A STUDY OF THE PHOSPHATASE TEST AND ITS USE AS AN INDICATION OF PASTEURIZATION

By

PETE J. MAGNINO, JR.

Bachelor of Science

Oklahoma Agricultural and Mechanical College

Stillwater, Oklahoma

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Thesis Approved:

James B. Mickle
Thesis Adviser

H. Olson
Faculty Representative

Robert Maddox
Dean of the Graduate School

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INTRODUCTION

The phosphatase test, when first developed in 1935, was thought to be an adequate test for the detection of underpasteurized milk. In recent years experiments and observations have shown that it might be otherwise.

Two different sources (36, 46) in the state of Oklahoma have reported "false positive" results with the phosphatase test, especially with high-temperature short-time pasteurization. In one instance, milk pasteurized at Muskogee, by the high-temperature short-time method, gave a negative test immediately after pasteurization, but a positive test after shipment to Tulsa.

According to various workers, reactivation of the enzyme occurs in some cases, especially in cream and when high pasteurization temperatures are used.

The primary objectives of this study were: (1) to learn more about the phosphatase test as applied to pasteurized milk; (2) to determine, if possible, some of the causes of "false positive" results; and (3) to learn whether or not these "false positive" results were caused by "reactivation" as described in the literature.

REVIEW OF LITERATURE

In 1933, Kay and Graham (31) published a method of determining the phosphatase content of milk and stated that the enzyme was sufficiently thermolabile to be destroyed completely when milk was heated to pasteurization temperatures. The Kay and Graham phosphatase test for detecting underpasteurized milk and pasteurized milk adulterated with raw was released in 1935 (32).

Graham and Kay (19) reported that active alkaline phosphatase was found in cows milk and would cause changes in the phosphoric ester content of milk upon standing at an optimum pH of 9.0. According to Morton (41), all true orthophosphate monoesters were rapidly hydrolyzed by milk phosphatase. Milk phosphatase is similiar to the phosphatases found in other parts of the body (38, 40, 69) and consists of an apoenzyme and a coenzyme (1). The apoenzyme appears to contain certain amino acids essential for phosphatase activity, according to Abul-Fadl and King (1). The enzyme is largely located at the fat globule-serum interface and is associated with an insoluble lipoprotein complex (38, 39, 69).

According to Burgwald (9) any milk which has been heated to over 60°C. for a sufficient length of time to destroy 96% of the phosphatase present is free of pathogenic organisms.

The principle of the phosphatase test is the addition of a small portion of milk to a large excess of phosphoric ester. During a standard period of incubation at an optimum pH and temperature; the phosphatase, if present, will hydrolyze the ester releasing phenol. The hydrolysis is stopped after a given period of time and the released phenol is determined colorimetrically.

The phosphatase test has undergone several modifications since its release and some of these are reviewed by Burgwald (9). The Sanders and Sager modification is used extensively today and is found in Standard Methods for the Examination of Dairy Products (2).

The principle of the test is the same as that of the Kay and Graham test, but the incubation period is only one hour and in place of Folin and Ciocalteu's coloring reagent, 2, 6-dibromoquinonechloroimide (BQC), as devised by Scharer (56), is used. Photometric determination of the liberated phenol, using a 610 mμ filter was first devised by Storrs and Burgwald (59) and perfected by Sanders and Sager (54).

The Sanders and Sager test will detect variations, from a standard of 143°F. for 30 minutes, of 1°F. less than the recommended temperature, five minutes less than the recommended holding time and 0.1% raw milk adulteration (9).

Horwitz, Knudsen, and Weiss (30), compared several phosphatase tests and concluded that the Sanders and Sager test with photometric determination of the phenol was the best procedure for detecting under-pasteurized or adulterated milk.

Several precautions are listed by various authors which must be followed to insure correct results with the phosphatase test. All glassware used must be washed in acid or alkaline cleanser and

thoroughly rinsed with distilled water (9, 10, 18, 52, 53). Phenol must not be stored in a laboratory where the test is being run and soaps containing carbolic acid or disinfectants containing phenol must not be used (32). Phenol contamination from plastic closures has been encountered (56) and rubber stoppers must be boiled in distilled water before using (37). BQC should be made daily and di-sodium phenol phosphate over three days old should not be used (32, 54). Whatman No. 42 filter paper was found to give the most satisfactory results (3) and when filtering, all the filtrate should not be allowed to filter through (9). Care should be taken to prevent contamination of the test with saliva (56) and erratic or false results can be caused by insufficient mixing throughout the test (52).

Sanders and Sager (55) established a unit of phosphatase as the intensity of blue color produced by one microgram, or part per million (p.p.m.) of phenol per 0.5 ml. of sample used. Using this standard they found that a negative phosphatase test resulted when not more than 2 units were produced by 0.5 ml. of milk. The minimum pasteurization standard, established by Scharer (56) for milk heated to 143°F. for 30 minutes was the blue color developed by 0.5 p.p.m. or 2.5 phosphomonoesterase units (2.5 gamma) in 5 ml. of milk. Phenol values higher than 4 p.p.m. per ml. of sample indicate underpasteurization of cows milk according to Standard Methods (2).

Hetrick and Tracy (27) established a straight line, semi-logarithmic relationship between the time and temperature required to inactivate phosphatase over a temperature range of 143°F. to 185°F. They expressed it by the formula $T = 174 - 9 \log t$: T being the temperature in degrees Fahrenheit and t the holding time in seconds. The Sanders

and Sager test was used to establish this formula and a value of one p.p.m. of phenol per ml. was used as a standard for inactivation.

A straight line graph also was prepared by Lear and Foster (34) for inactivation of phosphatase between 60°C. and 72°C. Hansen, Wood, and Thornton (23, 24) established a similar graph for temperatures between 153.3°F. and 160.7°F. This graph was represented by the equation $T = 171.84 - 9.66 \log t$.

Read, et. al. (50) found that a heating time and temperature of 0.25 second at 175.6°F. was sufficient to destroy completely pathogenic organisms in milk while phosphatase was inactivated in 0.25 second at 182.4°F. Micrococcus organisms are 99.99% destroyed at 144°F. for 55 minutes, 160°F. for 15 seconds and 165°F. for 4 seconds according to Speck (58). The same percentage destruction was obtained by Tobias, Herreid, and Ordal (61) at 143°F. for 30 minutes and 168.34° F. for 2.36 seconds assuming zero heating and cooling times. They also found that phosphatase could be inactivated to 4 p.p.m. phenol per ml. of milk at 169.7°F. for 2.36 seconds.

Temperatures between 140°F. and 160°F. required longer holding times to kill 99.99% of the pathogenic organisms and inactivate the phosphatase in cream than in milk, but in both instances a safety margin was maintained between bacterial destruction and phosphatase inactivation (25).

When pasteurizing milk at 143.5°F., Oldenbush, et. al. (43), found an ample margin of safety for pathogenic bacteria destruction. Dahlberg (12) established pasteurization standards from 140°F. to 160°F. and prepared margins of safety between bacterial thermal death points and standards for pasteurization which he found to be smaller at high-

temperature short-time intervals. Prucha and Corbett (48) also found this margin to be smaller at higher temperatures, but the safety margin between bacterial destruction and phosphatase inactivation was found to be wider at high temperatures.

It has been shown by several authors (7, 8, 47, 51, 63) that pasteurized cream will give a negative phosphatase test immediately after pasteurization but a positive test upon storage for a few days. Some "reactivation" occurred in cream pasteurized at temperatures of 62.2°C. and at storage temperatures down to 4°C.

On the other hand, Wister and Covington (64) found no increase in phosphatase activity of pasteurized cream and ice cream mix after storage of 24, 48, and 72 hours at pasteurization temperatures from 170°F. to 205°F.

Wright and Tramer (65) found inactivation of phosphatase to be practically instantaneous at temperatures over 80°C. In addition, milk heated at 85°C. and stored at 18 - 37°C. (optimum 30°C.) would give a positive phosphatase test after storage. This reappearance of phosphatase activity was termed "reactivation" and the phosphatase responsible was found to be identical to the alkaline phosphatase of raw milk.

Reactivation was found to occur in commercial milk heated to over 100°C. and cooled rapidly (65), but did not occur in commercial samples pasteurized at 161°F. for 15 seconds or 145°F. for 30 minutes in the laboratory (67).

No relation was found between reactivation and fat content, addition of ascorbic acid, metallic ions and amino acids or initial phosphatase content of raw milk. Removal of oxygen increased the reactivation while storage of raw milk increased the phosphatase content.

Reactivation did not occur when milk was heated sufficiently to denature the soluble protein (66).

Reactivation was shown by Fram (17) in milk heated to 260 - 280°F. for several seconds and stored at 88°F. for 18 hours. Reactivation did not occur in milk stored at 40°F. but occurred in skim milk heated to 162°F. for 16 seconds and after 2 hours in 20% cream pasteurized at high-temperature short-time intervals.

Read, et. al. (49) tested samples of milk immediately after pasteurization and upon storage at 46.4°F. for 24 hours. The milk was pasteurized at 180.1°F. to 184.1°F. for 0.25 second and at 176.9°F. to 179.8°F. for 0.50 second. These heat treatments gave 2.3 μ g. of phenol immediately after pasteurization. It was concluded that some reactivation was shown, but the results were very erratic.

Posthumus' (47) theory of reactivation states the enzyme is bound to the fat in such a way as to escape complete destruction during a very short heat treatment, but the active enzyme will diffuse into the serum upon storage and produce a positive test.

Three theories of reactivation were offered by Wright and Tramer (66): (1) reactivation occurs as the result of the reversion of the denaturing of the apoenzyme; (2) the coenzyme is inactivated upon heating but a new coenzyme is formed upon storage at certain time and temperature intervals; (3) heating the milk at certain temperatures breaks the bondage between the apoenzyme and coenzyme but a new bondage is formed at slightly higher temperatures.

Certain chemicals and metallic ions have been found to influence the phosphatase enzyme's activity. Wright and Tramer (68) found that ethylene diamine tetracetic acid would increase enzyme activity and

reactivation while magnesium, zinc, and manganese ions activated the enzyme. Copper and nickel ions as well as CO_2 were found to be inhibitory factors. Haab and Smith (21) found that magnesium ions did not increase the enzyme activity. Morton (41) stated that several phosphorus compounds caused slight inhibition of the enzyme's activity while iodine and cysteine caused strong inhibition. Magnesium salts were found, by Folley and Kay (15), to activate the phosphatase enzyme while Pett and Wynne (44) found the enzyme activity could be accelerated by arsenate or arsenite.

Churchill, et. al., (11) stated that antibiotics do not impair the reliability of the phosphatase test but Stotz and Hankinson (60) found that a small amount of antibiotic and a sub-standard pasteurization temperature could yield a false negative test.

Hammer and Olson (22) incubated bacterial cultures in sterile milk at 21°C. and 37°C. and found some strains produced phosphatase. Pseudomonas putrefaciens was an excellent producer and some Aerobacter groups gave positive results. Some authors (10, 17, 18, 33, 42) have found that a positive phosphatase test can result from bacteria but counts must reach several million. Sanders (53) found phosphatase activity was not caused by bacteria in fresh or reasonably fresh products but this type of activity was found in old butter, old cream, and cheese. A control test was used by Tramer (62) to demonstrate that bacterial phosphatase was produced by growing organisms. He incorporated di-sodium-para-nitrophenyl-phosphate into suitable media and measured colorimetrically the para-nitrophenol liberated by the organisms present.

The phosphatase content of raw milk has been found to vary from 950 - 3,000 p.p.m. for herd milk (20, 28) and 119 to 4,380 for

individual cows (20). An advance in lactation is accompanied by an increased concentration of phosphatase in milk (4, 16, 20, 28, 32) and mastitic milk has a very high phosphatase content (4). The maximum concentration for individual cows is reached approximately 180 days after parturition (16, 20).

Before accurate results can be obtained on raw milk, the substrate concentration must be higher than that used in testing pasteurized milk. In addition, a 20 minute hydrolysis period, at 38.5°C. is necessary and a pH of 10.32 ± 0.02 must be attained, according to Haab and Smith (21).

According to Ball (6), a major portion of the heat required for pasteurization may be furnished during the coming-up and cooling times when holding periods of 3.5 minutes or less are used.

Holland and Dahlberg (29) stated that a neglect to consider the time of holding before heating to high temperatures was an error often encountered in experiments on milk pasteurization.

An experiment by Herschdorfer (26) on the relationship of phosphatase destruction of heated milk cooled in air and ice water showed that milk cooled by air (at room temperature) had a higher phosphatase content than milk cooled in ice water to 5°C. immediately after heating.

EXPERIMENTAL METHODS

A. GENERAL PROCEDURE

The milk used in this experiment represented the night and morning milkings of the Oklahoma State University dairy herd. The milk was collected from the bulk storage tank at the dairy barn after completion of the morning milking, and care was taken to insure agitation of the milk before collecting it.

The milk was prepared for heating by sealing 13 ml. of it into a 16 x 150 m.m. soft glass test tube. The tubes were then placed in a water bath and the temperature of the contents was adjusted to 40°C.

Heating was accomplished in a bath filled with Fisher Stabilized Bath oil. A stirrer was placed in the bath to insure a constant temperature throughout the bath and the temperature was controlled by a Mercury Thermoregulator which had a sensitivity of $\pm$ 0.01°C.

When the bath had been adjusted to the desired temperature, the sealed tubes of milk were put into the bath. There they were turned end over end, by mechanical means, at a constant speed of approximately 3 r.p.m., while completely submerged in the oil. After the desired time interval had elapsed, the tubes were removed from the bath and allowed to cool to room temperature (29 $\pm$ 2°C.).

The milk was heated at 62.5°C., 65.0°C., 71.1°C., and 76.7°C. for various time intervals. These intervals, at each temperature, were

adjusted so that one produced milk that was underpasteurized, another produced milk that was pasteurized the minimum length of time, and the third produced milk that was overpasteurized.

Duplicate tubes of milk were heated at each time - temperature interval. One duplicate was tested immediately and one was stored at 32.2°C. for 24 ± 2 hours and then tested.

The phosphatase test used on all samples was the Sanders and Sager Method II (2). This procedure was modified by doubling all the dilutions to facilitate reading the samples colorimetrically. In addition, 0.2 ml. of BQC was added from a burette instead of the recommended 16 drops. All samples were run in duplicate and a blank was prepared for each set of duplicates.

The intensity of the blue color present in the tubes was determined colorimetrically with a Fisher Nefluoro-Photometer using a 610 mμ filter. Each milk sample was read, from the logarithmic scale, with the colorimeter standardized on the blank. To check for errors in the readings the colorimeter also was standardized on distilled water and colorimetric readings were made on both the samples and the blanks. However, only the readings made when the colorimeter was standardized on the blank were reported in the tables that follow.

The fat content of the milk was determined by the Babcock test, total solids by the Cenco method (35), ash by A.O.A.C. method (5), lactose by the method of Perry and Doan (45), and the pH was determined with a Beckman pH meter, Model H2, using a glass electrode.

B. MILK SAMPLE VARIATIONS

An effort was made to determine what variations occurred in the phosphatase content of the milk used in this work during different stages of handling. Samples were collected at the dairy barn storage tank after the evening and morning milkings: approximately 5:00 P.M. and 5:30 A.M. respectively. Samples of this same milk were collected from the holding vat in the college processing plant at 6:30 A.M. and 7:30 A.M.

These four raw milk samples were pasteurized separately at 62.5°C. for 24 minutes and tested immediately by the phosphatase test. The raw samples were then stored in the processing plant milk cooler at 45°F. for 24 hours. Duplicate samples of the stored raw milk were then heated to 62.5°C. for 24 minutes. One duplicate was tested immediately and one was stored at 32.2°C. for 24 hours before testing.

Two trials were conducted to determine the daily phosphatase variations in the milk samples used for this work. The milk used for these trials was collected from the college dairy barn bulk storage tank after the morning milking.

In trial 1, samples were collected for five consecutive days and a portion of each sample was heated to 62.5°C. for 24 minutes and tested for phosphatase inactivation immediately. The raw samples were stored at 45°F. for 24 hours and triplicate portions were heated from each sample. One replicate was tested immediately, one was stored for 24 hours at 32.2°C. before it was tested, and the last replicate was stored for 48 hours at 32.2°C. and then tested.

Trial 2 was conducted in the same manner as trial 1 except that duplicate portions of fresh raw milk were heated to 76.7°C. for 2.25 minutes. One duplicate was tested immediately and the other was tested after storage for 24 hours at 32.2°C.

C. HEATING AND COOLING CURVES

Heat curves were established to determine the length of time required for the milk to reach the temperature of the oil bath under the conditions of this experiment.

To do this a small thermometer which could be read to 0.1°C. was sealed into a 16 x 150 m.m. soft glass test tube filled with 13 ml. of water. The tube was placed in a water bath, the temperature of its contents adjusted to 40°C., and the tube was then put into the oil bath set at the desired temperature. Temperature readings were taken of the tube contents while it remained in the bath, as the temperature rose from 40°C. to the bath temperature. Five trials were run at each oil bath temperature (62.5, 65.0, 71.1, and 76.7°C.), then an equation was fitted to each set of data.

A cooling curve for each of the milk heating temperatures was established with the same test tube and thermometer assembly used to establish the heating curves. The curves for 62.5°C. and 65.0°C. were determined by heating the tube of water to 62.5°C. and 65.0°C. then removing the tube and allowing it to cool at room temperature. Readings of the temperature were taken as the tube's contents cooled to 40°C.

A graph of the 71.1°C. and 76.7°C. heating curves showed that the milk only reached 68.0 $\pm$ 0.5°C. when exposed to these temperatures

for time intervals that would assure minimum pasteurization. Thus a cooling curve with the oil bath set at 68°C. was used for these two oil bath temperatures. Four or more trials were run at each oil bath temperature and an equation was fitted to the data obtained for each temperature.

D. METHOD OF EXPRESSING RESULTS

The amount of phenol present in the heated milk was determined by comparing the colorimeter reading of the samples to those obtained from prepared phenol standards. Standards containing 1, 2, 3, 5, 7, 7.5, 9, 10, 13, 15, 20, and 30 p.p.m. of phenol were prepared and tested to evaluate the intensity of the blue color they produced by the phosphatase test used for milk. Two modifications of the milk test were used: the incubation period was omitted and the blank was prepared by using 2 ml. of distilled water, instead of boiled milk.

The data for the standards were collected by making several new stock solutions and at least two runs were made by two individuals from each stock solution.

Data were collected to see what relationships existed between the phosphatase content of the heated milk; and the pH and the phosphatase, fat, ash, and total solids content of the raw milk. The phosphatase content of the raw milk was determined by the same procedure used for the heated milk, but a dilution of 1 ml. of the raw solution in 19 ml. of color dilution buffer was made immediately before reading the samples colorimetrically.

RESULTS AND DISCUSSION

A. CONVERSION OF COLORIMETER READINGS TO PHENOL UNITS

A graph of a straight line calculated according to Snedecor (57) and represented by the formula $\hat{Y} = 0.59 + 1.2253X$ was used to convert all colorimetric readings taken during this experiment to parts per million of phenol. The data for this graph were from colorimetric readings of prepared phenol standards.

The first visible blue color in the milk was detected at a colorimetric reading of 5.0 ± 1.76. The mean of samples showing a slight visible blue was 5.0 and 1.76 was the 95% confidence interval prepared from the sample standard deviation of all duplicate milk samples run in this experiment (57).

The colorimetric reading of 5.0 ± 1.76, when converted, gave phenol values of 3.6 ± 1.3 p.p.m. Pasteurized milk throughout this experiment was referred to as any milk that had been heated, tested by the phosphatase test and produced less than 3.6 p.p.m. of phenol. However, any milk producing a phenol value between 2.3 and 4.9 was considered within the range of minimum pasteurization. This phenol value representing the point of minimum pasteurization approximates the values of 4.0 p.p.m. of phenol per ml. listed by Hansen, Wood, and Thornton (23, 24) and in Standard Methods (2).

The raw milk values are expressed in p.p.m. of phenol but these are relative values only, obtained by making a 1 - 20 dilution of the test solution filtrate with color dilution buffer immediately before reading them colorimetrically. After this procedure was established, it was found, according to some authors (21, 28), that more substrate must be used to determine the total phenol content of raw milk than was used for heated milk samples.

Later experimental work showed that determinations of the raw milk phenol content could be made by a 1 - 100 dilution of the raw milk with water and testing the mixture by the same procedure used for heated milk.

B. PHENOL CONTENT OF MILK HEATED AT FOUR TEMPERATURES FOR SELECTED TIME INTERVALS

Tables I to IV give the phenol content of the milk samples heated at 62.5°C., 65.0°C., 71.1°C., and 76.7°C. for selected time intervals. At each temperature the minimum holding time required to produce minimum pasteurization of the milk was found. Several trials were run at this time interval as well as at time intervals greater and less than this minimum.

The minimum times of pasteurization at the four temperatures appeared to be: 24.0 minutes at 62.5°C.; 9.5 minutes at 65.0°C.; 3.5 minutes at 71.1°C.; and 2.25 minutes at 76.7°C. However, the point of minimum pasteurization sometimes varied with different milk samples.

Early experimental work at 62.5°C. in November and December, 1956 (Samples D1 and E1) indicated the minimum time of pasteurization

to be 24 minutes. Additional experimental work in June, 1957 (C1) indicated milk was pasteurized when held for 23 minutes. The minimum time of pasteurization at 65.0°C. appeared to be 9.5 minutes in March (C2 and D2) but 60 days later, milk heated for 9.0 minutes gave values below the line of pasteurization. Two samples (E3 and F3) indicated the minimum time of pasteurization at 71.1°C. was 3.5 minutes, but three samples (B3, C3, and D3) run 30 days later were pasteurized after 3.0 minutes. Samples F4 and A6 - E6 established the minimum time of pasteurization at 76.7°C. to be 2.25 minutes, but three samples (C4, D4, and E4) indicated that the minimum time of pasteurization was 2.0 minutes.

Although complete data for the raw samples were not obtained on samples at the 62.5°C. temperature, and the raw values for the other temperatures did not show large variations, the author believes that these changes in pasteurization times are due in part to seasonal variations in the phosphatase content of the raw milk. The experimental error in this work (95% limits, $\pm$ 1.3 p.p.m. phenol) also may account for part of these variations in pasteurization times.

The incubated samples, throughout these tables gave more erratic values than did the same samples before incubation. However, no general trend toward higher or lower values after incubation was observed, and most variations were within the experimental error.

Several samples gave lower values after incubation than before but no value indicated the milk was underpasteurized before incubation but pasteurized afterward. Only five samples (F1, A2, D3, I3, and C4) gave phenol values that indicated the milk was pasteurized immediately after heating but underpasteurized upon incubation.

TABLE I

PHENOL CONTENT OF MILK HEATED IN A 62.5°C. BATH FOR SELECTED TIME INTERVALS[a]

Date	Sample Number	Time	Raw Diluted 1/20	Heated	
				Before Incubation	After 24 hr. Incubation
		(min)	(p.p.m.)	(p.p.m.)	(p.p.m.)
6/10/57	A1	22.5	6.9	7.7	6.3
6/11/57	B1	22.5	8.9	4.8	4.4
6/ 5/57	C1	23.0	9.6	1.2	3.0
11/28/56	D1	24.0	-	3.0	2.8
12/ 4/56	E1	24.0	-	4.8	6.9
10/21/56	F1	25.0	-	2.2	6.9
12/ 4/56	G1	25.0	-	2.8	2.4
11/28/56	H1	26.0	-	2.4	1.2
12/ 4/56	I1	26.0	-	2.8	2.6

[a]Each value represents the average of duplicates

TABLE II

PHENOL CONTENT OF MILK HEATED IN A 65.0°C. BATH FOR SELECTED TIME INTERVALS[a]

Date	Sample Number	Time	Raw Diluted 1/20	Heated	
				Before Incubation	After 24 hr. Incubation
		(min)	(p.p.m.)	(p.p.m.)	(p.p.m.)
5/24/57	A2	9.0	9.6	3.2	6.1
6/10/57	B2	9.0	6.9	5.2	4.8
3/ 4/57	C2	9.5	-	1.0	1.8
3/ 8/57	D2	9.5	9.8	4.0	8.1
3/ 8/57	E2	9.75	9.8	4.4	4.2
5/24/57	F2	9.75	9.6	2.0	3.2
6/10/57	G2	9.75	6.9	2.8	3.2

[a]Each value represents the average of duplicates

TABLE III

PHENOL CONTENT OF MILK HEATED IN A 71.1°C.

BATH FOR SELECTED TIME INTERVALS[a]

Date	Sample Number	Time	Raw Diluted 1/20	Heated	
				Before Incubation	After 24 hr. Incubation
		(min)	(p.p.m.)	(p.p.m.)	(p.p.m.)
6/11/57	A3	2.75	8.9	13.2[b]	4.0
5/22/57	B3	3.0	10.2	3.2	0.1
5/23/57	C3	3.0	10.2	1.8	0.4
6/10/57	D3	3.0	6.9	2.6	5.6
4/12/57	E3	3.5	5.2	3.2	2.4
4/15/57	F3	3.5	11.4	3.4	2.0
4/ 8/57	G3	3.75	5.0	2.4	2.6
4/12/57	H3	3.75	5.2	2.0	1.4
3/30/57	I3	4.0	5.0	2.6	4.0
4/ 8/57	J3	4.0	5.0	0.0	0.0

[a]Each value represents the average of duplicates

[b]Thought to be an error

TABLE IV

PHENOL CONTENT OF MILK HEATED IN A 76.7°C.

BATH FOR SELECTED TIME INTERVALS[a]

Date	Sample Number	Time	Raw Diluted 1/20	Heated	
				Before Incubation	After 24 hr. Incubation
		(min)	(p.p.m.)	(p.p.m.)	(p.p.m.)
6/12/57	A4	1.5	9.8	120.5	-
6/12/57	B4	1.75	9.8	35.1	28.1
5/15/57	C4	2.0	11.4	1.6	3.6
5/17/57	D4	2.0	7.3	4.0	8.1
6/11/57	E4	2.0	8.9	2.0	2.4
1/25/57	F4	2.25	-	2.0	2.4
5/14/57	G4	2.5	3.8[b]	2.0	2.0
6/11/57	H4	2.5	8.9	0.0	1.2

[a]Each value represents the average of duplicates

[b]Thought to be an error

A very noticeable illustration of the great variation in the phenol value that can be obtained by a slight change in the holding time is shown in table IV by samples A4 and B4. Both samples were run on the same day and both gave high phenol values. The blue color produced by sample A4 heated only 0.5 minute below the minimum time of pasteurization could not be distinguished visually from the color produced by the raw sample.

C. DAILY AND WITHIN-DAY VARIATIONS IN MILK SAMPLES

Two trials were run on milk samples collected for five consecutive days. Trial 1 was heated at 62.5°C. for 24 minutes and trial 2 at 76.7°C for 2.25 minutes.

Table V contains data for trial 1. These data show no trend toward higher phenol values after incubation of the raw or heated milk. The values obtained for this trial were somewhat erratic. However, they seem to indicate that if milk is heated at a time and temperature relationship which represents minimum pasteurization, the phenol values obtained from this milk will fluctuate, within the limits of experimental error, between pasteurized and underpasteurized.

The data for trial 2, run on daily samples heated at 76.7°C. for 2.25 minutes for five consecutive days, are found in table VI. All values were very low and none gave a value before or after incubation that indicated underpasteurization.

The phenol values were collected for the raw milk used in this trial but showed very little variation. These variations could have been due to variance in the technique of running the tests.

TABLE V

PHENOL CONTENT OF DAILY MILK SAMPLES HEATED

AT 62.5°C. FOR 24 MINUTES[a]

Date	Sample Number	Before Storage	Raw Stored at 45.°F. for 24 hrs. then Heated		
			Before Incubation	Incubated 24 hrs.	Incubated 48 hrs.
		(p.p.m.)	(p.p.m.)	(p.p.m.)	(p.p.m.)
2/11/57	A5	4.8	2.0	7.1	3.2
2/12/57	B5	2.0	4.4	4.0	6.0
2/13/57	C5	4.6	3.6	4.3	3.0
2/14/57	D5	2.6	2.2	3.4	2.8
2/15/57	E5	4.0	0.4	4.8	2.8

[a]Samples collected for five consecutive days

TABLE VI

PHENOL CONTENT OF DAILY MILK SAMPLES HEATED

AT 76.7°C. FOR 2.25 MINUTES[a]

Date	Sample Number	Raw Diluted 1/20	Heated	
			Before Incubation	After 24 hr. Incubation
		(p.p.m.)	(p.p.m.)	(p.p.m.)
5/27/57	A6	8.7	1.6	3.2
5/28/57	B6	8.7	1.0	0.0
5/29/57	C6	9.2	0.1	0.0
5/30/57	D6	7.9	0.1	0.8
5/31/57	E6	6.2	0.4	0.6

[a]Samples collected for five consecutive days

TABLE VII

PHENOL CONTENT OF MILK COLLECTED AT SELECTED TIMES DURING ONE DAY AND HEATED AT 62.5°C. FOR 24 MINUTES

Sample Number	Before Storage	Raw Stored at 45°F. For 24 Hours then Heated	
		Before Incubation	After 24 hr. Incubation
	(p.p.m.)	(p.p.m.)	(p.p.m.)
A7	5.6	3.2	4.4
B7	3.0	3.6	3.4
C7	4.8	3.0	4.2
D7	3.8	3.0	2.6

A7 collected at 5:00 P.M. after the evening milking (2/5/57) at the college dairy barn storage tank.

B7 collected at 5:30 A.M. (2/6/57) from the mixed evening and morning milkings at the college dairy barn storage tank.

C7 collected at 6:00 A.M. (2/6/57) at the college dairy plant holding tank.

C8 collected at 7:30 A.M. (2/6/57) at the college dairy plant holding tank.

TABLE VIII

COMPOSITION DATA OF MILK USED FOR

HEATING EXPERIMENTS

Date	pH	Fat	Total Solids	Lactose	Ash
		%	%	%	%
10/21/56	--	--	--	--	--
11/28/56	--	4.5	13.3	--	.749
12/ 4/56	--	4.5	13.6	--	.763
1/25/57	6.7	4.1	13.0	4.50	.725
2/ 6/57	6.8	4.25	12.9	4.66	.775
2/11/57	6.7	4.15	12.8	4.26	.751
2/12/57	6.7	4.2	12.6	4.02	.754
2/13/57	6.7	4.2	12.8	4.23	.746
2/14/57	6.7	4.2	12.8	4.32	.741
2/15/57	6.7	4.2	12.8	4.14	--
3/ 4/57	--	4.2	13.0	4.90	.740
3/ 8/57	6.7	4.2	12.6	4.00	--
3/30/57	6.7	4.0	13.0	5.40	--
4/ 8/57	6.8	4.0	13.2	4.90	--
4/12/57	6.8	3.9	13.1	4.40	--
4/15/57	6.8	3.8	13.2	4.55	--
5/14/57	6.8	3.9	12.8	5.03	--
5/15/57	6.75	3.8	12.7	5.42	--
5/17/57	6.75	4.4	13.0	5.30	--
5/22/57	6.7	3.8	12.4	4.87	--
5/23/57	6.75	4.0	12.5	4.72	--
5/24/57	6.7	3.9	12.8	4.55	--
5/27/57	6.7	4.0	12.6	4.65	--
5/28/57	6.7	3.8	12.6	5.00	--
5/29/57	6.75	3.6	12.4	4.72	--
5/30/57	6.8	3.9	12.5	4.55	--
5/31/57	6.8	3.8	12.3	4.52	--
6/ 5/57	6.7	3.8	12.3	5.07	--
6/10/57	6.7	3.6	12.2	5.25	--
6/11/57	6.65	3.7	12.0	5.07	--
6/12/57	6.7	3.7	12.1	5.15	--

Table VII contains data obtained from milk of four different sources during one day and heated at 62.5°C. for 24 minutes. The values show very little variation and only one (A7) was outside the limits of pasteurization.

D. COMPOSITION OF MILK SAMPLES

Table VIII contains the milk composition data collected from the samples used in this experiment. No relation between the composition data and variations in the phosphatase content of the raw or heated milk was observed. Therefore, no effort was made to calculate any such relationships.

E. CALCULATION OF HEATING AND COOLING CURVES

The preheating, holding, and cooling times for the milk heated at 62.5°C., 65.0°C., 71.1°C., and 76.7°C. were determined by fitting equations to the data obtained from the heating and cooling trials. The heating curves are represented by the formula:

$$T = B - (B - 40)\, e^{-kt}$$

when: T = temperature of water or milk in °C.

B = oil bath temperature in °C.

40 = temperature of tube contents before heating

k = constant

t = time in seconds

The constant, k, was calculated to be 0.01149 for 62.5°C., 0.01068 for 65.0°C., 0.01119 for 71.1°C., and 0.01117 for 76.7°C.

It was found that when milk was heated in a 71.1°C. or a 76.7°C. bath that the milk only reached 68.0 $\pm$ 0.5°C. at the time it was

pasteurized. Therefore a cooling curve starting at 68.0°C. was used for both of these high heat - treatments. Separate cooling curves were prepared for 62.5°C. and 65.0°C.

These curves are represented by the formula:

$$T = R + (B - R)\ e^{-kt}$$

when: T = temperature of water or milk in °C.

R = room temperature in °C.

B = oil bath temperature in °C.

k = constant

t = time in seconds

The k value was calculated as follows:

Temperature (°C.)	Trial 1	Trial 2
62.5	0.00213	0.00223
65.0	0.00203	0.00228
68.0	- - - -	0.00141

According to Farrall (13) the specific heat of milk, at temperatures between 62.5°C. and 76.7°C., is 0.93 ± .01. All heating and cooling curves in this experiment were run with water which caused the heating and cooling curves to be somewhat high. The thermometer used to establish these curves had a lag of approximately five seconds which caused the measured curves to be low. These two errors were in opposite directions, but no way was found to determine whether or not they exactly compensated each other. In view of this it was felt that the calculated equations were the most accurate estimate of the milk heating and cooling curves.

The combined heating and cooling curves showed that milk heated at 62.5°C. for 24 minutes required 5 minutes 50 seconds to rise from

40°C. to 62.5 ± 0.5°C. The milk was held at 62.5°C. for 18 minutes 10 seconds and cooled to 54 ± 0.5°C. in 2 minutes 15 seconds. When heated at 65.0°C. for 9.5 minutes the milk required 6 minutes 15 seconds to reach 65.0 ± 0.5°C. It had a holding time of 3 minutes 15 seconds and required 2 minutes 50 seconds to cool to 54 ± 0.5°C. Milk that was heated at 71.1°C. for 3.5 minutes and 76.7°C. for 2.25 minutes only reached 68.0 ± 0.5°C. and both cooled to 54 ± 0.5°C. in approximately 5 minutes 10 seconds.

F. EXPERIMENTAL ERRORS

The 95% confidence limits were calculated from the data used to plot the phenol standard curve (57). The limits of the regression line ($S\hat{Y}$) were ± 0.553 and for the mean of duplicate observations (SY) they were ± 3.75. These values appeared large, however, limits calculated from some of Burgwald and Giberson's data (10) gave values of ± 0.617 and ± 9.96 for $S\hat{Y}$ and SY respectively.

Toward the end of the experimental work, a slope-ratio assay procedure (14) was set up to determine differences between individuals and days. The procedure followed was the same as described earlier, but the tubes were randomized before adding BQC and were read colorimetrically in this randomized order.

In this experiment standard phenol samples (10 p.p.m.) were run against two unknowns, one a phenol standard of 7.5 p.p.m., and one a milk sample that had been pasteurized at 71.1°C. for 2.75 minutes. Duplicate determinations of 0.0, 0.5, 1.0, 1.5, and 2.0 ml. were run from each sample and the lines made by graphing the values for the

unknown samples were compared to a similar line prepared from the known sample. Duplicates were run by two individuals on separate days.

Statistical analysis showed the test on the phenol unknown to be valid, and there were no differences between individuals or days. However, due to a difference in the blanks, the test on the milk unknown was not valid. In all cases the lines made by graphing data from any one sample appeared to be linear.

G. OTHER PROBLEMS SUGGESTED BY THIS STUDY

During the course of this experiment several problems were found which appear worthy of further study. These revolve around the effects of preheating and cooling time - temperature relationships upon pasteurization and the phosphatase test.

The mathematical evaluation of these relationships and the use of these evaluations to help understand the phosphatase enzyme, and pasteurization, should be an interesting and worthwhile study.

SUMMARY AND CONCLUSIONS

The primary objective of this experimental work was to determine if reactivation of the phosphatase enzyme in milk would occur during incubation after milk had been pasteurized. In this study, the milk was heated at four temperatures (62.5°C., 65.0°C., 71.1°C., and 76.7°C.), tested immediately, and then tested again after 24 hours incubation at 32.2°C.

The minimum time of pasteurization was found at each of the four heating temperatures and these times were found to vary as the experimental work progressed. This variation was thought to be due in part to seasonal variations of the raw milk and in part to experimental errors.

The experimental error in this work was fairly large, the 95% confidence limits on the mean of duplicate milk samples being $\pm$ 1.3 p.p.m. of phenol. Even though this error appears to be smaller than that of some data in the literature, it may account for some of the false positive phosphatase tests that have been reported.

Erratic phosphatase values were obtained throughout the experiment before and after incubation, but values after incubation had more variation than those before incubation. However, most of these were within the limits of experimental error.

Some samples were declared pasteurized before incubation and underpasteurized afterward, but the variations between the two values

again were within the limit of experimental error. When tested before incubation, this milk gave values which indicated it was within the range of minimum pasteurization. No samples were found to be under-pasteurized before incubation and pasteurized afterward.

Milk collected for five consecutive days and samples collected at various times during one day showed no trend toward higher values after storage or incubation but it was noted that values fluctuated between pasteurized and underpasteurized and, for the most part, remained within the limits of experimental error.

There was no observed relationship between the phenol values obtained on the raw milk and the phenol values obtained after pasteurization. In addition, there were no apparent relationships between the composition values obtained for the milk samples and the phenol values of these samples after pasteurization.

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VITA

Pete J. Magnino, Jr.

Candidate for the Degree of

Master of Science

Thesis: A STUDY OF THE PHOSPHATASE TEST AND ITS USE AS AN INDICATION OF PASTEURIZATION

Major Field: Dairy Manufacturing

Biographical:

Born: March 20, 1933, Shawnee, Oklahoma

Undergraduate Study: Oklahoma A & M College, Stillwater, Oklahoma, 1950 - 1954.

Graduate Study: Oklahoma A & M College, 1956 - 1957.

Experience: U. S. Army, 1954 - 1956.

Member of: American Dairy Science Association, Red Red Rose, Dairy Science Club, Phi Sigma.

Date of Final Examination: July 1957