor changes only were observed, the time-temperature heat treatment relationships given in figure 1 are believed to be ample for prevention of lipolytic action induced by homogenization of raw milk.

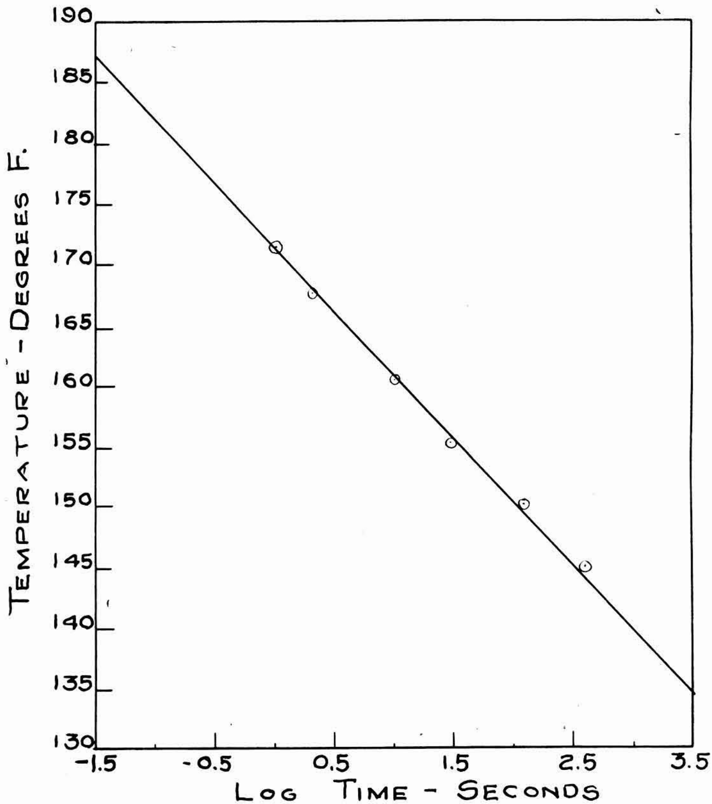

FIG. 1. Time-temperature relationship for the inactivation of the lipase enzyme in milk.

The data plotted in figure 1 show temperature to be a straight line function with log time, as was the case with the inactivation of the phosphatase (7). At 185° F. the time of inactivation is the same for both phosphatase and lipase, but the slope of the lipase curve is greater than that of the phosphatase; *i.e.*, as the temperature is lowered, lipase is inactivated with progressively less time of exposure than is the phosphatase.

If one extrapolates the curve in figure 1 to 30 minutes holding time, a temperature of 137° F. is indicated as necessary to prevent this induced type of lipolysis. Krukovsky and Sharp (10) found that a temperature of 135° F. for 30 minutes was required to prevent lipolysis induced by the warming and cool-

ing procedure of Krukovsky and Herrington (8); Tarassuk (14) found 130° F. for 30 minutes was sufficient to prevent the spontaneous type of lipolysis. Either there is a difference in the heat treatment necessary to inactivate the enzyme in the two systems, or perhaps the discrepancy could be explained by the differences in methods used to follow lipase activity or by the differences in the rates of heating to and cooling from the holding temperatures employed.

Effect of Rate of Heating

These samples were heated to the temperatures indicated within 5 seconds after homogenization. In unheated samples rancid flavor developed within 5 to 10 minutes after homogenization. With a slow rate of heating (5° F. per minute), such as would be the case if a commercial pasteurizing vat were used, the rancid flavor developed before the holding temperature was reached. If homogenization were done after heating, one could demonstrate the importance of heating rate. In one experiment, milk was heated at a constant rate of approximately 5° F. per minute and portions were homogenized as the milk was being heated. These were cooled immediately over the surface cooler and stored at 40° F. A temperature of 142° F. was sufficient to prevent the development of the rancid flavor for at least 48 hours. The results of previous experiments listed in table 1 show that a temperature of 185° F. without holding is necessary to prevent lipolysis when the heating is accomplished in 5 seconds. The effect of heat is accumulative and the temperature and holding time necessary to inactivate the enzyme will be determined by how rapidly one heats to and cools from the temperature at which the milk is being held.

Influence of Copper on Lipolysis Induced by Homogenization

Herrington and Krukovsky (6) found that additions of 0.2–0.4 p.p.m. copper reduced lipolysis almost 20 per cent. Krukovsky and Sharp (10) showed that in the absence of dissolved oxygen, copper in concentration of 2–8 p.p.m. had almost no inhibiting effect on lipolysis induced by temperature manipulation (8). Gould's (3) results showed copper to have no significant effect on the extent of lipolysis in raw milk induced by homogenization. In this study, holding of the milk at various temperatures was accomplished by use of a copper holding coil, so the milk possibly was contaminated with copper. The previous results secured in this study might not be truly representative of the effect of heat treatment alone, but might be due to the combined effects of heat and copper.

Accordingly, raw milk was heated (105° F.), homogenized, and immediately heated to 145° F. with the Mallory, as in previous experiments. The milk was collected in test tubes and immersed in a constant temperature (145° F.) bath, held for various times and cooled in ice water. Flavor and surface tension determinations were made after 44 hours of storage at 40° F. The results, as well as those secured by the copper coil holding method, are given in table 2. These results indicate that there was no appreciable difference in degree of lipolysis whether milk was held in the copper holding coil or in glass test tubes immersed in water bath. Similar results were secured at 153° F. It was found

in one trial that milk which was held in the copper coil for 10 minutes at 145° F. contained 8.5 p.p.m. copper. It is believed that this would be above the maximum amount of copper which would be present in any sample in the study because the coil had not been used for quite some time and the accumulated copper oxide was not thoroughly removed previous to the trial. The control milk not exposed to the coil contained 0.11 p.p.m. Even the 8.5 p.p.m. copper secured under the most drastic treatment is below the level (10 p.p.m.) which Gould (3) found to have no effect on lipolysis.

TABLE 2

Effect of heating homogenized raw milk to 145° F. and holding various lengths of time in test tubes vs. holding in copper holding coil on the extent of lipolysis after 44 hours of storage at 40° F.

Samples[a]	Time	dynes/cm. (20° C.)	Rancid flavor
	(sec.)		
1	200	41.5	1.0
1a	200	43.7	1.0
2	300	43.2	?
2a	300	43.9	1.0
3	400	43.8	?
3a	400	44.4	?
4	500	44.0	0
4a	500	44.5	0
Raw	not heated	37.1	3
Raw a	" "	37.9	3

[a] Sample numbers followed by *a* were held in glass tubes; others were held in copper coil.

SUMMARY

The holding times at various temperatures from 145–185° F. required to prevent lipolysis induced by homogenization of raw milk were determined. A semi-log relationship of temperature with time was observed. At 145° F., approximately one-third the time was required to inactivate the lipase as was required to inactivate the phosphatase (7), but at 185° F. the same time was required to inactivate both enzymes.

Added copper had no noticeable effect on the time-temperature relationships for inactivation of lipase under the conditions of these experiments.

The time required at any temperature to inactivate the lipase was found to vary with the rate of heating to and cooling from the holding temperature. When milk was heated at the rate of 5° F. per minute, instantaneous exposure at 142° F. was sufficient to inactivate the enzyme, while heating by means of the Mallory unit within 5 seconds required a temperature of 185° F. with instantaneous exposure.

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PROPERTIES OF THE COLOSTRUM OF THE DAIRY COW. II. EFFECT OF PREPARTAL RATIONS UPON THE NITROGENOUS CONSTITUENTS[1]

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The importance of colostrum in sustaining the health of neonatal dairy calves has received renewed emphasis in recent years, yet little is known concerning factors affecting the properties of this product. One phase of a study of the relation of preparturient dairy rations to the health of the cow, to the well-being of the newborn calf, and to the properties of colostrum included an investigation of the effect of level of protein intake on concentrations of nitrogenous constituents in the mammary secretions during the postpartum transition from colostrum to milk. This phase of the investigation is reported herein.

PROCEDURES

Feeding and management of experimental animals. Twenty pregnant heifers and cows were paired according to breed and to number and stage of gestation. The heifers included two pairs of Guernseys and one pair each of Holsteins, Ayrshires and Jerseys. The cows, all in their second gestation, included two pairs each of Holsteins and Ayrshires and one pair of Jerseys. All animals calved within the 5-month period from the middle of November, 1945, to the middle of April, 1946.

Seven weeks (average) before parturition, one cow of each pair was given a high-protein ration consisting of a concentrate mixture (approximately 25 per cent crude protein), Atlas sorgo silage and alfalfa hay. The other cow of each pair was fed a low-protein ration consisting of corn, Atlas sorgo silage and prairie hay. Silage and hay were fed in the ratio of 3 : 1 to the extent of the appetite of each animal, and concentrates were given at the rate of 10 lb. per 1,000 lb. body weight. The same quantity of concentrate was fed throughout the trial, the initial body weights being used as a base for establishing the level of feeding. Starting on the ninth day postpartum, all cows were changed to the regular herd ration consisting of alfalfa hay, sorghum silage and a concentrate mixture containing approximately 16 per cent crude protein.

Collection of samples and analytical procedures. The calves were not allowed to nurse. The mammary secretions were withdrawn as completely as possible by standard milking methods, either hand or machine, at approximately 12-hour intervals The first collection was made as soon as possible after parturition, usually within 4 hours. The total mammary secretions removed at each milking were well mixed before sampling. Colostrum and milk from each cow were ana-

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lyzed separately for each of the first six milkings and as composites for the seventh and eighth, the fifteenth and sixteenth, and the twenty-seventh and twenty-eighth milkings. Samples that were not analyzed immediately after collection were stored in a refrigerator at approximately 4° C. for a period not exceeding 4 days.

Rowland's method (14) was used to determine the nitrogen distribution. The only modifications of the method were the omission of selenium oxychloride as catalyst in the digestion procedure and the use of smaller samples for analysis. The amount of sample was reduced for two reasons: first, colostrum has a higher protein content than milk, which was the product for which the method was designed; and second, a micro-Kjeldahl apparatus was used in the distillation. The quantities of reagents for digestion and distillation were adjusted accordingly. Albumin and globulin nitrogen were not separated but were computed together by subtracting the values for non-protein nitrogen from those of non-casein nitrogen. Percentages of total protein and of casein were calculated from values for total nitrogen, non-protein nitrogen and non-casein nitrogen. Corrections were not applied to adjust for the volumes occupied by the precipitates of protein and fat.

RESULTS

Although considerable variation among the cows was observed, colostrum and early milk from animals of both groups had a similar total protein content and distribution of the components (fig. 1). While total protein, casein and albumin-globulin values for cows receiving the low-protein ration tended to be slightly higher during the first few milkings than were the values for corresponding samples from the high-protein group, the differences at none of the various periods were significant (t-test, $P = 0.05$). Concentrations of non-protein nitrogen were greater in both colostrum and early milk from cows receiving the high-protein ration than in these secretions from cows fed the low-protein ration. The differences, however, were significant only in samples representing the fourth, the fifth, the sixth, the seventh and eighth, and the fifteenth and sixteenth milkings. There was no significant difference in non-protein nitrogen of milk collected from the two groups on the fourteenth day (twenty-seventh and twenty-eighth milkings), which was 6 days after all cows had been changed to the regular herd ration.

Concentrations of the protein components tended to decrease logarithmically during the first four or five milkings of the transition period, the rate of change being similar for both groups (fig. 1). A markedly lower rate of change in the concentrations of protein components was evident by the sixth milking, the retardation seeming to occur earlier in the casein than in the albumin-globulin fraction. A subsequent gradual decline continued to the end of the second week, the time of final sample collection. The changes noted in total protein largely reflected changes in the albumin and globulin.

Non-protein nitrogen also followed a logarithmic decline which, except for the increases from the first to the second milkings, seemed to continue at approxi-

mately the same rate for the first 16 milkings. The physiological significance of the occurrence of higher values at the second than at the first milking is obscure. This increase was observed in colostrum of 14 of the 20 cows, and for each of the remaining animals the decreases from the first to the second milking were less than 0.002 per cent nitrogen.

Previous studies (6, 10), which indicate that colostrum from first-lactation cows is higher in vitamin A than that from cows in later lactations, suggested consideration of the relation of lactation number to the nitrogenous components of the initial mammary secretions. Analysis of the intra-group data (table 1) indicates that first-lactation cows receiving the high-protein ration secreted more albumin and globulin in the mammary products, particularly in the first three milkings, than did second-lactation cows of the same dietary group. These differences were reflected further in total protein. Concentrations of albumin-globulin nitrogen, however, were similar in the mammary secretions from first- and

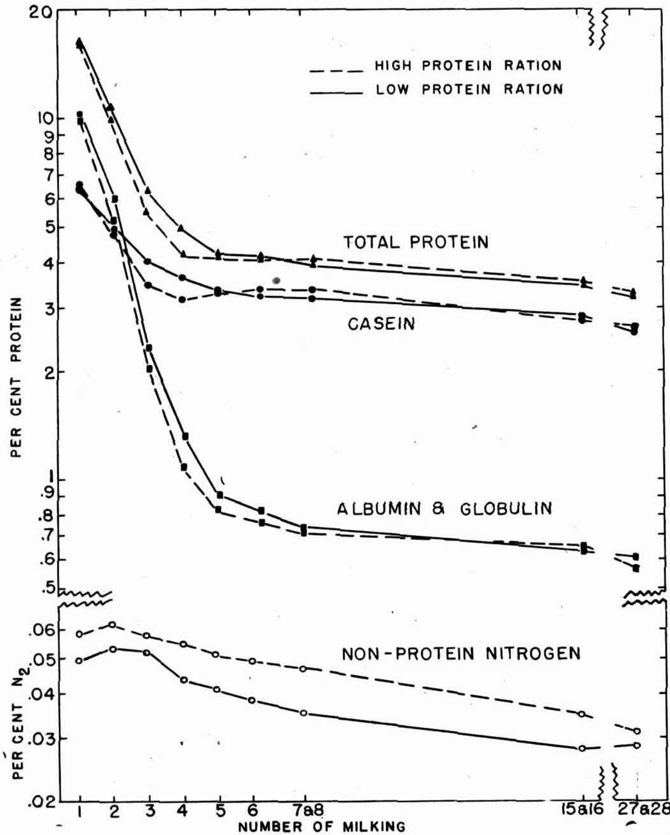

FIG. 1. Distribution of nitrogen fractions of colostrum and early milk from two groups of cows receiving high- and low-protein rations, respectively. Each group is composed of five first- and five second-lactation cows.

TABLE 1

Distribution of nitrogen fractions of colostrum and early milk from groups of cows receiving either a high- or a low-protein ration

Ration	Lactation	No. of milking 1	2	3	4	5	6	7 + 8[a]	15 + 16[a]	27 + 28[a]
		Total protein, %								
High protein	1[b]	19.86 ± 2.99[c]	13.03 ± 3.47	6.67 ± 1.39	4.52 ± 0.37	4.30 ± 0.41	4.30 ± 0.19	4.18 ± 0.29	3.60 ± 0.25	3.39 ± 0.19
	2	12.95 ± 1.60	6.81 ± 1.41	4.30 ± 0.82	3.95 ± 0.55	3.89 ± 0.36	3.96 ± 0.35	3.85 ± 0.29	3.42 ± 0.34	3.04 ± 0.40
Low protein	1	16.74 ± 1.73	10.95 ± 1.70	6.04 ± 1.10	4.46 ± 0.66	3.94 ± 0.32	3.90 ± 0.16	3.72 ± 0.26	3.44 ± 0.18	3.02 ± 0.38
	2	16.29 ± 5.12	10.39 ± 4.67	6.50 ± 2.09	5.42 ± 1.19	4.50 ± 0.74	4.32 ± 0.50	4.10 ± 0.44	3.47 ± 0.59	3.30 ± 0.16
		Casein, %								
High protein	1	6.81 ± 1.64	5.44 ± 0.97	3.75 ± 0.55	3.23 ± 0.17	3.36 ± 0.26	3.47 ± 0.16	3.43 ± 0.25	2.93 ± 0.16	2.75 ± 0.18
	2	6.24 ± 0.79	4.15 ± 1.24	3.20 ± 0.59	3.09 ± 0 42	3.17 ± 0.27	3.25 ± 0.30	3.18 ± 0.23	2.80 ± 0.26	2.56 ± 0.33
Low protein	1	6.32 ± 1.13	5.59 ± 0.79	3.66 ± 0.72	3.23 ± 0.35	3.10 ± 0.16	3.08 ± 0.34	3.05 ± 0.20	2.82 ± 0.09	2.47 ± 0.29
	2	6.19 ± 1.32	4.39 ± 0.72	4.31 ± 0.93	4.02 ± 0.76	3.54 ± 0.51	3.41 ± 0.44	3.30 ± 0.34	2.85 ± 0.54	2.65 ± 0.13
		Albumin plus globulin %								
High protein	1	13.06 ± 1.88	7.62 ± 2.76	2.90 ± 0.98	1.29 ± 0.31	0.94 ± 0.19	0.83 ± 0.13	0.74 ± 0.07	0.67 ± 0.10	0.62 ± 0.14
	2	6.73 ± 1.16	2.66 ± 0.19	1.15 ± 0.24	0.86 ± 0.14	0.72 ± 0.11	0.70 ± 0.13	0.67 ± 0.08	0.62 ± 0.08	0.48 ± 0.06
Low protein	1	10.44 ± 2.63	5.38 ± 1.33	2.31 ± 0.65	1.26 ± 0.32	0.85 ± 0.17	0.72 ± 0.13	0.67 ± 0.10	0.62 ± 0.10	0.55 ± 0.12
	2	10.10 ± 4.86	6.52 ± 4.81	2.30 ± 1.36	1.39 ± 0.65	0.97 ± 0.27	0.90 ± 0.19	0.80 ± 0.12	0.62 ± 0.06	0.65 ± 0.13
		Non-protein nitrogen, %								
High protein	1	0.061 ± 0.007	0.066 ± 0.009	0.062 ± 0.008	0.060 ± 0.012	0.054 ± 0.006	0.052 ± 0.008	0.049 ± 0.004	0.036 ± 0.004	0.031 ± 0.003
	2	0.055 ± 0.008	0.058 ± 0.009	0.053 ± 0.009	0.049 ± 0.006	0.048 ± 0.005	0.046 ± 0.003	0.044 ± 0.006	0.033 ± 0.006	0.031 ± 0.005
Low protein	1	0.047 ± 0.008	0.050 ± 0.008	0.051 ± 0.009	0.046 ± 0.006	0.042 ± 0.004	0.041 ± 0.002	0.039 ± 0.005	0.029 ± 0.002	0.032 ± 0.003
	2	0.052 ± 0.015	0.058 ± 0.015	0.050 ± 0.010	0.041 ± 0.009	0.039 ± 0.008	0.035 ± 0.007	0.031 ± 0.006	0.026 ± 0.003	0.024 ± 0.010

[a] Composite samples.
[b] Five cows in each group.
[c] Standard deviation.

second-lactation cows receiving the low-protein ration. Furthermore, albumin-globulin values for both heifers and cows of the latter group were between those of the two groups receiving the high-protein ration. Only small differences were noted between the casein concentrations of colostrum of first- and of second-lactation cows in either dietary group.

Levels of non-protein nitrogen in colostrum and early milk from first-lactation cows receiving the high-protein ration were higher than those from second-lactation cows. Similar comparisons of colostrum from cows of the two lactation groups receiving the low-protein ration indicated slightly higher non-protein nitrogen values for first-lactation cows only after the first two milkings.

Deviations from the mean of values of the nitrogenous constituents (table 1) are considerably greater during the early colostral period than later, as the composition of milk approaches normal, further indicating that colostrum is a more variable product than milk.

As might be expected from studies of normal milk (9), data (not shown) suggested that Guernseys and Jerseys produced colostrum of a slightly higher protein content than did Holsteins and Ayrshires. It is recognized, however, that too few animals were used to warrant conclusions relative to breed differences.

Observations of the development and condition of the mammary glands were made in conjunction with the present study. The severity of edema, as determined by palpation and by macroscopic examination, was more pronounced in heifers than in cows and did not seem to be associated primarily with the prepartal rations the animals received (17). Attempts to correlate the total protein and the albumin-globulin contents of early colostrum with the degree of edema were successful only to the extent that the average of each of these protein fractions was higher in colostrum from the ten cows judged to have the more severe edema than from the ten cows with the less severe edema. It also was found that rate of decline of total protein and of albumin-globulin contents in mammary secretions during the transition period was not related to degree of mammary congestion.

DISCUSSION

The results presented herein are in accord with previous reports indicating that the casein and the albumin and globulin contents of colostrum decrease as it changes to normal milk (3, 4, 5, 13, 15). Grimmer (5) pointed out that the proteins of colostrum tend to decrease according to a logarithmic curve during the transition period. In the present study, the decline persisted at the initial rate for only four to six milkings, a somewhat shorter interval than observed for tocopherols (11) and for vitamin A and carotenoids (10).

The effect of high-protein intake on the concentration of non-protein nitrogen of colostrum is similar to that reported for milk (7, 12). The increased protein consumption raised the non-protein nitrogen, not only of the colostrum but also of the blood serum (2). Determinations of the kinds of non-protein nitrogen in mammary secretions and in the blood serum were not made. Other investigators (1) found that in milk from cows fed iodinated casein, urea constituted

approximately one-half of the non-protein nitrogen; whereas in blood plasma, urea frequently accounted for two-thirds or more of the non-protein nitrogen. Results from feeding high- and low-protein rations to cows in normal milk production indicated that a major portion of the increase of non-protein nitrogen of milk from cows fed the former ration was attributable to urea (12).

Differences in the albumin-globulin concentrations in colostrum from first- and second-lactation cows receiving the high-protein ration are too large to be dismissed as a chance occurrence, but interpretation of results is obscured by the fact that similar differences did not occur in colostrum from the two lactation groups receiving the low-protein ration. Although protein quality is not considered to be an important nutritional factor in the case of ruminants, the fact that the proteins in the two diets were not the same might have contributed to differences in the nitrogen fractions of colostrum from cows of the two experi- perimental groups.

Antibodies, which are believed to be important for the well-being of the newborn, are transmitted from cow to calf through the globulins of colostrum (8, 16). Hypoproteinemia is reported to cause a decrease of antibodies and a lowered resistance to infection (16). This observation raises the question of whether first-lactation cows receiving the high-protein ration provided increased amounts of antibodies along with the high levels of albumin-globulin nitrogen of their colostrum.

SUMMARY

Twenty pregnant heifers and cows were paired according to breed and to number and stage of gestation. For 7 weeks (average) before parturition, one group received a high-protein ration and the other a low-protein ration. The rations did not effect a significant difference in the levels of total protein, of casein, and of albumin-globulin fractions of colostrum and early milk from the foregoing groups. Non-protein nitrogen levels were higher in colostrum and early milk from cows of the high-protein group, but the differences were significant only in samples after the first three collections postpartum.

The decrease in concentrations of the protein fractions of mammary secretions during the transition period tended to follow a logarithmic curve for the first four to six milkings, after which the rate of decline was less rapid. Changes in non-protein nitrogen seemed to continue at approximately the same logarithmic rate in samples representing the second through the sixteenth milkings. Rates of change of the nitrogenous constituents were similar in colostrum and in milk from cows receiving either the high- or the low-protein rations.

Analysis of intra-group data indicated that colostrum from first-lactation cows receiving the high-protein ration contained higher levels of albumin-globulin nitrogen than did second-lactation cows receiving the same ration. Only small differences were observed after the first four milkings. Similar differences between heifers and cows fed the low-protein ration were not evident; the values for both of these lactation groups were between those of the heifers and cows receiving the high-protein ration.

Deviations from the mean of values for nitrogen fractions for individual cows within the various groups were considerably greater during the early colostral period than later, as the composition of milk approached normal.

Total protein and albumin-globulin contents of early colostrum were related to degree of mammary edema only to the extent that the averages of each of these protein fractions were higher from the ten cows judged to have the more severe edema than from the ten cows with the less severe edema. The rate of decline of total protein and of albumin-globulin contents of mammary secretions during the transition period was not related to the degree of mammary edema.

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THE VALUE OF WINTER PASTURE AND SWEET POTATO MEAL FOR LACTATING DAIRY COWS[1]

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During recent years, considerable attention has been devoted to the value of winter grazing in the South as a means of reducing feed costs. In many of the southern states, it is estimated that the acreage utilized for winter pasture crops has more than doubled during the past 5 years. This important trend is destined to have a significant influence on the future of the dairy industry in the South.

Therefore, the present study was made in an effort to secure information about the comparative economic and physiologic value of winter grazing versus dry roughage feeding for lactating dairy cows under Georgia conditions. Furthermore, the experiment was so designed that additional information also could be secured on the feeding value of sweet potato meal. A previous study (5) showed no significant difference in the milk and butterfat production or in the liveweights of dairy cows when they were fed a grain mixture consisting of 36 per cent corn or sweet potato meal. Sweet potato meal also was observed to be as palatable as corn when fed in this proportion.

REVIEW OF LITERATURE

Various investigators have pointed out some of the beneficial effects of good grazing upon the quantity (13, 15) and quality (2, 3, 4, 6, 7, 9, 10, 11, 16, 17) of milk produced and the economic aspects (12, 18) of milk production during the seasons of the year when pasture can be provided.

Neel (13) calculated that Balboa rye provided grazing on an average of 169 days per winter in certain sections of Tennessee. A crimson clover and rye grass winter pasture at the University of Georgia Dairy Farm carried at least one cow per acre for 198 days during the winter months of 1946.

Hodgson *et al.* (8) observed an increase in milk production of about 25 per cent when the cows were changed from dry roughage feeding to pasture.

The work of Smith (18) showed that the dairy cow obtained 84.1 per cent of her total feed requirements from grazing during the pasture season of 204 days in the limestone area of southern Indiana. One hundred lb. of digestible nutrients were furnished by silage and hay at a cost of 92 cents and $1.08, respectively. On the other hand, 100 lb. of digestible nutrients were obtained from pasture at an average cost of 20.1 cents. Calculated on the average cost per 100 lb. of digestible nutrients of feeds other than pasture fed to all livestock

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over a 5-year period, permanent pasture provided $8.00 worth of feed at a cost of $1.56 per acre or 19.7 cents per 100 lb. of digestible nutrients.

In studies conducted by the United States Department of Agriculture (17), pasture furnished about 33 per cent of the total nutrients required by cows producing market milk, while constituting only about 14.1 per cent of the total feed cost. Moore at the Mississippi Station (13) reported that the feed cost was $1.21 per 100 lb. of milk produced when the cows were fed harvested roughages as compared with 69 cents (not including the cost of pasture) when the cows were provided pasture.

EXPERIMENTAL PROCEDURE

Fifteen cows of the University of Georgia Dairy Herd (three Guernseys and 12 Jerseys) were selected and balanced into three groups as evenly as practicable on the basis of age, breed, weight, current production, number of previous lactations, length of dry period and number of days fresh at the start of the experiment. These groups were divided further into five similar outcome groups. The cows were fed identical rations (ration *A*) during a 14-day preliminary period prior to being placed on the experimental ration (table 1).

TABLE 1

Rations used in study

Ingredients	Ration A	Ration B	Ration C	Ration D
Concentrate				
Ground corn	200	200	200	....
Sweet potato meal	. . .	....	....	200
Oats	200	200	200	200
Wheat bran	100	100	100	100
Soybean meal	150	....	....	...
Cottonseed meal	....	150	150	150
Steam bone meal	6	6	6	6
Salt	6	6	6	6
Roughage				
Silage, lb.	6 per cwt.	....	....	....
Hay, lespedeza	none	ad lib	a lib	ad lib
Winter pasture[a] (grazing hr. daily)	none	8	none	none

[a] Seeding rate was 5 bu. oats, 2 bu. barley and 20 lb. vetch per acre. Fertilizer (10–4–2) was applied at the rate of 200 lb. per acre at time of seeding on Oct. 28, 1946.

Rations *B* and *C* were composed of the same constituents except that grazing was provided in addition when the cows were being fed ration *B*. Rations *C* and *D* were of the same composition except that corn was supplied as the main source of carbohydrate in ration *C*, while sweet potato meal was the main source of carbohydrate in ration *D*.

The study was conducted during the winter of 1947 for three periods of 28 days each. A 4-day change-over period preceded each experimental period for the purpose of counteracting partially any carry-over effect that the previous ration may have caused and to give the cows an opportunity to become adjusted to the change in feed. The animals were quartered either in the stanchion barn or in a dry lot during the entire time of the study except one group which was

on a 22-acre winter pasture during the day. The pasture group (fed ration *B*) was permitted to graze approximately 8 hours each day, in addition to being fed lespedeza hay and concentrates.

Collection of milk samples. Samples of milk were collected from each cow during the 14-day preliminary period and scored for flavor for the purpose of eliminating any cow that might be giving abnormally-flavored milk. Composite evening and morning milk samples were taken from each feed group twice weekly during the experiment. The samples were placed in storage at a temperature of 40° F. and scored within 15 hours. The butterfat test of each cow's milk was determined biweekly.

Feed samples. A composite sample of the concentrate mixture fed during each experimental period was analyzed for the percentages of moisture, fiber, crude protein, ash, fat and N.F.E.

RESULTS

Physiological condition of the cows. The health and general condition of the cows throughout the study were excellent, except that two cows developed mastitis soon after the study was started. Milk from these cows was not used in the flavor study. Recurrence of this condition throughout the study made it necessary to calculate the milk and butterfat production data of these cows according to the Yates method (20).

The feces of the cows that were fed rations *C* (containing 30.2 per cent corn) and *D* (containing 30.2 per cent sweet potato meal) were firm in contrast to the loose, slightly watery feces of cows that received ration *B* (containing pasture). The liveweight data collected showed no definite trend for or against either of the three rations.

Feed consumption and palatability. All the rations were eaten readily the first time that they were offered. The total roughage consumption of the cows that were fed rations *C* and *D* was 8,003 lb. and 8,154 lb., respectively. Obviously, the small difference between the roughage consumptions of the two groups would be statistically non-significant.

When the cows were allowed to graze winter pasturage an average of 8 hours daily (ration *B*), they consumed only 4,358 lb. of dry roughage or 45.6 per cent (1.82 tons) less hay than did the cows on ration *C*. With hay selling at $35.00 per ton, the winter grazing had an average value of $4.25 per cow for a period of 28 days as a dry roughage supplement. This does not take into consideration the increase in milk production (to be discussed later) or the decrease in grain consumption which resulted when the cows went on pasture. The greatest percentage of the dry roughage consumed by the cows on ration *B* was eaten at night when green grazing was not available. Even a greater percentage of the nutrients would have been obtained from grazing had the pasture been seeded earlier.

Chemical analysis of feeds. The concentrate part of each experimental ration was analyzed chemically. These data (table 2) show that ration *B* and *C* (containing corn) had an average of 1.43, 0.83 and 1.05 per cent more moisture,

TABLE 2

Chemical analyses of concentrate mixtures[a]

Period	Ration	Moisture	Ash	Protein	Fat	Fiber	N.F.E.
		(%)	(%)	(%)	(%)	(%)	(%)
I	B and C	9.33	4.15	16.69	4.08	8.48	57.27
	D	7.88	5.10	16.25	3.24	8.79	58.74
II	B and C	8.90	4.21	19.06	4.21	9.10	54.52
	D	8.06	5.21	18.13	3.09	10.04	55.47
III	B and C	10.86	4.75	17.69	4.48	8.97	53.25
	D	9.97	4.96	16.56	3.30	9.71	55.50
Av.	B and C	9.97	4.37	17.81	4.26	8.85	55.01
	D	8.54	5.09	16.98	3.21	9.23	56.57

[a] Chemical analyses of feeds were made by the department of the state chemist.

crude protein and fat, respectively, than did ration *D* (containing sweet potato meal). On the other hand, ration *D* contained 0.72, 0.38 and 1.56 per cent more ash, fiber and N.F.E., respectively, than did rations *B* and *C*.

Milk and butterfat production. The lactation curves (fig. 1) represent the mean milk yields of outcome groups 2, 4 and 5. The data from outcome groups 1 and 3 were not included, because one of the cows in each of these groups, as pointed out earlier in the report, had recurring attacks of mastitis. A decided increase in milk production occurred in every instance when the cows were changed from dry roughage feeding to pasture. Conversely, when the animals were changed from winter grazing to dry roughage feeding, there was a sharp

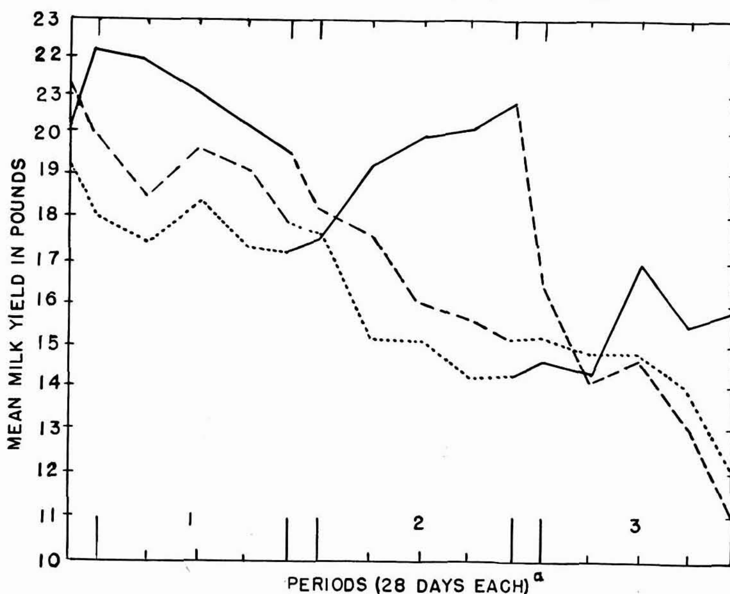

FIG. 1. Lactation curves showing the effect of the test rations on the cows during each of the experimental periods—a 4-day change-over period preceded each experimental period. (Ration B————; Ration C— — — — —; Ration D..................)

drop in milk production. The total milk production of the cows[3] fed rations *C* and *D* was 8,805 and 8,618 lb., respectively, while the milk yield of those that were fed ration *B* was 10,179 lb. The milk production of the cows while on winter grazing was 15.6 per cent greater than that produced when they were fed ration *C*. The milk yield of the cows fed ration *C* was 2.2 per cent greater than that of the cows fed ration *D*.

The statistical design which made it possible to analyze the milk and butterfat production data statistically was a latin square replicated five times (1, 19). Latin squares, as used in these analyses, refer to outcome groups. Analysis of variance gave an *F*-test (37.08) which indicated a highly significant difference between rations. The mean milk yields per cow when rations *B, C* and *D* were fed were 679, 587 and 575 lb., respectively. Application of the *T*-table to determine the least significant deviation revealed a highly significant difference between the population mean milk yield for rations *B* and *C*. Differences in milk yields between rations *C* and *D* were non-significant. The effect of rations on butterfat production was the same as that on milk production. The butterfat yield of the cows while on winter grazing was 18 per cent greater than that produced when they were fed the same type of concentrated mixture and dry roughage. On the basis of the prevailing price ($1.39 per lb. of grade A butterfat) at the time of the study, the increase in butterfat yield (78 lb.) in favor of ration *B* over ration *C* was worth $102.86 for the three experimental periods or an average of $6.86 per cow per period of 28 days. In considering the savings in dry roughage (valued at $4.25 per cow) and the increased production (valued at $6.86 per cow), the gross value of the pasture was $71.75 per cow for a period of 180 days. If the cost of providing the pasture is estimated at $35.00 per acre, the net returns to a dairy farmer as the result of including winter grazing in his feeding program would be approximately $36.75 per cow for a period of 180 days. This is a conservative figure because a considerable decrease in consumption of concentrates occurred when the cows went on pasture.

Milk flavor scores and criticisms. The milk produced by cows on winter grazing had a very distinct feed flavor. However, the flavor was very pleasing to the taste except when the cows were milked immediately after being removed from pasture. When the cows were withheld from pasture as much as 12 hours before being milked, the milk was preferred over that produced by cows on dry rations only. No difference was noted in the effect of sweet potato meal and corn on the flavor of milk when the roughage fed was identical.

Composite milk samples were secured from each of the evening and morning milkings four times during each of the experimental periods and scored separately for flavor. The mean flavor scores of the evening composite milk samples when rations *B, C* and *D* were fed were 36.2, 37.6 and 36.9, respectively. Samples with no criticism were scored 40 to 45. The *F*-test for ration effect was significant at the 5 per cent level. Application of the *T*-test for least significant differences revealed a significant difference between the mean flavor score of the evening composite milk samples when the cows were fed rations *B* and *C*. The significant

[3] Includes missing cow data calculated according to the Yates method (20).

difference in the flavor score of the milk from the cows fed these two rations appeared to be due to a strong grassy flavor which was characteristic of the milk drawn from cows immediately after being removed from pasture. The intensity of this flavor was not as great in the morning milk. The difference in the mean flavor score of evening composite milk samples when the cows were fed rations *C* and *D* was non-significant.

The mean flavor scores of the morning composite milk samples when rations *B*, *C* and *D* were fed were 37.4, 37.0 and 37.5, respectively. Statistical analysis revealed the differences between these data to be non-significant.

SUMMARY

Fifteen dairy cows of the University herd were used to study the value of winter pasture and sweet potato meal for lactating dairy animals during the winter of 1947. The project was conducted for three 28-day periods in accordance with the latin square design (1, 19). Analysis of variance was employed in analyzing the milk, butterfat and flavor data.

The cows that were on winter grazing consumed approximately 46 per cent less dry roughage and produced 15.6 per cent more milk and 18 per cent more butterfat than did the animals that were dry-lot fed. These differences in yields were highly significant.

The mean flavor scores of the afternoon milk samples were 36.2, 37.6 and 36.9 when the cows were fed ration *B* (containing pasture and corn), ration *C* (containing dry roughage and corn) and ration *D* (containing dry roughage and sweet potato meal), respectively. The differences in the flavor scores of the milk from cows fed rations *B* and *C* were statistically significant. The differences in the flavor scores of the milk from cows fed rations *C* and *D* were non-significant. The differences in the mean flavor scores of the morning milk samples from cows fed each of the rations were non-significant.

There was no significant difference in the amount of milk and butterfat produced or in the flavor score of the milk when the cows were fed a concentrate mixture consisting of 30.2 per cent of either corn or sweet potato meal. The animals ate one ration just as readily as the other. The sweet potato meal did not cause an excessive or objectionable laxative effect upon the digestive system of the cows.

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THE DETERMINATION OF BUTTERFAT IN ICE CREAM EMPLOYING MIXED PERCHLORIC AND ACETIC ACIDS

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The Babcock determination of butterfat in milk, cream and certain milk products such as skim milk (2) has been an established procedure for over 50 years. Probably no method of analysis has ever had a record remotely approaching the frequency with which the Babcock test has been applied in the dairy industry.

The unmodified Babcock butterfat test cannot be applied successfully to dairy products containing added sugar due to the charring action of the sulfuric acid. It was the purpose of the present work to show that the use of perchloric and acetic acids in place of sulfuric acid modifies the standard Babcock test to make it applicable to ice cream mix for the determination of butterfat. It can be applied without alteration of existing equipment and with marked improvements in speed, accuracy and simplicity. It diminishes the number of required manipulations per determination, as it is not necessary to add water and the bottle is centrifuged for only a 2-minute period. The increased cost of the mixed perchloric-acetic acid which it employs is more than justified by the saving in time and the abbreviation in operative details. Moderate variation in the amount of acid mixture used does not affect the accuracy of the test.

A mixture of 72 per cent perchloric acid and glacial acetic acid react to form two possible compounds (8), one with the ratio one molecule of perchloric acid to two molecules of acetic acid and the other compound with the molecular ratio of 1 to 1. Such mixtures are not hazardous to mix and may be stored without deterioration. At the boiling point, the acetic acid is evolved and may be thus separated from the perchloric acid. By the process to be described, no precautions other than those applied to the unmodified Babcock test are required. The usual care in the handling of strong mineral acids apply to both procedures.

Sugar is soluble in 72 per cent perchloric acid without charring. Butterfat is as insoluble in aqueous perchloric acid as it is in aqueous sulfuric acid. The proteins of milk and cream are soluble in perchloric acid. Since butterfat in the presence of 72 per cent perchloric acid tends to darken at temperatures near 100° C., thus making reading of the test difficult, it was found desirable to use a mixture of equal parts of 72 per cent perchloric acid and glacial acetic acid as a substitute for concentrated sulfuric acid in the application of the Babcock procedure to the testing of ice cream. The presence of sugar, ice cream stabilizers, flavors and egg products or chocolate does not interfere with the test.

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It is beyond the scope of the present work to give in any detail reference to former procedures which have been developed as substitutes for the original Babcock test as applied to the testing of ice cream. The literature on the subject is very voluminous (1, 2, 4, 5, 6, 7). In no published procedure was found any record of the use of perchloric acid for the purpose of modifying the Babcock test as applied to fat determination in ice cream or ice cream mix.

PROCEDURE

Apparatus and reagents. The perchloric acid-acetic acid mixture, which is the only reagent employed in this modification of the Babcock test, consists of equal parts of volume of 72 per cent perchloric acid ($HClO_4 \cdot 2H_2O$) and 95 per cent glacial acetic acid. Little heat is evolved from the mixing of these chemicals.

Standard Babcock equipment was used to measure the butterfat content by this method. Babcock 20 per cent ice cream test bottles graduated in 0.2 per cent were used throughout this study.

The Mojonnier test, a commercial adaptation of the official Roese-Gottlieb method (3), was employed to carry out control determinations described in this work. All samples tested were evaluated both by the Mojonnier method and the perchloric-acetic acid process simultaneously and the results compared.

The perchloric acid-acetic acid process. The procedure of the test is as follows:

(a) Weigh a 9-g. sample of ice cream mix (or melted ice cream) into a 20 per cent Babcock ice cream test bottle.

(b) Add approximately 30 ml. of the acid reagent (equal parts by volume of 72 per cent perchloric acid and glacial acetic acid) to the test bottle, rinsing the adherent mix off the graduated stem of the test bottle into the body of the bottle as the acid is added. The ingredients should all be at room temperature during mixing.

(c) Digest the ice cream and acid mixture by immersion in boiling water for 5 minutes. No color forms at first, but upon heating in boiling water the mixture turns progressively tan, brown and finally a deep chocolate color. The curd is completely dissolved in 1 to 2 minutes. The mixture should be agitated two or three times during the digestion period. After 5 minutes, the fat will be found as an immiscible supernatant layer.

(d) Add enough of the acid mixture to bring the fat into the calibrated stem of the bottle.

(e) Place the test bottles in balanced pairs in a standard Babcock test centrifuge and revolve at proper speed for 2 minutes. If the centrifuge is heated to 60° C., the per cent of fat can be read as soon as the sample is removed from the centrifuge. If an unheated centrifuge is used, the test bottles should be tempered by immersion in a water bath (130°–140° F.) to the top of the fat column for 5 minutes before reading. The reading of the fat column is made in the customary manner after the addition of glymol.

(f) Contents of the test bottles should be poured into a reservoir of water and then emptied in the sink drain for disposal. The test bottle is rinsed with hot water and is ready for a second test. No coating of insoluble calcium salts ever accumulates on the inner walls of the test bottle. All mineral salts present in cream are soluble in the acid mixture used.

RESULTS

Experimental results on plain vanilla ice cream as compared with the Mojonnier test. Thirty-one different samples of plain vanilla ice cream were subjected to test. These samples were from a wide variety of commercial sources or were

experimental ice creams prepared in the University of Illinois Dairy Technology laboratory. No attempt was made to record their composition. The results are shown in table 1. The maximum deviation between the new method and the

TABLE 1

The analysis of plain ice cream and ice cream mix by the perchloric acid-acetic modified Babcock test and comparison with Mojonnier values

Sample no.	Perchloric acid method		Mojonnier method	Maximum variation from Mojonnier	Average variation from Mojonnier
	No. of analyses	Av. B.F.	Av. B.F.		
		(%)	(%)	(%)	(%)
1	10	11.33	11.22	+ 0.23	+ 0.11
2	17	12.37	12.22	+ 0.23	+ 0.15
3	18	9.05	9.03	+ 0.12	+ 0.02
4	15	15.15	15.05	+ 0.13	+ 0.10
5	7	12.03	12.00	+ 0.17	+ 0.03
6	8	13.41	13.46	− 0.26	− 0.05
7	15	11.80	11.89	− 0.19	− 0.09
8	4	12.60	12.61	− 0.11	− 0.01
9	4	12.15	12.22	− 0.12	− 0.07
10	6	12.78	12.82	− 0.22	− 0.04
11	4	11.78	11.69	+ 0.11	+ 0.09
12	2	13.60	13.61	− 0.01	− 0.01
13	5	10.56	10.51	+ 0.09	+ 0.05
14	5	12.20	12.08	+ 0.22	+ 0.12
15	6	10.13	10.17	+ 0.13	− 0.04
16	8	11.20	11.08	+ 0.22	+ 0.12
17	4	10.68	10.65	+ 0.15	+ 0.03
18	4	12.18	11.89	+ 0.31	+ 0.29
19	37	12.42	12.49	− 0.19	− 0.07
20	8	12.42	12.30	+ 0.26	+ 0.12
21	8	12.48	12.44	+ 0.16	+ 0.04
22	6	12.37	12.29	+ 0.11	+ 0.08
23	12	12.51	12.21	+ 0.49	+ 0.30
24	10	12.62	12.28	+ 0.37	+ 0.34
25	10	12.70	12.43	+ 0.37	+ 0.27
26	10	12.77	12.54	+ 0.36	+ 0.23
27	8	12.91	12.64	+ 0.36	+ 0.27
28	4	12.33	12.31	− 0.11	+ 0.02
29	4	11.98	12.02	− 0.12	− 0.04
30	4	11.63	11.60	+ 0.10	+ 0.03
31	4	11.13	11.20	− 0.20	− 0.07
Summary	267				+ 0.07

Mojonnier process was + 0.49 per cent. The average algebraic difference was + 0.07 per cent.

Eight analyses of the same sample gave 11.2 per cent for six determinations, 11.1 for one determination and 11.3 for the remaining test. The Mojonnier test for this sample was 11.08 per cent.

The determination of butterfat in chocolate ice cream. The procedure as described was applied to the determination of butterfat in eight samples of chocolate ice cream with the results given in table 2. Control analyses were carried out using the Mojonnier method. Results of the test of chocolate ice cream samples indicate that the perchloric acid-acetic acid procedure is satis-

factory for use in the determination of butterfat in chocolate ice cream. The average variation between the two methods was – 0.11.

SUMMARY

A new reagent has been described for use in a modified Babcock butterfat analysis of plain ice cream and chocolate ice cream. The reagent consists of a mixture of equal parts by volume of 72 per cent perchloric acid and glacial acetic acid. The test requires only one centrifugation and a complete analysis can be accomplished in about 8 minutes. The results are in close agreement with those obtained by the Mojonnier method.

TABLE 2

The determination of butterfat in chocolate ice cream by the perchloric-acetic acid procedure

Sample no.	Perchloric acid method		Mojonnier method	Maximum variation from Mojonnier	Average variation from Mojonnier
	No. of analyses	Av. B.F.	Av. B.F.		
		(%)	(%)	(%)	(%)
1	7	14.36	14.37	– 0.17	– 0.01
2	12	13.28	13.44	– 0.54	– 0.16
3	2	20.05	20.14	– 0.14	– 0.09
4	5	11.00	11.07	– 0.17	– 0.07
5	4	10.10	10.01	+ 0.19	+ 0.09
6	5	15.42	15.12	+ 0.48	+ 0.30
7	7	10.89	11.10	– 0.30	– 0.21
8	7	12.53	13.26	– 0.86	– 0.73
Summary	49				– 0.11

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THE NUTRITION OF THE NEWBORN DAIRY CALF. II. EFFECT OF DIETARY TRYPTOPHAN ON THE URINARY EXCRETION OF NIACIN AND ITS METABOLITES BY YOUNG DAIRY CALVES

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Following the reports (6, 7) that tryptophan can replace niacin in affecting growth of laboratory animals on niacin-deficient rations, various investigators determined the effects of dietary tryptophan on the excretion of niacin and its metabolic products not only in the rat (10, 13) but also in the pig (8), horse (12) and man (11). In all cases the feeding of tryptophan resulted in a marked increase in the excretion of these substances, indicating a metabolic relationship between tryptophan and niacin. The nature and concentration of the excreted products suggest that there is a species variation in this respect. It has been found that there are large increases in the excretion of N^1-methylnicotinamide when tryptophan is fed to rats (13), pigs (8) and humans (11), whereas in the case of the horse (12), there is no significant increase in the excretion of this substance but an increase in free nicotinic acid and other non-methylated products.

In a previous communication (14) from this laboratory, a two-fold increase in the blood tryptophan of calves during the first 3 days of post-natal life was reported. This increase resulted from the ingestion of colostrum which was found to contain an average of 3.85 mg. of tryptophan per g. at the first milking. This is approximately five times that of normal milk on a wet-weight basis. In view of the existing knowledge that a metabolic relationship exists between tryptophan and niacin in the nutrition of the rat, pig, horse and man, and that calves do not require a dietary source of niacin when fed a synthetic milk diet (5), it was considered desirable to study the effects of feeding tryptophan to calves on a milk diet on the excretion of niacin and its derivatives.

EXPERIMENTAL PROCEDURE

Two calves were selected for this experiment and maintained on an exclusive milk diet from birth throughout the experimental period. Calf *A*, a Holstein, was put on the experiment 24 hours after birth. Calf *B*, a Guernsey, was assigned to the experiment at 40 days of age. This calf had been kept from the time of birth in a wire-bottomed pen. The usual procedure of feeding colostrum for the first few days was followed in both cases. When the urinary excretion of niacin and its metabolites was found to be fairly constant, each calf was fed 15 g. of L-tryptophan during a 3-day period. The weighed amount of L-tryptophan (2.5 g.) was dissolved in a small amount of dilute sodium carbonate

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solution and carefully mixed with the milk before each feeding. The calves were fed twice daily at 12-hour intervals.

Twenty-four-hour samples of urine were collected after 7.5 g. of L-tryptophan had been fed and again immediately following the last feeding when a total of 15 g. had been fed. Similar urine collections were made 2 and 7 days following the cessation of L-tryptophan feeding.

The amount of tryptophan in the milk consumed was determined each day and the amounts of niacin, other non-methylated products, N^1-methylnicotinamide and tryptophan were determined in each urine sample collected. N^1-methylnicotinamide was determined fluorometrically by the method of Huff *et al.* (4). A Coleman photofluorometer was used for the fluorescence measurement. Niacin and its non-methylated products were determined by the chemical procedure of Perlzweig *et al.* (9) with minor modifications. The *p*-dimethylaminobenzaldehyde method of Bates (1) as modified by Graham *et al.* (2) was used for the determination of tryptophan in both milk and urine. The Evelyn Photoelectric Colorimeter with appropriate filters was used for all determinations except N^1-methylnicotinamide.

RESULTS AND DISCUSSION

The effects of feeding L-tryptophan on the excretion of niacin and its metabolic derivatives are shown in table 1. It will be noted that following the ingestion of colostrum the excretion of N^1-methylnicotinamide was fairly high (table 1, calf *A*) but dropped rapidly, whereas the non-methylated products

TABLE 1

The effects of feeding L-tryptophan on the urinary excretion of nicotinic acid and its metabolites in dairy calves

Age	Tryptophan in the milk consumed	Urinary excretion			
		Nicotinic acid	Other non-methylated metabolites	N^1-methyl-nicotinamide	Total excretion
(days)	*(g./day)*	*(mg./day)*	*(mg./day)*	*(mg./day)*	*(mg./day)*
		Calf *A*, born April 7, 1948			
2	9.86	1.30	2.10	5.18	8.58
5	3.19	1.22	4.06	4.85	10.53
12	3.45	3.51	13.51	1.67	18.69
14[a]	3.96	3.38	14.17	1.91	19.46
16	4.50	3.84	32.79	2.65	39.28
18	3.62	3.62	48.04	3.35	55.01
21	4.05	4.05	34.01	2.55	40.61
27	4.30	3.10	12.10	2.54	17.74
		Calf *B*, born February 25, 1948			
41	2.03	0.43	5.82	3.12	9.36
48	2.61	0.80	4.30	1.22	6.32
58[b]	2.16	0.30	1.96	1.54	3.80
60[a]	1.86	0.42	2.13	1.52	4.07
62	2.58	2.50	13.70	1.92	18.12
63	3.41	3.15	22.95	1.35	27.45
66	3.05	1.27	5.83	1.39	8.49
69	3.20	0.84	3.61	1.70	6.15

[a] Following this collection 5 g. of L-tryptophan was fed daily for the next 3 days.
[b] Scoured.

increased appreciably. The difference between calves in the level of free non-methylated products excreted prior to the tryptophan feeding is not explainable, although it must be remembered that these calves differed in breed and age and in milk consumption. Therefore calf *A* received a larger daily amount of tryptophan.

Following the feeding of 15 g. of L-tryptophan, there was a three- to four-fold increase in the excretion of total nicotinic acid. There was no significant increase in the excretion of free niacin or N[1]-methylnicotinamide. The major portion of the increase was in the non-methylated products, and the maximum increase was noted in the collection immediately following the L-tryptophan feeding period. The data indicate that N[1]-methylnicotinamide is not the main metabolic product excreted by calves. In this respect, the calf is similar to the horse (3) but different from the rat, pig and man. The Illinois workers (5) found a relatively constant excretion of N[1]-methylnicotinamide in calves regardless of whether or not niacin was added to their diet. Following the cessation of L-tryptophan feeding the excretion of total nicotinic acid returned to normal for the individual in 3 to 7 days.

The results of the urinary excretion of tryptophan are presented in table 2.

TABLE 2

The effects of feeding L-tryptophan on the urinary excretion of tryptophan

Calf *A*			Calf *B*		
Age	Tryptophan in the milk consumed	Tryptophan excreted in the urine	Age	Tryptophan in the milk consumed	Tryptophan excreted in the urine
(days)	(*g./day*)	(*mg./day*)	(days)	(*g./day*)	(*mg./day*)
12	3.45	24.2	58	2.16	67.1
14[a]	3.90	25.5	60[a]	1.86	72.8
16	4.5	53.8	62	2.50	118.1
18	3.62	65.2	63	3.40	114.5
21	4.01	52.8	66	3.05	103.2
27	4.30	32.2	69	3.20	82.5

[a] Following this collection 5 g. of L-tryptophan was fed daily for the next 3 days.

Feeding 5 g. of tryptophan daily for 3 days resulted in an increase in the excretion of tryptophan in urine. This increase could account for only 1 to 1.5 per cent of the intake. It reasonably can be presumed that most of the ingested tryptophan was utilized in the body.

The facts that both colostrum and milk are poor in niacin but relatively rich in tryptophan, and that there is a marked increase in the urinary excretion of niacin and its derivatives following the ingestion of tryptophan suggest that dietary tryptophan serves as a precursor of the niacin required by the calf.

SUMMARY

Five g. of tryptophan were fed daily for 3 days to each of two dairy calves that had been maintained from birth on a whole milk diet. The amount of urinary excretion of nicotinic acid and its derivatives and tryptophan was determined on 24-hour samples and compared with similar data obtained previous to and following the tryptophan feeding.

Tryptophan feeding resulted in a three- to four-fold increase in the excretion of total free and combined nicotinic acid. There was little change in the excretion of free nicotinic acid and N^1-methylnicotinamide. The major portion of the increase was in the non-methylated products. The data indicate that N^1-methylnicotinamide was not the main metabolic product excreted by calves.

The urinary excretion of tryptophan accounted for only 1 to 1.5 per cent of the intake.

Increase in dietary tryptophan results in increased urinary excretion of total nicotinic acid, indicating that tryptophan serves as a precursor of niacin in the young calf as in other mammals thus far studied.

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THE OXIDIZED FLAVOR IN MILK AND DAIRY PRODUCTS: A REVIEW

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The oxidized flavor discussed in this review has been known by a number of descriptive terms. The terms most commonly used to describe the flavor are "cappy," "cardboard," "emery," "metallic," "oily," "oxidized" and "tallowy." The so-called tallowy flavor which develops in fluid milk is not the same as the tallowy flavor which develops in dried milk. The former generally is believed to be caused by an oxidation of the phosphatides, while the latter is caused

by an oxidation of the glycerides. The terms "cappy" and "cardboard" were used because early workers thought that the milk bottle cap was the source of the flavor, while other terms were used in an attempt to describe the taste. The presence of this flavor in milk has caused considerable concern in Europe for a number of years. More recently, it has become of increasing importance in the United States.

The development of the oxidized flavor in milk seems to be the result of a mild chemical oxidation of a minor constituent associated with the fat. A number of authors have presented data which indicate that an oxidation of the phospholipids is responsible for the development of the oxidized flavor. Hereafter, the oxidized flavor will be referred to as *the flavor.*

Golding and Feilman (73) were probably the first to make a study of the flavor in milk. However, Guthrie (88) refers to the work of White who, in 1901, scored butter which had a metallic flavor. The defect observed by White may have been due to active glyceride oxidation, because a metallic flavor usually precedes a tallowy flavor in butter. In this early publication, the author made a thorough study of the development and inhibition of the flavor in milk, skim milk, cream and buttermilk.

THEORIES

The enzyme theory. This theory was proposed by Kende (121) and accepted in whole or in part by the workers in this field for a number of years. He claimed to have isolated an enzyme which he called "oleinase", because it catalyzed the oxidation of the oleic radical of the fat. Sharp *et al.* (192) and Chilson (37) observed that heating milk destroyed an enzyme which oxidized ascorbic acid and at the same time destroyed the enzyme which promoted the development of the flavor. The fact that the flavor increases with decreased storage temperature and the observation that the addition of ascorbic acid (a normal constituent of milk) inhibits development have been used as arguments against this theory. The observation that the flavor may be promoted or inhibited by small changes in pH and E_h also seems to be an argument against the enzyme theory.

The chemical oxidation theory. The conditions under which the flavor develops are those that promote oxidation, such as the presence of air and contamination by metals that are known to be oxidation catalysts. The optimum conditions for development of the flavor are those that cause a mild oxidation, such as low storage temperatures, a limited supply of oxygen, a low concentration of certain oxidizing agents and a limited increase in E_h. It has been observed by many workers that if the intensity of oxidation is too great the flavor will not develop (83, 130, 131). Greenbank (83) postulated and presented data in support of his assumption that the flavor is the result of an intermediate oxidation product, and that the development of the flavor in milk may be inhibited by reducing or oxidizing agents. Recently, Krukovsky and Guthrie (131) have presented data which can be used to confirm the work presented by Greenbank (83). By the careful addition of hydrogen peroxide, these authors were able to promote or inhibit the development of the flavor. Milk so treated to inhibit the

flavor development then was made susceptible by the addition of ascorbic acid. This was repeated a number of times with the same result, but eventually the addition of ascorbic acid had no effect.

The addition of copper, a mild oxidizing agent, promotes development of the flavor, while a low concentration of ferric iron may promote the development of the flavor and a higher concentration inhibit it (83). The addition of reducing agents may inhibit development of the flavor or remove the flavor once it is formed (83). In the latter case, the flavored compound must be reduced before it has become bound to the fat or protein.

THE EFFECT OF THE CHEMICAL AND PHYSICAL PROPERTIES OF THE MILK

The chemical properties of the milk which have been considered as affecting the development of the flavor are E_h, pH, poising action and titratable acidity.

Oxidation-reduction potential (E_h). Tracy *et al.* (212) were among the first workers to show a relationship between the development of the flavor and the E_h. They found that the addition of copper caused an increase in E_h of the susceptible samples. These conclusions were verified by Thurston (204), Greenbank (82, 83), and Webb and Hileman (223). The latter were unable to find any relationship between the E_h and the development of the flavor when copper was added to milk from individual samples. The inhibition of the flavor by bacterial growth (15, 202, 203) and by heat has been attributed by Greenbank (83) to the lowering of the E_h. Greenbank's work has been confirmed by Josephson and Doan (119), and Gould and Sommer (78). Larsen *et al.* (133) found no correlation between the E_h and the inhibiting effect of homogenization. The addition of copper and iron to milk may cause a change in the E_h. Copper and ferrous iron are most effective in promoting the flavor (83). Ferric iron and hydrogen peroxide may inhibit if a sufficient concentration is employed (83). The use of poor feed increases the E_h and promotes development of the flavor, while green feed lowers the E_h and development of the flavor is inhibited.

Poising action. Poising is the resistance of milk to a change in E_h; it is analogous to buffering in the acid-base system. Greenbank (83) concluded that the variation in individual samples is a result of differences in poising. When poising is used as a criterion, according to Thurston's (205) classification, spontaneous milks are those which are very poorly poised, susceptible milks those poorly poised, and non-susceptible milks those well poised. The difference in poising between susceptible and non-susceptible samples seems to be confirmed by the data of Krukovsky and Guthrie (130) on the oxidation of ascorbic acid in susceptible and non-susceptible samples. Greenbank (81) proposed a test to detect susceptibility based on poising, which he claimed was 90 per cent accurate. Webb and Hileman (223) were able to predict with a fair degree of accuracy the susceptibility of samples by the rise in E_h after the addition of copper. The reduction of methylene blue in milk by light has been used as an indication of susceptibility (1, 62). Greenbank and Holm (86) have shown that methylene blue dissolved in butterfat is reduced by light.

Hydrogen ion concentration (pH). The effect of pH on the development of the oxidized flavor has not been studied extensively. Greenbank (83) found that an increase in pH of 0.1 was sufficient to inhibit development of the flavor for 24 hours. Although all samples developed the flavor after storage for 24 hours, the samples with increased pH developed less flavor. As a rule, an increase in OH ions accelerates oxidation and may prevent the development of the flavor (83). Anderson (4) presented similar data but attributed inhibition to the activation of an enzyme which destroys the flavor rather than to catalysis of oxidation by OH ions.

Titratable acidity. Brown and Dustman (22), in a study of 220 samples of milk, were unable to find any correlation between the titratable acidity and the development of the flavor when the milk was contaminated with copper. Anderson (4) found a relationship between the titratable acidity and the development of the flavor. Anderson and Triebold (2) observed that reducing milk of high acidity to 0.145 per cent acidity or lower was effective in inhibiting the flavor. Winter milk generally has a higher titratable acidity than summer milk, which would appear to be a positive correlation with the observation that winter milk is more susceptible than summer milk, but there probably are other changes that are more significant (4).

Color. The variation in the color of milk, especially the yellow color, has been correlated with the development of the flavor. The attempt to correlate color and the development of the flavor is probably a result of the fact that carotene has been thought to be an antioxidant. Anderson (6, 7) and Anderson *et al.* (10) were among the first to find a relationship between color and flavor. The yellow color of milk largely is due to the pigment carotene. Tucker *et al.* (220) found a good correlation between the intense yellow color and good flavor. Whitnah *et al.* (228) found that milk which was below the average color for the breed developed the flavor. However, they also found samples low in color which did not develop the flavor. See "Carotene," and "green feed" for additional discussion.

THE EFFECT OF MILK CONSTITUENTS

Glycerides. The glycerides of the fatty acids are reasonably stable at temperatures most conducive to the development of the flavor. When the glycerides oxidize, there is a measurable decrease in the iodine value. Kende (121) and Dahle (43) found a decrease proportional to the intensity of the flavor. Brown *et al.* (23) were unable to find any decrease. This has been confirmed by Swanson and Sommer (201). Since the flavor is thought to be the result of an oxidation of the phospholipids, no appreciable decrease in the iodine value should be expected, since the concentration of phospholipids in milk fat is low. The same authors found that the oxidized flavor is pronounced in butter, buttermilk, cream and milk, but found only a trace in butteroil. They found that oxidized butteroil when dispersed in skim milk has an off flavor but not an oxidized flavor. Dahle (44) mixed cream or butterfat with skim milk from cows that gave susceptible

milk and the flavor developed, but it developed more rapidly in the sample prepared with cream than in the one prepared with butteroil.

Phospholipids. Whole milk contains from 0.0038 to 0.2889 per cent phospholipids, according to Panzer (159). Early workers pointed out that the flavor developed most rapidly in the cream layer. Guthrie (88), as early as 1916, found that buttermilk from a susceptible cream developed a much stronger flavor than either the cream or the milk. The phospholipid content of milk and milk products (176) decreases in the following order: buttermilk > cream > whole milk > skim milk,—which has also been found to be the order of decreasing intensity of flavor development (88, 207). Thurston *et al.* (208) conclude that the oxidation of lecithin is the cause of the flavor. They arrived at this conclusion because removal of the hulls from milkfat globules, which then were redispersed, resulted in a milk which did not develop the flavor. Roland and Trebler (179) found a decreased sensitivity to copper-induced flavor when mechanical separation was employed. They attributed this to a redistribution of the lecithin between the fat and aqueous phases. Gould *et al.* (77) found no relationship between the lecithin content of the milk and the development of the flavor. Evans (60) found that lecithin (probably impure) was an antioxidant, an observation which has been confirmed by Holmes *et al.* (107) and Koenig (125). Ritter and Nusbaumer (174) found that both lecithin and cephalin of plant origin act as antioxidants. Olcott and Mattill (155) report that lecithin is not an antioxidant, but cephalin acts as such. Much of this work was done on fat substrates and may not be of great value here. Dahle and Palmer (53) conclude that spontaneous flavor, *i.e.* without metallic contamination, is due to the oxidation of the phospholipid fraction of the fat globule membrane. Josephson and Doan (119) found that a typical oxidized flavor develops when phospholipids and copper in suspension are heated together. Phospholipids plus protein developed the flavor without heat, but the intensity was greater when heat and copper were used. Swanson and Somner (201) found a decrease of 30 per cent in the iodine value of the phospholipids from milk which had developed the flavor. The data presented in these studies seem to indicate that oxidation of the phospholipids is the cause of the flavor.

Carotene. Milkfat contains 0.20 to 0.86 mg. of this yellow pigment per 100 g. of fat (176). The reason for the study of the effect of carotene on the development of this flavor is probably its purported antioxygenic activity. Briggs (20), Koenig (126) and Newton (154) conclude that carotene is an antioxidant, while other workers (19, 21, 87, 101, 156) conclude that it has no effect or is a prooxidant. However, most of this work has been done on glyceride substrates and may not be applicable in this work.

Brown *et al.* (30) concluded that some substance associated with the carotene is responsible for the development of the flavor. Trout and Schied (219) found no relationship between the carotenoid content and development of the flavor.

Vitamin A. The concentration of vitamin A in milk varies from 2.5 to 50.0 Sherman units per g. depending on the feed. It is practically all in the butterfat.

Booth *et al.* (18) found the concentration of vitamin A in summer milk was three times as great as in winter milk. Garrett *et al.* (67) found that feeds which increase vitamin A also may increase ascorbic acid. For additional discussion see "Green feed," and "supplements."

Ascorbic acid. In this review, the interest is greater in ascorbic acid than in vitamin C because the latter contains dehydroascorbic acid which does not influence the flavor (131). Milk has been found to contain as high as 26.5 mg./l. of ascorbic acid, according to Riddell *et al.* (168). It is reasonable to assume that ascorbic acid plays some role in the development of the flavor, because it is a reducing agent and has been reported to be an antioxidant. The ratio of the reduced form to the oxidized form may reflect the E_h of the milk because the ascorbic-dehydroascorbic acid system is reversible. Garrett *et al.* (67) found a relationship between the ascorbic acid content and the flavor of milk the day it was drawn. Hand and Sharp (98) found a good correlation between the oxidation of ascorbic acid and development of the flavor. Trout and Gjessing (217) found the ascorbic acid content greater in summer than in winter, which is the opposite of seasonal variation of the flavor intensity. This might indicate that ascorbic acid inhibits development of the flavor.

Whitnah *et al.* (228) found that the relationship between ascorbic acid content and development of the flavor varied in milk from different breeds. This is discussed under "Biological Factors." They found no relationship between vitamin C and development of the flavor in milk from cows within the breed. Sharp *et al.* (192) and Dahle (42) report there is such a relationship. Tucker *et al.* (220) found that a concentration of from 15 to 18 mg./l. was required to impart a good flavor to milk. Brown *et al.* (29) found that feeding KI reduced the ascorbic acid but had no effect on the intensity of the flavor. They made no study of the physical or chemical properties of the milk. Later, the same authors (25) found that the addition of 0.1 g./l. of KI would inhibit. A study of the passage of KI from the feed to the milk would be interesting. Recently, Krukovsky and Guthrie (130) concluded that ascorbic acid is a link in the chain forming the flavor. They based this conclusion on the observation that the oxidation of ascorbic acid by H_2O_2 inhibits development and milk so treated can be made to develop the flavor by adding ascorbic acid. Later, the same authors concluded that it is the "pressures" of ascorbic and dehydroascorbic acid which control the development of the flavor. Greenbank (85) explains these reactions in a slightly different manner. He concludes, according to his intermediate oxidation product theory, that when all the ascorbic acid is destroyed the E_h is high enough to produce a completely oxidized form of the causative agent which has no flavor. The addition of more ascorbic acid to the milk lowers the E_h so that the intermediate or flavored compound may form (83). The correlation of "pressures" of ascorbic-dehydroascorbic acid is another way of expressing the ratio of the reduced to the oxidized form. This ratio is the basis for E_h. These data may be used to confirm Greenbank's (82, 83) conclusion that the development of the flavor is related to the change in E_h. Guthrie *et al.* (92) found a general relationship

between factors which accelerates the rate of oxidation of ascorbic acid and development of the flavor.

Riboflavin (lactochrome) vitamin B_2. The green pigment in whole milk is concerned in a number of ways with biological oxidations, according to Ball (13). When combined with a specific protein, riboflavin becomes an enzyme. Such compounds are called flavoproteins. One of these is Schardinger's enzyme. It is not known whether it plays any part in the development of the flavor, but one interesting fact is that this enzyme is concentrated on the surface of the fat particles. Separating milk concentrates the flavoprotein in the cream and churning the cream concentrates it in the buttermilk (98, 101). It may be significant that flavoprotein is found in the following increasing order: Milk < cream < buttermilk, which is the order of increasing susceptibility to development of the flavor. The acceleration of the photochemical oxidation of ascorbic acid by riboflavin is discussed under "Irradiation."

Proteins, lactose, and salts. These constituents do not seem to be concerned in the development of the flavor, because they are stable towards oxidation under conditions most favorable to the development of the flavor (83).

BIOLOGICAL FACTORS

Biological factors are important because they influence the properties of the milk. In studying the biological factors, it is important that changes in constituents and properties be observed at the same time. Many conclusions found in the literature are not of much value because variables other than the one studied were not controlled.

Breed. The relationship of breed to the development of the flavor has been studied because certain breeds seem to synthesize carotene from their feed more readily than others and thus produce a more highly colored milk. Color has been thought to be related to the development of the flavor. Whitnah *et al.* (228) found that all the samples in which the flavor developed were below the breed average in intensity of color. However, they found samples low in color that did not develop the flavor. They found also that the average vitamin C content of milk from different breeds increased in the following order: Holstein, Aryshire, Guernsey and Jersey, while the spontaneous development of the flavor decreased in the same order (228). If ascorbic acid acts as a flavor inhibitor, the order of susceptibility given is correct.

Bacterial count. It has been known for some time that milk of low bacterial count is more susceptible than milk of high count, provided all other conditions are the same or similar. It has been postulated that the bacteria utilize the dissolved oxygen and form metabolic products which lower the E_h or are inhibitors. The growth of bacteria is one of the factors which inhibits development of the flavor at high storage temperatures. Thurston and Olson (209) found that milk stored at 38° F. had little bacterial growth and developed the flavor, while a sample of the same milk stored at 52° F. had considerable bacterial growth and did not develop the flavor. Roland *et al.* (178) found that the bacterial counts were generally lower in milk which developed the flavor than in

milk which did not develop it. Greenbank (83) found that bacterial growth inhibits the flavor development and decreases the E_h at the same time. Other workers have obtained similar results (1, 57, 202, 203). A number of workers have concluded that bacterial growth inhibits the development of the flavor (3, 53, 212). However, the evidence indicates that the number of bacteria required to exhaust the oxygen or reduce it to a concentration low enough to inhibit would be sufficient to produce serious bacterial defects (46, 64, 134).

Period of lactation. Brueckner and Guthrie (33) were among the first to study the relation of this factor to the development of the flavor, but they were unable to find any correlation. Rasmussen *et al.* (165) found that the ascorbic acid content of milk is relatively high during the early stages of lactation and decreases to a minimum in about 2 months but rises to a maximum during the latter stages. If ascorbic acid content is the controlling factor (130), milk from the middle of the lactation period should be the most susceptible and late lactation the least susceptible milk. This is contrary to the evidence of Corbett and Tracy (39), who found that milk from the first part of the lactation period is most susceptible, especially in the case of heifers.

Feed. Brueckner and Guthrie (33) were among the first to show that when cows are fed green feed they produce more stable milk than when they are fed dry feeds. This same conclusion has been made by a number of workers (27, 42, 53, 121, 204, 206). However, Hening and Dahlberg (104) found that feeding below the Morrison standard did not affect the flavor. They also found that feeding mangels or beet pulp had no effect on the flavor (103). Majer (137) found fresh "alp" hay in the ration inhibited development. Stebnitz and Sommer (198) found that when cows receive grass as a part of the ration the butterfat becomes more unsaturated and therefore more susceptible to oxidation, and the milk becomes less susceptible to development of the flavor. These data may be used to indicate that the fat is not responsible or that the liquid phase plays some part in controlling development of the flavor. Garrett (66) reports that green grasses or legumes preserved as silage or artificially dehydrated hay are especially desirable in preventing development of the flavor. Bartlett *et al.* (14) found molasses silage of immature grasses or legumes excellent in producing milk highly resistant to the flavor development. Babock and Haller (12) found that feeding different silages had no effect on the copper tolerances of the milk produced.

Dry feeds. The effect of feeds is dependent to a great extent on the preservation of their nutritive value in drying. Anderson (5) concludes that feeding good machine cured alfalfa changes poor milk to good milk and that poor alfalfa will do the reverse. Brown, *et al.* (30) found that feeding high quality alfalfa with alfalfa meal greatly reduced or eliminated metal induced flavor. Feeding of brown leafy alfalfa did not increase the tendency to develop the flavor. Dahle and Carson (47), on the other hand, found that feeding alfalfa hay produces milk more susceptible to the flavor than milk from cows fed on other roughages. Brown *et al.* (27) found that dry feeding increases the susceptibility and green feeding reduces it. Guthrie and Brueckner (90) found that dry feeds are not

the sole cause of the flavor, because milk from separate quarters of the udder developed different flavor intensities.

Supplements. The feeding of supplements is an attempt to supply essentials that are absent from poor feed. In correlating the feeding of these supplements, it is essential that a study of all the changes in the milk be made, otherwise, the conclusions may be misleading. The feeding of carrots is beneficial in preventing development of the flavor (9). Corbett and Tracy (38) fed cocoanut and corn oil and found that the iodine value of the milkfat was increased markedly, but there was only a slight change in susceptibility. Brown *et al.* (28) fed 1 lb. of cocoanut oil per day. The iodine value of the milkfat increased slightly and there was also a slight increase in susceptibility. One lb. of soybean oil increased the iodine value greatly and increased susceptibility to copper induced flavor. Prewitt and Parfitt (162) fed 14 cows ground soybeans, soybean oil, linseed meal, dried brewers yeast, and none of the milk from these cows developed the flavor spontaneously. However, milk from cows which were fed soybean oil or meal was least susceptible to metal-induced flavor. Brown *et al.* (27) fed 1 quart of lemon or tomato juice per animal per day and reduced susceptibility. The same authors found that feeding 0.5 g. of ascorbic acid per day reduces the tendency to develop the flavor. Brown *et al.* (29) fed 5 g. of KI per day and noted a marked decrease in the ascorbic acid content of the milk but no increase in metal-induced susceptibility. No study was made of changes in the other properties of the milk. Later, Brown and Olsen (25) found that 0.1 per cent KI added to milk would prevent development of the flavor.

Anderson *et al.* (9) found that feeding 8 lb. of carrots per day in the ration was more effective in inhibiting development of the flavor than the addition of 500,000 units of U.S.P. carotene. Whitnah *et al.* (229, 230) and Beck *et al.* (15) found a carotene supplement quickly corrected the tendency for the flavor to develop spontaneously. Brown *et al.* (24) found that a carotene supplement rendered the milk more resistant to the metal-induced flavor. Martin *et al.* (143) fed 1/3 g. of carotene per day for 15 days and increased the color of the milk 60 per cent, with a decrease in the flavor intensity. Brown *et al.* (29) studied the effect of ascorbic acid and carotene as supplements on the development of the flavor but did not study the effect of carotene in the ration on the ascorbic acid content of the milk.

Seasonal variations. Mattick (144) was one of the first to report a seasonal variation in the production of the flavor. He found that the flavor appeared in autumn, winter and spring but never in summer. More recently, the variation in susceptibility between winter and summer milk has been observed by many workers (5, 27, 33, 42, 90, 163, 204, 211, 217, 223). The greater susceptibility in winter, as observed by most workers, seems reasonable as shown by the discussion on green feed and its effect on the flavor. Anderson and coworkers (3, 4) found that the titratable acidity of milk is higher in winter than summer and concluded that there was a correlation between titratable acidity and development of the flavor.

THE EFFECT OF PROCESSING

Processing may change the properties of milk so as to inhibit or promote development of the flavor. In the discussion, an attempt will be made to point out changes in the properties of the milk which may influence the development of the flavor.

Heat treatment. Most workers conclude that pasteurization has little effect on the development of the flavor unless metallic contamination occurs. However, Dahle (43, 42) reported that heating milk to 145° F. for 30 minutes intensified the flavor. Gjessing and Trout (71) concluded that the ascorbic acid was less stable in milk pasteurized by holder methods than in milk pasteurized by using higher temperatures, especially when copper is present. Woessner *et al.* (231) concluded that 20 per cent of the asorbic acid was destroyed in the holder methods of pasteurization. They also concluded that a temperature of 167° to 185° F. for 15 seconds is required to stabilize ascorbic acid. The heat treatment necessary to inhibit development of the flavor is, according to Kende (121), 185° F. for 5 minutes, Sharp (191), 170° F. for 10 minutes, Dahle and Palmer (53), heating to 170° F. Kende (121) and Sharp (185) concluded that heating kills an enzyme which promotes the oxidation. Greenbank (83) found that heat treatment reduces the E_h and attributes retardation to a reduced E_h. This has been confirmed (78, 119). Gould and Sommer (78) and Gould (75, 76) have shown that sulphydryl compounds, which are of a reducing nature, are formed when milk is heated to temperatures above pasteurization and these compounds produce the cooked flavor.

Storage temperature. The effect of storage temperature on the development of the flavor is one of the factors which supports the theory that the reaction is a mild chemical oxidation and not enzymatic. The intensity of the flavor increases as the storage temperature decreases. This correlation is paralleled in many chemical oxidations and is contrary to the effect of variations in temperature on enzymatic reactions. Lowering the storage temperature should make the oxidizing conditions milder but has been observed to increase the intensity of the flavor (83). Tracy (210) was probably the first to observe that the flavor developed more rapidly at 4° C. than at 20° C. Greenbank (83) verified these results. Bell (15) observed that concentrated milk held at – 17° C. became oxidized more rapidly than at – 7° C., and also that the intensity at – 7° C. finally decreased while that at – 17° C. remained the same. However, Thurston and Olson (209) noticed an oxidized flavor in milk held at 38° F. which showed little bacterial growth. The same sample held at 58° F. showed considerable growth, and the flavor was not detected. Tracy *et al.* (212) found that milk incubated from 1 to 6 hours at 68° F. or at 90° F. and subsequently stored at 40° F. is less likely to develop the flavor than milk stored immediately at 40° F. A number of investigators have worked on the development of the flavor in cream (58, 149, 150, 184, 195). The inhibition at higher storage temperature has been attributed to a number of factors. Kende (121), Tracy *et al.* (212) and Greenbank (83) attribute the inhibition to a lowering of the E_h by bacterial growth. Tracy

et al. (212) also conclude that the bacteria use up the dissolved oxygen, but Sharp *et al.* (190) conclude that to do this there would be a deterioration in flavor due to excessive bacterial growth.

THE EFFECT OF METALLIC CONTAMINATION

Copper. Copper is a normal constituent of milk. However, contamination by this metal has been studied for years (11, 105, 126, 136, 172). Rogers *et al.* (177) probably were the first to conclude that copper contamination caused a more intense tallowy flavor in butter than did iron. Copper is an ideal catalyst for the development of this flavor, because it is a relatively mild oxidizing agent and high concentrations will not inhibit development of the flavor (83). Golding and Feilman (73) were probably the first to report the development of a metallic flavor in milk passed over a detinned cooler. Hunziker and Hosman (114) were among the first to point out that copper contamination produces a more intense flavor in milk than does contamination by iron. The mechanism concerned in the action of copper has been studied by a number of investigators. Osborne and Leavenworth (158) and Vandevelde (221) found that copper combines with protein more as an absorption complex than as a chemical compound. Olson and Brown (157) found that copper combines with the ascorbic acid anion and thereby promotes oxidation; Brown *et al.* (26) found that the intensity of the flavor was greater when copper was added after rather than before pasteurization. This has been confirmed by Greenbank (83).

Iron. This metal, like copper, is a normal constituent of milk. Contamination by iron is not as detrimental as contamination by copper. This has been observed by many workers (26, 83, 136). It has been shown that ferrous iron promotes the development of the flavor but requires a much higher concentration than does copper (83, 201). Ferric iron is less effective than ferrous iron and may even inhibit the flavor (83). Samples containing ferric iron were found to possess the flavor after storage for 24 hours but not for 48 hours (83). Hartman *et al.* (100) found ferrous iron lowered the E_h and did not cause as intense a flavor as copper. This has been confirmed by Swanson and Sommer (201). The effect of copper and iron on the development of the flavor has been studied by a number of workers (36, 41, 54, 55, 70, 72, 74, 80, 88, 93, 128, 142, 147, 148, 150, 171, 194, 195, 222, 225).

Other metals. Besides those already discussed, aluminum, lead, nickel, tin and zinc are the metals used in pure form or as alloys in dairy equipment. Nickel has been studied extensively by a large number of workers (26, 36, 41, 54, 55, 59, 63, 68, 70, 74, 93, 132, 146, 171, 215, 226, 227). Guthrie *et al.* (94) report the development of the flavor by nickel contamination in one case. Whitefield *et al.* (227) report a metallic flavor was caused by contamination by nickel. Fink and Rohrman (63) found that during pasteurization nickel may replace copper that is in solution and render the milk less likely to develop the flavor. Aluminum has been found by many workers to be without effect (63, 68, 94, 109, 112, 129, 171, 225, 227). Allegheny metal, chromium nickel steel and stainless steel are not corroded by milk and do not promote development of the flavor (109, 112, 113, 122, 164, 225, 226, 227).

Manganese, lead and zinc do not influence the oxidation of ascorbic acid and do not affect the flavor.

Irradiation. The exposure of milk and dairy products to light is a form of irradiation. The effect of light on the flavor of dairy products has been studied since 1890 (95). A large number of workers have observed the effect of sunlight on milk (36, 65, 140, 141, 145, 163, 206). The effect seems to be dependent upon the intensity, wave length and time of exposure (83). Hand *et al.* (97) have verified Hopkin's work (108) which indicated that the riboflavin of milk catalyzes the photochemical oxidation of ascorbic acid and itself is changed in the reaction to lumichrome. After the destruction of the riboflavin, the ascorbic acid is stable to the action of light (97, 108, 124). The addition of more riboflavin restores the reactivity (98). Burr (35) probably was the first to observe that exposure to light hastened the deterioration of milk and that dark bottles would prevent deterioration. Hammer and Cordes (96) confirmed Burr's work and added that copper and iron hastened the development of the flavor. Sharp *et al.* (187) found that part of the vitamin C is destroyed by sunlight if oxygen is present, but if the milk is deaerated the vitamin C is not destroyed. They also found that irradiation to produce vitamin D in milk decreases the vitamin C content from 3.4 to 1.7, 3.8 to 1, and 11.0 to 5.5 mg./l. Trout and Gjessing (217) found a slight destruction of vitamin C by irradiation. Guthrie *et al.* (92) found that paper bottles decrease the effect of sunlight on the oxidation of ascorbic acid and on the development of the flavor. On the contrary, Doan and Meyers (56) found that the flavor is more intense in milk stored in paper than in milk stored in glass bottles, but paper bottles did protect against development of burnt flavors. It would appear as if the difference here is one of light transmission or intensity of the incident light and that the bottles used by Doan and Meyers transmitted some of the shorter wave lengths which cause burnt flavor (83). Marquardt (139) found that 20 to 60 minutes in sunlight caused the flavor to develop in 24 hours and 2 hours caused a bleaching effect. According to Greenbank (83), light may inhibit, promote or have no effect on the development of the flavor, depending on the metallic contamination of the milk and the intensity of irradiation.

Dissolved gases. Fresh whole milk drawn without gaseous contamination contains dissolved gases of which 81.5 per cent is CO_2, 2.42 per cent O_2 and 16.54 per cent N_2 (168). Milking increases the O_2 content to 13.18 per cent. Guthrie (89) found that milk direct from the udder contains from 0 to 11 mg./l. of O_2. According to Sharp *et al.* (190), hand milking introduces 5.8 and machine milking 4.7 mg./l. of oxygen.

The effect of air on the deterioration of milk and dairy products has been studied for years (95, 117, 118).

Hartman and Garrett (99) found that the ratio of oxygen consumed to ascorbic acid oxidized increases progressively as the oxidative reaction proceeds. After the ascorbic acid is oxidized, there is a further consumption of O_2 presumably by the oxidation of the fatty substances. Greenbank (83) observed that aeration, and Sharp *et al.* (190) that deaeration inhibited development of

the flavor. These reactions are discussed under the heading "Prevention." Thurston *et al.* (208) and Greenbank (80) found that prolonged stirring inhibits the flavor. This may be assumed to be a form of aeration. Deoxygenation by bacteria is discussed under "bacterial count."

Homogenization. Tracy *et al.* (212) were probably the first workers to observe that homogenization retards the development of the flavor. More recently, Thurston *et al.* (208), Dahle (45) and Ross (180) observed the same effect. Trout and Gould (218) report that homogenization does not retard development when the copper contamination is too high. Larson *et al.* (133) confirmed the previous work but found no relationship between extent of inhibition and the changes in E_h. While there is not a direct correlation at every point between the flavor and E_h, the work does show that the E_h of the homogenized samples maintains a relatively high potential for at least a week, while the unhomogenized samples show a marked drop in potential after the first day (133).

METHODS OF PREVENTION

Aeration and deaeration. The results of a number of workers seem to prove quite conclusively that the development of the flavor may be inhibited by either aeration or deaeration (53, 80, 190). Greenbank (80) was able to inhibit the development of the flavor by aeration or addition of hydrogen peroxide. The same author was able to increase copper tolerance by aeration. Prolonged agitation, which is a form of aeration, has been found by Thurston *et al.* (208) to inhibit development of the flavor. Dahle and Palmer (53) were probably the first to conclude that removal of oxygen dissolved in the milk would prevent development of the flavor. More recently, this conclusion has been confirmed by other workers (91, 92, 97, 188, 189, 190). Sharp *et al.* (187) have developed a commercial method of deaeration which protects the milk for 7 days when contaminated with 0.1 mg./l. of copper. Brown *et al.* (32) found that the flavor developed most rapidly in vacuum capped bottles. Greenbank (83) found that deaeration would not protect against flavor development when relatively high concentrations of copper were present.

Elimination of metallic contamination. Much of the oxidized flavor in milk would be eliminated if metallic contamination did not occur. Roadhouse (175) found that passing 5 gallons of hot milk through a bronze pump caused the development of the flavor and loss of 2 points in score. Nickel in the equipment may replace copper in solution, according to Fink and Rohrman (63). The use of stainless steel and enameled equipment practically eliminates the possibility of the flavor developing from contamination.

Segregation. A simple method of preventing the development of the flavor is to eliminate individual samples of milk which are "spontaneous" or develop the flavor with low metallic contamination. Spontaneous milk may be detected by storing individual samples at 4.0° F. Susceptible milk of low copper tolerance may be detected by the increase in E_h after the addition of copper, according to Greenbank (81).

Antioxidants. Although the addition of antioxidants to milk is prohibited

by law, a number of workers have studied the action of these compounds in milk. Ritter and Christen (173) used a dried culture of bacteria which Kertesz (123, 124) called "*Reductobacterium frigidum neutrale*" and inhibited the development of the flavor. The authors isolated 5 to 7 per cent hydroquinone from the dried culture. Bird *et al.* (17) found that the higher the content of iron the less tendency for the flavor to develop. They believed that the iron combines in a ferrous form with the protein to serve as an antioxidant. Anderson (8) reported the use of pancreatic enzyme prevented development of the flavor whether metal contaminated or not. Russell and Dahle (182) found that concentrated or dried milk added to fluid milk acted as an antioxidant. Dried milk is more effective than concentrated milk. Ritter (169) found hydroquinone, metol and ascorbic acid inhibited development of the flavor. The effect of ascorbic acid and hydroquinone has been confirmed by Chilson (37), Dahle and Palmer (53) and Greenbank (83). Ascorbic acid sometimes is considered an antioxidant. While it does retard development, there remains the question, according to Greenbank (84), whether this action is that of a reducing agent to lower the E_h or to supply protons to regenerate the natural antioxidants in milk. Oat flour, known as Avenex, has been found by many workers to have antioxygenic properties (32, 34, 45, 51, 52, 153, 160).

Condensing and drying. Corbett and Tracy (39) report that concentrating milk to double the solids content prevented the development of the flavor in the condensed and reconstituted milk. The addition of concentrated or dried milk before pasteurization was more beneficial than addition after pasteurization. The addition of 0.2 per cent solids-not-fat had a noticeable effect on the flavor. Dahle and Folkers (49) and Ross (181) found that ice cream containing dry skim milk did not develop the flavor, but the flavor developed when the ice cream contained condensed skim milk. The inhibiting action of these products is probably due to the high heat treatment they received. Krukovsky and Guthrie (130), in a study of ascorbic acid as a key factor in the development of the flavor, concluded that complete oxidation of the ascorbic acid will inhibit development of the flavor. Previously, Greenbank (83) found that the addition of hydrogen peroxide would inhibit the flavor development but did not relate this action to the destruction of ascorbic acid. He concluded that the inhibition was the result of a more complete oxidation of the precursor, presumably to a tasteless form.

THE OXIDIZED FLAVOR IN DAIRY PRODUCTS

Cream. According to many workers, cream is more susceptible to the flavor development than milk. The fact that the phospholipid content of cream is greater than that of milk may be significant. Cream, which was susceptible to the flavor when incubated according to the method of Tracy *et al.* (212), scored 3.5 points higher than a sample of the same cream stored at 40° F. without incubation. Kooper (127) observed that cream held in rusty cans developed a metallic flavor. Another worker (11) confirmed this. A number of investigators have found that it is not so much the exact temperature, provided it is low,

as the metallic contamination in stored cream which induces the flavor development (58, 149, 150, 151, 184, 200).

Condensed milk. The development of the flavor is not as common in condensed milk as in milk or cream, but it has been observed by a number of workers (57, 69, 111, 167). Sommer and Gebhardt (197) report that the flavor of evaporated milk is destroyed in proportion to the copper content; Corbett and Tracy (40) reported that condensing to twice the solids content prevented development of the flavor in the condensed and the reconstituted milk. They attribute inhibition to the liberation of antioxidants derived from the proteins. The high heat treatment should inhibit the development of the flavor, according to other workers (78, 83, 119).

Ice cream. This product is probably less susceptible to the development of the flavor than cream. Strawberry ice cream seems to be very susceptible. However, Dahle *et al.* (47, 49) found pineapple ice cream just as susceptible. Dahle and Folkers (50) and Tracy *et al.* (213, 214) report that increased amounts of berries delayed the onset. They also found that soaking the berries in the mix retarded the development. Heating the berries used to 150°, 175° and 200° F. or autoclaving the berries at 15 lb. pressure for 15 minutes decreased the flavor in the order given but did not eliminate it. Dahle *et al.* (50) were unable to eliminate the flavor by heating berries for 1 hour at 180° F. Mudge and Tucker (151) reported that aeration of the berries for a considerable time resulted in a stale and unclean flavored ice cream. The presence of metallic salts in the berries has been given as a reason for the development of the flavor. Ross (181) reports that iron is a factor, and Iverson (115, 116) reports that it is not a factor in the development of the flavor. The latter postulates that ferrous iron combines with protein and acts as an antioxidant. Mack and Fellers (136) observed that the acidity of the berries induced flavor development. Tracy *et al.* (214) observed that the citric acid content had no effect. The latter authors found that apples, apricots, lemons, oranges, pineapples and peaches promote the flavor when in concentrations of 3 to 5 per cent. The work of Dahle and Josephson (51) indicates that vanilla ice cream will develop the flavor but not as readily as strawberry. This difference may be due to the vanillin which has antioxygenic properties. Schricker (193) found that the development of the flavor occurs with increasing intensity in the following order: chocolate, vanilla and strawberry. Chocolate is probably protected by the tannins in the chocolate which are antioxidants and as previously stated, the vanilla by vanillin. Dahle and Folkers (48) and Ross (181) found that dry milk has a tendency to inhibit but that in most samples containing condensed milk the flavor developed, probably because dry milk gets the higher heat treatment and contains more reducing substances. Ross (181) observed that condensed milk contained more copper and developed the flavor sooner; Dahle and Folkers (50) found that the flavor in milk or skim milk has little effect on the good flavor of the ice cream. Bird *et al.* (17) found more copper in condensed skim milk than in the skim milk powder; the ice cream made with the powder developed a stronger flavor than ice cream in which condensed

milk was used. Dahle and Josephson (51, 52) report that 0.3 per cent of Avenex was not quite sufficient to inhibit completely the development of the flavor in strawberry ice cream. Mueller and Mack (152) found 0.25 per cent sufficient to delay development of the flavor but 0.50 per cent was still more effective. Weckel (224) suggested the use of not more than 0.3 per cent for vanilla and 0.50 per cent for strawberry ice cream. Mack and Tracy (135) and Burke and Newman (34) and also Brown (31) found that 0.5 per cent of Avenex was sufficient to insure a fresh flavor.

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