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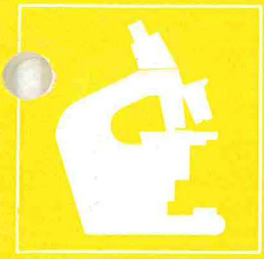





# *Journal of Food Protection*™

**An International Journal Concerned With An Adequate Food Supply That Is Safe, Wholesome, Nutritious, and Palatable**

"Recruit A New Member This Month"

64th Annual Meeting
Sioux City Hilton Hotel
Sioux City, Iowa
August 14-18, 1977

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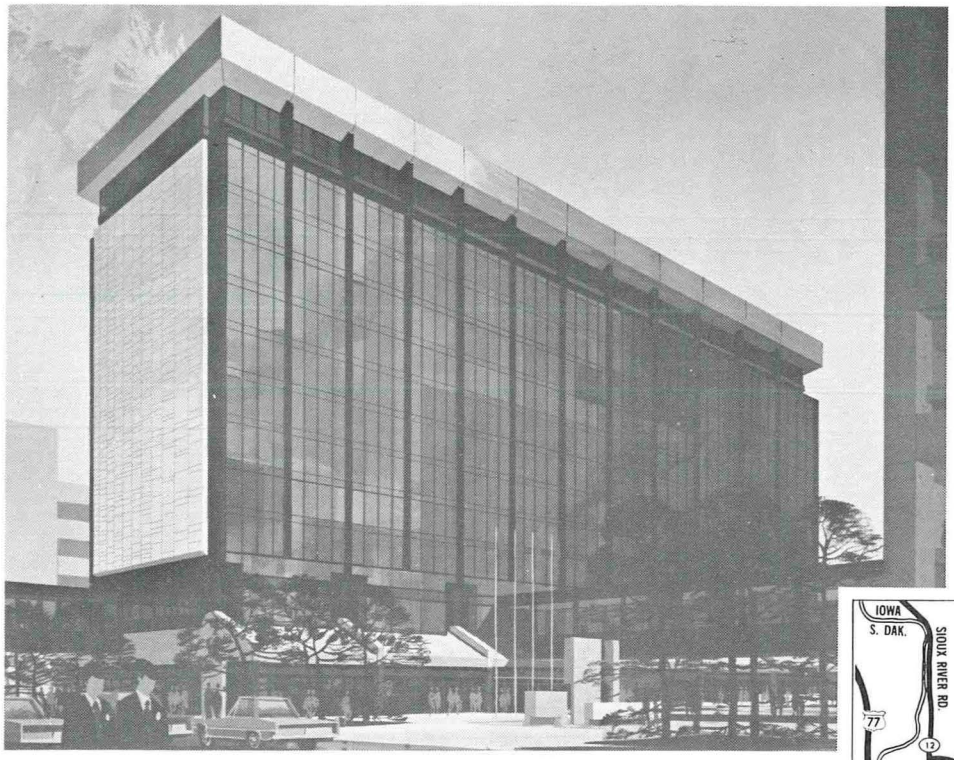

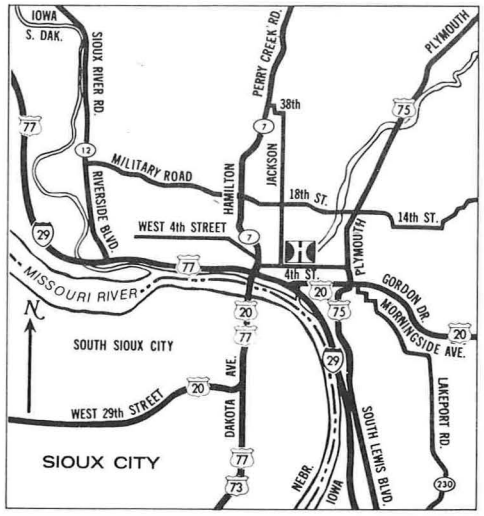


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64th Annual Meeting
August 14-18, 1977

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# *Journal of Food Protection*


(formerly Journal of Milk and Food Technology)

Official Publication

International Association of Milk, Food, and Environmental Sanitarians, Inc., Reg. U.S. Pat. Off.

Vol. 40 April, 1977 No. 4



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*Journal of Food Protection Vol. 40, No. 4, Pages 222-227 (April, 1977)*


# Effect of Various Combinations of Medium, Diluent and Incubation Conditions on Recovery of Bacteria from Manufacturing Grade and Grade A Raw Milk[1]

**B. E. LANGLOIS and CHAMRAS SANGHIRUM[2]**

*Department of Animal Sciences, Food Science Section*
*University of Kentucky, Lexington, Kentucky 40506*

(Received for publication December 19, 1975)

ABSTRACT

Recovery of microorganisms from manufacturing grade and Grade A raw milk was determined using 18 plating combinations which consisted of three media, three diluents, and two incubation temperatures. Plating conditions specified in *Standard Methods* for doing the Standard Plate Count was one of the 18 combinations used. Combinations studied consisted of Standard Methods Agar, Schaedler Agar, and Eugonagar as plating medium; phosphate buffered distilled water, 0.1% peptone water, and Ringer solution as diluent; and 28 C for 72 h and 32 C for 48 h as incubation temperature. Forty manufacturing grade and 40 Grade A raw milk samples were plated using each of the 18 combinations. Highest mean counts were obtained for both grades of milk with the combination of Standard Methods Agar, phosphate buffered distilled water, and 28 C for 72 h. Samples, diluents, media, and samples × diluents interaction had a highly significant ($P<.01$) effect on counts of manufacturing grade milk samples; while samples, media, and temperatures had a highly significant ($P<01$) effect on counts of the Grade A raw milk samples. Nonsignificant differences were obtained in counts of the manufacturing gradesamples with eight of 17 plating combinations when compared with counts obtained with *Standard Methods.* Counts for Grade A samples obtained with six of 17 combinations were similar to counts obtained with *Standard Methods.*

The microbial populaton of raw milk should give an indication of sanitation conditions during production and handling of milk. The Standard Plate Count (SPC) (*3*) is the method used to determine microbial populations of raw milk and to determine if the milk meets quality and microbial standards (*19, 21, 42*).

Plating medium, diluent, and incubation conditions required for determining the SPC of milk are detailed in *Standard Methods for the Examination of Dairy Products* (*3*). The 13th edition specifies use of Standard Methods Agar as the plating medium, phosphate buffered distilled water as the diluent, and incubation at 32 ± 1 C for 48 ± 3 h.

Investigators have reported that composition of the micro-flora of raw milk has been altered by the various changes which have occurred in the dairy industry during recent years (*22, 24, 27, 36, 38, 40, 45*). As a result, values obtained by the SPC may not indicate the microbial quality of the milk. Suggestions have been made by many researchers that changes should be made in the procedure for the SPC to obtain counts which would be more representative of the sample. Counts obtained for raw milk have been found to influenced by plating medium (*1, 7, 15, 30, 31, 35, 38, 46, 47*), dilution fluid (*5, 8, 9, 13, 18, 25, 34*), and incubation temperature and/or time (*1,4,11,15,16,17,20,23,24,28,29,32,33,37,39*).

This study was made to compare recovery of bacteria from two grades of raw milk using 18 different plating combinations. The combinations consisted of three media, three diluents, and two incubation conditions. Comparisons also were made between the plating combination used for the SPC and each of the other 17 plating combinations to determine if the recovery of bacteria from raw milk with any of the other plating combinations differed significantly from that obtained with SPC.

## MATERIALS AND METHODS

### *Samples*

Forty manufacturing grade and 40 Grade A milk samples were collected and analyzed during a 10-month period. The manufacturing grade milk samples were aseptically collected from properly agitated cans or tanker trucks upon their arrival at a cheese plant. No attempt was made to treat or analyze the can and tanker milk samples separately, since the study was designed to determine and compare recovery of bacteria in manufacturing grade raw milk as received at a cheese plant regardless of delivery method.

The Grade A milk samples were aseptically collected from properly agitated bulk tank milk at the University of Kentucky dairy farm. Only samples from this farm used to determine and compare recovery of bacteria in samples with low counts and produced under conditions superior to those of the average Grade A farm.

All samples collected were placed in sterile plastic bags for refrigerated transportation to the laboratory where they were held at 4 ± 1 C until analyzed. Samples were analyzed within 8 h of collection.

### *Plating procedure*

Except as described below, procedures given in the 13th edition of *Standard Methods for the Examination of Dairy Products* (*3*) were used to analyze the samples. Standard Methods Agag (Difco:SMA) (*3, 10*),

[1]*Published with the approval of the Director of the Kentucky Agricultural Experiment Station as journal article no. 75-5-123.*
[2]*Present address: Northeast Agriculture Center, Tha-Pha, Khon, kaen Thailand.*

Eugonagar (BBL:EA) (*6*), and Schaedler Agar (BBL:SA) (*6*) were prepared according to the directions of the manufacturer and used as plating media. Phosphate buffered distilled water, APHA, pH 7.2 (BBL:PB) (*3, 6*), Ringer solution (Oxoid:RS) (*26*), and 0.1% peptone water (PW) (*2, 12, 41*) were prepared according to the directions of the manufacturer or other investigators and used for preparing decimal dilutions of each sample. Plates prepared from each diluent and medium were incubated at 28 ± 1 C for 72 h or at 32 ± 1 C for 48 h.

Pour plates were made of each sample using each of the 18 plating combinations according to the scheme shown in Fig. 1. The sequence used for medium, diluent, and temperature was randomized to minimize errors inherent in the plating procedure. Counts of less than 30 colonies were used only when they were from the lowest dilution plated.

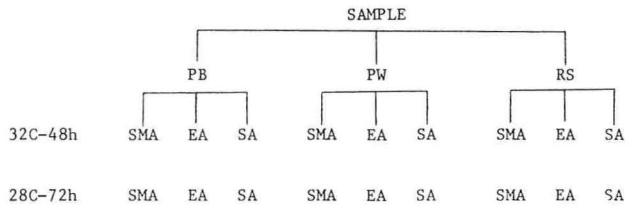


Figure 1. *Scheme used to plate each milk sample using eighteen combinations of medium, diluent and incubation temperature. PB = phosphate buffered distilled water; PW = 0.1% peptone water; RS = Ringer solution; SMA = Standard Methods agar; EA = Eugonagar; SA = Schaedler agar.*

*Analysis of data*

Data were analyzed using a Statistical Analysis System (SAS) program (*14*). The Waller-Duncan (*44*) procedure was used to determine if counts obtained with each medium, diluent, and incubation condition were significantly different from each other. Paired experimental analysis using the "Student's" t distribution test was used to determine if the results obtained with each of the 17 plating combinations differed significantly from those obtained with the SPC. Each grade of milk was analyzed separately.

## RESULTS AND DISCUSSION

Mean logarithms of counts and ranges of logarithms obtained for each of the 18 plating combinations are given in Table 1 for the manufacturing grade milk samples and in Table 2 for the Grade A samples. Samples which comprised each of the two grades of milk were analyzed separately.

The mean logarithms for manufacturing grade samples ranged from a high of 6.681 for the plating combination SMA, PB, and 28 C to a low of 6.379 for the combination SA, RS, and 32 C. The Grade A samples ranged from a high of 3.905 for the combination SMA, PB, and 28 C to a low of 3.712 for SMA, RS, and 32 C. The same plating combination gave the highest mean

TABLE 1. *Mean logarithms of counts and ranges of logarithms for the eighteen combinations of medium, diluent and incubation temperature obtained for the 40 manufacturing grade raw milk samples*

| | Diluents | | | | | |
|---|---|---|---|---|---|---|
| | PB[a] | | PW[b] | | RS[c] | |
| | Incubation temperatures (C) | | | | | |
| Media | 28 | 32 | 28 | 32 | 28 | 32 |
| SMA[d] | 6.681[g] (4.279-8.519)[h] | 6.664 (4.114-8.279) | 6.655 (3.982-8.699) | 6.600 (3.959-8.716) | 6.552 (4.079-8.255) | 6.488 (4.079-8.146) |
| EA[e] | 6.613 (3.881-8.544) | 6.617 (3.929-8.505) | 6.592 (3.908-8.699) | 6.508 (3.919-8.819) | 6.428 (3.895-8.176) | 6.435 (3.940-8.255) |
| SA[f] | 6.577 (3.991-8.362) | 6.603 (4.041-8.301) | 6.520 (3.944-8.613) | 6.536 (3.924-8.398) | 6.402 3.978-8.204) | 6.379 (3.969-8.230) |

[a]Phosphate buffered distilled water
[b]0.1% Peptone water
[c]Ringer solution
[d]Standard Methods agar
[e]Eugonagar
[f]Schaedler agar
[g]Mean count of 40 samples ($\log_{10}$)
[h]Range of counts obtained ($\log_{10}$)

TABLE 2. *Mean logarithms of counts and ranges of logarithms for the eighteen combinations of medium, diluent and incubation temperature obtained for the 40 Grade A raw milk samples*

| | Diluents | | | | | |
|---|---|---|---|---|---|---|
| | PB[a] | | PW[b] | | RS[c] | |
| | Incubation temperatures (C) | | | | | |
| Media | 28 | 32 | 28 | 32 | 28 | 32 |
| SMA[d] | 3.905[g] (2.833-5.114)[h] | 3.749 (2.792-5.000) | 3.865 (2.869-5.041) | 3.814 (2.708-5.079) | 3.882 (2.716-5.114) | 3.712 (2.681-5.079) |
| EA[e] | 3.856 (2.851-4.415) | 3.808 (2.732-4.447) | 3.875 (2.820-5.462) | 3.794 (2.851-4.362) | 3.880 (2.778-5.431) | 3.797 (2.716-4.398) |
| SA[f] | 3.844 (2.869-5.114) | 3.785 (2.792-5.079) | 3.760 (2.748-5.114) | 3.784 (2.690-5.041) | 3.839 (2.763-5.000) | 3.791 (2.748-4.991) |

[a]Phosphate-buffered distilled water
[b]0.1% Peptone water
[c]Ringer solution
[d]Standard methods agar
[e]Eugonagar
[f]Schaedler Agar
[g]Mean of 40 samples ($\log_{10}$)
[h]Range of counts obtained ($\log_{10}$)

logarithms for both grades of milk. Except for the plating medium, the combinations giving the lowest mean logarithms were similar for both milk grades. Schaedler agar was the plating medium in the combination giving the lowest mean logarithm for the manufacturing grade samples, while SMA was the medium in the combination for the Grade A samples. In general, regardless of the grade of milk the lowest count obtained for an individual sample was higher when SMA rather than either of the other two media was the medium in the plating combination.

As expected, counts were higher for the manufacturing grade samples than they were for the Grade A samples. Mean counts for the manufacturing grade samples were up to three-fold higher than those for the Grade A samples. The lowest count obtained for a manufacturing grade milk sample was one- to two-fold higher than the lowest count obtained for a Grade A sample. The highest count obtained for a Grade A sample was three- to four-fold lower than the highest count obtained for manufacturing grade sample. Depending on the plating combination, the range of counts obtained for the 40 manufacturing samples varied four-to five-fold, while the variation in the range of counts obtained for the 40 Grade A samples was two- to three-fold.

Analysis of variance of factors affecting counts is shown in Table 3 for the manufacturing grade samples and in Table 4 for the Grade A samples. Samples and media had a highly significant (P<.01) effect on counts obtained for both grades of milk. Diluents and samples × diluents interaction had a highly significant (P<.01) effect on the counts of the manufacturing samples, but non-significant effects on the counts of the Grade A samples. Incubation temperatures had a highly significant (P<.01) effect on the counts obtained for the Grade A samples, but a non-significant effect on the counts for manufacturing grade samples. Counts obtained for manufacturing grade samples were affected by the media x temperatures interaction at the 7% level. None of the other factors or interactions had a significant effect on the counts obtained for either grade of milk.

Mean logarithms of counts when the results obtained for each medium, diluent, and incubation temperature were combined are given in Table 5 for the manufacturing grade samples and in Table 6 for the Grade A samples. Highest mean logarithms for both grades of milk were obtained with SMA as the plating medium. Differences between the means of each of the three media for manufacturing grade samples were highly

TABLE 3. *Analysis of variance of factors affecting the counts obtained from the 40 manufacturing grade milk samples*

| Source | Degrees of freedom | F-value | P > F |
|---|---|---|---|
| Samples | 39 | 1118.94113 | P < .01 |
| Diluents | 2 | 79.77244 | P < .01 |
| Samples × diluents | 78 | 4.21307 | P < .01 |
| Media | 2 | 25.48565 | P < .01 |
| Temperatures | 1 | 0.39125 | NS[a] |
| Media × temperatures | 2 | 2.62840 | P < .07 |
| Diluents × media | 4 | 0.84692 | NS |
| Diluents × temperatures | 2 | 1.44233 | NS |
| Diluents × media × temperatures | 4 | 0.19837 | NS |

[a]NS = Non-significant

TABLE 4. *Analysis of variance of factors affecting the counts obtained from the 40 Grade A raw milk samples*

| Source | Degrees of freedom | F-value | P > F |
|---|---|---|---|
| Sample | 39 | 416.69695 | P < .01 |
| Diluents | 2 | 0.82534 | NS[a] |
| Samples × diluents | 78 | 0.86334 | NS |
| Media | 2 | 11.45409 | P < .01 |
| Temperatures | 1 | 106.31452 | P < .01 |
| Media × temperatures | 2 | 0.43121 | NS |
| Diluents × media | 4 | 1.39214 | NS |
| Diluents × temperatures | 2 | 0.43003 | NS |
| Diluents × media × temperatures | 4 | 0.81441 | NS |

[a]NS = Non-significant

TABLE 5. *Mean logarithms of counts for the 40 manufacturing grade milk samples when the results obtained for each medium, diluent, and temperature were combined*

| Number of determinations | Media | Mean count ($log_{10}$) |
|---|---|---|
| 240 | SMA[a] | 6.607[x] |
| 240 | EA[b] | 6.556[y] |
| 240 | SA[c] | 6.498[z] |
| | *Diluents* | |
| 240 | PW[d] | 6.580[x] |
| 240 | RS[e] | 6.447[y] |
| 240 | PB[f] | 6.634[z] |
| | *Temperatures (C)* | |
| 360 | 28 | 6.558 |
| 360 | 32 | 6.550 |

[a]Standard Methods agar
[b]Eugonagar
[c]Schaedler agar
[d]0.1% Peptone water
[e]Ringer solution
[f]Phosphate-buffered distilled water
[x,y,z]Means in column with different superscript differ significantly (P < .01)

TABLE 6. *Mean logarithms of counts for the 40 Grade A milk samples when the results obtained for each medium, diluent and temperature were combined*

| Number of determinations | Media | Mean counts ($log_{10}$) |
|---|---|---|
| 240 | SMA[a] | 3.851[x] |
| 240 | EA[b] | 3.835[x,y] |
| 240 | SA[c] | 3.815[y] |
| | *Diluents* | |
| 240 | PW[d] | 3.830 |
| 240 | RS[e] | 3.832 |
| 240 | PB[f] | 3.839 |
| | *Temperatures (C)* | |
| 360 | 28 | 3.865[v] |
| 360 | 32 | 3.802[w] |

[a]Standard Methods agar
[b]Eugonagar
[c]Schaedler agar
[d]0.1% Peptone water
[e]Ringer solution
[f]Phosphate-buffered distilled water
[v,w]Means in column with different superscript differ significantly (P< .01)
[x,y]Means in column with different superscript differ significantly (P< .05)

significant (P<.01). Unlike these results, a significant difference (P<.05) was obtained for the Grade A samples only between the means for SMA and SA.

Vera (*43*) indicated that SMA must not be too productive as a medium and permit recovery of all microorganisms because allowable microbial limits might have to be changed which could affect grading of milk and governmental regulations. She implied that requirements were such that SMA must give approximately the same results as media used decades ago. More productive medium could be made and would permit growth of more of the microflora present and/or more of the injured or damaged bacteria. Schaedler agar (*6*) and EA (*6*) appear to be attempts to develop media which are more productive than SMA. Schaedler agar originally was developed for enumeration of microflora of the gastrointestinal tract. Several modifications have been made of SA and it has been reported to be more productive than SMA (*6*). Eugonagar (*6*) was designed to permit growth of many bacterial species considered difficult to grow. This medium has been recommended for use in examination of meats, for control of food processing, and for determining total intestinal and oral counts.

Results of this study indicate that SMA was more productive than either SA or EA when used for examination of manufacturing grade and Grade A raw milk. Hartley et al. (*15*) obtained higher counts for manufacturing grade samples on SMA than on EA, while the reverse was obtained when Grade A milk samples were examined. Similar results have been obtained when a medium containing approximately the same composition as EA was compared with tryptone glucose beef extract milk agar (*30, 31*). Results of this study indicate that the medium specified in *Standard Methods* (*3*) gave better recovery of bacteria from raw milk than SA or EA.

The types of diluent to use in the routine analysis of milk and food products have been studied by many investigators (*5, 8, 9, 13, 18, 25, 34*). *Standard Methods* (*3*) specifies use of phosphate buffered distilled water. In England quarter-strength Ringer solution (*26*) is used, while 0.1% peptone water is recommended for microbial analysis involving meat, foods, and some pathogens (*2, 12, 41*).

In this study, highest mean logarithms were obtained for both grades of milk when PB was used as the diluent. Lowest mean logarithms were obtained with RS as the diluent with the manufacturing grade samples and with PW with the Grade A samples. Differences between mean count of each diluent were highly significant (P<01) for each other with the manufacturing grade samples. Unlike these results, non-significant differences were obtained between the means of each diluent for the Grade A samples. Oblinger and Kennedy (*25*) found that Butterfield's diluent (*8*) and peptone water appeared to offer more consistent recoveries of bacteria from a variety of food samples than distilled water and 0.85% NaCl. However, no diluent was found to be ideally suited for every sample analyzed.

Since the manufacturing grade samples represented both can and tanker milk, they should contain a wider variety in numbers and species of bacteria than the Grade A samples. Difference in the microflora comprising the two grades of milk could be responsible for differences obtained when the diluents within each grade of milk were compared. Researchers (*8, 18, 25, 34*) have found various factors which appear to influence the reliability or recovery of bacteria from food and milk. More research needs to be done to determine the reasons(s) for the differences obtained with the two grades of milk. These studies should involve treating separately can and tanker manufacturing grade samples and using Grade A samples from different sources.

Highest mean logarithms were obtained for both grades of milk when an incubation temperature of 28 C was used. The difference between the means for the two temperatures with the manufacturing grade samples was non-significant, but the difference was highly significant with the Grade A samples. Many investigators (*15, 16, 32*) have reported that the optimum temperature for recovery of microorganisms from milk is less than the 32 C specified in *Standard Methods* (*3*). Higher counts generally have been obtained at 28 C and in some instances at 21 C (*15*).

A reason for the non-significant differences between the counts at the two temperatures with the manufacturing grade samples may be the influence of the can milk samples and the fact that approximately 63% of the samples had counts which exceeded 3 million. This suggest the presence of large numbers of microorganisms, probably lactic acid bacteria or coliform, which would be capable of growing well at both temperatures. The Grade A samples were from a single farm with excellent practices and all the samples should have contained a similar microflora. This microflora had adequate cooling and should contain species which grow better at 28 than 32 C.

Optimum recoveries of bacteria from both grades of raw milk were obtained using the plating combination SMA, PB, and 28 C for 72 h. Except for incubation, other conditions are the same as specified for SPC. Results obtained in this study are similar to those obtained by others (*13, 16, 28, 32, 33*). Hartley et al. (*15*) reported that since some psychrotrophs do not grow at 32 C, better recovery is obtained with lower temperatures.

The procedure described in *Standard Methods* (*3*) for SPC specified use of SMA, PB, and 32 C for 48 h. The "Student's" t was used to compare results obtained with SPC and each of the other 17 plating combinations to determine if any of the other combinations gave results which were not significantly different from those obtained with SPC.

Results obtained with the manufacturing grade samples indicated that eight of the 17 combinations

TABLE 7. *Combinations of medium, diluent and temperature whose counts of manufacturing grade raw milk samples were not significantly different from those obtained using Standard Methods*

| Medium | Diluent | Incubation temperature | t-Value[a] |
|---|---|---|---|
| SA | PB | 32 | 1.7414 |
| SMA | PW | 32 | 1.4799 |
| SMA | PB | 28 | .4876 |
| SMA | PW | 28 | .1752 |
| EA | PB | 32 | .2713 |
| EA | PW | 32 | 1.7671 |
| EA | PB | 28 | 1.3307 |
| EA | PW | 28 | 1.7243 |

[a]"Student's" t distribution test
SA = Schaedler agar
SMA = Standard Methods agar
EA = Eugonagar
PB = Phosphate-buffered distilled water
PW = 0.1% Peptone water
RS = Ringer solution

(Table 7) gave results that were not significantly different ($P<.05$) from those obtained with SPC. Results obtained with six of the 17 combinations (Table 8) did not differ

TABLE 8. *Combinations of medium, diluent and temperature whose counts of Grade A raw milk samples were not significantly different from those obtained using Standard Methods*

| Medium | Diluent | Incubation temperature | t-Value[a] |
|---|---|---|---|
| SA | PB | 28 | .4250 |
| SA | PW | 28 | .5318 |
| SA | RS | 28 | .00168 |
| SMA | PW | 32 | 1.9458 |
| EA | PB | 32 | 1.5995 |
| EA | PB | 28 | .8070 |

[a]"Student's" t distribution test
SA = Schaedler agar
SMA = Standard Methods agar
EA = Eugonagar
PB = Phosphate-buffered distilled water
PW = 0.1% Peptone water
RS = Ringer solution

significantly ($P<.05$) from those obtained with SPC for the Grade A samples.

The grade of milk influenced whether or not a combination differed significantly from SPC. This would be expected, since numbers, species, and other factors all appear to influence recovery of bacteria from milk. More combinations containing EA or SA as plating medium gave counts similar to SPC for the Grade A samples, while for the manufacturing grade samples the combinations contained SMA or EA.

Ringer solution was not in any of the combinations with the manufacturing grade samples, but was in one of the combinations for the Grade A samples which gave results similar to SPC.

The temperature in the combination giving counts similar to those obtained for SPC were fairly equally divided between 28 and 32 C for both grades of milk.

The results obtained in this study indicate that best recoveries of bacteria occurred using the medium and diluent specified in *Standard Methods* (*3*) and incubation at 28 C for 72 h. Due to variation in numbers, species, and source of samples, combinations other than SPC may give results similar to those obtained by SPC. More analyses should be done before definite recommendations are made to change procedure for SPC for all grades of milk.

In this study 25 of the 40 manufacturing grade samples had counts by each of the 18 combinations which exceeded the Kentucky regulation (*21*) of $3 \times 10^6$/ml ($\log_{10}$ 6.48) for manufacturing grade milk. Only one of the Grade A samples exceeded the standard (*42*) of $1 \times 10^5$/ml ($\log_{10}$ 5.00) for an individual producer.

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# Effect of Using Milk as a Heating Menstruum on the Apparent Heat Resistance of *Bacillus stearothermophilus* Spores[1]

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ABSTRACT

Heat resistance at 121.1 C (250 F) of *Bacillus stearothermophilus* spores was studied using two heating menstrua. D values of 3.8 and 3.5 min were obtained when spores were heated in 0.01 M $PO_4$ buffer, pH 6.5, and in skimmilk, pH 6.5, respectively. With buffer as a heating menstruum, increasing the pH from 6.5 to 7.2 resulted in an increase in the D value from 3.8 to 4.1 min. When the pH of skimmilk was increased from 6.5 to 7.2, D values increased from 3.5 to 5.2 min. Skimmilk as a component of the enumeration medium inhibited germination and/or outgrowth of *B. stearothermophilus* spores; however, this inhibition was not influenced over the pH range of 6.0 to 7.2. Addition of 10% skimmilk, pH 6.5, to the medium for enumeration of spores heated in buffer at pH 6.5 or 7.2, in each instance reduced the number of spores that could be recovered but did not change the slopes of survival curves.

The heating menstruum has been shown to influence the apparent heat resistance of bacterial spores. Studies (*1, 6, 7, 8*) have shown that altering the concentration of sugar, salt, phosphate, acid, or a variety of other substances in the heating menstruum did influence the apparent heat resistance of certain spores. Segner et al. (*5*) found that *Bacillus stearothermophilus* spores suspended in 3:1 milk concentrate were heat inactivated at essentially the same rate as when they were suspended in phosphate buffer. Franklin et al. (*3*), however, note the *B. stearothermophilus* spores were more heat sensitive when heated in milk than in water.

The purpose of this investigation was to determine if using milk as a heating menstruum influenced the apparent heat inactivation of *B. stearothermophilus* spores and if carry-over of this milk into the enumeration medium affected the number of spores that could be recovered.

## EXPERIMENTAL PROCEDURES

### *Source of the microorganisms*

A culture of *B. stearothermophilus* (1518 smooth) was obtained from Dr. F. F. Busta at North Carolina State University, Raleigh. This organism is a faculative thermophile with an optimum growth temperature of 55 C.

### *Growth and sporulation of B. stearothermophilus*

*Vegetative growth.* Actively growing vegetative cells were prepared for inoculating the surface of the sporulation medium. One ml of a stock spore suspension was heat-shocked at 100 C for 20 min. One-tenth ml of this suspension was used to inoculate each of 10 flasks containing 100 ml of nutrient broth (Difco). After 14 h of incubation at 55 C, cells were removed from broth by centrifugation and resuspended to one-tenth the original volume with 0.01 M phosphate buffer, pH 6.5 (*4*). The cell suspension contained about $1 \times 10^9$ cells per ml.

*Sporulation on fortified nutrient agar.* Two hundred and fifty ml of nutrient agar (Difco) supplemented with 40 ppm $MnSO_4$ was poured into each of ten 17.8 × 27.9 cm pyrex dishes. Each dish was then covered with a double layer of aluminum foil and autoclaved at 121.1 C for 15 min. When the medium had solidified, each dish was inoculated by spreading 10 ml of the cell suspension evenly over the entire surface. After 72 h of incubation at 55 C, spores were harvested by washing the surface of each dish with 100 ml of cold sterile 0.01 M phosphate buffer, pH 6.5. Spores from 80 plates were collected, pooled, and washed in buffer three times by centrifugation, pouring off the supernatant fluid and resuspending the spores in fresh cold buffer. Spores were concentrated by centrifugation to give a final volume of 200 ml. The population of the final spore suspension was $4 \times 10^8$ spores per ml as determined by the agar plate method after heat shocking at 100 C for 20 min.

### *Enumeration medium*

Dextrose tryptone agar (Difco) plus 0.5% soluble starch was used as the recovery medium. Plates incubated at 55 C for 48 h and then counted.

### *Definition of D value*

D values were obtained from the straight line portion of the thermal death time curves and are numerically equal to the number of minutes required for the survivor curve to transverse one log cycle.

### *Heating methods*

*Oil bath system.* An oil bath system of heating was devised for studying heat inactivation at 121.1 C (250 F). Two oil baths were used in this system. A heat-up bath set 10 F above the desired heating temperature made it possible to bring the thermal death time tubes up to temperature much more rapidly than when only one bath set at the holding temperature was used. By means of a thermocouple inserted in one of the thermal death time tubes (TDTT), the temperature and time of the come-up period could be recorded. A Leeds and Northrup Speedomax G recording potentiometer was used to record the times and temperatures. When the TDTTs reached the desired holding temperature in the heat-up bath, they were immediately transferred to the hold bath. The TDTTs were removed from the holding bath at predetermined time intervals and immersed in cold water.

*Thermal death time tubes (TDTTs).* The thermal death time tubes were made from aluminum tubing of approximately 3-mm inside

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diameter and 120 mm long. Both ends were threaded so that a bolt could be used to seal each end.

*Heating menstrua.* Phosphate buffers were made using 0.01 M solutions of $KH_2PO_4$ and $Na_2HPO_4$ (*4*). These solutions were mixed in the proper proportions so as to yield the desired pH. The pH was checked using a Beckman Model H-2 pH meter.

The reconstituted milk used as the heating menstruum was prepared by adding 10 g of low heat nonfat dry milk solids (NDM) to 90 ml of deionized water and sterilizing at 121.1 C for 15 min. Skimmilk used as the heating menstruum was prepared from University of Minnesota herd milk taken from the bulk tank after agitation for at least 10 min and then separated using a DeLaval Separator, Model Number 618 and then sterilized at 121.1 C for 15 min. pH adjustments were done after sterilization.

Results reported are typical of those observed from replicate experiments. In this work three to five trials were done for each variable.

## RESULTS AND DISCUSSION

### *Effect of milk as a heating menstruum on heat resistance of spores*

When sterilized 10% reconstituted NDM was used as a heating menstruum in place of 0.01 M phosphate buffer, pH 6.5, a marked change was observed in the apparent heat resistance of *B. sterarothermophilus* spores. Figure 1 shows the survival curves for spores suspended in milk

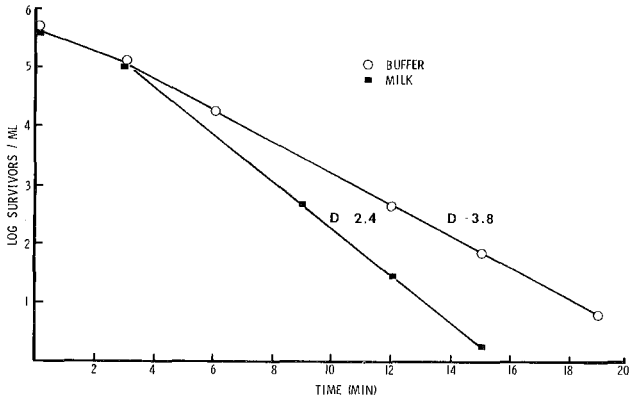


Figure 1. *Survivor curves at 121.1 C (250 F) for* B. stearothermophilus *spores suspended in reconstituted sterile nonfat dry milk (10%) and 0.01 M $PO_4$ buffer at pH 6.5.*

or buffer and then heated at 121.1 C (250 F). The decrease in the heat resistance of the spores heated in milk was reflected by a D value of 2.4 min as compared to 3.8 min for the same spores suspended in buffer. Further investigation showed that when milk was used as a heating menstruum, the pH was a critical factor. Figure 2 shows the survival curves for spores suspended in milk and buffer at pH 7.2. It can be seen that adjustment of the suspending menstruum from pH 6.5 to 7.2 had a major effect on the heat resistance of spores suspended in sterile 10% NDM, but only a minor effect on spores suspended in phosphate buffer. When sterile skimmilk was used, the same effect was noted. Figure 3 shows the effect of heating spores in skimmilk at pH 7.2, 6.5, and 6.0. It is interesting to note that with skimmilk at pH 6.5 and 7.2, the D values of 3.5 and 5.2 min, respectively, were higher than those obtained using 10% reconstituted

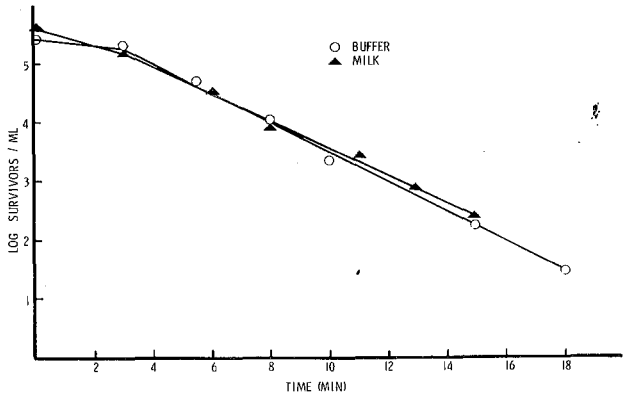


Figure 2. *Survivor curves at 121.1 C for* B. stearothermophilus *spores suspended in sterile reconstituted nonfat dry milk (10%) and 0.01 M $PO_4$ buffer at pH 7.2.*

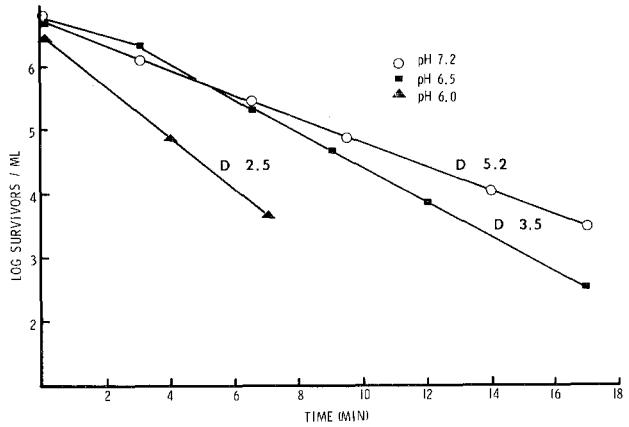


Figure 3. *The effect of pH on the survival at 121.1 C of* B. stearothermophilus *spores suspended in raw skimmilk.*

nonfat dry milk at the same pH. Regardless of the difference in D values, lowering the pH from 7.2 to 6.5 produced a similar change in heat resistance when either skim or reconstituted milk was used as the heating menstruum. If these differences were to be attributed to a pH effect alone, then spores suspended in buffer should have also shown the same response. Other workers have shown that when milk is heated to high temperatures a variety of chemical compounds may be formed, some of which may be inhibitory to micoorganisms. Therefore, it was concluded that changing the pH of the milk altered some other factor or factors in the milk and these changes were responsible for the differences in the apparent heat resistance of *B. stearothermophilus* spores heated in milk at pH 7.2 and 6.5.

### *Effect of inhibitors in milk on the apparent heat resistance of spores*

One of the factors investigated in this respect was inhibition of *B. stearothermophilus* spores by milk in the enumeration medium. Busta and Speck (*2*) and Franklin et al. (*3*) have reported that milk inhibited germination and/or outgrowth of *B. stearothermophilus* spores. The spores used in the previous studied were tested to determine the extent of inhibition induced by sterile

skimmilk. A spore suspension was heat shocked at 100 C in phosphate buffer for 20 min and plated on dextrose tryptone starch agar, both with and without addition of skimmilk sterilized by heating at 121.1 C for 15 min. Results in Table 1 show quite clearly that addition of 10% of this milk to the plating medium reduced the number of spores recovered.

TABLE 1. *The effect of the addition of skimmilk to the plating medium on the recovery of* B. stearothermophilus *spores*

| Medium | Number of colonies per plate[1] | % recovery |
|---|---|---|
| Dextrose tryptone starch agar | 76 | 100 |
| Dextrose tryptone starch agar + 10% sterile skimmilk | 16 | 21 |

[1]Average of duplicate plates, population of spores used was 76,000 per ml, $10^{-3}$ plate counted.

In the enumeration procedure for recovering spores heated in milk, a certain amount of milk would be carried over to the plates in the plating procedure. For low spore populations this could amount to as much as 1.0 ml per plate or approximately 10% of the plating medium. From Table 1 it can be seen that inhibition due to the milk could be a factor in determining apparent populations when the lower dilutions were plated.

If the above inhibitor was involved in the differences noted in the heat resistance between spores heated in milk and in phosphate buffer, then it should be sensitive to changes in pH as shown in Fig. 1 and 2. To determine the effect of pH on this inhibitor, sterile skimmilk was added to the enumeration medium and the medium was adjusted to various pH values aseptically with either sterile NaOH or HCl.

*B. stearothermophilus* spores were heat shocked at 100 C for 20 min and enumerated on dextrose tryptone starch agar with and without addition of milk. Results in Table 2 show that the pH of the plating medium does

TABLE 2. *The effect of the pH of plating media on the inhibition of* B. stearothermophilus *spores heat shocked at 100 C for 20 min*

| pH of the medium | Dextrose tryptone starch agar | Dextrose tryptone starch agar + 10% sterile skimmilk | Percent recovery[1] |
|---|---|---|---|
| 7.2 | 29,000 spores/ml | 5,000 spores/ml | 17 |
| 6.8 | 37,000 spores/ml | 9,000 spores/ml | 24 |
| 6.5 | 76,000 spores/ml | 16,000 spores/ml | 21 |
| 6.0 | 110,000 spores/ml | 27,000 spores/ml | 24 |

[1]Percent recovery was based on using the dextrose tryptone starch agar without added milk at each pH as 100%.

alter the number of spores recovered. However, the effect was opposite to that noted in the experiments where milk was used as a heating menstruum.

It is apparent that lowering the pH of the enumeration medium increased the number of spores that could be recovered whether milk was added to the medium or not. However, at any given pH, the ratio of the counts in the medium with milk and without milk was similar. Because the milk in the medium was at the same pH as the enumeration medium, it was concluded that the inhibitory property of the milk was not altered by changes over the pH range of 7.2 to 6.0.

To determine if heated spores reacted differently to milk in the recovery medium, spores were heated at 100 C for various times in 0.01 M $PO_4$ buffer, pH 7.2, or pH 6.5. The following media were used: dextrose tryptone starch agar (DTSA), and DTSA plus 10% sterile skimmilk. Figure 4 shows that addition of skimmilk

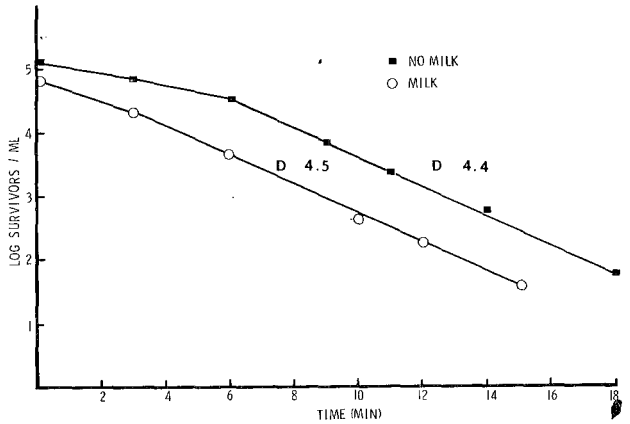


Figure 4. *The effect of adding 10% sterile skimmilk to the recovery medium for* B. stearothermophilus *spores heated at 121.1 C in 0.01 M $PO_4$ buffer, pH 7.2.*

reduced the number of spores that could be recovered at any point throughout the heat treatment by about 80%. The fact that the two survival curves shown in Fig. 4 had nearly the same slope indicated that the response of the heat treated spores to the inhibitory effect of the skimmilk in the recovery medium was not changed by more severe heat treatments.

Figure 5 shows that the survival curves for *B. stearothermophilus* spores heated in 0.01 M phosphate buffer, pH 6.5, had essentially the same response to skimmilk added to the recovery medium as spores heated in pH 7.2 buffer.

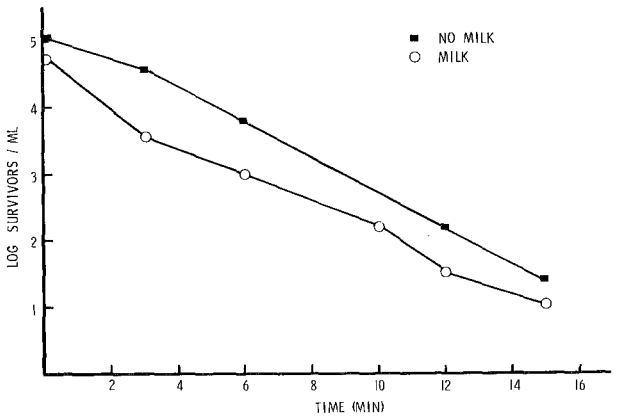


Figure 5. *The effect of adding 10% sterile skimmilk to the recovery medium for* B. stearothermophilus *spores heated at 121.1 C in 0.01 M $PO_4$ buffer, pH 6.5.*

Since the inhibitor in milk which prevents germination and/or outgrowth of *B. stearothermophilus* spores was stable to changes in the pH over the range of 6.0 to 7.2, and heat treatments ranging from 100 C for 20 min to

121.1 C for 15 min did not materially change the response of spores to this inhibitor, it seemed unlikely that the inhibitor was directly responsible for the difference in survival curves observed when spores were heated in milk at pH 6.5 and 7.2.

When milk was sterilized at pH 7.2 and the pH readjusted to 6.5 and 7.2 before being used as a heating menstruum, the number of spores surviving after 12 min at 121.1 C was reduced from 340,000 per ml to 45 and 2600 per ml, respectively. When milk was sterilized at pH 6.5, then readjusted to pH 6.5 and 7.2 and used as a heating menstruum, the number of spores surviving after 12 min at 121.1 C was reduced from 400,000 per ml to 25 and 1300 per ml, respectively. These data indicate that there was interaction between the spores and the milk during the heat treatment which was dependent on the pH of the milk during the heat treatments and not due to the effects of any inhibitory compound produced by previous heat treatments of the milk at different pH values.

## CONCLUSIONS

*B. stearothermophilus* spores were apparently more sensitive to heat inactivation when suspended in 10% reconstituted NDM than in 0.01 M phosphate buffer, pH 6.5. Increasing the pH of the reconstituted NDM heating menstruum from 6.5 to 7.2 resulted in a marked increase in the apparent heat resistance of the spores. Increasing the pH of the buffer from 6.5 to 7.2 had very little effect on the heat resistance of the spores.

Busta and Speck (*2*) and Franklin et al. (*3*) have shown that milk contains an inhibitor which prevents germination and/or the outgrowth of *B. stearothermophilus* spores. In this investigation it was found that addition of 10% sterile skimmilk to the recovery medium reduced the number of spores recovered by about 80%. This inhibitor was found to be stable to pH changes over the range of 7.2 to 6.0. It was also found that the severity of the heat treatment given the spores did not alter the response of spores to this inhibitor. It was concluded that the inhibitor in milk was not responsible for the differences in heat resistance noted between *B. stearothermophilus* spores heated in milk at pH 6.5 and 7.2.

The pH at which milk was sterilized did not alter it as a heating menstruum for *B. stearothermophilus* spores as long as the pH was readjusted before it was used as a heating menstruum.

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# Effect of Sporulation Media on the Heat Resistance of *Bacillus stearothermophilus* Spores[1]

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ABSTRACT

Based on D values obtained over the temperature range of 121.1 C (250 F) to 126.7 C (260 F), *Bacillus stearothermophilus* vegetative cells sporulated in the presence of milk were more resistant to heat inactivation than spores grown on nutrient agar fortified with $MnSo_4$. Spores grown in the presence of milk and heat-treated at 121.1 and 126.7 C had D values of 4.7 and 0.8 min, respectively. Spores grown on fortified nutrient agar and heat-treated at 121.1 and 126.7 C had D values of 3.7 and 0.55 min, respectively. No difference in heat resistance was observed between spores derived from vegetative cells grown in milk or nutrient broth when tested at 121.1 C.

The heat resistance of spores of a specific microorganism may vary greatly depending upon the sporulation environment (*1-7*). Spores harvested from microbiological media may not show the same inactivation rate as spores of the same strain sporulated in a natural environment. This fact makes it difficult to set adequate processing conditions without extensive large scale trials.

Several investigators (*1, 6, 7*) have shown that the sporulation medium can alter the heat resistance of spores. Much of the work reported in the literature has been done using spores grown on common bacteriological media. Because this study was primarily focused on factors important in dairy processing, the effect of sporulating *B. stearothermophilus* vegetative cells in the presence of milk solids was studied.

## EXPERIMENTAL PROCEDURES

*Source of the microorganism*

A culture of *B. stearothermophilus* (1518 smooth) was obtained from Dr. F. F. Busta at North Carolina State University, Raleigh. This organism is a facultative thermophile with an optimum growth temperature of 55 C.

Procedures for growing, sporulating, and heating the spores have been described in detail elsewhere (*6*). Essentially, the procedures consisted of growing vegetative cells in nutrient broth, sporulating them on the surface of nutrient agar fortified with 40 ppm $MnSO_4$ or milk agar medium. The milk medium consisted of an agar gel base prepared by adding 15 g of agar to 1 liter of deionized water and sterilizing at 121.1 C for 15 min. Two hundred and fifty ml of this base was dispersed in sterile containers to yield a surface area of 500 $cm^2$. When this base had solidified it was layered with 10 ml of sterile milk inoculated with vegetative cells of *Bacillus sterarothermophilus.* The milk was prepared by reconstituting 10 g of nonfat dry milk solids in 90 ml of deionized water sterilized at 121.1 C for 15 min, cooled, and inoculated with vegetative cells of *Bacillus stearothermophilus.* Heat treatments were done in an oil bath system using aluminum thermal death time tubes with a capacity of about 1.0 ml. Spores surviving the various heat treatments were enumerated on dextrose tryptone agar (Difco) plus 0.5% soluble starch.

Results reported are typical of those observed from replicate experiments. In this work three to five trials were run for each variable.

## RESULTS AND DISCUSSION

Heat resistance of *B. stearothermophilus* spores was determined over the temperature range of 250 F (121.1 C) to 260 F (126.7 C). Spores harvested from fortified nutrient agar were heated when suspended in pH 6.5, 0.01 M phosphate buffer. The results of these heat treatments are shown in Fig. 1. The straight line portion of each of these survival curves was used to determine the D values.

A second series of heat treatments was done using spores grown in the presence of milk. Figure 2 shows the survival curves and the D values for these studies using pH 6.5, 0.01 M phosphate buffer as the heating menstruum.

A difference in heat resistance can be seen if the D

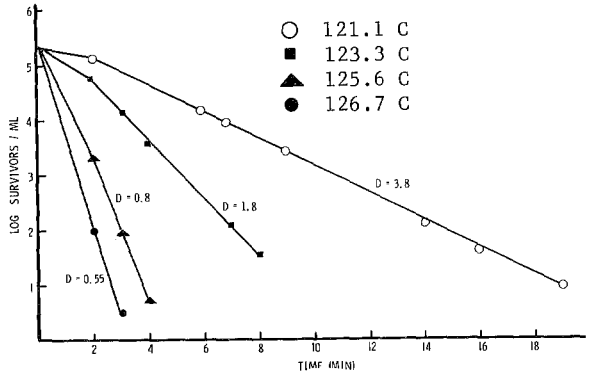


Figure 1. *Survival curves of* B. stearothermophilus *spores sporulated on fortified nutrient agar and heated in 0.01 M $PO_4$ buffer pH 6.5.*

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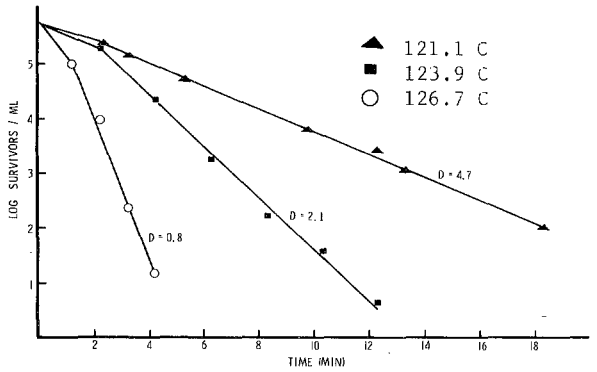


Figure 2. *Survival curves for* B. stearothermophilus *spores grown in the presence of milk and heated in 0.01 M $PO_4$ buffer pH 6.5.*

values for the spores grown in the presence of milk are compared with the D values of the spores grown on fortified nutrient agar. Fortified nutrient agar spores had D values of 3.8 min at 121.1 C and 0.55 min at 126.7 C whereas spores grown in the presence of milk had D values of 4.7 min at 121.1 C and 0.8 min at 126.7 C. Several other temperatures were tested but the data at 121.1 and 126.7 C provide common points for comparison. Although the differences observed are not large, they could result in processing problems. Furthermore, they point out the fact that the source of the spores should be considered when laboratory data are used to determine heat treatment values for actual processing conditions.

*Effect of vegetative growth medium on heat resistance of B. stearothermophilus spores*

Milk-produced spores used in the previous study were obtained from vegetative cells grown in nutrient broth and sporulated in the presence of milk. The question arose as to whether further increases in heat resistance could be induced by using milk for both the vegetative growth medium and the sporulation medium. A series of spore crops were made using milk (sterile 10% reconstituted nonfat dry milk) as the vegetative growth medium and sporulating these cells on both fortified nutrient agar and in the presence of milk. Heat resistance of these spores was compared to that of spores obtained from vegetative cells grown in nutrient broth and sporulated on these two media.

Heat resistance of these spores was tested at 121.1 C. Results in Table 1 show that the sporulation medium was the critical factor contributing to the increased heat resistance and that the vegetative growth medium had little or no effect on heat resistance of spores.

TABLE 1. *Effect of vegetative cell growth and sporulation media on the decimal reduction times of* B. stearothermophilus *spores at 121.1 C*

| Vegetative media | Sporulation media | D in minutes |
|---|---|---|
| Milk | Milk + agar | 4.3 |
| Milk | Fortified nutrient agar | 3.8 |
| Nutrient broth | Fortified nutrient agar | 3.8 |
| Nutrient broth | Milk + agar | 4.5 |

From results of these studies it becomes apparent that when using laboratory-grown spore crops to increase the spore load for testing thermal processing, an attempt should be made to obtain a spore crop that will have a heat resistance similar to that expected from the natural environment.

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# Residual Bisulfite in Iced *Penaeus* Shrimp[1]

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ABSTRACT

Shrimp dipped in sodium bisulfite solutions of 1.25 and 5.0% were analyzed for residual bisulfite during iced storage for up to 15 days. Most of the bisulfite was located on the shell and was readily leached away by the melting ice. A further reduction in bisulfite occurred when shrimp were rinsed in either water or hypochlorite solutions after storage, with the greatest reductions occurring during the hypochlorite rinses.

Black spotting (melanosis) of shrimp is a serious problem throughout the shrimp industry. Although it has little effect on organoleptic quality of shrimp, it seriously affects their appearance. To control black spotting, sodium bisulfite ($NaHSO_3$) is used to inhibit enzymatic oxidation of both tyrosine and dihydroxyphenylalanine thereby preventing darkening of the shell (*3, 5*). Since 1956, agencies such as the Florida State Department of Conservation (*2*) have recommended dipping shrimp in a 1.25% sodium bisulfite solution for 1 min to control black spot development. As with so many other control procedures where chemicals are applied by untrained individuals, the general feeling often prevails that "if a little is good, a lot is even better." This is especially true under certain conditions of harvest where the problem of black spotting may be the most serious due to the shortage of ice and/or extended stays at sea. Under these conditions excessive levels of bisultite can be applied to shrimp either as a dip or added directly to the shrimp as they are layered in ice.

The purpose of this study was to determine the fate of applied bisulfite during iced storage of shrimp and the effect of rinsing the treated shrimp with either water or a sodium hypochlorite (NaClO) solution, a strong oxidizing agent used to rinse and wash the shrimp in commercial practice.

## MATERIALS AND METHODS

Medium sized (31-40 count) fresh headless brown shrimp (*Penaeus aztecus*) obtained from Apalachicola, Florida, were used. A new lot of shrimp was obtained for each study. The freshly caught shrimp were packed in ice at dockside and immediately transported to the laboratory in Gainesville where they were treated and analyzed.

Shrimp were dipped for 5 min in either of two concentrations of sodium bisulfite (1.25 and 5.0%) and analyzed during 15 days of iced storage. Treated shrimp were packed separately in ice in styrofoam containers and stored at 2 C to promote slow melting of ice. The melt water was allowed to drain away from shrimp through holes in the bottom of containers and collected for analyses. The effect of using either water or sodium hypochlorite rinses on residual bisulfite levels in shrimp after storage was also examined. Shrimp for the rinsing experiments were placed in a wire basket and agitated for the specified time. The ratio of rinse to shrimp was held constant at 10 parts rinse to 1 part shrimp.

Chemical analyses for bisulfite were done according to the method of Monier-Williams (*1*).

## RESULTS AND DISCUSSION

Distribution of bisulfite on the shrimp is shown in Table 1. Regardless of the level of bisulfite used, most of the bisulfite was located on the shell, with a much smaller proportion found in the muscle. Bisulfite recovered from the muscle probably was absorbed in the area where the head was removed and the flesh was exposed. With the 1.25% $NaHSO_3$ dip, the level of bisulfite on the shrimp decreased to 5 ppm by the 15th day of storage. The levels found on shrimp dipped in the 5% bisulfite solution were considerably higher; however, they decreased rapidly in both the shell and edible muscle during iced storage.

TABLE 1. *Effect of iced storage on shell and muscle bisulfite levels[a] in shrimp*

| Pre-storage dip[b] | Post-storage rinse[b] | Days stored at 2 C | | | |
|---|---|---|---|---|---|
| | | 0 | 5 | 10 | 15 |
| 1.25% $NaHSO_3$ | Water | | | | |
| Shell | | 170[c] | 60 | 60 | 20 |
| Muscle | | 25 | 5 | 0 | 0 |
| Whole | | 65 | 20 | 20 | 5 |
| 5% $NaHSO_3$ | Water | | | | |
| Shell | | 1300 | 820 | 570 | 510 |
| Muscle | | 110 | 40 | 65 | 45 |
| Whole | | 440 | 290 | 210 | 220 |

[a]Expressed as ppm $SO_2$.
[b]Five minutes.
[c]Each value is the average of two studies.

Bisulfite is problably leached away by the melting ice and is not oxidized or irreversibly bound to the shrimp as demostrated by its recovery from the drip water collected from the iced shrimp. Following 24 h of storage, drip collected from two studies in which shrimp were dipped in 5% bisulfite showed recoveries of 980 and 760 ppm bisulfite.

When the treated shrimp were rinsed using either water or sodium hypochlorite, there was a further reduction in bisulfite levels indicating that the bisulfite is

[1]*Florida Agricultural Experiment Stations Journal Series No. 6194.*

loosely adsorbed to the shells and that a percentage of it is oxidized to non-volatile sulfates by the oxidation of the hypochlorite ion, as evidenced by the lower recoveries following the hypochlorite rinses. A similar reaction has been studied by Gleason et al. (*4*) in model systems and is known to occur.

Table 2 shows the magnitude of the effects of water rinsing, hypochlorite rinsing, and iced storage on shrimp dipped in a 5% bisulfite solution. When the freshly dipped shrimp received only a 5-min water rinse, there was a 41% reduction in bisulfite concentration. This reduction was further enhanced, by the presence of 500 ppm hypochlorite in water, to 52%. Following 14 days of iced storage the remaining bisulfite appeared to be less easily removed by a 5-min water rinse (12% reduction) but more reactive to the presence of hypochlorite (72% reduction). Over the 14 days of iced storage, even without post-storage rinsing, shrimp lost 94% of the bisulfite to the washing action of the melting ice. This reduction was increased to about 99% when the shrimp were rinsed in hypochlorite solution for 15 min.

TABLE 2. *Effect of iced storage and post-storage rinses on bisulfite levels*[a] *in shrimp dipped*[b] *in 5% sodium bisulfite*

| Post-storage rinse | Days stored at 2 C | | |
|---|---|---|---|
| | 0 | 7 | 14 |
| None | 2900[c] | 360 | 160 |
| 5-min water | 1700 | 260 | 140 |
| 15-min water | 1700 | 200 | 120 |
| 5-min NaClO (500 ppm) | 1400 | 210 | 45 |
| 15-min NaClO (500 ppm) | 1300 | 140 | 30 |

[a]Expressed as ppm $SO_2$.
[b]Five-minute dip.
[c]Each value is the average of two studies.

These data show that the bisulfite used to control black spot on shrimp is loosely bound on the shells. At reasonable levels of application, iced shrimp lose much of the adsorbed bisulfite to the drip water formed by the melting ice and to any subsequent rinsing that might take place. In addition, when treated shrimp are rinsed in hypochlorite solution, as is commercial practice, a further reduction takes place due to an oxidation of the bisulfite to non-volatile sulfates.

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# Evaluation of a Rapid Test for Antibiotic Residue in Milk Using Spores of *Bacillus stearothermophilus* var. *calidolactis*[1]

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ABSTRACT

Eleven analysts tested contaminated reconstituted (1:10) dry milk powders for penicillin residues using spores of *Bacillus stearothermophilus* var. *calidolactis* (Delvotest P method). Three types of responses were noted: positive, negative, and questionable. Prediction equations indicated that 95% of the time, analysts unfamiliar with the technique could detect positive results if penicillin concentrations in samples were 0.010 unit/ml or higher and positive and questionable results if the penicillin concentrations were 0.008 unit/ml. Increasing the reconstitution ratio from 1/11 to 1/4 increased the chances of detecting penicillin in milk powder. Penicillinase added to reconstituted penicillin-contaminated milks in all instances produced negative responses.

Many methods have been described for microbiological methods for detection of antibiotic residues in milk (review by Marth, *10*). Milk-impregnated discs on agar plates seeded with spores of either *Bacillus subtilis* (*2, 10, 18*) or with *Bacillus megaterium* (*14*) have been used.

Thermophilic bacteria have been used for rapid tests (2 1/2 h). Berridge (*3*) employed *Streptococcus thermophilus*. Igarashi et al. (*6*), using *Bacillus stearothermophilus*, showed this organism to be suitable for detecting antibiotics in raw milk by either the triphenyltetrazolium (TTZ) reduction method or by a disc assay. Galesloot and Hassing (*5*) indicated that as little as 0.0025 unit penicillin/ml of milk could be detected with *B. stearothermophilus* by a paper disc method. Kabay (*8*), using a cylinder cup assay, showed the organism to be sensitive to 14 antibiotics and several chemical preservatives; the minimum concentration of penicillin detected was 0.01 unit/ml. Terplan (*16*) and Jacquet and Riquier (*7*) used *B. stearothermophilus* var. *calidolactis* as the test organism in a disc assay. The latter workers indicated that this test could detect 0.0025 unit penicillin/ml. They found the method lacking in reproductibility when used for quantitative assay and suggested that it would be practical only if rigidly standardized. Forschner (*4*), on the other hand, showed this organism to be sensitive to 0.004 unit penicillin/ml in a disc assay when incubated 3 h at 70 C and used it successfully to quantitate penicillin. Picmanova et al. (*13*) also obtained good results with this variety of *B. stearothermophilus*. Romond et al. (*15*) indicated that at least one source of potential error could arise from the presence of antibiotic producing bacilli in milk, especially if incubation was extended beyond 2 1/2 h.

Van Os et al. (*17*) described a variation of the *B. stearothermophilus* var. *calidolactis* method. They used ampules containing agar seeded with spores, which, when nutrients were added, allowed germination and outgrowth of the organism with acid production showing up as an indicator change from purple to yellow. When antibiotics were present the indicator remained purple. Gist-Brocades, Delft-Holland, developed this method commercially as Delvotest P. Packard et al. (*12*) compared the *B. subtilis* disc assay (*1*) with the Delvotest P method and detected more positive raw milk samples with the latter.

The study reported here was a collaborative test to evaluate the reproducibility and sensitivity of the Delvotest P method for detection of penicillin in milk.

## MATERIALS AND METHODS

### *Samples and analysts*

Five antibiotic-contaminated [(determined by the FDA using the *Sarcina lutea* assay (*11*)] non-fat dry milk samples were obtained by the test coordinator (CNH) from supplies seized by the USDA, together with two samples free of antibiotics. The contaminated powders were diluted 1/2 (1:1) and 1/4 (1:3) with the antibiotic-free powder. The original, nondiluted powders gave a positive (+) reaction in the laboratory of the coordinator, while 1/2 dilutions gave questionable (±) reactions, and 1/4 dilutions gave negative (–) reactions.

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Artificially contaminated powders were prepared by mixing finely ground procaine penicillin G, tetracycline • HCl, or neomycin sulfate with control milk powder. The antibiotic level of the most concentrated powder was adjusted to that claimed to be within the sensitivity of the Delvotest P method and dilutions of 1/2 and 1/4 were made. The three actual concentrations of penicillin, as determined by the *S. lutea* assay for the three preparations, were 0.0040, 0.0020, and 0.0015 unit/ml on a reconstituted basis. Tetracycline concentrations were determined by the method of Kramer et al. (9) on dry milk powders containing approximately 520 mcg/g. These were diluted with antibiotic-free powder to give reactions of +, ±, and − when diluted 1/11 with water; the actual assayed values were, respectively, 0.192, 0.089, and 0.058 mcg/ml on a reconsituted basis. Neomycin in the same final dilutions gave +, ±, or − reactions, and, when assayed by a cylinder cup method (Oostendorp, personal communication) contained respectively, 26.5, 19.0, and 9.0 mcg/ml on a reconstituted basis.

The original and two dilutions (1/2, 1/4) of the five contaminated powders, the artificially contaminated (penicillin, tetracycline, and neomycin) and control powders, together with Delvotest P kits and penicillinase solution (containing $30 \times 10^6$ Kershey units/ml, prepared by the FDA laboratories, Washington, D.C.) were sent to the 11 participating analysts in the states of Indiana, Kentucky, Louisiana, Minnesota, Missouri, Ohio, Oregon, Tennessee, Texas, and Wisconsin.

*Reconstitution and assay*

Analysts were instructed to thoroughly mix duplicate 1-g portions of powder with 10 ml of water, add 0.1 ml to the ampules, incubate 2 1/2 h at 64-66 C, and record the results. If the agar remained completely purple, a + reaction was to be recorded; if zones of yellow and purple were noted, the reaction would be ±; a completely yellow agar would indicate −. The remaining portions of the diluted powders were refrigerated; those showing a + reaction were tested within 2 days for penicillin by adding two drops penicillinase to room temperature-warmed samples; after 10 min the test was repeated. The samples showing a ± or a − reaction in the initial test were reassayed using 1 g of powders and 3 ml of water (1/4 dilutions).

## RESULTS

*Types of responses obtained*

The numbers of +,±, and − responses obtained for each powder are in Table 1. The numbers of + responses of a total of 40 varied from five (analysts 7 and 8) to 16 (analyst 2) and the ± responses varied from three (analyst 2) to 18 (analyst 7). Negative results varied from 11 for analyst 11 to 28 for analyst 8. The two control powders, M and Z, gave no + and only one ± response. Those indicated as having a trace of antibiotics by the *S. lutea* assay (these were dilutions of powders known to be contaminated) gave four + responses, all from analyst 11, and 17 ± responses. Sample C showed only four + responses by the analysts, although originally it gave a + response in the coordinating laboratory. All + samples (of those known to contain penicillin( were tested for penicillin by the addition of penicillinase and became −.

*Increase in sensitivity with 1/4 dilution*

Milk samples originally exhibiting ± or − responses, when reassayed at 1/4 concentrations, showed that of 40 samples for which − responses were originally obtained, 16 (40%) remained −, 16 became ± and eight became +. Of 34 originally showing ±, in tests repeated at 1/4 levels, seven (21%) remained ± and 27 (79%) became +. The three samples showing a trace of antibiotic (samples P, R, and U) in the *S. lutea* assay gave an average of 96% + or ± responses in the 1/4 dilution but only 32% for the 1/11 dilutions. The + responses were 59% for samples diluted 1/4 as compared to 6% for samples diluted 1/11. The antibiotic-free powders, M and Z, showed no

TABLE 1. *Responses to penicillin-contaminated and control milk powders (1/11 dilution) using the Delvotest-P method*

| Sample[a] | Penicillin (unit/ml) | Original Test[b] | Analyst 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | Total + | ± | − |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C | 0.0040 | + | − | + | + | − | − | ± | ± | − | − | ± | + | 4 | 6 | 12 |
| B | 0.0020 | ± | − | ± | + | + | − | − | ± | − | − | ± | − | 3 | 4 | 15 |
| A | 0.0015 | − | − | − | − | − | − | − | − | ± | − | − | − | 0 | 1 | 21 |
| J | 0.0070 | + | + | + | + | + | + | + | + | + | ± | + | + | 19 | 3 | 0 |
| K | neg[c] | ± | ± | − | ± | ± | + | ± | ± | ± | ± | ± | ± | 3 | 18 | 1 |
| L | neg | − | − | − | − | ± | ± | − | − | − | ± | ± | − | 0 | 8 | 14 |
| N | 0.0086[d] | + | + | + | + | + | + | + | + | + | ± | + | + | 21 | 1 | 0 |
| O | 0.0052 | ± | ± | + | + | ± | ± | ± | + | + | − | ± | + | 10 | 9 | 3 |
| P | trace[d] | − | − | − | − | − | − | ± | ± | − | − | − | ± | 1 | 3 | 18 |
| Q | 0.0058 | + | + | ± | + | + | ± | ± | + | + | ± | ± | + | 9 | 12 | 1 |
| R | trace | ± | − | − | − | − | − | − | ± | ± | ± | − | ± | 1 | 7 | 14 |
| S | neg | − | − | ± | − | − | − | − | − | − | − | − | ± | 0 | 3 | 19 |
| T | 0.0086 | + | + | + | + | + | ± | ± | + | + | ± | ± | + | 14 | 7 | 1 |
| U | trace | ± | − | ± | − | − | − | − | ± | ± | ± | − | + | 2 | 7 | 13 |
| V | neg | − | − | − | − | − | − | − | − | − | − | − | + | 2 | 0 | 20 |
| W | 0.0084 | + | + | + | + | + | + | + | + | + | + | ± | + | 20 | 2 | 0 |
| X | 0.0040 | ± | ± | − | ± | ± | − | ± | ± | ± | − | ± | − | 0 | 15 | 7 |
| Y | neg | − | − | − | − | − | − | − | ± | − | − | − | ± | 0 | 4 | 18 |
| M | neg | − | − | − | − | − | − | − | − | − | − | − | − | 0 | 0 | 22 |
| Z | neg | − | − | − | − | − | − | − | − | − | − | − | − | 0 | 1 | 21 |
| | | + | 11 | 16 | 8 | 7 | 8 | 14 | 5 | 5 | 13 | 7 | 15 | 109 | | |
| | TOTALS | ± | 6 | 3 | 10 | 11 | 8 | 10 | 18 | 7 | 13 | 11 | 14 | 111 | | |
| | | − | 23 | 21 | 22 | 22 | 24 | 16 | 17 | 28 | 14 | 22 | 11 | 220 | | |

[a]Grouped samples are dilutions of same artificially or naturally contaminated powders.
[b]Determined in coordinator's laboratory before samples sent to analysts.
[c]No zone in *S. lutea* assay.
[d]Zone in *S. lutea* assay but not in range of tests.

increase in + or ± responses. Samples K, L, S, V, and Y, which were contaminated but gave no zones of inhibition in the *S. lutea* assay, gave 16.3% and 60.8% + or ± responses when diluted 1/11 or 1/4, respectively.

*Correlation of response with penicillin concentration*

Responses of analysts, arranged in order of increasing penicillin concentration (Table 2), showed an apparent correlation with antibiotic levels of the milk. At concentrations of 0.0084 unit/ml or greater almost 100% of the responses were either + or ± (84% were +). Samples with lower content of penicillin gave fewer + results. The ± results were most numerous in the intermediate penicillin (0.004 to 0.0058 unit/ml) samples.

TABLE 2. *Effect of penicillin concentration on the percentage of + or + and ± responses in milk powders diluted 1/11*

| Sample | Penicillin[a] unit/ml | Reponses: Number + | Number ± | Number − | Percent total + | Percent total +and ± |
|---|---|---|---|---|---|---|
| MZ | neg[b] | 0 | 1 | 43 | 0 | 2.3 |
| KLSVY | neg | 2 | 16 | 92 | 1.8 | 16.3 |
| P | trace[c] | 1 | 3 | 18 | 4.5 | 18.2 |
| R | trace[c] | 1 | 7 | 14 | 4.5 | 36.4 |
| U | trace[c] | 2 | 7 | 13 | 9.1 | 40.9 |
| A | 0.0015 | 0 | 1 | 21 | 0 | 4.5 |
| B | 0.0020 | 3 | 4 | 15 | 13.6 | 31.8 |
| C | 0.0040 | 4 | 6 | 12 | 18.2 | 45.4 |
| X | 0.0040 | 0 | 15 | 7 | 0 | 68.2 |
| O | 0.0052 | 10 | 9 | 3 | 45.4 | 86.3 |
| Q | 0.0058 | 9 | 12 | 1 | 40.9 | 95.4 |
| J | 0.0070 | 19 | 3 | 0 | 86.4 | 100.00 |
| W | 0.0084 | 20 | 2 | 0 | 90.9 | 100.00 |
| N | 0.0086 | 21 | 1 | 0 | 95.4 | 100.00 |
| T | 0.0086 | 14 | 7 | 1 | 63.6 | 95.4 |

[a]Samples A, B, C, and J, assayed by *B. stearothermophilus* var. *calidolactis* method (Gist-Brocades); others by *S. lutea* (FDA). These were assayed on a dry basis and the final concentrations obtained by calculation.

[b]No zone with *S. lutea;* probably less than 0.002 unit/ml.

[c]Zone with *S. lutea* but not assayable; probably between 0.002 and 0.004 unit/ml.

Samples other than A, B, C, and J of Table 2 were assayed by FDA using the *S. lutea* cylinder cup method on 1/4 dilutions of milk powder. The *S. lutea* method was not sensitive enough for A, B, C, or J samples. These, however, were assayed by Gist-Brocades using a cylinder cup method and spores of *B. stearothermophilus* var. *calidolactis.* The other samples were also assayed by Gist-Brocades, with the following results expressed as 1/11 water dilutions of the powders in units/ml penicillin: samples K (0.0028), L (0.0021), N (0.0056), O (0.0052), P (0.0030), Q (0.0050), R (0.0030), S (0.0020), T (0.0035), U (0.0021), V (0.0012), W (0.0053), X (0.0023), and Y (0.0013). The FDA *S. lutea* method (Table 2) showed no zone in K, L, S, V, or Y and a small zone beyond the range of the test for P, R, and U. The greatest discrepancy was in sample T with the FDA method showing more than twice the pencillin concentration of the Gist-Brocades test.

*Regression lines of type of response on penicillin concentration*

Prediction equations in the range of about 0.001 to 0.01 unit/ml for determining the probablility of obtaining a predetermined percentage of + or + and ± responses from various penicillin levels were derived using the data from Table 2 (from FDA and Gist-Brocades). The correlation coefficient (r) for both + and + or ± responses was 0.91 with 95% confidence limits of 0.82 to 0.95. The regression equation from the FDA data for determining penicillin concentration (X) needed to give a predetermined probability of desired + responses ($Y_1$) was

$$Y_1 = -25.2 + 12823.9\,X$$

and the equation for + and ± responses ($Y_2$) was

$$Y_2 = 7.69 + 11803.5\,X$$

By setting $Y_1 = 0.95$ (95% probability of obtaining only + responses), the corresponding penicillin level would need to be 0.0094 unit/ml; the same probability for $Y_2$ would require a penicillin concentration of 0.0074 unit/ml. Likewise for $Y_1 = 1.00$, X would be 0.0098 unit/ml, and for $Y_2 = 1.00$, X would be 0.0078 unit/ml.

Similar equations using the more sensitive methods of Gist-Brocades for determining pencillin levels gave a 95% probability value for $Y_1$ of 0.0072 unit/ml (the equation was $Y_1 = 2.943 + 17406.4\,X$, with $r = 0.87$ and confidence limits 0.67 and 0.95) and a value for $Y_2$ of 0.0056 unit/ml (the equation was $Y_2 = -1.55 + 17374.6\,X$, with $r = 0.81$ and confidence limits of 0.55 and 0.92).

*Other antibiotics*

In the 22 tests conducted, the three concentrations of tetracycline, 0.192, 0.089, and 0.058 mcg/ml, respectively gave + responses of 16, 0, 0; ± responses of 2, 2, o; and − responses of 4, 20, 22. The neomycin concentrations of 26.5, 19.0 and 9.0 mcg/ml, respectively, gave + responses of 20, 14, and 8; ± responses of 0, 7, 13; and − responses of 2, 1, 1.

## DISCUSSION

There are two areas in which antibiotic residue testing is important in milk. One is for incoming bulk tank raw milk where a simple, rapid, and sensitive test is necessary; the other is for dry milk where the method need not be as rapid but should still be simple and sensitive. The *S. lutea* test does not meet these criteria; it requires skilled personnel, is laborious and time-consuming, and requires considerable laboratory equipment. The *B. subtilis* disc assay, especially the rapid modification (*20*), is fairly simple but less sensitive than other tests. The *B. stearothermophilus* and *B. stearothermophilus* var. *calidolactis* tests appear better suited for qualitative field testing of milk. The Delvotest P modification (other variations could easily be developed by interested laboratories) is particularly simple, rapid, and sensitive.

The relative claimed sensitivities of these methods are 0.01 unit/ml penicillin for the *S. lutea* method (*11*); 0.05 for the *B. subtilis* disc method (*19, 20*); and 0.004 for the *B. stearothermophilus* var. *calidolactis* disc method as

reported by Forschner (*4*) and Van Os et al. (*17*). The latter workers claimed that samples with a concentration of 0.004 unit/ml gave 100% positive results. Similar results were obtained by one of us (CNH), probably because of familiarity with the technique. The study reported here was undertaken by analysts who were unfamiliar with the method; even they, however, reported a large proportion (83%) of + or ± responses (29% +). The concentration of penicillin which would produce + results 95% of the time (0.0072 and 0.0094 unit/ml from the regression equations using FDA data) indicated that the sensitivity of the Delvotest P method is greater than that of the *B. subtilis* disc method and approximates that of the *S. lutea* method. Reconstitution of dry milk powders to 1/4 instead of 1/11 would increase sensitivity.

Van Os et al. (*17*) indicated that the *B. stearothermophilus* var. *calidolactis* method could detect 0.40 mcg tetracycline/ml of milk. Our results on 22 tests, showing 16 + responses at the 0.192 mcg/ml level, support this estimate of sensitivity. Van Os et al. also indicated that neomycin gave 100% + responses at 22 mcg/ml. Our results were similar: 20 + responses of 22 tests at 26.5 mcg/ml. Lower concentrations under our conditions gave more ± rather than − responses. This type of response, also noted during the initial preparation of the neomycin powders in the coordinating laboratory, is probably due to slower diffusion but may also be due to pH effects as indicated by van Os et al. (*17*).

A problem with the Delvotest P method is interpretation of the ± reaction. Our results tend to indicate that the ± reaction should be considered +, since a large proportion of the ± responses became + in tests of the more concentrated 1/4 dilution. However, it is not practical to concentrate fluid milk under ordinary conditions. Laboratories experiencing uncertainty over the interpretation of the ± reactions in raw or dried milk dilutions could resolve this in another way by further diluting by 1/2 or 1/4; the reaction should then revert to −. If the reaction after dilution is still ±, then low levels of neomycin or other antibiotics might be present. The penicillinase test would verify presence of penicillin.

The present practice of assaying dry milk powders at a 1/4 dilution will give greater sensitivity as we demonstrated; however, for enforcement purposes, this practice is of doubtful value since the same sensitivity could not be attained in the parent raw milk. Dry milk should be assayed at 1/11 dilution, approximating that of raw milk.

Adoption of a method such as the Delvotest P modification of *B. stearothermophilus* var. *calidolactis* test should be considered only after all ramifications are considered. For instance, the greater sensitivity would undoubtedly mean that more milk would be condemned. What would be the impact on the producer or the distributor? The cost of this test would also be considerable (at present about 56 cents per test) if used on all farm bulk tanks. Perhaps this test should be used only for tank car lots of commingled milk where the greater sensitivity would be an advantage; while the less sensitive, cheaper, equally-as-rapid *B. subtilis* disc method could be used, as it now is, for farm bulk tanks. The *S. lutea* test appears to have no advantages over the *B. stearothermophilus* var. *calidolactis* test and has several disadvantages including extra time required, unstable bacterial culture, and the necessity for trained personnel. The ideal solution would be a disc method with *B. stearothermophilus,* similar to the *B. subtilis* method. This is now under consideration and is being studied in several laboratories.

## CONCLUSIONS

The *Bacillus stearothermophilus* var. *calidolactis* test (Delvotest P) is a good field technique for detecting antibiotics in raw milk or dry milk powder. Regression equations indicated that milk containing about 0.008 to 0.010 unit of penicillin/ml would be detected 95% of the time by untrained analysts.

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# Production of Tyrosine and Histidine Decarboxylase by Dairy-Related Bacteria

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ABSTRACT

Manometric and radiometric procedures were used to determine the ability of 38 dairy-related bacteria and four commercial starter preparations to produce tyrosine and histidine decarboxylase. All of the cultures had slight ability to cause release of $^{14}CO_2$ from carboxyl $^{14}C$-tyrosine and most released $^{14}CO_2$ from labeled histidine; however, because of inherent errors of the assay in detecting low levels of specific decarboxylase activity, the $CO_2$ release was not considered positive for specific decarboxylase activity unless the results were verified by the manometric technique. One strain of *Streptococcus lactis*, a *Micrococcus luteus* strain, and two *Leuconostoc cremoris* strains had active tyrosine decarboxylase systems. Only *Clostridium perfringens* and an *Escherichia coli* strain were found to produce histidine decarboxylase. None of the commercial starter cultures produced the enzymes. It was not determined whether tyramine or histamine was produced by the nonspecific decarboxylase activity.

Undesirable physiological reactions have been reported following ingestion of foods containing biologically active amines (*1, 7, 19, 27*). Rice et al. (*30*) discussed the roles of drug inducement and genetic tendencies in triggering these reactions.

Amines in food products are produced by specific amino acid decarboxylases, and amine build-up is the result of the growth of decarboxylase-positive bacteria under conditions favorable to enzyme synthesis and activity (*10, 25, 26, 29, 33*). Koessler et al. (*21*) postulated that decarboxylation may be a protective mechanism for bacteria against an acid environment. That is, when the hydrogen ion concentration becomes sufficient to threaten the life of the microorganism, a decarboxylase may be synthesized. In 1946, Gale (*12*) summarized several conditions that must be considered to optimize the synthesis of bacterial decarboxylases. These conditions included most of the following: (*a*) the organism must have the genetic potential to synthesize the enzyme; (*b*) the particular substrate amino acid needs to be supplied in the growth medium, as well as pyridoxine and nicotinic acid, except for histidine and glutamate decarboxylase (*2, 3, 14, 28*); (*c*) the growth medium must be acid, usually obtained by supplying a fermentable carbohydrate to the medium (*9, 17, 22, 25*); (*d*) the medium should be unbuffered (*3*); (*e*) the growth temperature should be $\leqslant$27 C; and (*f*) the culture should be harvested at either two-thirds maximal growth or at the end of active cell division (*3*). Acetone drying of bacteria cells is the standard method used to prepare bacterial decarboxylases for biochemical studies, and with the exception of ornithine decarboxylase, activities are not affected by the procedure (*12, 33*).

A survey for tyrosine decarboxylase activity in bacteria by Gale (*12*) included 800 streptococci; of these, approximately 500 synthesized the enzyme. Most of the 500 belonged to Lancefield's Group D and one culture was *Streptococcus lactis*. Koessler et al. (*21*) found five streptococci that produced tyrosine decarboxylase which included *S. lactis* from milk with the activity. Various workers have reported the enzyme to be present in *Escherichia coli* (*4, 11, 16*). Gale found tyrosine decarboxylase activity in nine of 100 cultures of this species. Lagerborg and Clapper (*22*) surveyed 33 lactobacilli and found one strain related to *Lactobacillus casei* or *Lactobacillus arabinosus* that released carbon dioxide from tyrosine. They found an unknown *Lactobacillus* species with the same activity, and another *Lactobacillus* species that decarboxylated both tyrosine and histidine, a most unusual find, since this is the only culture reported in the literature to possess both activities. Umezu (*34*) identified a tyrosine decarboxylase in a strain of *Leuconostoc mesenteroides* var. *Sake*. Blackwell and Mabbitt (*4*), after noting the presence of Group D streptococci (10,000 colony forming units/g of cheese) and *E. coli* in ripened cheese, concluded that these bacteria were the most likely source of the decarboxylase enzymes responsible for formation of tyramine in cheese. Recently, Rice and Koehler (*31*) surveyed several pediococci and lactobacilli common to sausage starters and found the bacteria lacking in tyrosine and histidine decarboxylase. A *Streptococcus* species isolated by these authors was an active tyrosine-decarboxylating organism.

Histidine decarboxylase is widely distributed in the genera *Escherichia, Salmonella, Clostridium, Bacillus,* and *Lactobacillus* (*20, 21, 27*). Evidence for the enzyme in *E. coli* include the finding by Gale (*12*) that 14 of 155 cultures possessed the enzyme and the report by Hanke

and Koessler (*16*) that six of 20 coliform cultures synthesized the enzyme. Havelka (*17*) noted that 71.4% of all *Enterobacteriacae* isolated from the imported marine fish produced histamine. Eggerth (*8*) found 40 strains of bacteria of intestinal origin capable of decarboxylating histidine. Histidine decarboxylase activity was found by Rodwell (*32*) in strains of *Lactobacillus pentoaceticus* and *Lactobacillus bifidus* as well and in *Lactobacillus* 30a and *Lactobacillus* 60. However, the number of dairy-type lactobacilli found to contain the enzyme has been small. Horakova et al. (*18*) isolated a *Lactobacillus* thought to to related to *Lactobacillus acidophilus* that decarboxylated histidine. In 1968, DeKoning (*7*) reported that a *Lactobacillus* was responsible for formation of toxic amounts of histamine in a Gouda Cheese. The author indicated that the lactobacilli were an unusual contaminant originating in the rennet.

The objective of the present study was to survey dairy starter cultures and dairy-related microflora for tyrosine and histidine decarobxylase activity. The study was conducted because bacterial decarboxylase studies have not included a variety of dairy-type bacteria. It was intended that the information would indicate the potential of dairy starters to contribute to tyramine and histamine build-up.

## MATERIALS AND METHODS

The 38 dairy-type cultures and the four starter cultures were obtained from Miles Laboratories, Chr. Hansens's Laboratory, Kraft Foods, and the Universities of Nebraska, Wisconsin, North Carolina State, and Georgia. Cultures were maintained in broth containing 1% of the following in distilled water: tryptone, yeast extract, and dextrose (TYEB). In addition, the TYEB contained 100 mg of tyrosine and histidine/liter. Cultures were activated at 27 C by repeated transfer in skim milk or TYEB allowing 24 h between each transfer. Cells grown in TYEB were harvested after 24 h of growth at 27 C. The decarboxylase assays were carried out on the preparations from the cultures grown in 10% skim milk and in the TYEB medium. In addition, assays were carried out on acetone-dried cells. *Clostridium perfringens* was carried and activated in an anerobic broth containing 29.8 g of fluid thioglycolate, 5 g of egg meat (Difco) and 14.5 of dextrose per liter, with mineral oil overlays.

Tyrosine and histidine decarboxylase activities were determined by an isotopic assay method adapted from the procedure of Levine and Watts (*23*) and by a manometric method. The isotopic procedure consisted of incubating 1.0 ml of cultured skim milk with 1.9 ml of 1 M sodium acetate buffer containing $2.5 \times 10^{-4}$ M tyrosine or histidine, pH 5.5, and 100 µl (0.125 µCi) of $^{14}$C-carboxyl tyrosine or histidine (CalAtomic, Los Angeles) at 27 C for 60 min in a reaction vessel (a polyethylene scintillation vial). All buffers contained $1.05 \times 10^{-4}$ M streptomycin sulfate to inhibit bacterial growth. All reactions were carried out in duplicate. Following incubation, 2 ml of 1.2 N perchloric acid was injected into the flasks to stop the reactions and to liberate dissolved $^{14}CO_2$. The liberated $^{14}CO_2$ was trapped on filter paper (3 MM Whatman, 1 × 3 cm rolled cylinders) containing hyamine hydroxide (New England Nuclear, Boston). The filter paper trap was suspended over the reaction solution by a paper clip inserted into a No. 2 rubber stopper. After shaking for 30 min in a Dubnoff Metabolic Shaker (Burrell Corp., Pittsburgh) to insure complete liberation and entrapment of dissolved $^{14}CO_2$, the filter paper was transferred to a scintillation vial containing 10 ml of a toluene scintillation fluor (4 g of 2,5-diphenyloxazola (PPO), 100 mg of P-bis-[2-(5-phenyloxazoyl)] benzene (POPOP), and 1000 ml toluene, all Beckman, Fullerton, California) and then counted in a Beckman Model LS-100 C Liquid Scintillation Counter. Controls for background correction consisted of reaction mixtures containing heat inacitvated culture media or acetone-dried cells. Reaction rates were reported as nMoles $CO_2$ released/min/mg of acetone dried cells, nMoles $CO_2$ released/min/ml of skim milk culture, or nMoles $CO_2$ released/min/ml of sonicated extract.

Manometric assays were carried out on a Gilson Differential Respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.). Enzyme preparation for the manometric assay consisted of acetone-dried cells grown in TYEB and resuspended in 0.5 M sodium acetate, pH 5.5. One milliliter of the acetate buffer and 1 ml of the cell suspension were added to the main compartment of the Gilson flask. One side arm of the flask contained 0.5 ml of substrate (0.5 M tyrosine or histidine in 0.5 M sodium acetate buffer, pH 5.5). Reaction components were allowed to equilibrate for 10 min at 27 C before the substrate was tipped in. Release of $CO_2$ were monitored for 60 min and results were reported as nMoles $CO_2$ released/min/mg of cells.

## RESULTS AND DISCUSSION

Data in Tables 1-3 show the ability of the various bacteria to decarboxylate tyrosine and histidine. The radiometric assay was used as a rapid method to screen bacterial cultures for decarboxylase activity. Skim milk cultures of all bacteria were checked by this method for decarboxylase activity. Other cell preparations, including

TABLE 1. *Release of $CO_2$ from tyrosine and histidine by* Lactobacillus *cultures as detected by isotopic and manometric assays*

| | Tyrosine | | | | Histidine | | | |
|---|---|---|---|---|---|---|---|---|
| | Isotopic | | | | Isotopic | | | |
| Culture | Skim milk culture[a] | Sonicated extract[b] | Acetone dried[c] | Manometric[c] | Skim milk culture[a] | Sonicated extract[b] | Acetone dried[c] | Manometric[c] |
| *L. acidophilus* | 1.0 | 0 | 0 | 0 | 2.5 | 0.01 | 0.04 | 0 |
| *L. acidophilus* | 0.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. bulgaricus* | 1.7 | 0 | 0 | 0 | 1.1 | 0 | 0 | 0 |
| *L. bulgaricus* | 0.8 | —[d] | — | — | 0.05 | — | — | — |
| *L. bulgaricus* | 0.3 | — | — | — | 0.15 | — | — | — |
| *L. bulgaricus* | 0.8 | — | — | — | 0 | — | — | — |
| *L. casei* | 0.9 | 0.04 | 0 | 0 | 1.5 | 0.09 | 0.08 | 0 |
| *L. casei* | 0.6 | 0 | 0 | 0 | 0.15 | 0.01 | 0 | 0 |
| *L. plantarum* | 0.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. plantarum* | 0.5 | 0 | 0 | 0 | 0.05 | 0 | 0 | 0 |
| *L. lactis* | 1.0 | — | — | — | 0.07 | — | — | — |

[a] nMoles $CO_2$ released/min/ml of skim milk culture.

[b] nMoles $CO_2$ released/min/ml of extract.

[c] nMoles $CO_2$ released/min/mg of acetone dried cells. Cells were grown in TYEM.

[d] Dash indicates activity was not determined.

sonicated cell extracts from TYEB and acetone-dried cells of bacteria grown in TYEB were assayed radiometrically. Because the radiometric method has been criticized by several researchers when used to assay low levels of decarboxylase activity (*13, 15, 24*), a manometric assay was also completed. The major criticism of the radiometric method concerns its sensitivity which permits detection of nonspecific release of $^{14}CO_2$ which can result in erroneous reporting of specific decarboxylase activity.

As indicated in Table 1, when the *Lactobacillus* cultures were assayed radiometrically directly from the skim milk media, most showed some capacity to cause $CO_2$ release from both tyrosine and histidine. This is also true for the other cultures shown in the remaining table. This low activity is probably due to non-specific $^{14}CO_2$ release. However, when the sonicated cell extracts or the acetone-dried cells were assayed radiometrically, no or greatly reduced $^{14}CO_2$ release was noted. The manometric assay showed the cultures to lack both tyrosine and histidine decarboxylase activity. All of the skim milk cultures of the other bacteria surveyed also showed at least slight ability to cause $CO_2$ release from tyrosine and histidine as detected radiometrically. Apparently the actively metabolizing skim milk cultures can promote such $CO_2$ release through mechanisms other than specific decarboxylation. Consequently, only those bacteria that showed positive results for $CO_2$ release from each of the cell preparations when assayed radiometrically and manometrically were considered as positive for specific decarboxylase activity.

Of the 20 *Streptococcus* cultures surveyed (Table 2), only one *S. lactis* strain and the *Streptococcus faecalis* var. *liquefaciens* strain possessed tyrosine decarboxylase. The ability of the *S. lactis* strain to decarboxylate tyrosine is atypical since strains of this species are described by *Bergey's Manual of Determinative Bacteriology* (*6*) as lacking the ability to synthesize tyrosine decarboxylase. However, other workers, including Gale (*12*) and Koessler et al. (*21*), have reported *S. lactis* strains that produced the enzyme. Another atypical feature apparent from the data (referring again to Bergey's classification) was the lack of tyrosine decarboxylase production by the two *S. faecalis* cultures (strains of this species provide the standard bacterial source of this enzyme). It is apparent from these data as well as from earlier studies by Gale (*11*) that all strains of *S. faecalis* do not decarboxylate tyrosine.

Histidine decarboxylase activity was absent in all of the streptococci. This was not surprising since tyrosine decarboxylase is the only decarboxylase normally produced by *Streptococcus* species (*12*).

Of other bacteria studied (Table 3), two strains of *Leuconostoc cremoris* and a *Micrococcus luteus* strain produced tyrosine decarboxylase. When assayed manometrically, one of the *L. cremoris* strains possessed the highest activity found in this study (287 nMoles $CO_2$/min/mg of actone dried cells). It must be emphasized, however, that optimal enzyme parameters were not used for each of the cultures and the specific activities can only be used as a means to identify those bacteria capable of synthesizing the decarboxylases. The data also indicate that most of the tyrosine decarboxylase-positive bacteria showed greater specific activities when assayed manometrically than when assayed by the radiometric procedure. This results from the low substrate concentration ($2.5 \times 10^{-4}$M used in the radiometric procedure compared to the saturating level

TABLE 2. *Release of $CO_2$ from tyrosine and histidine by* Streptococcus *cultures as detected by isotopic and manometric assays*

| | Tyrosine | | | | Histidine | | | |
|---|---|---|---|---|---|---|---|---|
| | Isotopic | | | | Isotopic | | | |
| Culture | Skim milk culture[a] | Sonicated extract[b] | Acetone dried[c] | Manometric[c] | Skim milk culture[a] | Sonicated extract[b] | Acetone dried[c] | Manometric[c] |
| *S. thermophilus* | 0.9 | 0 | 0 | 0 | 0.3 | 0 | 0 | 0 |
| *S. thermophilus* | 0.5 | —[d] | — | — | 0 | — | — | — |
| *S. thermophilus* | 0.5 | — | — | — | 0 | — | — | — |
| *S. thermophilus* | 0.5 | — | — | — | 0.15 | — | — | — |
| *S. thermophilus* | 0.5 | — | — | — | 0 | — | — | — |
| *S. lactis* | 0.8 | 0.01 | 0 | 0 | 0.4 | 0.01 | 0 | 0 |
| *S. lactis* | 0.5 | — | — | — | 0.05 | — | — | — |
| *S. lactis* | 1.1 | — | — | — | 0.45 | — | — | — |
| *S. lactis* | 0.8 | — | — | — | 0.15 | — | — | — |
| *S. lactis* | 1.0 | 0.07 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. lactis* | 12.6 | 7.8 | 13.3 | 76.2 | 0 | 0 | 0 | 0 |
| *S. lactis* | 0.2 | 0.03 | 0.02 | 0 | 0 | 0 | 0 | 0 |
| *S. faecalis* | 0.9 | 0 | 0 | — | 0.05 | 0 | 0 | 0 |
| *S. faecalis* | 0.4 | 0 | 0.01 | 0 | 0 | 0 | 0 | 0 |
| *S. liquefaciens* | 31.0 | 10.2 | 11.2 | 38.3 | 0.05 | 0 | 0 | 0 |
| *S. cremoris* | 1.2 | 0 | 0.01 | 0 | 0.15 | 0.01 | 0 | 0 |
| *S. cremoris* | 1.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. cremoris* | 1.0 | — | — | — | 0 | — | — | — |
| *S. cremoris* | 0.7 | — | — | — | 0.04 | — | — | — |
| *S. diacetylactis* | 0.7 | — | — | — | 0.26 | — | — | — |

[a] nMoles $CO_2$ released/min/ml of skim milk culture.
[b] nMoles $CO_2$ released/min/ml of extract.
[c] nMoles $CO_2$ released/min/mg of acetone dried cells. Cells were grown in TYEM.
[d] Dash indicates activity was not determined.

(0.5 M tyrosine) of substrate used in the manometric method. The Michaelis constant (Km) for *S. faecalis* tyrosine decarboxylase is known to be above the solubility limits of L-tyrosine (*5*). The low substrate concentration in the radiometric method was originally chosen by Levine and Watts (*23*) because it provided for suitable $^{14}CO_2$ release and minimized isotope requirements. Histidine decarboxylase activity was only found in *E. coli* and *C. perfringens* cultures (strains of these species provide the standard bacterial sources for this (enzyme). Histidine decarboxylase is known to occur in most strains of *C. perfringens* and has been frequently reported in *E. coli* strains (*12, 21, 27*. None of the commercial starters produced specific tyrosine or histidine decarboxylases when grown in skim milk or TYEB.

In conclusion, most bacteria used in starter cultures would not possess tyrosine or histidine decarboxylase activity. However, the few instances in which the enzyme might occur (expecially when the starter culture employs *L. cremoris*) should lead commercial starter manufacturers to survey their bacterial strains for these enzymes and eliminate any that have tyrosine or histidine decarboxylase activity. Further research is needed to determine whether tyramine or histamine are formed by the nonspecific activity. Such activity during the aging periods of fermented products could possibly lead to amine build-up. While radioisotope procedures offer a simple and rapid enzyme assay, results obtained with actively growing cultures or at low enzyme concentrations must be viewed with caution.

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TABLE 3. *Release of $CO_2$ from tyrosine and histidine by various bacteria and starter cultures as detected by isotopic and manometric assays*

| | Tyrosine | | | | Histidine | | | |
|---|---|---|---|---|---|---|---|---|
| | Isotopic | | | | Isotopic | | | |
| Culture | Skim milk culture[a] | Sonicated extract[b] | Acetone dried[c] | Manometric[c] | Skim milk culture[a] | Sonicated extract[b] | Acetone dried[c] | Manometric[c] |
| *L. cremoris* | 19.4 | 17.2 | 6.9 | 287 | 0 | 0 | 0 | 0 |
| *L. cremoris* | 6.8 | 3.8 | 6.3 | 42.0 | 0 | 0 | 0 | 0 |
| *L. cremoris* | 0.4 | —[d] | — | — | 0 | — | — | — |
| *L cremoris* | 0.5 | — | — | — | 0 | — | — | — |
| *L. dextranicum* | 0.8 | — | — | — | 0.15 | — | — | — |
| *L. mesenteriodes* | 0.6 | 0 | 0 | 0 | 0.25 | 0 | 0 | 0 |
| *P. shermanii* | 0.8 | 0 | 0.3 | 0 | 0 | 0 | 0 | 0 |
| *M. roseus* | 0.6 | 0 | 0 | 0 | 0.65 | 0 | 0 | 0 |
| *M. luteus* | 3.7 | 3.7 | 14.1 | 6.1 | 0 | 0 | 0 | 0 |
| *M. lysodeikicus* | 1.0 | 0 | 0 | 0 | 0.05 | 0 | 0 | 0 |
| *M. lacticum* | 0.5 | 0.04 | 0 | 0 | 0.05 | 0 | 0 | 0 |
| *E. coli* | 0.3 | 0 | 0 | 0 | 0.25 | 5.6 | — | 36 |
| *C. perfringens* | 0.4 | 0 | 0 | 0 | 11.2 | 5.6 | 0.7 | 54 |
| Klenzade #11 | 0.6 | — | 0.01 | — | 0 | — | 0.04 | — |
| Hansen #44 | 0.5 | — | 0.03 | — | 0.04 | — | 0.03 | — |
| Florida Dairies buttermilk | 0.8 | — | 0 | — | 0.8 | — | 0.1 | — |
| Florida Dairies cottage cheese | 0.4 | — | 0 | — | 0 | — | 0.04 | — |

[a]nMoles $CO_2$ released/min/ml of skim milk culture.
[b]nMoles $CO_2$ released/min/ml of extract.
[c]nMoles $CO_2$ released/min/mg of acetone dried cells. Cells were grown in TYEM.
[d]Dash indicates activity was not determined.

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# Experiments in Sanitizing Beef With Sodium Hypochlorite[1,2]

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**ABSTRACT**

Beef plate meat was sprayed with sodium hypochlorite (pH 6.0) from two sources, commercial and electroytically generated. Variables studied in two experiments were rate of flow of sanitizer, line pressure, speed of movement of meat through the sprays, and method and time of sanitization. Hypochlorite sprays reduced microbial counts significantly more than did water applied under the same conditions, but type of hypochlorite was unimportant. Maximum reductions in counts made immediately after sanitization approximated 97 and 93% as measured by swab and core sampling methods, respectively. Sprays were most effective when delivered in a single passage over meat at a rate of 2 rather than 10 cm/sec or in about seven successive passages at 10 cm/sec. Samples collected by coring and swabbing estimated microbial populations different from each other when the samples were taken after sanitized meat had been stored at 3 C for 48 h. Based on our findings we recommend the coring method.

We previously reported that sprays containing 200-250 mg of hypochlorite/liter reduced aerobic plate counts of micro-organisms up to 0.47 log (*2*) when samples of beef taken immediately after sanitization. Differences in log counts were smaller when samples were taken up to 48 h later.

Reductions in viable counts were disappointingly small in comparison with data reported by Emswiler et al. (*4*) and Kotula et al. (*6*) in which from 1.5 to 3 log reductions in numbers were observed depending on the experiment and time of sampling after sanitization.

In the experiment by Kotula et al. (*6*), meat was sanitized about five times as long as in our trials, electrolytically produced hypochlorite was used, the pH of the sanitizer was adjusted to 6.0, and samples were taken by swabbing. In a later experiment Emswiler et al. (*4*) showed that electrolytically produced sodium hypochlorite gave significantly lower counts than the same concentrations of calcium hypochlorite or chlorine dioxide. Because of these differences we did two experiments which are reported here.

## MATERIALS AND METHODS

*Experiment 1*

Sixty-four pieces of beef plate meat were obtained from a single source and stored frozen until used. Eight pieces were thawed and used in each of 8 days of testing. The surface of each piece was exposed to each other one to assure uniform distribution of contaminants.

Meat was placed on holding frames (*1*) and sanitized in the horizontal position with 200-250 mg of commercial sodium hypochlorite/liter at pH 6.0 (adjusted with acetic acid). Sanitizer was applied through nozzles 5001, 5002, 5004, and 5008 (Spraying Systems, Co., Wheaton, Il.). Conditions of application were sanitizer flow rates of 0.83 and 3.4 liters/min, line pressures of 3.5 and 14.0 kg/cm$^2$, and speeds of travel of meat past the nozzle of 2 and 10 cm/sec. Nozzle height above the meat was 40 cm. Immediately after sanitizing meat was drained for 60 sec in the vertical position. Samples were immediately taken and meat was stored uncovered at 3 C and a relative humidity of 86 ± 6%.

At each of the three sampling times each piece of meat was sampled by removal of four cores (2.54-cm diameter and 3-5-mm thickness) and by the swabbing of four adjacent areas (1 cm$^2$ each). Sampling times and locations are shown in Fig. 1. Cores were combined with 99 ml of

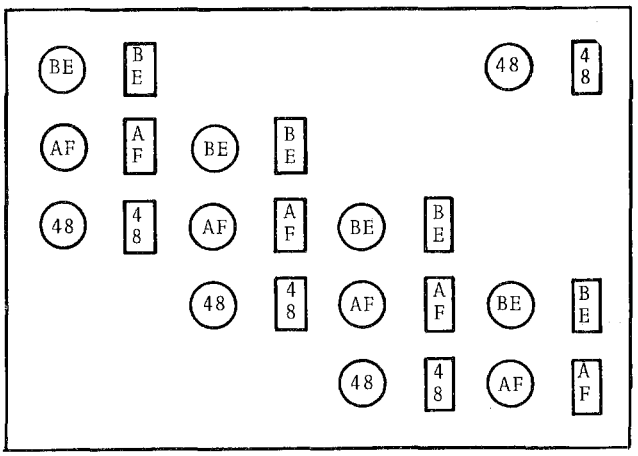


Figure 1. *Pattern of sampling by coring (circles-2.54 cm diameter, 3-5 mm thick) and swabbing (rectangles-1 cm$^2$) before (BE), immediately after (AF) and 48 h after (48) sanitizing.*

sterile phosphate buffered distilled water and blended at high speed for 60 sec in a Waring blendor. Swabs were rinsed in sterile buffered distilled water after they were rubbed three times over each template-outlined area. Samples were plated at appropriate dilutions, and counts were made according to *Standard Methods for the Examination of Dairy Products* (*5*) except that plates were incubated at 28 C for 72 ± 4 h.

[1]*Contribution from the University of Missouri Experiment Station, Journal Series No. 7511.*
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[3]*Department of Food Science and Nutrition.*
[4]*U.S. Department of Agriculture.*

The experiment was a randomized complete block design (2 × 2 × 2) with eight replications.

*Experiment 2*

Source and type of meat, sampling, plating, and counting procedures were the same as for Experiment 1.

The experiment was a randomized complete block design (3 × 3 × 3) with confounding of sanitizers, volume, and sanitizing time in the first, second, and third replications, respectively. In total, 81 pieces of plate meat were used. Nine pieces were thawed and placed on holding frames each day. The nine pieces were sanitized with commercailly available sodium hypochlorite (12%), electrolytically formed sodium hypochlorite or water control. The rate of application of hypochlorite was 200 to 250 mg/liter. Rates of flow of sanitizer, 1.7, 3.4, and 6.8 liters/min, were those delivered by nozzles No. 5002, 5004, and 5008 (Spraying Systems Co., Wheaton, Il.) at 14.0 kg/cm² pressure. Times of sanitizer application were 2, 15, and 30 sec per piece of meat which measured about 2 × 15 × 20 cm. Sanitizers were adjusted to pH 6.0 with acetic acid.

Aerobic plate counts (APC) were transformed into logarithms, and differences were calculated as follows for samples taken immediately and 48 h after sanitization.

$$\text{Difference in log counts} = \text{Log}_{10}\,\text{APC After} - \text{Log}_{10}\,\text{APC Before}$$

Data from both experiments were subjected to analysis of variance by the Statistical Analysis System (7) in an IBM 370 computer. Differences among means were determined by Duncan's multiple range test (3).

## RESULTS AND DISCUSSION

Designs of the two experiments allowed us to examine the effect of a relatively large number of variables with relatively small numbers of samples. However, the design made necessary the averaging of effects of individual variables over others. This tends to reduce the magnitude of effects of individual variables.

*Effects of pressure, flow rate, and speed of travel*

In Experiment 1 nozzles were used that would provide the same flow rates at each of the two pressures. This allowed us to separate the effects of pressure from those of flow rate and to show that 14.0 kg/cm² was significantly better than 3.5 kg/cm² ($P < 0.05$) based on swab samples collected 48 h after sanitizing (Table 1). Differences in log means of core counts representing the same meat were not significant. No effect of pressure was observed for counts (both core and swab) collected immediately after sanitizing. The higher volume of sanitizer, 3.4 liters/min, reduced counts significantly ($P < 0.05$) more than the lower volume, 0.83 liter/min, for both methods and times of sampling. Differences in mean log counts made after 48 h are shown in Table 1. Finally, significantly more bacteria were killed and/or removed when meat was moved at 2 cm/sec past the nozzle than when the speed was 10 cm/sec.

These observations were as expected and helped to further define physical limits within which spraying should be done. Data in Table 2 illustrate that the combination of 3.4 liters/min, 14.0 kg/cm², and 2 cm/sec was by far the most effective. Counts were reduced by more than 99% below the initial count.

TABLE 1. *Differences in mean log counts made 48 h after sanitizing averaged over type of hypochlorite as affected by pressure, volume, and speed of travel of meat*

| Treatment | Difference in log counts | |
|---|---|---|
| | Core[1] | Swab[1] |
| Pressure (kg/cm²) | | |
| 3.5 | -0.50[a] | -0.47[a] |
| 14.0 | -0.86[a] | -0.96[b] |
| Volume (liters/min) | | |
| 0.83 | -0.40[a] | -0.42[a] |
| 3.4 | -0.97[b] | -1.01[b] |
| Speed (cm/sec) | | |
| 2 | -1.11[a] | -1.02[a] |
| 10 | -0.31[b] | -0.34[b] |

[1]Each mean represents 32 observations, averaged over each other variable.

[ab]Means within each column and treatment with different superscripts are significantly different ($P < 0.05$) (3).

TABLE 2. *Differences in logs of microbial counts 48 h after sanitization as effected by flow rate, pressure and speed of travel of meat past the sanitizing nozzle and as determined by swab and core methods*

| Flow rate of sanitizer | Pressure of application | Speed of travel of meat | Method of sampling | |
|---|---|---|---|---|
| | | | Swab[1] | Core[1] |
| (liters/min) | (kg/cm²) | (cm/sec) | | |
| 0.83 | 3.5 | 2 | -0.55[b] | -0.34[b] |
| 0.83 | 3.5 | 10 | -0.13[b] | -0.16[b] |
| 0.83 | 14.0 | 2 | -0.51[b] | -0.50[b] |
| 0.83 | 14.0 | 10 | -0.49[b] | -0.61[b] |
| 3.4 | 3.5 | 2 | -0.81[b] | -1.10[b] |
| 3.4 | 3.5 | 10 | 0.37[b] | -0.42[b] |
| 3.4 | 14.0 | 2 | -2.58[a] | -2.18[a] |
| 3.4 | 14.0 | 10 | -0.27[b] | -0.17[b] |
| | | Mean | -0.67 | -0.68 |

[1]Each mean represents 8 observations.

[ab]Means in each column with different superscripts are significantly different ($P < 0.05$) (3).

*Effect of time of sanitizing and flow rate of sanitizer*

In Experiment 1 at the highest flow rate and pressure, meat that traveled through the sanitizing spray at 2 cm/sec had significantly greater differences in log counts than did meat that traveled at 10 cm/sec (Table 2).

Spraying the meat in Experiment 2 by passing it one time (2 sec) through the hypochlorite spray resulted in an average difference of -0.44 log, but passing it through the spray seven times (15 sec) increased the difference significantly to -1.18 log (Table 2). Fifteen passes (30 sec) failed to produce significantly greater reductions in counts (difference = -1.25 log) than did seven passes.

Spraying with 6.8 liters of sanitizer/min produced significantly ($P < 0.05$) greater differences in log counts than did spraying with 3.4 and 1.7 liters/min when samples taken immediately were considered (Table 3).

TABLE 3. *Differences in log counts of meat as affected by volume of sanitizer and method of sampling for samples taken immediately after sanitizing*

| Volume | Difference in log counts | |
|---|---|---|
| | Core[1] | Swab[1] |
| (liters/min) | | |
| 1.7 | -0.73[a] | -0.35[a] |
| 3.4 | -0.75[a] | -0.75[a] |
| 6.8 | -1.28[b] | -1.31[b] |

[1]Each mean represents 27 observations, averaged over type of sanitizer and time of application.

[ab]Means in each column with different superscripts are significantly different ($P < 0.05$) (3).

Flow rate was not a significant factor among samples taken 48 h after sanitization.

These data and those of Experiment 1 provide guidelines as to minimal times of exposure to hypochlorite. Thus, minimal exposure approximates a single passage at 2 cm/sec or seven passages at 10 cm/sec when line pressure is 14.0 kg/cm² and flow rate is 6.8 liters/min.

*Effects of method of sampling*

Results of Experiment 1 failed to show an effect of method of sampling on differences (log) in aerobic plate counts made before and after sanitization. However, the difference in log counts between samples taken by the swab method immediately and after 48 h approached significance at the 5% level.

In the second experiment the mean of the differences for samples taken by swabbing after 48 h was significantly greater than the other three means (Table 4). This meant that populations estimated by the swab and core methods 48 h after sanitization were different. Whereas, counts made by the swab method indicated continuing destruction of the microflora by hypochlorite, counts made by the core method suggested a small increase in viable counts during 48 h of storage. Kotula et al. (6) also observed that counts of samples taken by swabbing hypochlorite-treated meat were lower after 48 h of storage than immediately after sanitization. We previously reported (2) that hypochlorite had little or no residual effect on counts (core method). We now believe that the explanation for the discrepancy between our observations and those of Kotula et al. (6) is that different populations are observed when samples are taken by the swab and core methods. Whereas swabbing removes only those organisms on the surface, and only a variable portion of them, coring removes all of the cells from a given area. Organisms at the surface of the meat would be exposed to the greatest amount of sanitizer and be injured. A portion of the injured cells would likely recover on immediate transfer to plate count agar, whereas they could easily die when stored at an unfavorable temperature (refrigerated) for 48 h, especially if the humidity were low enough to allow for desiccation.

TABLE 4. *Differences in logs of microbial counts on meat surfaces as affected by sanitizing time and as determined by swabbing and coring methods immediately and 48 h after sanitizing*

| Time of sanitizing (sec) | Time of sampling: Immediate, Method of sampling: Swab[1] | Immediate, Core[1] | 48 h, Swab[1] | 48 h, Core[1] | Mean |
|---|---|---|---|---|---|
| 2 | -0.28[b] | -0.43[b] | -0.64[b] | -0.40[b] | -0.44[b] |
| 15 | -1.08[a] | -0.88[a] | -1.89[a] | -0.85[a] | -1.18[a] |
| 30 | -1.40[a] | -1.10[a] | -1.63[a] | -0.86[a] | -1.25[a] |
| Means | -0.91[x] | -0.80[x] | -1.39[y] | -0.70[x] | |

[1]Each mean represents 27 observations, averaged over type of sanitizer and volume of it.
[ab]Means in each column with different superscripts are significantly different ($P < 0.05$) (3).
[xy]Means on bottom line with different superscripts are significantly different ($P < 0.05$) (3).

In these experiments cores represented five times the surface area of swabs, and recovery by swabs was lower. However, this should not have been a factor in interpretation of residual effects of hypochlorite. Our procedures included exposing the surface to be sanitized of each piece of meat to each other one. This provided homogenous distribution of contaminants. Also, the amount of surface sampled by each method provided sufficient colonies to assure comparable accuracy in counting.

*Effects of type of hypochlorite*

Contrary to the report of Emswiler et al. (6) reductions in microbial populations on meat were practically the same regardless of whether electrolytically generated or commercial sodium hypochlorite were used (Table 5).

TABLE 5. *Differences in logs of microbial counts on meat sanitized with two types of hypochlorite and sampled by swabbing or coring immediately and 48 h after sanitizing*

| Treatment | Time of sampling: Immediate, Method of sampling: Swab[1] | Immediate, Core[1] | 48 h, Swab[1] | 48 h, Core[1] | Mean |
|---|---|---|---|---|---|
| Electro. HOCl[2] | -1.11[a] | -1.01[a] | -1.55[ab] | -0.80[a] | -1.12[a] |
| Com. HOCl[3] | -1.18[a] | -1.02[a] | -1.65[a] | -1.12[a] | -1.24[a] |
| Water (control) | -0.46[b] | -0.38[b] | -0.97[b] | -0.19[b] | -0.50[b] |

[1]Each mean represents 27 analyses, averaged over flow rate and time of application.
[2]Electrolytically generated sodium hypochlorite.
[3]Commercially produced sodium hypochlorite.
[ab]Means in each column with different superscripts are significantly different ($P < 0.05$) (3).

Means were practically the same for the two types of sanitizer for the respective times and methods of sampling. In these tests counts were reduced by slightly more than 90% by hypochlorite and by about 35% by water sprayed under the same conditions.

## CONCLUSIONS

Based on research reported herein, bacterial numbers on meat were reduced most when the highest pressure, 14.0 kg/cm², the highest flow rate, 6.8 liters/min, and the longest time of spraying, 15 sec, were used. Source of hypochlorite was unimportant. The coring method of sampling provided information on a different population of residual bacteria than did the swab method. We recommend coring.

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# A Practical Sensitive Test to Detect Penicillin in Milk

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ABSTRACT

The disc assay of the International Dairy Federation for determination of penicillin in milk has been modified in the following steps: preparation of seed broth, seeded plates, and standard solutions. The modified assay is sensitive and relatively rapid, requiring a 4-h incubation at 55 C. Approximately 0.004 unit of penicillin/ml of milk can be detected on plates stored for 7 days; with freshly prepared plates and standard solutions, as little as 0.002 unit of penicillin/ml can be detected.

The sensitivity of the American Public Health Association disc assay (*1*) for penicillin in milk is about 0.05 unit/ml, using *Bacillus subtilis* in the 14 to 24-h assay at 32 C. A more senstive test which requires less time and which is practical and economical is needed. The International Dairy Federation (IDF) at its meeting in Australia in October, 1970 approved publication of a disc assay procedure for detection of penicillin in milk; this procedure (*3*), which specifies *Bacillus stearothermophilus* var. *calidolactis* as the test organism, is more sensitive (< 0.0025 unit/ml) and requires less time than the *B. subtilis* assay presently undertaken in most dairy laboratories. Assay methods specified by the Association of Official Analytical Chemists include both a disc and a cylinder technique (*2*). The disc assay is sensitive to approximately 0.05 unit of penicillin/ml while the cylinder assay, which is more complex to use, is suitable for concentrations of less than 0.025 unit/ml.

We undertook some studies of the IDF procedure. Slight modifications were made to serve the practical needs of the dairy laboratory, i.e., in preparation of seed broth, standard solutions, and seeded plates. The test as described is sensitive, fits nicely into the 5-day work week, and provides assay plates for a 7-day period.

## MATERIALS AND METHODS

The test organism was *B. stearothermophilus* var. *calidolactis*. The stock culture was maintained in a screw-cap tube on a medium (slant) consisting of 2 g of yeast extract, 5 g of peptone, 1 g of meat extract, 5 g of sodium chloride, 15 g of agar, and 1000 ml of distilled water; the final pH was 7.4 ± 0.1. The stock culture was prepared by streaking and incubating the above medium for 48 h at 55 C; the culture was stored at 4 C. To start the test using the agar slant stock culture, transfer a loopful of slant culture to 50 ml of seed broth; incubate for 18 h and refrigerate for 5 days. This 5-day old culture is used to prepare seed broth as described below. The seed broth used to culture the organism contained 10 g of yeast extract, 20 g of tryptone, 0.5 g of glucose, and 1000 ml of distilled water; the final pH was 8.0 ± 0.1. The following regimen was followed to prepare the seed broth culture. Inoculate 50 ml of seed broth in a milk dilution bottle with 0.1 ml of a 5-day-old refrigerated broth culture and incubate for 18 h; transfer 1 ml of this culture to a second bottle containing 50 ml of seed broth and incubate for 6 h; transfer 1 ml of this culture to a third bottle with 50 ml of seed broth and incubate for 18 h. Prepare seeded plates from this culture. Store the 49 ml of seed broth which remain from the last transfer above at 4 C for 5 days; use this 5-day-old culture as the source of the inoculum for the first bottle. With the above procedure, the stock cell suspension counts of the seed broth ranged from 150,000 to 1,000,000/ml. Using our technique, we were unable to achieve the counts obtained by the IDF assay (50 to 100 million), but we were able to obtain clear zones of inhibition on the plates.

Plate Count Agar (PCA) adjusted to pH 8.0 ± 0.1 was used for preparing the seeded plate. Petri plates with bottoms having an inside diameter of 85 mm were used. To make a seeded plate, 12 ml of seed broth was added to 60 ml of PCA agar at 55 C; 7 ml of this mixture was used. Prepared plates were used immediately or stored for up to 8 days in a plastic sleeve at 5 C. No attempt was made to make the sleeve anaerobic.

The standard stock solution of potassium penicillin G solution was prepared to contain 10,000 units/ml in pH 6.5 buffer; the buffer was made by dissolving 8 g of monobasic potassium phosphate and 2 g of dibasic potassium phosphate in 1000 ml of distilled water, and adjusting the pH to 6.5. The stock solution was used immediately or stored at 5 C for not more than 24 h. On the day of use, 1 ml of the stock solution was diluted to 100 ml with pH 6.5 buffer; 1 ml of this solution was further diluted to 100 ml with buffer. Additional dilutions were made with sterilized (121 C for 10 min) pasteurized, antibiotic-free homogenized whole milk as follows: 1 ml of the second dilution was made up to 100 ml with milk; then 20-, 10-, and 5-ml volumes were each diluted to 50 ml with milk to obtain concentrations of 0.004, 0.002, and 0.001 unit of penicillin/ml, respectively.

For the assay, blank discs, ½ inch (1.27 cm) in diameter, were touched to the surface of the sample to allow the disc to soak up the sample. The edge of the disc was then touched three times on the underside of the petri dish lid; this provided a sterile surface and the milk spots could be easily removed with tissue before the top was replaced on the plate. The discs were placed on the surface of the agar as described in the *B. subtilis* assay (*1*). All plates were inverted and incubated at 55 C for 3.5-4 h. Sterilized, pasteurized, penicillin-free homogenized whole milk was used as the negative control.

## RESULTS AND DISCUSSION

Zone diameters were measured to the nearest millimeter. Averages of duplicate discs on triplicate plates are shown in Table 1. In only rare instances did the zone diameters vary by 1 mm.

The initial number of cells obtained on culturing by the procedure described is given in Table 1 for each trial. No explanation can be given for the range of cell numbers obtained, and no effort was made to study this aspect. The range in cell numbers actually provided the opportunity, without resorting to dilution, to determine a lower and an upper limit within which the assay could be undertaken on a practical basis. This 10-fold difference is of the same magnitude as that recommended for the *B. subtilis* disc assay (*1*).

The data show that the modified IDF assay can detect 0.004 unit of penicillin/ml in milk, using plates which have been stored for 8 days. The number of cells employed (150,000-1,000,000) did not influence the zone diameter when the diameter was measured to the nearest 1 mm; no effort was made to be more precise, as this represents a practical objective for most dairy laboratories.

A level of 0.002 unit/ml can be detected when fresh plates and fresh standard solutions are employed. At the 0.002 unit/ml level, clearly defined zones were observed for 66% of the assays with plates stored for 24 h and for 25% of the assays with plates stored for 7-8 days.

Clear zones of inhibition were readily seen at a penicillin concentration of 0.004 unit/ml in 3.5 h, but a 4-h incubation was needed to detect penicillin at 0.002 unit/ml. If zones are to be measured, a 4-h incubation period is mandatory to permit formation of a zone with a distinct edge.

All incubations were done at 55 C. Use of 55-C incubation is advantageous in that very few bacterial contaminants commonly found in a dairy laboratory will grow at this temperature. If, therefore, a poor technique is used in making culture transfers to seed broth, it is not likely that the contaminant(s) could grow and confuse interpretation of the test.

On the basis of these preliminary data it is apparent that the modified IDF test is (*a*) considerably more sensitive than either the official disc or the cylinder assay method for milk, (*b*) much simpler than the cylinder assay, and (*c*) readily adapted to the dairy laboratory, as it utilizes the basic disc assay technique which is familiar to technicians presently engaged in antibiotic assays. It is hoped that interested groups will be encouraged to investigate this procedure more fully to determine its suitability for incorporation in the next edition of *Standard Methods for the Examination of Dairy Products.*

### ACKNOWLEDGMENT

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TABLE 1. *Detection of known levels of penicillin added to milk*

| Penicillin concentration (unit/ml) | Zone diameters (mm) obtained at cell concentrations indicated | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 150,000/ml | | | 290,000/ml | | | 610,000/ml | | | 1,000,000/ml | | |
| | 1 (1)[a] | 1 (0) | 7 (1) | 0 (0) | 1 (1) | 8 (1) | 1 (1) | 1 (0) | 7 (1) | 0 (0) | 1 (1) | 8 (1) |
| 0.004 | 16[b] | 16 | 16 | 16 | 16 | 15 | 15 | 15 | 15 | 17 | 16 | 16 |
| 0.002 | 14 | 15 | 0 | 15 | 15 | 0 | 0 | 14 | 0 | 15 | 0 | 15 |
| 0.001 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Negative | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]The first figure indicates the age of the plate in days at the time of assay; the figure in parentheses indicates the age of the standard solution.
[b]Zone diameters are the average of two discs on each of three plates.

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# Effect of High Temperature Holding and Ice Storage on Protein, Non-Protein Nitrogen, Water, and Collagen Content of Penaeid Shrimp

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**ABSTRACT**

Five different sample lots of white shrimp (*Penaeus setiferus*) were divided into two groups. One group was kept at room temperature (23-24 C) and the other on ice until putrid odors began to appear. Protein and non-protein nitrogen (NPN), water, and collagen contents were measured initially and when putrid odors began to appear. Initial protein content ranged from 14.04-16.93% and water content ranged from 68.24-77.31%. Initial water and protein [(Total Nitrogen - NPN) × 6.25] had the relationship % water = 122.25 - 3.19(% protein), r = 0.998). At 23-24 C the NPN to TN ratio increased significantly ($p < 0.01$) in two lots and slightly in one lot. In ice stored shrimp NPN and protein nitrogen decreased by an average amount of 50% and 25%, respectively. During ice storage, water content increased significantly in all three lots of shrimp. Initial total collagen content ranged from 6.71-8.83 mg/g with the % soluble ranging from 11.92-45.22%. Soluble collagen increased significantly in two lots and decreased significantly in one lot at both room and ice-storage temperature.

Shrimp tail (abdomen) contains a high level of low molecular weight non-protein nitrogenous compounds (NPN) (*9*). Changes in levels of low molecular weight nitrogenous compounds in shrimp tails during storage have been reported by several investigators (*5, 9, 10, 15, 22*). Postmortem chemical content of shrimp tail is influenced by bacterial and enzymatic action (*9*) and leaching action of melting ice (*10, 17*).

Although the effect of postmortem ice storage on protein and collagen composition of shrimp has not been investigated, it would be of interest to know if changes in protein and collagen accompany changes in low molecular weight components. Thus the objectives of this study were to determine the changes of protein, non-protein nitrogen, water-soluble collagen content of shrimp spoiling under room temperature (23-24 C), and ice storage conditions.

## EXPERIMENTAL

*Materials*

White shrimp (*Penaeus setiferus*) were obtained live from a commercial dealer at four different times and designated to lots A, B, C, D, and E (Table 1 and 2). Shrimp were sacrificed, deheaded, and rinsed. Shrimp from each lot were then separated into two groups. Group I was placed in an ice chest as described by Cobb et al. (*12*). Group II was placed in a 1000-ml glass jar at room temperature (23-24 C). Shrimp were sampled (*a*) immediately after sacrifice and (*b*) when putrid odors began to appear (approximately 16 days postmortem in group I and 10-12 h postmortem in group II). Twenty-five or more shrimp were used for protein and water content determination at each sampling time on lots A, B, and C. Fourteen or more shrimp were used for soluble collagen content determination at each sampling time on lots C, D, and E. Shrimp were minced or powdered (collagen analysis) and stirred to achieve a homogeneous sample. Appropriate size samples of the mince were taken for chemical analyses.

Extracts for non-protein nitrogen determination were made by homogenizing one part shrimp mince with two parts 7% trichloroacetic acid solution. Total nitrogen (TN) and NPN were measured by the microkjeldahl procedure (*8*). Protein content was calculated from the relationship (TN — NPN) × 6.25. Moisture content was determined by heating shrimp tissue to a constant weight in a drying oven at 125 C for 2 h (*1*). Soluble and insoluble collagen were separated (*16*) and collagen determinations were made according to the procedure of Woessner (*25*) except that collagen content was calculated on the basis of 33.8 hydroxyproline residues/1000 total collagen residues (*21*).

The chemical data were statistically analyzed by using Dunnett's procedure according to Steel and Torrie (*20*).

## RESULTS AND DISCUSSION

Table 1 shows the protein, moisture, and nitrogen composition of freshly caught and slightly putrid shrimp (*P. setiferus*) tails held at two different temperatures. Three lots of shrimp tails were used. Initial protein content [(TN — NPN) × 6.25] varied from 14.04 to 16.93%. Initial moisture content varied from 68.24 to 76%. Water and protein content had the relationship: % water = 122.25 — 3.19(% protein) (r = 0.998). Initial non-protein nitrogen (NPN) content was approximately the same in all three lots, averaging from 0.64 to 0.68%. Initial total nitrogen (TN) content varied from 2.91 to 3.36%.

When the shrimp spoiled at room temperature, the weight percent of total nitrogen increased significantly ($p < 0.05$) in lot B (but not in lots A and C) because of

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moisture and/or drip loss. Non-protein nitrogen increased significantly ($p < 0.01$) in lots A and B and slightly increased in lot C. The ratio of NPN to TN increased significantly ($p < 0.01$) in lots A and B and slightly increased in lot C, with increases of 50, 18, and 7%, respectively. Moisture content remained about the same in lot A and decreased in lots B and C with a significant decrease only in lot B ($p < 0.01$). Protein content [(TN—NPN) × 6.25] decreased in lot A and increased in lots B and C. This caused the average amount of protein to remain constant.

In ice-stored shrimp, NPN and protein nitrogen decreased by an average of 50% and 25%, respectively, by the time spoilage had occurred. Moisture content increased significantly ($p < 0.01$) in all three lots (A, B, and C) of ice-stored shrimp. The predicted water content on the basis of protein content was 80.71, 85.05, and 82.88% vs. 83.58, 85.64, and 80.19% found, respectively.

The collagen content of shrimp tails before and after spoilage under two different storage conditions is shown in Table 2. Analyses were done on three lots (C, D, and E) of shrimp tails. The initial collagen content per g of shrimp tissue varied as follows: total collagen content ranged from 6.71 to 8.83 mg; soluble collagen content ranged from 1.05 to 4.0 mg; insoluble collagen content ranged from 4.83 to 7.75 mg, and percent soluble collagen ranged from 11.92 to 45.22%.

At room temperature, soluble collagen content increased significantly over the control ($p < 0.01$) by the time spoilage had occurred in lots C and D but decreased in lot E. The insoluble collagen content also changed unevenly with a highly significant increase in lot E, no significant change in lot D and a highly significant decrease in lot C. This caused the average level of insoluble collagen to remain approximately constant. The percent soluble collagen increased significantly in lots C and D and decreased significantly in lot E. Probably because of drip in the spoiled shrimp, the total collagen level appeared to increase in all three lots with the average total collagen increasing from 8.11 to 9.55 mg/g of shrimp tissue.

In the ice-stored shrimp, soluble collagen significantly

TABLE 1. *Protein, moisture and nitrogen composition of freshly caught and slightly putrid shrimp* (P. setiferus) *tails held at two different temperatures*

| Treatment | Lot[a] | % TN[b] | % NPN[c] | % Protein[d] | % Moisture | Average value of % TN | Average value of % NPN | Average value of % Prot. | Average value of % Moist. |
|---|---|---|---|---|---|---|---|---|---|
| Fresh | A | 3.36[e] | 0.65 | 16.93 | 68.24 | 3.08 | 0.66 | 15.18 | 73.85 |
| | B | 2.91 | 0.68 | 14.04 | 77.31 | | | | |
| | C | 2.97 | 0.64 | 14.56 | 76.00 | | | | |
| Spoiled, room temp. (23-24 C) | A | 3.38 | 0.98** | 15.04 | 68.86 | 3.31 | 0.88 | 15.18 | 72.46 |
| | B | 3.41* | 0.94** | 15.42 | 73.81** | | | | |
| | C | 3.13 | 0.72 | 15.08 | 74.72 | | | | |
| Spoiled, ice storage | A | 2.29** | 0.20** | 13.02* | 83.58** | 2.31* | 0.33* | 12.34* | 83.14** |
| | B | 2.05** | 0.19** | 11.66* | 85.64** | | | | |
| | C | 2.58 | 0.61 | 12.34 | 80.19** | | | | |

[a]Lot A = Average shrimp tail length 7 cm, obtained on 9/25/75.
B = Average shrimp tail length 4 cm, obtained on 10/10/75.
C = Average shrimp tail length 4 cm, obtained on 11/21/75.
[b]TN = Total nitrogen
[c]NPN = Non-protein nitrogen
[d]Protein = (TN − NPN) × 6.25
[e]Average of 3 analyses of a pooled sample of approximately 25 shrimp.
*$p < 0.05$
**$p < 0.01$

TABLE 2. *Collagen composition in freshly caught and slightly putrid shrimp* (P. setiferus) *tails held at two different temperatures*

| Treatment | Lot[a] | Soluble[b] collagen (mg/g) | Insoluble collagen (mg/g) | % Soluble collagen | Total collagen (mg/g) | Average (mg/g) Soluble collagen | Average (mg/g) Insoluble collagen | Average (mg/g) % Soluble collagen | Average (mg/g) Total collagen |
|---|---|---|---|---|---|---|---|---|---|
| Fresh | C | 4.00[c] | 4.83 | 45.22 | 8.83 | 2.30 | 5.81 | 27.97 | 8.11 |
| | D | 1.05 | 7.75 | 11.92 | 8.80 | | | | |
| | E | 1.80 | 4.91 | 26.76 | 6.71 | | | | |
| Spoiled, room temp. (23-24 C) | C | 8.13** | 1.59** | 83.51** | 9.72 | 3.50 | 6.05 | 36.09 | 9.55 |
| | D | 1.42** | 8.82 | 13.96* | 10.24* | | | | |
| | E | 0.94** | 7.75** | 10.79** | 8.69** | | | | |
| Spoiled, ice storage | C | 1.73** | 7.52** | 18.71** | 9.25 | 2.47 | 5.54 | 32.04 | 8.00 |
| | D | 2.51** | 5.25** | 32.34** | 7.76 | | | | |
| | E | 3.16** | 3.84* | 45.07** | 7.00 | | | | |

aLot C = Average shrimp tail length 4 cm, obtained 11/21/75, 10% molted shrimp.
Lot D = Average shrimp tail length 5 cm, obtained 2/25/76, > 30% molted shrimp.
Lot E = Average shrimp tail length 4 cm, obtained 2/25/76, > 30% molted shrimp.
[b]Collagen measurements were based on 33.8 hydroxyproline residues/1000 total collagen residues (*21*).
[c]Average of two analyses of a pooled sample of approximately 14 shrimp. All the values based on a fresh-weight basis.
*$p < 0.05$
**$p < 0.01$

decreased in lot C and significantly increased in lots D and E. Insoluble collagen significantly decreased in lots D and E and significantly increased in lot C. There was no significant difference in the total collagen content.

A number of factors could affect the proximate composition of freshly killed shrimp. Size may be important as smaller sized shrimp grow more rapidly and thus molt more rapidly than larger sized shrimp. In each lot, there was approximately 10% or more soft shell shrimp, suggesting either a recent molt or beginning molt. Lockwood (*18*) indicated an increase in moisture content of shrimp after molting. Differences in the salinity of the water from which the shrimp were taken could have affected the initial NPN levels (*7*). Glycine and proline contents, hence NPN, have a direct relationship with the salinity of the water.

Analysis of shrimp at the time putrid odors became evident indicated the maximum biochemical changes which might be expected during the useful life of the shrimp. Non-protein nitrogen increased during room temperature storage and decreased under ice storage. The above is in agreement with the work of Cobb et al. (*11*) which indicated an increase in free amino acids of white shrimp tails kept in a bag (no leaching by ice). In ice-stored shrimp a decrease in free amino acid and ammonia levels occur because of the leaching action of ice and probable bacterial metabolism (*6*). Dehydration of shrimp at room temperature can cause an increase in NPN levels. If dehydration was the only effect, then the NPN to TN ratio should remain constant. But the NPN to TN ratio changed in this study. Increases in moisture content in the ice-stored shrimp were probably due to postmortem pH increase and uptake of water from melting ice, which have been reported by a number of investigators (*2, 4, 14, 22, 24*).

The type and levels of microorganisms on the shrimp and endogenous enzymes or enzymatic contamination during the deheading process of the shrimp influence postmortem biochemical changes (*9*). Bauer and Eitenmiller (*3*) reported intracellular catabolism of proteins by enzymes (arylamidase) occurs in white shrimp. The loss of non-protein and protein nitrogen during ice storage suggested that these components were probably contained in interstitial fluids and/or that there was considerable cellular breakdown during the storage period.

The same factors which affect the protein composition of freshly killed shrimp could also affect the collagen composition. For instance, when shrimp molt they become very soft and expand to fit the new shell. This could cause changes in collagen content due to increases in water content. Also new collagen could be synthesized to accommodate increases in the size of muscles. Dutson (*13*) indicated that in beef there is no change in the amount of total collagen per gram of muscle tissue due to aging, but that soluble collagen content is decreased with increasing animal age. Similarly, the larger and probably older shrimp (nutrition has a considerable influence on shrimp size) contained a lower percent of soluble collagen than the smaller shrimp. However, shrimp of nearly equal size, but not necessarily equal age, contained different percentages of soluble collagen. Whether or not the stage of molting could affect the levels of soluble collagen is unknown.

The apparent increase in collagen content in shrimp kept at 23-24 C could be due to the amount of drip which collected in the bottom of the beakers in which the shrimp were kept. The increase of soluble collagen and a decrease of insoluble collagen in shrimp was possibly due to enzymatic hydrolysis of insoluble collagen. Iced shrimp from commercial sources have been shown to have connective tissue damage due to bacterial or enzymatic degradation within a few days after being caught by trawling (*19*). The decrease of soluble collagen and an increase of insoluble collagen is harder to explain as it occurred in one lot at room temperature and a different lot at ice-storage temperature. The decreases of soluble collagen could be due to leaching action of melting ice.

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# Organoleptic, Bacterial, and Chemical Characteristics of Penaeid Shrimp Subjected to Short-Term High-Temperature Holding

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ABSTRACT

White shrimp (*Penaeus setiferus*) were held at 0, 10, 20, 30, 37, and 44 C for 3, 6, and 24 h. Serious quality deterioration, as evidenced by off-color development (red and orange pigmentation) and off-odor development, was beginning to occur in shrimp held for 3 h at 30, 37, and 44 C, for 6 h at 20 C and for 24 h at 10 C. Red color development was evident in shrimp held at 30 and 37 C, orange color in those held at 44 C. Putrid odors appeared more rapidly in shrimp held at 37 than at 44 C where shrimp developed cooked-shrimp odors. Large increases in bacterial counts at 30-44 C (after 6 and 24 h) were usually accompanied by putrid odors. Tissue pH changes were erratic and small. Total volatile nitrogen (TVN), free amino acid nitrogen (AA-N), and urea production increased with storage temperature during the 3- and 6-h storage experiments. Musty and cooked-shrimp off-odors developed in the shrimp as a result of chemical and/or enzymic activity while putrid and sour odors were produced by bacteria growing in the shrimp.

A shrimp trawl contains an assortment of shrimp, fish, crabs, and often mud or debris. Some shrimp are killed immediately after entering the trawl, particularly if large quantities of fish are also caught. Trawling in warm tropical waters may continue as long as 12 h. Before icing, shrimp are separated from the trash (fish, etc.) and are usually deheaded. Thus several hours may elapse before the shrimp are iced. During this period the shrimp may be held at temperatures ranging from near freezing to above 40 C.

The effect of short-term high-temperature storage on shrimp quality has been investigated by Fieger et al. (*9*). However, the effects of short-term high-temperature storage need to be established more accurately as the above study was confined to only one temperature range. It was the purpose of this investigation to establish the effect of holding shrimp at temperatures from 0-44 C for short periods.

## EXPERIMENTAL

Shrimp were obtained live at Galveston, Texas, and sacrified by placing them in ice in well-insulated chests. The shrimp were rapidly transported to the laboratory where they were deheaded and washed in tap water. Shrimp were then blotted dry with paper towels, placed in sealed plastic bags to prevent dehydration and were rapidly equilibrated at the following temperatures: 0, 10, 20, 30, 37, and 44 C. Each lot of shrimp was held at these temperatures for 3, 6, or 24 h.

To reduce effects of bacterial action, some shrimp samples were dipped in a solution of 1 mg chloramphenicol/ml for 3 min to inhibit bacterial growth and then stored for 6 h at the previously specified temperatures.

### *Organoleptic analyses*

Organoleptic analyses were conducted by four trained judges. Shrimp were evaluated on the basis of color and odor description. The area of the shell covered by off-color development was estimated. Off-odors were noted and described. Shrimp were considered as unacceptable when three of the four judges rates them as unacceptable (putrid), and of questionable acceptability when one or two of the four judges rated them as unacceptable or of questionable acceptability.

### *Bacteriological analyses*

Initially and after incubation, 25 g of shrimp were blended with 225 ml of 0.1% sterile peptone solution in a Waring Blendor at 3000 rpm for 2 min. Appropriate dilutions were made in 0.1% peptone and 0.1-ml aliquots were placed onto Trypticase Soy Agar (TSA, BBL) using the spread plate method. Plates were then incubated at 25 C for 48 h.

### *Chemical analyses*

Total volatile nitrogen (TVN), amino acid nitrogen (AA-N), and trimethylamine nitrogen (TMN) were analyzed as previously described (*5*). The pH was measured by inserting a miniature combination electrode into comminuted whole tissue homogenates. Urea was determined by the procedure of Mellerup (*16*). Pooled samples of 10 shrimp were used for analyses. Each analysis was conducted in triplicate.

## RESULTS

The color and odor evaluations for shrimp held for 3, 6, and 24 h periods at 0, 10, 20, 30, 37, and 44 C (time-temperature experiments) are listed in Table 1. Red and orange colors, which are considered indicative of spoilage in raw white shrimp, were evident in shrimp held for 3 h or longer at 30,37, and 44 C, and in shrimp held for 24 h at 20 C. Red color developed in shrimp held at 20, 30, and 37 C while orange color developed in those held at 44 C. The amount of the off-color area increased with the time of holding. Melanosis (blackening) occurred in only one lot of shrimp held for 6 h at 37 and

[a]*Deceased August 30, 1976.*

44 C. Chloramphenicol treatment did not appear to influence off-color development.

Four types of off-odor development, musty, putrid, "cooked shrimp," and sour, were evident. Strong musty odors were detected in shrimp after 3 h at 30 and 37 C and in about 25% of the shrimp after 6 h at 30 C. Chloramphenicol-treated shrimp held for 6 h at 10, 20, and 37 C also had strong musty odor. Faint musty odor was detected in shrimp after 6 h at 20 C and after 24 h at 10 C. Putrid odors were evident in shrimp after 6 h at both 30 (75% of shrimp) and 37 C and after 24 h at 20 C. "Cooked shrimp" odors were evident in shrimp after both 3 and 6 h at 44 C and in chloramphenicol-treated shrimp after 6 h at 44 C. Sour odor was detected in chloramphenicol-treated shrimp after 6 h at 30 C.

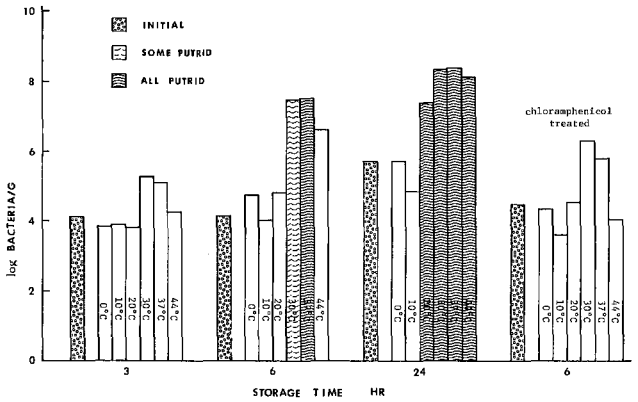


Figure 1. *Bacteriological analyses of shrimp stored for different time periods at different temperatures.*

Large increases in bacterial count occurred after 6 h at 30, 37, and 44 C and after 24 h at 20, 30, 37, and 44 C (Fig. 1). Some increases in bacterial population occurred in the chloramphenicol-treated samples after 6 h at 30 and 37 C but the bacterial levels were not as high as in the untreated samples. Development of putrid odors in most instances was related closely to development of high viable counts (after 6 h at 30 and 37 C; after 24 h at 20, 30, 37, and 44 C).

The tissue pH of the shrimp from the time-temperature experiments is shown in Fig. 2. Initial pH of the

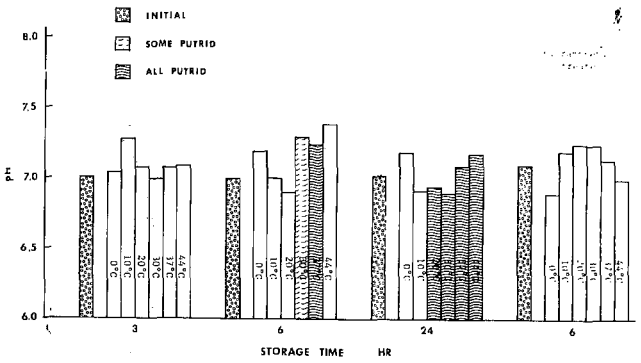


Figure 2. *Tissue pH of shrimp stored for different time periods at different temperatures.*

four groups of shrimp was in the narrow range of 7.05 to 7.2. There was no consistent pattern of pH change; changes were small, indicating that the increases in pH during postmortem storage, which have been reported by several investigators (*1, 2, 10, 23*), require time to develop.

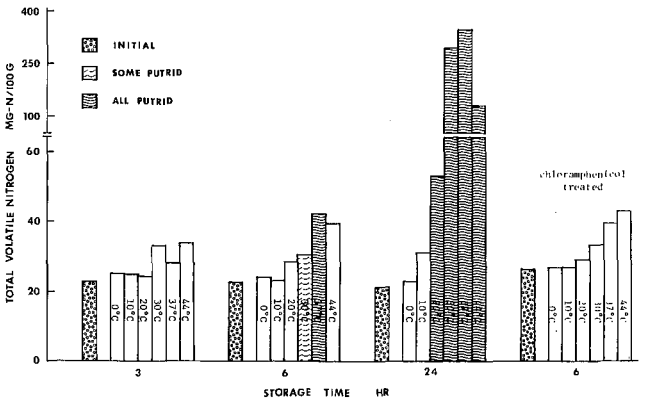


Figure 3. *Total volatile nitrogen (TVN) production in shrimp stored for different time periods at different temperatures.*

TABLE 1. *Color and odor evaluation of white shrimp* (P. setiferus) *held at different temperatures for different time periods*

| Temp (C) | Color | | | Odor | | | |
|---|---|---|---|---|---|---|---|
| | 3 h | 6 h[a] | 24 h | 3 h | 6 h | 6 h[b] | 24 h |
| 0 | Tan | Tan | Tan | Fresh | Fresh | Fresh | Fresh |
| 10 | Tan | Tan | Tan | Fresh | Fresh | Musty | Musty (Trace) |
| 20 | Tan | Tan | Red <5% | Fresh | Fresh to Musty (Trace) | Musty | Putrid with $H_2S$ |
| 30 | Red[c] <5% | Red >5%-<10% | Red 100% | Musty[d] | Musty[d] to Putrid | Sour | Putrid |
| 37 | Red <5% | Red >10%-<50% | Red 100% | Musty[d] | Putrid | Musty | Putrid |
| 44 | Orange <5% | Orange >50%-100% | Orange 100% | Cooked Shrimp | Cooked Shrimp | Cooked Shrimp | Putrid |

[a]Shrimp dipped in 1 mg chloramphenicol/ml had the same color development.
[b]Treated with 1 mg chloramphenicol/ml for 3 min.
[c]Percentages indicate area of shell involved.
[d]Shrimp which were rated as questionable acceptability.

Total volatile nitrogen content of shrimp held at various temperatures is illustrated in Fig. 3. Initial TVN levels ranged from 21-26 mg N/100 g. Large increases in TVN occurred in shrimp held for 24 h at 20, 30, 37, and 44 C. Measurable TVN increases occurred in shrimp held for 3 h at 30, 37, and 44 C and for 6 h at 20, 30, 37, and 44 C. All shrimp with musty odors had TVN levels ranging from 28 to 40 mg N/100 g. Distinct putrid odors were not evident in shrimp with less than 40 mg N/100 g. TVN levels of chloramphenicol-treated shrimp stored for 6 h were similar to those of untreated shrimp.

TABLE 2. *Trimethylamine production in white shrimp* (P. setiferus) *held at different temperatures for different time periods*

| Storage temp. (C) | Trimethylamine nitrogen content (mg N/100 g) | |
|---|---|---|
| | 6 h[a] | 24 h |
| 0 | 0 | 0 |
| 10 | 0 | 0 |
| 20 | 0 | 6.43[c] |
| 30 | 0.47[b] | 52.72[c] |
| 37 | 1.25[c] | 51.23[c] |
| 44 | 0 | 20.62[c] |

[a]Only 6 and 24-h data re shown as no trimethylamine was detected in 3-h samples and 6-h samples treated with 1 mg chloramphenicol/ml.
[b]Acceptability questionable.
[c]Putrid odors evident.

Trimethylamine nitrogen (TMN) production was evident in shrimp after 6 h at 30 and 37 C and after 24 h at 20 to 44 C (Table 2). TMN levels were not measurable in shrimp stored for 3 h and in chloramphenicol-treated shrimp. TMN was detected (0.47 mg N/100 g) in only one acceptable (questionable) sample of shrimp which had been held for 6 h at 30 C. The remainder of the shrimp containing measurable TMN levels had putrid odors.

Free amino acid nitrogen (AA-N) levels of shrimp held for 3 and 6 h at 0-44 C were higher than those of the control (initial) sample, particularly of those held at 37 and 44 C (Fig. 4). However, in samples held for 24 h,

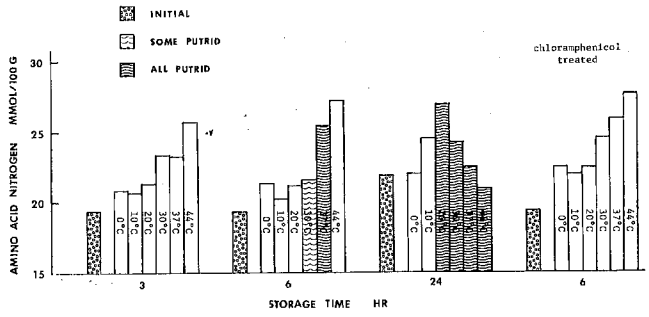


Figure 4. *Amino acid nitrogen (AA-N) production in shrimp stored for different time periods at different temperatures.*

AA-N levels increased as the temperature increased up to 20 C and then decreased as the temperature was increased. Chloramphenicol treatment did not appear to significantly affect AA-N production.

The TVN-AA-N ratio has been advocated as an indicator of shrimp quality (7). The TVN-AA-N ratio of shrimp used in the time-temperature experiments is presented in Fig. 5. The increases in the TVN-AA-N

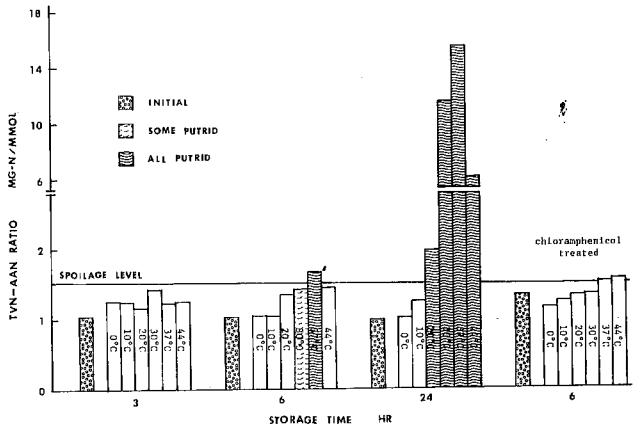


Figure 5. *TVN-AA-N ratio of shrimp stored for different time periods at different temperatures.*

ratio reflected the increases in the TVN levels. All samples with putrid odors exceeded the 1.5 mg N/mmole level previously established as spoilage level (7). All untreated samples, which were rated as of questionable acceptability, had TVN-A-AN ratios >1.3 but <1.5 mg N/mmole. Two chloramphenicol-treated samples which exceeded the 1.5 mg N/mmole level did not have putrid odors.

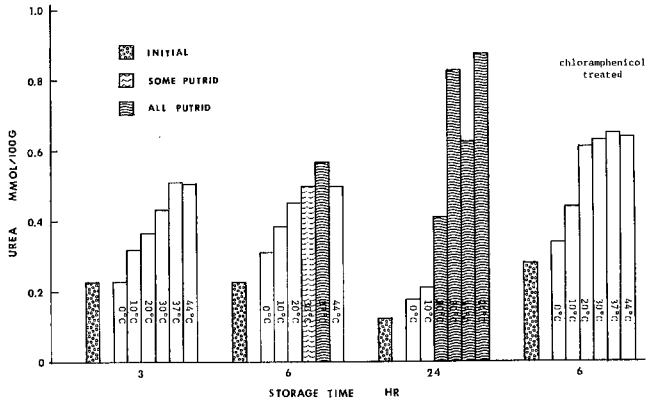


Figure 6. *Urea levels in shrimp stored for different time periods at different temperatures.*

Urea levels increased only slightly during the first 24 h of holding at 0 C (Fig. 6). Urea production increased with holding temperature except in the samples held for 24 h at 37 C and for 6 h at 44 C. Chloramphenicol-treated shrimp had higher urea levels than untreated shrimp.

## DISCUSSION

Holding shrimp for 3 h at 30-44 C caused serious quality deterioration. In general, approximately the same level of deterioration also occurred in shrimp held for 6 h at 20 C and 24 h at 10 C. Shrimp held at 44 C did not develop putrid odors as rapidly as those held at 30 and 37 C but had more off-color development. It is possible that those organisms responsible for the off-odor were not able to grow at 44 C.

Red or orange off-color development in shrimp is due to denaturation of astaxanthin-protein complexes and

oxidation of red astaxanthin to orange astaxin and/or to the presence of a cryptaxanthin-like yellow pigment (*13*). These experiments indicated that oxidation of astaxanthin probably occurred much more rapidly at 44 than at 37 C. Both the cooked shrimp odor and the orange color of the shrimp stored for 3 and 6 h at 44 C would have been acceptable in a cooked product but not in the raw product. Black, green, or brown off-colors in shrimp, which were virtually absent in this study, are due to melanin pigment development (*4*). Except in the presence of strong light, discoloration due to melanin pigment usually requires more than 24 h to develop.

Off-odor development in the shrimp appeared to be divided into two categories: (*a*) musty and cooked shrimp odors due to chemical and/or enzymatic activity and (*b*) putrid and sour odors due to bacterial activities. Cooked shrimp and musty odors occurred in some samples which had little increase in bacterial levels, while putrid shrimp odors occurred only in shrimp with high bacterial levels. Strong musty odors in shrimp with low bacterial levels were previously reported by Vanderzant et al. (*22*). Sour odors appeared only in the chloramphenicol-treated shrimp and probably resulted because of growth of microbial species other than those that develop on iced untreated shrimp.

Large increases in bacterial population as noted after 6 h at 30-44 C and after 24 h at 20-44 C clearly were associated with development of putrid odors. This most likely was caused by the development of gram-negative bacteria such as *Pseudomonas* sp. Development of this type of spoilage organism was controlled in the chloramphenicol-treated samples because putrid off-odors were not detected in these samples.

When the washing action of melting ice is absent, both TVN levels and the TVN-AA-N ratio appear to be good indicators of shrimp quality. Normally TVN levels do not increase significantly during the first few days of ice storage because melting ice tends to remove ammonia as rapidly as it is formed (*8*). Without the washing action of melting ice, the spoilage level was about 40 mg N/100 g rather than the 30 mg N/100 g level which is used in Australia and Japan (*17*). In addition, TMN production appeared to be a good indicator of spoilage and high ($>10^6$/g) bacterial levels. Fieger and Friloux (*10*) also indicated that TMN levels in Gulf shrimp increased when bacterial levels were high. The delay in TMN production in shrimp from the Gulf of Mexico in contrast to the production reported by Flores and Crawford (*11*) for Pacific Coast shrimp suggests that Gulf shrimp do not have trimethylamine oxide.

Tissue pH was not a good indicator of the quality of shrimp stored for 3-24 h at high temperatures. Recent studies have indicated that the postmortem pH of shrimp is determined by an initial drop in pH, the magnitude of which varies with the lot of shrimp (*24*).

Urea produced by the action of arginase (E.C. 3.5.3.1, L-arginine amidinohydrolase) could serve as a source for ammonia production. Urease (E.C.3.5.1.5, urea amino-hydrolyase) activities have been reported for crustaceans by several investigators (*3, 6, 12, 14, 15, 18-21*). Bacterial breakdown of urea may be an important source of ammonia, particularly during prolonged ice storage. Increased urea levels due to holding shrimp at high temperatures for short times could cause more rapid quality deterioration.

The present results indicate that TVN and the TVN-AA-N ratio are good indicators of changes in penaeid shrimp quality due to high temperature storage. In addition, TMN appears to be a good indicator of shrimp spoilage. If the shrimp had been subjected to a high temperature storage and then placed on ice, TVN would be a less reliable indicator than the TVN-AA-N ratio because of the washing action of melting ice (*7*).

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# SDS-Polyacrylamide Electrophoresis Pattern Alteration of Myofibrillar Proteins after Heating of Avian Leg and Breast Muscle [1]

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**ABSTRACT**

The SDS-polyacrylamide disc gel electrophoresis patterns of myofibrillar proteins from leg and breast avian muscle heated to 45, 50, 55, and 60 C were evaluated. Most of the detected bands were tentatively identified on the basis of molecular weight calculated from their Rm (relative mobility). Heating the tissue caused denaturation of protein which resulted in reduced intensity and ultimate disappearance of the bands as temperature increased. Bands identified as myosin were quite sensitive to heat compared to the greater resistance of the G-actin band.

Myofibrillar proteins are extremely important in determining postmortem tissue characteristics. These proteins are largely responsible for the muscle's water holding capacity (*10*); their postmortem alterations have a major influence on tissue tenderness (*29*) and their changes during heating regulate quality characteristics of cooked meat products (*23*).

Several studies (*8, 11, 33*) have evaluated the influence of heat on isolated myofibrillar proteins. Samejima (*27*) has shown that the effect of heat is different when the proteins are heated in the more complex structure of the tissue. The objectives of this research were to evaluate the influence of heating on the individual myofibrillar proteins in situ and to compare these proteins from two different types of avian muscle (light and dark). Other research (*16*) has indicated a difference in sarcoplasmic proteins from light and dark muscle.

The use of SDS (Sodium Dodecyl Sulfate)-polyacrylamide electrophoresis has proved (*31*) useful in separating individual myofibrillar proteins that differ in molecular weight. This technique has permitted isolation of new fractions as well as myofibrillar components previously separated by other techniques (*21*).

The treatment temperature range used in this study is based on previous research (*17*) of the loss of solubility during heating of avian muscle proteins.

## METHODS

Muscels from the breast (light) and leg (dark) of six domesticated White Leghorn chickens were removed 24 h (after rigor) postmortem. Samples (5 g) of ground tissue were placed in tubes and heated for 30 min in a water bath regulated at 45, 50, 55 or 60 C. The treatment temperature was terminated by removing the tubes from the hot water and placing them in an ice bath.

The myofibrillar proteins were extracted after the heat treatment by the method of Helander (*13*) as previously described (*17*). After extraction the 1.3 ionic strength of the extracting solution (1.0 M phosphate buffer, 1.1 M KCl) was lowered to .08 by diluting with distilled water. The precipitated myofibrillar proteins were separated by centrifugation and recovered in suspension with 3 ml of .01 M sodium phosphate

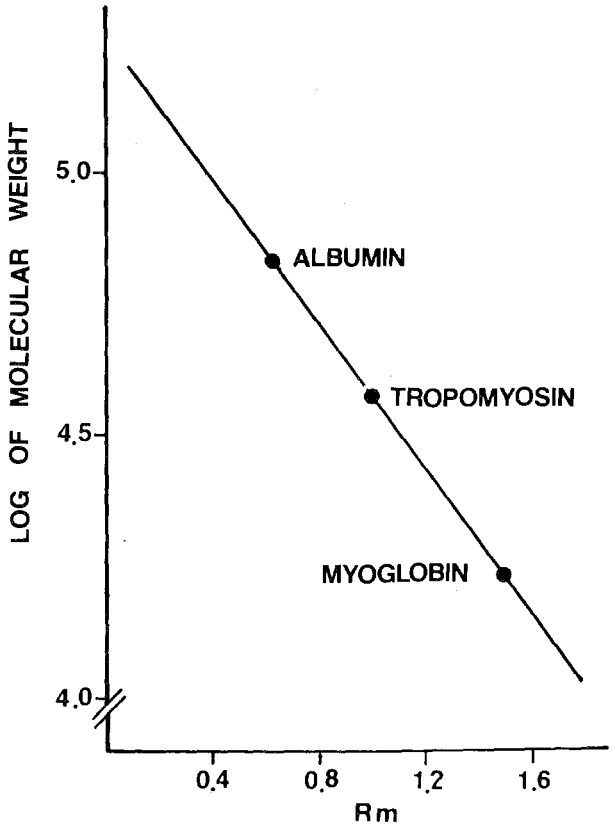


Figure 1. *Reference curve used for evaluation of molecular weight based on the Rm of the proteins separated using 8.5% polyacrylamide with SDS.*

[1] *Research conducted in Cordoba with statistical analysis and translation done in Columbus.*

[2] *Approved for publication as Journal Article No. 126-76 of The Ohio Agricultural Research and Development Center, Wooster, Ohio 44691.*

buffer (pH 7.1). Two ml of 0.1 M sodium phosphate buffer containing 2.5% of SDS and 2.5% of β-mercoptoethanol were added to the recovered proteins. This yielded a 1%SDS and 1% β-mercoptoethanol in the mixture. This solution was incubated 30 min at 40 C and 5 ml of 60% glycerol was added to increase the density. Volumes of 150 μl (containing 50 μg of proteins in control samples) were used for electrophoresis.

The gels were prepared as described by Hay et al. (*12*) and electrophoresis was done with a Canalco instrument applying a constant current of 5 mA per tube. Proteins used as reference for evaluation of molecular weight (MW) included bovine albumin (Calbiochem, A. Grade, MW-69,000), myoglobin (MW-17,000) isolated by water extraction and polyacrylamide electrophoresis separation in the laboratory from bovine diaphragm muscle and the tropomyosin (MW-36,000) appearing in the gels [Rm value of 1 (*12*)]. Figure 1 shows the curve used for calculating the MW of different fractions based on their Rm (Relative mobility) value. Molecular weights estimated from Rm values on SDS-polyacrylamide electrophoresis closely approximate values calculated using more complex techniques, with a maximum error of 10% (*31*).

## RESULTS

Figures 2 and 3 illustrate SDS-polyacrylamide gel electrophoresis patterns and band diagrams of myofibrillar proteins of avian muscle tissue after various temperature treatments. The relationship between Rm value and calculated molecular weight as well as tentative identification of these bands and the reference sources are shown in Table 1.

The most noticeable effect on heating to 45 C was the clearing of a moderately intense zone between Rm values of 0.15 and 0.45.

Heating to 50 C caused a considerable reduction in the intensity of the myosin band in both types of muscle. In breast muscle a band with Rm 1.7 (9,000 MW) disappeared.

Heating the breast muscle to 55 C caused the disappearance of the bands corresponding to myosin, α-actinin, M-protein, β-actinin and the 30,000 daltons protein. In dark muscle tissue, the myosin, M-protein and β-actinin disappeared and there was a reduction in the α-actinin and the 30,000 daltons component. In both types of tissue other bands were reduced in intensity.

After heating to 60 C there was almost total destruction of the myofibrillar proteins with only two weak bands remaining in the electrophoresis gel at the actin and tropomyosin sites.

## DISCUSSION

The slight difference in electrophoresis patterns noted between chicken leg and breast muscle tissue corresponds to those reported by Hay et al. (*12*).

The most obvious effect on the electrophoresis pattern due to the heat treatment was the progressive reduction of protein bands as the temperature increased and the almost total disappearance of the bands after heating to 60 C. This corresponds with previously observed losses in solubility (*17*) at elevated temperatures.

Woods (*32*) and Samejima et al. (*25*) indicated that after heating light meromyosin fragment #1 in solutions,

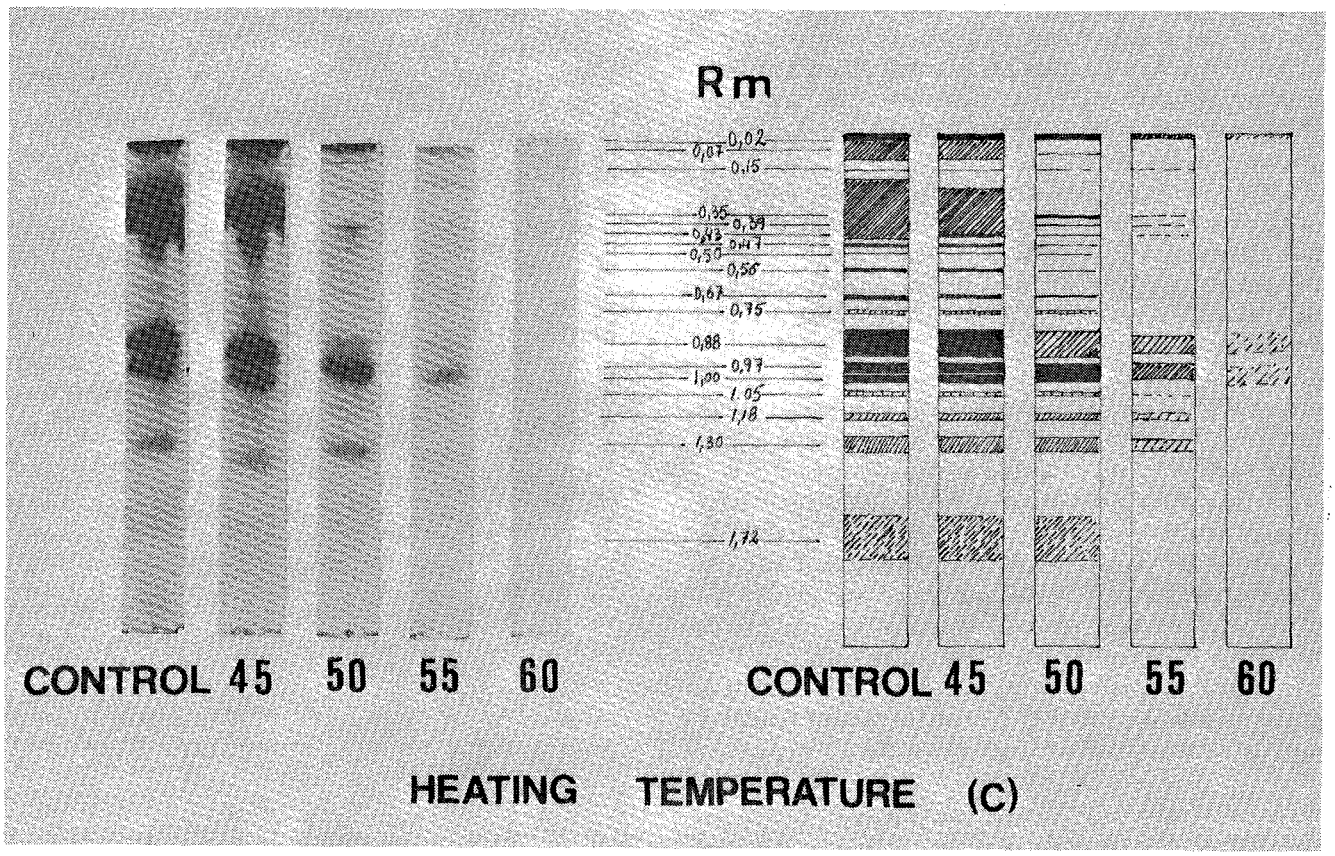


Figure 2. *Electrophoretic pattern and band diagram of proteins of myofibrillar fraction from chicken breast muscle after heating to different temperatures.*

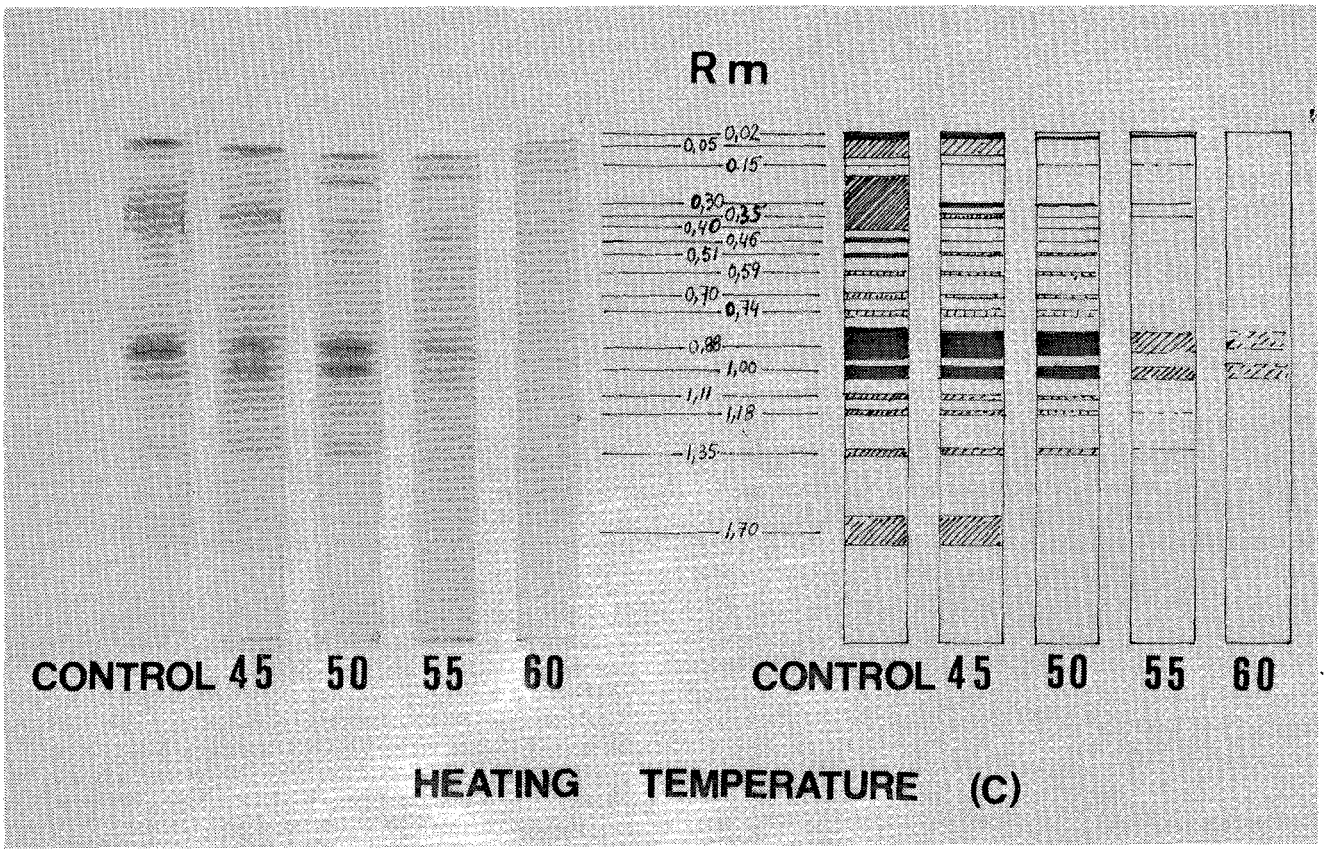


Figure 3. *Electrophoretic pattern and band diagram of myofibrillar proteins from chicken leg muscle after heating to different temperatures.*

viscosity decreased which they attributed to a depolymerizing effect. This effect would have led to the appearance of new bands after heating in our experiment. In more recent reports, Samejima and Yasui (*26*) have replaced the depolymerization theory with one involving the action of residual trypsin used in the preparative process.

The extreme sensitivity to heat of myosin in this study corresponds to the instability of this protein reported previously. Myosin in solution is very unstable even at 20 C and neutral pH (*15*). Smith (*30*) reported that rabbit myosin in solution was denatured at 40 to 45 C. When myosin was in combination with actin, as actomyosin, it was necessary to heat to 45 to 53 C for 3 to 12 min to obtain the same effect. These observations seem to indicate a protective effect of actin. In this research the

TABLE 1. *Relative mobility (Rm) value, calculated molecular weight and tentative identification of myofibrillar protein bands of leg and breast chicken tissue.*

| Rm | | Calculated molecular weight | Tentative identification | References |
|---|---|---|---|---|
| Leg | Breast | | | |
| .02 | .02 | 275,000 | — | |
| .07 | .05 | 213,000-220,000 | Myosin (heavy chains) | (*3, 14*) |
| .15 | .15 | 182,000 | *a*-actinin 6-S | (*6*) |
| — | .30 | 136,000 | — | |
| .35 | .35 | 123,000 | — | |
| .39 | .40 | 115,000-113,000 | *a*-actinin | (*9*) |
| .43 | — | 106,000 | — | |
| .47 | .46 | 99,000-100,000 | M-protein | (*4, 20*) |
| .50 | .51 | 93,500-91,000 | — | |
| .56 | .59 | 83,500-78,000 | | |
| .75 | .70 | 58,000-63,000 | *a*-actinin | (*19*) |
| .88 | .88 | 45,000 | G-actin | (*7, 12*) |
| .97 | — | 38,500 | TN-B[1] | (*2, 5*) |
| 1.00 | 1.00 | 36,000 | Tropomyosin | (*12*) |
| 1.05 | 1.11 | 32,000-29,000 | Degradation | (*27*) |
| 1.18 | 1.18 | 25,500 | LC-1[2] | (*28*) |
| 1.30 | — | 20,000 | LC-2[2] or TN-C[1] | (*2, 28*) |
| — | 1.35 | 18,000 | LC-2[2] or TN-C[1] | (*2, 28*) |
| 1.72 | 1.70 | 9,000-9,500 | — | |

[1]TN: tropomyosin
[2]LC: myosin light chains

actin band proved to be quite resistant.

The effect of heat on the remaining myofibrillar proteins has received very sparse attention in the literature. Arakawa et al. (*1*) have reported on the thermal behavior of troponin-tropomyosin system and $\alpha$-actinin. They reported that these proteins lost their activity after 16 h of heating at 40 C when the pH was 5.7. They also stated that with pH at 7.0 the $\alpha$-actinin was protected but the activity was lost for the troponin-tropomyosin.

Protein stability when subjected to heat is pH dependent, as pointed out by Penny (*22*) and Young (*34*), who reported this when studying the heat denaturation of actin and actomyosin. Probably the difference in original pH between the leg and breast muscle of the chicken (*17*) could explain the slight differences in changes in electrophoresis patterns encountered in this system after heating to 55 C.

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# Present Status of and Trends in Tuberculosis and Brucellosis in Cattle

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ABSTRACT

Tuberculosis and brucellosis have been greatly reduced in the cattle population. The reduction has led to complacency in the eradication effort. In each of these cooperative State-Federal Eradication Programs there have been modifications of program procedures as husbandry practices changed. Surveillance at slaughter is currently the basis for location of herds affected with either of these diseases. A lesion resembling a tubercle found by the meat inspector on postmortem examination starts a tracing procedure leading to a tuberculin test of the herd from which the lesioned animal originated. A blood sample for brucellosis is also collected from adult cattle at slaughter and sent to the laboratory. Any reactors likewise are traced and herds tested. Effectiveness of the surveillance is dependent on accurate identification of slaughter animals. This has improved, but needs further improvement. Dairy herds benefit from additional surveillance through the milk ring test which has proven to be very effective when conducted three or four times each year on bulk milk samples collected at dairy plants. Use of *Brucella abortus* strain 19 vaccine decreased with reduction of Federal funds supporting vaccine purchase and administration. Vaccination is currently being encouraged especially for cattle moving into the more heavily infected areas in the South. New immunizing agents and diagnostic procedures are being studied for brucellosis. The comparative cervical test is being widely used to eliminate problems relating to sensitivity to mycobacteria other than those causing tuberculosis in cattle.

We in the Animal and Plant Health Inspection Service are concerned with eliminating the organisms capable of causing brucellosis and tuberculosis from the milk and food products available to the population. Our effort in cooperation with each of the States and the livestock industry is directed at eradicating the diseases from the livestock population. We appreciate the solid support from others who have a responsibility in this industry.

There has been a tremendous reduction in both tuberculosis and brucellosis in the cattle population. The number of tuberculous herds has been below 100 for each of the last 10 years; less than 50 in recent years. The incidence of brucellosis nationwide is about 0.5% of the total cattle population. With both diseases, most herds currently being found infected are beef cattle. Looking back a few years, it appears that we expected to glide through to eradication with little effort. We should have known that finding the last infected animal is the most difficult.

Program procedures in each of these cooperative State-Federal programs have been adjusted to changes in husbandry practices. The major change has been that both campaigns now depend on screening of cattle at slaughter. For tuberculosis, the meat inspector examines the carcass for lesions resembling tuberculosis; for brucellosis, a blood sample is collected and sent to a cooperative State-Federal laboratory for serological testing.

Success of the screening is dependent on proper identification of the animal and thus providing the ability to trace the infected animal to its herd of origin. Although approximately 12 million adult cattle are slaughtered with special identifying back tags, there needs to be an improvement in the precision of the identification.

## CONTROL OF TUBERCULOSIS

As a result of post mortem inspection at slaughter, 1518 lesions which in some aspect resembled tuberculosis were sent by meat inspectors to our Veterinary Services Laboratory for further examination. The large number of samples submitted is encouraging. It indicates that meat inspectors are aware that although they have a very high rate of accuracy in diagnosing tuberculosis, an occasional lesion not grossly identifiable as tuberculosis will, in fact, be a tubercle. In 1975, 200 of the lesions were tubercles and tracing to the herd of origin was inaugurated (Fig. 1).

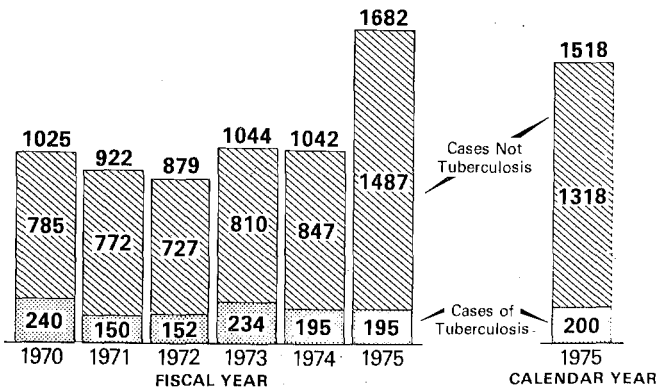



Figure 1. *Tuberculosis traceback investigations, regular kill.*

It is unfortunate that 71 of these cases could not be traced to the herd from which they originated.

The success in tracing the infected animal is very dependent on the accuracy of identification of the animal when it was started on its movement to slaughter. Properly identified, 81% of the herds were quickly located, without identification, 37%. Not only is the inability to find the infected herd a problem, but the tremendous number of man-hours spent in trying to find the herd of origin of the diseased animal is a serious waste of resources. It may take 100 times longer to find the source of the inadequately identified animal and over and above that is the frustration of coming up at a dead end.

Although this is a disappointing effectiveness, 79% of the adult animals showing lesions were traced to their herd of origin. The poorest tracing ability occurred in the animals which had been in feed lots. A number of these were of Mexican origin (Fig. 2). Accuracy in identification is improving as a result of a regulation now in effect which requires all adult cattle moving interstate to slaughter be identified with a black tag capable of identifying the herd of origin. It has been recommended by the U.S. Animal Health Association that each State have a comparable requirement for intrastate movements. Several have such requirements.

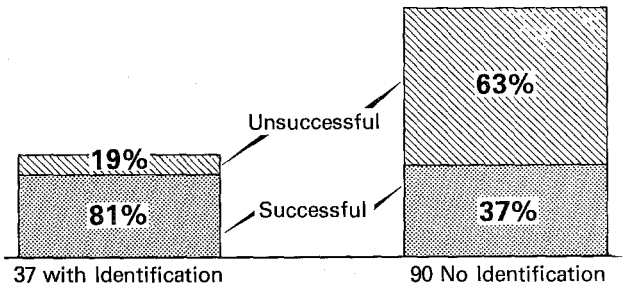



Figure 2. *Two hundred tuberculosis cases (regular kill), animals identified and unidentified, calendar year 1975.*

Tracing animals found tubercular at slaughter led to 14 of the 41 herds found infected in 1975. Epidemological tracing of animals sold from these herds led directly to an additional 13 herds. Eight were the result of retest of herds previously infected. Only one herd was found tuberculous as a result of testing to meet milk ordinance requirements (Fig. 3).

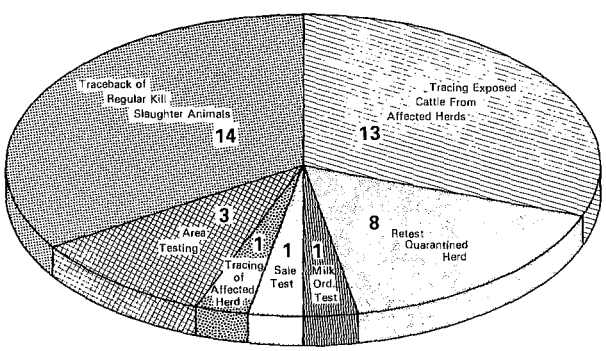



Figure 3. *Methods of locating tuberculous herds initially detected in calendar year 1975.*

In recent years one of the most beneficial adjustments to the program has been depopulation of the entire herd when there is evidence of spread of tuberculosis within the herd. Tuberculosis can be a slow-moving chronic disease or it may be relatively acute. Depopulation of the negative animals that had been in association with the diseased animals eliminates any further spread of the disease. The herd owner is indemnified for the negative exposed cattle which are slaughtered under inspection. During 1975, 50 herds were known to be infected. This included those known infected from previous years; 26 were completely depopulated (Fig. 4).

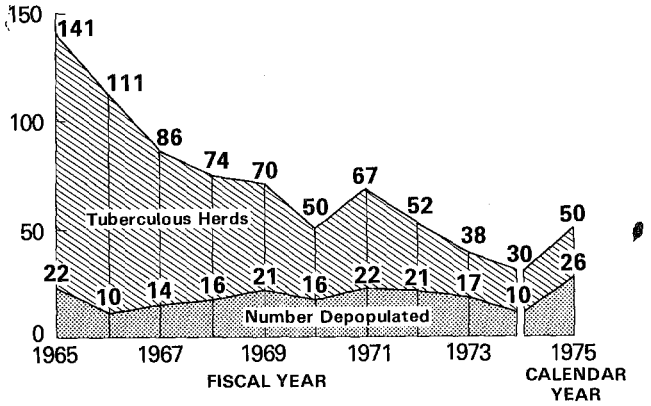
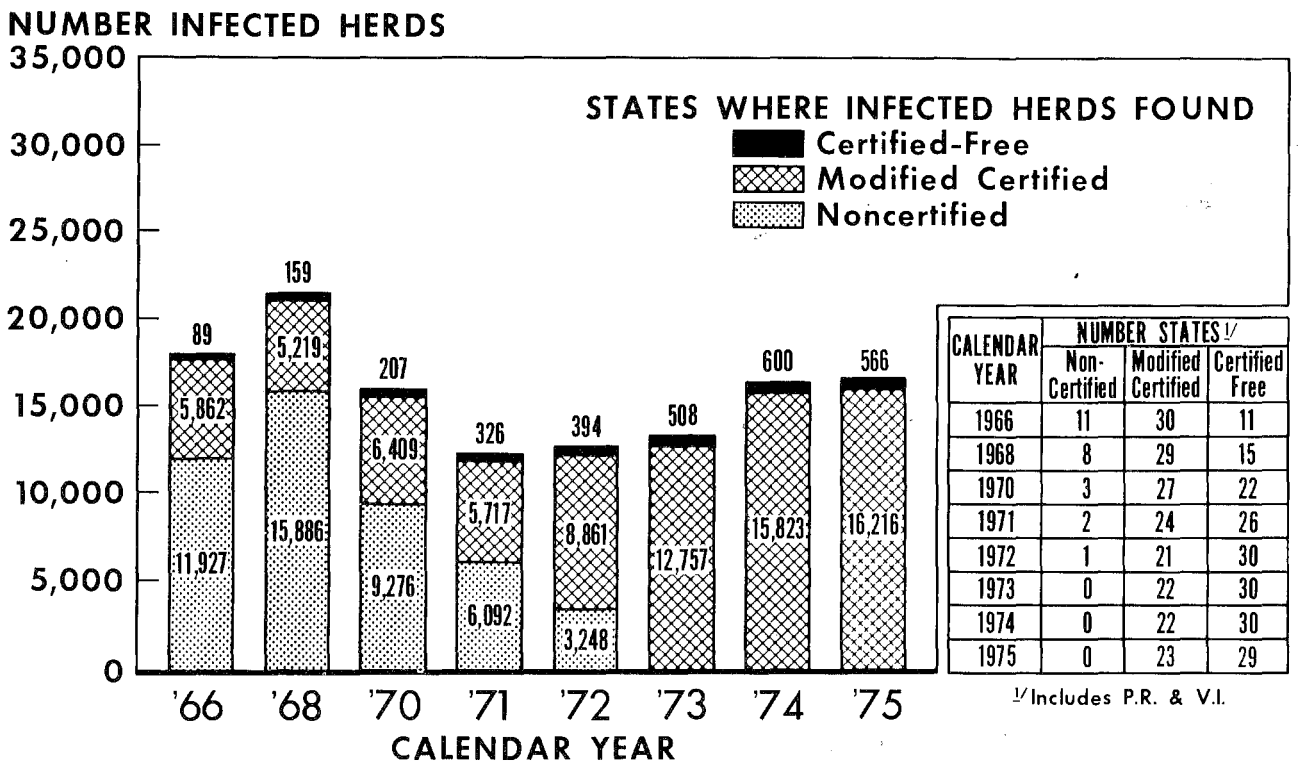



Figure 4. *Number of tuberculous herds and those depopulated, 1965 through calendar year 1975.*

There have been some problems associated with diagnosis of bovine tuberculosis. A very small percentage of tuberculous cattle do not respond to a tuberculin test. They can cause problems, because they may spread the disease to other cattle in the herd. Unfortunately, this is the way that type of animal is most often located. When a herd is depopulated, we may find a non-reactor with extensive lesions on post mortem inspection. In contrast, we have the animal responding to a tuberculin test in which no lesions are disclosed on post-mortem examination. In an infected herd, this is understandable; the lesions could be very early infection, microscopic lesions. The greater problem has been responses to the tuberculin test as a result of exposure to organisms related to but other than the cause of bovine tuberculosis. One of the more serious of these is the organism causing avian tuberculosis or closely related organisms. These are the 'no gross lesion cases' which have been not only an inconvenience, but an economic problem to the producer and regulatory agencies. This problem is being reduced and will be nearly eliminated as greater use is made of a comparative cervical test. In this test, biologically balanced tuberculins, one produced from *Mycobacterium bovis,* and one produced from *Mycobacterium avium,* are injected into adjacent areas in the neck of the animal. Skin thickness measurements are recorded before the tuberculins are injected intradermally and again when the results are read at 72 h. Animals showing a response to the avian tuberculin greater than the response to mammalian tuberculin are




| CALENDAR YEAR | NUMBER STATES 1/ | | |
|---|---|---|---|
| | Non-Certified | Modified Certified | Certified Free |
| 1966 | 11 | 30 | 11 |
| 1968 | 8 | 29 | 15 |
| 1970 | 3 | 27 | 22 |
| 1971 | 2 | 24 | 26 |
| 1972 | 1 | 21 | 30 |
| 1973 | 0 | 22 | 30 |
| 1974 | 0 | 22 | 30 |
| 1975 | 0 | 23 | 29 |

1/ Includes P.R. & V.I.

Figure 5. *Brucellosis infected herds found in non-certified, modified certified, and certified-free states.*

considered to be free of bovine tuberculosis. It is working effectively; we have not had outbreaks of bovine tuberculosis in herds where these responding animals were allowed to remain. The test is done on cattle which show a suspicious response to the standard mammalian tuberculin caudal fold tuberculin test when there is no reason to suspect that the herd is infected. To avoid interference from the previous tuberculin injection, the comparative test must be done within 10 days of the previous test or delayed for 60 days. This procedure has been adopted by the U.S. Animal Health Association and is now incorporated in the program procedures.

## CONTROL OF BRUCELLOSIS

Brucellosis eradication has been through an obstacle course for a few years. There was no smooth glide to completion of the eradication, but there was complacency. Brucellosis funds and manpower were diverted to emergency activities, one after another, for several years. The incidence increased; States that had been without infected herds or very nearly free of the disease again found infected animals resulting from movements of animals from areas where less progress in eradication has been made. This reversal in the trend in 1972 must be used as a learning experience. There was an increase from about 12,000 infected herds in 1971 to over 16,000 in 1975 (fig. 5). It is obvious that brucellosis eradication or for that matter eradication of any disease is not an easy task. There is again a slow decrease in the number of infected herds, but there can be no relaxation in the effort until eradication is achieved.

In spite of the bleak picture there has been tremendous progress in eradication of brucellosis. When organized testing was begun in the mid 1930's, about 11% of the cattle tested were found infected. Today about 0.5% of the cattle and less than 1% of the herds in the nation are infected.

The South Central and Gulf States have the highest rate of infection. Some of these States did not exert the early effort that was expended in the nothern States which had a high level of disease at the inauguration of the program. In some of the northern States, nearly one half of the herds were infected; they cleaned up. In 1975 one State, Texas, had 35% of the infected herds found in the nation, but even with that number there has been progress made in Texas. A review of the 85 counties in Texas with the highest infection at the time the first test of the county was completed revealed over 22,000 infected herds in those counties. In April, 1976 those same counties had just over 2,000 infected herds, a 90% reduction. It is encouraging that 27 States each had less than 30 infected herds during the year; together, they account for 2% of the nation's infected herds. Some States have been without a single infected herd for several years (Fig. 6).

The increasing number of infected herds throughout the country in recent years is being found as a result of increased surveillance through the market cattle testing program, by improving efficiency at the packing plant in blood sample collection, and perhaps of greater importance, collection of blood samples of cattle moving through markets. Testing the live animal at the market

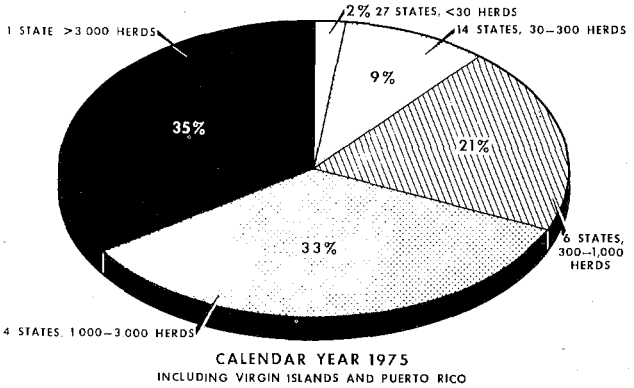



Figure 6. *Distribution of brucellosis reactor herds (percent of total reactor herds found).*

has the added benefits of improved identification of the herd of origin, because the animal has not yet changed ownership and when the infected cow and exposed animals from the same source are taken out of marketing channels at that point, they do not contribute to spread of brucellosis to a clean herd. Over 13.5 million cattle were bled in the market cattle testing program in 1975. Nearly 5 million of these were live cattle tested at markets (Fig. 7). There is duplication in that some cattle going to slaughter are bled at the market and again at the slaughterhouse. This is acceptable; limited duplication is preferable to missing a part of the population.

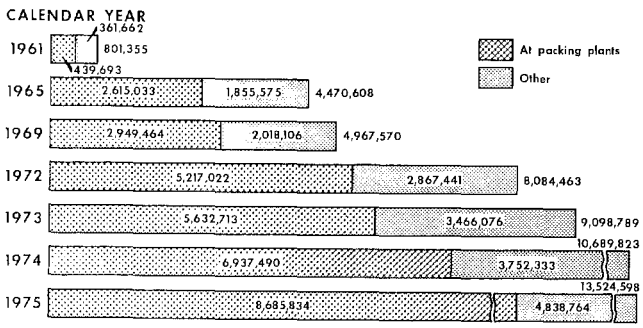



Figure 7. *Market cattle brucellosis testing program (cow's blood tested).*

With dairy cattle, we have an excellent surveillance system to supplement slaughter sampling, the Milk Ring test. It is done with no inconvenience to the dairyman, three or four times a years. It is inexpensive; it is sensitive. Perhaps the sensitivity could to some extent be considered a disadvantage since about one half of the herds showing a positive ring test reveal no infected cattle when the herd is blood tested. Efforts have been made to increase the specificity of the test without success. Reduction of the sensitivity to the point that it would allow false negatives would be hazardous. The test has been adjusted to changes in management of dairy herds. When the test was incorporated into program procedures in 1952, most herds were relatively small; samples were collected from cans as milk was delivered to the dairy plant. Today the cans have become valuable antiques; the samples are collected from the same sample the plant uses for quality, milkfat, or solids determination. The test can be adjusted to detect an infected cow in a maximum of 750 milking cows.

The Milk Ring test is without a doubt the greatest single factor contributing to reduction of brucellosis in the dairy cattle population. It has eliminated the repeated testing of herds free of the disease and it provides assurance that the herd is free of brucellosis. During 1975, 0.28% of the tests were positive which, when considering the false positives, would indicate 0.14% of the tests revealed infected herds (Fig. 8).

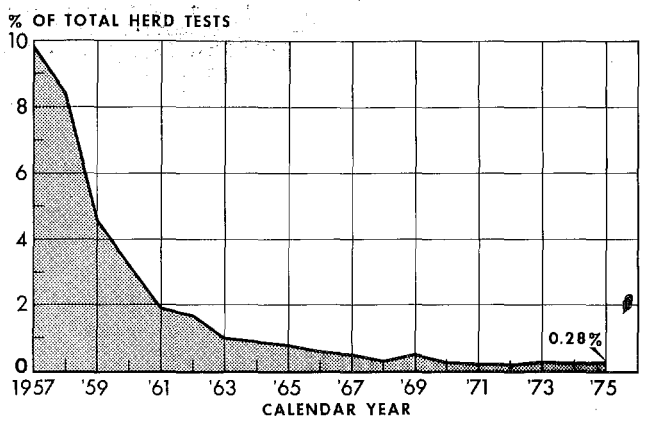



Figure 8. *Milk ring testing. Percentage of positive tests.*

Use of *Brucella abortus* strain 19 vaccine in the eradication of brucellosis has been controversial since its introduction into program procedures in 1941. Some States encouraged its use, some discouraged vaccination. For a dozen or more years, the U.S. Department of Agriculture made available vaccination of heifer calves at no cost to the owner in States desiring that support. As high as 90% of the calves were vaccinated in a few States. Considerably less vaccination was done in the southern States. In 1964, about 7.25 million calves were vaccinated. That was the highest number ever reached in any year; even this was less than 50% of the heifers to be retained as breeding animals. Included in that percentage were many heifers going into feedlots, vaccinated to make subsequent movement within regulations easier. They did not contribute to building a more resistant adult cattle population (Fig 9).

In the years after 1964, intensification of hog cholera eradication and emergencies drew some support from brucellosis eradication; some program activities had to be curtailed. Several States had reduced brucellosis to a very low level. Titers resulting from vaccination were confusing diagnosis. Market cattle testing was developing and needed additional funds. Accordingly, it was agreed that support of calf vaccination would be reduced, market cattle surveillance would be increased, and stricter controls applied to movements of cattle which had been exposed to brucellosis. Withdrawal of funds supporting vaccination did reduce the number of calves vaccinated. Surveillance did not increase as rapidly as planned, nor were exposed animals adequately controlled. In spite of continuing to recommend vaccination where there was a high probability of

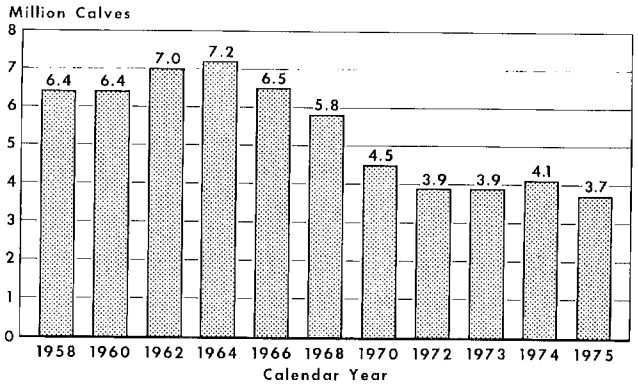



Figure 9. *Calves vaccinated in brucellosis eradication program.*

exposure, lack of Federal funds to provide vaccine and its administration to calves slowed the use of vaccine, especially in the South where it was and still is especially desirable. For the past 2 years, the Department has been urging increased vaccination of heifer calves unless the State feels vaccination is not desirable. There has been special emphasis on vaccination of dairy heifers which have a possibility of moving into the southeastern States. California has reemphasized its requirement that any dairy cattle imported into the State must have been officially calf-vaccinated.

Changes in dairy cattle management have resulted in larger herds. Concentration of large numbers of cattle in small areas is conducive to a more rapid spread of brucellosis within a herd. Even though *Brucella abortus* strain 19 vaccine is only 65% effective in increasing resistance, it has been beneficial in slowing the spread of brucellosis in a herd. In general, herds with high level vaccination have been more readily freed of disease than the herds with little or no vaccination.

Some herds respond very slowly or fail to respond to efforts to eradicate the disease. In the low incidence States, depopulation of the dairy herd is less appealing to the dairyman than to most beef producers, because of the limitation on Federal indemnity of $50.00 for grade cattle or $100.00 for registered cattle. Although this is adequate for most beef herds, replacement of dairy cattle can rarely be accomplished with the salvage received from slaughter plus indemnity. Most States supplement Federal indemnity, but even then the total received is considerably below replacement cost of a good dairy cow.

Progress made in eradication of brucellosis supports the belief of most scientists that brucellosis can be eradicated with proper application of the information available today. Since inauguration of the program, however, we have been seeking means to make eradication an easier process. Introduction of Strain 19 vaccine in 1941, after its development and extensive field testing by the Department, and incorpation of the Milk Ring test in 1952, modified from Danish procedures, and the development of more specific serological tests in the 1960's, are examples of those efforts. We are continuing to search for better methods. Since 1963, we have been conducting controlled studies and field trials with a widely publicized vaccine, a killed *Brucella abortus* strain 45/20; we have not found it advantageous either as an aid to diagnosis as reported in some countries or as an immunizing agent. We are currently conducting trials using Strain 19 vaccine in adults cows. These results should be available later this year. We have a study under contract with the University of California, Davis, using a French vaccine, killed *Brucella melitensis* strain H 38, which has been reported to free infected cattle of brucellosis. At Michigan State University, we have a contract to study the possibility of development of rapid resistance in cattle which may be exposed. This could be useful in the outbreak of brucellosis in an area free of the disease. In the event these studies bring forward new advantageous tools, they would be incorporated into program procedures. The Department is currently sponsoring a Review of the Cooperative State-Federal Brucellosis Program by a technical commission of independent scientists and also by the National Academy of Science. Their reports should be available in about 18 months. Their recommendations will be a factor in the direction of future program activities. Until then it is planned that current procedures be continued. To most effectively use the resources available, there will be selective intensification of activities dependent on the incidence of brucellosis in the States with the greatest infection remaining. There will be continuing effort on the control of movement of exposed cattle. This should give greater protection to the areas with very little disease, allowing them to be freed of the disease. In the high incidence States the intensification of effort will be on calf vaccination, developing a population with greater resistance in preparation for intensification of testing and removal of infected animals. Those States with levels of infection varying between low and high will systematically move into increasing program activity. Through such procedures as testing all cattle at markets and tracing to herds of origins with test and removal of reactors, it is anticipated that about 3 years in a State will reduce the incidence of brucellosis to a very low level at which the disease can readily be eradicated. The livestock industry is supporting this proposed plan. Two States will begin the intensified program in 1976. In each succeeding year, an additional three or four States will begin the intensive program. The last States will enter an intensified testing program in 1981. After several years of intensive vaccination, brucellosis should be eradicated by 1988.

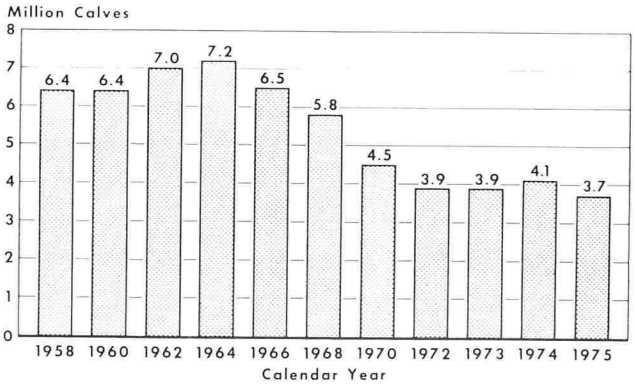



Figure 9. *Calves vaccinated in brucellosis eradication program.*

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*Journal of Food Protection Vol. 40, No. 4, Pages 270-271 (April, 1977)*


# Request for Comments on Method to Detect Penicillin in Raw Milk as Proposed for Inclusion in *Standard Methods for the Examination of Dairy Products*

J. W. MESSER[1], L. L. CLAYPOOL[2], G. A. HOUGHTBY[1], E. M. MIKOLAJCIK[3], E. L. SING[4], and E. H. MARTH[5]

The Intersociety Council approved for publication the following proposed addition to *Standard Methods for the Examination of Dairy Products.*

## CHAPTER 9.
## DETECTION OF INHIBITORY SUBSTANCES IN MILK

9.6 Special Methods

9.64 Qualitative agar diffusion test to detect penicillin in raw milk. A sensitive qualitative agar diffusion test (Delvotest P) to detect penicillin in raw milk has been described (*5*) and has been successfully used in some laboratories (*3, 4*).

9.641 EQUIPMENT AND SUPPLIES

a) *Seeded agar ampules:* Ampules of plain agar seeded with *Bacillus stearothermophilus* var. *calidolactis.* Store at 0-4.4 C.

b) *Nutrient tablets:* Each containing 0.5 mg of tryptone, 5.0 mg of glucose, 2.0 mg of nonfat dry milk and 0.025 mg of bromcresol purple. Store at room temperature.

c) *Forceps:* For transferring nutrient tablets.

d) *Dosing syringe with single service disposable tips:* For sampling and dispensing 0.1-ml samples of milk.

e) *Penicillinase, conc*: Available from biological supply houses. Store at 0-4.4 C.

f) *Water bath*: Thermostatically controlled.

g) *Metal or wire rack*: For holding ampules.

h) *Non-fat dry milk*: Antibiotic-free.

i) *Penicillin:* Crystalline sodium or potassium penicillin G. Store at -10 C. Do not use beyond expiration date.

9.642 CONTROLS

As a check on the proper functioning of reagents both penicillin-positive and -negative control samples must be included with each series of samples tested by the screening and confirmatory procedures. A single positive and negative control will suffice for each series of samples tested.

9.6421 *Negative control:* The negative control is prepared as follows: Autoclave 100-ml aliquots of reconstituted (100 g in 1000 ml of distilled water) antibiotic-free non-fat dry milk in screw capped Pyrex dilution bottles for 10 min at 121 C. Cool rapidly and store at 0-4.4 C. When tested, this milk must produce yellow coloration of the entire solid test medium.

9.6422 *Positive control:* The penicillin-positive control is prepared by dissolving an accurately weighed portion of crystalline sodium or potassium penicillin G in sufficient 1% phosphate buffer, pH 6.0 (2 g of dipotassium phosphate and 8 g of monopotassium phosphate per liter) to give a known concentration of 100-200 units per ml. This solution may be stored refrigerated for no more than 2 days. From this solution, prepare a reference concentration of 0.006 unit per ml with antibiotic-free reconstituted non-fat dry milk (9.6421). Dispense 5 ml into 15 × 100-mm tubes. Cap and freeze at -10 C. This positive control may be stored at -10 C for 6 months (1). For use thaw at room temperature. When tested this milk must produce purple coloration of the entire solid test medium.

9.643 PROCEDURE

9.6431 *Screening procedure:* Place the required number of ampules for the samples to be tested in a suitable rack. Identify each ampule legibly and indelibly. Remove the

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[2]*Mid-America Farms, Inc., Springfield, Missouri 68505.*
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[4]*Mosely Lavoratories, Inc. Indianapolis, Indiana 46201.*
[5]*Department of Food Science, University of Wisconsin, Madison, Wisconsin 53706.*

tip of each ampule. With the clean forceps, add one nutrient tablet to each ampule. With the syringe, add 0.1 ml of a well mixed (5.062) milk sample or positive or negative control sample to each identified ampule. Use a clean disposable tip for each sample and control transferred. (Note: Extreme care must be taken not to contaminate the multiuse syringe when sampling and dispensing). Place the rack containing the ampules in a water bath at 63-66 C for 2.5 h. Remove the rack containing the ampules from the water bath and observe the color of the solid medium. A purple coloration of the entire or part of the solid test medium in any of the milk sample ampules indicates the presence of substances inhibitory to the test organism. Samples of raw milk which give a purple coloration of part or all of the solid medium must be heated to 82 C for 2-3 min (2), cooled, and retested as in 9.6432.

9.6432 *Confirmatory procedure:* Add 0.05 ml of penicillinase conc (9.641e) to 5 ml of the heated, cooled test sample(s) found positive in 9.6432. Inoculate separate identified ampules with 0.1 ml of the penicillase-treated, and untreated heated sample. Incubate as in 9.6432.

9.6433 *Interpretation:* A yellow coloration of the entire solid medium of the test ampule containing the penicillinase-treated portion of the sample and purple coloration of part of or the entire solid medium containing the untreated heated portion of the same sample is a positive test for penicillin. (Note: If the sample contains heat stable inhibitory substances and pencillin, a false-negative penicillin confirmation by this test may occur). A purple coloration of part of or the entire solid medium of the ampule containing the penicillinase-treated portion of the sample and a purple coloration of part of or the entire solid medium of the ampule containing the untreated heated portion of the same sample may indicate the presence of an inhibitory substance. Assay by other methods before interpretation.

## DISCUSSION

This proposed addition to *Standard Methods for the Examination of Dairy Products* describes a new more sensitive qualitative test to detect penicillin in raw milk which requires less time, is practical, and is readily adoptable by dairy laboratories.

## COMMENTS REQUESTED

Interested persons are invited to submit comments and data in writing regarding this proposal within 90 days after publication of this announcement. Comments should be addressed to Dr. E. H. Marth, Chairman, Intersociety Council on Standard Methods for the Examination of Dairy Products, Department of Food Science, University of Wisconsin, Madison, Wisconsin 53706, USA.

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*Journal of Food Protection Vol. 40, No. 4, Pages 272-273 (April, 1977)*


*A Field Topic*

# Freezing Points and Milk Adulteration

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*The Pennsylvania State University*
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(Received for publication August 18, 1976)

ABSTRACT

The freezing point study of raw and retail milk samples as reported in the *Journal of Milk and Food Technology* (July, 1976) has continued for retail samples. The Pennsylvania Department of Agriculture adopted a regulation which considers milk with a freezing point of above -0.525 C to be adulterated. A list of causes of added water was prepared and distributed to producer and processor groups in Pennsylvania.

Results of a freezing point study of 2,000 herd samples of milk and 243 samples of processed milk were reported in the July, 1976 issue of this journal. Checking of retail samples has continued with results each month quite variable. From 0 to 22% of the samples have had freezing points above -0.525 C. Almost 500 additional retail samples have been checked than were earlier reported. There are indications that similar results could be found in other states than Pennsylvania.

Two things have been done as a result of the freezing point study. First the Pennsylvania Department of Agriculture adopted a regulation which states that milk found to have a freezing point above -0.525 C shall be considered adulterated, unless proven to be free of added water. This applies to both herd and retail samples. The burden of proof is on the dairy farmer or processor. No supervised milking samples are required.

Both dairy farmer and processor groups supported the regulation when it was proposed. The regulatory agency has enforced this freezing point standard since its' adoption in the spring of 1976.

The second result of the study was the preparation of a list of possible causes of added water. It has been distributed to producer and processor groups in Pennsylvania and is available throughout the northeastern states as a guideline of the Northeast Dairy Practices Council.

## CAUSES

1. Access to various sources of feed and water does not usually change the freezing point. As long as cattle have reasonable access to the necessary quantities of feed and water, freezing points are within acceptable limits. Milk from cows which do not have access to adequate feed or water may show a different freezing point. However, unless they are in a starved condition, the freezing point usually remains acceptable. Changes in the types of feed may have a slight effect, but milk will still have a freezing point within the normal range.
2. Sickness or disease does not usually change the freezing point of milk from an individual cow or a herd. This includes mastitis where the appearance and composition of the milk may differ greatly from normal.
3. Milking systems should be properly installed to permit good drainage. Joints and gaskets should give a relatively smooth inner surface. Slopes for permanent lines should be at least 1 ½ inches per 10 ft.
4. Plastic milk or conveyor hoses should drain to the floor. They must be disconnected from the bulk tank during all rinsing and washing cycles. A warm air drier should be used daily on all lengths of plastic hose which are more than 8 ft long.
5. Use proper procedures for washing udders and teats. Wash with a sanitizer solution and paper towels or cow cloths. Dry udder and teats by removing all excess moisture.
6. Follow accepted procedures for rinsing, washing, and sanitizing the pipeline milker or transfer station. Disconnect the pipe from the bulk tank before starting. Do not attempt to rinse the line and then shift the pipe when milky color disappears.
7. Do not dip teat cups in water or sanitizer unless the vacuum is off.
8. Do not rinse off the top of a bulk tank with a rinse hose. Water may splash under the covers or porthole lids. Use a sponge or paper towel to remove spilled milk.
9. Leave the bulk tank valve open after washing and sanitizing to let solutions drain before adding milk.
10. Disconnect the hose to the bulk tank truck before rinsing the bulk tank with water. Instruct milk haulers not to permit entry of any water into milk.
11. In plants be sure to time all changes when you follow milk with water or vice versa. This may vary from 2 to 10 min. Water inadvertently left in lines, tanks, and equipment sometimes means that first filled containers may show added water.
12. Do not use paper gaskets which may prevent proper operation of air operated valves.
13. Install processing equipment such as clarifier, separator, pumps, lines, and balance tank to permit maximum draining.
14. Avoid diversion of HTST once milk enters the system until all water has been flushed from the balance tank and entered the

system.

15. Allow equipment to drain almost completely before following with water.
16. Observe automatic cleaning systems to be sure cycles are completed and all tanks drain thoroughly.
17. Prevent moisture condensation on ceilings or piping from dripping into milk in bulk tanks or processing vats. Eliminate piping over milk tanks with covers.
18. Use moisture-free equipment for sampling milk. Rinse a dipper in milk at least twice before collecting a sample. Use sampling containers which do not leak.
19. Prevent all means of water getting into milk by intentional or accidental means.
20. The practice of mixing vitamin concentrates with 5 to 10 gal. of water before adding it to milk storage tanks should be changed.

## CONCLUSIONS

Added water is both illegal and an economic loss to the dairy industry. Receiving plants should institute and carry out programs for checking samples of all loads, and if necessary individual producer samples. Follow up on all suspicious cases and determine the causes. Take positive action to correct and prevent any added water in milk.

ACKNOWLEDGMENTS

Presented at the 63rd Annual Meeting of the International Association of Milk, Food, and Environmental Sanitarians, Arlington Heights, Illinois, August 8-11, 1976.

*Journal of Food Protection Vol. 40, No. 4, Pages 274-276 (April, 1977)*


# 3-A Sanitary Standards for Colloid Mills

Number 36-00

*Formulated by*
*International Association of Milk, Food and Environmental Sanitarians*
*United States Public Health Service*
*The Dairy Industry Committee*

It is the purpose of the IAMFES, USPHS, and DIC in connection with the development of the 3-A Sanitary Standards program, to allow and encourage full freedom for inventive genius or new developments. Colloid mill specifications heretofore or hereafter developed which so differ in design, material, and construction, or otherwise, as not to conform to the following standards but which, in the manufacturer's or fabricator's opinion, are equivalent or better, may be submitted for the joint consideration of the IAMFES, USPHS, and DIC at any time.

A.

*SCOPE*

A.1

These standards cover the sanitary aspects of colloid mills for processing liquid dairy products.

A.2

In order to conform with these 3-A Sanitary Standards, colloid mills shall comply with the following design, material and fabrication criteria.

B.

*DEFINITIONS*

B.1

*Product:* Shall mean the dairy product which is processed in the colloid mill.

B.2

*Colloid Mills:* (Referred to hereinafter as "mills") Shall mean equipment which will produce a colloidal suspension.

B.3

*Product Contact Surface:* Shall mean all surfaces that are exposed to the product and surfaces from which liquids and/or solids may drain, drop, or be drawn into the product.

B.4

*Non-Product Contact Surface:* Shall mean all other exposed surfaces.

B.5

*Mechanical Cleaning or Mechanically Cleaning:* Shall denote cleaning, solely by circulation and/or flowing chemical detergent solutions and water rinses onto and over the surfaces to be cleaned, by mechanical means.

B.6

*Engineering Plating:* Shall mean plated to specific dimensions or processed to specified dimensions after plating.[1]

C.

*MATERIALS*

C.1

Product contact surfaces shall be of stainless steel of the AISI 300 series[2] or corresponding ACI[3] types (see Appendix, Section E.), or metal which under conditions of intended use is at least as corrosion-resistant as stainless steel of the foregoing types, and is non-toxic and non-absorbent except that:

C.1.1

Bushings, rotors and stators may also be made of (1) non-toxic hardenable, corrosion-resistant stainless metal which may be covered with an engineering plating of nickel or chromium or (2) hardenable carbon steel covered with an engineering plating of nickel or chromium.

C.1.2

Rubber and rubber-like materials may be used for gaskets, seals, and parts used in similar applications.

C.1.3

Rubber and rubber-like materials when used for the above specified applications shall comply with the applicable provisions of the 3-A standard for rubber and rubber-like materials, Number 18-00.

C.1.4

Plastic materials may be used for gaskets, seals, and parts used in similar applications.

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[1] *QQ-C-320a-Federal Specification for Chromium Plating (Electrodeposited), July 26, 1954. (Available from General Services Administration, Seventh and D Streets N.W., Room 1643, Washington, D.C.].*
*QQ-N-290-Federal Specifications for Nickel Plating (Electrodeposited), April 5, 1954, and Amendment 1, December 13, 1961, (Available from General Services Administration, Seventh and D Streets N.W., Room 1643, Washington, D.C.).*

[2] *The data for this series are contained in the following reference: AISI Steel Products Manual, Stainless & Heat Resisting Steels, December 1974, Table 2-1, pp. 18-19. Available from: American Iron & Steel Institute, 1000 16th Street N.W., Washington, D.C. 20036.*

[3] *Alloy Casting Institute Division, Steel Founders' Society of America, 20611 Center Ridge Road, Rocky River, Ohio 44116.*

C.1.5

Plastic materials when used for the above specified applications shall comply with the applicable provisions of the 3-A standard for plastic materials, Number 20-00, as amended.

C.1.6

Where materials having certain inherent functional properties are required for specific applications, such as seals, carbon and/or ceramic materials may be used. Carbon and ceramic materials shall be inert, non-porous, non-toxic, non-absorbent, insoluble, resistant to scratching, scoring, and distortion when exposed to the conditions encountered in the environment of intended use and in cleaning and bactericidal treatment.

C.2

Non-product contact surfaces shall be of corrosion-resistant material or material that is rendered corrosion-resistant. If coated, the coating used shall adhere. Non-product contact surfaces shall be relatively non-absorbent, durable and cleanable. Parts removable for cleaning having both product contact and non-product contact surfaces shall not be painted.

D.

*FABRICATION*

D.1

Product contact surfaces shall be at least as smooth as a No. 4 finish on stainless steel sheet free of imperfections such as pits, folds and crevices. (See Appendix, Section F.).

D.2

Permanent joints in product contact surfaces shall be continuously welded. Welded areas shall be at least as smooth as a No. 4 finish on stainless steel sheets free of imperfections such as pits, folds and crevices.

D.3

Product contact surfaces not designed to be mechanically cleaned shall be easily accessible for cleaning and inspection either when in an assembled position or when removed. Removable parts shall be readily demountable.

D.4

Mills that are to be mechanically cleaned shall be designed so that all product contact surfaces of the mill and all non-removable appurtenances thereto can be mechanically cleaned and are easily accessible for inspection.

D.5

Product contact surfaces shall drain when the mill is disassembled.

D.6

Connections in product contact surfaces shall conform to the applicable provisions of the 3-A standard for sanitary fittings, Number 08-17 and/or to the applicable provisions for welded sanitary product-pipelines found in the 3-A accepted practice for permanently installed sanitary product-pipelines, Number 605-02.

D.7

Thermometer connections, when provided, shall conform to the applicable provisions of the 3-A standard for instrument fittings, number 09-07.

D.8

*Radii*

Internal angles of 135° or less on product contact surfaces shall have radii of not less than 1/4 inch except:

D.8.1

Smaller radii may be used when required for space or essential functional reasons such as rotor serations.

D.8.2

The radii in gasket grooves or gasket retaining grooves other than those for standard 1/4 inch and smaller O-Rings shall be not less than 1/8 inch.

D.8.3

The radii in grooves for standard 1/4 inch O-Rings shall be not less than 3/32 inch and for standard 1/8 inch O-Rings shall be not less than 1/32 inch.

D.8.4

When the functional reasons the radius must be less than 1/32 inch, in such applications as flat sealing surfaces, the product contact surface of this internal angle must be readily accessible for cleaning and inspection.

D.9

Gaskets shall be removable. Gasket retaining grooves shall be no deeper than their width.

D.10

There shall be no threads on product contact surfaces.

D.11

Coil springs having product contact surfaces shall have a least 3/32 inch openings between coils, including the ends when the spring is in a free position.

D.12

Shaft seals shall be of the packless type, sanitary in design with all parts readily cleanable.

D.13

The minimum thickness of engineering plating shall be 0.0002-inch for all product contact surfaces except that when the parts listed in C.1.1 that are to be plated are other than stainless steel, the minimum thickness of the engineering plating shall be 0.002-inch.

D.14

*Supports:* The means of supporting a mill shall be one of the following:

D.14.1

*With legs:* Legs shall be smooth with rounded ends and have no exposed threads. Legs made of hollow stock shall be sealed. Legs shall provide a clearance between the lowest part of the mill, with the exception of the leg itself, and the floor of at least 4 inches when the mill outlines an area in which no point is more than 12½ inches from the nearest edge or a clearance of at least 6 inches when any point is more than 12½ inches from the nearest edge.

D.14.2

*With casters:* Mills which are portable may be

equipped with casters. Casters shall be easily cleanable, durable and of a size that will permit easy movement of the mill.

D.15

Guards required by a safety standard that will not permit accessibility for cleaning and inspection shall be designed so that they can be removed without the use of tools.

D.16

Non-product contact surfaces shall be readily cleanable and shall be free of pockets and crevices and those to be coated shall be effectively prepared for coating.

## *APPENDIX*

E.

*STAINLESS STEEL MATERIALS*

Stainless steel conforming to the applicable composition ranges established by AISI[2] for wrought products, or by ACI[3] for cast products, should be considered in compliance with the requirements of Section C.1 herein. Where welding is involved the carbon content of the stainless steel should not exceed 0.08 percent. The first reference cited in C.1 sets forth the chemical ranges and limits of acceptable stainless steel of the 300 series. Cast grades of stainless steel corresponding to types 303, 304, and 316 are designated CF-16F, CF-8, and CF-8M, respectively. These cast grades are covered by ASTM[4] specifications A296-68 and A351-70.

F.

*PRODUCT CONTACT SURFACE FINISH*

Surface finish equivalent to 150 grit or better as obtained with silicon carbide, is considered in compliance with the requirements of Section D.1 herein.

These standards are effective August 24, 1977.

[4] *Available from American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.*

*Journal of Food Protection Vol. 40, No. 4, Pages 277-279 (April, 1977)*


# 3-A Sanitary Standards for Continuous Blenders

Number 35-00

*Formulated by*
*International Association of Milk, Food and Environmental Sanitarians*
*United States Public Health Service*
*The Dairy Industry Committee*

It is the purpose of the IAMFES, USPHS, and DIC in connection with the development of the 3-A Sanitary Standards program, to allow and encourage full freedom for inventive genius or new developments. Continuous blender specifications heretofore or hereafter developed which so differ in design, material and construction, or otherwise, as not to conform to the following standards but which, in the manufacturer's or fabricator's opinion, are equivalent or better, may be submitted for the joint consideration of the IAMFES, USPHS, and DIC at any time.

A.

*SCOPE*

A.1

These standards cover the sanitary aspects of continuous blenders used for combining and/or mixing either wet or dry, (1) dairy products or (2) a dairy product(s) with an edible non-dairy product(s) and includes that portion of any part integral with the blender such as hoppers and valves, which is in contact with the product. It does not pertain to batch-type blenders.

A.2

In order to conform with these 3-A Sanitary Standards, continuous blenders shall comply with the following design, material and fabrication criteria.

B.

*DEFINITIONS*

B.1

*Product:* Shall mean the dairy product(s) and/or other ingredient(s) which are combined and/or mixed in this equipment.

B.2

*Continuous Blenders:* (Referred to hereinafter as "blenders") Shall mean equipment in which two or more products are continuously added, the products combined and/or mixed by mechanical means and the blend of products discharged continuously.

B.3

*Product Contact Surface:* Shall mean all surfaces that are exposed to the product and surfaces from which liquids and/or solids may drain, drop, or be drawn into the product.

B.4

*Non-Product Contact Surface:* Shall mean all other exposed surfaces.

B.5

*Mechanical Cleaning or Mechanically Cleaning:* Shall denote cleaning, solely by circulation and/or flowing chemical detergent solutions and water rinses onto and over the surfaces to be cleaned, by mechanical means.

C.

*MATERIALS*

C.1

Product contact surfaces shall be of stainless steel of the AISI 300 series[1] or corresponding ACI[2] types (See Appendix, Section E.), or metal which under conditions of intended use is at least as corrosion-resistant as stainless steel of the foregoing types and is non-toxic and non-absorbent, except that:

C.1.1

Rubber and rubber-like materials may be used as a coating or covering on rotors and may be used for gaskets, seals, wiping paddles, and parts used in similar applications.

C.1.2

Rubber and rubber-like materials when used for the above specified applications shall comply with the applicable provisions of the 3-A standard for rubber and rubber-like materials, Number 18-00.

C.1.3

Plastic materials may be used as a coating or covering on rotors and may be used for gaskets, seals, wiping paddles, bearings, augers, inspection portcovers, and parts used in similar applications.

C.1.4

Plastic materials when used for the above specified applications shall comply with the applicable provisions of the 3-A standard for plastic materials, Num-

[1]*The data for this series are contained in the following reference: AISI Steel Products Manual, Stainless & Heat Resisting Steels, December 1974, Table 2-1, pp. 18-19. Available from: American Iron & Steel Institute, 1000 16th Street, N.W. Washington, D.C. 20036.*

[2]*Alloy Casting Institute Division, Steel Founders' Society of America, 20611 Center Ridge Road, Rocky River, OH 44116.*

ber 20-00, as amended.

C.1.5

Rubber and rubber-like materials and plastic materials having product contact surfaces that are a coating or a covering, shall be of such composition as to retain their surface and conformation characteristics when exposed to the conditions encountered in the environment of intended use and in cleaning and bactericidal treatment.

C.1.6

Where materials having certain inherent functional properties are required for specific applications, such as seals, carbon and/or ceramic materials may be used. Carbon and ceramic materials shall be inert, non-porous, non-toxic, non-absorbent, insoluble, resistant to scratching, scoring, and distortion when exposed to the conditions encountered in the environment of intended use and in cleaning and bactericidal treatment.

C.1.7

Silver soldered or brazed areas and silver solder or braze material shall be non-toxic and corrosion-resistant.

C.2

Non-product contact surfaces shall be of corrosion-resistant material or material that is rendered corrosion-resistant. If coated, the coating used shall adhere. Non-product contact surfaces shall be relatively non-absorbent, durable and cleanable. Parts removable for cleaning having both product contact and non-product contact surfaces shall not be painted.

D.

*FABRICATION*

D.1

Product contact surfaces shall be at least as smooth as a No. 4 finish on stainless steel sheets free of imperfections such as pits, folds and crevices. (See Appendix, Section F.).

D.2

Permanent joints in metallic product contact surfaces shall be continuously welded. If it is impractical to weld, they may be silver soldered or brazed. Welded areas on product contact surfaces shall be at least as smooth as a No. 4 finish on stainless steel sheets free of imperfections such as pits, folds and crevices.

D.3

Rubber or rubber-like materials and plastic materials having product contact surfaces that are a coating or covering shall be bonded in such a manner that the bond is continuous and mechanically sound, and so that when exposed to the conditions encountered in the environment of intended use and in cleaning and bactericidal treatment, the rubber or rubber-like material or the plastic material does not separate from the base material.

D.4

Product contact surfaces not designed to be mechanically cleaned shall be easily accessible for cleaning and inspection either when in an assembled position or when removed. Removable parts shall be readily demountable.

D.5

Blenders that are to be mechanically cleaned shall be designed so that all product contact surfaces of the blender and all non-removable appurtenances thereto can be mechanically cleaned and are accessible for inspection.

D.6

Product contact surfaces of non-removable parts shall be self-draining except for normal clingage.

D.7

Fittings and valves having product contact surfaces that are furnished by the blender manufacturer shall conform to the applicable provisions of the 3-A Standard for sanitary fittings, Number 08-17 and/or to the applicable provisions for welded sanitary product-pipelines found in the 3-A accepted practice for permanently installed sanitary product-pipelines, Number 605-02, as amended.

D.8

Instrument connections, when provided, shall conform to the applicable provisions of the 3-A Standard for instrument fittings, Number 09-07.

D.9

Internal angles of 135° or less on product contact surfaces shall have radii of not less than 1/4 inch, except smaller radii may be used when required for space or functional reasons such as in sealing ring grooves and in the way the rotor fits. When the radius is less than 1/32 inch, the product contact surface of the internal angle must be readily accessible for cleaning and inspection.

D.10

Gaskets shall be removable. Gasket retaining grooves shall be no deeper than their width. The radius of any internal angle in a gasket retaining groove shall be not less than 1/8 inch, except that the radius may be 3/32 inch where a standard 1/4 inch O-Ring is to be used and the radius may be 1/32 inch where a standard 1/8 inch O-Ring is to be used.

D.11

There shall be no threads on product contact surfaces, except where necessary for attaching the rotor to the shaft. When a thread is necessary it shall be an Acme thread, an illustration of which is shown on 3-A drawing number 3A-100-30 in the 3-A standard for sanitary fittings, Number 08-17. The thread shall have (1) not more than 10 threads per inch and (2) basic major diameter of not less than 1/2 inch. The length of the nut shall not exceed three-quarters of the thread basic major diameter and the nut shall be of the open type.

D.12

Coil springs having product contact surfaces shall have at least 3/32 inch openings between coils, including the ends when the spring is in a free position.

D.13

Openings through either hinged or removable covers, to which conections are not permanently attached, shall be flanged upward at least 3/8 inch. All sanitary pipelines and other appurtenances furnished by the blender manufacturer that will enter through the cover shall be fitted with a permanently attached sanitary umbrella deflector that tightly overlaps the edges of the opening. Other openings shall have a removable cover.

D.14

Covers shall pitch to an outside edge(s).

D.15

Agitators, including the complete seal, shall be readily demountable for cleaning. Non-removable parts having product contact surfaces shall be designed so that the product contact surfaces are readily cleanable and inspected from the inside of the blender. Seals for an agitator shaft shall be of a packless type, sanitary in design, with all parts readily accessible for cleaning.

D.16

*Supports:* The means of supporting a blender shall be one of the following:

D.16.1

*With legs:* Legs shall be smooth with rounded ends and have no exposed threads. Legs made of hollow stock shall be sealed. Legs shall provide a clearance between the lowest part of the blender, with the exception of the leg itself, and the floor of at least 4 inches when the blender outlines an area in which no point is more than 12½ inches from the nearest edge or a clearance of at least 6 inches when any point is more than 12½ inches from the nearest edge.

[3]*Available from the American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.*

D.16.2

*With casters:* Blenders which are portable may be equipped with casters. Casters shall be easily cleanable, durable and of a size that will permit easy movement of the blender.

D.17

Non-product contact surfaces shall be readily cleanable and shall be free of pockets and crevices, and those to be coated shall be effectively prepared for coating.

## *APPENDIX*

E.

*STAINLESS STEEL MATERIALS*

Stainless steel conforming to the applicable composition ranges established by AISI[1] for wrought products, or by ACI[2] for cast products, should be considered in compliance with the requirements of Section C.1 herein. Where welding is involved the carbon content of the stainless steel should not exceed 0.08 percent. The first reference cited in C.1 sets forth the chemical ranges and limits of acceptable stainless steel of the 300 series. Cast grades of stainless steel corresponding to types 303, 304, and 316 are designated CF-16F, CF-8, and CF-8M, respectively. These cast grades are covered by ASTM[3] specifications A296-68 and A351-70.

F.

*PRODUCT CONTACT SURFACE FINISH*

Surface finish equivalent to 150 grit or better, as obtained with silicon carbide, is considered in compliance with the requirements of Section D.1 herein.

These standards are effective August 24, 1977.

# News and Events

## "Applied Foodservice Sanitation" Taught in Korean Language

Classes in the Korean language on the "Applied Foodservice Sanitation" course, developed by the National Institute for the Foodservice Industry, are taught in a classroom at Chicago's Korean Association by instructor Hae D. Park. Forty-seven Korean foodservice operators and employees are enrolled in two classes each week. In addition to Korean, examinations for the course are available in Chinese, Italian, Greek and Spanish translations to foodservice managers and supervisors preparing for mandatory sanitation certification which becomes law in the Windy City in 1978. More than 2,500 students have been certified in Chicago, in cooperation with the Chicago Board of Health, at four City College branches: Loop, Wright, Southwest, and Olive-Harvey.

NIFI is the not-for-profit foundation established by the industry to advance foodservice management standards through education.

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## Minnesota Sanitarians Association Hold Outstate Educational Spring Meetings

*Tuesday, April 19, Albert Lea, Minnesota*

5:30 p.m. Social Hour at Universal Milking Machine Co.
6:30 p.m. Dinner at the Skyline Supper Club

*Thursday, April 21, 1977, Holiday Inn, Alexandria, Minnesota*

5:30 p.m. Social Hour
6:30 p.m. Dinner

Both programs will feature Dr. Michael Pullen, D.V.M., Associate Professor and Extension Meat Hygienist from the University of Minnesota College of Veterinary Medicine, speaking on "Trichinosis in Man and Animal." Charles B. Schneider, Minnesota Department of Health, will review the progress on "Registration of Sanitarians in Minnesota."

Members and non-members are invited to attend.

## New Hay Marketing Standards Proposed

A new system for establishing feeding value and equitable market pricing of hay has been proposed by the Hay Marketing Task Force of the American Forage and Grassland Council.

The new system consists of five market grades plus a sample (unmarketable) grade. It's meant to replace an outdated and cumbersome system devised in the 1940's, according to U.S. Department of Agriculture researcher Gordon C. Marten.

There are two primary determinants of hay quality in the new system. They are concentrations of acid detergent fiber (to estimate digestibility) and neutral detergent fiber (to estimate intake potential by ruminant animals). Other characteristics that can alter a hay grade established by these two assays are crude protein concentration, moisture level, and degree of contamination with injurious foreign material.

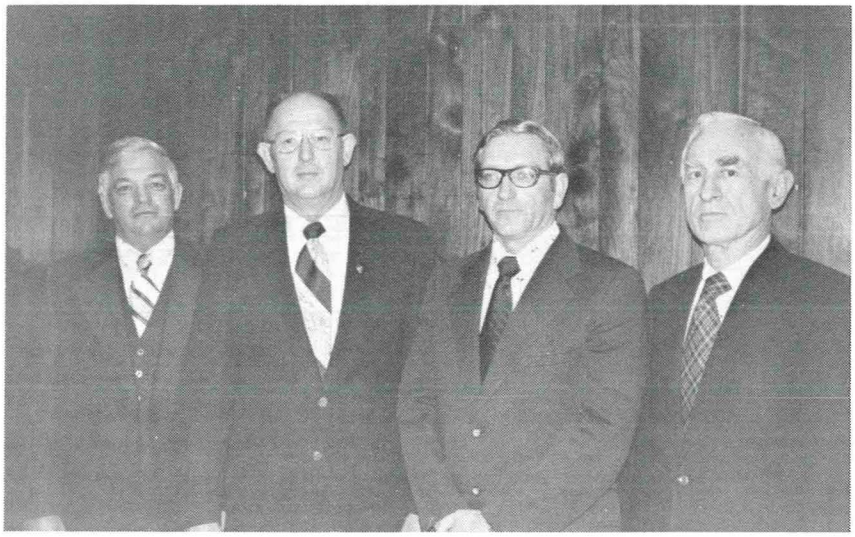
A "relative feed value" is derived from estimating digestible dry matter intake for each hay lot. Then an equitable price for the hay can be calculated from a localized base price for Grade 4 hay with a relative feed value of 100. Relative feed values would range from a high of 140 percent for early cut legumes to about 83 percent for late cut grasses.

Faster tests for fiber and crude protein are needed to make the system workable for small lots of hay in transit. Marten says a new technique called infrared reflectance analysis is being developed to meet this need.

The Federal Grain Inspection Service of USDA must react favorably to the proposal before the new quality standards can be officially used for hay marketing. Marten spoke at a recent meeting of the Minnesota Forage and Grassland Council in St. Cloud, Minn. CA.IA.D.L.

## News and Events

## National Mastitis Council Elected Officers for 1977



L to R—Boyd Cook, James Welch, Dr. R. D. Mochrie and J. C. Flake.

The National Mastitis Council held it's annual meeting in Louisville Kentucky March 21-23, 1977. James R. Welch, Klensade Products Division, Economics Laboratory, Inc., St. Paul Minnesota, was named president for the coming year. The meeting covered all segments of the Mastitis problem. Highlighting the meeting were discussion by Dr. John Milne, of the National Dairy Laboratory, Ruakurd Agriculture Centre, Hamilton, New Zealand. The meeting was well attended.

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## NCIMS Meeting

The sixteenth meeting of the National Conference on Interstate Milk Shipments will be held at Stouffer's Cincinnati Towers, Cincinnati, Ohio, May 22-26, 1977. The main topics of discussion will be a discussion of the five documents of the Food and Drug Administration and the proposed revisions of the constitution and the addition of a set of by-laws.

H. H. Vaux, Indiana State Board of Health, is Chairman of the organization. Advance registration forms and hotel room reservation cards can be secured by writing to: J. C. McCaffrey, Executive Secretary, 3306 Glouster Street, Sarasota, Florida.

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## New, Mod. IV, Shielded Ultra Pure Water Still

Details on the new, Mod. IV Ultra Pure Water Still with housing have just been released by Gilmont Instruments, Inc., Great Neck, N.Y. The Mod. IV delivers all the product purity and volume of the Mod. III (1.8 liters/hr. of up to 95% conductivity water), and has the additional advantage of a protective, acrylic cabinet. This allows for convenient portability of the still (while set-up), from one location to another. The all-acrylic enclosure provides clear observation of the operating still, while the sliding front panel permits selective adjustments during operation.

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## Calendar of Events

OCTOBER 17-20, 1977. 91st annual meeting of the Association of Official Analytical Chemists. Marriott Hotel, Twin Bridges, Washington, D.C. 20001. For further information contact: L. G. Ensminger, Executive Secretary, AOAC, Box 540 Benjamin Franklin Station, Washington, D.C. 20044.

## 1977 Annual Dairy Fieldmen's Conference

The 35th Annual Pennsylvania Dairy Fieldmen's Conference is going to be held on June 14 and 15 on the campus of The Pennsylvania State University. An evening program for June 13 is also planned. All meetings will be held in the J. O. Keller Conference Center. Topics to be included this year include plans of the Pennsylvania Department of Agriculture, the problem of added water in milk, feeding practices and milk quality and a discussion of milk pricing. Other topics will include regulatory monitoring of herd health, problems and responsibilities of milk haulers, calf housing and management, somatic cell counting problems and programs, regulatory guidelines for manure management, field programs to meet I.M.S. demands and antibiotic detection and penalty programs.

All questions related to the conference should be sent to: Agricultural Conference Coordinator, J. O. Keller Conference Center, University Park, PA 16802.

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## Edgar Davis Elected President of Food Service Group

Edgar L. Davis, director food service marketing, Berkel Inc., La-Porte, Indiana, has been elected president of the Society for the Advancement of Food Service Research.

Other officers elected at the recent 32nd conference of the organization, held at the University of Houston, Houston, Texas, are: vice president, Gordon D. Bell, national products manager, Lincoln Manufacturing Co., Fort Wayne, Indiana; and three members of the board of directors—Ronald M. Kochman of Kochman Associates, Morton Grove, Illinois; Jane Trishman, consulting dietitian, Urbana, Illinois; and Mary R. DeMarco, director of dietetics, Cleveland Metropolitan General Hospital, Cleveland, Ohio.

## News and Events

## National Association of Dairy Fieldmen Elect Officers

Back row L-R—Phillip Hermsen, Edward Kaeder, Walt Suntken, Front row L-R—Gerald Shick, Earl Brancel, Sidney Beale.

The affiliated National Association of Dairy Fieldmen held a meeting in conjunction with the National Mastitis Conference in Louisville, Kentucky.

The meeting was held February 21, 1977. The fieldmen present held a open discussion with officers and other interested people from IAMFES. It was concluded that the annual meeting of the NADF will be held in conjunction with the annual meeting of the IAMFES to be held in Sioux City, Iowa August 14-18, 1977. A special program has been planned for the fieldmen on August 17 which will be followed by their business meeting.

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## FACULTY POSITIONS

### Food Process Engineer/Food Scientist

Ph.D. with documented excellence in food engineering and food processing research. Industrial experience desirable. Undergraduate and graduate teaching, research, and service responsibilities. Rank and salary open.

### Food Science/Management

Assistant Professor: Ph.D. in Food Science with background in food systems management. Demonstrated research capability. Teaching courses in food processing, management, and quality evaluation; accompanying research and service responsibilities. Salary open.

Positions available September 1, 1977. Send resume', official transcripts, and names of four referees before April 21, 1977, to Dr. R. J. H. Gray, Chairperson, Search Committee Food Science and Human Nutrition, College of Home Economics, University of Delaware, Newark, DE 19711. (302) 738-8407.

The University of Delaware is an Affirmative Action/Equal Opportunity Employer

## Press Release

At the recent annual meeting of the National Clay Pipe Institute's Board of Directors, Darold W. Taylor was appointed vice president for government relations in the institute's Washington office.

Mr. Taylor came to the institute four years ago as their director of engineering.

Before coming to the institute, Mr. Taylor served a number of years with the U.S. Public Health Service in the engineering and environmental health field. Mr. Taylor has had wide experience in working with federal and state environmental health agencies.

The institute's Washington office is located at 1130 Seventeenth Street, N.W., Washington, D.C. 20036, Telephone (202) 296-5270.

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## CLASSIFIED ADS

### For Sale

Single Service milk sample tubes. For further information and a catalogue please write, Dairy Technology, Inc., P.O. Box 101, Eugene, Oregon 97401.

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## NOTICE

### Second Printing

"PROCEDURES TO INVESTIGATE FOODBORNE ILLNESS"

The overwhelming response to the publication of the 3rd Edition of "Procedures to Investigate Foodborne Illness' has resulted in a tempory shortage of the publication. The 3rd Edition's second printing will be off the press March 1, 1977, and orders for the publication will be filled and processed as soon as possible after that time.

The prices quoted for "Procedures" in the first printing will remain in effect for the second printing. However, the cost of pads of investigational forms has been lowered as follows:

Forms A, D, E, G and H—$2.35 per pad of 100

Forms B, C and F—$3.25 per pad of 100

# Association Affairs

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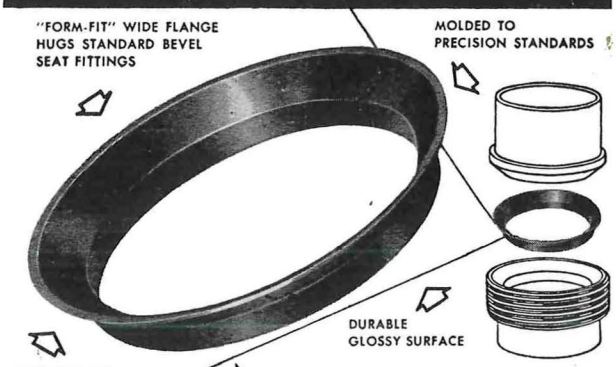


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**CLEVELAND, OHIO 44113**

# A Better Milk Harvest Through Good Milking Practices

*By Dr. John R. Campbell*
*Professor of Dairy Husbandry*
*University of Missouri—Columbia*

For the corn producer, the most important harvest he makes occurs only once a year—when he goes into the fields with his corn picker. But, for the dairyman, the most important harvest takes place two, and in some cases, three times a day, every day of the year. And, the use of good milking practices helps dairymen to have a good harvest every time they milk their cows. Additionally, a complete milk harvest today will help the cow produce more milk tomorrow.

**Milk-making Cells Work Harder With Use**

The milk-making (epithelial) cells work the hardest immediately following milking because that is when intramammary pressure is the lowest. At each milking a hormone called prolactin or lactogen is released and its effect is to cause the milk-making cells to go back to work. But, if through poor milking practices, some of the milk is left in the udder, intramammary pressure mounts faster and this, in turn, slows down milk secretion. Research indicates that milk secretion each hour following milking is approximately 90 to 95 percent of that of the preceding hour. But as the udder fills, this percentage decreases. Naturally, milk left in the udder following milking will shorten the period of time that the milk-making cells work at maximal capacity.

Repeated failure to remove milk from mammary glands causes the milk-making cells to become inactive. Thus, for maximal milk production, the milk secreting cells must be challenged... and that means removing all of the milk possible at each and every milking. Although incomplete milking will not have a big detrimental effect in one or two milkings, it sure will over a period of several days. Not only will the milk left in the udder not be harvested and, therefore, not be sold, it will, in addition, accelerate the cow's decline in level of production and, thereby contribute to unprofitable dairying.

**Persistency: A Slower Decline Means More Profit**

A cow reaches her peak production about two months into the lactation. After this, a natural, gradual decline in milk production occurs. The relationship between milk given one month compared to that produced the next is known as persistency. Persistency can be improved if good milking practices are used to assure a full harvest of the milk crop at each milking.

**Eight Steps Toward Getting a Full Milk Harvest**

Good cow milking practices include eight steps which, when done properly, will achieve the fullest possible harvest of your valuable milk crop.

**1.** Environment: Provide a comfortable, stress-free environment.
**2.** Proper Stimulation: A vigorous massage of the mammary glands will help insure complete letdown of milk.
**3.** Strip Foremilk: Stripping acts to further stimulate the cow and, at the same time, eliminates much of the bacteria-laden first milk.
**4.** Timely Application of Milking Machine: This should be done one minute after starting stimulation to take advantage of maximal letdown.
**5.** Adjust Machine: Proper forward/downward adjustment is important for complete milking.
**6.** Remove Teat Cups as Quarters Milk Out: Avoid over-milking which can lead to tissue irritation and mastitis.
**7.** Dip Teats: Teat dipping gives the teat end the protection it needs against mastitis-causing bacteria while the streak canal regains its full constriction.
**8.** Maintain Equipment Properly: Properly adjusted and maintained equipment is an essential step toward better milking.

The amount of milk a cow produces in a lactation from the time she freshens until she stops lactating is determined by a number of factors; some can be controlled—others cannot. However, good milking practices will go a long way toward helping you achieve more nearly the full potential of your cows' production. In other words, good milking will give a more complete harvest of your most important crop—and that means more profit.