Present Status of the Phosphatase Test*  
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A description of an improved phosphatase test for pasteurization, applied to Cheddar cheese and fluid milk, was published in 1946 (12). During the research work it became evident that milk phosphatase is much more stable than had been generally supposed, retaining a large proportion of its activity almost indefinitely in raw or under-pasteurized products. It seemed, therefore, that the quantitative measurement of the enzymic activity of most dairy products should be an index of the enzymic activity of the milk used, and therefore an index of pasteurization of the milk. Accordingly, the scope of the work was enlarged to attempt to develop a unified method applicable to various dairy products.

The application of the phosphatase test as an index of pasteurization is based on the following facts, determined by earlier research work of others: All normal milk that has not been pasteurized contains a phosphatase enzyme. This milk enzyme is inactivated by heating for a prescribed time at a temperature a few degrees higher than that required to kill the organism that causes tuberculosis, which, as pointed out by North and Park (10), is the most heat-resistant of the pathogenic organisms that may occur in dairy products. Therefore, a negative phosphatase test provides an index of the adequacy of pasteurization, indicating indirectly that the product was heated sufficiently to kill any pathogenic organisms that might have been present.

According to researches of Theobald Smith (14), the tuberculosis organism is killed in milk by heating at 140° F. for 15 to 20 minutes, and in cream by heating at that temperature for a slightly longer time. North and Park repeated Smith's experiments and verified the results. They determined the thermal death points of this organism also at various other temperatures, and found, for example, that it is killed in milk by heating at 160° for approximately 20 seconds. The results of these experiments, and similar experiments by others with various organisms, were the basis of the present specifications for pasteurization.

A positive phosphatase test involves two reactions, the chemical equations for which were given previously (11). The first is hydrolysis of disodium phenyl phosphate (known as the substrate, and added in the test) by the phosphatase, producing free phenol. The second is chemical combination of phenol with 2,6-dibromo (or -dichloro) quinone chlorimine (known as BQC, and added in the test), forming blue indophenol. The quantity of phenol is determined by measuring the intensity of the blue color, either with a colorimeter or by comparison with visual standards.

Detailed directions for the laboratory method for testing various dairy products are published in the December 1947 issue of the Journal of Dairy Science (13) and will not be repeated here. The following discussion is presented in three parts: First, difficulties encountered in conducting earlier tests; secondly, the manner in which the test was modified and im-

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proved to yield reliable, quantitative results on various products; and thirdly, some of the results obtained.

**Difficulties in the Test**

Late in 1944, certain members of the cheese industry and of the Research Committee of the National Cheese Institute requested information from these laboratories as to whether it would be possible to develop a test that would show reliably whether the milk used in making Cheddar cheese had been adequately pasteurized. Two methods that were available, and most recommended for the test on milk, were the Kay-Graham method and the Scharer method. There was also available an advance copy of a report (3) of research on the application of a modified Scharer test to Cheddar cheese the results of which indicated that the test on raw-milk cheese tended to be negative in many instances after a curing period of three to six months, and also that the optimal pH for phosphatase activity in the test could be maintained by mixing the samples of cheese with a buffer solution before testing.

There were available in our laboratories approximately 375 Daisy-style Cheddar cheeses, made in pasteurization experiments in which exact records of the heat treatment of the milk had been obtained. In the preliminary tests numerous difficulties were encountered. In using the methods then available, "false negative" results (negative results on samples of cheese made from raw milk) were encountered very frequently, and values much lower than those obtained on milk were encountered generally, especially with cured cheese.

Determinations made with a Beckman pH meter showed that the pH values during incubation were, in many instances, so low as to be outside the range of activity of the phosphatase enzyme in the test. The buffers in use for testing milk were not adequate to bring the reaction in the tests within the optimal pH range. Moreover, different dairy products have different buffering capacities, and the buffering capacity of cheese increases with age. It was therefore necessary to modify both the concentration and the alkalinity of the buffer for each type of product, and also for cheeses of different ages.

Earlier tests involved preliminarily diluting the sample with water, or with a mixture of butyl alcohol and water, and then using a portion of the liquid material in testing, in order to decrease the buffer capacity of the sample to a level corresponding with that of the relatively weak buffers then available. It was found that this practice of testing only the liquid portion excluded most of the enzymic activity from the test, and produced erroneous and low results. Tests on samples of cheese and on the filtrates prepared therefrom showed that the enzymic activity is associated more with the solids (fat and also casein) than with the aqueous phase. Moreover, preliminarily grinding the samples with a mortar and pestle or with a blender was laborious and apparently unnecessary.

Experiments showed that phosphatase activity is inhibited by certain negative ions, including phosphates and citrates. The sodium buffers used in earlier tests did not remove these ions from solution, and it was desirable, especially in testing process cheese, to use as buffer a reagent that would precipitate these ions.

Difficulty was encountered in the use of lead acetate, recommended in an earlier procedure, as a protein precipitant. The filtrates were frequently cloudy, due to (a) insufficient removal of the proteins from some samples, and (b) the frequent presence of colloidal lead hydroxide in the filtrate, requiring further adjustment with alkali to clear up the filtrate. It was found to be very essential that the protein material be removed as completely as possible, and that the filtrates be clear, in order to obtain uniform, maximal color.
development, sensitivity, and precision in the readings.

The use of lead as a precipitant yielded an excessive amount of interfering yellow color when BQC was added, and an insufficient amount of blue color, requiring the use of more BQC than should be necessary otherwise and thus further increasing the amount of yellow off color.

It was found also that lead tends to cause some catalytic decomposition of the substrate, with a resulting tendency toward misleading, slightly positive controls.

In the methods available, adequate provision had not been made for adjusting the pH accurately, in testing cheese, for uniform and maximal production of blue color without excessive development of yellow color.

The tests available were not quantitative over a sufficiently wide range to measure phosphatase activity accurately in all samples.

An incubation period of 10 or 30 minutes was found to be inadequate for precise results and desirable sensitivity. On the other hand, an incubation period as long as 24 hours was considered too long for practical purposes.

The Kay-Graham procedure was found not applicable for use with cured cheese, sour buttermilk, butter, and other stored or ripened products, principally for two reasons: First, the Folin-Ciocalteu reagent used for color development was developed originally as a specific reagent for the detection of tryptophane and tyrosine, two amino acids which are liberated when hydrolysis of casein occurs. Therefore, the blue color was excessively intense in all controls prepared in testing ripened products. Moreover, the intensities of blue color in tests employing the Folin-Ciocalteu reagent on fresh, raw samples were relatively less than those in tests employing BQC. Secondly, the preparation of the Folin-Ciocalteu reagent in the laboratory is time-consuming and complicated.

As a result of these difficulties it was necessary to investigate as thoroughly as possible the chemistry of the test. In lieu of a presentation of the voluminous research data, a brief description of the details and improvements is given below.

**Improvements in the Test**

A flow-sheet diagram of the test is presented in figure 1.* The new improvements are outlined as follows:

1. Barium borate-hydroxide is used as a buffer, instead of sodium tetaborate, to aid in precipitating the proteins and especially to precipitate phosphates and citrates, which interfere with the activity of the phosphatase enzyme. In controlled experiments on samples of milk, the use of barium resulted in an average increase of approximately 12 percent in the quantity of phenol liberated by the enzyme.

2. The barium borate-hydroxide buffer has a high buffering capacity in the pH region in which the enzyme is most active. The concentration and the alkalinity of the barium buffer are specified for each type of product and are so adjusted as to yield the optimal pH of approximately 10, or within a range of 9.85 to 10.2 (in no case should the pH be below 9.5) during incubation, thus improving the quantitative accuracy. For example, one specified concentration of this buffer suffices for testing uncured Cheddar and most other uncured cheeses, and it is only necessary to dilute it with water for testing products of lower buffering capacity, such as milk, cream, chocolate drink, cheese whey, cottage cheese, and cream cheese. More concentrated solutions of this buffer, specified in the method, are used for testing cheeses with higher percentages of nonfat solids, such as Parmesan, and for aged cheeses.

Folley and Kay (4) showed that, with a concentration of substrate similar to that used in these experiments,
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FIGURE 1. Flow-sheet diagram of the improved phosphatase test

the optimal pH for the activity of the enzyme is approximately 10.0. In testing milk and cottage cheese, Horwitz (6) found the average optimal pH to be 9.91. In our experiments with milk, it was found to be in the region of pH 10.0 to 10.05. Horwitz pointed out that the pH of incubation in testing milk by an earlier method was found to be considerably lower than the optimum, i.e., as low as 9.3–9.4.

The correct pH during incubation is apparently the most important condition for a successful, reliable test.

3. A protein precipitant containing zinc (for testing ripened products) or zinc and copper (for fresh products) has been introduced, replacing the lead precipitant used earlier. The use of the new precipitant yields uniformly clear filtrates. More important, the zinc-copper precipitant increases the brilliance and the intensity and rate of development of the blue color and decreases the interfering yellow color produced by a given quantity of BQC. This was proven by spectrophotometric analyses (12) of the colors.

4. A sodium metaborate-sodium chloride buffer, known as the color development buffer, is added to the filtrate. It serves to dilute somewhat the interfering compounds present, and it also adjusts the pH to 9.3–9.4, which was found to be optimal for the development of the blue color with a minimum
of yellow color. The use of sodium chloride in the buffer also aids in reducing the yellow color.

5. A dilution method has been introduced to make possible the full color development from all the phenol present, at the same time reducing the amount of off color, thus increasing the precision and making the test quantitative even for raw samples.

Preliminary preparation of samples with a mortar and pestle or with a blender has not been found necessary. With the alkalinity and concentration of the buffers adjusted to give reliable results for samples of any given type, it is generally unnecessary to determine and adjust the pH when testing. All reagents are prepared by weighing, and the need for primary standards or solutions of designated normalities has been eliminated.

SUMMARY OF RESULTS

No attempt will be made here to present the voluminous data obtained in studying the optimal conditions in each step in the test and in analyzing several thousands of samples of products made under known conditions of heating. For example, more than 1,500 samples of various cheeses, and more than 3,000 samples of milk and cream, have been tested. Some of the data were presented earlier (12), and only a few of the results that seem most important will be discussed.

Table 1 lists examples of the products tested, and shows the range of phosphatase values found in tests on a considerable number of raw samples of each product. The method was found to give reliable results as an index of pasteurization on each of the products listed.

In connection with these data it should be pointed out that the unit of measurement is the intensity of blue color produced by 1 microgram (0.000001 gram, designated by the symbol \( \gamma \), or "gamma") of phenol. It is possible to detect visually, in the test, the intensity of the blue color produced by 1 microgram of phenol, equivalent to 1 unit of phosphatase activity, and, by means of a photoelectric colorimeter, to detect 0.5 unit. Tests on many samples of raw milk show average activities greater than 1,000 units per 0.5 milliliter. The test is sufficiently sensitive to show an increase in the reading due to the presence of as little as 1 pound of raw milk added to 2,000 pounds of properly pasteurized milk, and the addition of raw cream to pasteurized cream in the proportion of 1 pound in 5,000. It is sufficiently sensitive to detect a decrease of 1°F. below

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>PHOSPHATASE ACTIVITY FOUND IN RAW MILK PRODUCTS</th>
</tr>
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<tbody>
<tr>
<td>Product, other details</td>
<td>Range of phosphatase values</td>
</tr>
<tr>
<td>Milk, 4%</td>
<td>900-1,300 units per 0.5 ml.</td>
</tr>
<tr>
<td>Skim milk</td>
<td>600-800 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Cream, 20%</td>
<td>1,800-2,600 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Cream, 40%</td>
<td>3,500-5,000 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Ice cream mix</td>
<td>1,400-2,200 &quot;  &quot; &quot;</td>
</tr>
<tr>
<td>Sherbert mix</td>
<td>700-900 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Butter</td>
<td>600-800 &quot; 0.5 g. &quot;</td>
</tr>
<tr>
<td>Butter serum</td>
<td>4,000-5,000 &quot; 0.5 ml. &quot;</td>
</tr>
<tr>
<td>Buttermilk, sweet</td>
<td>4,500-5,500 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Buttermilk, sour (pH 4.3)</td>
<td>1,400-1,600 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Cheese, uncured Cheddar</td>
<td>600-900 &quot; 0.25 g. &quot;</td>
</tr>
<tr>
<td>1-year-old Cheddar</td>
<td>300-600 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>uncurd Swiss</td>
<td>100-400 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>Cottage curd</td>
<td>200-450 &quot; 0.5 g. &quot;</td>
</tr>
<tr>
<td>Cheddar cheese whey</td>
<td>400-500 &quot; 0.5 ml. &quot;</td>
</tr>
<tr>
<td>Goat milk—regular test for milk</td>
<td>60-105 &quot; &quot; &quot;</td>
</tr>
<tr>
<td>special test for goat milk</td>
<td>350-600 &quot; 1.5 ml. &quot;</td>
</tr>
</tbody>
</table>
a standard pasteurizing temperature, or, at a given temperature, an equivalent shortening of the holding time necessary for adequate pasteurization.

Data obtained in tests on milk and on products made from the same milk show that the activity of the enzyme is greater in cream than in milk, increasing with increasing percentages of fat, and less in skim than in whole milk; that there is more than twice as much activity in 1 gram of fresh cheese as in 1 gram of the milk used, and less in cheese whey than in milk; and that the activity is low in butter and high in fresh buttermilk from sweet cream, and in butter serum. Samples of cultured buttermilk stored for one week at 45°F, with a pH of 4.3, still retained about one-third of the original activity of the fresh buttermilk.

The activity of the phosphatase was found to be much lower in goat milk than in cow milk. A special modification of the test for use with goat milk, with an increase in the quantity of sample and in the period of incubation, is given in the description of the method.

In order to determine effects of different heating conditions on the extent of inactivation of the enzyme, pasteurization experiments were carried out under accurately controlled conditions. A laboratory pasteurizer was assembled, comprising a tubular metal coil 30 feet long and \( \frac{3}{8} \) inch internal diameter, with a metal chamber at the end, immersed in a water bath with temperatures controlled thermostatically with a variation not greater than ±0.2°F. Fluid samples were forced into the coil under pressure, and removed by suction into tubes immersed in ice water. The holding time at the bath temperature was controlled with an error not greater than ± 3 seconds. In addition to tests on fluid dairy products, 3-gram samples of cheese were ground and placed in heat-sealing metal foil envelopes, which were squeezed to a thickness of 1 millimeter, sealed, and immersed in the bath. The time required for the temperature of the samples of cheese to reach that of the bath was not more than 18 seconds. A four-junction thermocouple was used to determine the temperature of samples.

Some of the results are shown in figure 2. The experimental data for phosphatase destruction show that, at any given temperature and different heating periods, the rate of destruction of the enzyme is very rapid at first and diminishes to a relatively slow rate with time. It was found also that a straight line results when the logarithms of the times of heating, to produce negative tests, are plotted against the corresponding temperatures. Van Bever (15) and others have shown that a similar straight-line relationship exists with respect to the destruction of bacteria by heat.

Holding periods found necessary to produce negative phosphatase tests, i.e., not more than 2 units per 0.5 milliliter of whole milk, were: 37 minutes at 143°F, 30 minutes at 143.6°F, 24 seconds at 160°F, and 15 seconds at 161.7°F, respectively.

The temperature required to produce a negative test 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 percent cream than for whole milk, about 4.5°F higher for ice cream mix (containing 15 percent fat) than for whole milk, and about 5.7°F higher for milk 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.

Negative tests were obtained at considerably lower temperatures and shorter holding times in Cheddar cheese than in milk—e.g., at 130°F in approximately 13 minutes and at 140°F in slightly less than \( \frac{3}{4} \) minute in cheese at pH 5.3. Mixing alkalis or emulsifying salts with the cheese had some effect in stabilizing the enzyme against heat. For example, the temperature required to produce a negative test in approximately \( \frac{3}{4} \) minute was 150°F in samples of cheese containing 1.5 percent an-
hydrous disodium phosphate and having a pH of 5.56.

Although these data do not pertain to destruction of bacteria by heat, they should be useful in studies on pasteurization, in pointing out equivalent effects produced by heating for given times at any temperature in the pasteurization range.

Concerning the problem of so-called "false positive" tests, phosphatase activity caused by microorganisms has not been found in this work in any fresh or reasonably fresh products. It has been encountered in some samples of old butter and old cream, on the surfaces of some soft and semi-soft ripened cheeses (Limburger, Liederkranz, Camembert, and, to a less extent, brick), and in several specific cultures of microorganisms. Such activity, in the samples tested to date, is indicated by blue color in the controls or blanks which have been heated slightly beyond the pasteurizing temperature to destroy the milk enzyme, as described in the method.

The discovery that phosphatase produced by certain microorganisms is inactivated at a higher temperature than milk phosphatase was reported in 1939 by Neave (9), and in 1942 and 1943, in tests with certain other microorganisms, by Buck (2), Kaplan (8),

![Figure 2. Time of heating at different temperatures found necessary to reduce phosphatase activity to 2 units per 0.5 ml. in milk and in other fluid dairy products.](http://meridian.allenpress.com/jfp/article-pdf/11/2/67/2393906/0022-2747-11_2_67.pdf)
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As Kaplan and also Barber and Frazier pointed out, this difference in inactivation temperatures apparently may provide a means of differentiating between "false positive" tests caused by at least some microorganisms and true positive tests caused by the milk enzyme.

Accordingly, if the test itself is positive and if any blue color is noted in the control that has been heated slightly beyond pasteurization (we recommend heating the control momentarily to 185°F.) before testing, the presence of microbial phosphatase can be suspected. To test further for microbial phosphatase, the following procedure is recommended: Using another portion of the sample, increase the pH to nearly 10 by adding 1 milliliter of the appropriate barium buffer (without substrate), heat it for 5 minutes in the tube in boiling water, cool the contents, and conduct the determination in the usual manner. If this control is negative, it indicates that the blue color in the original control was due to microbial phosphatase, i.e., a "false positive" sample.

Collaborative work was done in 1946, under the Association of Official Agricultural Chemists, by Gilcreas on the phosphatase test applied to hard cheese, and by Horwitz on the test applied to soft, unripened cheeses. In Gilcreas' work (5), both a modified Kay-Graham method and the method discussed here were investigated, with tests on a large number of samples of Cheddar cheese, some of which had been cured for as long as 4 years. The results showed, as had been found also in our work, that the modified Kay-Graham procedure could not be adapted satisfactorily as an index of pasteurization in testing cheese. Gilcreas found that the Sanders-Sager method provides a reliable estimate of the inactivation of the enzyme by heat, and thus of the degree of heat treatment of the milk from which hard cheese was made. He recommended at that time that the Association adopt it as a tentative method.

Horwitz (7) likewise found that this method could be applied successfully in testing soft cheeses.

At the annual meeting of the above-named Association in October 1947, as a result of the collaborative work and additional results reported, this laboratory procedure was adopted as an official method; first action, for use with fluid milk and cream, Cheddar cheese, and the soft, unripened cheeses. It was adopted as a tentative method for the other dairy products mentioned.

It can be stated with assurance that if the milk, cream, or fluid mixture used is negative, the cheese, butter, ice cream, or sherbet manufactured from it will also be negative, unless there is contamination with a raw product after pasteurization and unless a "false positive" condition develops. Therefore, negative tests on the milk or other fluid products themselves should generally suffice in a manufacturing plant.

DISCUSSION AND CONCLUSIONS

A description is presented of modifications and improvements in the laboratory phosphatase test for pasteurization, and application of the test to virtually all dairy products. The test has been found to be reliable when applied to milk, cream, butter, buttermilk, ice cream mix, sherbet, chocolate drinks, Cheddar and most other types of cheese, cheese whey, and (with less sensitivity) goat milk. A description of the method is published in the December 1947 issue of the Journal of Dairy Science.

In the case of fluid milk and cream, it is well known that pasteurization improves the keeping quality. With respect to these and other products that are consumed fresh, the knowledge that the products have been pasteurized offers further assurance to consumers that the products are safe.

The improved test for pasteurization and demonstration of its reliability makes it possible to determine on practically all dairy products whether or not the milk used has been pasteurized.
It is believed that the test will be useful to the dairy industry in checking the effectiveness of pasteurization procedures for controlling the quality and for safeguarding public health.

REFERENCES


