A STUDY OF CERTAIN FACTORS WHICH INFLUENCE PHOSPHATASE REACTIVATION IN MILK

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INTRODUCTION

The phosphatase test was first developed by Kay and Graham in 1935 and since that time has been adopted universally as a means of detecting underpasteurized milk or pasteurized milk which had been adulterated with raw milk. In recent years the test has not proven entirely satisfactory, perhaps due to changes in methods of pasteurization. There have been cases when commercially pasteurized milk or cream, which was negative to the phosphatase test immediately after pasteurization, developed a positive reaction after a few days of incubation. This change in phosphatase activity during incubation, which was often termed reactivation, made it possible to draw erroneous conclusions concerning the previous heat treatment of the milk or cream.

Many authors concluded that reactivation occurred only at high pasteurizing temperatures of 190.4°F. or above. Recent work from this laboratory, however, indicated that false-positive phosphatase tests after incubation also occurred occasionally at heating temperatures of 144.5°F. for 24 minutes and 170.06°F. for 2 minutes and 34 seconds. Further, it was found that the amount of reactivation at these temperatures was so small that customary testing methods often do not detect it. Therefore, statistical analysis of the data was necessary to demonstrate this small amount of reactivation.

The cause of reactivation, whether small or large, still was not completely understood and the reasons for the occasional reactivation at comparatively low heating temperatures were particularly obscure. It was

thought that the time and temperature used to heat the milk as well as the time and temperature of incubation after heating would have some bearing on these questions.

The primary objectives of this study were: a) to determine the influence of incubation temperatures on the reactivation of milk phosphatase, b) to study the influence of the length of the incubation periods on the reactivation of milk phosphatase, and c) to determine the effects of overheating the milk on the amount of reactivation which occurred during subsequent incubation.

REVIEW OF LITERATURE

The phosphatase test was first applied to milk by Kay and Graham (29) in 1935. They found that the phosphatase was sufficiently thermolabile to be "destroyed completely" when milk was pasteurized. Burgwald (7) mentioned in his literature review on the phosphatase test that Gilcrease and Davis were the first to publish results of the test in the United States. This was the start of the phosphatase test and since that time it has been modified and improved.

Apparently, the phosphatase enzyme is found in all raw milk (1,13, 27,28,35,36). There are two phosphatases in nature, alkaline and acid; the alkaline phosphatase being the most active of the two (36,37,38,64, 65,66,67). The amount of phosphatase contained in the milk of individual cows varies (13,16,17,56). Milk from cows in the early stages of lactation had a low phosphatase content (2,37,38) while milk from abnormal (mastitic) udders usually had a higher phosphatase content than the milk from normal udders (2,56).

That the phosphatase enzyme was associated with fat was shown by Kay and Graham (28,29). It was not fat-soluble, but was present in the thin protein layer which covered the globules or was adsorbed to the fat globule in such a way that the greater part was released into the aqueous phase (buttermilk) on churning the cream to butter. Morton (37,39,40) and Van Klinkenberg (61) prepared alkaline phosphatase from cow's intestine, kidney, and vaginal mucosa. They studied the phosphatase activities of these organs and compared the results with the activity of the

alkaline phosphatase from cow's milk. It was found that the phosphatase activity of normal cow's milk was very low as compared to that from these organs. Morton (38) compared two of these enzymes: one obtained from milk and one obtained from intestinal mucosa. He found that both enzymes were colorless, unconjugated proteins and were substantially free of organic phosphate and nucleotides or related compounds. The two enzymes had similar tyrosine and tryptophane contents and sometimes contained small amounts of carbohydrates. The optimum pH for the enzyme was found to be 10.0 - 10.5 (10,18,32,33,34,35,58,65).

The alkaline phosphatase (phosphomonoesterase) was capable of splitting phosphoric acid esters, in alkaline medium, into phosphoric acid salts and their corresponding alcohols. The principle of the phosphatase test according to Storrs and Burgwald (56) involved the addition of a small amount of raw milk to an excess of substrate containing disodiumphenylphosphate and incubating the mixture at 30°C. from 15 minutes to one hour (depending upon the test). The action of the phosphatase, if present, upon this substrate resulted in the liberation of phenol. The amount of phenol liberated then was determined colorimetrically as indophenol blue and this served as an estimate of the amount of phosphatase present, thereby, indicating the efficiency of pasteurization. The optimum pH for the development of the indophenol blue color has been found to be 9.3 - 9.4 (13,23,28,41,51,63).

All the phosphatase tests have the same general principle. The differences between tests usually involved the period of incubation or the reagents used. The Kay and Graham test (29) used a Lovibond tintometer for measuring the color after a 24 hour incubation period. They set a limit of 2.3 Lovibond units (L.B.U.) as a standard since no sample which

had been properly pasteurized at 145°F. for 30 minutes gave a color exceeding this limit. Storrs and Burgwald (56) and others (3,6,8,55) found that 2.3 L.B.U. was a satisfactory limit for milk pasteurized at 143 F. for 30 minutes using Kay and Graham's method. In 1937 Scharer (54) reported a modification of the phosphatase test which was shorter than Kay and Graham's method and required only one hour of incubation in place of 24. Scharer (54) also developed a rapid test for use in the field. This field test could be completed in 10 to 15 minutes.

The Sanders and Sager (49) test is used extensively today and is outlined in Standard Methods for the Examinations of Dairy Products (2). The principle of the test is the same as that of the Kay and Graham test, but the incubation period was only one hour. In place of Folin and Ciocalteau's coloring reagent, 2,6-dibromoquinonechlorimide (B.Q.C.), as advised by Scharer (54), was used. Photometric determination of the liberated phenol using a 610 mμ filter was first advised by Storrs and Burgwald (56) and perfected by Sanders (53). Burgwald (7) reported that the Sanders and Sager test would detect variations of 1°F. less than the recommended 143°F. and 5 minutes less than the recommended holding time of 30 minutes. It also would detect as little as 0.1% raw milk adultration.

Horwitz (26) compared several phosphatase tests and concluded that the Sanders and Sager test with photometric determination of the phenol was the best procedure for detecting underpasteurized or adultered milk. Sanders (53) established a unit of phosphatase as the intensity of blue color produced by one microgram, or one 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 p.p.m. of

phenol was produced by 0.5 ml. of milk. Phenol values higher than 4 p.p.m. per ml. of sample indicated underpasteurization of cow's milk according to standard methods (2).

Magnino (34) defined minimum heat treatment as that time which was required at any given temperature, to produce milks which liberated 2.3 - 4.9 µg. phenol per ml. of milk. This definition was used in this work. He also found that the visible blue color (caused by phenol and B.Q.C.) disappeared when milk contained 2.3 - 4.9 µg. phenol per ml.

It has been found by several authors (4,5,6,7,30,35,45) that pasteurized milk and cream will give a negative phosphatase test immediately after pasteurization but a positive test upon storage for a few days. Eddleman and Babel (11) ran phosphatase tests on samples of raw skim milk, whole milk, and cream heated at temperatures of 167 to 284°F. They found that the samples heated at temperatures above 190.4°F. were negative to the phosphatase test immediately after heat treatment, but showed sufficient phosphatase activity after 24 hours of incubation at 86°F. to be classed as underpasteurized.

Wright (63) and Wright and Tramer (64,65) also studied the reactivation of milk phosphatase following heat treatment. Their results showed that samples of sterile milk stored for a week or more at temperatures varying from 22 - 37°C. gave high L.B.U. values. Samples which were laboratory pasteurized before storage gave high L.B.U. values after storage, but this value was less than half of that given by the milk samples which were stored as received. Samples which were laboratory pasteurized after storage gave only slightly higher L.B.U. values after further storage. Maximum increases in L.B.U. values occurred after storage at 30°C. which confirmed the work of other workers (45,47,48,58,62) that the

optimum storage temperature for the reactivation of phosphatase was 30°C.

Wright and Tramer (64,65,66) also proved that the phosphatase developed during storage was identical to the alkaline phosphatase of raw milk and was not of bacterial origin. It was also shown that considerable variation occurred in the degree of reactivation of different milks. From the examination of a number of milks, it was concluded that this variation was related to the milk itself. According to Wright and Tramer (67) no relation was found between reactivation and the fat content or original phosphatase content of the milk. Neither was there any relationship between reactivation and the addition of ascorbic or amino acids. Removal of oxygen increased reactivation while storage of raw milk increased the original phosphatase content (11,65).

Posthumus (45) published his theory of reactivation which indicated that the enzyme was bound to the fat in such a way as to escape complete destruction during a very short heat treatment, but the active enzyme would diffuse into the serum upon storage and produce a positive test. Three theories of reactivation were offered by Wright and Tramer (65): a) reactivation was due to a reversion of the denaturation which the apoenzyme undergoes during heating, b) the coenzyme was inactivated by pasteurization, but upon storage at certain periods and temperatures was replaced by a new coenzyme which together with the apoenzyme would cause reactivation, c) heating the milk at normal pasteurization temperatures broke the bondage between the apoenzyme and coenzyme, but at temperatures a little higher than normal a new bondage was formed.

Hetrick and Tracy (23,24) established a straight line semi-logarithmic relationship between the time and temperature required to inactivate phosphatase over a range of 143 to 185°F. They expressed it by

the following formula: $T = 174 - 9 \log t$. T is the temperature in degrees Fahrenheit and t is the holding time in seconds required to inactivate the enzyme at (T) temperature. The Sanders and Sager (49) test was used to establish this formula with a value of one p.p.m. of phenol per ml. being used as the standard for inactivation. Others (9,19, 20,21,31,32,33,51,52,54) also have reported that a straight line results when the logarithms of the time of heating were plotted against the corresponding temperatures.

Read, et al. (47) found that heating for 0.25 seconds at 175.6°F. was sufficient to destroy completely the pathogenic organisms in milk while phosphatase was inactivated in 0.25 seconds at 182.4°F. According to Tobias, (59) Micrococcus organisms were 99.99% destroyed at 143°F. for 30 minutes and 168.34°F. for 2.3 seconds assuming zero heating and cooling times. They also found that phosphatase could be inactivated at 169.7°F. for 2.36 seconds and have a phosphatase test value of 4 p.p.m. phenol per ml. of milk or less.

It was found by many investigators (15,26,31,43,44,50) that at temperatures between 140°F. and 160°F. longer holding periods were required to kill pathogens and inactivate phosphatase in cream than in milk. In both milk and cream, however, a safety margin existed between bacterial destruction and phosphatase inactivation. Dahlberg (9) and Holland and Dahlberg (25) reported on the safety margins which existed between bacterial thermal death points and pasteurization standards. They found these safety margins were smaller at high pasteurization temperatures than at low ones. Prucha and Corbett (46) also found this margin was smaller at higher temperatures, but the safety margins between bacterial destruction and phosphatase inactivation was found to be wider at higher temperatures.

Churchill, et al. (8) stated that antibiotics did not impair the reliability of the phosphatase test but Stotz and Hankinson (57) found that small amounts of antibiotics and a substandard pasteurization temperature could yield a false negative phosphatase test.

Hammer and Olson (19) incubated bacterial cultures in sterile milk at 21°C. and 37°C. and found that some strains produced phosphatase. Psedomonas putrefaciens was an excellent producer and some Aerobacter groups gave positive results. Some authors (6,7,14,22,31,42) have found that bacteria could cause positive phosphatase tests, but counts must be in the millions. A control test was used by Tramer (60) to demonstrate that bacterial phosphatase was produced by growing organisms. He incorporated di-sodium para-nitro-phenyl-phosphate into suitable media and measured colorimetrically the para-nitro phenol liberated by the organisms present.

Reactivation of alkaline milk phosphatase was affected by the presence of chemical and metallic ions (13,46,67). Ethylene diamine tetra-acetic acid would increase reactivation as would magnesium, zinc and manganese ions. Copper, nickel and cobalt ions were found to be inhibitory factors. Morton (38,40) stated that several phosphorous compounds caused slight inhibition of the enzymatic activity while iodine and cysteine caused strong inhibition. Pett and Wynne (44) found that the enzyme's activity could be accelerated by arsenate or arsenite.

EXPERIMENTAL METHODS

A. GENERAL PROCEDURE

The milk used in this study represented the night and morning milkings of the Oklahoma State University dairy herd which was composed of four breeds, Ayrshire, Guernsey, Holstein and Jersey. The milk was collected from the bulk storage tank at the dairy plant after the morning milking. Care was taken to insure proper agitation of the milk before sampling. The milk was standardized to 4% fat and 14 ml. samples of it were then pipetted into 10 x 120 mm. screw-capped test tubes. In preparation for heating, these tubes were placed in a water bath, the temperature of the contents was adjusted to 98°F. and that temperature was maintained for a period of not less than 15 minutes.

The tubes were then submerged in a preheated oil bath. The temperature of the bath was controlled by a mercury thermogulator within a range of 169.9 - 170.3°F. and constant temperature throughout was insured by the use of a stirrer. During heating, the sample tubes were rotated at a speed of approximately 3 r.p.m. After the desired time interval had elapsed, the tubes of milk were removed from the oil bath and immediately immersed in ice water. They were then ready to be tested for phosphatase activity. A modification of the phosphatase test of Sanders and Sager (49) was used in this work to determine the phosphatase activity of the samples. This modification consisted of doubling the sample size and amounts of reagents used. The intensity of the blue color present was

determined at 610 mμ using a Beckman model B spectrophotometer. The samples were read while in 8 x 80 mm. cylindrical glass colorimeter tubes with the instrument standardized on a reagent blank. The data were recorded as optical density.

A statistical "validity test" procedure as described by Finney (12) was used to evaluate the data obtained and to check its validity.

The design for this analysis required duplicate aliquots of 0.0, 0.5,1.0 and 2.0 ml. of milk to be taken from each sample. All of these were diluted to a volume of 2.0 ml. with distilled water before being tested. A standard containing 7.5 p.p.m. of phenol was run with each sample. Single aliquots of 0.0,0.5,1.0 and 2.0 ml. of the phenol solution also were measured and diluted to a volume of 2.0 ml. with distilled water. The phosphatase test was then run on all 12 aliquots at the same time.

The relative phosphatase acitvity of the milk samples was calculated by dividing the slope of the line representing the milk sample by the slope of the line representing the standard. The validity of each set of data used in this study was checked (12) and if any sample did not meet the requirements for linearity, blanks or intersection of the lines, the data for that sample were discarded.

B. EXPERIMENTS

The experimental work in this study was divided into four parts, called experiments. The purposes of experiments 1,2 and 3 were to determine the influence of: a) incubation temperatures and b) the length of the incubation period on the reactivation of the phosphatase enzyme. The purpose of experiment 4 was to see if overheating (exposing the samples to a higher temperature or for a longer period of time than was necessary to produce milks with a relative phosphatase activity of less than 1.3) would affect the reactivation of the phosphatase enzyme.

Experiments 1,2, and 3 were composed of six trials each and for each trial 3 to 4 replicates were used. In each trial, the first set of tubes was tested for phosphatase activity immediately after heating. The other sets were tested after selected periods of incubation. The milk samples of experiment 1 were incubated at 90°F. for 0,1 and 2 days. The incubation temperature for the samples in experiment 2 was 72°F. and these were stored for 0,1,2,3 or 4 days. The milk samples of experiment 3 were incubated at 52°F. for 0,1,2,3,4,6 or 7 days.

In experiment 4, four replicate milk samples were used in each trial. Two of these replicates (A and B) were given a minimum heat treatment and the other two (C and D) were overheated. In trials 1,2 and 3, the overheated samples were heated at 170.06°F. for 15 minutes instead of 2 minutes and 34.7 seconds and in trials 4,5 and 6, they were heated at 176.3°F. for 5 minutes instead of at 170.06°F. for 2 minutes and 34.4 seconds. All the samples were incubated at 72°F. for 0,1,2,3 or 4 days. The data recorded in the tables which follow are not complete in all cases. Each experiment originally contained six trials with three or four replications in each. One reason for the missing data is that the milk often

coagulated before it had been incubated the described length of time. For example, in experiment 1, the samples coagulated after 48 hours of incubation and could not be tested thereafter. Statistical analysis indicated that results for some of the other samples were not valid. A code for numbering the milk samples in this study was adopted. This system was a concise way of identifying the samples and made it easier to find any particular sample in the tables that follow. Using sample numbers 1204 and 2311 as examples, the first digit of each sample number represents the experiment, 1 and 2 respectively. The next digits of the numbers, 2 and 3, stand for trials 2 and 3. The 04 and 11 are the code numbers for the samples. Thus, sample 1204 was the fourth sample in trial 2 of the first experiment, and sample 2311 was sample 11 in trial 3 of the second experiment.

RESULTS AND DISCUSSION

Table 1 shows an example of the calculations involved in the statistical analysis of the data obtained from the samples in this work. Graphs of representative data are shown in Figures I to V. These are presented in the hope that they will give a clearer picture of the data obtained from each sample, the lines which were calculated from this data, and the way in which these samples reacted to different temperatures and incubation periods. All of the original data obtained in this study are recorded in Tables I to IV in the appendix. These data have met the validity test requirements for linearity, blanks and intersection of the lines. The heating of the milk samples in the oil bath was described by the equation: $T = B-(B-40)e^{-kt}$.

Where T = temperature of milk in °C.,
B = oil bath temperature = 76.7° ± 0.1°C.,
40 = temperature of the tube contents before heating in °C.,
k = constant - 0.01660, and
t = time in seconds (at temperature T)

The constant, k, was calculated to be 0.0166. By substituting in the above equation, it was found that at 2 minutes and 29.0 seconds and 2 minutes and 37.3 seconds, the temperature of the milk only reached 164.4°F. and 167.0°F. respectively. This range of time (2 minutes and 29.0 seconds to 2 minutes and 37.3 seconds) was the minimum required for heating the milk samples at an oil bath temperature of 170.06°F. to initially inactivate the phosphatase enzyme. Thus, all the heating involved occurred during the so called "preheating" period and no holding time at 170.06°F. was involved.

TABLE 1

EXAMPLES OF THE CALCULATION INVOLVED IN THE VALIDITY TEST.
MEAN SQUARES FOR THE SAMPLES IN
EXPERIMENT 1, TRIAL 2

Source	d.f.	Sample		
		1204	1205	1206
Linear	2	28.0251[e]	44.6822[e]	376.3003[e]
Blanks	1	0.6749	2.4083	0.29999
Intersection	1	2.7777	0.4444	1.7777
Quadratic	2	0.3839	0.0267	2.5634
Error	5	1.7583	0.9584	7.2486

[e] $P < 0.5$

The raw data used to calculate the heating curve are shown in Table V also in the appendix.

A cooling curve was made to determine the time required to cool the milk from 165°F. to 104°F. This was done by heating the sample tubes of milk in a water bath to 165°F., then the samples were removed and immediately immersed in ice water and cooled to 104°F. The time required to cool the samples to 104°F. was found to average 20.4 seconds and the equation of the cooling curve was a straight line.

The time required for minimum heat treatment in this work ranged from 2 minutes and 29.0 seconds to 2 minutes and 37.3 seconds. (footnotes of Tables 2 to 5). This time was found to vary from experiment to experiment, and these variations were thought to be at least partly due to variations in the unpasteurized raw milk as sampled from the holding vat.

The influence of the incubation temperature on relative phosphatase activity of the samples for the first three experiments are shown in Tables 2,3 and 4. Average daily increases in relative phosphatase activity during incubation were 0.80,0.82 and 0.20 for the samples incubated at 90°F.,72°F. and 52°F. respectively. It appears that there was no distinct difference between the samples incubated at 90°F. and those incubated at 72°F. However, the samples incubated at 52°F. showed less increase in relative activity with time than did the samples incubated at the other two temperatures. The influence of the length of incubation period on relative phosphatase activity can also be shown by the data in Tables 2,3 and 4. In general, it was found that as the incubation period increased, the phosphatase activity of the samples also increased. It was desirable to know whether these milk samples were pasteurized according to the usual interpretation of the test in terms of p.p.m. phenol. As

TABLE 2

EXPERIMENT 1, TRIALS 2 AND 5
RELATIVE PHOSPHATASE ACTIVITY OF MILK HEATED AT 170.06°F. FOR A MINIMUM TIME[a] AND INCUBATED AT 90°F. FOR SELECTED PERIODS OF TIME

Sample No.	Days Incubation	Relative Activity	Sample No.	Days Incubation	Relative Activity
1204	0	0.41	1509	0	0.43
1205	1	0.69	1510	1	0.80
1206	2	2.89	1511	2	1.14

[a]The minimum time required to produce milk which liberated 2.3 - 4.9 μg. phenol per ml. was 2 minutes and 30.3 seconds for trial 2 and 2 minutes and 29.0 seconds for trial 5.

TABLE 3

EXPERIMENT 2, TRIALS 1 - 6
RELATIVE PHOSPHATASE ACTIVITY OF MILK HEATED AT 170.06°F. FOR A MINIMUM TIME[a] AND INCUBATED AT 72°F. FOR SELECTED PERIODS OF TIME

Sample No.	Days Incubation	Relative Activity	Sample No.	Days Incubation	Relative Activity
2101	0	0.83	2412	0	1.08
2102	1	1.25	2413	1	3.47
2103	3	1.21	2415	3	5.78
2104	4	3.31			
2205	0	0.36	2516	0	0.45
2206	1	1.50	2517	1	3.00
2207	2	3.40	2518	2	0.87
2308	0	0.45	2619	0	1.02
2309	1	0.75	2620	1	1.98
2310	2	0.46	2622	4	4.49
2311	3	1.16			

[a]The minimum time required to produce milk which liberated 2.3 - 4.9 μg. phenol per ml. for these trials was as follows:

(1) 2 minutes, 32.8 seconds
(2) 2 minutes, 33.9 seconds
(3) 2 minutes, 37.3 seconds
(4) 2 minutes, 37.2 seconds
(5) 2 minutes, 36.2 seconds
(6) 2 minutes, 36.2 seconds

TABLE 4

EXPERIMENT 3, TRIALS 1,2,4,5 AND 6
RELATIVE PHOSPHATASE ACTIVITY OF MILK HEATED AT 170.06°F. FOR A MINIMUM TIME[a] AND INCUBATED AT 52°F. FOR SELECTED PERIODS OF TIME

Sample No.	Days Incubation	Relative Activity
3101	0	0.75
3102	1	0.70
3103	2	1.00
3204	0	0.74
3205	2	2.20
3206	3	3.72
3411	0	1.16
3412	2	398.23[b]
3413	4	1.74
3414	5	19.38[b]
3515	0	1.46
3516	2	6.56
3517	4	5.95
3518	6	6.17
3619	0	8.03[b]
3620	2	0.90
3621	6	0.53
3622	7	0.59

[a]The minimum time required to produce milk which liberated 2.3 - 4.9 γ g. phenol per ml. for these trials was as follows:

(1) 2 minutes, 29.1 seconds
(2) 2 minutes, 30.0 seconds
(4) 2 minutes, 32.2 seconds
(5) 2 minutes, 33.1 seconds
(6) 2 minutes, 33.0 seconds

[b]An unreasonable value, thought to be an error.

mentioned earlier, Magnino (34) found minimum pasteurization to range from readings of 2.3 to 4.9 p.p.m. phenol per ml. of milk. Since a 2.0 ml. milk sample and a 7.5 p.p.m. phenol standard were used in this work, the relative phosphatase activity representing the upper limit of Magnino's range then becomes: $\frac{4.9 \times 2}{7.5} = 1.3$

The relative phosphatase activity of most milk samples became greater than 1.3 after an incubation period of one to two days. Thus, after being stored this long, most of the samples in experiments 1,2 and 3 would have been declared underpasteurized according to the usual interpretation of the phosphatase test. The data obtained in experiment 4 (Table 5) showed that the relative phosphatase activity of the samples which were given a minimum heat treatment increased more during incubation than did the relative activity of the overheated samples. The average daily increase in relative phosphatase activity for samples heated at minimum time was 0.30 and for the overheated samples it was 0.11. These results seem to indicate that overheating the milk samples slowed their phosphatase reactivation during subsequent incubation. Except for sample 4103, the overheated samples never developed a relative phosphatase activity as great as 1.3. On the other hand, all but one set of the samples given minimum heat treatments (4204-4207) had relative phosphatase activities of 1.3 or more after two days of incubation.

To answer the question as to whether reactivation was of chemical or bacterial origin, some of the reactivated samples were smeared on standard plate count agar. It was found that the bacterial growth of the smeared samples was very low and it would seem impossible to conclude that the phosphatase activity after incubation was of bacterial origin.

TABLE 5

EXPERIMENT 4, TRIALS 1 - 6
RELATIVE PHOSPHATASE ACTIVITY OF MILK HEATED FOR A MINIMUM TIME[a] OR OVERHEATED[c] AND INCUBATED AT 72°F. FOR SELECTED PERIODS OF TIME

Sample No.	Days Incubation	Heat Treatment	
		Minimum	Overheated
4101	0	1.58	0.85
4102	1	2.66	1.05
4103	3	2.76	1.48
4204	0	1.10	0.53
4206	2	0.89	0.58
4207	3	1.11	1.07
4308	0	0.72	0.54
4309	1	1.26	0.94
4310	2	1.72	0.59
4411	0	0.41	0.52
4412	2	1.70	0.87
4413	3	1.64	0.85
4414	4	1.38	0.69
4515	0	0.72	0.58
4516	1	1.54	0.82
4517	2	2.44	0.92
4618	0	0.90	0.77
4619	1	1.11	0.84
4620	2	1.40	0.83
4621	3	1.10	0.87

[a]The minimum time require to produce milk which liberated 2.3 - 4.9 µg. phenol per ml. for these trials was as follows:

(1) 2 minutes, 35.5 seconds
(2) 2 minutes, 34.3 seconds
(3) 2 minutes, 34.3 seconds
(4) 2 minutes, 34.9 seconds
(5) 2 minutes, 34.1 seconds
(6) 2 minutes, 34.2 seconds

[c]Overheated milk in this experiment referred to milk heated at 170.06°F. for 15 minutes in trials 1,2 and 3 and milk heated at 176.3°F. for 5 minutes in trials 4,5 and 6.

SUMMARY AND CONCLUSIONS

The objectives of this study were to determine the influence on phosphatase reactivation of: a) incubation temperatures, b) incubation times and c) overheating the milk.

Four experiments were conducted. Experiments 1,2 and 3 were designed to determine the first two objectives, and the samples in these three experiments were incubated at 90°F.,72°F. and 52°F. respectively. The samples were tested for phosphatase activity after 0,1,2,3,4,5,6 or 7 days. The milk samples in these experiments were exposed to the minimum heat treatment required to inactivate the phosphatase.

Experiment 4 was designed to determine the third objective of this study. Two of the four replications in this experiment were exposed to a minimum heat treatment while the other two were exposed to a higher temperature or for a longer period of time than was necessary to inactivate the phosphatase.

The Sanders and Sager procedure was used to determine the phosphatase activity of the milk samples in this study and the statistical "validity check" procedure of Finney was used to evaluate the data obtained.

From this study it can be concluded that:

a) The relative phosphatase activity of milk samples incubated at 72°F. and 92°F., increased faster during incubation than did samples incubated at 52°F.

b) The relative phosphatase activity of the milk samples continued to increase as the period of incubation increased.

c) After one to two days incubation, relative phosphatase activity of most milk samples in this study became greater than 1.3, the point at which they would have been declared underpasteurized by standards commonly accepted in the industry.

d) Exposing the milk samples to a higher temperature or for a longer period of time than the minimum time necessary to inactivate the enzyme was found to slow the increase in relative phosphatase activity of milk phosphatase during subsequent incubation.

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TABLE I
EXPERIMENT 1, TRIALS 2 AND 5
COLORIMETER READINGS OF MILK HEATED AT 170.06°F. FOR A MINIMUM TIME[a] AND INCUBATED AT 90°F. FOR SELECTED PERIODS OF TIME

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk		Phenol Standard
			A	B	
1204	0	0	0.000	0.015	0.010
		½	0.000	0.015	0.040
		1	0.005	0.025	0.050
		2	0.030	0.060	0.095
1205	1	0	0.020	0.000	0.000
		½	0.040	0.025	0.045
		1	0.050	0.040	0.060
		2	0.070	0.080	0.095
1206	2	0	0.015	0.020	0.005
		½	0.040	0.090	0.020
		1	0.110	0.170	0.045
		2	0.225	0.255	0.095
1509	0	0	0.020	0.020	0.030
		½	0.005	0.015	0.045
		1	0.035	0.030	0.065
		2	0.040	0.070	0.115
1510	1	0	0.015	0.010	0.015
		½	0.030	0.005	0.045
		1	0.035	0.015	0.055
		2	0.110	0.075	0.100
1511	2	0	0.010	0.010	0.005
		½	0.030	0.000	0.025
		1	0.075	0.060	0.060
		2	0.180	0.110	0.120

[a]The minimum time required to produce milk which liberated 2.3 - 4.9 μg. phenol per ml. was 2 minutes and 30.3 seconds for trial 2 and 2 minutes and 29.0 seconds for trial 5.

TABLE II
EXPERIMENT 2, TRIALS 1 - 6
COLORIMETER READINGS OF MILK HEATED AT 170.06°F. FOR A MINIMUM TIME[a] AND INCUBATED AT 72°F. FOR SELECTED PERIODS OF TIME

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk		Phenol Standard
			A	B	
2101	0	0	0.005	0.005	0.000
		½	0.015	0.015	0.040
		1	0.025	0.025	0.050
		2	0.085	0.080	0.085
2102	1	0	0.005	0.005	0.005
		½	0.045	0.020	0.025
		1	0.080	0.040	0.055
		2	0.160	0.100	0.100
2103	3	0	0.005	0.005	0.010
		½	0.000	0.000	0.025
		1	0.040	0.045	0.040
		2	0.110	0.135	0.085
2104	4	0	0.005	0.005	0.005
		½	0.070	0.030	0.015
		1	0.140	0.110	0.035
		2	0.210	0.200	0.075
2205	0	0	0.010	0.000	0.025
		½	0.010	0.015	0.040
		1	0.020	0.020	0.055
		2	0.040	0.045	0.095
2206	1	0	0.005	0.010	0.000
		½	0.025	0.040	0.015
		1	0.040	0.080	0.035
		2	0.085	0.140	0.080
2207	2	0	0.000	0.005	0.010
		½	0.060	0.080	0.025
		1	0.140	0.170	0.045
		2	0.260	0.280	0.090
2308	0	0	0.010	0.022	0.035
		½	0.015	0.000	0.032
		1	0.015	0.020	0.060
		2	0.057	0.045	0.150

TABLE II (CONTINUED)

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk		Phenol Standard
			A	B	
2309	1	0	0.000	0.000	0.005
		½	0.012	0.010	0.022
		1	0.030	0.027	0.055
		2	0.095	0.067	0.105
2310	2	0	0.150	0.025	0.002
		½	0.000	0.000	0.015
		1	0.010	0.010	0.052
		2	0.040	0.050	0.115
2311	3	0	0.010	0.000	0.012
		½	0.012	0.010	0.022
		1	0.067	0.050	0.055
		2	0.150	0.110	0.102
2412	0	0	0.000	0.012	0.025
		½	0.032	0.027	0.035
		1	0.060	0.057	0.040
		2	0.122	0.090	0.104
2413	1	0	0.005	0.000	0.002
		½	0.060	0.125	0.032
		1	0.102	0.260	0.067
		2	0.195	0.490	0.105
2415	3	0	0.002	0.015	0.020
		½	0.130	0.135	0.030
		1	0.310	0.285	0.050
		2	0.590	0.510	0.102
2516	0	0	0.010	0.005	0.010
		½	0.010	0.020	0.035
		1	0.020	0.030	0.065
		2	0.045	0.060	0.095
2517	1	0	0.005	0.010	0.005
		½	0.070	0.070	0.030
		1	0.160	0.110	0.045
		2	0.240	0.200	0.095
2518	2	0	0.010	0.005	0.000
		½	0.015	0.010	0.030
		1	0.030	0.025	0.060
		2	0.095	0.120	0.110

TABLE II (CONTINUED)

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk		Phenol Standard
			A	B	
2619	0	0	0.000	0.020	0.000
		½	0.050	0.030	0.035
		1	0.080	0.060	0.070
		2	0.140	0.125	0.130
2620	1	0	0.010	0.000	0.010
		½	0.055	0.075	0.020
		1	0.090	0.120	0.075
		2	0.200	0.200	0.105
2622	3	0	0.025	0.015	0.025
		½	0.055	0.180	0.045
		1	0.150	0.360	0.070
		2	0.280	0.640	0.115

[a]The minimum time required to produce milk which liberated 2.3 - 4.9 µg. phenol per ml. for these trials was as follows:

(1) 2 minutes, 32.8 seconds
(2) 2 minutes, 33.9 seconds
(3) 2 minutes, 37.3 seconds
(4) 2 minutes, 37.2 seconds
(5) 2 minutes, 36.2 seconds
(6) 2 minutes, 36.2 seconds

TABLE III
EXPERIMENT 3, TRIALS 1,2,4,5 AND 6
COLORIMETER READINGS OF MILK HEATED AT 170.06°F. FOR A MINIMUM TIME[a] AND INCUBATED AT 52°F. FOR SELECTED PERIODS OF TIME

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk		Phenol Standard
			A	B	
3101	0	0	0.035	0.015	0.015
		½	0.010	0.030	0.035
		1	0.025	0.045	0.070
		2	0.095	0.090	0.110
3102	1	0	0.025	0.005	0.025
		½	0.015	0.035	0.025
		1	0.020	0.050	0.035
		2	0.040	0.090	0.100
3103	2	0	0.015	0.005	0.025
		½	0.040	0.005	0.035
		1	0.065	0.055	0.055
		2	0.120	0.110	0.115
3204	0	0	0.015	0.015	0.000
		½	0.025	0.030	0.025
		1	0.040	0.065	0.035
		2	0.092	0.105	0.160
3205	2	0	0.010	0.000	0.025
		½	0.045	0.045	0.025
		1	0.105	0.075	0.045
		2	0.195	0.150	0.080
3206	3	0	0.000	0.025	0.000
		½	0.080	0.085	0.035
		1	0.120	0.170	0.045
		2	0.270	0.330	0.085
3411	0	0	0.000	0.000	0.030
		½	0.015	0.010	0.025
		1	0.045	0.060	0.060
		2	0.130	0.150	0.105
3412	2	0	0.005	0.010	0.010
		½	0.205	0.205	0.010
		1	0.450	0.370	0.040
		2	0.710	0.610	0.070

TABLE III (CONTINUED)

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk		Phenol Standard
			A	B	
3413	4	0	0.015	0.005	0.015
		½	0.055	0.050	0.035
		1	0.090	0.085	0.055
		2	0.185	0.170	0.105
3414	6	0	0.025	0.015	0.015
		½	0.240	0.230	0.045
		1	0.440	0.440	0.055
		2	0.750	0.750	0.112
3515	0	0	0.015	0.000	0.015
		½	0.035	0.015	0.035
		1	0.075	0.065	0.055
		2	0.170	0.165	0.100
3516	2	0	0.000	0.000	0.005
		½	0.065	0.155	0.025
		1	0.095	0.290	0.050
		2	0.145	0.500	0.085
3517	4	0	0.020	0.005	0.005
		½	0.170	0.105	0.035
		1	0.280	0.165	0.065
		2	0.500	0.300	0.105
3518	6	0	0.025	0.005	0.001
		½	0.070	0.230	0.035
		1	0.140	0.450	0.075
		2	0.260	0.800	0.110
3619	0	0	0.015	0.020	0.025
		½	0.010	0.040	0.040
		1	0.020	0.050	0.055
		2	0.065	0.105	0.095
3620	2	0	0.010	0.000	0.000
		½	0.030	0.030	0.040
		1	0.045	0.035	0.055
		2	0.095	0.100	0.100
3621	6	0	0.000	0.005	0.000
		½	0.000	0.030	0.020
		1	0.000	0.050	0.035
		2	0.005	0.080	0.085

TABLE III (CONTINUED)

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk		Phenol Standard
			A	B	
3622	7	0	0.010	0.010	0.025
		$\frac{1}{2}$	0.020	0.010	0.035
		1	0.030	0.020	0.055
		2	0.100	0.030	0.100

[a]The minimum time required to produce milk which liberated 2.3 - 4.9 µg. phenol per ml. for these trials was as follows:
(1) 2 minutes, 29.1 seconds (5) 2 minutes, 33.1 seconds
(2) 2 minutes, 30.0 seconds (6) 2 minutes, 33.0 seconds
(4) 2 minutes, 32.3 seconds

TABLE IV
EXPERIMENT 4, TRIALS 1 - 6
COLORIMETER READINGS OF MILK HEATED A MINIMUM TIME[a] OR OVERHEATED[c] AND INCUBATED AT 72°F. FOR SELECTED PERIODS OF TIME

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk Minimum Heat A	Milk Minimum Heat B	Milk Overheated C	Milk Overheated D	Phenol Standard
4101	0	0	00.025	00.025	00.005	00.005	00.040
		½	00.045	00.000	00.000	00.000	00.010
		1	00.085	00.040	00.025	00.075	00.020
		2	00.155	00.075	00.030	00.075	00.080
4102	1	0	00.010	00.005	00.025	00.025	00.010
		½	00.095	00.070	00.040	00.015	00.025
		1	00.175	00.110	00.020	00.065	00.060
		2	00.315	00.240	00.115	00.125	00.105
4103	3	0	00.015	00.015	00.000	00.005	00.000
		½	00.045	00.115	00.015	00.025	00.010
		1	00.075	00.210	00.050	00.080	00.050
		2	00.125	00.340	00.120	00.160	00.095
4204	0	0	00.010	00.010	00.015	00.000	00.010
		½	00.035	00.050	00.025	00.005	00.005
		1	00.055	00.070	00.045	00.025	00.050
		2	00.095	00.120	00.065	00.045	00.110
4206	2	0	00.010	00.000	00.010	00.025	00.010
		½	00.010	00.035	00.005	00.010	00.035
		1	00.035	00.060	00.035	00.040	00.065
		2	00.065	00.120	00.065	00.055	00.095
4207	3	0	00.000	00.000	00.000	00.000	00.010
		½	00.045	00.035	00.025	00.010	00.030
		1	00.085	00.065	00.045	00.045	00.045
		2	00.150	00.095	00.120	00.150	00.120
4308	0	0	00.015	00.005	00.005	00.005	00.000
		½	00.015	00.025	00.020	00.005	00.025
		1	00.030	00.035	00.025	00.025	00.045
		2	00.075	00.085	00.060	00.055	00.115
4309	1	0	00.005	00.015	00.000	00.025	00.010
		½	00.015	00.020	00.030	00.020	00.045
		1	00.075	00.090	00.070	00.050	00.060
		2	00.140	00.180	00.110	00.120	00.120

TABLE IV (CONTINUED)

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk Minimum Heat A	Milk Minimum Heat B	Milk Overheated C	Milk Overheated D	Phenol Standard
4310	2	0	00.015	00.000	00.005	00.015	00.005
		½	00.055	00.060	00.005	00.015	00.040
		1	00.105	00.135	00.035	00.030	00.060
		2	00.175	00.240	00.085	00.075	00.125
4411	0	0	00.000	00.020	00.015	00.000	00.015
		½	00.005	00.005	00.010	00.015	00.045
		1	00.035	00.030	00.020	00.010	00.055
		2	00.045	00.040	00.065	00.065	00.110
4412	2	0	00.010	00.015	00.005	00.020	00.000
		½	00.080	00.045	00.020	00.040	00.035
		1	00.120	00.060	00.045	00.050	00.050
		2	00.220	00.125	00.100	00.110	00.110
4413	3	0	00.000	00.000	00.015	00.015	00.015
		½	00.055	00.025	00.025	00.040	00.045
		1	00.110	00.060	00.040	00.055	00.060
		2	00.220	00.120	00.080	00.100	00.100
4414	4	0	00.005	00.010	00.005	00.015	00.015
		½	00.008	00.035	00.030	00.010	00.035
		1	00.130	00.050	00.040	00.030	00.060
		2	00.220	00.110	00.085	00.075	00.115
4515	0	0	00.015	00.015	00.000	00.005	00.015
		½	00.015	00.030	00.030	00.020	00.045
		1	00.040	00.065	00.050	00.025	00.065
		2	00.080	00.105	00.075	00.080	00.125
4516	1	0	00.015	00.015	00.000	00.000	00.005
		½	00.045	00.045	00.040	00.030	00.030
		1	00.060	00.075	00.060	00.050	00.045
		2	00.125	00.160	00.075	00.075	00.100
4517	2	0	00.005	00.005	00.005	00.005	00.010
		½	00.030	00.090	00.010	00.025	00.030
		1	00.090	00.145	00.030	00.050	00.050
		2	00.160	00.260	00.085	00.100	00.105
4618	0	0	00.015	00.015	00.005	00.005	00.020
		½	00.035	00.030	00.025	00.030	00.025
		1	00.055	00.055	00.050	00.045	00.050
		2	00.085	00.085	00.075	00.075	00.100
4619	1	0	00.005	00.000	00.000	00.015	00.015
		½	00.030	00.040	00.030	00.035	00.035
		1	00.065	00.060	00.045	00.045	00.060
		2	00.140	00.095	00.085	00.100	00.105

TABLE IV (CONTINUED)

Sample No.	Days Incubation	Ml. of Milk or Phenol Standard Per 2 Ml. Sample	Milk Minimum Heat A	Milk Minimum Heat B	Milk Overheated C	Milk Overheated D	Phenol Standard
4620	2	0	00.020	00.005	00.005	00.005	00.005
		½	00.030	00.050	00.030	00.010	00.030
		1	00.055	00.100	00.040	00.040	00.050
		2	00.090	00.100	00.080	00.085	00.095
4621	3	0	00.000	00.015	00.005	00.005	00.005
		½	00.015	00.020	00.000	00.000	00.020
		1	00.040	00.045	00.035	00.035	00.040
		2	00.095	00.100	00.070	00.080	00.085

[a]The minimum time required to produce milk which liberated 2.3 - 4.9 µg. phenol per ml. for these trials was as follows:

(1) 2 minutes, 35.5 seconds (4) 2 minutes, 34.9 seconds
(2) 2 minutes, 34.3 seconds (5) 2 minutes, 34.1 seconds
(3) 2 minutes, 34.3 seconds (6) 2 minutes, 34.2 seconds

[c]Overheated milk in this experiment referred to milk heated at 170.06°F. for 15 minutes in trials 1,2 and 3 and milk heated at 176.3°F. for 5 minutes in trials 4,5 and 6.

TABLE V
DATA INVOLVED IN CALCULATING THE HEATING CURVE

Time (Seconds)	Temperature (°C.)	Time (Seconds)	Temperature (°C.)
0	40.00	75	69.60
10	45.00	75	69.00
10	46.00	80	69.60
15	46.50	90	70.00
15	47.00	90	70.00
20	50.00	100	71.00
25	52.10	105	71.00
30	55.20	120	72.00
30	55.30	120	71.80
40	61.10	120	71.80
40	60.00	150	73.80
45	61.20	150	73.20
45	60.10	160	75.20
50	61.80	165	75.40
50	61.00	180	76.20
60	65.00	180	76.00
60	64.00	200	76.10
60	63.80	220	76.30
		240	76.60

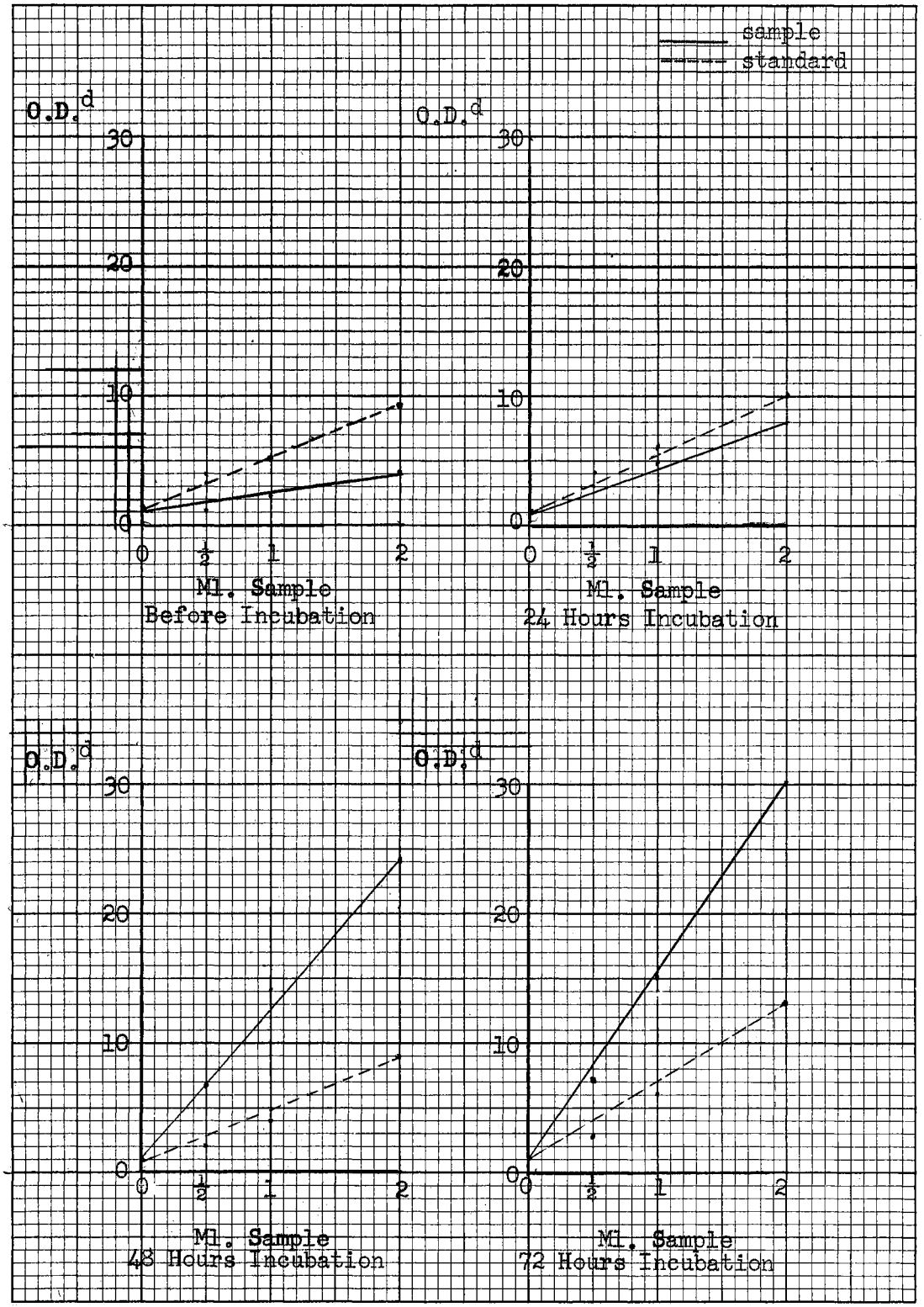

Figure I. Experiment 1, Trial 2. Relative Phosphatase Activity of Milk Heated for a Minimum Time at 170.06°F. and Incubated at 90°F. for Selected Periods of Time.

[d]O.D. = Optical Density

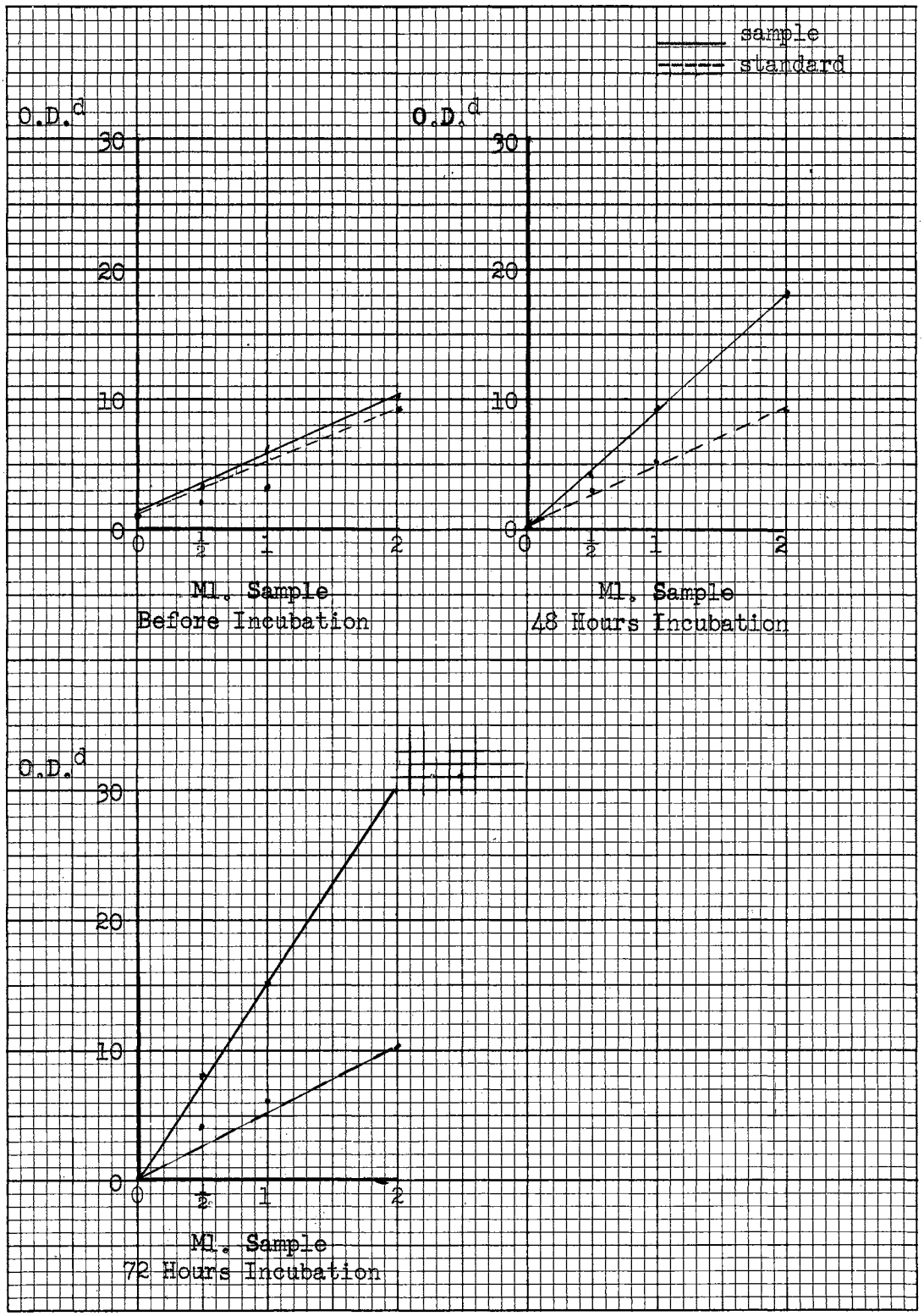

Figure II. Experiment 2, Trial 2. Relative Phosphatase Activity of Milk Heated for a Minimum Time at 170.06°F. and Incubated at 72°F. for Selected Periods of Time.

[d]O.D. = Optical Density

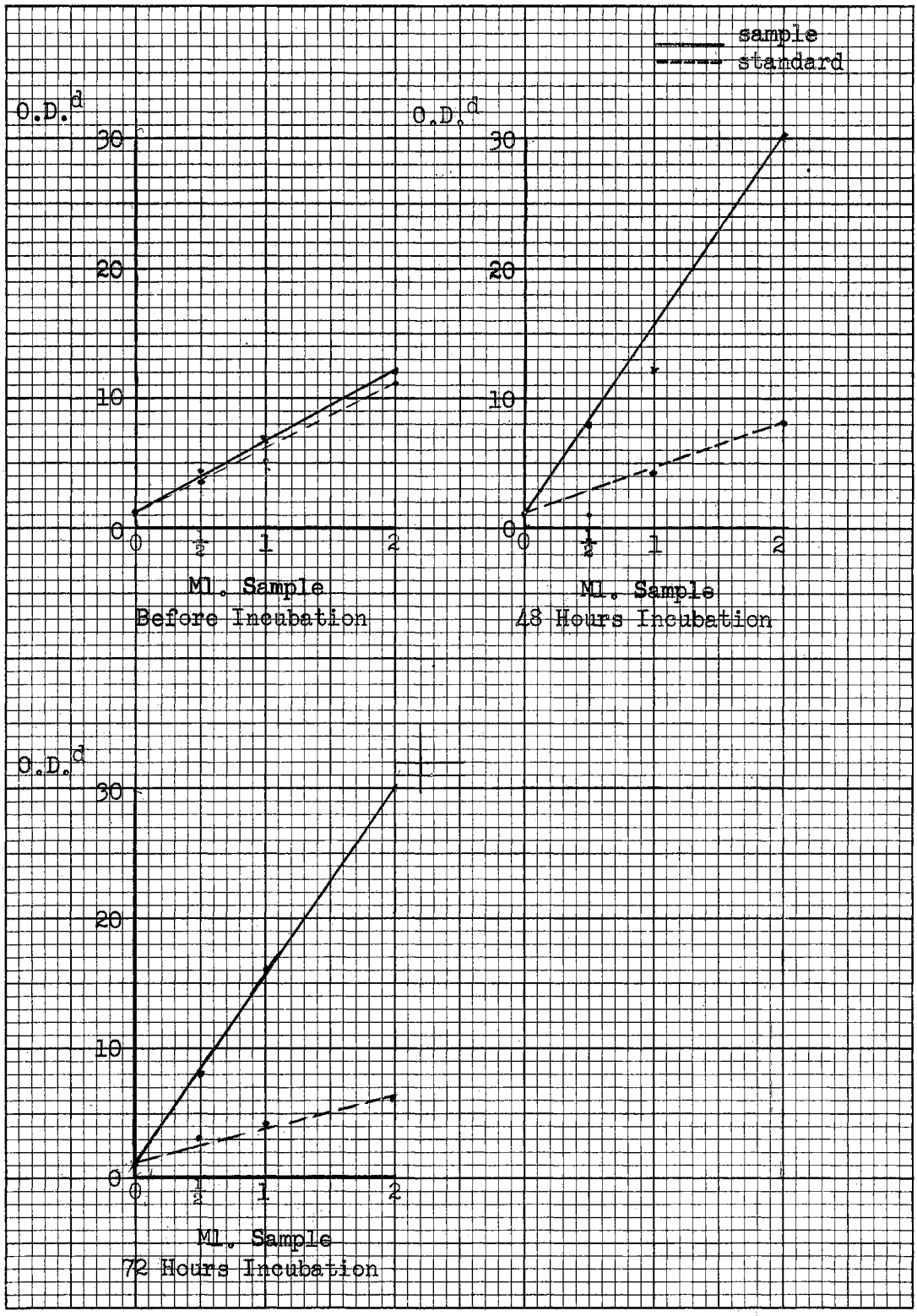

Figure III. Experiment 3, Trial 5. Relative Phosphatase Activity of Milk Heated for a Minimum Time at 170.06 F. and Incubated at 52 F. for Selected Periods of Time.

[d]O.D. = Optical Density

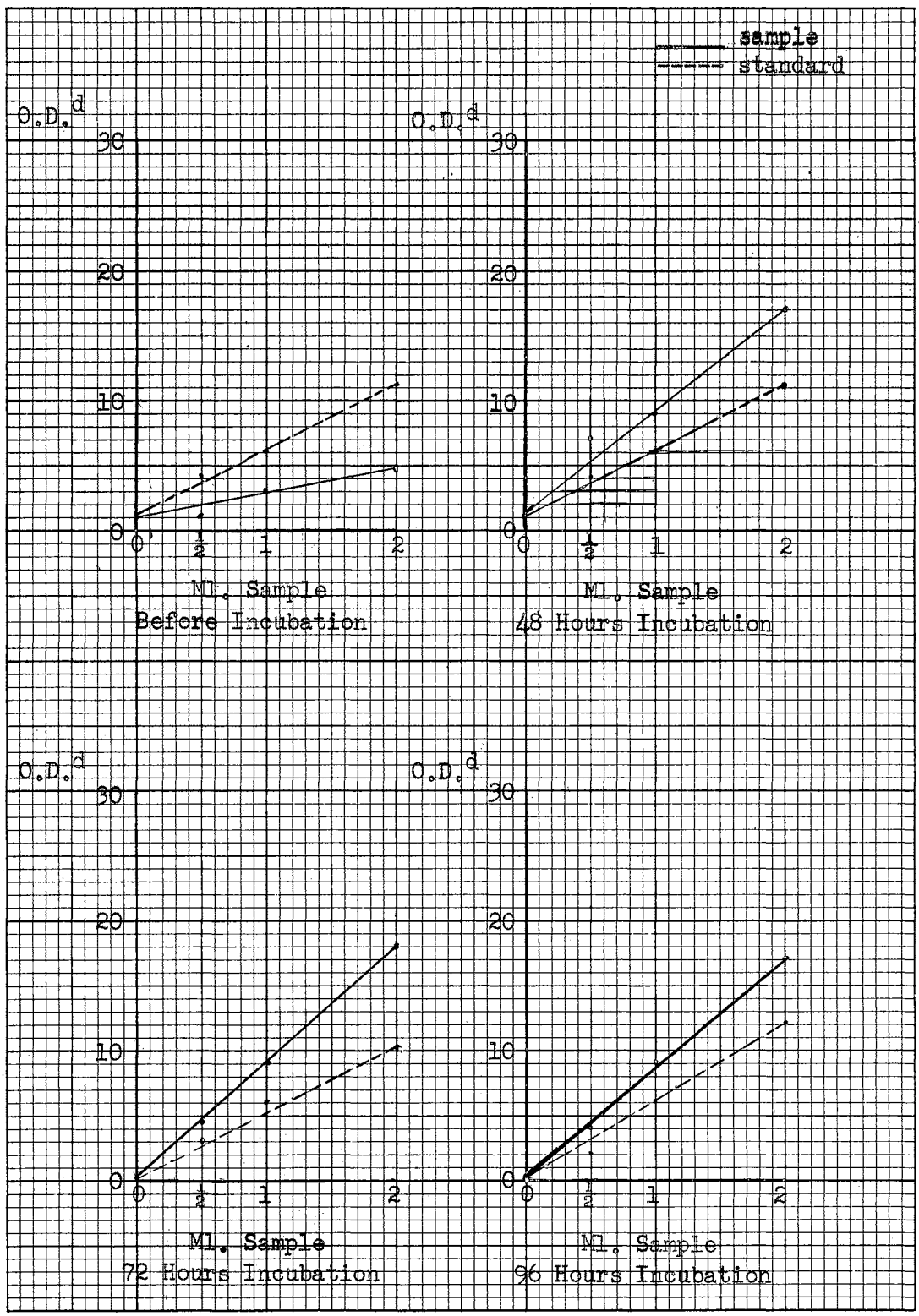

Figure IV. Experiment 4, Trial 4. Relative Phosphatase Activity of Milk Heated for a Minimum Time at 170.06° F. and Incubated at 72° F. for Selected Periods of Time.

[d]O.D. = Optical Density

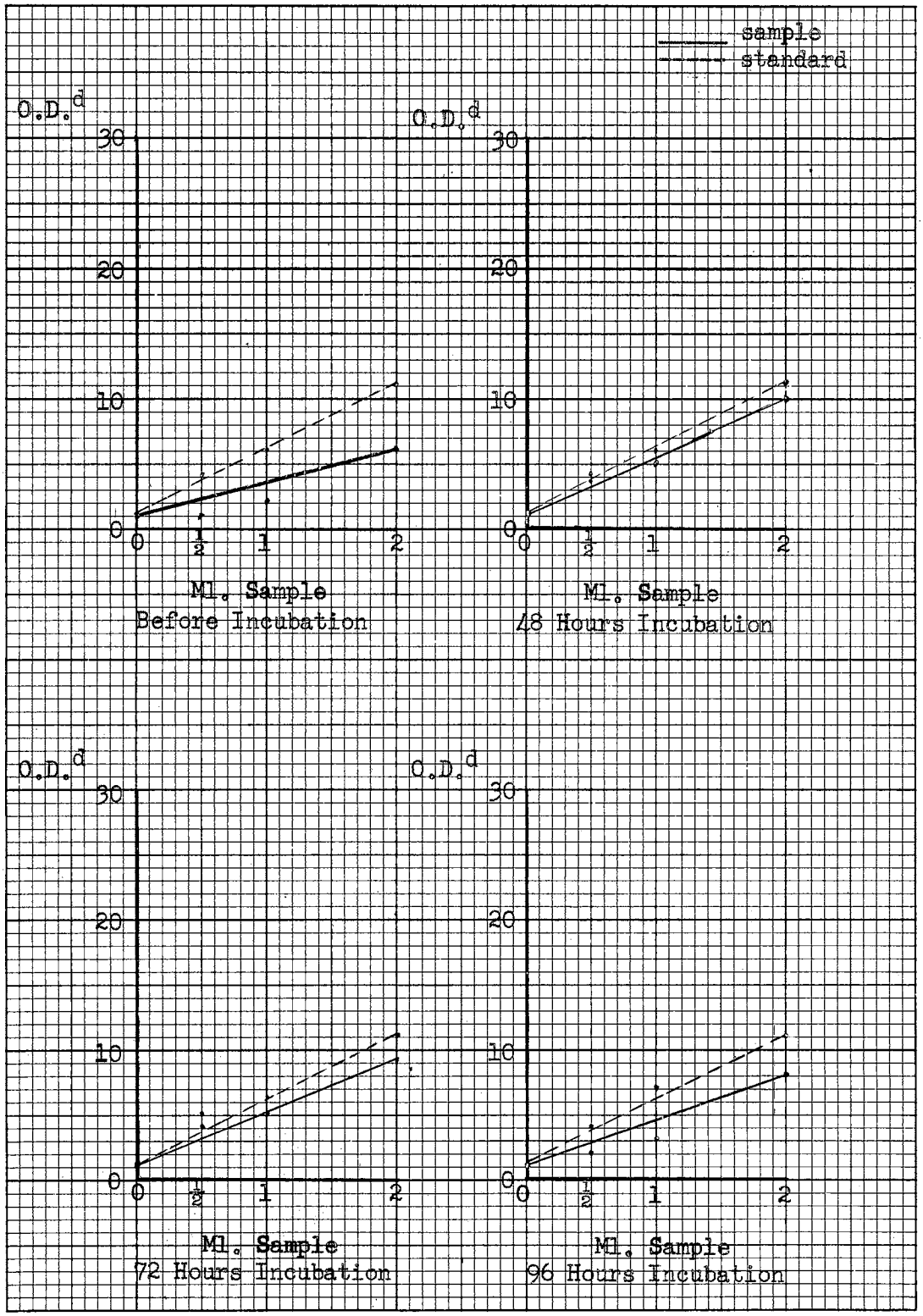

Figure V. Experiment 4, Trial 4. Relative Phosphatase Activity of Milk Overheated and Incubated at 72°F. for Selected Periods of Time.

dO.D. = Optical Density

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