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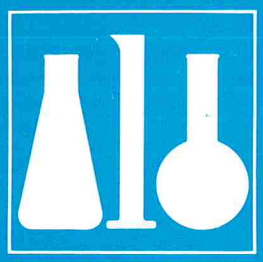

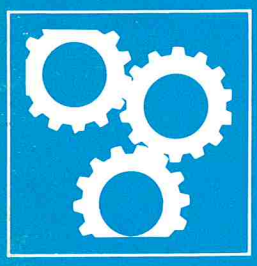

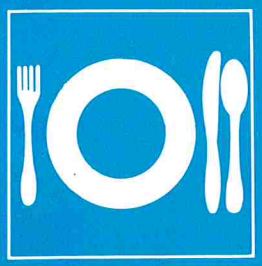

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Antimicrobial Effect of Butylated Hydroxyanisole and Butylated Hydroxytoluene on *Staphylococcus aureus*[1]

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ABSTRACT

The antimicrobial effect of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on three enterotoxigenic strains of *Staphylococcus aureus* in Brain Heart Infusion broth (BHI) was evaluated by turbidity measurements. Also, the interaction of these compounds with pH and NaCl on growth of *S. aureus* strain 100 was measured. Inhibition of *S. aureus* growth increased with an increase in the concentration of BHA and/or BHT. Complete inhibition of *S. aureus* growth occurred in BHI with 1.12 μmole of BHA/ml or 0.70 μmole of BHT/ml as well as with a combination of 0.25 μmole of both BHT and BHA/ml. Inhibition of *S. aureus* growth by BHA or BHT was substantial at pH 7.0 and with 2% NaCl. When 0.84 μmole or greater of BHA/ml and 0.47 μmole or greater of BHT/ml were added to BHI, growth of *S. aureus* 100 was inhibited to the extent that enterotoxin A could not be detected after 24 h of incubation.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used an antioxidants in foods and packaging materials. Recent reports show that these antioxidants also possess antimicrobial properties. Ward and Ward (*15*) found that a 1% concentration of BHT was slightly inhibitory to *Salmonella senftenberg*. Chang and Branen (*3*) reported that BHA had an antimicrobial effect against *Staphylococcus aureus*, enteropathogenic *Escherichia coli*, *Salmonella typhimurium* and *Aspergillus parasiticus*. *S. aureus* was the most sensitive of the bacteria tested and a 10^6 inoculum was totally inactivated by 150-200 ppm of BHA. Shih and Harris (*8*) reported that *S. aureus* and *E. coli* were inhibited in Trypticase Soy Broth (TSB) containing 100, 150, 200 and 400 ppm of BHA after 24 h of incubation. The amount of inhibition increased as the concentration of BHA increased. Recently VanTassell et al. (*14*) also reported that the growth of *S. aureus* in TSB was delayed for 36 h by 100 ppm of BHA while viable cell numbers decreased to less than 1 per ml within 3 h in the presence of 200 ppm BHA.

Microbes other than *S. aureus* are also affected by BHA and/or BHT. Surak (*12*) reported that both BHA and BHT were inhibitory to *Tetrahymena pyriformis*. Roback et al. (*6*) reported that *Vibrio parahaemolyticus* was inhibited in Trypticase Soy Broth containing 2.5% NaCl and 50 ppm BHA. Fung et al. (*4*) tested six toxigenic and six non-toxigenic strains of *Aspergillus flavus* to determine the effect of BHA and BHT on growth inhibition, spore formation, pigmentation and aflatoxin production. No inhibitory effect on growth and toxigenesis was observed with BHA (0.005-0.020 g per plate) while BHT (0.005-0.020 g per plate) gave no visible inhibitory effects.

The purpose of this study was to further define the antimicrobial properties of BHA and BHT toward *S. aureus*. The concentrations of BHA and BHT necessary to inhibit growth and enterotoxin production of *S. aureus* cultures and the influence of pH and NaCl on this inhibition were determined.

MATERIALS AND METHODS

Cultures

All enterotoxigenic strains of *S. aureus* (100, S-6, 361) used in this study were preserved in the dried form on porcelain beads using the method of Hunt et al. (*5*). Seed cultures were prepared by inoculating beads and incubating 18 h at 37 C. In the inhibition studies, BHI broth (100 ml) containing BHA and/or BHT was inoculated with a 1% inoculum from the seed cultures and incubated 24 h at 37 C.

BHA and BHT solutions and medium

The BHA and BHT used in this study was obtained from Eastman Kodak Company, Kingsport, Tennessee. One percent solutions of BHA and BHT were prepared by dissolving the antioxidants in 95% ethyl alcohol. Appropriate quantities of these solutions were added to BHI broth before sterilization at 121 C for 15 min. Preliminary experiments indicated that addition of these quantities of ethyl alcohol did not influence growth of *S. aureus*.

Growth measurement

Culture growth was measured turbidometrically using a Klett-Summerson coloriphotometer with a green filter (No. 54) of 520-580 nm wavelength. One hundred ml of BHI broth were added to a 250-ml Erlenmeyer flask with a Klett tube attached to the flask neck. The control flasks contained only BHI medium, whereas the experimental medium contained BHI plus BHA and/or BHT. The medium was inoculated, incubated without shaking and at specific intervals the flasks were agitated, tipped to fill the Klett tube and the turbidity was measured. Initial experiments indicated that maximum cell numbers and Klett readings were reached in 24 h in the control medium and in the medium containing BHA and/or BHT. Thus all experiments were terminated after 24 h and a Klett reading made at that time. Each trial was replicated and the two Klett readings were averaged. Percent inhibition was calculated by the following formula:

$$\%\text{inhibition} = \frac{(\text{Klett reading of control} - \text{Klett reading with BHA and/or BHT}) \times 100}{\text{Klett reading of control}}$$

[1]*Scientific Paper No. 4456. College of Agriculture Research Center, Pullman, WA 99164, Project 0168.*

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Enterotoxin production

After 24 h of incubation, 100 ml of the inoculated BHI broth was extracted for enterotoxin assay. The final extract volume was 0.2 ml. The extract was analyzed for enterotoxin by the microgel diffusion technique as described by Casman and Bennett (*2*). This technique has a sensitivity of 0.1 or 0.25 μg of enterotoxin per 100 g of substrate. In the work reported herein, no attempt was made to quantify the amount of enterotoxin present in the extract.

Effect of pH and NaCl

BHI broth containing BHA and/or BHT was adjusted before sterilization to pH 3, 4, 5, 6, 7, 8, or 9 using either 1.0 N NaOH or 1.0 N HCl. To determine the effect of NaCl, the BHI broth was prepared to contain 0, 2, 5, 10 or 15% NaCl and adjusted to pH 7 before sterilization.

RESULTS AND DISCUSSION

Three strains of *S. aureus* were tested against both BHA and BHT in Brain Heart Infusion (BHI) broth to determine if the antioxidants possessed antimicrobial properties (Table 1 and 2). Klett readings of the control cultures using *S. aureus* strain B were approximately 450 after 24 h of incubation at 37 C and the corresponding plate count was 3×10^{10}/ml. When 0.28 μmole and BHA/ml was added, the Klett readings were 400 after incubation and a plate count of 2×10^{9}/ml was obtained. A Klett reading of 350 was obtained and a plate count of 2×10^{8}/ml when 0.23 μmole of BHT/ml was added to the medium. When both BHA and BHT were added to the medium, Table 3, 0.28 μmole/ml and 0.23 μmole/ml, respectively, the initial Klett reading was 50 and after 60 h of incubation at 37 C, the Klett readings was still 50. The initial and concluding plate count was approximately 3×10^{7}/ml. Similar results were obtained with all three strains of *S. aureus* tested.

As the concentration of BHA or BHT was increased, the percent of inhibition also increased and total inhibition occurred with 1.12 μmoles of BHA/ml and 0.70 μmole of BHT/ml. In all the studies reported herein, BHT was more inhibitory than BHA. This observed difference in inhibitory action could be related to the greater lipolytic nature of BHT (*10*). Aaloto (*1*) also reported that as lipolytic nature of the phenols increases, the antimicrobial activity also increases. Similarly, Snipes et al. (*9*) reported that the ability of BHT to inactivate viruses was related to its lipolytic properties and its ability to associate with the lipid-containing membranes of the viruses. However, Fung et al. (*4*) reported BHA was more inhibitory to *A. flavus* than BHT. This is probably partially due to the lack of lipid material on the cell wall of *A. flavus*. These same workers also reported that BHA may be altering cell permeability, thus allowing leakage of macromolecules and may interact with cell membrane protein to cause disruption of membrane structure.

A combination of BHA and BHT had a greater inhibitory effect against *S. aureus* strain 100 than when either BHA or BHT was added alone (Table 3). Although 0.5 μmole of BHA/ml showed a 50% inhibition, a combination of 0.1 μmole of BHA/ml with 0.4 μmole of BHT/ml or 0.28 μmole of BHA/ml and 0.23 μmole BHT/ml resulted in 100% inhibition. The reason for this increased activity is not known; however, a similar synergistic effect has been noted in antioxidant activity when 0.1% BHA plus 0.1% BHT were used (*11*).

Detectable levels of enterotoxin A were produced by *S. aureus* strain 100 after 24 h in BHI broth alone, whereas enterotoxin was not detected after 24 h when 0.89 μmole or greater of BHA/ml, or 0.45 μmole or greater of BHT/ml, or a combination of 0.28 μmole BHA/ml plus 0.23 μmole BHT/ml were added.

Table 4 contains measurements of *S. aureus* strain 100 when incubated 24 h at 37 C in BHI medium adjusted to pH 3, 4, 5, 6, 7, 8 and 9 when 0.56 μmole BHA/ml or 0.45 μmole BHT/ml were added. Best growth occurred in the control medium (no BHA or BHT present) at pH 8 and 9 with gradual decrease down to pH 5 and then a slight increase in growth was noted at pH 3 and 4. When BHA or BHT were included in the medium, the least amount of inhibition occurred at the extreme pH values (3 and 9). Approximately 40 to 80% inhibition occurred when pH was between 5 and 8 with the 80% occurring with BHT at pH 7. Tompkin et al. (*13*) also reported

TABLE 1. *Inhibitory effect of BHA on three toxigenic strains of* Staphylococcus aureus *incubated 24 h at 37 C*[a].

BHA		% Inhibition		
ppm	mole/ml	100	S-6	361
0.0	0.00	0[b]	0	0
50	0.28	22	10	28
75	0.42	51	60	61
100	0.56	66	72	87
125	0.70	84	90	90
150	0.84	93	97	95
175	0.98	93	97	95
200	1.12	100	100	100

[a]BHI broth used at pH 7.0.
[b]Each value in the calculated mean of two replications.

TABLE 2. *Inhibitory effect of BHT on three toxigenic strains of* Staphylococcus aureus *incubated 24 h at 37 C*[a].

BHT		% Inhibition		
ppm	mole/ml	100	S-6	361
0.0	0.00	0[b]	0	0
50	0.23	42	23	39
75	0.35	65	56	76
100	0.45	97	93	94
125	0.58	97	93	94
150	0.70	100	100	100

[a]BHI broth used at pH 7.0.
[b]Each value is the calculated mean of two replications.

TABLE 3. *Inhibitory effect of the combination of BHA and BHT on the growth of* Staphylococcus aureus *strain 100 incubated 24 h at 37 C*[a].

mole/ml		
BHA	BHT	% Inhibition
0.0	0.0[b]	0
0.5	0.0	50
0.4	0.1	78
0.3	0.2	85
0.2	0.3	93
0.1	0.4	100
0.0	0.5	95
0.5	0.1	100
0.25	0.25	100

[a]BHI broth used at pH 7.0.
[b]Each value is the calculated mean of two replications.

TABLE 4. *Inhibitory effect of 0.56 μmole BGA/ml or 0.45 μmole BHT/ML in Brain Heart Infusion broth at different pH values on the growth of* Staphylococcus aureus *strain 100 incubated 24 h at 37 C*[a].

pH	Klett reading			Percent inhibition
	Control	BHI + BHA	BHI + BHT	
3	220	180		18
4	220	100		56
5	100	60		40
6	120	40		67
7	300	140		53
8	450	240		47
9	420	380		10
3	420		380	10
4	220		160	27
5	30		10	67
6	150		100	33
7	150		30	80
8	450		200	56
9	450		400	11

[a]Each value is the calculated mean of two replications.

maximum growth and enterotoxin A inhibition of *S. aureus* strain 100 at pH 7.0 in a medium containing sodium nitrite. The inhibition decreased as the medium was made more acidic or basic.

The data in Table 5 are the Klett readings and percent inhibition when *S. aureus* strain 100 was grown in BHI medium (pH 7.0) containing various concentrations of NaCl and BHA or BHT. As the NaCl concentration increased from 0 to 15%, without addition of BHA or BHT, the growth rate of *S. aureus* decreased, especially at the 10 and 15% levels of NaCl. The addition of 0.56 μmole BHA/ml, in the absence of NaCl, resulted in a 19% inhibition of growth. However, when 2% NaCl was included with the BHA, a 95% reduction in growth occurred and approximately the same reduction occurred when 5, 10 and 15% NaCl was included. When 0.45 μmole BHT/ml was added, an 87% reduction in growth occurred (Table 5). When NaCl was included, the percent inhibition ranged from 73 to 97%.

Addition of 2% or more NaCl enhanced the antimicrobial activity of BHA on *S. aureus* strain 100. The inhibitory effect of BHA, when in the presence of 2% NaCl, could be of importance in many food products since a 2% concentration of NaCl is commonly used (*7*). More research is necessary to determine the interactive effects of antioxidants, NaCl and pH on inhibition of *S. aureus*.

In conclusion, these studies indicate that BHA and BHT have marked antimicrobial effects on growth of three strains of *S. aureus* and enterotoxin production of one strain of *S. aureus*. A combination of BHA and BHT appeares to provide more effective inhibition than when the antioxidants are used alone. Both BHA and BHT are effective against *S. aureus* strain 100 in the range of pH and NaCl concentration encountered in food products. To ascertain the real effect of BHA and BHT in foods, commercial food items with and without BHA and BHT should be tested. Also, BHA and BHT can be added to food items known to be without these compounds and tested against *S. aureus*.

TABLE 5. *Inhibitory effect of sodium chloride and BHA or BHT in Brain Infusion broth on growth of* Staphylococcus aureus *strain 100 incubated 24 h at 37 C*[a].

% NaCl	μ mole/ml BHA	Klett reading	Percent inhibition due to:[b] NaCl	BHA	BHA + NaCl
0	0	540	0		
2	0	340	37		
5	0	440	19		
10	0	100	82		
15	0	60	89		
0	0.56	440		19	
2	0.56	20		94	95
5	0.56	40		91	91
10	0.56	60		40	86
15	0.56	20		67	95

% NaCl	μ mole/ml BHT	Klett reading	Percent inhibition due to:[b] NaCl	BHT	BHT + NaCl
0	0	600	0		
2	0	450	23		
5	0	400	33		
10	0	140	77		
15	0	40	97		
0	0.45	80		87	
2	0.45	60		87	90
5	0.45	160		60	73
10	0.45	40		71	73
15	0.45	20		50	97

[a]Each value is the calculated mean of two replications.
[b]% inhibition due to NaCl:

$$\%\text{ inhibition} = \frac{\text{Klett reading of control} - \text{Klett reading with NaCl} \times 100}{\text{Klett reading of control}}$$

% inhibition due to BHA or BHT:

$$\%\text{ inhibition} = \frac{\text{Klett reading of control} - \text{Klett reading with BHA or BHT} \times 100}{\text{Klett reading of control}}$$

% inhibition due to NaCl and BHA or BHT:

$$\%\text{ inhibition} = \frac{\text{Klett reading of control} - \text{Klett reading with NaCl and BHA or BHT} \times 100}{\text{Klett reading of control}}$$

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Enhanced Aflatoxin Production by *Aspergillus flavus* and *Aspergillus parasiticus* after Gamma Irradiation of the Spore Inoculum

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ABSTRACT

Distilled water plus 0.1% surfactant suspensions of spores of *Aspergillus flavus* and *Aspergillus parasiticus* were exposed to several radiation levels of cobalt-60 gamma rays. Spores of *A. flavus* isolate M-141 were exposed to radiation levels of approximately 16, 90 and 475 Krads and inoculated onto a sterile rice substrate which was then monitored for aflatoxin production. In this initial trial with *A. flavus* M-141, aflatoxins B_1 and M production on rice increased as radiation dose increased. At the highest dose, this increase was more than 50 times higher than the non-irradiated controls. Spores of an aflatoxin G_1-producing *A. parasiticus* isolate, M-1094, were exposed to 90, 215 and 430 Krads and resulted in increased production of aflatoxins G_1, B_1, and M. Aflatoxin production by M-1094 was highest at the low and medium dose levels. Irradiation of spores of this isolate with 430 Krads produced no observable spore germination or growth on rice and no detectable aflatoxin after 1 week of incubation at 27 C. Atypical colonies from irradiated spores were selected and their mycotoxin production was determined. Increase in aflatoxin production by these strains, as compared to non-irradiated controls, was 67:1 for aflatoxin B_1, 136:1 for B_2, and 138:1 for M. This potential for greatly increased mycotoxin production must be considered when food is irradiated or when a high production of aflatoxins is desired.

During the decade of the '60s *Aspergillus flavus* and its metabolites, the aflatoxins, first came to the attention of the scientific community. This was mainly due to the occurrence in England and parts of Africa of what appeared to be a new disease of turkeys, ducklings, and young pheasants (*2,3*). In the interim there has been an abundance of research on the structure, biosynthesis, toxicology, detoxification, microbiology, and control of aflatoxins (*6,7,11,12*). In addition to their toxicity, aflatoxins were found to be among the most potent of the known carcinogens; therefore any information relative to induced changes in amounts produced by *A. flavus* and *A. parasiticus* are of great interest to those concerned with hazards to human health.

The relationship of aflatoxins to food safety must be considered when methods of food preservation are being selected. Gamma irradiation of foods has been proposed as a means of food preservation, along with either sterilizing or pasteurizing doses. However, until studies published and/or reported by Jemmali and Guilbot (*8,9,10*) and Schindler and Noble (*13*), demonstrating an increase in aflatoxin production after irradiation, there was no information about the effects of gamma radiation on the production of aflatoxins by *A. flavus* or *A. parasiticus*. Although more recent publications (*1,4,5*) have reported similar studies, in certain instances these results differ from ours.

MATERIALS AND METHODS

All exposures in this study were carried out at the U.S. Naval Research Laboratory cobalt-60 irradiation facility in Washington, DC. The radiation source consisted of 20 rods containing ^{60}Co of $\simeq$ 7.5 kilocuries total activity. These were arranged in a cylindrical configuration 5.5 inches high by 3.25 inches (14.0 × 8.3 cm) inside diameter and positioned at the bottom of a pool of water 25 ft (7.6 m) deep. Samples consisted of spore suspensions in distilled H_2O plus 0.1% Tween 80 surfactant (and/or dosimeter solutions) contained in 35 ml (2.5 × 5.0 cm) polyethylene screw-capped vials placed inside a hermetically sealed stainless steel container 9 inches (22.9 cm) tall with a 3-inch (7.6 cm) diameter. The containers were lowered into position by means of an extension rod manipulator and irradiated by exposure to the source.

The standard ASTM "Ferrous-Cupric Dosimeter" method (*14*) was used to determine the absorbed gamma-ray dose in the irradiated samples. The ferrous sulfate-cupric sulfate solution, prepared according to the standard method, was contained in vials of borosilicate glass approximately 4 cm long by 1.6 cm wide (0.05-cm wall thickness) and flame sealed.

The actual exposure conditions for the spore suspensions were closely approximated with the dosimeter solutions by placing two ampules in tandem in the center of a water-filled polyethylene bottle and exposing them for 10 min within the center position of the gamma ray source. After exposure, the ampules, along with an unexposed control, were returned to the FDA laboratory for reading. The absorbance of each dosimeter solution was measured in a Beckman Model DU spectrophotometer at a wave length setting of 305 nm and a slit width of 0.5 mm. From the color change of the dosimeter solutions in the water bottle, a mean value of 4.28×10^4 rads/min ± 1.37% was calculated. This value was then used for computing the absorbed dose of the spore suspensions exposed to the ^{60}Co source.

Aflatoxin-producing isolates used in these experiments were FDA cultures *A. flavus* M-141 and *A. parasiticus* M-1094 (USDA, NRRL 3251 and NRRL 2999, respectively). Culture M-141 was originally isolated in this laboratory from a moldy California walnut sample; isolate M-1094 was originally obtained from a Uganda peanut sample. Spores were harvested from Czapek agar slants kept at ambient (72 ± 2 F) laboratory temperatures.

All spore suspensions except the mutant strain received three radiation treatments at low, medium and high dosage levels, plus a control treatment of no irradiation. Exposure levels were ca. 16, 90 and 475 Krads for *A. flavus* (M-141) and 90, 215 and 430 Krads for *A.*

[1]*Division of Microbiology.*
[2]*Division of Training and Medical Applications.*

parasiticus (M-1094). All treatments were replicated. After irradiation of the approximately 22-ml spore suspension of each sample, inoculations were made onto a substrate composed of 25 g of polished white rice plus 25 ml of distilled H_2O, previously autoclaved for 20 min at 121 C in 500-ml wide mouth Erlenmeyer flasks. Inoculations were made with 4 ml of spore suspensions per flask, except for the delayed inoculation trial in which only 1 ml of isolate M-141 was used and in the mutant strain experiment in which spores were removed from Czapek agar test tube slants with a transfer needle. A mutant strain experiment was initiated when two distinct colony phenotypes of strain M-141 were noticed on the rice substrate of those flasks inoculated with irradiated spores. Light green colonies similar to those growing in the flasks inoculated with non-irradiated spores (controls) and dark green colonies strikingly different in appearance from the original M-141 strain were observed. Three lines originating from strain M-141 were established in test tube slants by transferring spores, using a sterile transfer needle, from the control (non-irradiated) colonies and from the two types of colonies growing from spores irradiated with 475 Krads. In all instances, the molds were allowed to grow for 1 week in Biological Oxygen Demand incubators at 27 C. In all trials, treatments were replicated 4-6 times. All flask contents were extracted by heating with 150 ml of $CHCl_3$ on a steam bath until hot $CHCl_3$ vapors were driven through the cotton plug of the flask, followed by two more extractions with 100-ml portions of unheated $CHCl_3$. These three extracts were then combined, filtered through No. 588 filter paper (Schleicher & Schuell, Inc., Keene, NH), and evaporated to dryness over steam. Aflatoxins recovered from each flask were visually quantitated by spotting measured amounts of known dilutions in $CHCl_3$ onto activated thin layer glass plates coated to about 0.25-mm thickness with silica gel G-HR (Merck, Darmstadt, Germany). Appropriate standard spots containing known amounts of aflatoxins were included on each plate. Plates were then developed in equilibrated tanks with a solvent system of MeOH + $CHCl_3$ (7 + 93), dried and examined over long wave ultraviolet light.

RESULTS AND DISCUSSION

In the initial experiment with the *A. flavus* strain M-141, the production of aflatoxin B_1 was significantly higher at the 0.01 level of significance using a one-way analysis of variance. With Duncan's New Multiple Range Test (*15*) it was determined that amounts of aflatoxins produced after each treatment of medium and high levels of irradiation were significantly different from each other and from all other treatments. There was no significant difference between the control and low radiation treatments (Fig. 1). The results for aflatoxin M were similar, although at a much lower aflatoxin level (0.9 μg for the control compared to 49.9 μg for the high radiation). For both aflatoxins B_1 and M, the increase at the high irradiation level was more than 50 times higher than the non-irradiated control. There was no production of aflatoxins B_2, G_1, or G_2. The results of the delayed inoculation experiment also showed increased production of aflatoxin as radiation dose increased. However, at the medium dose level, aflatoxin B_1 production was more than double that produced when inoculation occurred immediately after irradiation (Fig. 1). In addition, aflatoxin G_1 was also produced and averaged 1,266 μg/flask (49 mg/g of rice) at the medium dose (90 Krad) level, with all five replications positive for G_1. All other treatments were negative for aflatoxin G_1 production. Three days after inoculation, colony growth was inversely proportional to radiation dose level in both the above trials, i.e., the least growth occurred at the high

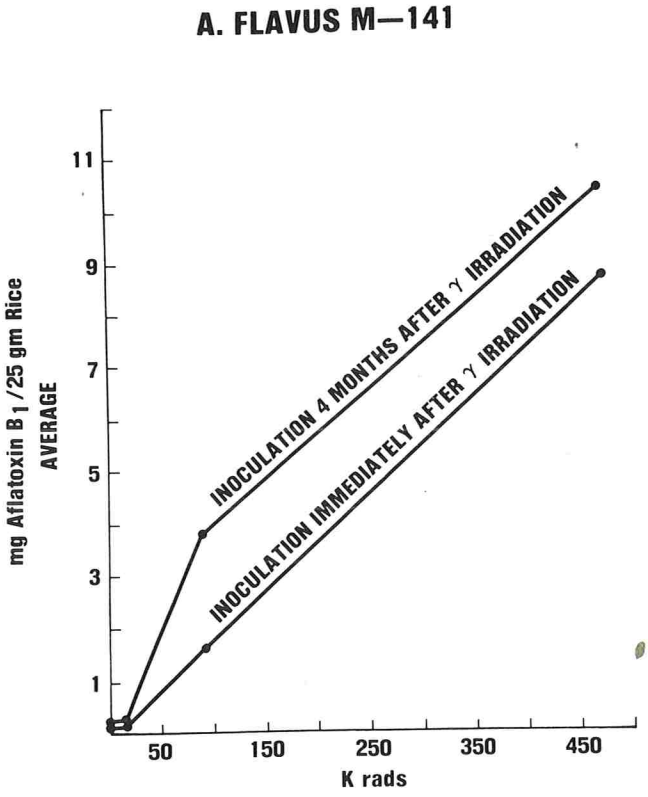

Figure 1. *Aflatoxin B_1 production by* A. flavus *spores growing on white rice immediately after and 4 months after gamma radiation.*

dose level. At 7 days this growth pattern was reversed, with the heaviest growth occurring in colonies from spores that had received the high and medium levels of radiation. In addition, the spores of these colonies were a darker green color.

Irradiation of the spores of *A. parasiticus* (M-1094) caused a strikingly different picture of aflatoxin production (Fig. 2). Duncan's test was again used to determine which treatments were or were not significant. Increases in the production of aflatoxins B_1 and G_1 were greatest at the low and medium dose levels and were not significantly different from each other. Both the control (non-irradiated) and the high radiation dose levels were significantly different from all other treatments (Fig. 2). Aflatoxin M production was similar for the different treatments but at much lower levels of average production/25 g rice (15 μg for the control and 26 μg for both the low and medium levels of irradiation).

In the trials conducted to test the hypothesis that increases in mycotoxin production after irradiation of spores were probably due to the formation of mutants, the effects on mycotoxin production were even more extreme (Fig. 3). There were no significant differences in aflatoxin production between the normal non-irradiated and irradiated lines ($p > 0.10$) and a highly significant increase ($p < 0.001$ using t-test on logarithmic transformation of data) in mycotoxin production by the mutant irradiated strain. Compared to the non-irradiated controls the ratios were 67:1 for aflatoxin B_1, 136:1 for aflatoxin B_2, and 138:1 for aflatoxin M.

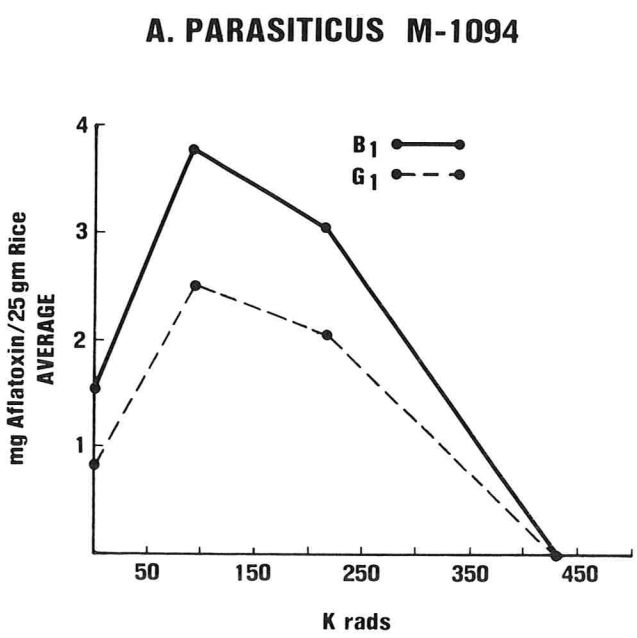

Figure 2. *Aflatoxin B_1 and G_1 production by* A. parasiticus *spores growing on white rice after gamma irradiation.*

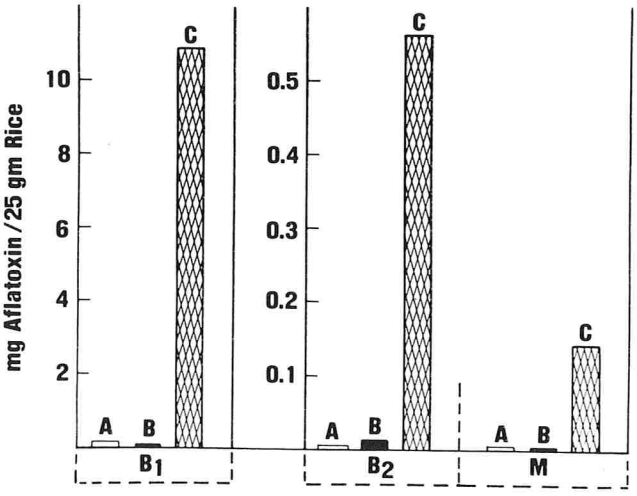

Figure 3. *Production of aflatoxins B_1, B_2 and M by* A. flavus *after gamma irradiation and mutant colony selection.*

These experiments indicate that gamma radiation of aflatoxin-producing molds probably causes an increase in aflatoxin production. This effect may be quite variable, depending on the species and strains irradiated, i.e., maximum aflatoxin production by M-141 occurred at the highest radiaton level of 475 Krads, while M-1094 demonstrated maximum production after exposure to the low and medium levels of radiation (90 and 215 Krads) and zero production of toxins after irradiation at the high dose level (430 Krads).

Increases in mycotoxin production after spore exposure to gamma radiation seem to be due to mutations. If separated from the mixed colonies arising from irradiated spores, these darker green mutants have considerably higher rates of mycotoxin production compared to both the non-irradiated lines of the original isolate and the non-mutant lines picked from irradiated colonies. These results should be considered when irradiation of food products is being contemplated or when a higher production of aflatoxins is needed.

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Use of Total and Differential Somatic Cell Counts to Differentiate Potentially Infected from Potentially Non-infected Quarters and Cows and Between Herds of Various Levels of Infection

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ABSTRACT

The objective of this study was to ascertain the value of variables measured on bulk tank and composite milk samples as predictors, respectively, of the level of mastitis in herds and individual cows. The standard of comparison was the results obtained by bacteriological examination of the secretion from individual mammary quarters. It was found, whether sampling at the herd or cow level, that of the three variables measured on each sample, namely somatic cell count, percentage of cell volume in channel 8 (includes cells with individual volumes from 89.2 to 178.3 μm^3) and presence or absence of *Streptococcus agalactiae*, that the ability of the first two variables to discriminate (predict) was not significantly improved by the inclusion of the latter variable. Using values for the former two variables as measured on bulk tank milk and collected at the time of quarter sampling, it was possible to correctly classify 45.5% of the study herds into one of the three quarter infection rate categories considered, namely, < 10, 10-25 or $> 25\%$. The percentage of herds correctly classified was increased to over 80% by inclusion of five monthly counts recorded previous to the time of quarter sampling. Using the same two variables but measured on composite milk samples, 79.4% of cows were correctly classified as being either positive or negative when a positive cow was defined as one having at least one positive quarter. The overall percentage of cows correctly classified decreased from 79.4 to 77.9% when an attempt was made to distinguish between negative cows and cows with one positive quarter. However, this overall percentage progressively increased to 92.7% as the definition of a positive cow was changed from one having one positive quarter to one having two, three or four positive quarters. The procedure shows promise for monitoring the level of mastitis infection in either dairy herds or cows but requires further refinement before consideration could be given to its implementation.

Bacteriological examination of the secretion from the individual mammary quarter of the bovine has traditionally been the approach taken for assessing its infection status, and, when considered collectively, the infection status of the cow and the level of infection in the herd. However, examinations of this latter type are time-consuming and expensive and hence impractical for large scale monitoring. Therefore, increased attention is being given to the use of samples collected at the cow (composite milk samples) or herd (bulk tank milk samples) level as a means of monitoring (*1,4,5*) the level of intramammary infection in individual dairy herds.

The objective of this study was to ascertain the value of variables measured on bulk tank and composite milk samples as predictors, respectively, of the level of intramammary infection in herds and the infection status of individual cows. The reason for restricting the variables measured to those related strictly to milk were twofold. First, because bulk tank milk samples are collected in Ontario on a monthly basis for quality testing, they were available. Second, since a mechanism exists for collection and automated processing of milk samples, it would be possible, providing the methods to be evaluated proved useful, to carry out large-scale mastitis monitoring with little additional input.

To determine the predictive value of the variables measured on composite and bulk tank milk samples, it was necessary to use a standard for comparison purposes. For this reason individual quarter and or composite samples were collected from each cow in the study and the secretion subjected to bacteriological examination as described below. The standard itself has an associated error rate and hence future references to positive or negative quarters or cows, as determined by the standard, should be interpreted as potentially positive or negative.

MATERIALS AND METHODS

Collection of milk samples

Two groups of herds were used for this study. Group A comprised 44 herds and came from an area approximately 50 to 75 miles west of Guelph, Ontario, whereas the 10 herds of Group B all came from within a 10-mile radius of the city.

Bulk tank and individual quarter foremilk samples were collected from each of the Group A herds on one occasion by an Ontario Ministry of Agriculture and Food (OMAF) fieldman. These samples were gathered at one point and delivered to the Ontario Veterinary College (OVC) in refrigerated vans by OMAF personnel. In addition, the Group A herds had had bulk tank somatic cell count determinations made at the OVC laboratory on a monthly basis for not less than 5 months previous to the time of quarter sampling.

Individual quarter foremilk and composite samples were collected from all milking cows in each of the 10 Group B herds. The composite sample for each cow was collected immediately following quarter sampling and was formed by collecting in a single screw-top vial approximately equal volumes of milk from each milking quarter. Six of the Group B herds were sampled as described on two occasions 1 week apart, whereas the remaining four herds were sampled weekly for 3 weeks.

Examination of milk samples

Each quarter sample from the cows of Group A herds was divided into two subsamples, one of which was submitted to the regional OMAF Veterinary Services Branch (VSB) Laboratory for mastitis diagnosis. Quarters were classified as negative if a negative California Mastitis Test (CMT) reaction or a trace reaction was found and no pathogens were isolated, or as positive if a CMT 1 or more reaction was detected and pathogens were isolated. Individual quarters that could not be classified as being either positive or negative were not considered in the analysis phase of the study.

The other sub-sample, as well as the bulk tank sample for each herd, was retained at the OVC laboratory for somatic cell counting. These latter samples were fixed, diluted and counted with a Model TA Coulter Counter (Coulter Electronics Ind., Hialeah, Florida) as described by Newbould (*2*).

The Model TA Counter, as well as counting the total number of somatic cells present, also sizes the counted particles into 16 groups or channels. Newbould (*2*) reported that with milk from infected quarters the greatest change occurred in the percentage of cell volume in channel 8. This channel includes those cells with individual volumes from 89.2 to 178.3 μm^3, which is equivalent to spheres with diameters between 5.54 and 6.98 μm. Newbould (*2*) also reported that most of the cells in channel 8 are neutrophils and pointed out that the reason the sizes are rather smaller than expected for neutrophils is due to shrinkage of the cell following formalin fixation. Using the above technique, the somatic cell count (SCC) and percentage of total cell volume in channel 8 (PCH8) was determined for each sample.

Quarter and composite samples from Group B herds were processed at the OVC laboratory. The procedures used in this instance were the same as those outlined previously except that quarters shedding *Streptococcus agalactiae* were classified as being positive regardless of the CMT reaction, and cell count determinations were made using the newer model Coulter Milk Cell Counter with attached TA.

On the basis of the CMT reactions and the bacteriological findings, quarter (QIR) and cow infection rates (CIR) were calculated for each herd. For this latter determination a cow was considered positive if at least one of her quarters was found to be positive, and negative only if all four quarters were negative.

Analysis

Discriminant analysis (*3*) was used in an attempt to differentiate, on the basis of various combinations of the herd and bulk tank variables listed in Table 1, between Group A herds with various levels of quarter and cow infection rates. For this latter purpose three categories for each of QIR and CIR were used. The upper threshold for each of the three QIR categories was respectively 10, 25 and 100% and for CIR 20, 50 and 100%.

A second series of discriminant analyses was made using the data gathered from the Group B herds. This analysis was used in an attempt to differentiate between positive and negative cows by means of the variables SCC, PCH8 and the presence or absence of *S. agalactiae* (PASTR) determined for each composite sample. Further to this, an attempt was made to differentiate between negative cows and cows classified as being positive but which had one, two, three or four positive quarters.

A third series of discriminant analyses was made again using Group B herd data. This analysis was done in an attempt to differentiate between positive and negative quarters by means of the variables SCC, PCH8 and PASTR determined for each quarter sample.

TABLE 1. *Herd and bulk tank milk variables used in the study of bovine mastitis in Ontario.*

Variable syntax	Variable definitions and/or code(s)
NOCOWS	Number of cows quarter sampled
PASTR	Presence (= 1) or absence (= 0) of *Streptococcus agalactiae*
SCCB	Bulk tank somatic cell count at time of quarter sampling
SCC1...SCC5	Bulk tank somatic cell counts taken respectively 1 to 5 months previous to SCCB
PCH8B	Bulk tank percentage of cell volume in channel 8 at time of quarter sampling
PCH8,1...PCH8,5	Bulk tank percentages of cell volume in channel 8 taken respectively 1 to 5 months previous to PCH8B

RESULTS

Eight of the 44 Group A herds had a QIR less than 10%, 10 had between 10 and 25% inclusive and 26 had a QIR greater than 25% (Table 2). On the basis of CIR, six herds had less than 20% of cows infected, 10 had between 20 and 50% inclusive and 28 had a CIR greater

TABLE 2. *Means of variables and results of discriminant analyses when the Group A herds*[1] *were grouped on the basis of quarter and cow infection rates.*

Variable[2]	Quarter infection rate (%)			Magnitude of discriminant co-efficient	Cow infection rate (%)			Magnitude of discriminant co-efficient
	< 10	10-25	> 25		< 20	20-50	> 50	
NOCOWS	25.6	32.0	37.1	-0.04	25.0	30.6	36.9	0.04
PASTR	0.4	0.6	0.7	-0.10	0.3	0.5	0.7	0.16
SCCB($\times 10^3$)	480.3	543.6	653.4	0.43	528.7	462.1	659.7	-0.42
SCC1($\times 10^3$)	373.3	773.8	691.9	0.33	393.5	402.8	797.3	-0.07
SCC2($\times 10^3$)	262.2	602.5	729.7	-0.33	223.9	433.0	765.1	0.29
SCC3($\times 10^3$)	360.9	577.0	651.7	0.17	347.2	518.6	654.7	-0.47
SCC4($\times 10^3$)	277.9	406.7	654.7	-0.09	299.0	379.0	633.1	0.16
SCC5($\times 10^3$)	112.8	357.2	609.4	-0.40	93.1	311.5	594.4	0.30
PCH8B	16.6	32.4	33.5	-0.28	16.8	24.8	34.9	0.22
PCH8,1	15.6	26.4	35.1	-0.21	15.5	22.5	35.1	0.27
PCH8,2	21.0	29.6	35.0	0.02	18.7	27.3	35.3	0.14
PCH8,3	13.9	26.0	36.3	-0.31	12.7	20.3	36.9	0.59
PCH8,4	15.3	22.0	33.3	-0.28	19.5	22.2	31.1	-0.10
PCH8,5	8.3	21.6	26.4	0.03	8.5	19.8	25.7	-0.11
No. of herds	8	10	26		6	10	28	
Discriminant function group centroids	1.45	0.38	-0.59		-1.55	-0.72	0.59	
% of herds correctly classified	87.5	80.0	80.8		83.3	90.0	85.7	

[1]See text for description.
[2]See Table 1 for definitions and codes.

than 50%. There appeared to be a general upward trend in herd size, as measured by the variable NOCOWS, as the QIR and CIR increased from the low, to the intermediate, to the high level (Table 2). This overall trend was also observed for each of the variables PASTR, SCC and PCH8.

In general, and based primarily on the absolute magnitude of the discriminant co-efficients, the monthly measurements for each of SCC and PCH8 were the variables best able to differentiate between the three infection levels, whether classified by QIR or CIR (Table 2). There did not appear, however, to be any trend among the monthly SCC or PCH8 determinations. That is, the discriminant co-efficients did not consistently increase or decrease as the interval between the time of quarter and bulk tank sampling increased. It was decided, therefore, to investigate the discriminating ability of various combinations of the latter variables.

In this regard, six combinations of variables were used and are listed along with the results of the respective analysis in Table 3. The results of analyses one and two are the same, with 81.8 and 86.4% of the 44 Group A herds being correctly classified when categorized respectively by QIR and CIR (Table 3). These results confirm the results of the previous analysis, that is, that the variable NOCOWS did not increase the predictive ability of the discriminant function.

Analysis three (Table 3) is based solely on the discriminating ability of the variables SCC and PCH8 which were measured on the bulk tank sample at the time of quarter sampling. This analysis resulted in only 45.5 and 52.3% of the 44 herds being correctly classified when categorized by QIR and CIR, respectively.

Analyses four to six differ from analyses one to three only by addition of the variable PASTR. Inclusion of this latter variable did not significantly alter the discriminating ability of the respective previous functions. The overall discriminating ability of the six discriminant functions was slightly higher when herds were categorized by CIR in comparison to that achieved by QIR.

The bacteriological examination of individual quarter samples from the cows of Group B herds resulted in 897 quarters being classified as positive and 3210 as negative or, when expressed on a per cow basis, in 425 positive and 571 negative cows (Table 4). Composite milk samples from positive cows and milk from positive quarters had a higher mean SCC, PCH8 and proportion of samples shedding *S. agalactiae* (PASTR) than did negative cows or quarters (Table 4). The variable PCH8, again based primarily on the absolute value of the discriminant co-efficient, appeared to be the variable best able to differentiate between positive and negative cows and quarters (Table 4).

The results of the analyses in which an attempt was made to differentiate between negative cows and cows classified as being positive because of at least one positive quarter or which had one, two, three or four positive quarters is presented in Table 5. Two discriminant functions were used: the first of these included the variables SCC and PCH8 and the second included these latter two variables plus the variable PASTR. The inclusion of the variable PASTR in the discriminant function did not significantly increase its ability to distinguish between positive and negative cows (Table 5). For example, the overall percentage of cows correctly classified as being positive or negative, when a positive cow was defined as one having at least one positive quarter, was increased only 1.7% (from 79.4 to 81.1) by the inclusion of the variable PASTR.

Considering the first of the two discriminant functions only (Table 5), and considering the case of a positive cow being defined as one having at least one positive quarter,

TABLE 3. *Percentage of herds correctly classified by the discriminant function for each of six combinations of variables when the Group A herds*[1] *were grouped on the basis of quarter and cow infection rates.*

Analysis	Variables[2] included	Quarter infection rate (%)			Overall by QIR	Cow infection rate (%)			Overall by CIR
		< 10	10-25	>25		< 20	20-50	> 50	
1	NOCOWS SCCB,SCC1...SCC5 PCH8B, PCH8,1...PCH8,5	87.5	80.0	80.8	81.8	100.0	80.0	85.7	86.4
2	SCCB, SCC1...SCC5 PCH8B, PCH8,1...PCH8,5	87.5	80.0	80.8	81.8	100.0	80.0	85.7	86.4
3	SCCB, PCH8B	75.0	60.0	30.8	45.5	50.0	30.0	60.7	52.3
4	NOCOWS, PASTR SCCB, SCC1...SCC5 PCH8B, PCH8,1...PCH8,5	87.5	80.0	80.8	81.8	83.3	90.0	85.7	86.4
5	PASTR SCCB, SCC1...SCC5 PCH8B, PCH8,1...PCH8,5	87.5	70.0	80.8	79.6	66.7	90.0	85.7	84.1
6	PASTR SCCB, PCH8B	87.5	50.0	34.6	47.7	66.7	30.0	57.1	52.3

[1]See text for description.
[2]See Table 1 for definitions and codes

it can be seen that 66.6% of the positive cows and 89.0% of the negative cows (79.4% of all cows) were correctly classified. The overall percentage of cows correctly classified decreased from 79.4% to 77.9% when an attempt was made to distinguish between negative cows and cows with one positive quarter. However, the overall percentage of cows correctly classified progressively increased from 77.9 to 92.7% as the definition of a positive cow was changed from one having one positive quarter to one having two, three or four positive quarters. That is, the discriminant function was better able to distinguish between cows having four positive quarters and negative cows than between cows having one positive quarter and negative cows.

DISCUSSION

In these studies, variables measured on milk samples which were collected at two levels, namely the herd (bulk tank) and cow (composite), were evaluated as predictors respectively of the level of mastitis in dairy herds and the mammary health status of individual cows. Sampling at either of these latter two levels offers the potential advantage, compared to individual quarter sampling, of being less expensive with respect to both time and other resources.

The results of these studies indicate, whether sampling at the herd or cow level, that of the three variables measured on each milk sample, namely somatic cell count (SCC), percentage of cell volume in channel 8 (PCH8) and the presence or absence of *S. agalactiae* in the sample (PASTR) that the ability of the first two variables to discriminate was not significantly improved by inclusion of the variable PASTR. This finding has practical importance as cell count determinations, that is, SCC and PCH8, are automated and are less costly to do than are bacteriological examinations.

The finding that the variables SCC and PCH8, measured on the bulk tank sample at the time of quarter sampling, only correctly classified 45.5% of herds (on a QIR basis) indicates that the procedure does not provide a reliable measure of the level of intramammary infection in herds and agrees with what others (*4,5*) using SCCs only, have found. However, the percentage of herds correctly classified was increased to over 80% when the results of five previous monthly SCC and PCH8 bulk tank determinations were included in the analysis. In areas where SCCs on bulk tank milk samples are currently routinely used as a means of monitoring the level of infection in individual herds, these additional monthly counts are readily available and could be used with no additional effort. Although the variables SCC and PCH8 were identified here in a retrospective study, the intent of this study was to use such a function in a prospective sense, that is to predict the status of herds with respect to mammary health.

As with any monitoring technique, some errors are likely to occur. In this study and using the variables SCC and PCH8 only, approximately 15% of herds were incorrectly categorized into one of the three cow infection rate (CIR) categories considered (i.e. <20, 20-50,

TABLE 4. *Means of variables and results of discriminant analyses when the quarters and cows of Group B herds[1] were grouped on the basis of infection status.*

Variables[1] included	Cow infection status[2]		Magnitude of discriminant co-efficient	Quarter infection status		Magnitude of discriminant co-efficient
	Positive	Negative		Positive	Negative	
SCC ($\times 10^3$)	1096.7[3]	262.3[3]	-0.14	1552.8	262.4	-0.26
PCH8	21.6[3]	6.5[3]	-0.84	26.3	7.0	-0.76
PASTR	0.17[3]	0.0[3]	-0.23	0.18	0.0	-0.27
No. of cows or quarters	425	571		897	3210	
% of cows/quarters correctly classified by discriminant function	69.4	89.8		81.3	91.8	

[1]See text for description.
[2]Determined by quarter sampling.
[3]Determined for composite milk sample.

TABLE 5. *Number of positive and negative cows and percentage of cows correctly classified by discriminant function for each of two combinations of variables and five definitions of positive cow.*

Variables[1] included	No. positive quarters for positive cow	Number of cows		Percent cows correctly classified		
		Positive	Negative	Positive	Negative	Overall
SCC	1-4	425	571	66.6	89.0	79.4
PCH8	1	167	571	59.3	83.4	77.9
	2	114	571	66.7	89.5	85.7
	3	74	571	79.7	90.2	89.0
	4	70	571	88.6	93.2	92.7
SCC	1-4	425	571	69.4	89.8	81.1
PCH8	1	167	571	59.3	83.7	78.2
PASTR	2	114	571	67.5	91.2	87.3
	3	74	571	78.4	92.3	90.7
	4	70	571	85.7	95.8	94.7

[1]See text for description.

> 50%). It should be pointed out, however, that most herds that were incorrectly classified were classified in the directly adjacent category and that they were, for the most part, on the borderline between two categories. Misclassifications of this type might be minimized by use of averaging techniques, for example, use of rolling averages rather than actual monthly values. However, the authors did not have sufficient data to explore this possibility. The percentage of herds correctly classified might also be increased by inclusion of additional data; for example, average cow age and milk production.

At the individual cow level, as was true at the herd level, inclusion of the variable PASTR in the discriminant function did not significantly increase the discriminating ability of the function. The variable PCH8 based primarily on the absolute magnitude of the discriminant coefficient, appeared to be the variable best able to differentiate between positive and negative cows and quarters (Table 4).

The finding that the discriminant function was better able to distinguish between negative cows and cows with four positive quarters (92.7% of cows correctly classified) than between negative cows and cows with one positive quarter (77.9% of cows correctly classified) is not surprising and is primarily a function of dilution. That is, the secretion from a positive gland in a cow with only one positive quarter is greatly diluted by the milk produced by the other three normal glands and hence the presence of the positive gland is hard to detect at the cow level. This is illustrated by the fact that of the 167 cows in the study with one positive gland, only 99 (59.3%) were correctly classified (Table 5). The percentage of cows correctly classified might be improved by inclusion in the discriminant function of variables such as cow age and stage of lactation.

Further studies are underway with the objective of determining the value of including additional variables such as those mentioned above. If the predictive value of the functions so derived are improved, prospective studies will be initiated to validate or refute the findings.

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of other very damaging species such as singlet oxygen and hydroxyl radical. On the other hand, the inability of nucleosides and nucleotides to photogenerate detectable $O_2^{\cdot}$ in contrast to their corresponding bases requires further investigation.

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Production of Phospholipase C by Nine Strains of *Clostridium perfringens* at 37 C and at a Constantly Rising Temperature[1]

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ABSTRACT

Nine strains of *Clostridium perfringens* were compared for phospholipase C production in autoclaved ground beef (AGB) at 37 C. Enzyme production is reported as units per colony forming unit (CFU). Phospholipase C concentrations were determined by the hemolysin indicator method and colonies were observed on Tryptose Sulfite Cycloserine agar. Enzyme production by each of the nine strains was significantly different. The increase and decrease of viable cells of *C. perfringens* in AGB exposed to temperatures rising constantly at a rate of 4.1 C/h was accompanied by the appearance and disappearance of phospholipase C. This demonstrated that *C. perfringens* can produce detectable levels of phospholipase C when grown during dynamic increasing temperatures.

Clostridium perfringens foodborne illness occurs upon ingestion of a large number of viable organisms (*3,11*). To establish this organism as the causative agent, it is necessary to estimate the number of viable *C. perfringens* in a suspect food by using quantitative cultural methods. When frozen or held under prolonged refrigeration, *C. perfringens* cells rapidly lose viability (*2,18*), making it difficult or impossible to obtain reliable counts. Harmon and Kautter (*6,7,8*) have suggested the hemolysin indicator plate test as an index of growth of this organism in foods which have been refrigerated or frozen. This method involves quantifying the extracellular phospholipase C (alpha toxin) produced by *C. perfringens*. The quantity of phospholipase C detected may then be used to estimate the extent of previous growth of the organism (*7*).

Long-time low-temperature (LTLT) cookery is used extensively in industry, foodservice and the home. *C. perfringens* can grow to high population levels under LTLT conditions (*19*). One objective of this research was to determine if phospholipase C would be produced at detectable levels in simulated LTLT environments. Since many foodborne disease strains of *C. perfringens* have been reported to produce phospholipase C at low levels (*9,10,14*), the second objective was to compare phospholipase C production by nine strains of *C. perfringens*, eight of which are associated with foodborne illness.

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MATERIALS AND METHODS

Test organisms and culture preparation

C. perfringens strains NCTC 8238, NCTC 10240 and ATCC 3624 were obtained from D. A. Adams, North Carolina State University, Raleigh NC; NCTC 8798 was obtained from R. G. Labbe, University of Wisconsin, Madison, WI; NCTC 8239, S-40, S-45, and NCTC 9851 were obtained from the culture collection, University of Minnesota, St. Paul, MN and ATCC 13124 was obtained from American Type Culture Collection, Rockville, MD.

The method of Willardsen et al. (*19*) was used with slight modifications in preparation of stock cultures and test cultures. Test cultures were prepared by transferring 0.1 ml of a freshly thawed stock culture to 12 ml of laboratory-prepared Fluid Thioglycollate medium (FTG) and incubating at 37 C for 20 h. FTG was no more than 1 week old and, if not freshly prepared, was steamed 10 min and cooled immediately before use. After incubation in FTG, a composite of eight strains (all listed strains except ATCC 13124) was prepared as detailed by Willardsen et al. (*19*). Single strain studies were conducted on each strain by using the appropriate dilution of the 20 h FTG culture. Dilutions were made with 0.1% peptone water.

Growth and enumeration media

The autoclaved ground beef (AGB) test medium was prepared according to the method of Willardsen et al. (*19*). The ground beef was frozen for a maximum of 6 months at -30 C. Four batches (fat content 17.0, 20.5, 23.9 and 28.5%) were used during the duration of this research. Before use, the tubes were tempered to the desired initial temperature (37 C for constant temperature strain comparisons, or 25 C for constantly rising temperatures studies), and inoculated with a 0.1 ml of inoculum diluted in 0.1% peptone water to the desired cell population. The diluted inoculum was carefully injected into the bottom of the AGB tubes with a 13-cm long 15 gauge needle.

The enumeration medium was laboratory-prepared Tryptose Sulfite Cycloserine (TSC) agar (*17*) prepared as detailed by Willardsen et al. (*19*). The TSC agar was made no more than one day before use and stored at room temperature.

Enumeration procedure

At each appropriate sampling time, the ground beef sample was aseptically transferred to an 18 × 30 cm, 3½-mil polyethylene bag using a sterile 23-cm chrome letter opener. The sample was diluted to 100 ml with sterile distilled water (85 ml), and macerated for 60 sec in a Colworth 400 Stomacher (*16*). Serial dilutions were made into 9.9 or 9.0 ml of 0.1% peptone (Difco), and a 0.1-ml portion of the appropriate dilution was spread over the surface of TSC agar and overlaid with TSC agar without egg yolk. The inoculated plates were incubated at 37 C overnight in anaerobic jars (BBL Gaspak System) which were evacuated to 15 psi vacuum and flushed to 5 psi, three times, with a 90% N_2-10% CO_2 mixture or with 100% N_2.

Extraction and quantification of phospholipase C

The method described by Harmon and Kautter (*8*) was employed, with a slight modification for extraction of the enzyme. Phospholipase C was extracted after homogenization of a 25-g portion of AGB culture, with 100 ml of 0.4 M NaCl, buffered at pH 8 with 0.05 M

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), for 1 min in a Colworth 400 Stomacher (*16*). Portions of two AGB cultures (15 g each) were used to obtain 25 g. Samples were removed from the test tube with a sterile 23-cm chrome letter opener. The layer of fat which developed on the surface of the AGB was removed and discarded before sample removal. After centrifuging the homogenate at 20,000 × *g* for 20 min at 4 C, the supernatant fluid was decanted through Whatman No. 1 filter paper to remove the remaining fat and meat solids. The supernatant fluid was sterilized by filtration through a Seitz Filter (No. L6, Hercules Division, Republic Filter Corp., Milldale, CT) and the sterile extract was concentrated to less than 10 ml by dialysis overnight against polyethylene glycol (PEG; molecular weight 20,000) at 4 C. The dialysis tubing (No. 3737-D32, A. H. Thomas Co., Philadelphia, PA, molecular weight cutoff 12,000) was autoclaved in distilled water for 15 min at 121 C and rinsed once with sterile physiological saline, or rinsed three times with sterile physiological saline, before contact with the sterilized extract. After dialysis, the tubing was rinsed with tap water to remove the PEG. Samples concentrated to a volume of less than 5 ml were placed into physiological saline solution for 1-2 h to permit easier and more quantitative removal of the extract from the dialysis sac. The concentrated extract was collected in a sterile 10-ml volumetric flask. The dialysis sac was rinsed 2 or 3 times with ca. 1-ml quantities of sterile physiological saline solution, and the washings and extract were brought to a volume of 10 ml. Care was taken throughout the procedure to recover all the AGB extract and washings.

The hemolytic activities of the extracts were measured by means of a modification of the hemolysin indicator (HI) plate method described by Noyes and Easterling (*13*) and employed by Harmon and Kautter (*8*). Packed human red blood cells were washed and used in preparation of the HI plates (*17*). The red blood cells were obtained from the American Red Cross Blood Bank, St. Paul, MN. Plates were allowed to dry overnight at room temperature and used the following day, or stored overnight in an airtight plastic bag at 4 C for use 2 days later. Just before use, test wells were cut in the agar by applying a vacuum to a thin-walled stainless steel tube (3 mm O.D., No. 9 surgical tubing) plunged into the agar (*17*). Eight two-fold dilutions of the concentrated extract were made with sterile physiological saline solution, using a fresh serological pipette for each dilution. The undiluted extract and eight dilutions were placed in the wells of duplicate HI plates, along with anti-alpha toxin and diluent controls (*17*). *C. perfringens* (*welchii*) Type A diagnostic sera (anti-alpha) was purchased from Wellcome Reagents, Ltd., Beckenham, England. Aseptic procedures were used in handling the extract. The plates were incubated for 24 h at 37 C and examined for hemolytic zones around the wells. All hemolytic zones were measured in mm (width from edge of well) with calipers. The hemolytic zones produced by phospholipase C on HI plates were clear and easily differentiated from the unreacted red blood cell region.

Standard curve

A lyophilized standardized preparation of Type A test enzyme (alpha toxin, E.C.3.1.4.3., phospholipase C, lecithinase C) from *C. perfringens* was purchased from Sigma Chemical Co., St. Louis, MO.

The Sigma commercial enzyme preparation was used as a standard. An initial 1:10,000 dilution was made with filter-sterilized HEPES, followed by a 1:10 dilution in physiological saline solution. Two-fold dilutions of the commercial enzyme-HEPES-saline dilution were made with sterile physiological saline solution. These dilutions were added to HI plates and incubated in a manner identical to the unknowns. Standard curves were constructed for use with each experimental trial by plotting the log activity of the standard phospholipase C dilutions in units/ml versus the reaction zone size in mm. Regression analysis was employed to construct the line of best fit.

Experimental apparatus and design

Water baths and temperature maintenance and control apparatuses for 37 C constant temperature as well as 4.1 C/h-constantly rising temperatures have been described by Willardsen et al. (*19*).

AGB was inoculated with either a single strain or the eight-strain composite of *C. perfringens*. The AGB inoculated with the single strains was incubated at 37 C for 4.5-5 h to make comparisons among strains. AGB inoculated with the eight strain composite or with strain ATCC 13124 was exposed to a linear temperature increase of 4.1 C/h. Colony forming units on TSC were determined on samples taken every 10-20 min. Samples were plated immediately to determine the viable count. Samples expected to be greater than 10^6 CFU/g (for strain comparisons, the target population was 10^8 CFU/g) were assayed for phospholipase C. Samples monitored for phospholipase C activity were placed immediately into ice water to prevent further growth of the organisms. The samples were held at 4 C overnight, with extraction of the enzyme the following day. Quantification was by comparison of unknown sample dilutions resulting in 1-mm reaction zones with the standard curve. If a 1-mm zone was not present, the dilution which would have resulted in a 1-mm zone was calculated from the zone sizes of the two closest dilutions. The calculated dilution was then compared to the standard curve.

For one trial of the three with ATCC 13124 at 37 C, the phospholipase C was quantified with an estimated value for the Sigma standard, enzyme activity which produced a 1-mm reaction zone (reference activity), rather than a freshly prepared standard curve. The estimated enzyme activity was the average of reference activities of Sigma phospholipase C for 15 standard curves constructed in previous experiments. The arithmetic average of the 15 reference activities was 7.8×10^{-3} units/ml, with a standard deviation of 4.4×10^{-3} unit/ml. Reference activities ranged from 1.7×10^{-3} to 1.6×10^{-2} unit/ml. The phospholipase C unit per CFU calculated with the estimated reference activity, 7.8×10^{-3} unit/ml, was between the results of the other two ATCC 13124 trials. Subsequent experiments using the same average, rather than a standard curve, also yielded results in agreement with duplicate and triplicate trials which were analysed with standard curves (data not presented).

Statistical analyses

Analysis of variance was used to detect differences in the phospholipase C production of the nine strains examined. A clustering analysis method described by Scott and Knott (*15*) as appropriate for grouping means in the analysis of variance was employed in an attempt to divide the strains into groups based upon their level of phospholipase C production.

RESULTS AND DISCUSSION

A comparison of the production of phospholipase C by each of the 9 strains of *C. perfringens* in AGB at 37C is presented in Fig. 1. The data are presented as units of phospholipase C per colony forming unit to make the comparison on a per cell basis. In each instance the populations were between 5×10^7 and 3×10^8 CFU/g. By direct microscopic observation, an average colony forming unit consisted of 2-3 cells.

Analysis of variance revealed that there was no significant difference from the first to second replication of the experiment, while there were significant differences between units/CFU for each of the nine strains ($p < 0.05$). An attempt was made to group the nine strains by ability to produce phospholipase C according to a clustering of means procedure (*15*). The mean enzyme units/CFU of the nine strains could not be separated into two or more groups ($p < 0.05$); therefore, they were considered as one group. This reveals that although each of the strains can be considered different from the other eight strains in its ability to produce phospholipase C in AGB at 37 C, the differences between any two or more groups formed were not clear enough to merit a division with 95% confidence with this limited amount of data.

Many investigators have reported differences in phospholipase C production between strains (*1,4,5,12*).

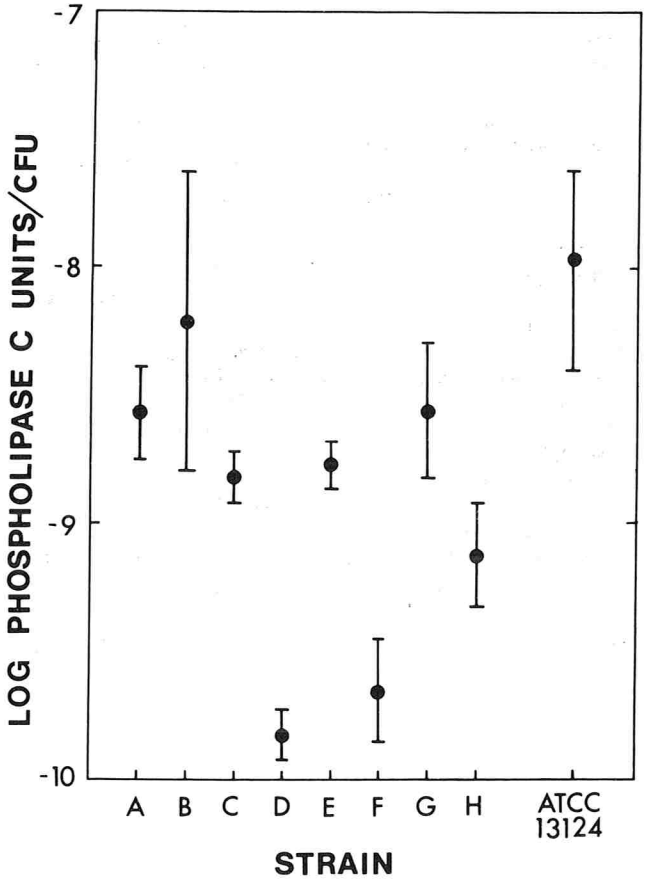

Figure 1. *Phospholipase C production per colony forming unit for nine strains of* Clostridium perfringens *grown in autoclaved ground beef at 37 C. Strains examined were: S-40 (A); S-45 (B); ATCC 3624 (C); NCTC 8238 (D); NCTC 8239 (E); NCTC 8798 (F); NCTC 9851 (G); NCTC 10240 (H); and ATCC 13124. Data presented are the averages and ranges of 2-3 determinations.*

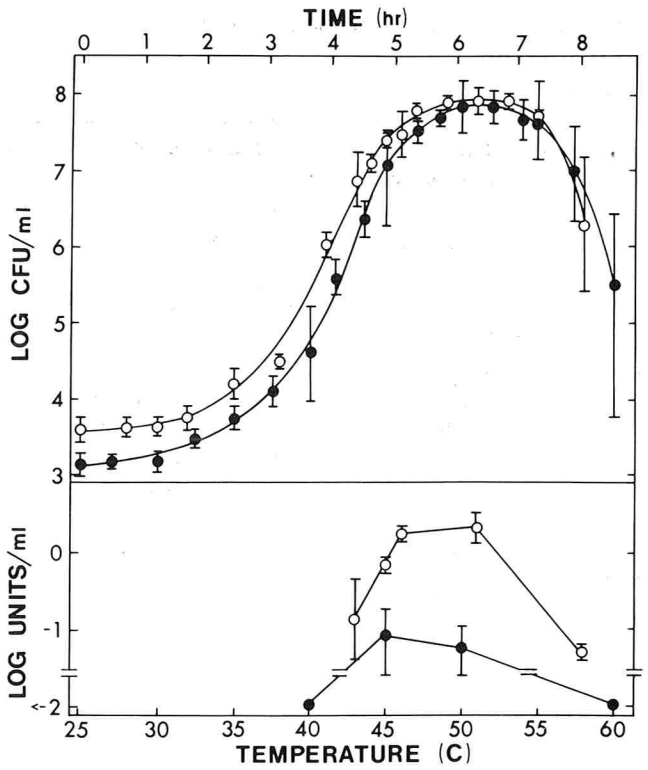

Figure 2. *Growth and phospholipase C production by ATCC 13124 and an eight-strain composite of* Clostridium perfringens *in autoclaved ground beef exposed to a 4.1 C/h temperature increase. Data presented are averages and ranges of duplicate and quadruplicate trials with ATCC 13124 (○) and the composite (●) respectively. The phospholipase C activity of the composite at 40 C is an estimated value.*

NCTC 8239 (strain E) has been reported (*1*) to produce small amounts, and ATCC 13124 was recognized (*12*) as producing large amounts of phospholipase C. However, the data presented here describe the strain comparison of phospholipase C production by *C. perfringens* on a units/CFU basis. From our observation on a cellular basis, NCTC 8238 (strain D) and NCTC 8798 (strain F) produced small amounts of phospholipase C. ATCC 13124 produced a large amount of phospholipase C on a cellular basis.

C. perfringens has been observed to grow to high populations during dynamic temperature conditions (*19*). Production of phospholipase C by *C. perfringens* during growth at constantly rising temperatures has not been reported. Strain ATCC 13124 was studied singly, and the remaining eight strains were studied as a composite during exposure to dynamic temperatures increasing at linear rates of 4.1 C/h. The eight-strain composite was identical to that studied by Willardsen et al. (*19*) under the same conditions.

Temperature-based growth curves and phospholipase C activity curves for *C. perfringens* ATCC 13124 and the composite are presented in Fig. 2. Data presented are averages of duplicate and quadruplicate trials for ATCC 13124 and the composite, respectively. The graphs represent viable cell numbers and enzyme activity during a linear temperature increase of 4.1 C/h. The elapsed time from initiation of heating (25 C initial temperature) is shown on the upper axis. Growth of ATCC 13124 and the eight-strain composite were similar, and agree with reports by Willardsen et al. (*19*). The phospholipase C activity of the eight-strain composite is estimated to be 0.013 unit/ml at 40 C. Harmon and Kautter (*7*) indicate that populations of ca. 10^6/g are needed to attain detectable phospholipase C levels. Our experiments with the eight-strain composite of *C. perfringens* also indicate a requirement for about 10^6 CFU/g to attain measurable phospholipase C. The estimated 0.013 unit/ml activity at 40 C is just above the detection limit under the test conditions employed, making the estimate of enzyme activity at 40 C valid.

Growth of the single strain was initiated at about 32 C. Rapid growth was observed to about 50 C with inactivation beginning at temperatures above approximately 52 C. Phospholipase C activity showed an increase, leveling and decline which appeared to correspond closely to the population level. Growth and phospholipase C production by the eight-strain composite were similar to strain ATCC 13124. With the eight-strain composite, the phospholipase C was again observed to parallel the growth pattern, increasing as CFU's increased, then leveling and declining at high

temperatures. This comparison accentuates the ability of ATCC 13124 to produce phospholipase C at high levels which is in agreement with Möllby et al. (*12*). For example, from 45 to 58 C, populations of ATCC 13124 nearly identical to the eight-strain composite yielded phospholipase C activities 0.5 to 1.5 logs higher than did the composite. High phospholipase C levels were expected from ATCC 13124, based upon the comparison of the nine *C. perfringens* strains.

In conclusion, all nine strains studied produced detectable levels of phospholipase C in AGB at 37 C, although comparable populations resulted in a wide ranged of enzyme activity. There was a large difference in the ability of various strains to produce phospholipase C on a per cell basis. This work demonstrates that *C. perfringens* can produce detectable levels of phospholipase C during exposure to dynamic temperatures, which are representative of LTLT cooking conditions for beef and other products. These data do not indicate, nor were they intended to show, that all high and low phospholipase C producing strains of *C. perfringens* would yield detectable phospholipase C when grown in AGB at simulated LTLT conditions. Rather, the study demonstrates that detectable phospholipase C can and may be produced under these conditions. During a constantly rising temperature rate of 4.1 C/h in autoclaved ground beef, the appearance and disappearance of phospholipase C parallels the increase and decrease of *C. perfringens* populations. The decrease in phospholipase C activity upon exposure to high temperatures indicates that heat has a detrimental effect upon either synthesis or stability of phospholipase C. Further studies are underway in this laboratory to ascertain the effect of high temperature on phospholipase C.

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Photogeneration of Superoxide Anion upon Illumination of Purines and Pyrimidines in the Presence of Riboflavin: Structure-activity Relationships

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ABSTRACT

Photogenerated superoxide anion might be involved in the oxidative deterioration of foods. For this reason, purines, pyrimidines and related compounds were illuminated with fluorescent light in the presence of riboflavin to examine their capacity to photogenerate superoxide anion (measured from suppression of its reduction of nitro blue tetrazolium by superoxide dismutase). Superoxide anion was photogenerated in the presence of guanine, xanthine, 6-thioguanine, thymine, uracil, 6-methyl uracil, orotic acid and 5- as well as 6-amino uracil but not in the presence of 24 other related compounds examined. Replacing the oxygen at the 6-position of guanine with sulfur or attachment of an amino group to the 5- or 6-carbon of uracil greatly increased superoxide anion generation as compared to guanine and uracil, respectively. The attachment of a carboxyl group at the 6-position of uracil augmented superoxide anion photogeneration to a much smaller extent and thymine and 6-methyl uracil did not yield any more superoxide anion than did uracil. In general, only those compounds which had an oxo group at the 6-position of purines or the 4-position of pyrimidines, and either an oxo or an amino group in the 2-position of either ring served as substrates for photogeneration of superoxide anion. Additionally, presence of purines and pyrimidines in an enol and/or amino form and an unsubstituted imidazole ring for purines were required for photogeneration of superoxide anion.

Superoxide anion ($O_{2\cdot}^{-}$) is a common cellular metabolite formed as a result of the univalent reduction of molecular oxygen during many biochemical reactions (*3*). It might also be generated in photosensitized reactions (*2*) upon illumination of biological systems with visible light. $O_{2\cdot}^{-}$ can undergo secondary reactions to form other active oxygen species such as hydrogen peroxide, singlet oxygen and hydroxyl radical (*3,9*). The last two species are very strong oxidizing agents. $O_{2\cdot}^{-}$ and/or the other active oxygens derived from it have been implicated in the oxidative destruction of biological material (*3*), in oxygen toxicity (*6*), and in a variety of photodynamic effects manifested as phototoxicity, mutations, aging or carcinogenesis (*10*). The importance of active oxygen species in initiating oxidative changes in foods has been recently reviewed (*7*).

$O_{2\cdot}^{-}$ can be generated in an aqueous medium at neutral pH when certain amino acids, in the presence of catalytic amounts of riboflavin as a sensitizer, are exposed to fluorescent light (*8*). Upon illumination, riboflavin absorbs visible light at a wavelength around 450 nm to become electronically excited to its triplet state. In the presence of an appropriate substrate and at sufficient oxygen concentration, triplet riboflavin can initiate redox reactions, being fully reduced in the process (*2*). Spontaneous reoxidation of this photoreduced riboflavin by molecular oxygen results in the formation of $O_{2\cdot}^{-}$ (*5*).

It has been reported (*1,4*) that, in addition to certain amino acids, several other aliphatic and aromatic nitrogen compounds can photoreduce riboflavin. It is also known that nucleic acids are subject to photosensitized oxidation (*10*). It was therefore considered likely that purines and pyrimidines have the potential to photoreduce riboflavin, thereby producing $O_{2\cdot}^{-}$. The photogeneration of small amounts of $O_{2\cdot}^{-}$ in milk serum via photoreduction of riboflavin by orotic acid present in milk has been observed previously (*8*). The studies reported here were performed to determine if other purines and pyrimidines served as substrates for riboflavin-sensitized photogeneration of $O_{2\cdot}^{-}$. The photogeneration of $O_{2\cdot}^{-}$ by monomeric bases from nucleic acids could be responsible in part for the oxidative changes (odors and off-flavors) which occur rapidly upon exposure of certain foods to visible light. Structural aspects of purines and pyrimidines which are important for photogeneration of $O_{2\cdot}^{-}$ were investigated.

MATERIALS AND METHODS

Reagent-grade chemicals and deionized water were used in all assays. All photoinduced reactions were carried out in 10-ml pyrex glass test tubes placed in an aluminum foil-lined box. The upper part of the box was fitted with a 15-watt cool white fluorescent bulb placed 8 cm from the test tubes. The test tubes were thus subjected to 5200 lux of light (compared to 100,000 lux for sunlight on a sunny day) for 1 h at 25 C; the temperature of the tube contents did not rise above 29 C. Samples kept in the dark were incubated under the same conditions, except that they were wrapped with aluminum foil to protect them from light. The reaction mixture, contained in 4.5 ml of air-equilibrated 50-mM phosphate buffer, pH 7.8, included 12.8 μM of riboflavin, 0.1 mM of purine, pyrimidine or related compound and 0.5 mM of nitro blue tetrazolium. Controls contained the same reagents in addition to 30 μg of bovine erythrocyte superoxide dismutase, added to the reaction mixture before illumination was initiated. Additional reagent blanks having riboflavin or the nitrogen-base omitted from the reaction mixture were illuminated using the same conditions. The photogenerated $O_{2\cdot}^{-}$ was measured by the nitro blue tetrazolium (NBT) assay, as previously described (*8*).

RESULTS AND DISCUSSION

Thirty-three compounds, including imidazole, purine or pyrimidine bases and related compounds, all at 0.1-mM concentration, were evaluated for their capacity to photogenerate $O_{2}^{\cdot -}$ in a riboflavin-photosensitized reaction. Among the compounds studied, three of the purine bases (guanine, xanthine and 6-thioguanine), and six of the pyrimidine bases (thymine, uracil, 6-methyl uracil, orotic acid, 5-amino uracil and 6-amino uracil) were sources of $O_{2}^{\cdot -}$, whereas 24 other related compounds did not yield any measurable $O_{2}^{\cdot -}$ (Table 1). Moreover, none of the compounds studied formed detectable $O_{2}^{\cdot -}$ in the absence of light nor in the presence of light when riboflavin was omitted, indicating that both light and sensitizer were necessary for the reaction to occur. On the other hand, a relatively small amount of $O_{2}^{\cdot -}$ (about 5-8 μM) was measured in reagent blanks containing riboflavin and nitro blue tetrazolium, but devoid of the nitrogen bases. It is likely that at the concentration used, riboflavin served as both a sensitizer and as an oxidizable substrate (*4*). This background quantity of $O_{2}^{\cdot -}$ was subtracted from the total $O_{2}^{\cdot -}$ measured to compute the final yield of $O_{2}^{\cdot -}$ emanating from the nitrogen-bases. As indicated in Table 1, the purines and pyrimidines sensitive to photooxidation generated between 10-80 μM of $O_{2}^{\cdot -}$ after 1 h of illumination.

TABLE 1. *Quantity of superoxide anion photogenerated by purines and pyrimidines in the presence of riboflavin upon illumination with fluorescent light for one hour.*

Compound[a]	Superoxide anion (μM)
Purines:	
Guanine	15.3 ± 1.0
Xanthine	31.9 ± 2.0
6-Thioguanine	85.5 ± 13.3
Pyrimidines:	
Thymine (5-methyl uracil)	11.4 ± 2.3
Uracil	11.9 ± 2.2
6-Methyl uracil	15.4 ± 0.9
Orotic acid	27.0 ± 1.1
5-Amino uracil	64.9 ± 4.7
6-Amino uracil	66.7 ± 10.8

[a]The results are expressed as mean ± standard deviation of four measurements. The following nitrogen-bases did not generate any detectable superoxide anion: imidazole, adenine, adenosine, adenosine 5'-triphosphate, 8-azaguanine, 8-azaxanthine, caffeine, guanosine, guanosine 5'-triphosphate, hypoxanthine, purine, theobromine, theophylline, uric acid, cytosine, cytidine, cytidine 5'-triphosphate, dihydroorotic acid, 2,4-dihydroxy-1,3-dimethylpyrimidine, pyrimidine, thymidine, thymidine 5'-triphosphate, uridine, uridine 5'-triphosphate.

Among the bases commonly occurring in nucleic acids, only guanine, thymine, and uracil generated $O_{2}^{\cdot -}$, whereas adenine and cytosine did not yield $O_{2}^{\cdot -}$. None of the corresponding ribonucleosides or ribonucleotide triphosphates yielded any detectable $O_{2}^{\cdot -}$. Most of the purines and pyrimidines which photogenerated $O_{2}^{\cdot -}$ had oxo groups at the 2- and the 6-positions of the purine ring or the 2- and the 4-positions of the pyrimidine ring. However, an amino group can substitute for the oxo group at the 2-position of purines; for example, guanine photogenerated about 50% of the $O_{2}^{\cdot -}$ which was formed in the presence of xanthine. Examination of the structures indicated that the capacity of purine and pyrimidine bases to undergo photosensitized oxidation depended on the capacity of the nitrogens in the 1- and 3-positions of the purine or pyrimidine rings to contribute to keto-enol or imino-amino tautomerism. Those compounds which had oxo groups in potentially reactive positions, but which could not undergo tautomerization because of methylated nitrogens in the 1- and 3-positions (theophylline, caffeine, theobromine and 2,4-dihydroxy-1,3-dimethyl-pyrimidine) did not photogenerate $O_{2}^{\cdot -}$. The double bond between positions 5 and 6 of the pyrimidines apparently was required for photoreaction since dihydroorotic acid did not undergo the photooxidation which was evident for orotic acid. Although imidazole itself was not a substrate for the photogeneration of $O_{2}^{\cdot -}$, the presence of an unmodified imidazole ring in the purine structure was important for the reaction. For example, 8-azaxanthine, 8-azaguanine, guanosine, and uric acid did not yield any measurable amount of $O_{2}^{\cdot -}$, whereas $O_{2}^{\cdot -}$ was photogenerated in the presence of xanthine and guanine.

The results further suggest that presence of purines and pyrimidines in an enol and/or amino form is required to univalently reduce riboflavin with subsequent formation of $O_{2}^{\cdot -}$. The purine or pyrimidine radical resulting from donation of an electron to riboflavin would be stabilized by resonance. If the resulting radicals were less stabilized by resonance, transfer of an electron from the enol group of the purine or pyrimidine to riboflavin would be much less likely. Thus a decreased possibility for resonance might explain why dihydroorotic acid, for example, did not generate any detectable $O_{2}^{\cdot -}$. Similarly, Zenda et al. (*11*) observed that photodegradation of purines in the presence of methylene blue was favored by an enol and/or amino structure with respect to the N-1 and N-3 positions.

Certain modifications of the purines and pyrimidines increased $O_{2}^{\cdot -}$ formation (Table 1). The capacity to photogenerate $O_{2}^{\cdot -}$ was increased several-fold by replacing the oxo oxygen at the 6-position of guanine with sulfur to give 6-thioguanine. Introduction of an amino group at the 5- or 6-position of uracil to yield 5- or 6-amino uracil also greatly increased the photogeneration of $O_{2}^{\cdot -}$. The attachment of a carboxyl group at the 6-position of uracil (orotic acid) augmented $O_{2}^{\cdot -}$ photogeneration to a much smaller extent than did the amino group at the 5- or 6-position, whereas 5-methyl uracil (thymine) and 6-methyl uracil did not yield any more $O_{2}^{\cdot -}$ than did uracil.

The photogeneration of $O_{2}^{\cdot -}$ in the reaction mixture containing many commonly-occurring nitrogen bases and riboflavin could have ramifications for a number of biological systems ranging from deterioration of foods to the etiology of skin cancer and photo-induced drug reactions. Not only would the nitrogenous base be photooxidized and the $O_{2}^{\cdot -}$ be formed, but also generation of $O_{2}^{\cdot -}$ might result in subsequent formation

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A Research Note

A Method for Decreasing Sampling Variance in Bacteriological Analyses of Meat Surfaces[1,2]

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ABSTRACT

Viable counts of bacteria are often high in some areas and low in adjacent areas of the same surface of fresh meat. The present study indicated that rubbing meat surfaces together before sampling reduces variation among bacterial plate counts of pieces of beef plate meat. Counts before rubbing ranged from 2 to 6,187/cm², whereas counts after rubbing ranged from 15 to 2,043/cm². The reduced sample variance allowed for fewer samples to be taken in studies of cleaning and sanitizing of fresh beef.

Surfaces of dressed carcasses of meat animals would be essentially sterile were it not for the bacteria deposited on them from their hides and from equipment, personnel and the environment. Numbers and distribution of microorganisms on surfaces of fresh meat vary with the method of dressing and cleanliness of the environment.

In previous research the numbers of microorganisms/cm² of fresh meat surface were highly variable, ranging from logs of 3.42 to 8.86/cm² (*1*). Viable counts of bacteria were high in some areas and low in others. Thus both the area sampled and the number of samples had to be large. In some research situations, an alternative to using large numbers of samples might be to rub each meat surface to be sampled against each of the other sampling surfaces before sampling to more evenly distribute the microorganisms. The present paper reports on distribution of microorganisms on strips of fresh beef plate meat before and after the surfaces of the meat were rubbed together.

MATERIALS AND METHODS

Nine pieces of beef plate meat (13 × 18 × 1.3 cm) were obtained from a commercial source (24-h postmortem), placed in plastic bags, and frozen. Three pieces were thawed and used in each of the three replications.

Initially, from each piece of meat in each of three replications six meat-core samples (2.54-cm diameter, 3-mm approximate depth) were removed at random according to the sampling procedure shown in Fig. 1. Each core was placed in a separate blender jar with 99 ml of buffered distilled water and blended for 1 min. Then the sampling surfaces of each piece of meat were exposed to each of the other pieces of meat within the replicate by rubbing the surfaces together. In that procedure, each piece of meat was twice laid on and pulled across the surface of each of the other pieces of meat. After the exposure, another six cores were removed and treated as described above.

Blended samples were plated in duplicate and consecutive dilutions. Plates were incubated for 72 h at 28 C. Colonies were counted and results recorded. The data were analyzed by use of the Statistical Analysis System (*2*) on an IBM 370 computer.

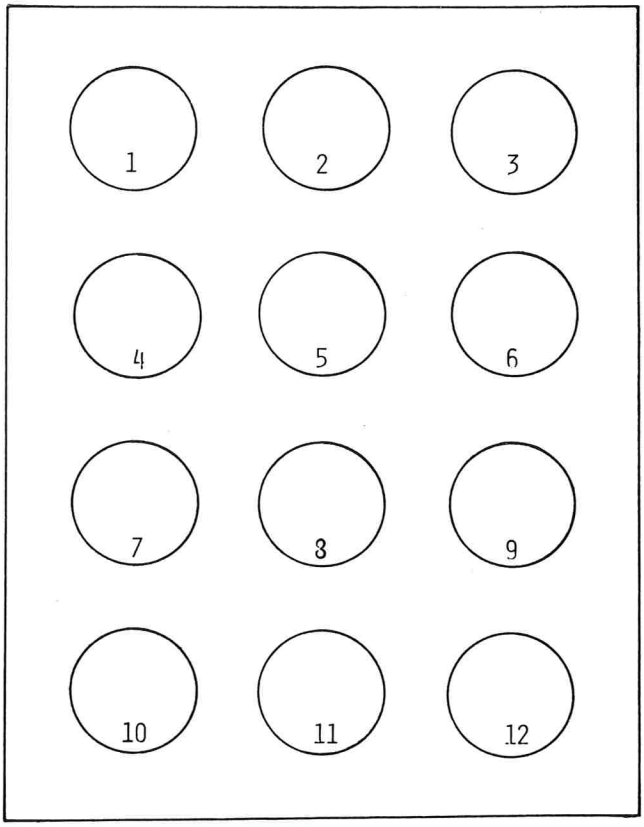

Figure 1. *Sampling pattern for sample variance study. Six core samples were elected at random from each piece of meat both before and after the surfaces of the meat pieces were rubbed together.*

[1] *Contribution from the University of Missouri Experiment Station, Journal Series No. 8261.*
[2] *Mention of a trade name or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.*
[3] *U.S. Department of Agriculture.*
[4] *Agricultural Experiment Station.*
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The data were analyzed (*a*) separately for each of three pieces of meat within each of three replicates, (*b*) by replicate and (*c*) overall. In each instance, the null hypothesis tested was that the dispersion of colony counts before rubbing is the same as the dispersion after rubbing. If the dispersions before and after rubbing for a particular piece of meat or for a particular replicate were different, the null hypothesis was to be rejected. For example, if one or more of the colony counts before rubbing was lower than the lowest count after rubbing and one or more of the before-rubbing counts was also higher than the highest count after rubbing, then the counts before rubbing would be more dispersed than the counts after rubbing.

RESULTS AND DISCUSSION

Mood's test (*3*) was used to test the above null hypothesis nonparametrically. The assumption of normality required by the usual parametric F-test for equality of variance could not be made because a few high counts caused the distribution of colony counts before and after rubbing to be skewed to the right (Table 1). The number of counts less than 20/cm² changed from seven to one because of rubbing.

Table 2 shows the minimum and maximum colony counts for the cores removed before and after rubbing, as well as the results of Mood's test. The null hypothesis of equal dispersion was rejected for one piece of meat in each of the three replicates, for all meat pieces combined in the first two replicates, and overall. Ranges of counts in the third replicate were smaller with minimums higher and maximums lower than for the first two replicates. With this smaller range of counts, it was more difficult to detect a difference in dispersion caused by rubbing.

Data in the two columns under "Numbers of counts lower (or) higher" verify the claim that rubbing reduced variance. Of six cores taken before rubbing from meat two, replicate one, three counts were lower and two higher than those of any core taken after rubbing. The same was true for replicate three, meat three. Overall, of the fifty-four cores removed before rubbing nine had lower colony counts and one had a higher colony count than any core removed after rubbing. Thus, the overall range was significantly reduced by rubbing. Counts before rubbing ranged from 2 to 6,187/cm², whereas counts after rubbing ranged from 15 to 2,043/cm².

It was not surprising that the colony counts before rubbing tended to be lower than the counts after rubbing. Before rubbing, relatively few areas had high counts. Since sampling was random, the areas of highest count may not have been sampled. After the meat pieces were rubbed together, bacteria from the few high count areas were more evenly distributed over the surface of the meat.

The data gathered indicated that the rubbing of meat surfaces together tends to reduce the variance of bacteria counts. Therefore, such rubbing reduces the number of samples needed for any specific cleaning and sanitizing study.

TABLE 1. *Frequency distribution of colony counts before and after rubbing, replicate 1.*

Interval of colony counts (per cm²)	Number of cores	
	Before	After
0-9	5	0
10-19	2	1
20-29	2	5
30-39	1	3
40-49	0	2
50-99[a]	2	3
100-199[a]	2	2
200-299[a]	2	0
300 and over[a]	2[b]	2[c]
Total	18	18

[a]Change in interval size.
[b]Before values are 1,125 and 6,187.
[c]After values are 320 and 2,043.

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TABLE 2. *Minimum and maximum colony counts for the cores taken before and after rubbing and the results of Mood's Test for three pieces of meat for each of 3 replicates.*

Replicate	Piece of meat	Number of cores	Before rubbing, bacteria/cm²		After rubbing, bacteria/cm²		Number of counts[a]		Mood's test
			Min	Max	Min	Max	Lower	Higher	
1	1	6	6	6,187	36	2,043	1	1	
	2	6	2	1,125	15	46	3	2	*
	3	6	9	94	20	77	3	1	
	all	18	2	6,187	15	2,043	7	1	**
2	1	6	15	336	24	710	1	0	
	2	6	3	258	55	191	4	1	*
	3	6	21	858	73	493	1	1	
	all	18	3	858	73	710	5	1	*
3	1	6	22	76	34	151	5	0	
	2	6	13	65	23	76	2	0	
	3	6	19	90	29	71	3	2	*
	all	18	13	90	23	151	5	0	
Overall		54	2	6,187	15	2,043	9	1	**

*Means $P < .05$
**Means $P < .01$
[a]Number of colony counts before rubbing that were lower than the smallest or higher than the largest of the counts after rubbing.

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A Research Note

Isolation of *Yersinia enterocolitica* from Pig Tonsils

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ABSTRACT

Yersinia enterocolitica serotype 0:5 was isolated from the tonsils of 8 of 27 butcher hogs subjected to normal slaughtering procedures. Isolation through enrichment in cold mannitol broth or a bile medium at 4 C for 10-60 days was equally effective. Enrichment in modified Rappaport broth at 25 C for longer than 2 days sharply decreased recovery of *Y. enterocolitica* from tonsils. The type of enrichment medium also had a marked effect on the recovery efficiency of different strains of *Y. enterocolitica* from ground meat.

In recent years, *Yersinia enterocolitica* and closely related bacteria have been isolated with increasing frequency from various foods such as meats, poultry, seafoods, vegetables and raw milk (*2,6,7,8,11,12,16,17, 19*). Many of the food isolates differ in some biochemical characteristics from typical clinical strains responsible for human yersiniosis (*2,6,9*). In spite of the presence of *Y. enterocolitica* or related bacteria in certain foods, there has been only one documented outbreak of foodborne infection of *Y. enterocolitica* in the United States (*20*). This incident was caused by consumption of contaminated chocolate milk. Among meat animals, swine are considered by many investigators as a potential source of clinically significant types of *Y. enterocolitica*. European, Canadian and Japanese specialists (*1,14,15, 18,21,22,23*) have reported isolation of human serotypes from cecal contents, lymph nodes and throats of pigs. For example, *Y. enterocolitica* serotype 0:3 was recovered from 47 of 426 throat swabs of pigs (*21*) and from 75 of 142 pork tongues (*22*). This information indicates that swine may be a potential reservoir for human infection with *Y. enterocolitica*. This note describes isolation of *Y. enterocolitica* from pig tonsils and the recovery efficiency of three enrichment media in tandem with plating on bismuth sulfite agar.

MATERIALS AND METHODS

Samples

Palatine tonsils were removed by surgical excision from market weight (200-250 lb) butcher hogs after the animals had been subjected to normal slaughtering procedures (electrical stunning, exsanguination, scalding, dehairing, evisceration). Animals were obtained from the Texas A&M Meats Laboratory and from a commercial meat packing plant. The tonsils were placed immediately in 100-ml diluent bottles containing 10 ml of 0.1% sterile peptone (Difco). The surfaces of the tonsils were rinsed thoroughly with the peptone broth. No attempt was made to break up the tissue. The peptone rinse was examined for *Y. enterocolitica* immediately after collection of the samples and after storage for 14 days at 4 C. An additional 10 ml of 0.1% sterile peptone was added to each sample before refrigerated storage.

Isolation of Y. enterocolitica

One-ml portions of the peptone rinse were placed in 10 ml of modified Rappaport broth [MRB, (*4*)], cold mannitol broth [CMB, (*3*)] and a bile enrichment medium [BEM, (*13*)]. Enrichment in MRB was at 25 C for 2 to 60 days, in CMB and in BEM at 4 C for 10 to 60 days. After appropriate intervals, a loopful of enrichment broth was streaked on a bismuth sulfite agar (BS) plate (Difco). In addition, a loopful of the peptone rinse was streaked immediately on a BS agar plate. Plate incubation was at 25 C for 3 to 4 days. Suspicious black colonies were picked from the plates and placed on slants of tryptic soy agar (Difco) at 25 C. Each isolate was subjected to the tests shown in Table 1. Test procedures and composition of the media and test reagents were as described in the *Compendium of Methods for the Microbiological Examination of Foods* (*4*). Serotyping of the isolates was carried out through the courtesy of Dr. T. J. Quan (Center for Disease Control, Fort Collins, Colorado). Testing for tissue invasiveness by the Serény test was done by Dr. D. L. Zink, Department of Veterinary Microbiology, Texas A&M University.

RESULTS AND DISCUSSION

Y. enterocolitica was recovered from the tonsils of 8 of 27 pigs (Table 2). Only three of the samples were positive when the peptone rinse of the tonsils was streaked directly on BS agar. Holding the rinse fluid for 14 days at 4 C increased the number of positive samples to six. Isolation through enrichment in CMB or BEM at 4 C for 10 to 60 days was equally effective. With enrichment in MRB at 25 C, however, the number of samples positive for *Y. enterocolitica* was greatest after 2 days and decreased sharply after 10 days. Characteristics of the isolates of the 8 positive samples are presented in Table 1. On the basis of these characteristics, they were assigned to Niléhn biotype 3 (*4*). They belonged to serotype 0:5. All of the isolates were Serény test (kerato-conjunctivitis in guinea pigs) negative. *Y. enterocolitica* serotype 0:5 has been previously isolated from pork and throat swabs of pigs (*1,14*). Although the potential public health significance of the cultures isolated from pig tonsils is not yet known, their biochemical characteristics are similar to those of many typical clinical isolates. A

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TABLE 1. *Characteristics of* Y. enterocolitica *isolated from pig tonsils.*

Test	Reaction
Gram reaction	–
Cytochrome oxidase	–
Urease (25 C)	+
Phenylalanine deaminase (25 C)	–
Arginine dihydrolase (25 C)	–
Lysine decarboxylase (25 C)	–
Ornithine decarboxylase (25 C)[a]	+
Citrate (25, 36 C)	–, –
Motility (25, 36 C)	+, –
Voges-Proskauer (25 C)[a]	+
β-galactosidase (25 C)[a]	+
Nitrate (36 C)[a]	+
TSI (25, 36 C)	A/A
Indole (36 C)[a]	–
Esculin (25, 36 C)[a]	–, –
Lactose O/F (25 C)[a]	+
Salicin (36 C)[a]	–
Rhamnose (25 C)	–
Raffinose (25 C)	–
Melibiose (25 C)	–
Sucrose (25 C)[a]	+
Trehalose (36 C)[a]	+
Sorbitol (36 C)[a]	+
Sorbose (36 C)[a]	+
Xylose (25 C)[a]	+

[a]Niléhn tests for biotype.

previous study (5) has shown that heating of beef to internal temperatures of 60 to 62 C destroys large numbers of *Y. enterocolitica*. Hence, properly heated pork should not present a public health problem with respect to *Y. enterocolitica* unless it is recontaminated after heating.

In preliminary experiments, the recovery efficiency of the enrichment media (BEM, CMB, MRB) was tested. Meat homogenates were prepared by mixing in a Stomacher-400, 10 g of ground meat with 90 ml of 0.1% sterile peptone. To meat homogenates were added 1 ml of appropriate dilutions of a 24-h-old brain heart infusion (BHI, Difco) culture of *Y. enterocolitica*. One ml of these inoculated meat homogenates then was added to each of the three enrichment media. Enrichment in MRB was carried out at 25 C for 4, 7, 14, 21, 30 and 60 days; in CMB and BEM at 4 C for 7, 14, 21, 30 and 60 days. Isolation and identification of *Y. enterocolitica* was as described for pig tonsils. Distinct differences in biochemical characteristics (esculin hydrolysis, carbohydrate utilization) allowed differentiation between added and naturally occurring *Y. enterocolitica*. Three cultures were used: AYE-11, serotype 0:3, isolated from cecal contents of swine in Japan (obtained from Armour Food, Scottsdale, AZ); 2635, serotype 0:8, isolated from chocolate milk (Center for Disease Control, Atlanta, GA); and 84, serotype 0:5, isolated from pig tonsils at Texas A&M University. Based on agar plate counts of 24-h-old BHI cultures, concentrations of viable cells of *Y. enterocolitica* per enrichment tube were calculated to range from: 0.007-740 (AYE-11), 0.006-570 (2635) and 0.009-940 (84).

Recovery efficiency of *Y. enterocolitica* on BS agar was dependent upon culture and type of enrichment medium. With AYE-11, *Y. enterocolitica* was recovered from MRB with an initial concentration of 7 cells by enrichment for 4 to 60 days. However, recovery of this culture from BEM (740 cells per tube, 0-7 days) or CMB (74 cells per tube, 30 days) was less efficient. With cultures 2635 and 84, recovery was more effective in BEM (6-9 cells, 21-60 and 60 days) or in CMB (6-9 cells, 14-21 and 21-30 days); in MRB, *Y. enterocolitica* was recovered from tubes initially containing 57-94 cells. Recovery of *Y. enterocolitica* by direct streaking of meat homogenates on BS agar was commensurate with the small amount of sample (0.01 ml) placed on the plate. Fewer naturally occurring *Y. enterocolitica* and *Pseudomonas* spp. appeared on BS plates streaked from MRB, which made examination of the plates and picking of suspicious black colonies easier. With enrichment in BEM or CMB for 7 days or longer, a large number of *Pseudomonas* spp. and naturally occurring *Y. enteroco-*

TABLE 2. *Recovery of* Y. enterocolitica *from pig tonsils by direct streaking of peptone wash on BS agar and by enrichment in MRB, CMB and BEM and subsequent streaking on BS agar.*

		Enrichment in															
		MRB (days at 25 C)						CMB (days at 4 C)					BEM (days at 4 C)				
Sample	Direct streaking on BS agar	2	10	14	21	30	60	10	14	21	30	60	10	14	21	30	60
1[a]	–	+	–	–	+	–	–	+	+	+	+	+	+	+	+	+	+
1[b]	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
2[a]	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
2[b]	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
3[a]	–	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+
3[b]	–	+	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+
4[a]	–	+	+	–	–	–	–	–	–	–	–	–	+	+	+	+	+
4[b]	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+
5[a]	–	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
5[b]	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
6[a]	+	+	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+
6[b]	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+
7[a]	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
7[b]	+	+	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
8[a]	+	–	+	+	+	+	+	+	+	+	+	+			ND[c]		
19 other samples	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a]Sample was examined immediately.
[b]Sample was examined after storage in 10 ml of 0.1% peptone for 14 days at 4 C.
[c]ND: not done.

litica (esculin +, salicin +) were frequently present on the BS plates.

Results of this study, as well as those of others (*10,16,17*), indicate that the effectiveness of isolating *Y. enterocolitica* from foods or clinical specimens depends upon the ability of the selective enrichment and plating media to support growth of the strain(s) involved. Present methods for detection and enumeration of low numbers of *Y. enterocolitica* added to various foods are not very efficient and often produce erratic results. For this reason, use of several enrichment and plating media simultaneously for isolation of unknown strains of *Y. enterocolitica* from foods is highly recommended.

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Lactase and Starter Culture Survival in Heated and Frozen Yogurts[1]

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ABSTRACT

Yogurt customarily contains large numbers of viable starter culture, although some yogurt is heat-treated to inactivate the starter. It was found that heating to inactivate the starter also inactivated lactase. This could have a disadvantageous effect on lactose-intolerant consumers. Manufacture of frozen yogurt did not have any appreciable damaging effect on the yogurt culture or lactase activity.

Yogurt is believed to possess special nutritional attributes even though supporting scientific evidence has been lacking. Its consumption as a stable part of the diet has seemed to be associated with populations having greater longevity. Recent studies have provided some evidence for beneficial roles of yogurt in human digestion and physiology. For example, yogurt cultures produce certain metabolites during their growth in yogurt manufacture that allow the milk proteins to be digested and absorbed more readily than the protein in its unaltered form (*2,11*). Certain of these metabolites (*10*) also have a definite antagonistic effect against foodborne pathogens (*12*) which, if allowed to enter the yogurt, could be inhibited by the metabolites and thus be prevented from having a damaging effect on the host. It also has been shown that lactose-intolerant individuals may be able to consume yogurt without the adverse effects experienced when regular milk is consumed (*7*), because of the cultures contained in the yogurt (*8,9*).

There has been a controversy for some years as to whether or not yogurt should necessarily contain viable cells of the starter bacteria. Possibly those who contend that the bacteria need not be alive in yogurt derive their conclusions from the fact that *Lactobacillus bulgaricus* and *Streptococcus thermophilus* used as starter for yogurt do not survive in the intestinal tract. Those who proposed that viable cultures must be contained in yogurt have relied on the fact that historically yogurt always contained viable cultures and any treatment to destroy the culture would alter the basic identifying characteristic associated with yogurt. This position has received considerable opposition in recent years because of longer shelf-life requirements needed for yogurt during its distribution and storage. Therefore, a growing tendency has been to heat yogurt, whereby starter cultures in the yogurt are inactivated. Also, frozen yogurt has become a popular product, but information about survival of starter culture in the product is not available.

This study reports the effects of heating and freezing on culture viability and lactase activity in yogurt.

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MATERIALS AND METHODS

Yogurt sources

Starters were prepared by subculturing plain commercial yogurt and by combining isolates of *L. bulgaricus* and *S. thermophilus* obtained from yogurt.

Yogurt was prepared using sterile 12% non-fat milk (NFM) inoculated with 2% starter and incubating at 43 C. Acid production was monitored by an automatic pH Analyzer (Beckman Instruments, Inc., Fullerton, CA) connected to an Electronic 15 Strip Chart Multipoint Recorder (Honeywell Industrial Products Groups, Philadelphia, PA). When the desired pH was attained, the yogurt was refrigerated overnight before making further observations.

Commercial brands of plain and flavored yogurts and frozen yogurts were purchased from local stores. Expiration dates were checked to insure that samples were within the date specified. Samples were transported in an ice chest and assayed on the day they were obtained.

Plain yogurt, frozen yogurt and production line samples of frozen yogurt were obtained from a local processing plant. Production line samples of frozen yogurt included the plain yogurt, the yogurt base mix, combination of the yogurt plus mix, and the yogurt and mix with the flavoring added.

Heat treatment of yogurt

A 10-g sample of yogurt was weighed into a sterile 125-ml Erlenmeyer flask. Flasks were immersed in a Metabolyte Water Bath Shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) and agitated at 250-300 rpm. Time required to reach the test temperature was 2 min. Temperature of the yogurt was monitored using a YSI Model 42 Tele-thermometer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) with the probe inserted through a stopper in the flask. At intervals, the flasks were removed and cooled in an ice water bath.

Colony counts were made using procedures basically as described in *Standard Methods for the Examination of Dairy Products* (*1*), with certain modifications. Initial dilutions were made in phosphate buffer and subsequent dilutions were made in blanks containing 0.1% non-fat milk solids (NFMS) and 0.1% antifoam B (Sigma Chemical Co., St. Louis, MO) in distilled water. Blanks were autoclaved at 121 C for 20 min.

Enumeration of starter bacteria

Total numbers of starter bacteria were enumerated on either MRS agar (*4*) or lactic agar (*6*). MRS broth (Difco) was rehydrated as directed and 1.5% granulated agar (BBL) was added. The media were autoclaved at 121 C for 15 min. MRS agar plates were placed in anaerobe bags and flushed with CO_2 (MRS CO_2). Lactic agar in plates was overlaid with lactic agar. All plates were incubated 3 days at 37 C and counted with the aid of a Quebec Colony Counter.

Sonication of samples for lactase assay

Sonication was employed to release the intracellular enzyme as suggested by Kilara and Shahani (*8*). A 10-g sample of yogurt was diluted to 100 ml using NaH_2PO_4/K_2HPO_4 buffer, pH 7.0. A portion of this was subjected to sonic disruption using a Branson Model S110 Sonifier (Branson Instrument, Inc., Stamford, CT) at power setting #2 for 8 min.

Lactase assay

Lactase activity was determined using ortho-nitrophenyl β galacto-pyranoside (ONPG) as the substrate (*3,9*). Tubes containing 4-ml volumes of 0.005 M ONPG (Sigma Chemical Co., St. Louis, MO) were tempered to 37 C; water was used for the blanks. Lactase activity was measured by adding 1 ml of yogurt sample diluted in 0.1 M phosphate buffer pH 7.0; after incubating 10 min at 37 C, 5 ml of 0.5 M Na_2CO_3 was added to halt the reaction. Release of orthonitrophenol (ONP) was measured spectrophotometrically at 420 nm. Quantitation was obtained from a standard curve of ONP versus optical density. A unit of lactose activity was defined as the release of 1 μmole ONP per minute per gram of yogurt. All assays were made in duplicate.

RESULTS

A preliminary survey was made on commercial samples of yogurt and frozen yogurt for lactase activity, starter culture count, and pH (Table 1). In regular yogurt, there were considerable differences in level of lactase activity. Differences were smaller in pH and colony counts, with the exception of one sample which had 0.08 U of lactase activity and a markedly lower colony count; other samples of this brand of yogurt gave similar results. The frozen yogurts showed much more variability in levels of lactase activity, pH, and starter counts.

Experiments were conducted to determine the effects of storage time on regular and on frozen yogurt. Samples of each type were obtained on the day of their manufacture in a commercial operation. The unfrozen was stored at 1 C, and the frozen at -16 C. There was a progressive decrease of about 50% in the lactase activity and a small decrease in colony counts in the unfrozen yogurt during the 20-day period (Fig. 1); in the frozen yogurt, there was no decrease in lactase activity, and only a slight decrease in colony counts.

TABLE 1. *Lactase activity and starter viability in regular and frozen commercial yogurt.*

Type/Flavor	Lactase (Units)	Colony forming units/g (Log_{10})[a]	pH
Regular unfrozen[b]:			
Plain	2.90	8.94	3.9
Strawberry	2.75	8.82	4.0
Plain	3.50	9.04	4.2
Strawberry	2.70	8.92	4.1
Plain	3.25	9.18	4.0
Strawberry	2.65	8.98	3.9
Plain	4.15	9.15	4.1
Lemon	0.08	5.66	4.3
Frozen[c]:			
Vanilla	0.60	4.51	6.1
Strawberry	0.92	6.26	5.6
Banana	1.95	7.58	5.7
Strawberry	0.94	8.32	5.6
Orange	0.79	7.32	5.0
Boysenberry	2.80	7.68	4.1
Strawberry	4.80	7.64	4.2
Strawberry	1.22	5.85	4.5

[a]Regular enumerated on lactic agar; frozen on MRS-CO_2.
[b]Plain samples and fruit flavors from different processors.
[c]Samples mostly from different processors.

Strains of *L. bulgaricus* and *S. thermophilus* isolated from commercial yogurt were combined and used as a starter. The combined strain culture was grown in 12% NFM, using 43 C incubation until pH values of 4.6 and 4.2 were obtained in individual batches. Samples of these yogurts were then heated at 60, 63 and 70 C, and residual levels of lactase and starter culture were determined after heat up (0 time) and after 1, 2 and 3 min at the test temperatures. Different batches of yogurt were prepared for each test temperature. Batches prepared for studying the effect of pH were from the starting materials, and testing was done the same day on batches at pH 4.6 and 4.2. Heat was more damaging to the lactase and starter in the yogurt at pH 4.2 than in yogurt at pH 4.6. At 60 C, survival of lactase and starter persisted through the 3-min heating period, although both had been reduced appreciably. At 65 C, lactase and starter persisted for 1 min at pH 4.6, but not in yogurt at pH 4.2. At 70 C, lactase was not detected beyond the heat-up period (2 min), and the culture was unable to survive 1 min.

Yogurt was also prepared by subculturing two brands of commercial yogurt (for starter) in 12% NFM; incubation at 43 C was maintained to pH 4.2. In the resulting yogurt, comparable inactivation of lactase and starter at 60 and 65 C were observed. This was interpreted as confirmation of data (in Table 2) obtained using mixed strains of *L. bulgaricus* and *S. thermophilus*.

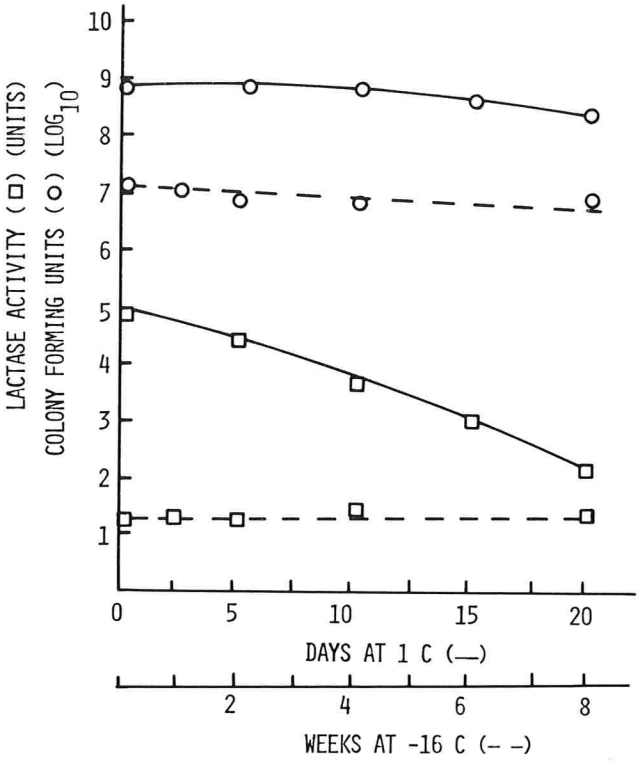

Figure 1. *Lactase activity and starter culture viability during storage at 1 C and - 17 C.*

Refrigerated storage at 1 C of the heated yogurt resulted in no reactivation of lactase during a 20 day period.

Data (Table 1) obtained from analyses of frozen yogurt purchased from retail stores suggested that manufacturing procedures influenced starter viability and lactase activity more than did frozen storage (Fig. 1). Therefore, samples were taken from the main steps in the formulation and manufacture of frozen yogurt at a commercial operation. The starting yogurt was the source of lactase, culture and acidity, as none was contributed by the yogurt mix (Table 3). Addition of the mix to the yogurt had a diluting effect on lactase activity and colony counts; the effect from flavor addition was less marked. Freezing had no effect on lactase activity, but did lower colony counts somewhat. Storage of the frozen yogurt at -16 C maintained the original level of lactase activity and colony counts decreased only slightly (Fig. 1) during an 8-week period.

TABLE 3. *Lactase activity, starter count and pH of production line samples during the manufacture of frozen yogurt.*

Line samples	Lactase activity[a] (units)	Colony forming units[b]/g (log_{10})	pH
Yogurt	3.90	8.73	4.0
Mix	0	3.0	6.4
Yogurt + mix	2.50	8.59	4.3
Yogurt + mix + flavor	2.05	8.54	4.3
Frozen product	2.10	8.40	4.3

[a]Samples were sonicated.
[b]Enumerated on lactic agar.

DISCUSSION

There has been some interest in the yogurt industry to heat-treat yogurt to inactivate the yogurt culture and arrest further acid development during distribution and storage. Heat treatment could not be expected to eliminate yeast and mold contaminants that would enter the product during packaging operations. In view of the present study, any increase in shelf-life obtained by a heat treatment must be weighed against inactivation of the lactase resulting from the heating. This becomes especially important if yogurt is to be used by individuals intolerant to lactose.

It has been shown by Goodenough and Kleyn (*8*) that rats fed natural yogurt containing viable yogurt culture were able to digest lactose more efficiently than animals fed other experimental diets, including pasteurized yogurt. Kilara and Shahani (*9*) concluded that lysing of the yogurt culture cells, as occurs in the intestinal tract, releases lactase from the cells which hydrolyzes lactose contained in the dairy product that has been consumed. Such studies point to the importance of maintaining the activity of the enzyme present in the cells of the yogurt culture. Lactase contained in the cells of the yogurt culture could enable lactose-intolerant persons to consume these products with no subsequent problems. This probably explains the observations by Gallagher et al. (*7*) who showed that lactose-intolerant people were able to digest yogurt, buttermilk and cottage cheese without adverse consequences.

In its proposal to establish new identity standards for different cultured milk foods, the Food and Drug Administration (*5*) has proposed that yogurt be either heat-treated or not heat-treated after the product has been cultured. The proposal stated that "the commissioner believes it is in the best interest of the consumer to preserve the food in its traditional form, i.e., containing live microorganisms and to provide for labeling to inform consumers when yogurts has been heat-treated after culturing". In further explanation, the FDA proposal stated "except for destroying the microorganisms, these foods retain essentially the same characteristic attributes." Considering the fate of lactase in heated yogurt it now is obvious that heated yogurt does not retain attributes present in nonheated yogurt, especially for lactose-intolerant persons.

Labeling of yogurt as "heated" when the heating is done to inactivate the culture, would not be a satisfactory manner of informing consumers that the product contains neither live culture nor lactase. Consumers generally consider heat treatment and pasteurization of milk products to be beneficial, especially when associated with the lowering or absence of bacterial populations. It is now obvious that this would not apply to yogurt. Actually, it would be desirable to prolong or increase lactase activity for many consumers.

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TABLE 2. *Effects of heat on inactivation of lactase and starter culture in yogurt at pH 4.6 and 4.2.*

		Lactase (U)			Colony counts/g[b] (log_{10})		
	Time (min)[a]	60 C	65 C	70 C	60 C	65 C	70 C
Yogurt pH 4.6	Unheated	3.25	5.00	4.70	8.81	9.04	8.95
	0[c]	2.65	0.45	0	8.73	8.36	5.11
	1	2.05	0.11	0	8.58	6.04	< 1.0
	2	1.27	0	0	8.38	2.92	< 1.0
	3	0.96	0	0	8.28	< 1.0	< 1.0
Yogurt pH 4.2	Unheated	4.40	5.80	6.00	8.92	9.18	9.08
	0[c]	3.15	0.36	0	8.83	8.26	2.58
	1	1.90	0	0	8.49	< 3.0	< 1.0
	2	0.52	0	0	7.78	< 1.0	< 1.0
	3	0.38	0	0	6.72	< 1.0	< 1.0

[a]Time held at treatment temperature; come-up time 2 min.
[b]Enumerated on lactic agar.
[c]Heated only until designated temperature was reached.

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Milk Films Exposed to High Humidity: Studies with Electron Microscopy and Electrophoresis[1,2]

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ABSTRACT

Stainless steel plates, which are similar to milk contact surfaces, were dipped in fresh raw milk. The residual film was dried (37 C and 10% to 20% relative humidity) for 30 min. Treated plates were then exposed to 100% relative humidity for 30 min at 37 C. Scanning electron microscopy revealed splotches of fat on surfaces of dried films and the humidified films had a more aggregated and porous appearance than films that were dried only. The incidence of granulated lactose was greater among humidified samples than among nonhumidified samples. Discontinuous polyacrylamide gel electrophoresis revealed that α- and β-caseins resisted rinsing from plates on which dried films were exposed to 100% relative humidity but not from plates on which films had been dried only.

In a previous paper (*3*) we reported that amounts of protein left on stainless steel plates after rinsing were increased by exposure of dried films of milk to 100% relative humidity. Optimal temperature was about 37 C, and amounts of soluble calcium directly affected the reaction.

High humidity exists in milk processing equipment such as pipelines, tanks, pasteurizers and separators. If such conditions cause buildup of tenacious soil, energy and detergent requirements will be increased. The efficacy of halogenated sanitizers may also be affected. Thus cleaning and sanitizing can be impaired.

In the study presently reported we used scanning electron microscopy and electrophoresis to help explain the phenomenon. Our objectives were to determine the mechanism by which solubility of films was decreased and to identify components of the affected films.

MATERIALS AND METHODS

Scanning electron microscopy

Ten square plates of stainless steel (304, No. 4 finish), 6 × 6 mm, were immersed 3 cm in cold (5-7 C) milk or skim milk and then dried for 30 min at 37 C and 10 to 20% relative humidity (RH). Five of the squares were subsequently exposed for 7.5 min to 100% RH at 37 C. They were then redried at 10 to 20% RH for 30 min. Five were not exposed to 100% humidity before redrying; they were redried only at 10 to 20% RH for 30 min. Three each of the exposed and unexposed samples were then rinsed with distilled water and allowed to air dry. Two of each were left unrinsed.

Plates were mounted on aluminum studs (1.5-mm diameter) with adhesive-backed copper conducting tape (3M Co.). The studs were placed in a sputterer-coater apparatus (Model PE 5000, International Scientific Instruments) and coated with gold to 720-μm thickness, according to manufacturer's instructions. Samples were examined in a scanning electron microscope (Model SMS 2-1, International Scientific Instruments) and photographs were prepared.

Preparation of samples for electrophoresis

Milk. Sucrose (0.5 g) was dissolved in 10 ml of milk, after which a few crystals of the tracking dye, bromophenol blue (Reagent ACS, Eastman Kodak Co.) were added. Twenty microliters of the mixture was transferred to the slots (1-cm wide) of gels to serve as controls.

Samples of rinse solutions. Fifty stainless steel plates (10 × 10 cm, No. 4 finish) were soiled in milk. Twenty-five were exposed three successive times to 100% RH for 7.5 min and to drying at 37 C, 10 to 20% RH, for 30 min. The remaining 25 were dried only at 37 C for 112.5 min. Soiled plates were rinsed with 350 ml of distilled water, five at a time, in a washing vessel. The volume of water in the vessel was readjusted to 350 ml between rinsings of groups of five plates within each set of 25. Rinsings were concentrated in a rotary evaporator (Rotavapor R, Büchii Laboratoriums-Technik AG) at 37 C and 710 mm (28 in.) vacuum and with the condenser at 0 C. The solution was evaporated to 50 ml in about 4 h, and 9 g of Lyphogel (Gelman Instrument Co.) was added to further concentrate the solution. After storge at 5 C for at least 6 h, the volume of the solution was about 5 ml and the protein concentration was about 2%. A few crystals of bromophenol blue and 0.25 g of sucrose were then added. Fifty microliters of the solution was introduced into gels.

Samples of residue. Samples of residue were obtained from exposed and unexposed plates used in the preparation of rinse samples. Plates were thoroughly rinsed by spraying them twice with distilled water, then they were air dried. We spread distilled water (0.2 ml/side/plate) evenly on plates and loosened soil by vigorously rubbing the plates with our fingers while wearing rubber gloves. Washings from each plate in a treatment were collected in a 25-ml flask. Sucrose and bromophenol blue were added to the washings in the flask.

Electrophoresis

Discontinuous vertical gel electrophoresis was done with an EC Model 470 unit (EC Apparatus Corp.) and an EC 454 power supply. The unit was cooled by a constant-temperature circulator (Model FK, Polyscience-Haake, Inc.).

Preliminary studies disclosed that the following buffers, gels, and techniques were satisfactory:

Buffers. (*a*) *Running buffer, pH 8.9:* 46 g of Tris and 4 ml of hydrochloric acid (HCl) were diluted to 1 l with distilled water. (*b*) *Spacer buffer, pH 6.7:* 7.5 g of Tris and 4 ml of HCl were diluted to 1 l with distilled water. (*c*) *Electrode buffer, pH 8.3:* 1.2 g of Tris and 5.8 g of glycine were dissolved in 2 l of distilled water. pH was adjusted by

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[1]*Contribution from the University of Missouri Experiment Station, Journal Series No. 8218.*

[2]*Reference to a company or product name is for specific information only and does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.*

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adding either of the two reagents.

Gels. Spacer and running gels were prepared with 5 and 10% solutions, respectively, of Cyanogum 41 (Fisher Scientific). To each 100 ml of solution were added 0.1 ml of N,N,N',N'-tetramethylethylenediamine (TMED) and 1 ml of 1:100 dilution of Tween 80 (Rohm and Haas). Ammonium persulfate (0.1 g/100 ml) was added with vigorous stirring just before use.

Conditions of electrophoresis. Eight 1-cm slots were formed in the gels. Temperature was maintained at about 7 C by circulating cold water. Current was set at 50 ma and 200 volts until the dye front reached the running gel (about 30 min), after which it was increased to 70 ma and 330 volts. We terminated electrophoresis when the dye front reached 5 cm from the distal edge of the gel (about 3 h).

Staining and destaining. Amido Black 10 (Eastman Kodak Co.) was dissolved in a solution composed of 45 parts methanol, 10 parts acetic acid, and 45 parts distilled water such that the concentration of the dye was 0.25%. We stained the gels for 45 min and then destained them in the solution described above without the dye. The destaining solution was changed three times during the first hour. When gels had cleared substantially, we clarified them further by soaking them in a solution of 5% aqueous glycerin for 2 h. The gels were photographed and then scanned with a densitometer or preserved by keeping them in destaining solution, or both.

Identification of the tenacious protein components

Identification of components of the tenacious fraction was based primarily on comparisons of electrophoretic patterns of the tenacious samples with those of standards of known composition in the same gel.

A set of 25 plates was dipped into milk at 5 C, dried, and exposed to high humidity. Nontenacious components were rinsed from the plates with distilled water. Tenacious film was removed by vigorous scrubbing and collected as described previously. Milk used to soil the plates was used as the control in electrophoresis. Skim milk separated from the remaining milk was cooled to about 5 C, then acidified with 0.1 N hydrochloride acid, while stirring, until pH 4.6 was reached. This skim milk was stored at 5 C for 30 min, after which precipitated casein was filtered with cheese cloth. The casein was washed five times with distilled water (1 L/washing) to remove whey. It was then resuspended in a solution of sodium hydroxide (pH 11.0) for resolubilization. We adjusted the pH of this solution to 6.7 with 0.1 N HCl. The whey (pH 4.6) was kept at 5 C for 60 min and then refiltered in a Büchner funnel with Whatman No. 40 filter paper. The pH of the filtrate was adjusted to 6.7. Acidification, filtration and pH adjustments of whey were repeated to increase purity. Ten milliliters of the whey, casein suspension and the original milk were prepared for electrophoresis as before. The tenacious sample was prepared similarly. We introduced milk (20 μl), casein suspension (50 μl), whey (100 μl), and tenacious samples (100 μl) in duplicate into the gel slots, and performed electrophoresis.

Electrophoretic patterns of the tenacious fraction were compared with patterns of α-, β- and κ-caseins (Missouri Experiment Station Chemical Laboratories) and commercially prepared isolates of α-, β- and γ-caseins (United States Biochemical Corp.). Isolates were suspended in distilled water to which sucrose and bromophenol blue were added. The quantity of protein applied to the gel was 1 mg.

RESULTS AND DISCUSSION

Figures 1 and 2 are scanning electron photomicrographs of whole milk films on the surfaces of stainless steel plates. The film-coated surface pictured in Fig. 1 was not exposed to high humidity, and that in Fig. 2 was so exposed. The milk film-stainless steel interfaces were photographed to show contrast (at arrow) between clean and soiled surfaces. A thicker film remained on the humidified plate than on the nonhumidified plate. The difference in thickness was visible to the naked eye, if examined at an oblique angle under sufficient light. We

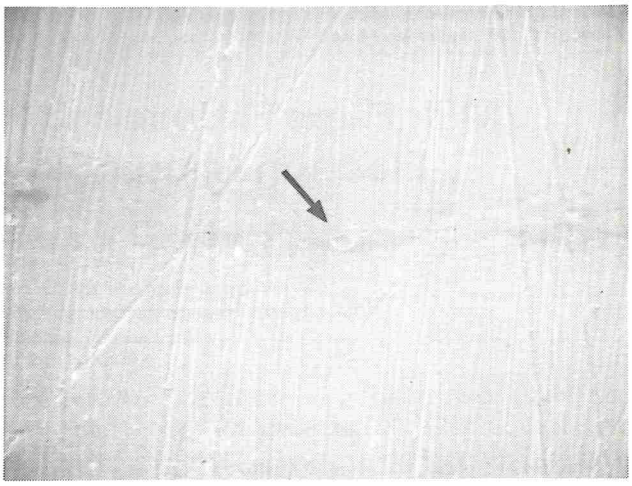

Figure 1. *Scanning electron photomicrograph (1000X) of unexposed, rinsed plate surface. Arrow shows depth to which plate was immersed in whole milk.*

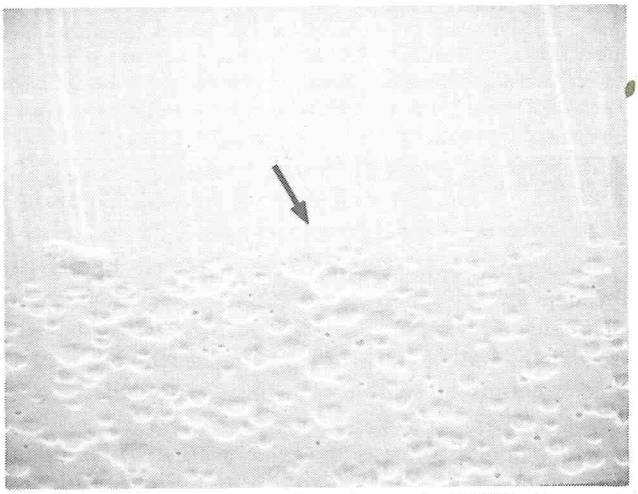

Figure 2. *Scanning electron photomicrograph (1000X) of exposed, rinsed plate surface. Arrow shows depth to which plate was immersed in whole milk.*

theorized that the crater-like structures visible in Fig. 2 were probably left by collapsing fat globules as the film dried. This theory was confirmed by the observation of few such "craters" in the film of skim milk (Fig. 3). Films produced from the same milk before separation are shown in Fig. 4 and 5. The small holes were probably produced by collapsing air bubbles. Objects within circles are probably lactose granules.

Smooth layers (arrows) visible on the periphery of the craters were probably fat and remnants of the fat globule membrane. The appearance of these layers contrasted with the porous appearance of the uncratered portion, especially in the exposed sample (Fig. 5), for which better resolution was achieved than with the unexposed sample. These materials covered and surrounded the craters. They are more clearly visible in the enlarged photo (Fig. 6) of the exposed sample than in the original photo (Fig. 5). These photographs (Fig. 4, 5 and 6) indicate that fat in the milk film is present largely in the coalesced, de-emulsified form, instead of in globular form. Collapse of fat globules was probably due to the rupture of

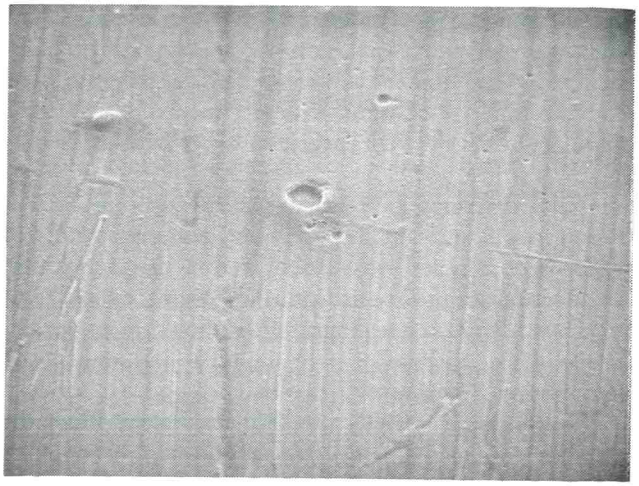

Figure 3. *Scanning electron photomicrograph (1000X) of exposed and unrinsed skim milk film on stainless steel plate.*

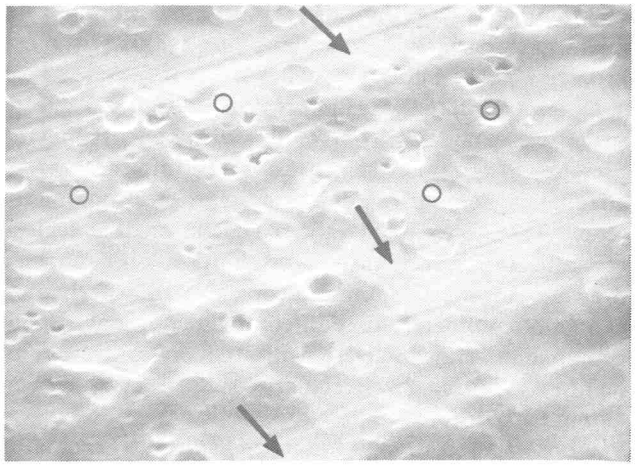

Figure 4. *Scanning electron photomicrograph (1000X) of unexposed and unrinsed whole milk film on stainless steel plate. Arrows indicate edges of layers formed from ruptured fat globules. Objects in circles are probably lactose granules.*

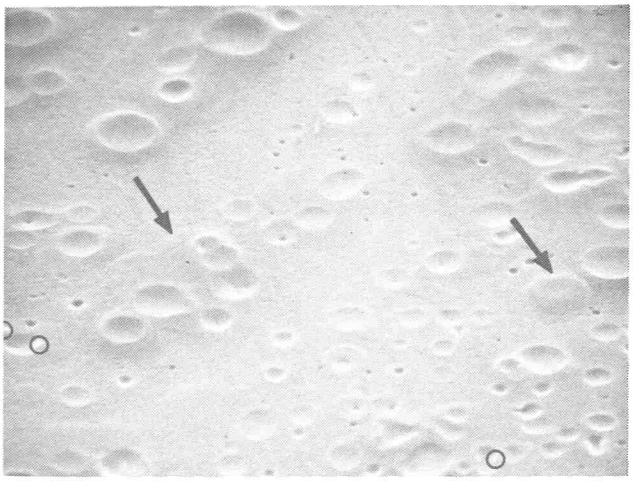

Figure 5. *Scanning electron photomicrograph (1000X) of exposed and unrinsed whole milk on stainless steel plates. Arrows indicate edges of layers formed from ruptured fat globules. Objects in circles are probably lactose granules.*

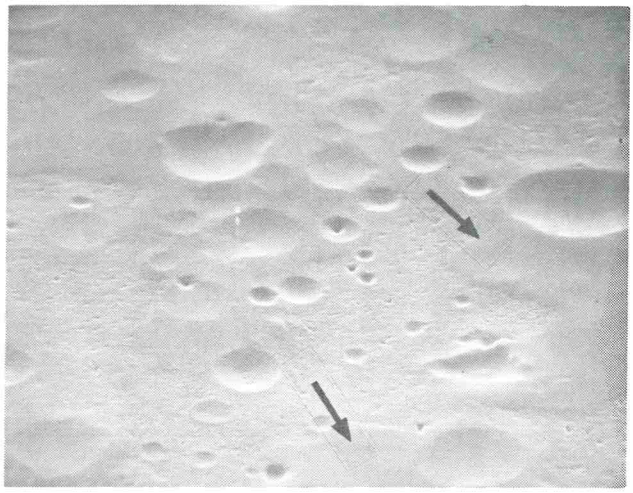

Figure 6. *Scanning electron photomicrograph (2000X) of unexposed and unrinsed whole milk film on stainless steel plates. Arrows point to layer that was probably fat or fat globule membrane.*

membrane upon the rapid loss of moisture during drying of the film. During drying, moisture is lost, the film shrinks and its viscosity increases. Thus the milk fat globule membrane may be stressed, possibly causing the globules to rupture. King (*2*) explained that the moisture content of milk powder in an atmosphere of high humidity reaches a critical level at which fat is freed as moisture is absorbed.

It was calculated, as follows, that about one-third of the total fat globules were visible on the surface of the films in Fig. 4 and 5. Assuming that the milk had approximately equal protein and fat content, measurement of protein indicated a fat content of 35 $\mu g/cm^2$. Then, since the milk initially contained 3.5% fat, it follows that the amount of whole milk per cm^2 of plate surface was 1000 μg. Assuming that 1000 μg of milk equals 1 μl and taking a median estimate of 2.25×10^9 globules per milliliter of milk (*1*), the number of globules on the disc was derived, vis., $10^{-3}(2.25 \times 10^9) = 2.25 \times 10^6/cm^2$. This milk was spread in an area of 1 cm^2 and was viewed at a magnification of 1000 ×. The area photographed was 1.43×10^4 μm^2 (125 μm long × 114 μm wide when viewed at a 45° angle). Thus, the microscopic field represented only 1.43×10^4 $\mu m^2/1 \times 10^8$ $\mu m^2/cm^2 = 1.43 \times 10^{-4}$ cm^2. This factor was multiplied by the number of fat globules per cm^2 (2.25×10^6) to estimate the number of globules (3.2×10^2, or 320) in one microscopic field. Since we counted only about 100 craters on each film surface, less than one-third of the fat globules were on the surface. The remainder were distributed throughout the film layer. Distribution within the film was confirmed by Fig. 7, which exhibits empty globular spaces within the film.

Figure 7 (whole milk film) and Fig. 8 (skim milk film) show differences in structure between unexposed (A) and exposed (B) samples. Exposed samples were more porous and aggregated than unexposed samples. Although it appears that exposed film would be more readily accessible to rinse water because of pores and could be

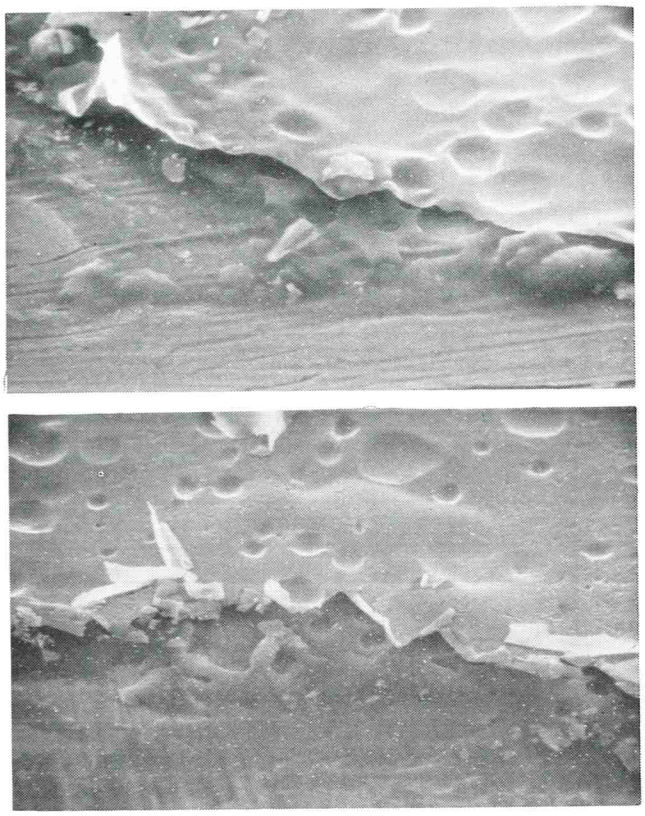

Figure 7. *Scanning electron photomicrographs (2000 ×) of unrinsed, unexposed (top) and exposed (bottom) whole milk films on stainless steel plates before rinsing. Films were broken with a needle before examination.*

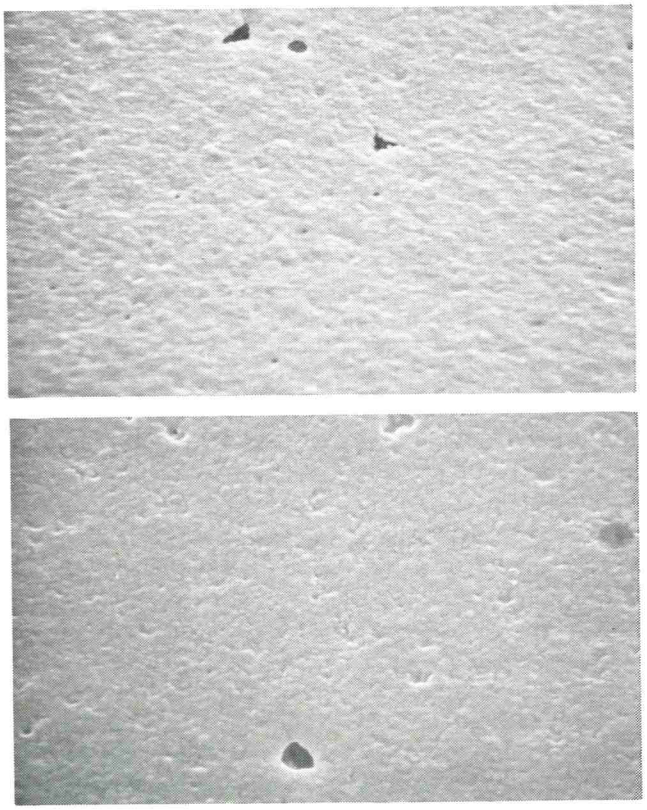

Figure 8. *Scanning electron photomicrographs (7000 ×) of unexposed (top) and exposed (bottom) unrinsed skim milk films on stainless steel plates before rinsing.*

washed from the plates more easily than unexposed film, such was not the case. Exposed samples were more tenacious than unexposed ones. This result suggested that the tenacity of films is determined more by their solubility than by their accessibility to rinsing water. Figure 8 illustrates the aggregating effect of high humidity that may have led to protein insolubilization. The greater porosity of exposed samples than of unexposed samples was probably due to aggregation and denaturation of casein micelles during exposure to high humidity and to evaporation of moisture on drying. The particles visible in both exposed and unexposed samples were likely casein micelles.

The photomicrographs in Fig. 7 suggest that fat globules ruptured during drying before treatment of plates because even the unexposed film exhibited craters. They also show spaces probably left by fat globules within the film. The fat globule membrane likely ruptured on drying and, in the exposed sample, which was more porous than the unexposed sample, fat migrated farther into the film, thus making it more hydrophobic than unexposed film.

Despite the likelihood that lactose crystallized due to successive exposures to high humidity and drying, the presence of crystals, even in exposed samples, was not adequately demonstrated by scanning electron microscopy. Therefore, a generalized conclusion that lactose crystallization affects protein insolubilization cannot be drawn. However, submicroscopic crystal formation cannot be completely discounted. In dairy products, milk components may interfere with the crystallization of lactose, resulting in irregularly shaped crystals and clumping (5).

Films exposed to high humidity had more granulated amorphous lactose than did unexposed samples. Several patches (Fig. 9) were observed in 3 of 12 exposed samples, but only a single patch was observed in 1 of 12 unexposed samples. Granules generally appeared in small areas which were near the lowermost edge of plates

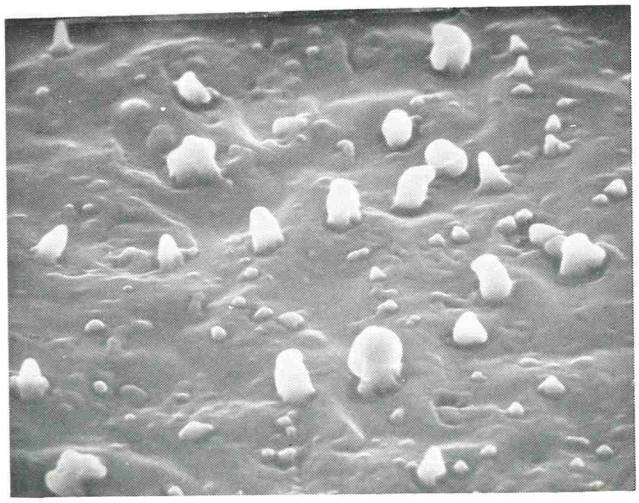

Figure 9. *Scanning electron photomicrograph of a patch of amorphous lactose localized at the bottom of an exposed, unrinsed plate (2000 ×).*

during drying. The general area involved was the last to dry because plates were held vertically in the drying chamber. One possible explanation for the greater incidence of these patches in exposed samples than in unexposed samples is that lactose was probably washed down by moisture condensing on plate surfaces during exposure to high humidity and became concentrated in this area.

Electrophoretic patterns of the concentrated rinses

Appreciable differences were observed between electrophoretograms of the milk sample and the rinse samples. Figure 10 shows a few components missing in each type of rinse, suggesting that they were either left on plates or lost during concentration. However, there was practically no difference between the patterns of samples obtained by washing unexposed and exposed plates. This observation led to the suspicion that both rinse samples underwent high humidity treatment during concentration. Indeed, some material was left on the inside walls of the evaporating flask. This film was more pronounced with unexposed than with exposed rinse samples. The concentration of protein was always higher in unexposed than in exposed samples because more soil was rinsed from unexposed than from exposed plates. This protein became tenacious when dried partially and exposed continually to 100% RH during rotary evaporation.

Electrophoretic patterns of the sticky sample, of rinses and of milk

Complete collection of rinse samples was facilitated when evaporation was localized in the bottom of the evaporating flask during concentration. Localization was achieved by orienting the evaporating flask almost vertically. Patterns were similar in strips (electrophoretograms) 1 and 2, of Fig. 11, which were the milk control and the concentrated rinse from unexposed plates, respectively. Thus, practically all of the proteins on these plates were removed merely by rinsing. However, differences were marked between a second milk control (strip 6) and the rinse from exposed plates (strip 7). Some fractions were obviously missing in the latter, probably the ones rendered tenacious by high humidity. When the samples collected by rubbing unexposed and exposed plates were introduced into strips 3 and 8, respectively, and electrophoresced, fractions were present in exposed samples (strip 8) but absent in unexposed samples (strip 3). Fractions observed in exposed samples were those missing from rinses of the same plates. Results clearly verified that exposure of plates to high humidity rendered tenacious some fractions of the protein. Densitometric tracings (Fig. 12) of the electrophoresced strips (strips 2 and 7 of Fig. 11) illustrate differences between concentrated rinses from unexposed and exposed plates. Two peaks, indicated by arrows, were practically absent in the rinse from exposed plates.

Identity of the sticky fractions

Electrophoretic mobilities of fractions of the sticky protein closely resembled those of whole casein (Fig. 13).

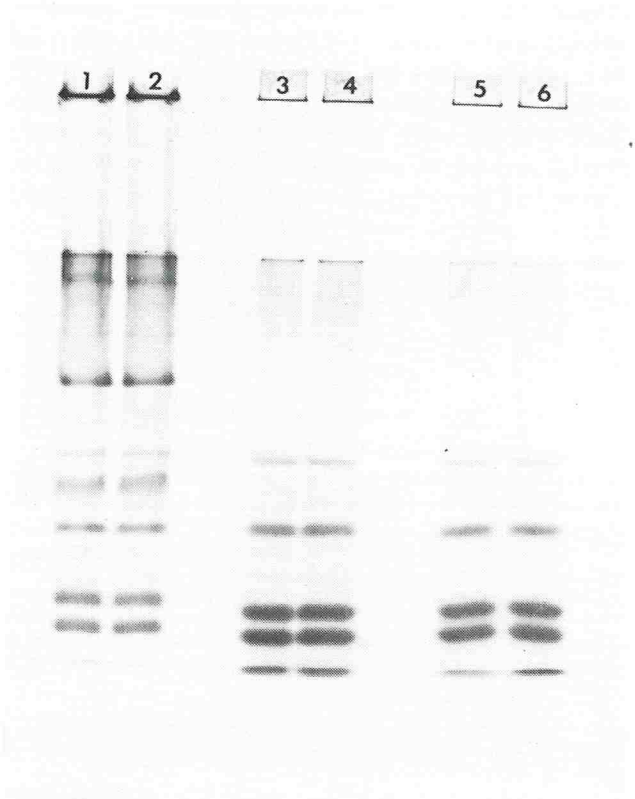

Figure 10. *Electrophoretograms of milk (1 and 2), concentrated rinse from unexposed plates (3 and 4), and concentrated rinse from exposed plates (5 and 6).*

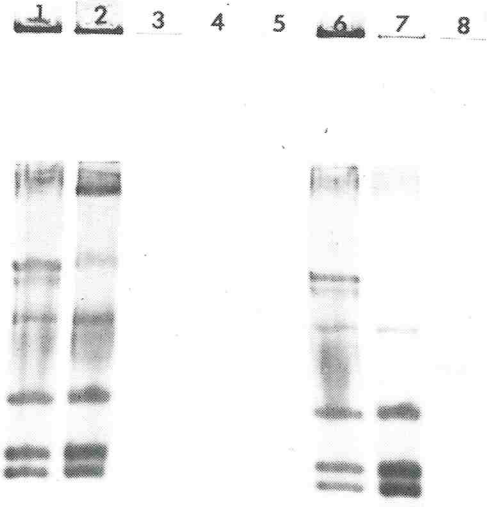

Figure 11. *Electrophoretograms of milk (1 and 6); concentrated rinse from unexposed plates (2); concentrated rinse from exposed plates (7); and tenacious sample from exposed plates (8).*

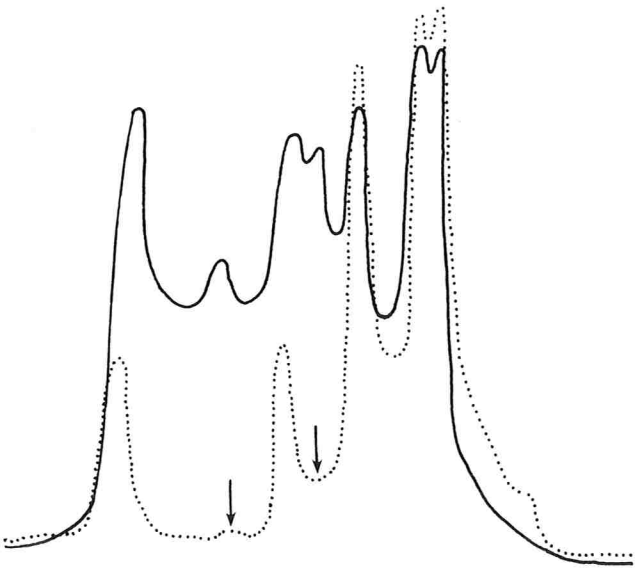

Figure 12. *Densitometric tracings of typical patterns of concentrated rinses from exposed (dotted line) and unexposed (solid line) plates. Arrows point to supposed location of components missing from the sample from the exposed plates, a-casein at the left and β-casein at the right.*

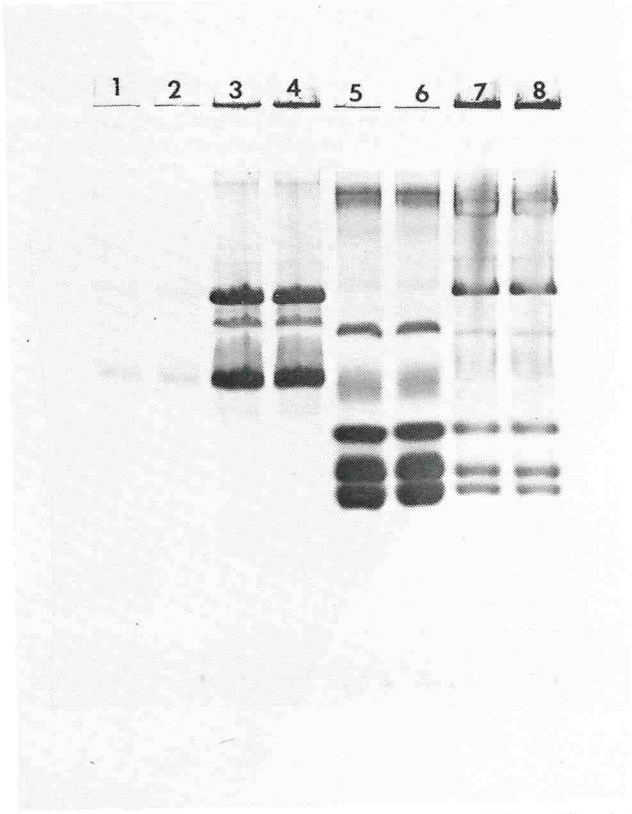

Figure 13. *Electrophoretograms of tenacious sample (1 and 2); whole casein (3 and 4); whey (5 and 6); and milk (7 and 8).*

Electrophoretic patterns determined for both whole casein and whey protein in this experiment were similar to those described in previous reports (*4,6*).

The electrophoretic pattern of the sticky fraction was compared with those of individual casein components (Fig. 14). Although the *a*- and *β*-caseins were not electrophoretically pure (each was contaminated with the other), *a*-casein predominated in the alpha standard, and *β*-casein predominated in the beta standard.

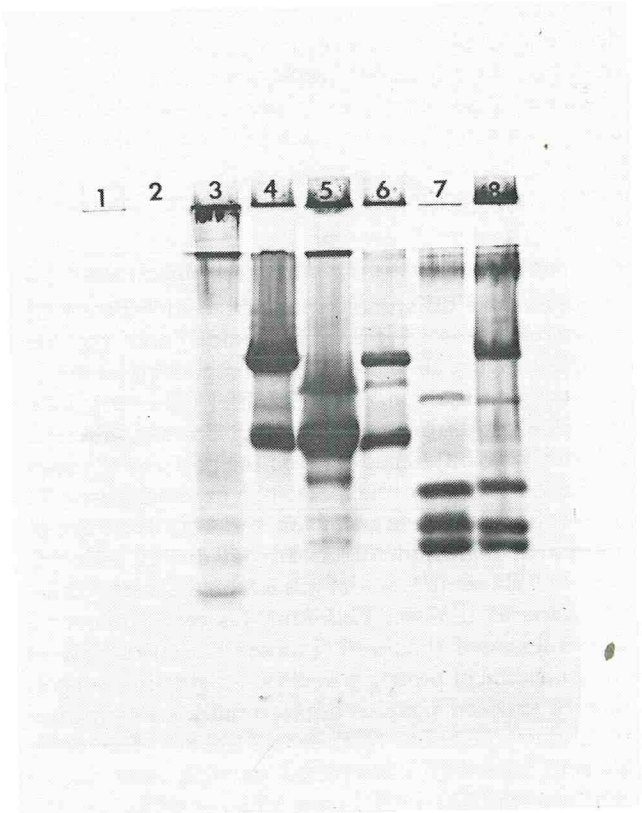

Figure 14. *Electrophoretograms of tenacious sample (1), κ-casein (3), β-casein (4), a-casein (5), whole casein (6), whey (7), and milk (8). Standards prepared in the laboratory.*

The electrophoretograms in Figure 15 show how the sticky fractions matched with *a*-, *β*- and *γ*-casein standards commercially available. They also verified that the two major fractions involved in the reaction to high RH were the *a*- and *β*-caseins. The whole casein micelle was likely involved. However, *γ*-casein could not be detected electrophoretically in a tenacious sample under conditions of this study. This can be attributed largely to the lower proportion of the *γ*-casein in milk than of the *a*- and *β*-caseins.

CONCLUSIONS

Based on the results of research reported here and in our previous paper (*3*), as well as on our knowledge about the relationship between various components of milk films and their physicochemical properties, the following is our concept of the mechanism of tenacious film formation.

Drying of milk film decreases the integrity of the fat globule membrane. Shrinking of the film during drying generates lateral forces that flatten globules against the supporting medium and stress the membrane, causing it to rupture. This contention is supported by the observation that the diameter of some craters left by globules far exceeded thickness of the film, and coalesced fat covered the surface in and around craters.

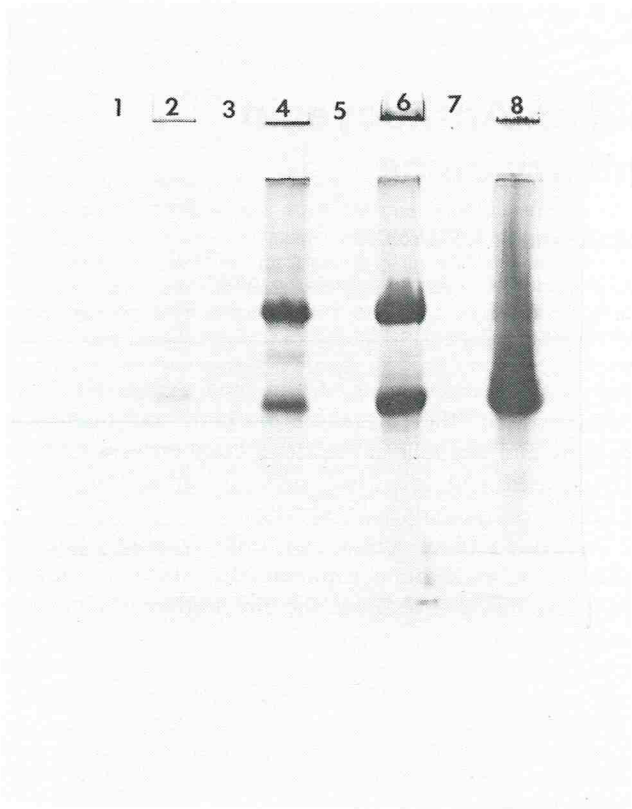

Figure 15. *Electrophoretograms of tenacious sample (2), γ-casein (4), β-casein (6), and a-casein (8). Standards obtained commercially.*

This covering should increase tenacity of film because of the repellency against rinsing water afforded by fat on the film surface.

Exposure to high humidity probably hydrates film sufficiently to allow mobility and aggregation of casein micelles. Subsequent treatments (exposures and drying) enhance aggregation and compaction. Surface area accessible to rinsing water is thus reduced, thereby decreasing the films wettability, solubility, and rinsability. In the course of hydration and swelling of the film, bubbles are likely trapped. These burst and leave holes upon drying. Although the holes could increase films' accessibility to water during rinsing, protein particles in their immediate vicinity are well aggregated and possess low wettability. Therefore, films exposed to high humidity, though exhibiting greater porosity than unexposed films, showed higher resistance to rinsing than unexposed films.

ACKNOWLEDGMENTS

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Effect of Cinnamic Acid on Anthocyanin Stability in Cranberry Juice

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ABSTRACT

The direct effect of cinnamic acid on anthocyanin stability in cranberry juice without added ascorbic acid was investigated. The concentration of cinnamic acid was determined throughout the storage study by high-performance liquid chromatography (HPLC), and results indicate no loss of cinnamic acid either due to pasteurization or storage. Cinnamic acid was found to have no significant effect on anthocyanin stability in cranberry juice during storage at ambient temperature at the levels used in this study.

Enzymic browning of fruit juices brought about by the interaction of o-diphenol oxidase with substrates which are naturally present is usually controlled by addition of ascorbic acid during juice preparation before pasteurization (*7*). Cinnamic, p-coumaric and ferulic acids were also found to be effective in controlling browning of apple juice and it was considered that cinnamic acid might be an inexpensive, useful alternative for the control of enzymic browning during the preparation of other fruit juices (*7*).

The findings of Walker (*7*) are in agreement with those of Pifferi et al. (*5*) who reported m- and p-coumaric acids to be strong inhibitors of sweet cherry o-diphenol oxidase.

It is generally recognized that when both ascorbic acid and anthocyanins are present in a fruit juice, any inhibition in the rate of oxidation of ascorbic acid will give protection to anthocyanins as well.

Clegg and Morton (*3*) found phenolic acids such as chlorogenic acid and p-coumaric acid, as well as flavonols, to have a protective effect on ascorbic acid in model systems. This led them to suggest that phenolic extracts of black currants or pure flavonols may be added advantageously to less stable sources of ascorbic acid which would also stabilize anthocyanins.

Flavonols have the disadvantage of being only slightly soluble in water (*6*) and tend to precipitate out of solution after pasteurization. By use of a surface active agent, such as Tween 80, the protective effect of the added flavonol was not increased by the increased solubility (*3*).

One does not ordinarily think of the occurrence of browning in cranberry juice during its preparation because the extent to which browning does occur is masked by the presence of anthocyanin pigments. Conditions of juice preparation do create opportunity for oxidative reactions involving phenols, and it is to be expected that browning reactions are occurring during pre-pasteurization steps. Chan and Yang (*1*) have demonstrated the presence of both polyphenolase enzyme and phenolic substrate in cranberries, and Chu (*2*) isolated and identified some of these fluorescent phenolic compounds in cranberries.

Ascorbic acid was not added to the cranberry juice in this study, as is done commercially, since our major objective was to investigate the direct effect of cinnamic acid on anthocyanin stability.

MATERIALS AND METHODS

Chemicals

The cinnamic acid used in this experiment was obtained from Mallinckrodt Chemical Works, St. Louis, Mo., and was used without further purification.

Treatment of the cranberry juice

Frozen, unpasteurized first press cranberry juice was obtained from Ocean Spray, Inc. (Hanson, Massachusetts), and was stored at - 20 C until ready for use. The juice was thawed and filtered through a large Büchner funnel with a double layer of Whatman No. 1 filter paper. Filter papers were coated with an analytical filter aid to accelerate the filtration. The juice was then diluted with an appropriate amount of double distilled, deionized water to give a final anthocyanin concentration of 7.25 mg/100 ml.

Before pasteurization of the juice, the following treatments were applied. About 8 liters of juice were divided into four equal portions of exactly 2 liters each and the following treatments were given: (*a*) cranberry juice alone (control), (*b*) cranberry juice plus cinnamic acid (3 mg/100 ml), (*c*) Cranberry juice plus cinnamic acid (6 mg/100 ml) and (*d*) cranberry juice plus cinnamic acid (9 mg/100 ml). The cinnamic acid was dissolved in a small volume of alcohol to keep it in solution, and the same amount was added to the controls.

Pasteurization

The juice to be pasteurized was drawn from the 2-liter volumetric flask to a variable speed pump. From the pump the juice was sent through 36 inches of 1/4 inch OD stainless steel, thin walled tubing coiled into loops which was submerged in a bath of hot glycerol. The temperature of the bath was controlled by a bimetallic immersion thermoregulator. The temperature of the bath was adjusted to 260 F which gave a temperature of 190 F to juice coming out of the tubing. After heating, the juice was filled to overflowing into 100-ml glass bottles, the caps were twisted on and the bottles were then inverted. After about 2 min, the bottles were placed on ice to cool and then stored in boxes at room temperature.

Anthocyanin and cinnamic acid determination

Anthocyanin and cinnamic acid contents for each treatment were determined at regular intervals of 0, 1, 2, 4, 8 and 16 weeks on duplicate samples. Anthocyanin content was determined using the pH differential method of Fuleki (*4*). Cinnamic acid concentration was determined by comparison of high-performance liquid chromatographic (HPLC) peak heights to that of a standard curve. The following HPLC conditions were used in the analysis:

Column: 4.6 mm × 25 cm analytical column packed with 10 μm Spherisorb Octadecylsilyl (ODS) reversed-phase packing.
Mobile phase: methanol-acetic acid-water (30:5:65).
Flow rate: .7 ml/min.
Pressure: 750 psi.
Detection: UV at 280 nm

Cinnamic acid standard curve

A series of dilutions from 10 to 100 ppm were prepared from a 100-ppm stock solution of cinnamic acid in a 1-liter volumetric flask. The acid was dissolved in approximately 50 ml of alcohol to keep it in solution. Appropriate volumes were pipetted into 100-ml volumetric flasks and made up to volume with distilled deionized water. Peak heights were recorded from the resulting HPLC chromatograms. This serial dilution was repeated two more times on the same day using freshly prepared mobile phase of water-acetic acid-methanol (30:5:65) and again the resulting peak heights from the chromatograms were recorded. The test of significance among regression lines for the cinnamic acid standard curve showed that the lines were not significantly different at the 1% level. The standard curve was found to be a straight line in the concentration range used in this study. The standard curve was repeated at the end of the 16-week storage study.

RESULTS AND DISCUSSION

Control samples of cranberry juice injected into the chromatograph gave a phenolic profile which allowed for comparison throughout the storage study. It was interesting that, with the exception of the anthocyanins, the phenolic profile did not change during the 16-week storage period.

Table 1 shows the percent recovery for cinnamic acid throughout the 16-week period of storage under ambient conditions. Percent recovery ranged from 93 - 118%, indicating that there was no loss of cinnamic acid either due to pasteurization or storage. The high recoveries for the 2nd, 4th and 8th weeks are, in part, due to the inherent variability of the instrumental response from week to week. Addition of an internal standard to the samples is recommended since this would eliminate apparatus and procedure errors which could account for the variability in percent recovery observed.

The o-diphenol oxidase system in cranberry juice is apparently not a factor in the browning of diluted cranberry juice before pasteurization since the control samples of cranberry juice did not differ colorimetrically from the treatments at the onset of the study. This would seem to indicate that there is no need for the short term treatment of cranberry juice with added cinnamic acid to control enzymic browning in the pre-pasteurization steps of juice production.

Table 2 shows the effect of cinnamic acid on the total anthocyanin stability of cranberry juice during a 16-week storage period. Cinnamic acid was shown to have no significant effect, as shown by analysis of variance, on anthocyanin stability in cranberry juice during storage, at the levels used in this study.

TABLE 1. *Recovery of cinnamic acid added to diluted cranberry juice.*

Time (weeks)	Cinnamic acid (ppm) Amount added	Amound found	% Recovery[a]
0	30	28	93 ± 1
	60	60	100 ± 3
	90	87	96 ± 1
1	30	28	93 ± 3
	60	58	97 ± 5
	90	85	94 ± 1
2	30	33	110 ± 5
	60	70	116 ± 3
	90	103	114 ± 1
4	30	35	116 ± 2
	60	71	118 ± 3
	90	103	114 ± 2
8	30	34	113 ± 1
	60	68	113 ± 3
	90	95	105 ± 7
16	30	31	103 ± 4
	60	65	108 ± 3
	90	90	100 ± 3

[a]Standard deviation based on duplicate samples.

ACKNOWLEDGMENTS

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TABLE 2. *Effect of cinnamic acid on stability of anthocyanins from cranberry juice during storage.*

Cinnamic acid level (ppm)	Storage time (Weeks) 0	1	2	4	8	16
	Total anthocyanin (mg/100 ml)					
0	7.25	7.24	7.21	5.95	4.54	3.14
30	7.30	7.25	7.24	5.92	4.47	3.05
60	7.27	7.25	7.14	5.92	4.23	3.04
90	7.25	7.24	7.19	5.90	4.36	2.74

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Simple Method for Anaerobic Inoculation of Foods in Cans, Retort Pouches, Glass Jars or Semi-Rigid Containers

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ABSTRACT

Cured silicon sealant (SS) on lids of cans and glass jars, on retort pouch and semi-rigid container surfaces was employed for providing a port of entry during inoculation of foods while maintaining anaerobiosis. Following inoculation, the SS was covered by molten wax and aluminum foil. Anaerobic growth in the packs was demonstrated by gas formation by several species of clostridia. Staphylococci also grew anaerobically.

Artificial inoculation of cans is necessary to determine the growth and action of bacteria in canned foods. A variety of approaches has been developed to achieve this: inoculation of cans before (*5*) or after processing (*3*) by piercing of lids for inoculation and resealing with solder (*7*), gluing on of metallic squares with an epoxy resin (*1*) or using a rubber diaphragm (*1*). None of these methods, however, meet the need of maintaining an anaerobic environment in commercially processed cans during and after inoculation. The present approach, involving use of a silicon sealant, allows for maintenance of anaerobic conditions for post-thermal process inoculation.

MATERIALS AND METHODS

The uncoded can lid was cleaned with acetone and sterilized with sodium hypochlorite solution (*4*) or 2% iodine in 70% alcohol. These methods proved to assure sterility in several hundred inoculated units. After drying the cans in a laminar flow cabinet, a large droplet of sterility-tested transparent viscous silicon sealant (SS) (Dow Corning, General Electric) was squeezed from a tube onto the can lid and flattened to give dimensions of 1-1.5 cm in diameter and 0.6-0.8 cm in height (Fig. 1a). The SS on the can was allowed to cure under a steady flow of sterile air (or under aseptic conditions) at room temperature for at least 48 h before inoculation. Just before inoculation, the SS and the can lid are pierced with a presterilized, 5-cm long, gauge 13 hypodermic needle containing flowing sterile nitrogen. Oxygen-free sterile nitrogen was obtained by passing a high-purity nitrogen gas containing less than 0.5 ppm oxygen through a 10% sodium hydrosulfate solution (*5*) at pH 7.5 in a sparger which was connected by 0.3-cm ID sterile rubber tubing to a presterilized 0.45-μm pore size, 2.5-cm diameter membrane filter in a stainless steel filter holder equipped with a gauge 13 hypodermic needle. The gauge 13 hypodermic needle was led through the center of a size 11, concave hard rubber stopper (Fig. 2 shows assembly) to cover the SS and to permit only sufficient penetration into the can's head space to replace the vacuum with free flowing nitrogen. The hypodermic needle was pressed through the SS and the can lid by the palm of the sterile gloved hand. After the vacuum was replaced with nitrogen, the gauge 13 hypodermic needle was withdrawn and the SS prevented leakage of nitrogen or penetration by air. The organism to be inoculated was

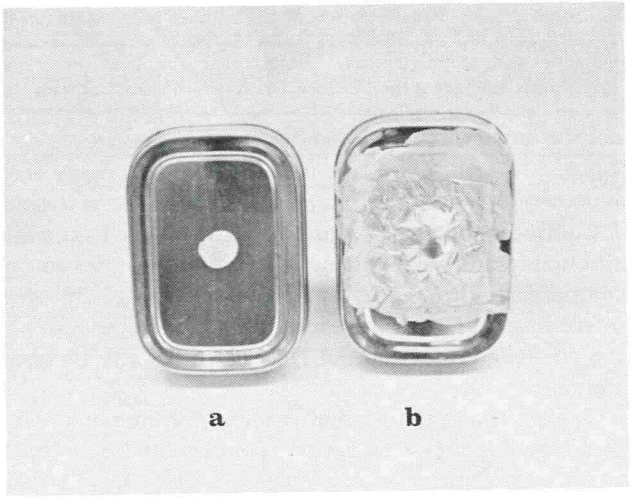

Figure 1. *Anaerobic inoculation method for cans: (a) application of silicon sealant (SS) (Dow Corning) to the center of can before replacement of vacuum by oxygen-free nitrogen; (b) SS covered with molten wax and heavy gauge (0.002 cm thickness) aluminum foil after inoculation.*

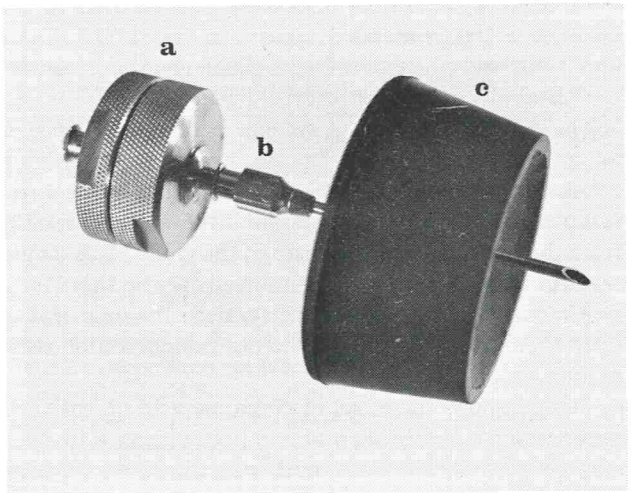

Figure 2. *Filter holder (Millipore) (a), gauge 13 hypodermic needle, (b), and concave size 11 rubber stopper (c) assembly for penetration of can and introduction of nitrogen free oxygen into can for anaerobic inoculation.*

washed, suspended and diluted in 0.1% sodium thioglycolate medium to the desired number of organisms per can in 0.1-ml amounts delivered by a sterile 1-ml capacity tuberculin syringe and a gauge 25½ hypodermic needle. The organisms were inoculated through the SS and hole in the can lid. Immediately after inoculation, sterile hot parafin wax (60-65 C) was poured over the SS, which was then covered with a

5.5-cm heavy duty (0.002-cm thickness) aluminum foil premoulded to the shape and size of the SS (Fig. 2b, 3b and 4). The excess hot parafin (Fig. 1b, 3b and 4) was removed quickly by pressing down the aluminum foil around the mound of the SS. The wax served as an adhesive and the aluminum foil as an effective oxygen barrier to prevent entry of oxygen by diffusion during prolonged storage. Silicon sealant, and other plastics normally used for food packaging are permeable to gases during storage (2). Ten cans with cured SS may be conveniently prepared and inoculated at one time without changing the needles, except that they are flamed between each penetration. For subsequent examination, each can was opened aseptically by (*a*) removing the aluminum foil, (*b*) scraping off the wax and (*c*) removing the SS from the can lid with a scalpel while holding the can inside a sterile plastic bag to prevent possible spread of microorganisms during deflation. With careful manipulation gradual deflation can be achieved, otherwise if a new hole were made on the reverse side of the can by piercing the can, gases would be released explosively. The can was then opened with a presterilizied Bacti-disc cutter (Continental Can Company), using the same hole made with the gauge 13 hypodermic needle.

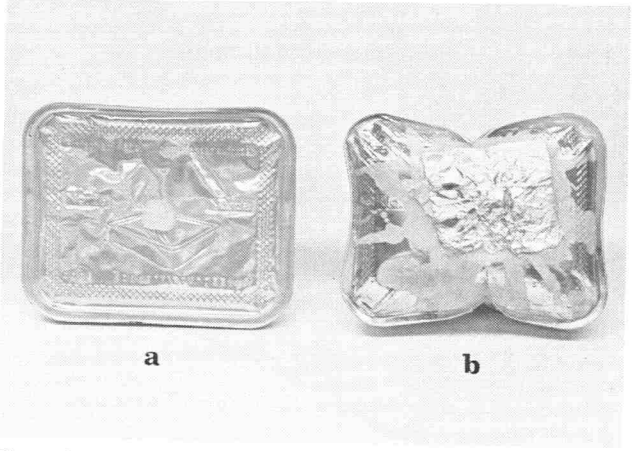

Figure 3. *(a) Semi-rigid container containing fish stew, in center with silicon sealant, before inoculation; (b) same container after inoculation with* Clostridium botulinum *Type E, and incubation at 35 C for 5 days.*

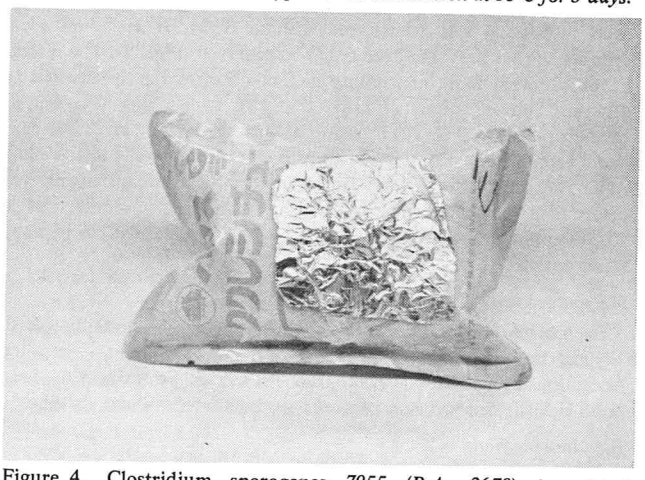

Figure 4. Clostridium sporogenes *7955 (P.A. 3679) inoculated retortable pouch containing vegetable soup after 4 days of incubation at 35 C.*

RESULTS AND DISCUSSION

This method has been used successfully (anaerobic and facultative anaerobic organisms grew well in the inoculated packs, maintaining anaerobic environment and sterility in inoculated packs for up to 6 months). This method is particularly useful for inoculating into the headspace of cans and other containers to simulate post-processing leakage-type conditions; anaerobic as well as facultative organisms can be introduced into containers to study their rate of growth and toxin production in an oxygen-free environment. Facultative organisms under anaerobic, that is, extreme conditions may exhibit different activities from those under aerobic conditions. Anaerobes such as *Clostridium botulinum* types A, B, and E, *Clostridium perfringens, Clostridium sporogenes* ATCC 7955 (P.A. 3679) and a facultative anaerobe, *Staphylococcus aureus*, were inoculated into canned salmon and sardines by this method. Hard swells occurred after 4 days of incubation at 35 C when the just-named *Clostridium* species were inoculated (slight swelling could be detected after 24 h). *S. aureus* inoculated into canned sardines and salmon did not produce appreciable amounts of gas after 6 months of incubation at 20-25 C; however, small amounts of carbon dioxide were detected by gas detection tubes (Dräger-werk-AG-Lubeck, F.R. of Germany; Gastec Corporation, Tokyo, Japan). Uninoculated sterile cans filled with nitrogen showed no signs of leakage after 6 months. Using the above method with slight adaptations, other hermetically sealed containers such as glass jars with metallic lids, retortable pouches and semi-rigid containers can be inoculated.

Glass jars containing strained beef with vegetables (pH 6.2), and meat purees and semi-rigid containers and retortable pouches with fish stew (Fig. 3b), vegetable soup (Fig. 4), vacuum-packed sterilized omelet (meat and cheese) and frankfurter sausages were inoculated with the above organisms. Glass jars were inoculated through the metal caps after replacement of vacuum with nitrogen. Inoculated cans, glass jars, retort pouches and semi-rigid containers were incubated in transparent plastic bags as a precaution against bursting. Containers with a relatively thin wall, such as retort pouches and some types of semi-rigid containers, can be inoculated directly through the SS and container wall without need for a gauge 13 needle as the walls of these containers offer little resistance to a gauge 25½ hypodermic needle during inoculation. Since these containers contained no vacuum, there was no necessity for introduction of nitrogen to replace the vacuum. In retortable pouches and semi-rigid containers, growth of clostridia was demonstrated by swelling (Fig. 3b and 4) and distortion of the container after 4-6 days of incubation at 35 C; metallic caps of glass jars were blown off.

This method, besides maintaining anaerobic conditions in the cans' contents and in the head-space, is also useful for capturing accumulated gases in the container. These gases can be analyzed subsequently or they may be passed through gas-detection tubes which can provide a qualitative or a semi-quantitative analysis during deflation of the can. Liquid products can be sampled through the SS during storage by re-entering the container with a sterile needle and by drawing contents into a syringe.

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Control of Foaming during Microbiological Analysis of Foods and Recovery of Indicator Organisms

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ABSTRACT

Persistent foam formation occurring during blending of jelly powder (JP), whole egg powder (WEP) and skim milk power (SMP) was demonstrated. Reduction of foaming was uniformly best achieved by using the Colworth Stomacher 400 (STOM) for mixing or blending under a vacuum (VAC). Two hundred, 1000 and 6000 ppm of Dow Corning Antifoam Agent (DAF) was necessary to reduce foaming of JP, SMP and WEP, respectively. *Escherichia coli* was recovered in similar numbers from JP, SMP and WEP by normal blending (N), STOM, VAC or by the application of 200 ppm of DAF. Significantly lower recovery of *Staphylococcus aureus* from JP resulted when N or VAC was employed and from SMP when STOM was used. *Streptococcus faecalis* showed significantly lower recoveries from JP and WEP when STOM or VAC and DAF at 200 ppm were used.

Emulsification of proteins and peptides has been shown to be the major cause of foam formation in culture media (*6,12,15*). Most foods analysed in the laboratory contain proteins and peptides and, as a consequence, blending with diluents often causes excessive foaming. Despite this, most of the currently available food microbiological analytical methods either do not deal with the problem of foaming (*1,3,7,10,19*), or only suggest shorter blending time, or waiting 2-3 min for the foam to disperse after blending (*11*). In the fermentation industry, several groups of chemicals have been used (*5,9,14*) for suppression of foaming. Dow Corning Antifoam AF (DAF) emulsion, belonging to the silicon type antifoam group (*5*), is considered to be non-metabolizable (*9*) and non-toxic (*13*). It is recommended by the *Compendium of Methods for the Microbiological Examination of Foods* (APHA) (*2*) in a concentration of 1-2 drops of a 10% solution of antifoam to 300 ml of food sample. The final concentration of this solution is about 5-10 ppm of antifoam in the diluted food which was found to be inadequate for high speed blending of some foods. Consequently, we determined suitable foam-inhibiting concentrations of this antifoam agent for these foods, and whether or not they may adversely affect recovery of indicator organisms. We also investigated other means of avoiding foaming by preparing food homogenates with the exclusion of air by using the Colworth Stomacher 400 (A.J. Seward and Co., Ltd., Blackfriars Road, London, England) (*8,11,16,18*) and by blending under a vacuum.

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MATERIALS AND METHODS

Foods

Commercially available jelly powder (JP) containing sugar, gelatin, adipic acid, trisodium citrate, artificial and natural flavor, fumaric acid salt, food color, tri-calcium phosphate and lactic acid, commercially available instantized skim milk powder (SMP) and whole egg powder (WEP) spray dried for institutional use were the foods used in these experiments.

Of the first two foods, several packages of each were mixed by tumbling to provide sufficient quantities for all the experiments. Whole egg powder was obtained in bulk and mixed by tumbling to obtain unformity. Background counts were determined for each product in triplicate; these were in the range of 0.0001 to 0.01% with respect to the inoculum, and therefore, negligible as the statistical analyses were done at the 95% significance level.

Determination of foaming capacity of foods

Eleven grams of each food were blended with 99 ml of 0.1% peptone water for 1 min in an Osterizer blender at 15,000 rpm. After blending, each suspension was transferred without delay to a 250-ml glass cylinder so that the foam head could be observed (Fig. 1a). The effects of the different foam controlling methods, i.e. as the application of different concentrations of DAF (Fig. 1b), stomaching (Fig. 1c) or vacuum blending (Fig. 1d) by using a Waring Blendor at 20,000 rpm and a vacuum apparatus were determined experimentally in the same way. The most effective concentration of the antifoam agent was determined for each food by adding increasing quantities of a 20,000 ppm stock solution of DAF to a food and to a 0.1% peptone water mixture.

Microorganisms

Escherichia coli, and *Staphylococcus aureus* were incubated in Tryptic Soy Broth (Difco) for 18-20 h at 35 C in a controlled environment incubator shaker (New Brunswick Scientific Co., Inc., N.J., U.S.A.). *Streptococcus faecalis* was incubated without shaking.

Blending methods

(The blending methods were those usually employed in laboratories with similar equipment). The above cultures were diluted 100-fold with 0.1% peptone water at room temperature and refrigerated at 4 C while waiting to be blended, then blended with the food in the following manner:

(*a*) Ninety-nine ml of inoculated peptone water was added to 11 g of food and blended in an Osterizer blender fitted with a blending blade assembly and a 1-pint Mason jar. The blending was carried out at 15,000 rpm for 1 min.

(*b*) Ninety-eight ml of inoculated peptone water and 1.1 ml of 20,000 ppm DAF were added to the 11 g of food in a Mason jar to obtain a final antifoam concentration of 200 ppm. This concentration

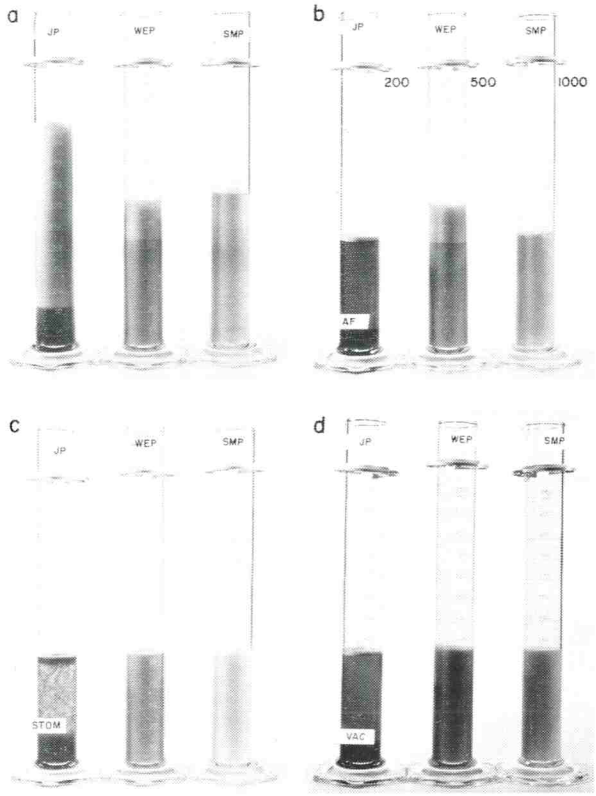

Figure 1. *(a) Foam formation during blending of 99 ml of peptone water diluent with 11 g of jelly powder (JP), whole egg powder (WEP), or skim milk powder (SMP) in an Osterizer blender at 15,000 rpm; (b) control of foaming of JP, WEP and SMP by application of 200, 500 and 1,000 ppm Dow Corning Antifoam AF, respectively; (c) control of foaming by stomaching JP, WEP and SMP; (d) control of foaming of JP, WEP and SMP by blending under a vacuum of 25 inches of Hg in a Waring Blendor at 20,000 rpm. Blending was done for 1 min for a, b, c, and d conditions.*

of DAF was used in all experiments with all three foods as it reduced the foam maximally in the food (JP) which produced most foam. Blending was carried out as above.

(c) Ninety-nine ml of inoculated peptone water were added to 11 g of the food in a sterile Colworth Stomacher 400 bag. Entrapped air (Fig. 2c) was excluded by sliding down (Fig. 2a and b) the filled stomacher bag on the edge of the Colworth Stomacher 400 to form a continuous liquid from the food to the top of the bag (Fig. 2a, b, c and d show process, and results). The sample was "stomached" for 1 min.

(d) A laboratory gas valve was attached to the top of a Waring Blendor so that the Blendor could be evacuated during blending. Blendings were then carried out under a vacuum of about 25 inches of Hg. Liquid impingment in a phenolic solution was employed to prevent the escaping of aerosolized bacteria. In this blending, the 1:10 dilution was obtained by aspetically adding 22.0 g of food and 198 ml of inoculated peptone water into the Blendor. Blending was done at 20,000 rpm for 1 min.

During the serial dilution of foods, care was taken that the first sample from the blended food homogenate was withdrawn from below the interface of any amount of foam and the underlying liquid.

For recovery of organisms from 0.1% peptone water, the same blending methods were used as for foods.

Statistical analysis

Each experiment for each organism and each food and 0.1 peptone water was analyzed separately. A split plot experimental design was first analyzed by analysis of variance (ANOVA) (*17*). Each possible pair of blending/medium combination means were simultaneously compared by the Bonferroni T-test (*4*) at an overall error rate of 0.05 using standard errors derived from the ANOVA table. In the statistical

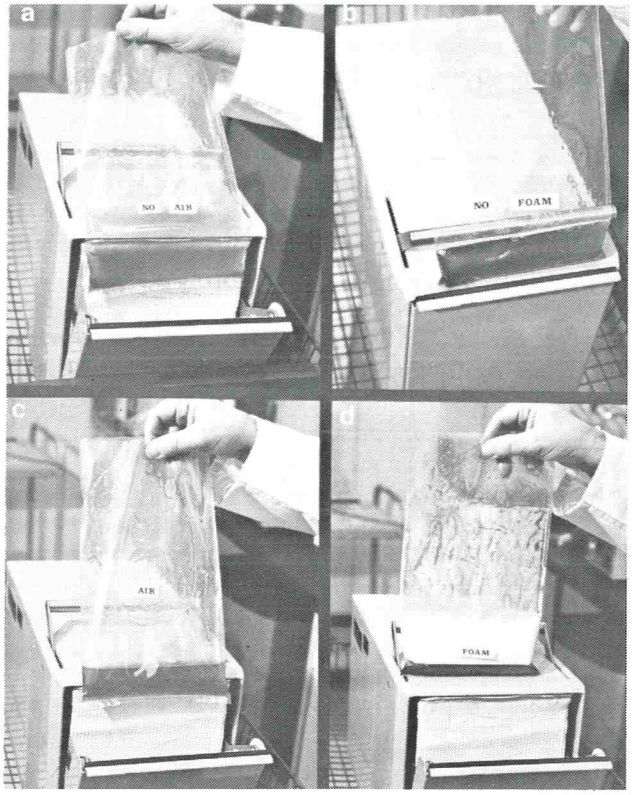

Figure 2. *Stomaching 11 g of jelly powder (JP) with 99 ml of 0.1% peptone water diluent (PWD) for 1 min; (a) exclusion of air from Stomacher 400 bag before stomaching (NO AIR), (b) absence of foam (NO FOAM) after stomaching, (c) inclusion of air (AIR) over JP and PWD slurry before stomaching, (d) production of foam (FOAM) after stomaching JP and PWD slurry in presence of air.*

design, the blocks were the days, the main plots were the blending methods, which were randomized (except for a few initial experiments where blending methods were rotated from day to day), and the sub-plots within the mean plots were the media. For each combination of organism, food, peptone water and blending method, 10 trials were carried out.

Determination of recoveries of indicator organisms

After blending, the food suspension or inoculated 0.1% peptone water was serially diluted in 0.1% peptone water and pour-plated on Plate Count Agar (PCA) for aerobic colony counts, on Violet Red Bile Agar (VRB) for *E. coli*, on m-Enterococcus medium (m-ENT) for *S. faecalis*, and on Mannitol Salt Agar (MSA) for *S. aureus*. *S. aureus* was also counted on spread plates of Baird-Parker Agar (BP). All media used were manufactured by Difco.

RESULTS AND DISCUSSION

Determination of foaming capacity

The method described above and shown in Fig. 1a, 1b, 1c and 1d proved to be highly reproducible. Consequently, there was no necessity for a large number of experiments for statistical analysis. When the foods were blended without any control, JP always produced the greatest quantity and the most persistent foam, WEP and SMP produced moderate amounts of foam; when DAF was used in 200 and 1000 ppm concentrations, foaming of JP and SMP was reduced maximally, whereas 500 ppm of DAF in WEP did not reduce foaming at all, as compared to normal blending (Fig. 1a and 1b). Six

thousands ppm of DAF was required to reduce foaming in WEP. Stomaching and vacuum-blending reduced foaming nearly equally in all three foods (Fig. 1c and 1d).

Recovery of indicator organisms

Jelly Powder. Effects of the foam controlling methods, non-selective (PCA) and selective media on recovery of *E. coli, S. aureus* and *S. faecalis* from JP are shown in Fig. 3A. There was no significant difference in recovery of *E. coli* using the different foam controlling blending methods. However, VRB gave significantly lower recovery than PCA. Recovery of *S. aureus* depended on the foam controlling method employed. The highest recoveries were obtained on all three plating media by using DAF at the 200 ppm level, but recoveries using the Colworth Stomacher 400 were not significantly lower. Blending without anti-foam and vacuum-blending gave significantly lower recoveries of *S. aureus* than the other two foam controlling methods. Baird-Parker agar gave significantly higher recovery of *S. aureus* than the two other media. There was no significant differences between recoveries of *S. faecalis* whether blending was done with or without the antifoam agent. Stomaching and vacuum-blending resulted in mean recoveries of *S. faecalis* significantly lower than those by normal blending and blending with antifoam. Plate Count Agar gave significantly higher recoveries of *S. faecalis* than m-Enterococcus medium.

Skim milk powder. As shown by Fig. 3B, the efficiency of recovery of *E. coli* from SMP was similar to that of the JP, in that there was no significant difference among foam-controlling methods. PCA, however, was a significantly better recovery medium than VRB. In the recovery of *S. aureus* from SMP, all blending methods were nearly equal with the exception of stomaching which gave a significantly lower recovery on all three media. There was no significant difference in recovery of *S. aureus* on PCA, MSA and BP. Recovery of *S. faecalis* from SMP was equally efficient by all four blending methods, but PCA gave a significantly better recovery than m-ENT.

Whole egg powder. As shown by Fig. 3C, the efficiency of recovery of *E. coli* from WEP was similar to the recoveries of *E. coli* from JP or SMP in that there was no significant difference among the blending methods. VRB gave significantly lower recoveries of *E. coli* than PCA. However, there were no significant differences in recovery of *S. aureus* from WEP by the four different blending methods. MSA gave significantly lower recoveries than PCA and BP. Blending without antifoam, blending under vacuum or stomaching had statistically equivalent means for recovering *S. faecalis* from WEP. When antifoam was added the recovery of *S. faecalis* was significantly lower than by the other blending methods. PCA gave significantly lower recoveries than m-ENT.

Peptone water. Recoveries of *E. coli* from peptone water (Fig. 3D) were similar to those observed with the three foods: there was no significant difference in recoveries from one blending method to another but

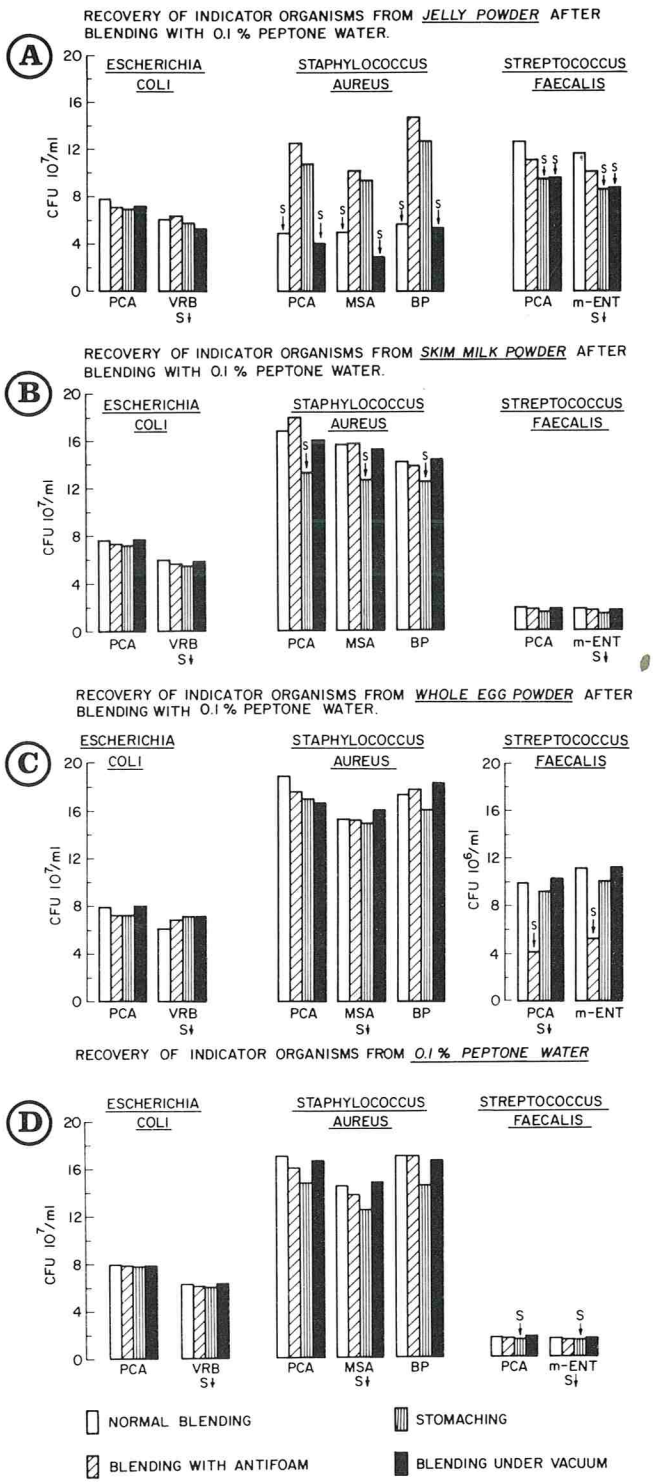

Figure 3. *Recovery of* Escherichia coli, Staphylococcus aureus *and* Streptococcus faecalis, *from jelly powder (A), skim milk powder (B), whole egg powder (C), and from 0.1% peptone water (D) after blending for 1 min without foam control (□), blending with 200 ppm DOW Corning AF Antifoam (▨), Stomaching (▥), blending under a vacuum of 25 inches of Hg (■). Recoveries on Plate Count Agar (PCA), Violet Red Bile Agar (VRB), Mannitol Salt Agar (MSA), Baird-Parker Agar (BP) and the m-Enterococcus agar (m-ENT). Significantly (S) lower, (↓) recovery at 0.05 level. CFU: colony forming units.*

significantly lower recoveries were obtained on VRB than on PCA. With respect to *S. aureus* recoveries from peptone water, the closest resemblance was observed

with recoveries from WEP. There was no significant difference among blending methods when the recovery was on the same media either on PCA, BP or MSA; however, MSA gave significantly lower recoveries than PCA or BP. Recoveries of *S. faecalis* from peptone water were significantly lower with stomaching as compared to blending without antifoam or blending under a vacuum. Recoveries of *S. faecalis* were significantly lower on m-ENT than on PCA.

CONCLUSIONS

As seen from these results, *E. coli* was recovered equally well by blending with or without antifoam, by stomaching or by blending under a vacuum. *S. aureus* and *S. faecalis* recoveries varied with food and foam controlling methods, although under certain circumstances one foam controlling method did not differ significantly from the other. The 0.1% peptone did not seem to interfere with recovery of *E. coli* and *S. aureus* when any of the four blending methods was used; however, recovery of *S. faecalis* was significantly reduced when stomaching was employed, and this may have accounted for lower recovery of this organism from JP. Consequently, significant differences in recoveries from the three foods are mainly due to the inherent characteristics of the food in question.

By trial and error the most suitable concentration of DAF for the reduction of foaming can be determined for each food. DAF at 200 ppm did not affect recovery of any of the three organisms from JP and SMP or from 0.1% peptone water; only *S. faecalis* showed significantly lower recovery from WEP when DAF at 200 ppm was used when compared to the other blending methods.

Recovery of *E. coli* on VRB was significantly lower from all three foods and from the 0.1% peptone water diluent when compared to PCA. Recovery of *S. aureus* from WEP and 0.1% peptone water on MSA was lower than on PCA or on BP. Lower recoveries of *S. faecalis* from JP, SMP, and from 0.1% peptone water were observed on m-ENT, but PCA gave lower recovery from WEP. Generally speaking, inhibitory substances in selective media may restrict growth of some of the population of organisms they are supposed to be selective for. Further discussion of this is beyond the scope of the present paper.

ACKNOWLEDGMENTS

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Effects of Sampling Procedures on *Salmonella* Recovery from Fresh Water Catfish

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ABSTRACT

To determine the effectiveness of different sampling procedures, 200 frozen catfish known to be contaminated with *Salmonella* were divided into 350 samples. Variables in sampling included anterior and posterior portions of fish, blending, immersion, swabbing, rinsing and incubation at elevated temperatures. The composite of blended anterior and posterior samples incubated at 43 C and immersion of whole fish incubated at 35 C showed the highest number of positive samples, 50% and 42%, respectively. The contact method of swabbing (14%) and rinsing (14%) were the least effective of the methods examined. The anterior (visceral cavity area) portions of the fish seemed to be more highly contaminated (38% positive) than the posterior portion (26% positive). These data show that the sampling procedure can greatly affect recovery of *Salmonella* from fresh water catfish. Overall levels of *Salmonella* were low and the hazards of cross-contamination with other foods seem remote.

Salmonella has been reported in fresh water or marine species of fish (*1,3,10,11,13,14*) and is generally associated with fecal contamination of water. Testing for *Salmonella* is usually initiated by either swabbing (*4,12,16*), rinsing (*7,8,21*) or the blending (*2,22*) of a sample in pre-enrichment or selective enrichment broths. A recent study of *Salmonella* detection on dressed frog legs by blending, immersion of whole legs, maceration with a Stomacher, and by rinsing showed no significant difference in recovery for the first three methods, whereas rinsing was significantly inferior (*2*). The present study compares the recovery of *Salmonella* from fresh water catfish as a function of sampling and sample incubation.

MATERIALS AND METHODS

In this study, 350 samples from 200 frozen catfish known to be contaminated with *Salmonella* were tested for the presence of *Salmonella* by various sampling procedures. Method I consisted of dividing a fish in half by severing it just behind the anal vent. The tail portion (posterior-IP) and the visceral portion (anterior-IA) were then weighed and blended separately in nine parts of lactose broth (LB). One-half of each homogenate was incubated at 35 C for 24 h and the remaining halves were combined and reblended for homogeneity. This composite sample was then halved and one-half incubated at 35 C for 24 h (composite-IC). Method IC served as the control method for this study. The other half of the composite homogenate was incubated at 43 C for 24 h (composite-IC43). Method II consisted of complete immersion and incubation of the entire fish in 1 part LB (w/v). Method III included shaking the entire fish in 1 part LB (w/v) for 1 min and removing it before incubation. Method IV consisted of swabbing the entire surface area of the fish using two dacron swabs moistened with LB and placing them in 20 ml of LB. The LB pre-enrichment broths for Methods II, III and IV were incubated at 35 C for 24 h. Ten samples were analyzed daily by each method. Pre-enrichment was followed by the selective enrichment of 1-ml portions of LB in 10 ml of tetrathionate (TET) and 10 ml of selenite-cystine broth (SEL). Brilliant green agar with sulfadiazine, bismuth sulfite agar and *Salmonella-Shigella* agar with 1% sucrose and 0.65% agar added, as recommended by Sperber and Deibel (*19*), were streaked from TET and SEL. Plates were incubated for 24 h at 35 C. Suspect *Salmonella* colonies as described in the *Bacteriological Analytical Manual for Foods* (BAM) (*22*) were picked from each plate to triple sugar iron agar slants and motility-indole-lysine (MIL) deeps (*23*). MIL is a modification of Ederer and Clark's (*9*) motility-indole-ornithine medium (MIO) with lysine substituted for ornithine. MIL was prepared by adding 1% trypticase and 0.2% agar to Falkow lysine broth (*23*). This medium allowed for biochemical differentiation of *Salmonella* from indole-positive *Edwardsiella*. Incubations were at 35 C for 24 h at each step of the isolation procedure. Suspect *Salmonella* isolates were further tested for methyl red, Voges-Proskauer reactions, utilization of citrate on Simmon's citrate agar and for acid production in 0.5% mannitol in purple broth base. Serological confirmation of *Salmonella* was performed with poly-O antisera using the slide agglutination method. All media used in this study were BBL with the exception of SS agar from Difco.

RESULTS AND DISCUSSION

The percentage of *Salmonella*-positive samples with different methods of sampling is given in Table 1. An analysis of variance was done to determine if there was a difference in the recovery rate of the methods. The F value obtained indicated a significant difference between the sampling methods. Duncan's new multiple range test was done to determine which methods were significantly different. The methods with different superscripts were significantly different. Method IC43 had the highest recovery rate, but was not significantly different from Method II. The effectiveness of the increased incubation temperature recommended by several workers (*5,6,15,17, 18,20*) is also applicable for *Salmonella* detection in catfish. No significant difference existed between Methods II, IA and IC (control). The control method was based on the procedure in the BAM manual (*22*) which calls for a 25-g portion from an unspecified area of the fish to be blended in nine parts of LB with incubation at 35 C. The present study indicates that a significant difference exists between the presence of *Salmonella* in

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TABLE 1. *Recovery of* Salmonella *from fresh water catfish sampled by body portion, homogenization, immersion, rinse, swab, and incubation at 43 C (50 samples each).*

Type of sample[1]	Positive (No.)	Positive (%)	Negative (No.)
IA[a] - Anterior portion (LB-homogenate)	19	(38)	31
IP[b] - Posterior portion (LB-homogenate)	13	(26)	37
IC[a] - Composite sample (IA & IP - LB-homogenate)	19	(38)	31
IC43[d] - Composite at 43 C (IA & IP - LB-homogenate)	25	(50)	25
II[d] - Whole fish (LB-immersed & incubated)	21	(42)	29
III[c] - Rinse (LB-rinse)	7	(14)	43
IV[c] - Swab (LB)	7	(14)	43

[1]a,b,c,d Sampling methods with different superscripts are significantly different.

the visceral cavity and the tail area of catfish. This could influence the *Salmonella* recovery rate if indiscriminate selections are made of portions of the fish. A higher incidence of *Salmonella* in the visceral cavity can be expected because of increased chances of contamination from the viscera and from increased handling during processing. The presence of *Salmonella* on the tail area would most likely result from cross-contamination during processing (*23*).

Maximum recovery of *Salmonella* from catfish, according to the present study, would be either by immersion of the whole fish or the visceral cavity portion of large fish in LB followed by incubation at 43 C. The statistical analysis indicates that recovery of *Salmonella* by incubation at 43 C was not significantly different than that obtained by Method II, although a higher recovery rate was noted at 43 C. Method II probably is more practical because of the simplicity in sample preparation and the minimal equipment needed for incubation. The sample can be collected and pre-enriched in the same plastic pouch and incubated in a dry-heat incubator, whereas a water bath is required for incubation at 43 C.

Results from a study by the U.S. Food and Drug Administration on the effect of sample methodology on *Salmonella* isolation from frog legs were in general agreement with those of this study (*2*). The methods the agency compared were immersion of whole frog legs, blending, use of the Stomacher and rinsing. The agency found no significant difference between the first three methods, but recovery was significantly lower with rinsing. The agency did not use 43 C as an incubation temperature.

With methods IA, IP, IC and IC43, *Salmonella* were detected in 41 (82%) of the 50 fish samples. Theoretically, with a *Salmonella*-positive IA or IP sample, one would expect the corresponding IC and IC43 samples to be positive. However, this only occurred with 11 of 19 IA-positive samples and with 9 of 13 IP-positive samples. This indicates that 20 (62.5%) of the 32 positive-IA and -IP samples had sufficient numbers of *Salmonella* to be recovered in the corresponding composite sample. Of the 25 positive-IC43 samples only 13 were positive as IC samples. Although the IC43 method had the highest recovery (25 positives), it missed 16 (33.3%) of the 41 positive fish. This indicates that although a large percentage (82%) of the fish were *Salmonella*-positive, the number of *Salmonella* present on each fish was at a relatively low level and unevenly distributed. In addition, the poor recovery (14%) obtained with contact methods (rinse and swab) indicates that very few *Salmonella* are dislodged from the surface area of the fish.

Salmonella in raw foods such as catfish, red meats, poultry and frog legs is of concern because of its cross-contamination potential to foods consumed raw and/or its survival in foods that receive marginal heat treatment. The poor recovery by contact methods (III and IV) coupled with low numbers indicated by Methods IA and IP (when compared to IC and IC43) negate catfish as a potential *Salmonella* hazard. The FDA had concluded this, based primarily on its 1977 study (*1*). In its opinion however, this was caused by the procedure used to process catfish. The procedure described was developed by this laboratory (*23*) and to our knowledge was in operation in only one plant. The procedure was part of a complete quality assurance program submitted to the FDA for approval to resume processing after being enjoined. This procedure was proven to be effective in producing *Salmonella*-free carcasses. Additional research (*23*) has shown that the presence of *Salmonella* in live fish is directly related to the level of *Salmonella* in the water. *Salmonella* was found to be continuously present in low numbers, but under certain conditions of increasing temperature, large stocking rate and increased fish body weight, the number of *Salmonella* in the water rapidly increased. The presence of *Salmonella* on the skin and in the viscera of live fish resulted in *Salmonella*-positive carcasses when normal processing procedures were used to dress catfish. Results of the present study indicate that the method of sampling can have a great effect on detection of *Salmonella* in catfish. Although *Salmonella* were present in a large percentage of the samples in this particular lot, methods are available (*23*) to reduce or eliminate *Salmonella* from dressed catfish.

ACKNOWLEDGMENTS

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Method for Predicting Minimum Detectable Residual Alkaline Phosphatase in High-Temperature, Short-Time Processed Dairy Products

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ABSTRACT

High-temperature, short-time (HTST) processed milk, cream and buttermilk were mixed with small portions (0 to 0.6%) of the raw milk product to obtain desired levels of residual alkaline phosphatase. Samples were subjected to the differential test to discern reactivation and analyzed for phosphatase activity by the rapid colorimetric test. The experimental data were fitted to a linear statistical model to determine the minimum detectable residual phosphatase (E_0) in the product. These observed values and the computed expected values were highly correlated, with a rank correlation coefficient of 0.956, which was significant at $a = 0.05$ level. The values of [E_0] varied depending upon the extent of phosphatase reactivation in the HTST product when the residual phosphatase was zero. As the differential values of reactivation (reactivated [E] of the control sample minus the reactivated [E] of diluted sample containing magnesium) increased, the [E_0] increased also. In general, the [E_0] in cream was greater than that in milk. A method is proposed for predicting [E_0] in liquid HTST products.

Alkaline phosphatase is a naturally occurring enzyme (E) in milk. Standard methods (visual and colorimetric) are available for differentiating reactivated from residual phosphatase in high-temperature, short-time processed (HTST) milk and cream (*1,2*). In the routine testing of dairy products, the differential test is performed only when the conventional test is positive and a determination is needed as to whether these results are due to reactivated or residual phosphatase. For regulatory purposes, no action is taken if the differential test is positive for reactivated phosphatase. However, if the differential test is positive for residual phosphatase, attempts are made to determine problem areas and, if necessary, to take regulatory action.

A reactivated phosphatase in an unrefrigerated product will yield test results that are like those of residual phosphatase. Thus, a false-positive test may be obtained when a sample capable of reactivation has been allowed to stand longer than about 4 h at 16 C, or 2 h at 21 C. The differential test is therefore not applicable to such abused products (*6*).

Various factors, including initial phosphatase concentration and processing temperature, affect reactivation of phosphatase in HTST products (*9*). It has been reported that the reactivation of phosphatase in commercially UHT-processed heavy cream > half and half > milk (*5*). Therefore, prediction of small amounts of residual phosphatase in a product containing highly reactivatable phosphatase may be difficult to determine whether the samples are analyzed by the visual or the colorimetric methods (*1,2*). In addition, as indicated elsewhere (*9*), in the differential test the dilution ration of 1 + 5 for the samples with added magnesium (Mg) is not a constant factor, and thus occasional failure of the method to predict the differential test correctly may be expected.

The purpose of this study was to determine the minimum detectable levels for residual phosphatase and to estimate the variations of the differential test for several dairy products.

MATERIALS AND METHODS

Materials

Raw milk and 35% cream were collected from a local milk plant. Cream was standardized to 12% fat with milk. Buttermilk was prepared by churning raw cream in a Waring blender[1].

Method

To produce reactivatable products, milk, cream and buttermilk were heated to 93.3 to 112.8 C for < 1 sec in a continuous-flow heat exchanger (*7*). The heated products were stored overnight at 4 C before reactivation was studied.

Heat-processed milk, cream and buttermilk were mixed with small portions (0.0, 0.2, 0.3, 0.4, 0.5 and 0.6%) of raw counterpart milk product to obtain desired levels of residual phosphatase. Duplicate portions were analyzed for residual phosphatase (*7*). Then the samples were reactivated with and without addition of magnesium acetate for 60 min at 34 C and analyzed for residual and reactivated phosphatases (*7,8*).

With the observed results, a graph was constructed relating the difference between reactivated phosphatase of the sample without Mg, [E_{RC}], and the reactivated phosphatase of the counterpart sample containing Mg and diluted 1 + 5, [E_{RS}], to the residual phosphatase. The experimental points were fitted by linear regression, assuming that both variables have error terms, and the value of residual phosphatase at [E_{RC}] − [E_{RS}] = 0 was computed which gave the minimum residual phosphatase, [E_0], in the product.

RESULTS AND DISCUSSION

Differential test rationale

The criterion is based on comparing results in testing a diluted (1 + 5) sample containing added Mg with the test results of an undiluted sample without the added Mg after storing at 34 C for 1 h. If the diluted sample shows

[1]*Mention of commercial product does not imply endorsement by the Food and Drug Administration.*

less phosphatase activity than the undiluted sample, the test is considered positive for residual phosphatase. If the diluted sample shows greater phosphatase activity than the undiluted sample, the test is considered positive for reactivated phosphatase (*1*).

Observed data

To calculate the $[E_0]$ in a given HTST product, the observed values were used for $[E_{RC}] = w_2$ and $[E_{RS}] = w_1$ at each level of residual E added to the product. An estimate of the $[E_0]$ occurs when $x = [E_{RC}] - [E_{RS}] = 0$. A linear regression can be computed for the functional relation:

$$y = \beta_0 + \beta_1 + (e - \beta_1 d)$$

where: β_0 = intercept
β_1 = slope
$x = [E_{RC}] - [E_{RS}] = w_2 - w_1$
y = residual [E]
d,e = error terms for x and y

Both x and y are measured with error, so the estimated regression coefficients b_0 and b_1 were computed (*3*), with the assumption that the ratios of variances $\sigma_d^2/\sigma_e^2 = 2$. The intercept b_0 is the estimate of $[E_0]$ when $x = 0$. The relationship between x and y in all samples tested was highly correlated with correlation coefficients (r) ranging from 0.95 to 1.00. The slopes of the regression lines varied as follows: milk 1.08 to 1.51, with an average of 1.22 ± 0.14; cream, 1.09 to 1.53, with an average of 1.29 ± 0.13; and buttermilk, 1.35.

Data in Table 1 show that the values of $y_0 = [E_0]$ depended on the product, as follows: milk, 1.16 to 2.95 μg phenol/ml; cream, 1.79 to 8.36 μg phenol/ml; and buttermilk, 4.85 μg phenol/ml. Data for buttermilk are limited but it is believed that the variations in y_0 would be similar to those observed for milk and cream.

Estimated data

An estimate of the expected data for $[E_0]$ can be obtained by taking measured values of $[E_{RS}] = w_{10}$ and $[E_{RC}] = w_{20}$, when $y = 0$ and by assuming what the residual [E] is for $y > 0$. For each addition of Δy to the control sample, the diluted sample containing Mg would be increased by $\Delta y/6$. Thus,

$$w_1' = w_{10} + y/6 \qquad (2)$$

for the diluted sample containing Mg, and

$$w_2' = w_{20} + y$$

for the control sample. The values for w_1' and w_2' are the predicted reactivated E, $[E_R]$, of w_1 and w_2 if it is assumed that these $[E_R]$ are dependent on the dilution factor.

The increment that will correspond to $[E_0]$ is:

$$y_0 = 1.2\,(w_{10} - w_{20}) \qquad (4)$$

Typical graphic presentation of the data is shown in Fig. 1. The expected values for all samples are presented in Table 1.

The observed and expected $[E_0]$ for all samples were compared (*4*). A Spearman's rank correlation coefficient (r_s) of 0.956 was obtained, which indicates a significant correlation at the $a = 0.05$ level.

Because the differential test uses a dilution ratio of 1 + 5 for samples containing Mg, the theoretical slope of the regression line relating x and y is 1.2 (eq. 4). The slope based on expected values of x and y is 1.19 ± 0.02, which is similar to the theoretical value. The slope based on observed values for x and y was 1.26 ± 0.13, which is not significantly different from the expected value. The observed and expected values (slopes) are close for milk, but not for cream and buttermilk. Variations in the observed slopes are related to errors in sample dilution, analysis of samples for E, etc. However, for control purposes, the slope can be experimentally determined, or the theoretical value of 1.2 can be used (eq. 4) to determine $[E_0]$ in an HTST product.

Other data

Kleyn and Ho (*5*) have published some reactivation data for commercially UHT-processed heavy cream and

TABLE 1. *Relationship between reactivated and minimum detectable residual alkaline phosphatase in high-temperature, short-time processed liquid dairy products.*

Sample	Processing temperature (C)	Reactivated phosphatase (μg phenol/ml)		Minimum detectable residual phosphatase (μg phenol/ml)	
		Sample without Mg	Diluted samples containing Mg	Observed	Expected
Milk	104.4	2.80	4.50	1.66 (± 0.08)[a]	2.02
Milk	104.4	3.50	6.10	2.95 (± 0.07)	3.12
Milk	93.3	3.66	4.62	1.65 (± 0.05)	1.15
Milk	105.0	2.68	4.23	1.64 (± 0.06)	1.86
Milk	112.8	1.74	2.78	1.16 (± 0.05)	1.25
Milk	98.9	2.83	4.82	2.15 (± 0.06)	2.39
Milk	104.4	1.64	3.65	2.51 (± 0.03)	2.41
Milk	104.4	1.75	3.72	2.51 (± 0.03)	2.36
Cream	104.4	3.90	7.63	4.65 (± 0.13)	4.48
Cream	104.4	3.30	7.39	4.94 (± 0.12)	4.91
Cream	104.4	6.27	11.58	6.55 (± 0.17)	6.37
Cream	98.9	4.27	6.22	2.95 (± 0.09)	2.34
Cream	98.9	1.94	3.77	1.79 (± 0.08)	2.20
Cream	100.0	4.09	6.92	3.83 (± 0.08)	3.40
Cream	111.0	3.08	6.92	5.09 (± 0.07)	4.61
Cream	104.4	6.78	11.96	8.36 (± 0.42)	6.22
Buttermilk	105.0	2.70	6.34	4.85 (± 0.09)	4.37

[a]Standard deviations are enclosed in parantheses.

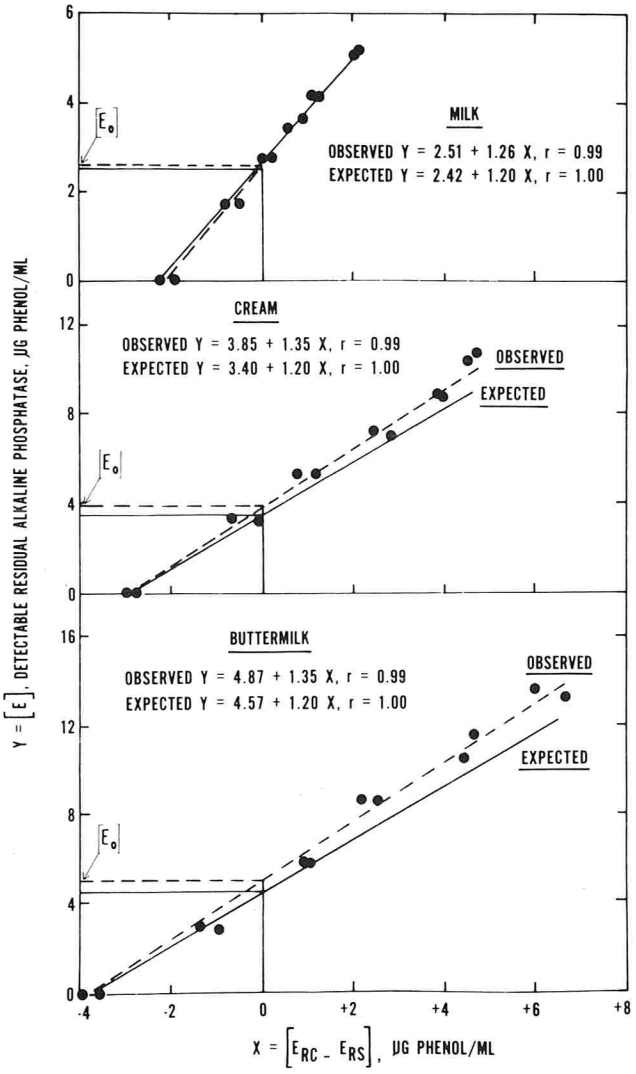

Figure 1. *Relationship between differential reactivated alkaline phosphatase and detectable residual alkaline phosphatase in dairy products.*

half-half. These data are based on magnesium chloride reactivation of the enzyme and a 1-h test for enzyme analysis (*1*). If the data are satisfactory and if eqs. 2 and 3 are applied, the estimated $[E_0]$ in the samples shown in Table 2 are obtained.

Since the results in Table 2 are based on a 1-h test, a residual E of 2.3 µg phenol/ml is used as a limit for adequate pasteurization. Therefore, all samples show high $[E_0]$ except for heavy cream sample no. 4 and half-and-half sample nos. 2, 3, 5, 6 and 8. The relationship between $x = (w_2 - w_1)$ and $y = E$ is represented by $y = 0.02 - 1.20\ x$, with a correlation coefficient of $r = -1.00$. The slope of the computed line is 1.20, which is as expected for a 1 + 5 dilution system.

These investigations have provided information concerning the usefulness of the method for determining $[E_0]$ with respect to a given UHT or HTST product that shows a positive, conventional test only. As indicated earlier, the differential test is not applicable to HTST samples that show a false-positive conventional test for phosphatase because they have been allowed to stand longer than about 4 h at 16 C or 2 h at 21 C. For control purposes, the test can be performed as described under Method, or a theoretical value of 1.20 for the slope of eq. 4 can be used to determine $[E_0]$. The positive results from the conventional test are then assessed in terms of the computed $[E_0]$. Further confirmation as to the presence or absence of phosphatase in a HTST milk product must be made by means of established microbiological testing, etc. to determine the cause and effect relationship.

TABLE 2. *Estimated minimum detectable residual phosphatase (µg phenol/ml) for commercially UHT-processed heavy cream and half-and-half.*

	Heavy cream			Half-and-half		
Sample	E_{RC}[a]	E_{RS}[a]	E_0	E_{RC}[a]	E_{RS}[a]	E_0
1	12.5	20.2	9.24	0.9	4.6	4.44
2	6.2	20.6	17.28	0.5	2.1	1.92
3	3.0	20.6	21.12	0.9	1.2	0.36
4	11.8	12.8	1.2	2.3	8.5	7.44
5	—	—	—	2.1	3.2	1.32
6	6.4	16.3	11.88	0.6	2.5	2.28
7	12.5	15.6	3.72	1.2	3.8	3.12
8	—	—	—	1.0	1.8	0.96
9	6.9	16.2	11.16	1.3	5.3	4.80
10	8.8	15.4	7.72	2.2	4.4	2.64

[a]Data are printed, with permission, from Kleyn, D. H. and C.-L. Ho. 1977, J. Assoc. Offic. Anal. Chem. 60:1389-1391.

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Screening Kulfi for Staphylococcal Enterotoxins with the Thermonuclease Test

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ABSTRACT

Forty kulfi samples collected from local push-cart vendors and restaurants were screened for thermonuclease and staphylococcal enterotoxins. Viable staphylococcal counts were also determined in these samples. Thermonuclease was detected in four kulfi samples. The incidence was, however, more (15%) in samples from push-cart vendors as compared to those from restaurants (5%). Enterotoxins were also detected in thermonuclease-positive samples. The incidence of enterotoxins A and B was 10%. Enterotoxins C and D were not detected in the samples. Of 81 isolates of staphylococci obtained from kulfi samples, seven exhibited thermonuclease, coagulase and enterotoxin production. Ten percent of thermonuclease-positive isolates came from kulfi samples collected from push-cart vendors as compared to 6.5% from restaurant samples. The incidence of enterotoxin A producing staphylococci was 8% and 3.2% in samples from push-cart vendors and restaurants, respectively. One of the coagulase-positive isolates from restaurant samples failed to produce either thermonuclease or enterotoxins.

Kulfi is a frozen indigenous milk product popular in many parts of North India (*21*) and Pakistan (*10*). This product is prepared from cow, buffalo or mixed milk which is concentrated to approximately half of its volume in a large open pan (karahi), kept over a fire hearth (chula). The concentrated milk is cooled for a while at ambient temperature. Sugar at the rate of 15-20% is then added to milk and stirred for thorough mixing. Spices and nuts (cardamum, almonds, pistachio, cashew) or fruit juices (mango) are added to the sweetened milk and mixed. The mixture is then filled in conical containers and frozen in an earthen pot containing an ice-salt mixture. The frozen product is consumed either as such or along with semya (prepared from wheat flour, drawn in the form of long threads and immersed in water before use). Kulfi is marketed either by halwais (sweetmeat makers) in restaurants or from push carts by roadside vendors.

The hygienic quality of kulfi is yet to be known to many people. In a recent investigation, Rao (*16*) has isolated various types of microorganisms including staphylococci from this product. *Staphylococcus aureus* has gained importance in recent years in view of its ability to grow and produce enterotoxins in foods, including milk and milk products (*7,9,19*), leading to food poisoning outbreaks. A large number of coagulase producing staphylococci have been reported to produce thermostable deoxyribonuclease (*12,13*) and a close correlation between the *S. aureus* growth and thermostable DNase (thermonuclease) and between thermonuclease and enterotoxin production has been very well established in dairy products (*6,19*). Cords and Tatini, Park et al. and Batish et al. (*3,6,15*) have recommended thermonuclease test as a rapid and reliable method for the detection of staphylococcal enterotoxin in dairy products.

In the present investigation, an attempt has been made to assess the quality of market kulfi samples by determining staphylococcal population as well as thermonuclease.

MATERIALS AND METHODS

A total of 40 samples of kulfi were collected according to Standards Methods (*1*) from the Karnal market during summer (March to June, 1978). These included 20 from push-cart vendors and 20 from restaurants. Samples obtained from push-cart vendors had a candy-like appearance containing a wooden stick and the product was frozen in a conical iron container. Kulfi sold in restaurants was available in either metal or plastic containers. The containers, sealing material (rubber bands) and the earthern pot are shown in Fig. 1.

Kulfi samples were examined for staphylococcal counts on Staphylococcus medium 110 (*5*). Thermonuclease was extracted (*20*) and the extract was boiled for 15 min. The thermonuclease test was carried out on toluidine blue DNA agar medium (*11*). The thermonuclease-positive samples were tested for the presence of enterotoxins by the microslide gel diffusion technique (*4*). The standard antisera of the enterotoxins A, B, C and D were obtained from Dr. M. S. Bergdoll.

The isolates of staphylococci obtained from kulfi samples were characterized on the basis of anerobic glucose and mannitol fermentation (*2*), coagulase production (*17*), thermonuclease (*11*) and enterotoxin production (*4*).

RESULTS AND DISCUSSION

Of 40 samples of kulfi examined in the current study, four were positive for presence of thermonuclease (Fig. 2). The incidence was, however, more (15%) in kulfi samples obtained from push-cart vendors than from restaurants (5%). Data in Table 1 show that a fair correlation between detectable thermonuclease and viable counts of staphylococci in samples was not possible. The results of the present study indicate that kulfi samples from push-cart vendors had low viable counts of staphylococci (95×10^3 per g) which are not suggestive of a potential danger but contained detectable levels of thermonuclease (zone diameter, 12.2 mm). Viable staphylococcal counts (14×10^3 per g) were also less in thermonuclease-positive samples (zone diameter.

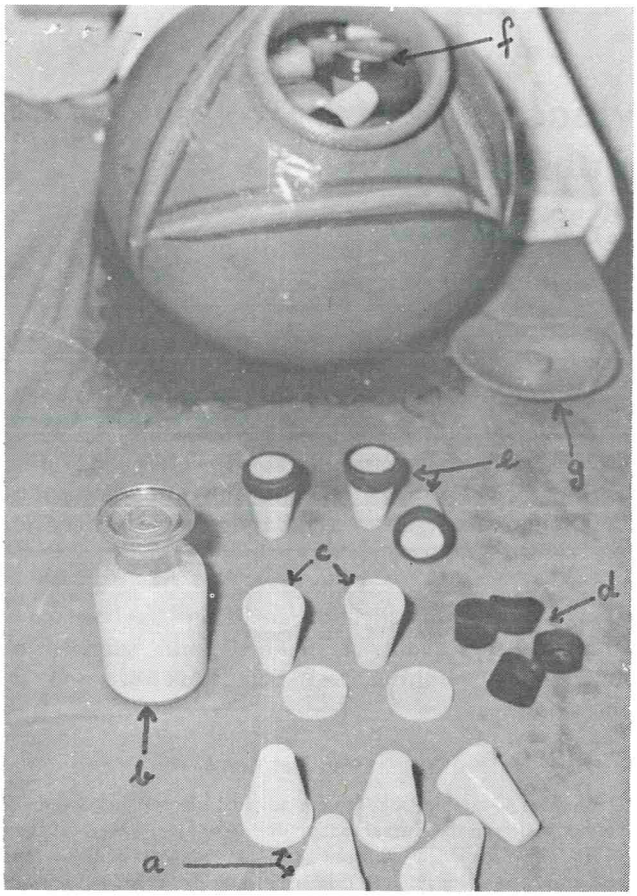

Figure 1. *Equipment and accessories used for preparation of kulfi. a. Plastic cones before filling. b. Milk mixture. c. Cones containing milk mixture. d. Sealing material. e. Sealed plastic cones. f. Freezing of kulfi contained in sealed plastic cones in an earthen pot filled with ice-salt mixture. g. Lid of earthen pot.*

8.5 mm) obtained from restaurants. Similar findings were reported (*3,19*) for butter, cheese, non-fat dry milk, dried malted milk and baby food samples. In an earlier survey conducted on several samples of kulfi from the Karnal market, Ghosh (*9*) observed staphylococcal counts ranging between 43×10^2 and 59×10^3 per g with an average of 139×10^2 per g in the samples.

Among the types of enterotoxins encountered in the market samples of kulfi, enterotoxin A predominated, followed by enterotoxin B. Both the types of enterotoxins (A and B) were detected in kulfi samples which also contained thermonuclease. In the present study, the incidence of enterotoxins A or B in kulfi samples from both the sources was about 10%. Enterotoxins C and D were not detected in any of the samples examined during this study (Table 1).

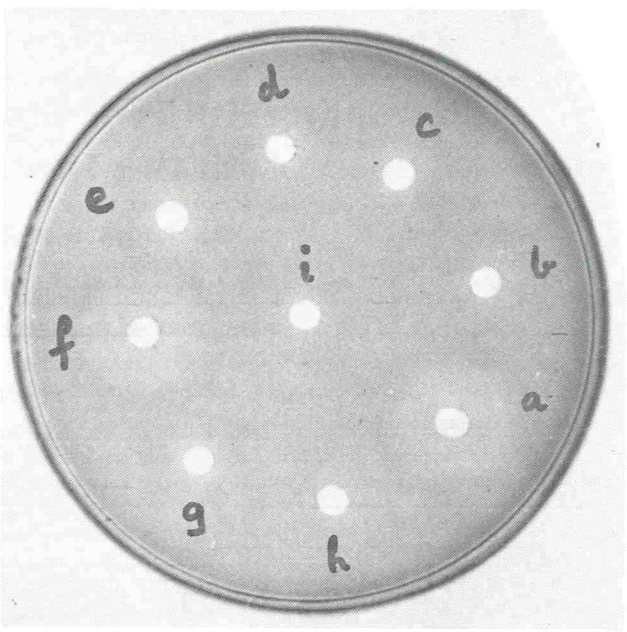

Figure 2. *Thermonuclease detection in kulfi samples. a. and f. Thermonclease-positive. b. to e, g, & h. Thermonuclease-negative. i. Negative control (sterile milk).*

A total of 50 isolates of staphylococci was collected from 20 samples of kulfi obtained from push-cart vendors and 31 isolates from 20 samples received from restaurants. Among 81 isolates examined, seven were positive for thermonuclease, coagulase and enterotoxins. In regard to the incidence of thermonuclease-producing strains of staphylococci from different sources tested, 10% (5/50) of the isolates were positive; only 6.5% (2/31) of the isolates from restaurant samples were positive. It is possible that enterotoxigenic staphylococci might have gained entry into milk at some stage of processing. According to Ghosh (*9*), incidence of enterotoxigenic staphylococci was more in frozen milk products like ice-cream (10.2%) and kulfi (6.7%) than in other dairy products.

Data in Table 2 indicate that each of the seven thermonuclease-producing isolates of staphylococci showed production of either enterotoxin A or B. The incidence of enterotoxin A produced by staphylococcal isolates in kulfi samples from push-cart vendors and restaurants was 8% and 3.2%, respectively. One isolate was positive for enterotoxin B from each source of sample. Enterotoxins C and D were not detected in any of the thermostable DNase-producing isolates examined during the current study.

One (out of three) of the coagulase positive isolates obtained from restaurant sample failed to produce either

TABLE 1. *Incidence of thermonuclease and enterotoxins in kulfi samples.*

Source of kulfi sample	Incidence of thermonuclease: No. of samples positive[a]	Percent	Zone diameter (mm)[b]: Range	Average	Staphylococcal counts/g in thermonuclease positive samples: Range	Average	Types of enterotoxin: A	B	C	D
1. Push-carts	3[c]/20	15	9.5 - 14.5	12.2	63×10^3-11×10^4	95×10^3	+	–	–	–
2. Restaurants	1/20	5		8.5		14×10^3	–	+	–	–

[a]The numerator indicates number of positive samples and the denominator indicates total number of samples.
[b]This includes the diameter of well (4 mm)
+ = Present; – = Absent
[c]All three samples were positive for enterotoxin. (Sensitivity of microslide assay = 0.5 μg/ml)

TABLE 2. *Distribution of staphylococcal isolates on the basis of thermonuclease, coagulase and enterotoxin production.*

Kulfi samples		Number of staphylococcal isolates	Characteristics of isolates					
					Type of enterotoxin			
Source	Number		Thermonuclease	Coagulase	A	B	C	D
			(Number of isolates)					
1. Push-carts	20	50	5 (10%)	5 (10%)	4 (8%)	1 (2%)	0	0
2. Restaurants	20	31	2 (6.5%)	3 (9.7%)	1 (3.2%)	1 (3.2%)	0	0

thermostable DNase or enterotoxins. According to Rayman et al. (*17*), some staphylococcal strains capable of producing coagulase did not produce thermonuclease and enterotoxins. In contrast, Omori and Kato (*14*) demonstrated clearly certain coagulase-negative *S. aureus* strains which could produce enterotoxins. Hence the sole character of coagulase production in staphylococci cannot be considered as an indicator of enterotoxigenicity.

During the manufacture of kulfi, milk is subjected to a substantial heat treatment (boiling for 1 h); staphylococci are not known to survive such drastic heat treatments. The possibility of these organisms gaining entry into milk before freezing through fingers and other sources cannot be ruled out. These organisms, after attaining several millions, might have produced both the thermonuclease and enterotoxins in milk at ambient temperatures. In a similar study, Ghosh (*9*) found that enterotoxin A and B were detectable in milk when staphylococci reached a population of about 42 to 43 million per ml. Very recently, several other workers (*8,18*) have indicated that staphylococci grow well in milk and produce thermonuclease and enterotoxins under favorable conditions.

In the light of the above reports, the findings of the present investigation suggest that market kulfi can serve as a vehicle of enterotoxigenic staphylococci causing food poisoning outbreaks, and thermonuclease test can be used as a rapid and reliable screening method for indicating the likely presence of enterotoxins, thus warranting enterotoxin analysis.

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Precision of the Pro-Milk Method in Routine Determination of Protein in Dairy Testing Laboratories[1]

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ABSTRACT

In a collaborative study of the Pro-Milk Mark II dye-binding method for determining protein in milk, repeatability (r) was found to be 0.053% protein, and reproducibility (R) 0.215% protein at the 95% confidence level. Standard deviations of these two measures of performance were 0.0188 and 0.0761, respectively. Repeatability was influenced by sampling error, mis-readings, equipment performance and routine control. Reproducibility was influenced by the same factors and, in addition, accuracy of calibration, with Kjeldahl as the reference method. Results of this study indicate the need to centralize laboratory calibration and to calibrate equipment over a wide range of protein levels.

Renewed interest in pricing and purchase of milk on a protein basis has been observed in the U.S. in recent years. Some plants, where state regulations allow, already purchase milk on this basis. Certain European countries have been involved in such programs for several years. For routine testing, dye-binding and infra-red analysis appear to be the most common methods used. In either instance, appropriate standardization and calibration are essential to accurate, precise testing. In a recent collaborative study of the Pro-Milk device, certain factors were uncovered which relate to precision of this dye-binding method. They appeared to the authors to be significant and worthy of note.

MATERIALS AND METHODS

Collaborative study

Six laboratories, five commercial and one university, were involved in this study. All had prior experience in analyzing protein in milk by the Pro-Milk, Mark II manual method (Foss America, Inc., Fishkill, NY 12524).

Duplicates of 15 milk samples were prepared from nonfat dry milk, with protein level varying incrementally from 2.55-4.12%. All samples were preserved by addition of 0.1% potassium dichromate. Samples were randomized before shipment to participating laboratories. Each laboratory, therefore, received 30 samples (15 duplicates) numbered 1-30, but previously re-ordered in random sequence. Samples were refrigerated before and after, but not during shipment. All samples were tested within 5 days of shipment.

Since the main purpose of the work was to derive a realistic evaluation of the precision of the Pro-Milk method under practical operating conditions, no attempt was made to standardize the various Pro-Milk units. No specific directions for testing the samples were given over and above the need to do the work within 24 h of receipt of samples.

Definitions and statistical evaluation:

Precision of an analytical method involves two different aspects, repeatability and reproducibility. By International Standards Organization definition (ISO __/ /DIS 5725), repeatability (r) is the value below which two single test results (duplicates) obtained with the same method on the same sample under the same test conditions may be expected to lie at a probability of 95%. If σ_r is the standard deviation (s.d.) of repeatability, $r = 2.83 \times \sigma_r$.

Reproducibility has the same definition as above except that test conditions are different, that is, two different laboratories are involved. Given σ_r as the standard deviation (s.d.) of reproducibility, $R = 2.83 \times \sigma_r$.

Values of σ^2_r and σ^2_R can be obtained from an analysis of variance of test results of q samples (at different protein levels) analyzed by p laboratories doing n replications. Assuming that the laboratories involved are a random selection of the total laboratory population, and with protein levels fixed, a two-factor mixed model analysis of variance can be calculated. From this model, the variance of error is an unbiased estimate of the variance of repeatability (σ^2_r), and variance of reproducibility (σ^2_R) becomes the sum of the following variances: (*a*) between laboratories (σ^2_L), (*b*) interaction of laboratory × sample ($\sigma^2_{L \times S}$) and (*c*) repeatability (σ^2_r): $\sigma^2_R = \sigma^2_L + \sigma^2_{L \times S} + \sigma^2_r$.

RESULTS AND DISCUSSION

Analysis of variance of the data obtained in this study is shown in Table 1. The values indicate a highly significant laboratory effect likely brought about by differences in calibration of the various instruments. The data also point out a highly significant laboratory × level interaction. This presumes a difference in the slope of the calibration curves.

Expected mean square values (shown in Table 2) provide the components of variance needed to calculate r and R. The standard deviation of repeatability is 0.0188 and repeatability (r) is 0.053% protein. The standard deviation of reproductibility is 0.0761, and reproducibility (R) 0.215% protein.

Data in Table 3 illustrate the highly significant laboratory effect in the analysis of variance. Regression formulae are given for each laboratory. Except for laboratory number 1, which displays a signficantly different calibration compared to the others, agreement is relatively good. However, it is also clear that the instruments were calibrated only at the medium protein level. At this level (3.2%), the estimated laboratory readings differ by no more than 0.05% (laboratory 1 excluded). At higher and lower protein levels, agreement

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TABLE 1. *Analysis of variance[1] of results of protein determinations in several laboratories using the Pro-Milk method.*

Source of variation	Degrees of freedom	Mean square	F ratio with degrees of freedom	Expected mean square
Samples (level)	14	3.08603198	1082.17* (14/70)	$\sigma^2_r + n\sigma^2_{L\times S} + nq\sigma^2_S$
Laboratories	5	0.125964556	358.19* (5/90)	$\sigma^2_r + np\sigma^2_L$
Laboratories × sample	70	0.002851698	8.11* (70/90)	$\sigma^2_r + n\sigma^2_{L\times S}$
Error	90	0.000351666		σ^2_r

[1]Using a mixed model in which 15 milk samples (p = 15) at fixed protein levels were analyzed in duplicate (n = 2) by six laboratories (q = 6) assumed to reflect a random selection.
*Significant at 0.005 level.

TABLE 2. *Estimated values of the components of total variance (variance and standard deviation in % protein).*

Source of variation	Variance	Standard deviation
Laboratories	$\sigma^2_L = 0.0041870$	0.0647
Laboratories × sample	$\sigma^2_{L\times S} = 0.0012500$	0.0353
Error	$\sigma^2_r = 0.00035166$	0.0188

TABLE 3. *Regression equations and estimated values at three different protein levels for six laboratories using the Pro-Milk Mark II method.*[1]

Laboratory	Regression equation	Estimated value of y for x = 2.60	x = 3.20	x = 4.00
1	y = 0.968 x + 0.232	2.75	3.33	4.10
2	y = 0.970 x + 0.072	2.59	3.18	3.95
3	y = 0.9186 x + 0.216	2.60	3.16	3.89
4	y = 1.1121 x − 0.379	2.51	3.18	4.07
5	y = 0.9819 x + 0.062	2.61	3.20	3.99
6	y = 1.0493 x − 0.203	2.52	3.15	3.99

[1]Data were obtained on 15 milk samples ranging in protein from 2.55 to 4.12%. In the above equations, x is the overall mean and y is the laboratory test value.

is much poorer; i.e., 0.10% at 2.6% protein, and 0.18% at 4.0% protein.

The above data are in relatively good agreement with those reported by others (*1,3,*). In particular, Sherbon (*3*) found a standard deviation of error of 0.0279 and a standard deviation for laboratories of 0.0721 for the Pro-Milk device. Thus, repeatability of the unit may be considered relatively good. It is emphasized, though, that data from the present study, showing a repeatability of 0.053% protein, represent the equipment potential under routine laboratory operations. This value is somewhat higher than the true repeatability, which is usually considered to be 0.03%. To approach or achieve this latter precision, the following details should be considered: (*a*) sampling error should be minimized, possibly by using very accurate syringe samplers of the Cornwall type, (*b*) technician mis-readings must be avoided (several such errors were noted in this study) and (*c*) liquid flow should be checked frequently because partial blockage of the circuit causes slow drifting of the reader needle. Also, to assure good repeatability from day to day, a milk control sample is essential. Such samples can be stored for a 2-week period, provided that they are of good bacteriological quality, split into several sub-samples, preserved with potassium dichromate or mercuric chloride and kept refrigerated (4 C). A new sub-sample would be used to check the equipment each time it is put in use.

Other factors affect reproducibility; that is, agreement with the reference standard. Regular calibration against the Kjeldahl method is necessary because dye purity varies from lot to lot, and also because the relation between Pro-Milk and Kjeldahl (N × 6.38) may be influenced by seasonal variation in the proportion of non-protein nitrogen (NPN). Therefore, any variation in Kjeldahl determinations will be reflected in the calibration of the instrument.

To overcome this problem, centralization of laboratory calibration becomes necessary. Some countries centralize the calibration directly and/or check laboratory results routinely to monitor calibration. A simple method developed in France by Grappin and Jeunet (*2*), uses two standard reconstituted milk samples, one at high and one at low protein level. These samples, checked in the central laboratory, are circulated to other laboratories and used to gauge present calibration. The reference instrument of the central laboratory is itself calibrated against the Kjeldahl results over a wide range of milk samples. Unpublished data indicate the potential to reduce differences between laboratories to within 0.05% protein. The present study strongly indicates this potential. Instruments used by three of the laboratories (2, 3 and 4) were in fact calibrated against a central laboratory. At 3.2% protein level, data are in very good agreement (see Table 3). A program involving regular distribution of milk samples at different protein levels between cooperating laboratories would be expected, therefore, to significantly reduce between-laboratory error.

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Sensory Aspects of UHT Milk Combined with Whole Pasteurized Milk[1]

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ABSTRACT

The objective of this study was to evaluate the sensory quality of mixtures of whole pasteurized milk (WPM) containing from 0 to 75% ultrahigh-temperature (UHT) processed milk indirectly heated. Thirty experienced panelists were used to make multiple comparison tests between samples. From 75 to 87.5% of the panelists were able to distinguish differences between samples of UHT milk and WPM in multiple comparison tests. About 86% of the consumer panelists were neutral toward or liked WPM, whereas only 56% of the consumer panelists were neutral toward or liked mixtures containing 75% UHT milk. These studies show that people can accurately identify UHT milk and that they prefer WPM to indirectly heated UHT milk.

The effects of many factors on the sensory quality of sterile ultrahigh-temperature (UHT) milk have been studied. A number of these factors have a negative effect on the flavor of sterile UHT milk. Flückiger (*4*) demonstrated the negative effects of oxygen and light on UHT milk flavor. Increased storage time (*8*) and storage temperature (*6*) may also have deleterious effects on UHT milk quality. Heat resistant proteases produced by psychrotrophic bacteria in raw milk may survive UHT processing and cause development of bitter flavor and gelation in sterile milk (*16*). The method of UHT sterilization (direct vs. indirect) may also influence milk quality; Rossikhina et al. (*11*) observed that the effects of the direct method (steam injection) were less marked than those of the indirect method (milk heated by contact with hot metal surface). Also, UHT milk of reduced fat content shows less sensory change during storage (*12*).

Although many factors have been shown to impact on the sensory quality of UHT milk, little work has been done to quantitate the ability of people to discriminate between UHT and whole pasteurized milk (WPM), or to evaluate their degree of preference for each. Using triangle tests with 1000 people, Nahra and Facas (*9*) observed that from 41 to 47% of the individuals could correctly identify UHT milk. The marketing division of the Canadian Department of Agriculture has also evaluated the organoleptic quality of UHT milk (*1*); it was reported that 40% of the persons questioned do not perceive any difference between UHT and WPM, and that 25% noticed a very slight difference. Fram and Westhoff (*5*) conducted triangle tests to determine differences between sterile UHT and conventional pasteurized milk. Preliminary results of their study show that less than half of the respondents could tell the difference between sterilized and pasteurized milk.

The objective of the work reported here was to determine both the ability to detect differences between UHT and WPM, as well as to determine consumer preferences for UHT milk in relation to WPM. Mixtures of different ratios of UHT and WPM were evaluated to strengthen the tests for differences between the two types of milk, as well as to determine the feasibility of blending these milks for masking any off-flavors associated with UHT milk.

MATERIALS AND METHODS

Samples of UHT whole milk were obtained from two commercial firms; one U.S. and one Canadian supplier. UHT milk from each source had been sterilized by indirect heating. Milk from Source A had been heated to 282 F for 9-13 sec, whereas that from Source B had been heated to 293 F for 2 sec. Until the time of testing, all samples were refrigerated to maintain freshness. Sensory evaluations were completed within 3 months after UHT processing. For comparative work, freshly pasteurized milk was obtained from a local dairy.

A multiple comparison test (Fig. 1) was used for each brand of UHT milk to see if panelists could discriminate between whole pasteurized

MULTIPLE COMPARISON ANALYSIS

Name _____ Date _____

QUESTIONNAIRE:

You are receiving samples of _____to compare for _____ You have been given a reference sample, marked R, to which you are to compare each sample. Test each sample; show whether or not it is different than the reference for the characteristic being evaluated. Then mark the amount of difference that exists.

Sample Number	____	____	____	____
Identical to R	____	____	____	____
Different than R	____	____	____	____
DEGREE OF DIFFERENCE FROM R, IF ANY				
Slight	____	____	____	____
Moderate	____	____	____	____
Much	____	____	____	____
Extreme	____	____	____	____

COMMENTS: Any comments you may have about the samples may be made here:

Figure 1. *Questionnaire used for analysis of consumer discrimination between 0%, 25%, 50% and 75% ratios for UHT milk with whole pasteurized milk.*

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and UHT milk. In the first multiple comparison test, panelists evaluated milk samples containing 0, 25, 50 and 75% UHT milk from Source A mixed with fresh pasteurized whole milk. They compared samples to a whole pasteurized milk reference, "R". In the second multiple comparison test, panelists did likewise, but the samples contained UHT milk from Source B. To facilitate data analysis, the values 1 through 4 were assigned to the degree of difference terms, slight, moderate, much and extreme, respectively.

Samples were contained in 1-oz. plastic portion cups and randomly coded with three digital numbers. Panelists were served in individual panel booths. The number of participants for each test was 30. Samples were measured, mixed and held refrigerated (7 C) in portion cups 1 h before presentation to the panelists. Samples were removed from 7-C refrigeration and stood at room temperature approximately 15 min before tested by panelists.

A hedonic preference test was given to 58 consumers in the University of Nebraska dairy store. The tests were given in an open booth inside the dairy store. Panelists rated acceptability of seven different milk samples on a nine-point hedonic scale, ranging from "like extremely" to "dislike extremely" (Fig. 2). The seven samples consisted of: WPM mixed with each source of UHT milk in 25, 50 and 75% concentrations and one sample of 100% WPM. Half-ounce portions of each sample were served in 1-oz. plastic, odorless cups at approximately 7 C. Samples within each set were served in random orders. For data analysis, ratings on the nine-point scale were assigned the values of 1 to 9, with "like extremely" assigned the value of 1.

The data were subjected to analysis of variance, and Duncan's Multiple Range Test for differences between treatment means (*13,14*).

HEDONIC RATING
METHOD

Date _____ TASTER _____
PRODUCT _____

Taste test these samples and check how much you like or dislike each one. Use the appropriate scale to show your attitude by checking at the point that best describes your feeling about the sample. Please give a reason for this attitude. Remember, you are the only one who can tell what you like. An honest expression of your personal feeling will help us.

SAMPLE CODE							
Like Extremely							
Like Very Much							
Like Moderately							
Like Slightly							
Neither Like Nor Dislike							
Dislike Slightly							
Dislike Moderately							
Dislike Very Much							
Dislike Extremely							

COMMENTS:

Figure 2. *Questionnaire used to analyze consumer preference for milk samples containing 0%, 25%, 50% and 75% ratios of UHT milk, for both Source A and Source B brands.*

RESULTS

Figure 3 illustrates the results of the multiple comparison test. Panelists' ability to detect differences between both UHT milk sources and the reference increased as the percentage of UHT milk in the sample increased. Between 75 and 87.5% of the panelists could detect differences at the 75% UHT milk level. Half of the panelists had difficulty in matching WPM to an identical reference sample; however, the degree of difference between the samples and the reference increased significantly as the percent UHT milk increased. Analysis of variance indicated that increasing the concentration of each source of UHT milk in the samples from 0 to 75% had a positive linear effect on the degree of difference taste panelists detected between test samples and the reference (Tables 1 and 2).

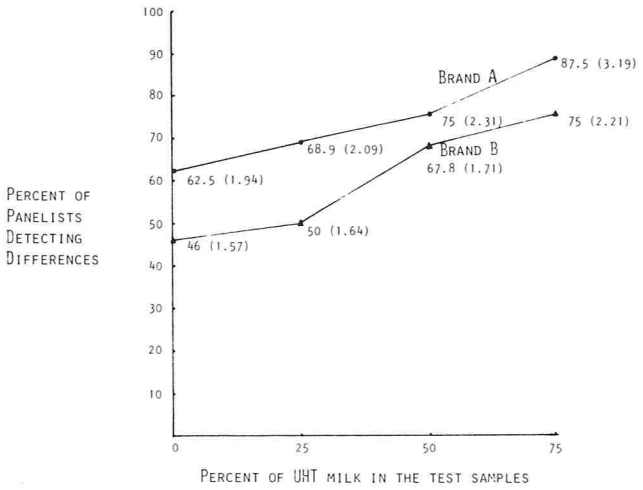

Figure 3. *Effect that increasing the ratio of Brand A (•) or Brand B (△) UHT milks had on taste panelists' ability to detect differences between a whole pasteurized milk reference and the test samples. Degree of difference (maximum = 4; minimum = 0) given in parentheses.*

Varying statements about flavor characteristics were made by panelists who participated in the multiple comparison tests. The detected off-flavors were sometimes described as having an evaporated milk flavor.

TABLE 1. *Analysis of variance of the effect varying the ratio of Source A (UHT) milk to pasteurized whole milk (0%, 25%, 50%, and 75%) had on taste panelists' ability to detect differences between test samples and a pasteurized whole milk reference.*

Source of variation	Degrees of freedom	Sums of squares	Mean square	F Value
Ratio of Source A UHT milk to pasteurized whole milk	3	29.9	9.97	8.93**
Linear	1	25.2	25.2	22.59**
Quadratic	1	4.13	4.13	3.70^{ns}
Cubic	1	0.56	0.56	0.50^{ns}
Error	124	138.34	1.1157	
Total	127	168.24		

**Probability of greater F value < .01.
^{ns}Not significant.

Some claimed the UHT milk was thicker and creamier, while others interpreted the flavor as cooked and chalky.

Results from the consumer preference test (Table 3) showed that as the percentage of UHT milk in each sample increased, the percentage of panelists disliking the sample also increased. Duncan's multiple range test was used to evaluate the differences between the mean scores of the samples in the preference test (Table 4). Addition of 25% UHT milk did not significantly alter flavor. Two-thirds of the panelists liked, to some degree, samples containing 50% UHT milk. However, as the percentage of UHT milk in the samples increased above this level, the average preference score decreased significantly.

TABLE 3. *Effect of varying the ratio of two types of UHT milk (Source A and Source B) to whole pasteurized milk (0%, 25%, 50% and 75% UHT milk) on the percent of consumer panelists liking or disliking the product.*

Panelist reaction	Treatments			
	Whole pasteurized	25% UHT	50% UHT	75% UHT
% of panelists liking product	72.4	75	67	49.6
% of panelists neutral	13.8	12.9	11.3	6.2
% of panelists disliking product	13.8	12.1	21.7	44.2

TABLE 4. *Effect of varying the ratio of two types of UHT milk (Source A and Source B) to whole pasteurized milk (0%, 25%, 50% and 75% UHT milk) on milk quality when measured on a nine-point scale by consumer type panelists.*

Treatment	Average preference score	Duncan's Multiple Range Test (5 percent level)
Whole pasteurized	3.60	┼
25% Source A	3.62	┼
25% Source B	3.67	┼
50% Source A	4.05	┼
50% Source B	4.19	┼ ┼
75% Source A	4.52	┼ ┼
75% Source B	4.60	┼

DISCUSSION

As with the earlier studies (*1,5,9*), it has been demonstrated that many people can distinguish between UHT and WPM and further that a large percentage of people prefer WPM over UHT milk. These studies by design ask panelists to identify differences and state preferences for samples; therefore, differences and preferences are somewhat prejudiced relative to how milk is normally consumed.

Thirty percent of the fluid milk sold in Europe is in the form of unrefrigerated UHT milk (*7*), indicating that consumer preference for WPM over UHT milk is not great. Factors other than sensory attributes may account for the increased production of UHT milk in Europe; milk processing plants are typically located far from central cities and the cost of energy is higher than in the United States. These considerations apparently outweigh any flavor preferences.

Nahra and Facas (*9*) have stated that for more than 2 years the University of Maryland sold UHT milk in conventional containers as regular milk. During the entire time no negative comments were received. Coupled with the European experience, this observation indicates that the flavor problems of UHT milk are minimal.

Even though marketing information would indicate that UHT milk has an acceptable flavor and is therefore salable, a number of strategies have been proposed to improve UHT milk flavor such as: alterations in packaging (*8*), addition of antioxidants (*17*), treatment with immobilized sulfhydryl oxidase (*15*), addition of L-cystine (*2*), and a pre-UHT heat treatment to inactivate heat resistant proteases (*16*). A procedure for adding a β-galactosidase preparation into UHT milk to hydrolyze the lactose has been described (*10*), with a thought that increasing the sweetness of milk by hydrolyzing the lactose could be used to counter-balance the cooked flavor of UHT milk.

Many people can identify differences between UHT and WPM, and a sizeable proportion of people prefer WPM over UHT. Whether or not these observations can be translated into consumer preference for milk as it is normally consumed seems to be in question; therefore, research efforts to improve UHT milk flavor also appear to be in question.

In the U.S., problems with the flavor quality of sterile UHT milk are of secondary importance to regulatory considerations. The FDA has defined ultra-pasteurized milk as milk that must be refrigerated (*3*). Failure to eliminate the necessity for refrigeration defeats the principal advantage of aseptic UHT milk processing, even though safety and wholesomeness are not apparent problems. Until there is full regulatory acceptance of the UHT process for aseptically packaged fluid milk, the

TABLE 2. *Analysis of variance of the effect varying the ratio of Source B (UHT) milk to pasteurized whole milk (0%, 25%, 50%, and 75%) had on taste panelists' ability to detect differences between test samples and a pasteurized whole milk reference.*

Source of Variation	Degrees of freedom	Sums of squares	Mean square	F Value
Ratio of Source B UHT milk to pasteurized whole milk	3	9.28	3.09	4.41**
Linear	1	8.26	8.26	11.8**
Quadratic	1	0	—	—
Cubic	1	1.03	1.03	1.47**
Error	108	75.43	.7	
Total	111	84.71		

**Probability of greater F value < .01.

findings of research efforts aimed at improving the quality of UHT milk cannot be fully utilized.

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Methods to Detect Abnormal Milk — A Review

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ABSTRACT

Screening and confirmatory methods for detecting abnormal milk, mastitic milk, or milk of high somatic cell count are reviewed. Those procedures reviewed in some detail include the Catalase Test, Brabant Mastitis Reaction, pH and chlorine analysis, Ruakura Rolling Ball Viscometer method, California Mastitis Test (CMT), Wisconsin Mastitis Test (WMT), Optical Somatic Cell Count (OSCC), Direct Microscopic Somatic Cell Count (DMSCC), and Electronic Somatic Cell count (ESCC). Other detection methods are tabulated.

Many methods have been developed to screen dairy herds for subclinical and clinical mastitis. A number of confirmatory tests are also available. Table 1 gives most of the latest abnormal milk detection methods. Giesecki and Van den Heever (*21*) have extensively reviewed the literature on methods used to detect subclinical mastitis. A few of these methods have been further refined relatively recently. Somatic cell counts are used by regulatory agencies as a criterion for ascertaining abnormal milk. If the cell count exceeds a given number, action is taken to alleviate the problem. A discussion of some of the more recently developed tests follows:

TABLE 1. *Abnormal milk detection methods.*

Method	Reference(s)
Albumin/globulin ratio	*60*
Behavior of osmotically active substances	*54*
Blood-serum albumin in whey	*48*
Brabant mastitis test (BMR or BMT)	*39,68,100*
California mastitis test (CMT)	*2,19,25,55,56,64,68,74,78, 98,99*
Casein number	*82,85*
Catalase (or Roundy) mastitis test	*18,64,98,99*
Chloride ion	*30,61*
Chloride-lactose number	*44,45*
Coulter Counter (Electronic somatic cell count-ESCC)	*13,17,23,24,26,29,31,37,42,* 43,52,63,70,71,75,77,86,91, *97,99*
Dimastin test	*8,9,47*
Direct microscopic somatic cell count-DMSCC	*4,17,22,44,52,53,54,64,65, 76,84,91*
Electrical conductivity	*14,49,50*
Electronic counters	*92*
Flow-through cytophotometer	*94*
Fossomatic (fluorescense-optical technique)	*16,29*
Freezing point depression	*81*
Lactose content	*79*
Mastirapid mastitis test	*41,44*
Membrane-filter DNA	*3,5,33*
Milk quality gauge (MQG)	*10*
Modified whiteside test (MWT)	*98,99*
Negretti's test and Disk flotation technique	*18*
NK mastitis test	*34,35,36,40*
Optical somatic cell count (OSCC)	*44.83,89,90,96,100*
pH	*100*
Prescott-Breed cell count (smear method)	*41,73*
Ruakura rolling ball viscometer	*17,57,58*
Rennetability of milk	*79*
"Sofia" mastitis test	*62*
Use of difference in lactose content and refractometer number	*87*
Wisconsin mastitis test (WMT)	*1,10,22,32,46,59,66,69, 80,91,93,98,99*

CATALASE TEST

This test is a laboratory screening test used to detect abnormal milk (*98,99*). The test assays the amount of catalase present and this is an indirect measure of the somatic cell count of raw milk. Somatic cells contain a relatively large amount of catalase. In this test, hydrogen peroxide is converted to water and oxygen by the action of catalase. Fermentation tubes have been used to measure the amount of oxygen liberated during the reaction by displacement of the milk as the gas accumulates. Catalase content of normal milk is low, except at the beginning and end of lactation.

For this test to be of practical value, the results must be related to a reference standard. The standard most frequently used is the Direct Microscopic Somatic Cell Count (DMSCC). Since factors other than somatic cells may also affect the Catalase Test, interpretation of the results can be difficult. Various amounts of free catalase and/or bacterial catalase may occur in milk even though the detectable somatic cell count is low. There is no direct relationship between results of the Catalase and DMSCC tests; however, a general workable relationship has been established. Normal milk will generally generate less than 10% oxygen. Twenty percent or more oxygen suggests the presence of 500,000 or more somatic cells/ml.

BRABANT MASTITIS REACTION TEST (BMR)

Jaartsveld (*38*) determined that the capillary flow rate of a mixture of 0.6 ml of milk and 0.4 ml of Teepol No. 414 (10%) or sodium lauryl sulfate (3%) can be related to the somatic cell content of milk. The test is based on the reaction of a detergent and DNA in cell nuclei with the production of a viscous mass. The viscosity is determined by measuring the flow rate from the capillary tube. The BMR was first designated the California Mastitis Tube

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Test (CMTT) and was later called the Brabant Mastitis Reaction Test (abbreviated BMR or BMT).

Roughley et al. (*80*) modified this method and reported the results in terms of a milk gel index. The correlation coefficient (r) between the BMT and the Breed smear method (*73*) was 0.851 (*7*). Dijkman et al. (*15*) found that the BMT was useful for screening purposes but was of questionable use for grading farm milk, mainly because of poor accuracy and reproducibility in the 500,000 to 1 million somatic cell count range. The test also correlated poorly with the ESCC and DMSCC methods.

pH TEST

The pH of normal milk is 6.6 to 6.7. Mastitic milk may have a pH 6.8 or above (*6*). Methods of testing pH of milk employ the use of Bromthymol Blue (BTB) or Bromcresol Purple (BCP) as indicators in solution or on pH paper. When using BTB, abnormal milk causes a color change from grass green to blue-green; with the BCP, the change is from light to deeper purple. These tests provide only limited information and their results correlate poorly with those of the California Mastitis Test (CMT) and Catalase Test (*6,100*).

CHLORINE TEST

Milk-secreting cells are unable to prevent serum chloride from entering milk during a mastitis infection. Since sodium chloride is osmotically active, a compensating decrease in lactose content of milk occurs. For this reason, the chlorine content or the chlorine/lactose ratio can be used as indicators of abnormality. Chlorine content of an acidified milk mixture, at pH 2.0, can be measured by titrating silver nitrate to a potential of + 250 mv as determined by use of a pure silver electrode and pH meter (*30*). Each ml of the silver nitrate solution corresponds to 0.0100% of chlorine in a 10-ml milk sample. Chlorine content of over 0.14% indicates mastitic milk, and 0.12% is considered normal (*6*). This test, like the pH test, gives only limited information in detecting subclinical mastitis.

RUAKURA ROLLING BALL VISCOMETER

The Raukura rolling ball viscometer (*17,58*) can be used to measure the viscosity of the milk-reagent mixture resulting from use of the California Mastitis Test reagent. The viscosity of the milk-reagent mixture is inversely related to the distance of travel of a stainless steel ball down an inclined tube during a pre-determined time lapse. The correlation coefficient between the inverse of the viscometer reading and the DMSCC is 0.92. In a scale range of 1-10, the viscometer has a sensitivity in predicting somatic cell counts at levels of 150,000 to more than 1 million cells per ml. Duirs and Cox (*17*) have shown that the RBV has a correlation of 0.84 with the DMSCC and the Electronic Somatic Cell Count (ESCC- Coulter Counter) methods. They have concluded that with adequate operator training, to minimize variation in testing techniques, the RBV provides a satisfactory and simple alternative to the DMSCC and ESCC methods when used on "fresh" milk samples.

The RBV has grown in popularity in New Zealand, the country in which it was developed. The RBV technique is not as yet widely used elsewhere, although considerable interest is being shown in its potential (*58*).

CALIFORNIA OR RAPID MASTITIS TEST (CMT OR RMT)

The CMT (*19,25,55,56,74,78*) was derived from the Modified Whiteside Test (MWT) and is an efficient screening test for mastitis and abnormal milk. It is simple to do and can be used in the barn as a cow-side test. The CMT reagent is a neutral detergent (alkyl arylsulfonate) which makes it possible to add a pH indicator (bromcresol purple) to evaluate the alkalinity of milk in addition to estimating cell counts (*27*). The reaction of detergent and DNA in cell nuclei produces a viscous mass. Viscosity, as with the RBV, relates to the total somatic cell concentration.

The CMT test is conducted using a white plastic paddle with four shallow cups in which milk is collected from each of the four quarters. After adding an equal amount of reagent to each cup, the paddle is rotated to mix the milk and reagent thoroughly. The mixture is scored by using seven different symbols: — (no reaction), T (trace reaction), 1,2,3 +, and y. Generally a score of 1 is considered as indicative of presence of 500,000 or more somatic cells per ml, a score of 2 as 1 million or more cells per ml, and a 3 score of over 5 million cells per ml. Symbol + shows that the pH of the milk is 7.0 or over, while the symbol y (yellow) means the mixture is acidic (*20,100*). The + or y symbol is added to the score whenever the reaction is alkaline or acidic.

Milk collected for later CMT testing should be refrigerated, (but not frozen (*76*) to prevent bacterial growth, and should be tested within 24 to 36 h. Boric acid (1.5%) may be used as a bacteriostatic agent. Since other preservatives, such as potassium dichromate, formalin and mercuric chloride alter the DNA, no CMT reaction occurs when they are used (*100*).

As a subjective procedure, CMT is more difficult to standardize between analysts and laboratories than methods such as the WMT, ESCC and DMSCC. Read et al. (*78*), in an evaluation of five screening tests (CMT, Catlase, Milk Quality Test, MWT, and WMT) and two confirmatory test (ESCC and DMSCC), noted that all screening tests studied showed considerable variability. These researchers suggested that screening tests could be used to indicate whether or not a bulk milk might have an unsatisfactory somatic cell count. A confirmatory test should be made to determine whether the sample in question exceeded the somatic cell standard of 1.5 million somatic cells per ml.

WISCONSIN MASTITIS TEST (WMT)

The WMT uses the CMT reagent diluted 1:1 with distilled water. It is based on an increase in milk-reagent

viscosity. The viscosity is determined by measuring the amount of the mixture remaining in a special test tube after a 15-sec outflow through an orifice in the cap. The measuring gauge, placed next to the test tube, is calibrated to determine the height in mm of the remaining mass or in somatic cell concentration per ml. The WMT score correlates well with the DMSCC method. Workers have found a 0.89 (*78*), and 0.85 (*91*) correlation coefficient between these two methods and a value of 0.88 between the WMT and the ESCC (Coulter Counter) (*91*). The dimensions of the WMT test tubes are 12.5 × 125 mm, with matching caps having an orifice of 1.15 mm. and an orifice in the side of the test tube at 65 mm from the outside bottom (*93,100*). Advantages of this test are as follows: (*a*) WMT permits examination of a large number of samples per unit of time — there is no delay for results and retests may be made rapidly, (*b*) the test uses a readily available, inexpensive, stable and noncorrosive reagent, (*c*) the test can be used to estimate cell content of bulk, bucket or quarter milk samples, and (*d*) the test uses inexpensive and simple equipment.

Disadvantages of this test (*98*) are as follows: (*a*) the WMT does not lend itself to cow-side use as readily as the paddle type tests, (*b*) the test has no permanent reaction that can be examined or re-examined at leisure, as the dried or stained smears for the DMSCC, and (*c*) milk must be refrigerated promptly and preferably tested on the day of collection. The reaction diminishes slowly on storage at 32 to 40 F and milk scores appear lower than they actually are (*32*).

A WMT value of 11 mm or over suggests that the milk has more than 500,000 somatic cells/ml. A WMT value of 20 or over suggests that the milk has greater than 1.5 million somatic cells/ml. In the U.S., this level (*66,67,98*), if confirmed by DMSCC, is considered an actionable level. Ginn (*22*), in a commercial laboratory, confirms the highest 10% of the WMT scores greater than 21 mm. Of 22,553 such tests, 9.95% or 2,243 were "confirmed" between May 1 and December 31, 1971. Only 0.68% had a DMSCC of greater than 1.5 million per ml of milk.

Table 2, adapted from reference (*98*), shows the "significance" of somatic cell counts over a range of counts. Among the tests mentioned, the WMT is the most objective.

TABLE 2. *Significance of somatic cell counts of milk over a range of counts.*[a]

Somatic cell count/ml	Significance
0 - 250,000	No pathogenic bacteria present, no mastitis. Negative reactions by MWT[b] and CMT tests. WMT readings of 5 or less. Less than 20% oxygen for Catalase Test.
250,000 - 500,000	Considered normal milk (no pathogenic bacteria) May show trace (T) reaction by CMT. (A precipitate begins to form, thickening into a gel as concentration of cells increases.) Catalase — less than 20% oxygen MWT — (trace reaction) WMT — (5-13 mm)
500,000 - 1,000,000	Some mastitis or other abnormality is present. CMT — weak positive (*1*) (a distinct precipitate forms, but no gel, and may be reversible, disappearing upon continued movement of the paddle). Catalase — (20-30% oxygen) MWT — trace reaction; about 40% of the tests may still show negative. WMT — (11-19 mm)
1.0 - 1.5 million	Milk is abnormal, either from mastitis or for other reasons. CMT — [weak positive (*1*)] Catalase — (30-40% oxygen for counts to 2 million) MWT — (83% of tests show 1 + reaction) WMT — (17-22 mm)
1.5 - 2.0 million and higher	Milk is abnormal CMT — positive (1 to 2). Mixture thickens immediately, with some suggestion of gel formation. Upon swirling, mixture tends to move toward the center, leaving bottom of outer cup edge exposed. Catalase — (40% and over) over 2 million. MWT — (1 + to 2 +) WMT — (20-25 mm)

[a]Adapted from reference (*98*).
[b]Key: (MWT): Modified Whiteside Test, (WMT): Wisconsin Mastitis Test, (CMT): California Mastitis Test, Catalase Test.

OPTICAL SOMATIC CELL COUNT (OSCC)

A semi-automatic cell counting system, developed commercially by Technicon Instruments Corporation (Terrytown, NY) (*89,90*) involved continuous flow analysis. This system, the Technicon Optical Somatic Cell Counter II (OSCC II), has the precision of the DMSCC, a coefficient of variation of 3%, correlation coefficient of 0.96 with the DMSCC, and ability to evaluate 120 samples per hour (*90*).

The OSCC II measures light scattering that occurs as somatic cells pass through a light beam of an electronic microoptical system. Scattered light pulses are transformed into electrical pulses by a photomultiplier tube. The number of somatic cells corresponds to the electrical pulses. All larger entities, especially fat globules, have to be dissolved before counting. Before analyzing, the samples must be pretreated with a formalin fixative at a rate of 0.05 ml per 4 ml of milk sample. Fixation is carried out at room temperature (20-23 C) for 18 h, or 55 C for 45 min (*90,96,100,101*).

Wang and Richardson (*101*) simplified sample preparation for the OSCC II by using a Fisher Auto Diluter Model 250 (Fisher Scientific Company, Pittsburgh, PA). This improvement made the OSCC II test simpler and more time-saving, with no loss in accuracy. These workers established the workability of the OSCC II system in a central milk testing facility involved in the routine testing of Dairy Herd Improvement (DHI) milk samples.

The A/S N. Foss Electric firm (Hillerød, Denmark), has developed the "Fossomatic" (*28*) test, which determines the number of somatic cells in milk by automated and continuously operating fluorescence microscopy. The correlation with the DMSCC is high (*16,29*). The Fossomatic principle involves the formation of a fluorescent complex from a dye bound between DNA

of the cell nucleus and ethidium bromide, which emits a strong fluorescence when excited with light from a xenon lamp within a certain wavelength range. Other particles may also fluoresce, but at a different wavelength and intensity. Thus their influence can be optically filtered out.

After heating to 40 C, the milk samples are inserted into racks which transfer the samples to the stirrer and subsequent pipetting position. A sample of 200 μl is taken and transferred to the turret where it is diluted 1:20 with 1.8 ml of dye solution and 2.0 ml of buffer. After extensive stirring at 60 C, a sample of 20 μl is applied to a rotating disc by a microsyringe, providing a liquid layer of 0.5 mm width and 10 μm thickness. The available sample volume represents a liquid layer approximately 3500 mm long which is exposed to the optical system.

Blue light of a wavelength up to 580 nm is directed toward the rotating disc, exciting the cell complex to emit fluorescent light with a wavelength from 590 nm and up. An optical system transfers the light pulses to a rectangular slit where a photomultiplier tube transforms the light into electronic signal. This signal is fed to a digital display, an oscilloscope and a printer. The result multiplied by 1000 gives the cell content per ml (*28*).

DIRECT MICROSCOPIC SOMATIC CELL COUNT (DMSCC)

Prescott and Breed (*73*), developers of the first microscopic procedure for examination of milk films, identified two procedures: (*a*) the Direct Microscopic Count (DMC) and (*b*) the Direct Microscopic Leucocyte Count (DMLC) to enumerate bacterial cells and leucocytes, respectively. Work completed by many investigators has indicated that the term "somatic cells" is more indicative of those body cells in milk generally associated with inflammation of the cow's udder than the more specific term "leucocyte." Accordingly, the Direct Microscopic Somatic Cell Count (DMSCC) (*99*) discussed below includes body-cell-counting procedures developed by Prescott and Breed (*73*), Levowitz (*99*), and Brazis et al. (*4*).

In the DMSCC, the microscope is first adjusted and calibrated so that the microscopic factor (MF) may be determined (*4,54*). A film containing 0.01 ml of milk is spread over a 1-cm^2 area on a glass microscope slide and then fixed and stained with a modified methylene blue reagent. The total cell count is determined by averaging the cell number in 10-60 fields, depending upon the number per field, and then multiplying this average by the MF.

The DMSCC is usually considered the reference standard for other methods for detecting abnormal milk. As a confirmatory test, it is relatively rapid, permitting a microscopic determination of somatic cells in 10 to 15 min. Milk films can be prepared quickly, stained, examined later and kept for re-examination. Tentative identification of bacterial flora may be made during examination of the milk film, although bacteria responsible for mastitis are not usually observed in fresh, unincubated milk.

The DMSCC has several disadvantages (*99*) which limit its usefulness. Measuring the small quantity (0.01 ml) of milk is difficult. Foam may cause inaccurate measurement by both glass pipettes or metal syringes (syringe may also add metal filings). Correct preparation of stain or proper staining of slides are necessary. If the stain is not prepared properly, the film will erode during the rinsing stage. If the film is not properly dried, the film will crack. Enough time must be allowed for correct staining. The small amount of milk examined in this test tends to limit its precision because of uneven spreading of milk over the 1-cm^2 area of slide; high or low counts may result. Failure to count the required number of fields, when a specified number of individual fields are recommended for the field counting procedure, also reduces precision. Use of a double tally assists in enumeration of fields counted as well as the number of somatic cells in each field. Distinguishing among nucleated and non-nucleated body cells, dirt and/or other artifacts can be quite difficult. Too little or too much light, along with inexperience with the method, can cause problems in the recognition of nucleated body cells generally. Error may occur in the arithmetical calculation of counts. Fatigue, a common problem to laboratory technicians reading many slides consecutively, reduces precision. Initial cost of equipment is relatively high in comparison to that of the CMT, MWT, and Catalase tests, but is less than that for OSCC and ESCC instruments.

A comparison (*78*) of the abnormal milk confirmatory procedures showed the Electronic Somatic Cell (ESCC) to be more precise, with approximately one-fourth the replicate log standard deviation of the DMSCC (0.012 vs. 0.047). Thompson et al. (*91*) showed that the coefficients of variation computed at the DMSCC count near one million were 15.6% (DMSCC), 6.3% (WMT) and 4.2% (ESCC).

The DMSCC, used as a confirmatory method, in conjunction with WMT as a screening test, proved to be quite successful in a dairy quality control laboratory (*22*). Modifications of the reticle-strip counting method of the DMSCC (*4*) were made at this laboratory, thereby reducing the total time of making films and reading to an average of 9.6 min per milk sample (*22*). The modified method was accurate and was easily understood by the technicians.

Like other abnormal milk detection methods (WMT, Catalase, and ESCC), milk samples for DMSCC should not be frozen before analysis. Freezing significantly reduces test scores (*76*). Holding samples in a refrigerator at 4 C for up to 4 days has no significant effect on the DMSCC results (*72*). Some workers feel that rapid testing of milk samples, a necessary requirement for other detection methods (MQG, WMT, CMT, ESCC), should be broadened to include all tests, including DMSCC, as a general rule (*72,99*) for greatest precision and accuracy.

ELECTRONIC SOMATIC CELL COUNT (ESCC)

Except for the DMSCC and the OSCC, tests described thus far do not provide particularly accurate results;

most are subjective, and all of them take considerable time. The DMSCC is somewhat more accurate than other methods, but it is a fatiguing test and is not suitable for dairy herd improvement testing (*100*).

Application of an Electronic Somatic Cell Counter (ESCC), namely the Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) for estimating somatic cells in milk has been studied. There are many models of Coulter Counters available, each with specific operating and sample preparation requirements. Researchers have found success with the Coulter Counter Models Z_B, Z_{BI}, Z_F, FN or equivalent.

The Coulter Counter is based upon the "Coulter Principle" (*11*). As particles or cells pass through an aperture and displace an equal volume of electrolyte, the resistance in the path of current changes. This results in corresponding current and voltage changes. The quantity (magnitude) of this change is directly proportional to the volumetric size of the particle or cell. The number of changes within a specific length of time is proportional to the number of particles or cells within the suspension.

During development of the method using the Coulter Electronic Counter, the major problems encountered were interference of fat globules and ascertaining the lower threshold setting. The fat globules, in larger numbers and in overlapping distribution, caused counting errors (*71*). Phipps (*70*) observed that incomplete fat dispersal following chemical treatment could produce higher counts in the region of low cell concentrations. However, elimination of fat globules as interfering compounds was achieved by a centrifugation technique (*71*) or by dispersal by chemical treatment (*95*). The chemical method requires that the milk be treated beforehand with formalin, which renders the cells resistant to a fat-dissolving reagent mixture. Phipps (*70*) reported that a minimum time for formalin treatment was 24 h and maximum time was about 4 days at room temperature. Most laboratories found this treatment too time consuming and thus have opted to use the compound, Somafix (Coulter Electronics, Inc., Hialeah, FL) (*52*). Three drops of this formalin-based reagent will rapidly "fix" a 10-ml raw milk sample when the mixture is incubated at 60 C for 6.5 min (1.5-min warm-up time + 5-min reaction time). When DMSCC was correlated with a chemical method and with a centrifugal method of preparing samples for Coulter Counter testing, Pearson et al. (*68*) obtained correlation coefficients of 0.966 and 0.930, respectively.

Studies on the instrument setting referred to as the "lower counting threshold" were of major importance in correlating the ESCC to the DMSCC. Tolle et al. (*94*) suggested an optimum threshold of 4.5-5.0 μm in the calculation of the lower threshold setting. Dijkman et al. (*15*) found a value of 5 μm (65.5 μm^3 - average somatic cell particle size) to agree well when the ESCC counts were compared to the DMSCC. Macaulay et al. (*52*) found that the 4.43 μm value was useful in giving an ESCC to DMSCC correlation coefficient of 0.973. A procedure published in the 14th edition of the *Standard Methods for the Examination of Dairy Products* (*97*), calls for a value of 4.4 μm (44.6 μm^3). Kinsman (*42,43*) also found that this value was useful in accurate Coulter Counter standardization.

The Coulter Counter method has many advantages (*97*). Analysis time per milk sample is minimal. After fixing, samples can be stored. The ESCC device yields cell-size distribution as well as count data.

The Coulter Counter method has few disadvantages (*97*). Instrument cost is high, but may be offset by rapid amortization. Good laboratory cleanliness techniques are required for the instrument and the glassware. Instrument calibration and standardization are needed.

Coulter Counters are semi-automatic because they require further preparation steps beyond cell fixation. Coulter Electronics has recently developed an automatic unit named the "Milk Cell Counter" (*12*). This unit requires that the samples be "fixed" before entry into the system. It tests a maximum of 210 milk samples per h, loaded up to 50 at a time in a sample rack. Essentially the same procedure (*37,97*) is used as with the semi-automatic models. The somatic cell count is printed on a teleprinter and can also be interfaced with a computer. Modified versions of the original units are being used in England and Scotland to do routine cell counts. A unit is currently undergoing tests at the Dairy Quality Control Institute (St. Paul, MN).

Table 3, adapted from Northern (*64*), is a comparison of six common abnormal milk detection methods. The

TABLE 3. *Comparison of abnormal milk screening tests.*[a]

Test parameter	Relationship between tests in terms of test parameter[b]
Accuracy	ESCC[c] = DMSCC > WMT > CMT > CATALASE > WHITESIDE
Simplicity	CMT > WHITESIDE > WMT > CATALASE > DMSCC > ESCC
Equipment cost	ESCC + DMSCC > CATALASE > WMT > ESCC > DMSCC > CMT > WHITESIDE
Repeatability	ESCC > DMSCC > WMT > CATALASE > CMT > WHITESIDE
Objectivity	ESCC > DMSCC > WMT > CATALASE > CMT = WHITESIDE
Total labor required	CATALASE > DMSCC > ESCC > WHITESIDE > WMT > CMT
Specialized training	ESCC > DMSCC > CATALASE > WMT > WHITESIDE > CMT
Time to run test	DMSCC > CATALASE > ESCC > WMT > WHITESIDE = CMT

[a]Adapted from Northern (*64*).
[b]Assume fifty samples are tested.
[c]Key: DMSCC - Direct microscopic somatic cell count, ESCC - Electronic somatic cell count (Coulter Counter), WMT - Wisconsin mastitis test, CMT - California mastitis test, CATALASE - Catalase test, WHITESIDE - Whiteside test.

ESCC, though more costly than the other methods presented, is preferred in terms of repeatability of the test results and objectivity of the test method. Fossomatic and other methods not compared in this table would likewise be more objective and repeatable than screening-type procedures shown.

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Preparation of Lactobacilli for Dietary Uses

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ABSTRACT

Increased use of lactobacilli as dietary supplements has emphasized the need for preparations of those cultures that possess the attributes of cell viability, resistance to bile and capability of establishing in the intestinal tract. Incompatibility of different species of lactobacilli may limit development of products designed to overcome objectionable, or impart improved flavor to cultured foods. Stresses that are imposed on cultures during their preparation and distribution must minimize damage to cells which would detract from the foregoing characteristics.

Preparation of *Lactobacillus acidophilus* cultures as dietary adjuncts originated with research conducted about the turn of this century. Following publication of Metchnikoff's theory on the role of lactobacilli in prolongation of life (*14*), research studies on this subject accelerated greatly.

Some of the most outstanding earlier studies describing the attributes of *L. acidophilus* were reported by Kopeloff (*11*) and by Rettger et al. (*20*). The studies described the use of this microorganism in humans needing therapy for various types of intestinal illnesses. The successes obtained presented evidence on the benefits of consuming *L. acidophilus*, particularly by persons encountering intestinal maladies.

In retrospect, it is not surprising that the enthusiasm which developed for the potential values of consuming *L. acidophilus* exceeded the understanding that was necessary for preparation of the culture in reliable forms for establishment in the intestinal tract. Products often contained too few viable cells, and insufficient attention was given to culture identity or its ability to withstand the intestinal environment. Such factors could have contributed to the lack of consistency in results reported by different workers on the effects of feeding acidophilus milk. A subsequent apathy developed in the medical profession about the value of any product containing *L. acidophilus*, and demands for it from consumers became minimal.

RENEWED INTEREST

After World War II, interest was renewed in the intestinal microflora and its effect on health. This has been evidenced by the greatly increased number of research studies reported on the subject in recent years (*1,22,24*). These studies have covered wide ranges of subjects (*2,3,8*) and efforts have been made to understand the normal intestinal microflora of humans from infancy (*13*) through senility (*19*). Lactobacilli appear to constitute a large portion of the microflora present in the small intestine (*16*). Although their numbers are large in the colon, the numbers of other bacteria, particularly the anaerobes, are so high that the lactobacilli constitute a smaller percentage of the total (*15*). It is believed by many, however, that the metabolic functions of the lactobacilli may cause them to be more important than others that are present in much larger numbers.

The microflora of the intestinal tract appears to develop in a random manner in the newborn infant. In breast-fed infants bifidobacteria quickly gain dominance (*13*). Thereafter, many factors affect the flora that develops after weaning. When physiological functions are satisfactory, it can be assumed that the microflora has developed in a manner whereby it and the host benefit mutually. However, it is known that many factors adversely affect the balance existing among the microbial types: antibiotics, infections, diet and possibly others not recognized currently. The lactobacilli appear to be particularly vulnerable to such factors and their absence has been associated with different intestinal maladies. There is increasing evidence to suspect that maintenance of the lactobacilli in desirable numbers in the intestine can be accomplished by fortifying or supplementing the diet with appropriate species.

ACIDOPHILUS MILK

Acidophilus milk was the first dietary product developed containing large numbers of *L. acidophilus*. It was natural that milk should be used as a carrier for the microorganism because soured or cultured milk had been an important component in the diet of many people. High populations of *L. acidophilus* could be obtained by growing the cells in milk and their viability maintained over a reasonable period. This product, however, never gained popularity because flavors developed in its manufacture caused it to be unappetizing. As a result, Acidophilus milk was used primarily as a therapeutic agent. The extensive care needed in producing Acidophilus milk and restricted demand for it caused high manufacturing costs. Many factors made it an unattractive product for the dairy industry to market.

OTHER PRODUCTS CONTAINING *LACTOBACILLUS ACIDOPHILUS*

Other types of foods, such as confectionery products, sherbet and jellies, were used as carriers for the

microorganism to overcome objections to the flavor of Acidophilus milk. These, however, did not prove to be successful, primarily because of the viability of the *L. acidophilus* was not satisfactorily maintained. Other milk products have been developed, especially in Europe, in an effort to offer *L. acidophilus* to consumers in more appetizing forms. A culture for the manufacture of bioghurt has been marketed in Germany, and contains *L. bulgaricus* and *Streptococcus thermophilus* in addition to *L. acidophilus*; after a few transfers only *L. bulgaricus* and *S. thermophilus* are present in any significant numbers. Another culture used for this product apparently contains *L. acidophilus* and *Streptococcus lactis*. No data have been available to assess the ability of *L. acidophilus* to remain viable in any significant numbers in products made with these cultures. Biogarde is a product in Germany which contains *L. acidophilus* and bifidobacteria among others, such as *S. thermophilus*. The cultures apparently are propagated separately, but in the final product only 1% of the flora is *L. acidophilus* and bifidobacteria. There is no evidence that *L. acidophilus* can be cultivated successfully in any significant numbers with *L. bulgaricus*, *S. thermophilus* or *S. lactis*. The latter three species, along with certain others that have been tried, grow much more rapidly, and *L. acidophilus* is reduced to a very insignificant portion of the final population.

Products containing a mixture of milks cultured separately with different starter bacteria and with *L. acidophilus* have been developed in efforts to overcome the difficulty in growing *L. acidophilus* with other milk culturing bacteria. In Switzerland, such a product is Aco-yogurt. In this product, a quantity of milk culture of *L. acidophilus* is added to yogurt before packaging so that the final product contains $4\text{-}6 \times 10^7$ *L. acidophilus* per 200 ml of final product. A similar product, A-38, has been developed in Denmark. Milk which has been cultured with a normal butter starter (buttermilk) is mixed with milk cultured with *L. acidophilus* in a ratio of 9:1; a ratio of 8:2 also can be used without affecting the flavor of the product. It is claimed that the butter starter results in the product having the consistency of a junket and that the product at the time of distribution to the consumer contains about $3\text{-}10 \times 10^6$ *L. acidophilus* per ml of product. Unfortunately, data are not available on the number of viable cells of *L. acidophilus* in these products; in view of its susceptibility to other lactic acid bacteria, it is uncertain whether or not these products contain sufficient numbers of *L. acidophilus* to be of any consequence in the intestine.

In Japan, Yakult is a milk cultured with a different intestinal lactobacillus (*17*). It is made from skim milk or non-fat milk solids, sweetening agents and flavoring which is accomplished by the addition of fruit juices and flavoring essences. The culture is a special isolate of *Lactobacillus casei* (strain Shirota) used to produce the desired acidity. The product is maintained under refrigeration. Extensive studies have been conducted on the product and in feeding trials using Yakult beneficial effects of consuming large numbers of the lactobacilli have been reported (*25*). The manufacturers of this product have more recently made an additional milk beverage containing bifidobacteria.

LACTOBACILLUS ACIDOPHILUS ADDED TO PASTEURIZED MILK

Myers (*18*) grew *L. acidophilus* in a sterile medium, harvested the cells and then added them to pasteurized milk; this provided a population of *L. acidophilus* comparable to that in regular acidophilus milk. His carefully conducted study showed that this process resulted in an unfermented acidophilus milk that had the same flavor as regular pasteurized milk and that at 2 to 5 C the original milk flavor and *L. acidophilus* count persisted for 7 days. Similarly, Duggan et al. (*4*) reported on a frozen concentrate of *L. acidophilus* for preparation of a palatable acidophilus milk. The authors proposed that the concentrate containing the 'daily requirement' of viable cells, could be stored in 5- or 10-ml quantities and added to a pint or quart of whole milk for daily consumption.

Studies through 1959 dealing with effects of feeding *L. acidophilus* on intestinal microflora were based on the premise of having the lactobacilli be the predominant species present in the intestinal contents. Presumably this originated with the theories proposed by Metchnikoff that were based on the 'replacement' of the putrefactive types of microorganisms by the lactobacilli. The predominance of the lactobacilli in fecal contents was obtained by the consumption of about 1 quart of acidophilus milk per day containing approximately 1×10^{11} of *L. acidophilus*. While such a large number of lactobacilli undoubtably could cause great increases in the numbers of this microorganism in the feces, it should be noted that the earlier cultural methods used to study the fecal microflora would not detect strict anaerobes. Refinement of methodology for culturing strict anaerobes has revealed the presence of many of these bacteria in fecal contents. In the well functioning intestinal tract, presumably the different groups of microorganisms exist in a balance which favors their beneficial interactions and which is conducive to proper functioning of the intestinal tract. In the feeding of *L. acidophilus*, it is now believed that the effort should be directed at replenishing the lactobacilli so that their balance among the types present can be maintained. Generally, it has been found that 1×10^8 to 1×10^9 *L. acidophilus* per day is adequate to effect its increase in the feces (*7,9*).

In 1975, we developed a product using pasteurized low fat milk to which *L. acidophilus* is added. The culture, a human isolate possessing bile resistance, is grown in a milk medium, harvested, resuspended in milk and frozen at - 196 C. The culture in the form of a concentrate is added to cold pasteurized milk in a surge tank, mixed well, packaged, and then maintained below 40 F, and distributed in the normal channels used for milk. Several million viable and bile-resistant cells of *L. acidophilus*

are present per ml and the population can be maintained for 2-3 weeks with proper refrigeration; the flavor of the milk is unaltered by the culture. Feeding trials have demonstrated that humans showed a significant increase of the lactobacilli in fecal contents after this milk has been consumed (*7*).

QUALITY OF MICROBIAL SUPPLEMENTS

Pharmaceutical products have been marketed as sources of *L. acidophilus*. These products contain dried preparations of the microorganism, and viability can be maintained over many months, particularly with refrigerated storage. The drying, however, stresses cells sufficiently to cause injury (loss of bile resistance) to many. Different types of food products have been sold also in "health food" stores as sources of *L. acidophilus*. Unfortunately, many of these products have been found to contain a very low population of viable lactobacilli; bile resistant lactobacilli were found in some products, but *L. acidophilus* was rarely present (*5,6,24*).

Much improvement is needed in the qualities of microbial supplements designed for use as dietary sources of intestinal lactobacilli. Methods are now available that allow definitive identification of most species (*12,22*). Technological expertise is available for the large scale production of these cultures, whereby viability can be assured for extended periods. Attention must be given to the physiological state of the cultures, which can be affected by culturing and storage conditions. Evidence now suggests that such conditions can impose stresses that can injure many of the viable cells; a manifestation of the injury is loss of bile resistance (*10*). Such injury could have an adverse effect on the ability of affected cells to reside in the intestinal tract, since intestinal lactobacilli are bile-resistant and non-intestinal species lack this property.

More specific attributes of microorganisms probably will be recognized that can be used advantageously for selection of cultures for use in the intestinal tract. Studies to date have dealt primarily with the lactobacilli, but the roles of others, such as the bifidobacteria are not well understood. Factors that determine the capability of cultures to associate with the intestine are not elucidated. Some types appear to be able to attach tenaciously to the intestinal epthelium, and this may be an important factor in identifying microorganisms that can have value in the intestine. It is reasonable to assume that mechanisms controlling implantability of microorganisms will be identified, and means be developed to maximize these capabilities. Considering the important roles of the intestinal tract in the nutrition of humans, and the intimacy of microorganisms with the entire gastrointestinal tract, it is reasonable to predict that increasing efforts will be made to assure the maintenance of a favorable balance in the microflora through use of dietary supplements of certain microorganisms. To accomplish this, it will be important for the microbiologist to develop products that contain cultures possessing the capabilities to perform the roles desired.

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Application of Radioimmunoassay for Detection of Staphylococcal Enterotoxins in Foods

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ABSTRACT

The staphylococcal enterotoxins can be iodinated with chloramine-T, lactoperoxidase or gaseous iodine. Low concentrations of enterotoxin, chloramine-T and ^{125}I are recommended to avoid possible damage to the enterotoxin. The antigen-antibody complex can be separated from the unreacted enterotoxin by antibodies adsorbed onto tubes or bromoacetyl-cellulose or by precipitation of the complex with a second antibody or protein A cells. As little as 1 ng of enterotoxin per gram of food can be detected in food extracts with the solid-phase tube method or by precipitation of the antigen-antibody complex with protein A cells.

Investigations of the use of radioimmunoassay (RIA) for assay and detection of the staphylococcal enterotoxins was initiated in 1963 by Dr. Robert A. Monroe at the National Dairy Laboratories (now Kraft, Inc.). He was unable to perfect a method for its use before he moved to Nuclear-Chicago but in 1968 he again became interested in this subject. He determined that RIA for the enterotoxins was feasible and recommended that anyone interested should pursue the investigations. As it turned out Dr. Howard M. Johnson of the Food and Drug Laboratories in Cincinnati had already initiated work on RIA as an assay method for the staphylococcal enterotoxins. Arrangements were made for Monroe, Johnson and Bergdoll to meet at the Annual Meeting of the American Society for Microbiology in Minneapolis in 1971 to discuss Johnson's progress and the future possibilities of RIA for enterotoxin assay. In November of 1971 Johnson and his coworkers published their first paper on the subject (*16*). Earlier, Dickie (*7*) of the Canadian Food and Drug Directorate had suggested RIA as a possible method for detection of the enterotoxins and Gruber and Wright (*11*) had demonstrated that enterotoxin B (SEB) could be iodinated with ^{131}I without damage to the toxin.

Very briefly, in RIA a given antigen (in an unknown sample) is competing with a given amount of the same antigen labeled with a radioactive nucleotide for antibodies specific for the antigen in question. The amount of antigen present in the unknown sample is determined from a standard curve prepared from the results obtained when known amounts of unlabeled antigen are used in the system. There was little doubt RIA could be used for the assay of staphylococcal enterotoxin but proof was needed that the small amounts of enterotoxin often present in foods involved in food poisoning outbreaks could be detected by this method. The initial studies were done with the purified enterotoxins. Sensitivities reported by Johnson et al. (*16*) and Collins et al. (*5*) were 1 to 5 ng/ml and 10 ng/ml, respectively. The difference in sensitivity reported by the two groups were due to the size of sample used in the two procedures, that is, 1 ml versus 0.1 ml. The sensitivity of 1 ng/ml was adequate provided it could be transferred to 1 ng/g of food as this is the level of sensitivity achievable with the extraction-concentration-microslide (EPMS) methods (*4,29*).

One of the first reports on the use of RIA for the assay of enterotoxin in foods was a comparative study by Bennett et al. (*1*) on use of the EPMS method (*4*), the reversed passive hemagglutination method (RPHA) and RIA for detection of enterotoxin A (SEA) in various food materials. They found the RHPA method to be unreliable, which later was confirmed by Bergdoll et al. (*2*). The results with the other methods showed that the minimal amount of enterotoxin detectable by RIA was 10 ng/g of food while as little as 1 ng/g could be detected by the EPMS method. This was disappointing but subsequent reports by Park et al. (*27*), Johnson et al. (*15*) and Bukovic and Johnson (*3*) showed that with the methods they used, much smaller amounts were detectable. Park et al. (*27*) were able to detect 1.25 ng of SEA per gram when it was added to cheese. They used two volumes of buffer in their extraction procedure. Johnson et al. (*15*) were able to detect SEA and SEB and Bukovic and Johnson (*3*) were able to detect enterotoxin C (SEC) in a variety of foods when the toxin was added at a level of 1 ng/g, with the exception of SEA in cheese. It was necessary to use 2 ml of buffer to extract the toxin from cheese while only 1 ml/g was required for the other foods with which they worked.

The results of Park et al. (*27*), Johnson et al. (*15*) and Bukovic and Johnson (*3*) were within the range of sensitivity for methods for detection of the enterotoxins. The results obtained in the study by Bennett and his collaborators (*1*) are understandable when one takes into account that the RIA method of Collins et al. (*5*) was used in the study. As was mentioned earlier, the sensitivity of this method was approximately one-tenth that of the method of Johnson et al. (*16*) because of the smaller volume of sample used. A report by Jarvis (*13*)

that the minimum amount of SEB she could detect in cheese and dried milk was 6 ng/g is difficult to explain. She used the method of Johnson et al. (*15*).

IODINATION OF TEST MATERIAL

The first consideration in the use of RIA is iodination of the substance to be assayed. Within limits, the higher the specific activity one achieves, the easier it should be to detect the substance with which one is working. Initially, Johnson et al. (*16*) obtained a specific activity of 40 μCi/μg of toxin but later Kauffman and Johnson (*17*) showed that the binding capacity of this toxin was lost much faster than toxin with a specific activity of 4 μCi/μg. Niskanen and Lindroth (*23*) reported that SEA labeled to a specific activity of 68 to 300 μCi/μg was partially damaged and aggregated during the labeling and storage. They stated that the damage did not appear to be greatly dependent on the specific activity of the toxin, although they did not compare their toxin to that of a relatively low specific activity (4 to 20 μCi/μg). Enterotoxin with too low a specific activity (< 1 μCi/μg) may make detection of the toxin more difficult. It is recommended that a specific activity of 5 to 15 μCi/μg be the one of choice.

The method of Greenwood et al. (*10*) [described in detail by Hunter (*12*)], with chloramine-T as the oxidizing agent, has been the basis of most of the procedures used for iodination of the enterotoxins. In the basic method (*12*) 5 μg of protein, 2 mCi of ^{125}I and 100 μg of chloramine-T in 100 μl of solution were used. The reaction was carried out at room temperature for 30 sec because longer exposure to the reactants resulted in increased damage to the protein. There was considerable variation in the conditions used by the different investigators, as is illustrated in Table 1. Apparently each group found their methods satisfactory for their purposes.

The major points of concern in the iodination are: (*a*) oxidation of the enterotoxin by the chloramine-T with possible denaturation, (*b*) aggregation of the toxin which would decrease the reaction with the specific antibodies and (*c*) the effect of high specific activity on the toxin during storage. It is not known exactly how the iodination affects each of these but in any event it is best to use the mildest conditions that will give adequate specific activity. We have chosen the relatively mild procedure of Freedlender et al. (*9*) for our work. In this procedure 10 μg (originally was 15 μg) of chloramine-T in 0.5 ml is added dropwise, over a 1-min period, to 2.5 ml of phosphate buffer, 0.05 M at pH 7.5, containing 15 μg enterotoxin and 1 mCi of ^{125}I. The reaction is allowed to continue for 10 min so that adequate iodination takes place. The mixture is kept in an ice bath during the reaction. Addition of the dilute solution of chloramine-T slowly to an ice-cooled solution reduces possible damage of the toxin to a minimum. Also, the low concentration of iodine (332 μCi/ml) along with the small amount of enterotoxin (5 μg/ml) undoubtedly reduces damage to the toxin as well as possible aggregation. It is difficult to say what the important factors are. Orth (*25*) has attempted to show that the critical factor in loss of antigen-antibody reaction is the electromotive force (EMF) of the iodination. The activity was lost rapidly if the EMF was allowed to go above 250 mV. The amount of chloramine-T added in his method probably did not exceed 20 μg as he stated that a few microliters of a 2-μg/ml solution was added. The total volume of the reaction mixture was 1.5 ml which contained 1 mCi ^{125}I and 1 mg of enterotoxin. The reaction required about 30 min and apparently was done at room temperature. The specific activity of his toxin was not given, but from information in the paper one can assume that it was 1 μCi or above per microgram. He was unsuccessful with the method of Johnson et al. (*16*) in which 3 to 25 μg of chloramine-T was added to 50 μg of SEB and 2 mCi of ^{125}I in a volume of 300 μl.

We have information that the iodination can be done with a high concentration of chloramine-T without apparent damage to the toxin. Freedlender suggested we use 10 mg of chloramine-T in our iodinations instead of the 15 μg recommended in this method (*9*) because we were experiencing some difficulty in iodinating the enterotoxin. Apparently due to the other conditions of the method, the enterotoxin was not affected by the high concentrations of chloramine-T (3.3 μg/ml). We have observed no adverse effects on any of the enterotoxins and have been able to use them even longer than the usual 60-day half-life of the ^{125}I. Dickie has used the Freedlender et al. (*9*) method with the lower concentrations of chloramine-T successfully (private communica-

TABLE 1. *Iodination of staphylococcal enterotoxin.*

Investigator[a]	Volume (μl)	Enterotoxin (μg)	Chloramine-T (μg)	^{125}I (μCi)	Time[a]	Temperature[a]	Specific activity (μCi/μg)
1	100	5	100	2000	30s	R	
2	300	50	5	2500	2-10m	R	40
				250			4
3	60	8	88	1000	45s	4 C	115
4	100	30	100	3000	2m	R	40
5	75	5	90	1000	15-30 s	R	
6	1500	1000	2-20	1000	30 m	R	
7	3015	15	10,000	1000	10 m	4 C	8-15
8	3015	15	15	1000	10 m	4 C	

[a]1, Hunter (*12*); 2, Kauffman and Johnson (*17*); 3, Niskanen and Lindroth (*22*); 4, Dickie et al. (*8*); 5, Robern et al. (*33*); 6, Orth (*25*); 7, Miller et al. (*21*); 8, Freedlender et al. (*9*); s, sec; m, min; R, room temperature.

tion), hence, it probably would be advisable to use 10 to 15 μg rather than the 10 mg. The best guess is that the high concentration of the toxin used by Orth (*25*) resulted in aggregation of the toxin when the EMF was increased above 250 mV. Aggregation has been shown by Kauffman and Johnson (*17*) and Robern et al. (*32*) to greatly reduce the reactivity of the enterotoxin. It seems reasonable to expect increased aggregation when larger concentrations of toxin are used. It would appear from the work in our laboratory that it is best to use a dilute solution of toxin. There is a definite advantage in being able to use dilute solutions because normally only a few micrograms of labeled toxin will be needed during the 60-day half-life of the ^{125}I. One soon learns that the purified enterotoxins are valuable commodities. We are not passing judgement on the different iodination procedures that have been used, but for an investigator just beginning in the field we would recommend the use of the Freedlender et al. (*9*) method.

Another method that has been used to a limited extent is the lactoperoxidase procedure of Thorell and Johansson (*34*). Robern et al. (*33*) applied it successfully to SEC_2 and Orth (*25*) found it useful for the iodination of SEA and SEC. The results we obtained with the method were inconsistent. Also, we were unsuccessful in our attempts to iodinate with the Enzymobeads (Bio-Rad Laboratories) which contained lactoperoxidase and glucose oxidase. The enzyme methods appear to be mild procedures for iodination and no doubt are good ones for those who have success with them. Gaseous iodine has been used in the iodination of the enterotoxins (*6,11,20*). Apparently this is a satisfactory method but extra care is needed in carrying out the iodination as it is somewhat hazardous.

SEPARATION OF ENTEROTOXIN – ANTIBODY COMPLEX FROM TOXIN

The second consideration in the use of RIA is separation of the enterotoxin-antibody complex from unreacted toxin. The first vehicle chosen for this purpose was polystyrene tubes to which the specific antibodies were adsorbed (coating). After reaction of the enterotoxin with the antibodies, the solution containing the unreacted toxin is removed and the tubes counted. Coating the tubes appeared to be relatively easy and once coated they could be stored indefinitely, or so it seemed. From the beginning, differences in duplicate tubes were apparent as the coating from tube to tube lacked uniformity. This could be remedied by using a larger number of tubes for each sample; for example, Orth (*26*) used six tubes per sample. Another problem began to appear and that was an apparent decrease in the amount of labeled toxin taken up by the antibodies. It was discovered that on standing some of the antibodies were sluffing from the surface of the tubes or being replaced by proteins in the food extracts. Orth (*26*) suggested that the sluffing could be overcome by coating the tubes shortly before use and Pober and Silverman (*28*) found treating the tubes with bovine serum albumin (BSA) resolved the second problem. These may be satisfactory solutions, but some of the convenience of using tubes was lost.

The second method proposed was the use of acetylbromocellulose (BAC) as the solid phase (*5*). One hundred microliters of the enterotoxin samples was added to tubes containing 0.5-ml portions of the BAC-antibody complex. Ten microliters of the ^{125}I-enterotoxin was added and the tubes shaken for 15 min at room temperature and then for 2 h at 4 C. One and one-half milliliters of cold borate BSA buffer was added to each tube and the tubes were centrifuged. The radioactivity in 1 ml of the supernatant fluid was determined and the amount of unbound labeled antigen calculated. This method is rapid but lacks the sensitivity of the tube method because of the smaller volume of sample used.

The third method proposed was the addition of a second antibody (double-antibody) to separate the enterotoxin complex from the unreacted toxin. In this instance, antibodies prepared in goats to rabbit gamma globulin were used as the second antibody. Robern et al. (*33*) presented a method for the assay of SEC_2 in which they were able to detect as little as 0.33 ng of SEC_2 per milliliter of reconstituted dehydrated soup. Later Robern et al. (*32*) reported that this method was satisfactory for detection of SEA and SEB in fermented sausage at the level of 5 ng/ml of extract. Lindroth and Niskanen (*19*) were able to detect 2 to 5 ng of SEA per milliliter of extract from minced meat and sausage, using sheep anti-rabbit gamma globulin adsorbed onto cellulose. The double-antibody method is an attractive method and one which we thought might be the one of choice. In our experiments, we encountered problems with variations in the commercial anti-rabbit gamma globulin from lot to lot. Also, great care must be used in removing the supernatant fluid as some of the precipitate may be lost. Robern and Gleeson (*31*) overcame this problem by adding aqueous polyethylene glycol to precipitate the double-antibody complex. The method does lack sensitivity as it currently is being performed because of the smaller volumes of sample that are being used. To increase the volume to obtain a sensitivity equal to the tube method, larger quantities of reagents would be needed, which would add to the cost.

Niyomvit et al. (*24*) reported another approach in which they used Sepharose 4B as the solid phase. They passed the unknown through the Sepharose 4B column followed by the labeled enterotoxin. The toxin was dissociated from the antibodies with NH_4OH at a pH of 10.5. The least amount of SEB they could detect in milk was 2.2 ng/ml and for hamburger it was 6.3 ng/ml of extract. This indicates a lack of sensitivity but maybe this could be overcome with further work. An analysis can be done within a few hours. Our experience with affinity chromatography has been that the enterotoxin-antibody complex is difficult to disassociate.

The method which we have presented (*21*) involves use of protein A cells to separate the antigen-antibody complex from the unreacted toxin. Metzger and Johnson (*20*) proposed this method for the detection of the enterotoxins in 1977 at the ASM Meeting in New Orleans. As they were not primarily interested in developing the method for detection of enterotoxin in foods, we pursued the work in our laboratory because we felt that it had great promise. The protein A cells for use in the precipitation of the complex were prepared easily in the laboratory from *Staphylococcus aureus* Cowan-I strain. After washing, the cells were formaldehyde-treated and heat-inactivated (*18*). They were frozen or freeze-dried and stored for use as needed. The cells in either state could be kept indefinitely. All that was required was that an excess of cells be added to assure the precipitation of the complex. The reaction occurs within minutes although the reaction was allowed to take place for 20 min to assure that all of the complex was precipitated. One beneficial aspect of the method is that the complex can be precipitated from larger volumes of the reaction mixture than is used in the double-antibody method. This makes possible an increase in the sensitivity of the method. We use 1 ml, which is the volume employed in the tube method, although we are satisfied that even larger volumes can be used. As little as 0.3 ng/ml was statistically significant.

The method was easily adapted to foods of all kinds as is illustrated in Table 2. As those who have worked with the extraction of enterotoxin from foods know, not all foods can be treated in the same way. Liquid foods can be analyzed rather easily as usually no treatment is required. Solid foods are extracted with either 1.5 or 2.0 ml of water, the pH is adjusted to 4.5 for some foods, and the mixture is centrifuged. The pH of the extract is adjusted to 7.5, treated with chloroform and recentrifuged. One milliliter of the extract is used for analysis. We were able to detect 1 ng of enterotoxin or less per gram of food in all the foods we tested. The method has been used to detect enterotoxin in naturally contaminated foods, ones in which we encountered difficultly in detecting the enterotoxin by the CPMS method (*29*) which can under certain circumstances be used to detect as little as 1 ng/g of food. Since publication of our paper (*21*), we have encountered a problem with unheated meat samples because of the presence of gamma globulin which interferes with precipitation of the enterotoxin-antibody complex by the protein A cells. This problem was overcome by treating the meat extract with protein A cells before doing the analysis. The interference, as might be expected, was more pronounced when the samples were concentrated (by ultrafiltration) to confirm results that were borderline. We have been able to detect enterotoxin in foods naturally contaminated when it was not possible to detect it by other means.

TABLE 2. *Detection of staphylococcal enterotoxin in foods.*

Food	Extract[a] (ml/g)	Enterotoxin (1.25 ng/g)	Enterotoxin found ng/ml	Enterotoxin found ng/g
Ham	2.0	SEA	0.57	1.14
Milk		SEA	0.95	0.95
		SED	0.67	0.67
Sausage	1.5	SEA	0.44	0.66
		SED	0.56	0.84
Cheese	1.5	SEA	0.80	1.20
		SEE	0.69	1.04
Pudding	1.0	SEC_1	1.30	1.30
		SED	0.78	0.78
Boiled egg	1.5	SEB	0.42	0.63

[a]Acid-base extraction was used for all foods except the water extraction was used for the boiled egg.

IN CONCLUSION

We feel that RIA is a useful technique for detection of enterotoxin in foods. An assay can be accomplished within one working day provided the iodinated toxins are available. We doubt that RIA will ever be widely used because of the need for expensive equipment and the requirement for purified enterotoxins which are scarce and expensive. It is not a method one would use for an occasional assay as the iodinated toxins can be used only for about 60 days. It would be most useful when a relatively large number of assays are to be done or for someone who is doing routine work such as in an analytical laboratory. It is not recommended for assay of culture supernatant fluids because there are better methods such as the optimum sensitivity plate (OSP) method (*30*) or the microslide for doing this. With these methods one can compare the unknown directly with the known toxins and the sensitivity of the reactions is sufficient to classify the staphylococci as enterotoxigenic. In addition, interference from protein A and other substances in the culture supernatant fluids may be encountered. We noted this problem when we were attempting to test culture supernatant fluids from strains that produced unidentified enterotoxins against cross-reacting antibodies. The interfering substances including protein A could be removed by passing the culture supernatant fluids over an affinity chromatography column containing human gamma globulin. We are satisfied that protein A does not interfere in the assay of enterotoxin in foods because too small amounts would be present. At least 125 ng of protein A must be present before any effect is noted.

RIA can be a useful tool in doing certain types of research such as determining conditions necessary for production of enterotoxin in foods as one can detect lower levels of toxin than by the ECMS methods. We are using it in our study of cross-reacting antibodies because only one antigenic site is necessary to obtain a reaction in RIA while two are necessary to obtain a precipitin reaction. Preliminary experiments indicate that cross-reacting antibodies do exist which can be used to detect more than one enterotoxin. This may include some of the unidentified ones. Johnson et al. (*14*) showed only slight cross-reactivity between the enterotoxins but cross-reactions between the enterotoxins are very dependent on the antisera one is examining. Cross-reacting antibodies are produced by a relatively few rabbits.

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Why are Grade-A Surveys Necessary?

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ABSTRACT

Evaluation of large milk processing plants has brought about the need for interstate shipments of raw and processed milk throughout the United States. Uniform procedures developed under the Interstate Milk Shipment Conference have brought about uniform standards and inspection programs. This uniformity has improved the quality of milk and eliminated the duplication of inspections through the practice of reciprocity between states.

Some individuals feel the Grade A milk supply in the United States in over-regulated. To some extent, I can agree with them. However, when we look back a few years we see a tremendous change in the marketing trends and consumer acceptance for Grade A milk and milk products. First, no food surpasses milk as a single source of those dietary elements needed for proper health - especially by our children and senior citizens (*1*). Second, milk is a potential disease carrier, to say nothing of the potential for milk to become contaminated with pesticides, antibiotics and other adulterants.

Before 1938 milk accounted for over 25% of all disease outbreaks caused by food and water. Today the incidence of milkborne illness has been reduced to less than 1% of all foodborne disease outbreaks (*2*).

Before World War II, individual communities were by-and-large responsible for their own milk supply. Most cities of any size had at least one milk bottling plant and most all of the raw milk purchased by these plants traveled relatively short distances. It was common for milk produced one day to be hauled to the plant that same day or early the next morning. Most plants processed the milk the same day it was received and home delivery put this same milk on the doorstep or in the ice box many times within 24 to 36 h after milking. Very little milk moved from one county to another much less from state to state.

Closing of many small bottling plants brought about the need to move milk (raw and processed) long distances from the point of production to the processor and on to the consumer. Milk company representatives will tell you today that a minimum of a 10-day shelf-life is needed to furnish the public a safe, wholesome product with an acceptable flavor. Many companies process milk with a much longer shelf-life.

CHANGES CAUSED BY MOVEMENT OF MILK

This movement of milk intra- as well as interstate brought about many changes in the operation of regulatory programs. No longer were milk producers and processors being regulated by an inspection agency in the county or city in which they lived and worked, but other counties and states were becoming involved as they were now receiving this milk into their jurisdiction.

As processing plants became larger, they covered more and more territory with their sales. This involved many more regulatory agencies and soon milk inspectors were crossing not only county lines but state boundaries as well. Some areas of the country had very stringent inspection laws and others had virtually none. In any event a lot of duplication of inspections was taking place (Fig. 1, Fig. 2).

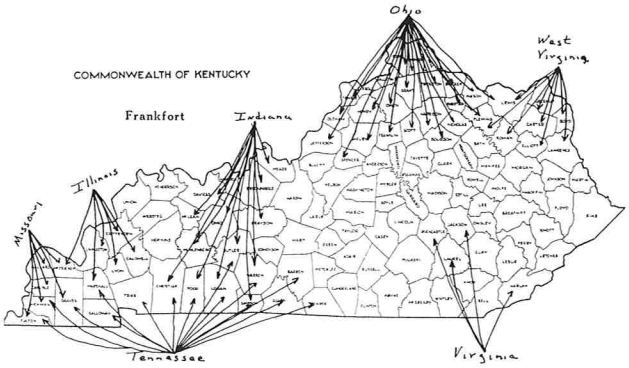

Figure 1. *Out-of-State Inspections in Kentucky before 1972.*

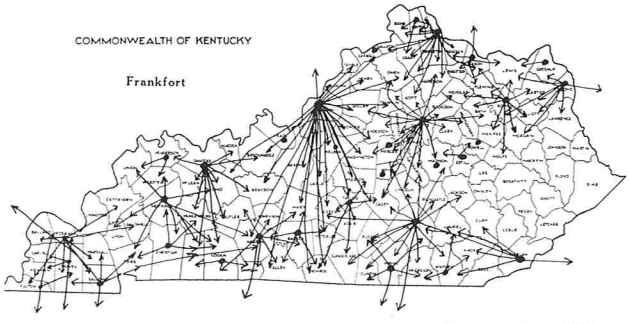

Figure 2. *Local Health Department Inspections Areas before 1972.*

To provide assistance to states, the Food and Drug Administration developed and published a *Standard Milk Ordinance* in 1924. An accompanying code was published in 1927. This *Pasteurized Milk Ordinance and Code* was for state adoption (*3*). (This differs from the Federal Food, Drug and Cosmetic Act which is Federal law administered by Federal people.)

A few states did adopt this early *Pasteurized Milk Ordinance*; however, uniform inspection which allowed for the free movement of milk was not developing rapidly. Much of the milk moving from state-to-state was of unknown or questionable quality and this pointed to the need for a national program which would regulate the nation's milk supply on a uniform basis and allow free movement of milk between municipalities and states. In 1950, at the request of the Association of State and Territorial Health Officers, the Surgeon General called a National Conference on Interstate Milk Shipments to discuss the problems of duplication of inspections, lack of uniformity and difficulty of moving milk interstate.

The conference developed a plan, utilizing a cooperative State/Federal approach for evaluation of the sanitation compliance status of milk supplies of interstate milk shippers and for dissemination of such information between states. This first conference was attended by representatives of industry, state health and state agriculture departments of 22 states and the District of Columbia (*4*). Several basic agreements came out of this first conference:

1. The PHS *Pasteurized Milk Ordinance* or it's equivalent would be used by participating states as the basic milk sanitation standard.
2. PHS rating methods would be used as the procedure for determining the degree of compliance with the basic standard.
3. Only those milk supplies which were under full-time supervision of a regulatory agency should be eligible for certification under the program.
4. Supplies would be rated by milk sanitation rating officers of the state in which the supply is located. These rating officers would be standardized by the PHS and issued a certification certificate.
5. The state rating agency would report all survey results to the federal agency for publication on an *Interstate Milk Shippers List*, which would be available on a quarterly basis to all regulatory agencies through the United States.
6. No shipper's rating would be published without his written permission.
7. PHS/FDA would monitor the rating system and do check rating to insure that all state rating agencies were conducting the required ratings according to established procedures.

Since the 1950 Conference 15 additional conferences have been held. The 17th conference will be in Louisville in May of 1979. Many modifications have been made since the first conference; however, the basic agreements still are in effect. Since 1950, the Interstate Milk Shippers (IMS) program has grown to include participation by 49 states and the District of Columbia. (Hawaii is the only state not participating in the program.) As of January 1, 1979, there were 1,975 listed shippers, representing approximately 155,000 Grade A producers.

The cooperative program for certification of Interstate Milk Shippers continues to expand, not only by the continued increase each quarter in the number of shippers participating, but also in terms of national interest and acceptance. The Veterans Administration has used the program to purchase milk and milk products for its hospitals throughout the country. Since July 1, 1966, only milk from sources which are approved under this program has been served on interstate carriers (airlines, ships, etc.). Recently the Department of Defense drafted specifications to use the program in its joint regulations for purchase of fresh whole milk. Public health service hospitals, Indian hospitals and schools under Federal specifications now use the program.

The program has improved the sanitary quality of milk being shipped in interstate commerce; it has stimulated a high degree of uniformity in the interpretation and application of sanitary standards between states; it has improved milk laboratory control methodology and it has eliminated the need for the costly and wasteful practice of multiple inspections for two or more states on single sources of milk.

With adoption by the Kentucky Legislature of the *1972 Kentucky Milk and Milk Products Act* and the requirements of this act, no outside state agency presently comes into Kentucky to inspect milk plants or producers' dairies and we do not travel outside of Kentucky. Presently, through the cooperation of states in the IMS program, Kentucky milk moves into many states with little difficulty. With Kentucky being a shipping state, that is, we produce more milk than we consume, it is very important to dairy farmers in this state that their cooperatives and plants can move milk into areas where the milk can be sold as Class 1. Kentucky enjoys a good national reputation for production of good quality milk.

CERTIFICATION PROGRAM

Briefly, the program for certification of plants and producers for the IMS Program is as follows. In addition to the routine inspection program, each milkshed is surveyed or evaluated once every 14 to 18 months. A milkshed is a group of dairy farms producing milk in a given area or attached to specific milk plant, receiving or transfer station. Each group of producers in a milkshed and the plant, transfer or receiving station is assigned a rating based on the findings during the survey. The evaluation or survey of a milkshed consists of:

1. Selection of a statistical number of producers for inspection.
2. Assigning of a rating score to each producer inspected based on the violations found at the time of the inspection. Each item on the inspection sheet is assigned a numerical score. Violations may range from 10 to 1 point, depending on the seriousness and public health significance of the violation. (Cleaning of Equipment - 10; Water Supplies and Toilet Facilities - 5; Cleanliness of Milkhouse - 3; Cleanliness of Milking Area and Cowyard - 2; and

Walls and Ceilings, Lighting Feed Storage and Etc. - 1 point)

3. The producer's quality record for the past year is also reviewed. Failure to meet quality standards also affects the rating assigned the producer.

4. The survey officer reviews the enforcement program being carried out by the inspector. In other words, the inspector assigned to the area is also being surveyed or checked to determine if he is carrying out the requirements of the regulation regarding inspection frequency, issuance of notices when required and suspension of producers not meeting requirements.

5. Scores of all producers inspected during the survey are averaged together. The average rating for the milkshed must be 90% or above to be approved for interstate milk shipment.

6. Plants and receiving and transfer stations are also assigned a rating in much the same way as producers. They must also maintain a 90% rating or above.

Milk from milksheds which fail to maintain a 90% sanitation and enforcement rating may not be received by plants which sell milk and milk products in interstate commerce. In Kentucky this includes practically all pasteurization plants.

Even though surveys require a few more inspections for those producers who are selected on the survey, the benefits from the survey ratings are for the producer. Without an approved rating, his milk could not move into interstate commerce. Also, without a rating procedure and an organization such as the Interstate Milk Shippers Conference, movement of milk between states would not be permitted unless those states receiving the milk sent their inspectors to evaluate the supply. In the past, when some of this was done, many of the receiving states charged the dairy industry for these inspections. Also, in some areas requirements were established which made it virtually impossible for milk to move into that state when the milk was not produced within the state.

Recently Smathers stated: "A dairy farmer who is consistently above 90 always is a welcome member in a milk supply" (5). This is certainly true. Not only does failure by an individual producer to meet the Grade A requirements affect his milk, but it may also affect that of other producers and the producer association. This is in addition to the money lost by the producer because of high somatic cell counts which lower production and cause milk to be diverted to Class II and III markets or cause milk to be disposed of because it is adulterated or is below the quality standards.

In closing, we can provide an answer to the question, "Why are Grade A surveys necessary?" Surveys not only have proven by past history to improve the overall quality of the nation's milk supply, but they have benefitted the dairy farmer tremendously by allowing free movement of milk and milk products throughout the United States.

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Gordon, Morris and Packard, *con't from p. 64*

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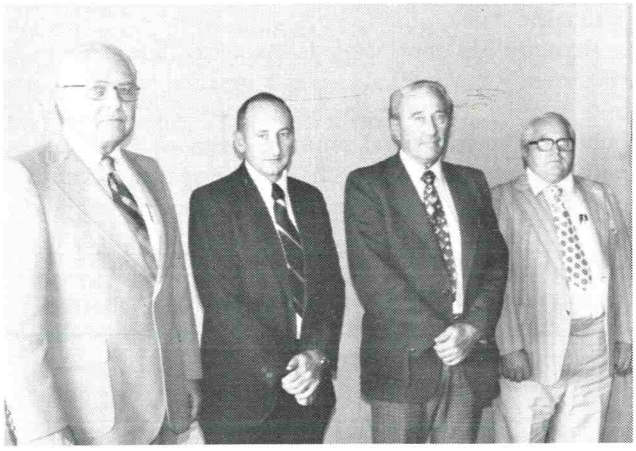
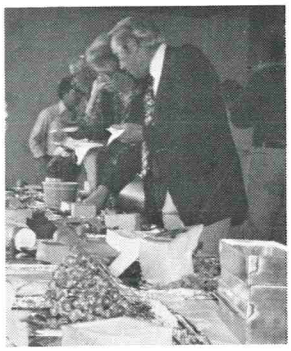
News and Events/Coming Events



Washington officers, above, left to right, are: Bill Roth, President; Lloyd Leudecke, Secretary-Treasurer; George Andrews, President-Elect; and Stan Harriman, Past President. Oregon officers who served for 1978-79 will serve again for 1979-80.



Above, left: Dr. Richard A. Scanlan, Professor of Food Science, Oregon State University, speaks on "Nitrosamines in Foods: An Update," at the joint Washington-Oregon meeting. Right, a cheese smorgasbord prepared by the Oregon State University Food Tech Club was a social highlight of the meeting.

Scenes from Washington-Oregon Joint Meeting
(for story, see p. 77)

Jan. 19---MIDWEST MEAT PROCESSORS SEMINAR. Kansas State University. Contact: Dave Schafer, Extension Specialist, Weber Hall 115, KSU, Manhattan, KS 66502.

Feb. 3-5---SANITATION THROUGH DESIGN, FDA Food Industry Workshop. San Diego, CA, Hanalei Hotel. Contact: Food Sanitation Institute, Environmental Management Association, Harold C. Rowe, Executive Director, 1701 Drew St., Clearwater, FL 33515 813-446-1674.

Feb. 10-13---1980 INTERNATIONAL EXPOSITION FOR FOOD PROCESSORS. St. Louis, MO. Contact: T. J. Gorman, Food Processing Machinery and Supplies Association, Suite 700, 1828 L St., N.W., Washington DC 20036.

Feb. 10-13---NATIONAL FOOD PROCESSORS ASSOCIATION, Annual Meeting. Convention Center, St. Louis, MO. Contact: National Food Processors Association, 1133 20th St. NW, Washington, DC 20036.

Feb. 12-13--OREGON DAIRY INDUSTRIES CONFERENCE, Valley River Inn, Eugene, OR. Contact: Mary K. Moran, ODI Secretary, Room 100, Wiegand Hall, Oregon State University, Corvallis, OR 97331, 503-754-3131.

Feb. 24-29---TENTH ENVIRONMENTAL ENGINEERING IN THE FOOD PROCESSING INDUSTRY CONFERENCE. Asilomar Conference Grounds, Pacific Grove, CA. Sponsored by the Engineering Foundation. Fee: $250, double occupancy, $300 single occupancy. Contact: Engineering Foundation, 345 E. 47th St., New York, NY 10017, 212-644-7835.

March 3-5--GOOD MANUFACTURING PRACTICES FOR THE FOOD INDUSTRY, shortcourse. Sponsored by the Center for Professional Advancement. Contact - Mary Sobin, Dept. NR, Center for Professional Advancement, P.O. Box H, East Brunswick, NJ 08816, 201-249-1400.

March 10-12--SENSORY EVALUATION METHODS. IFT shortcourse, Portland, OR. Contact - Dan E. Weber, IFT, 221 N. LaSalle St., Chicago, IL 60601, 312-782-8424.

March 17-19--CANADIAN FOOD PLANT SANITATION SEMINAR. Toronto, Ont. Sponsored by American Institute of Baking and Bakery Council of Canada. Contact - Carol Lyon, AIB, 1213 Bakers Way, Manhattan, KS 66502, 913-537-4750.

March 24-26--FOOD MICROBIOLOGY. shortcourse. Sponsored by the Center for Professional Advancement. Contact - Mary Sobin, Dept. NR, Center for Professional Advancement, PO Box H, East Brunswick, NJ 08816, 201-249-1400.

March 24-28--MIDWEST WORKSHOP IN MILK AND FOOD SANITATION. The Ohio State University, Columbus, OH. Contact - J. Lindamood, Dept. of Food Science and Nutrition, 2121 Fyffe Rd., The Ohio State University, Columbus, OH 43210.

March 25--DAIRY INDUSTRY CONFERENCE. Scheman Building, Iowa State University, Ames, IA. Contact - V. H. Nielsen, Dept. of Food Technology, Iowa State University, Ames, IA 50011.

Mar. 26-28---CONFERENCE ON WASTEWATER TREATMENT TECHNOLOGIES FOR THE CONTROL OF TOXIC/HAZARDOUS POLLUTANTS. Stouffer's Cincinnati Towers, Cincinnati, OH. Contact: Kenneth A. Dostal, IERL-Ci, EPA, Cincinnati, OH 45268.

Mar. 27-28---1980 MEAT INDUSTRY RESEARCH CONFERENCE. Ramada O'Hare Inn, Chicago, IL. Contact: Dr. John Birdsall, Director of Scientific Activities, American Meat Institute, P.O. 3556, Washington, DC 20007.

Mar. 31-April 2---AMERICAN CULTURED DAIRY PRODUCTS INSTITUTE ANNUAL TRAINING SCHOOL AND JUDGING CONTEST, Hilton Airport Plaza Inn, Kansas City, MO. Contact: C. Bronson Lane, ACDPI, P.O. Box 7813, Orlando, FL 32854.

April 14-16---5th ANNUAL FOOD SERVICE SYSTEMS SEMINAR AND EXPOSITION. Sheraton O'Hare Hotel, Chicago, IL. Contact: G. E. Livingston, Food Science Associates, Inc., 595 Fifth Avenue, New York, NY.

April 21-25---SCANNING ELECTRON MICROSCOPY/1980. McCormick Inn, Chicago, IL. Contact: O. Johari, Scanning Electron Microscopy Inc., P.O. Box 66507, AMF O'Hare, IL 60666.

April 27-May 1--ISF/AOCS WORLD CONGRESS. Joint meeting of American Oil Chemists' Society and the International Society for Fat Research. New York Hilton, New York City. Contact - American Oil Chemists' Society, 508 S. Sixth St., Champaign, IL 61820.

April 30---USE AND ABUSE OF FOOD SUBSTANCES---TECHNOLOGICAL AND HEALTH IMPLICATIONS, Pick Congress Hotel, Chicago. Sponsored by Illinois State Medical Society, Chicago Nutrition Association and Chicago Section of Food Technologists. Contact: Therese Mondeika, 312-751-7624.

Milt Held Dies

Milton Held, San Carlos, CA, died November 17 of complications after suffering an aortic aneurysm.

Captain Held was retired as Sanitarian Director of the U.S. Public Health Service in nine western states. He was a member of the Episcopal Church of the Epiphany, San Carlos.

He graduated from Iowa State University in 1928 where he was a member of Sigma Alpha Epsilon fraternity.

He was born in Hinton, IA, and had lived in San Carlos since 1960. He retired in 1970 after 30 years of work in public health.

He was also a member of 32° Scottish Rite, and Landmark Lodge #103 AF&AM, Sioux City, IA. Held was a lifetime member of the Iowa Milk, Food and Environmental Sanitarians Association, Inc., and was a past president of IAMFES.

Captain Held was a life member of the Commissioned Officers Association of the U.S. Public Health Service.

He is survived by his wife, Nina; three sons, Marc, Portland, OR, John, Seattle, WA, and Phil, Atherton, CA; a sister, Gladys Rosburg, Los Angeles; and four grandchildren.

Burial was at the Skylawn Memorial Park, San Mateo, CA.

The family suggests that memorial contributions be made to the Church of the Epiphany, M. E. Held Memorial Fund.

NAMA Council Lauds Jones, Association

NAMA's Automatic Merchandising Health-Industry Council (AMHIC) held its 23rd annual meeting in Chicago in conjunction with its National Convention/Exhibit.

Karl K. Jones represented IAMFES, as he has done for a number of years. G. Richard Schreiber, President of AMHIC, noted in a letter to Earl Wright, "We always count heavily on Karl for his guidance and are never disappointed."

"Please express to your Association officers my appreciation for providing representation since 1957 on our advisory council. The vending industry has made great strides in consumer protection over the years, thanks in large part to the cooperation of IAMFES and the other public health associations who provide delegates to the Health-Industry Council," he added.

NOTICE

USPHS Inactive Reservists - Sanitarian Category

The Sanitarian Career Development Committee, USPHS, is attempting to locate members of the Inactive Reserve. Those inactive reservists who have not been contacted please write:

Executive Secretary, SCDC
Cmdr. Leo Snyder
Executive Secretary
Bureau of Medical Services
6525 Belcrest Road
W. Hyattsville, MD. 20782.

Jack Walsh, Former WPI Head, Dies

John T. Walsh, 69, former Executive Director of the Whey Products Institute, died November 10, 1979. Jack had been in declining health since spring, and bedridden since June. Funeral services were in Mt. Prospect, IL, with burial in Des Plaines, IL.

Jack first joined the staff of the American Dry Milk Institute in 1938 as a laboratory assistant. Subsequently he served in almost every capacity, being named Assistant Executive Director in 1955 and Executive Director in 1959, the position he held for 17 years until retirement on June 30, 1976. He was a major factor in the formation and growth of the Whey Products Institute in 1971.

He is survived by his wife, Hilda, a daughter Kathy, and twin grandsons Roger and Raymond.

Washington, Oregon Affiliates Meet Together

The Washington and Oregon affiliates held a joint annual meeting this year and much effort was directed toward planning the 1981 IAMFES Annual Meeting that the two Associations will sponsor.

Each group held its own business meeting following a joint luncheon. Earl Wright, IAMFES Executive Secretary, discussed 3A Standards and the function and operations of the 3A Symbol Program at that luncheon.

Dr. Richard Scanlan of Oregon State University presented an update on nitrosamines in foods. He noted that the newest additions to the list of foods containing the suspected carcinogen are beer and scotch.

Prior to the Annual Awards Banquet, a cheese smorgasbord was served by Oregon State University food technology students. At the banquet, Dr. James Oldfield of Oregon State University showed slides and spoke on his trip through China. He assessed the agricultural status of China and noted the Chinese are attempting to improve their agricultural programs in many different areas.

The Washington affiliate established a scholarship for a junior student enrolled in food technology with a dairy emphasis.

Officers of the Washington affiliate elected for 1980 were the following: President, Dr. Bill Roth, President-elect, George Andrews, Past President, Stan Harriman, Secretary-Treasurer, Dr. L. O. Luedecke. Oregon officers from 1978-79 will serve again for 1979-80.

For photos, see p. 76

Extension Publications Available

"Safe Handling and Storage of Natural Cheese," is one of several publications available through the Cooperative Extension Service of the University of Nebraska-Lincoln.

To obtain the publications, write:

Dr. Stan Wallen
Extension Food Scientist
116 H.C. Filley Hall
Univ. of Nebraska-Lincoln
Lincoln, NE 68583

Minnesota Sanitarians Hear Varied Program

A variety of current subjects attracted a good attendance to the Sanitarian's Conference of the Minnesota Association of Sanitarians.

"Energy---Use and Conservation on Dairy Farms," by Paul Shea of the Minnesota Power and Light Co., "A Look at State-Wide Certification of Food Service Managers," by John W. Urbach, Senior Health Planner in the Hennepin, Minnesota County Community Health Department, and "STOP Program Update," by Larry Stowe, Professor of Large Animal Clinic Sciences at the University of Minnesota, were just three of the many presentations during the two-day conference, held at the Earle Brown Continuing Education Center of the University of Minnesota.

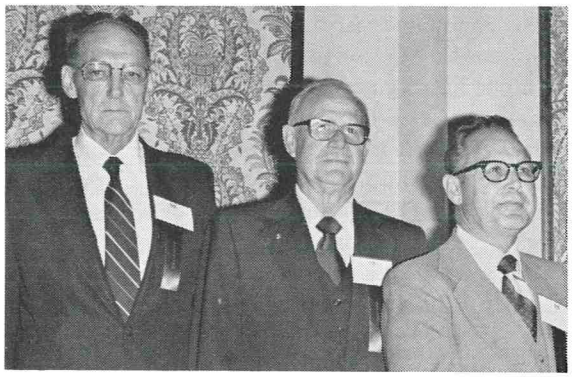
"Mastitis, The Big Picture," was an all-morning session presented by Nelson Philpot, Professor of Dairy Production and Bacteriology, at Louisiana State University.

A series of presentations on microwave ovens — functions, surveillance for potential hazards, and commercial application and uses — was given opposite the mastitis program.

"Alternatives to Nitrites in Cured Meats," by F. F. Busta, Professor of Food Science and Nutrition, and "What Consumers Say They Want on a Food Label," by Isabel Wolf, Extension Specialist in Foods and Nutrition, both of the University of Minnesota, were also presented.

Winner of the 1979 Minnesota Sanitarian's Achievement Award was Hugh Munns, Manager of Member Services for Mid-America Dairymen. Munns has served actively on the IAMFES Farm Methods Committee, National Milk Producers Federation, National Conference on Interstate Milk Shipments, National Mastitis Council, Minnesota Sanitarians Association, Minnesota Mastitis Council, and Minnesota Department of Agriculture, among other groups.

Joseph Olson was honored by the Minnesota Association with its Honorary Life Membership. He was similarly honored by IAMFES at this year's International Annual Meeting.



Above, left, Minnesota officers, left to right, include: Roy E. Ginn, Sec.-Treas.; James A. Rolloff, Past Pres.; and Douglas E. Belanger, President. Above, right: Arnold Ellingsen, left, presents the Minnesota Sanitarians Award to Hugh Munns. Right: Shown at the Annual Meeting of the Wisconsin Association are, left to right: John Gerberich, President; the Wisconsin Associate are, left to right: John Gerberich, President; Earl Wright, Exec. Sec., IAMFES; and Norm Kirschbaum, Past Pres.

Joint Meeting Held for Wisconsin Associations

"Awareness for Wellness" was the theme of the First Annual Joint Educational Conference of the Wisconsin Association of Milk and Food Sanitarians, Wisconsin Dairy Plant Fieldman's Association, Wisconsin Dairy Technology Society, Wisconsin Environmental Health Society and the Wisconsin Institute of Food Technology. This marks the first time that WEHA has met jointly with WAMFS and sets the precedent for the International meeting to be held in Milwaukee July 26-31, 1981. The conference was held in Madison September 19-20.

Wednesday's session opened with a keynote address by Terry Willkom, Deputy Secretary of the Wisconsin Department of Health and Social Services. He emphasized "prevention rather than cure." The general session was followed by separate dairy and general environmental health sections. Organizational business meetings concluded the day. Thursday's activities started with a general session, followed by separate food and on-site waste sections.

Dr. John Gerberich, University of Wisconsin-Eau Claire, was presented with the WAMFS President's gavel by Norman Kirschbaum, outgoing President.

John Collier, Southeast Regional Supervisor, Food Division, WDAT-CP, was honored as the WAMFS "Sanitarian of the Year." Louie Nachreiner of Nachreiner Lab, Plain, WI was presented with the "Fieldman of the Year" award sponsored by Monarch Chemical Co. Darrell Farmer was honored as WEHA "Sanitarian of the Year."

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The program at the banquet featured awards presentations, of course, but also a presentation by Oscar Snyder, Extension Specialist in Food Service Management at the University of Minnesota. He showed slides and discussed "The Kitchens of Europe." Food sanitation standards in European restaurants needn't be constantly checked for the most part, Snyder said. It's a matter of pride and professionalism that the standards be met, he explained. As European sanitarians usually appear at a restaurant only to investigate a foodborne disease outbreak, European restaurant owners, when asked about sanitation inspections, reply, "I guess there's a sanitarian downtown somewhere..."

19th ANNUAL NATIONAL MASTITIS COUNCIL MEETING

February 18-20, 1980
Louisville, Kentucky

Tuesday - February 19
8:00 a.m. - 5:00 p.m. Registration

GENERAL SESSION

8:45 a.m. Call to Order
8:50 President's Address
L. H. Schultz - Madison, Wisconsin
9:00 Etiology of Bovine Teat End Lesions
R. L. Sieber* - Madison, Wisconsin and R. J. Farnsworth - St. Paul, Minnesota
Relationship of Teat End Lesions to Intramammary Infections
R. J. Farnsworth* and R. L. Sieber
10:00 Break
10:15 Methods of Mastitis Detection Including the Rolling Ball Viscometer and Electrical Conductivity Meter
G. F. Duirs - New Zealand
10:45 Effects of Vacuum Level and Pulsation Ratio on Udder Health
D. M. Galton* and D. E. Mahle - Baton Rouge, Louisiana
11:05 Current Issues of Concern to FDA Relative to Mastitis Control
L. M. Crawford - Washington, DC
11:35 Questions
12:00 NMC Luncheon

GENERAL SESSION

1:15 p.m. Environment and Udder Health on Norwegian Dairy Farms. Evaluation of Data and Application of Results in Organized Mastitis Control
G. Bakken - Norway
1:45 Effects of Feeding Fermented Mastitic Milk to Calves
J. E. Keys, Jr* - Beltsville, MD,
L. V. Schaffer and R. K. McGuffey* - Brookings, SD
2:15 Current Emphasis on the Control of Mastitis in New Zealand
G. F. Duirs - New Zealand
2:45 Film - "The Mastitis Case" - New Zealand
3:15 Break

CONCURRENT SESSIONS - CURRENT TECHNOLOGY

3:30 p.m. - 5:30 p.m. Section A - Environmental Factors in Mastitis Control

Recycled Manure Solids as a Free-Stall Bedding for Lactating Dairy Cows and Its Association with Mastitis
S. D. Allen*, M. Wallentine, K. Hoopes, S. Austin and P. Burch-Logan, Provo, Utah.

Bacterial Populations in Bedding Materials and Coliform Mastitis Incidence
E. J. Carroll and D. E. Jasper* - Davis, California

A Practical Low-Cost Method of Heat Sanitizing of Teat Cups
C. N. Dobbins, Jr.*, E. B. Shotts, Jr., W. K. Whitehead, and E. J. Lloyd - Athens, Georgia

How the Dairy Industry Can Benefit From a Somatic Cell Program
T. C. Everson - Baraboo, Wisconsin

Effects of Premilking Udder Preparation on Milk Quality
D. M. Galton - Baton Rouge, Louisiana

Questions and Discussion

3:30 p.m. - 5:30 p.m. Section B - Clinical Aspects of Mastitis Control

Antibiotic Resistance Patterns and Trends of Common Mastitis Pathogens
J. N. Davidson - Ithaca, New York

Immunization Against Bovine Mastitis
J. W. Pankey - Homer, Louisiana

Mastitis Control: Who? How? Why? - A Practitioner's Approach
B. Anderson - Loysville, Pennsylvania

Antibiotic Residues in Milk
B. W. Maxey, C. C. Miller and G. H. Swenson* - Kalamazoo, Michigan

Questions and Discussion

7:45 p.m. - 10:00 p.m. Evening Program

GENERAL SESSION

Estimates of Iodine Consumption and Implications for Human Health (Based on the AMA Iodine Workshop, November 1979)
R. Hemken - Lexington, Kentucky

"Methods of Enhancing Adoption of Mastitis Control Procedures In The U.S.": A report of the Education Committee,
- Introductory Remarks - R. Webber - Washington, DC and J. Reeder - Arlington, Virginia
- Procedures for Improving Udder Health - A. N. Bringe - Madison, Wisconsin
- Reference Index of Herd Health Information - H. Munns - St. Paul, Minnesota and K. Kirby - Fort Atkinson, Wisconsin
- Development of a Training Seminar for Field Personnel - W. N. Philpot - Homer, Louisiana

Wednesday, February 20

8:00 a.m. Registration

GENERAL SESSION

8:15 a.m. Panel Discusion: Milking Systems - Design, Installation and Maintenance,
Sponsored by the Milking Machine Manufacturers Council of the Farm and Industrial Equipment Institute
- Challenges & Opportunities of the 80's - R. C. Dawson - Chicago, Illinois
- Behind the Scene of Milking System New Product Development - J. Scolaro - Chicago, Illinois
- Successful Milking System Installations - P. Lammert - Washington, Missouri
- A Positive Approach to Milking System Service and Maintenance - R. Gray - Poughkeepsie, New York

10:15 Break
10:30 Milking Equipment Evaluation - A Component of Herd Mastitis Control,
P. Blackmer - Upland, California
11:20 Final Questions
11:35 NMC Business Meeting
12:30 p.m. NMC Board of Directors Luncheon and Meeting
2:30 Final Adjournment

Seminar Proceedings Available

Proceedings are available from the "Seminar on Examining Dairy Policy Alternatives." The event, jointly sponsored by the Milk Industry Foundation (MIF) and the International Association of Ice Cream Manufacturers (IAICM), was held in September. Future dairy policy alternatives in the Federal Milk Marketing order and Milk Price Support program were investigated at that seminar by milk processors, dairy cooperative representatives, government officials, and university economists.

Copies of the proceedings are available from the MIF or IAICM, 910 17th St., N.W., Washington, DC 20006, 202-296-4250.

NSF Seminars Scheduled

Two-day seminars scheduled by NSF include sanitation aspects of design and construction of foodservice equipment, and facility plan preparation and review.

Los Angeles, CA	Jan. 21, 22
Kansas City, MO	Feb. 11, 12
Indianapolis, IN	Mar. 17, 18
Philadelphia, PA	Apr. 21, 22
New York, NY (Area)	May 12, 13

Participants may attend one or both of the two seminars held at each location.

For more information, contact:
Education Service
National Sanitation Foundation
P.O. Box 1468
Ann Arbor, Michigan 48106
313-769-8010

Wisconsin Meeting,

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A number of scholarship awards were also presented to students.

The WAMFS-WEHA Joint Committee on Education reported that a Master's Degree program in Environmental Health will be implemented in the University of Wisconsin system starting in September, 1980.

CLASSIFIED ADS

Position Available

Academic staff position — University of Wisconsin-Eau Claire inviting applications for academic position teaching graduate and undergraduate in Environmental and Public Health program. Position for academic year August 25, 1980 to May 24, 1981, with possibility of renewal. Qualifications: Doctorate in Public Health or DMV/MD with a masters in Public Health area with emphasis in administration: Salary negotiable; vita, complete official transcripts, three letters of recommendations required. Apply to Dr. John B. Gerberich, chairman, Division of Allied Health professions, University of Wisconsin-Eau Claire, Eau Claire, WI 54701.

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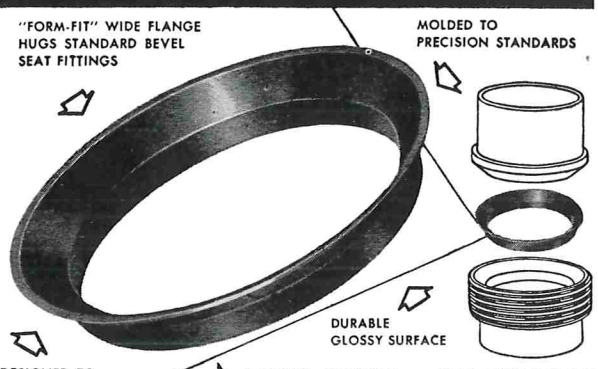

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NEOPRENE GASKET for Sanitary Fittings

Check these SNAP-TITE *Advantages*

- Tight joints, no leaks, no shrinkage
- Sanitary, unaffected by heat or fats
- Non-porous, no seams or crevices
- Odorless, polished surfaces, easily cleaned
- Withstand sterilization
- Time-saving, easy to assemble
- Self-centering
- No sticking to fittings
- Eliminate line blocks
- Help overcome line vibrations
- Long life, use over and over

Available for 1", 1½", 2", 2½" and 3" fittings.
Packed 100 to the box. Order through your dairy supply house.

THE HAYNES MANUFACTURING CO.
4180 Lorain Avenue • Cleveland 13, Ohio

HAYNES
SELF-CENTERING
SNAP-TITE® *Gaskets*

SIZES 1" - 1½"
2" - 2½" - 3" - 4"

*MADE FROM
TEFLON®

"The Sophisticated Gasket"

THE IDEAL UNION SEAL FOR BOTH VACUUM AND PRESSURE LINES

Gasket Color . . . slightly off-white

SNAP-TITE self-centering gaskets of TEFLON are designed for all standard bevel seat sanitary fittings. They SNAP into place providing self-alignment and ease of assembly and disassembly.
HAYNES SNAP-TITES of TEFLON are unaffected by cleaning solutions, steam and solvents. They will not embrittle at temperatures as low as minus 200° F. and are impervious to heat up to 500° F.

FOR A FITTING GASKET THAT WILL OUT-PERFORM ALL OTHERS...

Specify . . . HAYNES SNAP-TITES of TEFLON

- *TEFLON ACCEPTED SAFE FOR USE ON FOOD & PROCESSING EQUIPMENT BY U. S. FOOD AND DRUG ADMINISTRATION*

* Gaskets made of DuPont TEFLON ® TFE-FLUOROCARBON RESINS

THE HAYNES MANUFACTURING COMPANY
4180 LORAIN AVENUE • CLEVELAND, OHIO 44113

A HEAVY DUTY SANITARY LUBRICANT

Available in both
SPRAY AND TUBE

All Lubri-Film ingredients are approved additives and can be safely utilized as a lubricant for food processing equipment when used in compliance with existing food additive regulations.

ESPECIALLY DEVELOPED FOR LUBRICATION OF FOOD PROCESSING AND PACKAGING EQUIPMENT

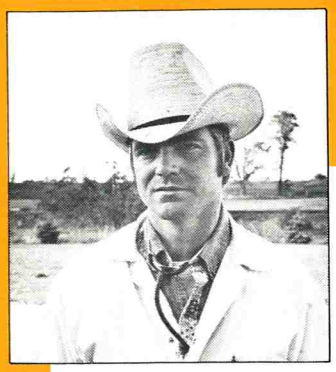
For Use in Dairies — Ice Cream Plants — Breweries — Beverage Plants — Bakeries — Canneries — Packing Plants

SANITARY • NON TOXIC • ODORLESS • TASTELESS

SPRAY — PACKED 6 — 16 OZ. CANS PER CARTON
TUBES — PACKED 12 — 4 OZ. TUBES PER CARTON

THE HAYNES MANUFACTURING CO.
CLEVELAND, OHIO 44113

SOMEONE YOU SHOULD KNOW IN THE DAIRY INDUSTRY



Dr. Bruce Beachnau, Veterinarian

Dr. Beachnau is an active member of many professional veterinary organizations. He began his current practice, the Portland (Michigan) Veterinary Service in 1967. It has grown to four veterinarians serving more than 600 dairymen and 5,000 cows in a thirty mile radius from Portland. The practice is a three-way partnership with Dr.'s Ken McCrumb and Gerald Segerland and services both domestic and farm animals. It is based on scheduled herd health which has helped dairymen avoid problems through the use of preventive medicine. Dr. Beachnau offers his views on the importance of on-going herd health programs.

Ever since I was a boy and milking cows on my dad's farm, I saw the importance of taking care of them properly. Taking care of them means more than just milking and feeding, and that's even more important with the improved cows and higher efficiency of today's dairy industry. If a dairyman wants to make money, given today's costs, he just can't afford to go without the services of a veterinarian on a regular herd health program. I have had dairymen tell me, "I don't have a herd health problem." Most of these dairymen don't realize they have a problem until we point out certain factors like cows that he thinks are bred and aren't which are costing him money every day. And subclinical mastitis problems resulting in lost milk and slower milking.

What We Do

We get personally involved in the dairyman's operation. Our goal is to catch a problem before it can get a solid foothold on a herd. We look at everything from sanitation to milking equipment, feeding program and record keeping. We also look at the breeding program and give recommendations on improving any part which is lacking. We encourage dairymen to get on a herd health program with visits at least every four weeks. On larger herds of more than 150, we recommend a visit once every two weeks to assure every cow gets enough attention to avoid problems.

Our program includes the following services:

- Checking every cow which freshened sinced last visit.
- Checking for mastitis and infected udders.
- Checking entire reproductive tract for abnormalities, cystic ovaries, retained placentas, infections, etc.
- Check bred cows for pregnancy.
- Examine cows that are return breeders.
- De-horn calves at four to eight weeks of age.
- Vaccinate for Bangs at two to seven months of age.
- Check feeding program.
- Discuss any problems cropping up since last visit.
- Look at milk production records for sudden drops.

In addition to these other services, we culture mastitis problem cows and recommend dry cow treatment. We also want to find out why these cows have mastitis to make sure there isn't a condition that could spread. Sometimes an inadequate or worn out milking system is a cause. A few years ago, I'd say about 70% of our herds had this problem; today, in our area, it's probably down to about 40% operating with less than capacity systems.

Not An Easy Way To Go But Worth It

If you think a herd health program makes life easier, it's not so. Like anything worthwhile, it takes planning and extra effort to make herd health productive. The first three or four months dairymen become very irritated with the program, but when they start planning for the day of our visit, they get in the groove and realize its importance. An average stop takes about two hours and many dairymen feel it's the most valuable two hours they can spend because it's preventive medicine.

On most herds we see rolling herd production go up at least 1,000 pounds of milk or more by simply clearing up the reproductive tract which all means more milk and more money for the dairyman.

I have seen that every dollar a dairyman invests in a herd health program should return between $20 and $30 in higher income. That alone makes the program one of the best investments he can make. A definition of what our ultimate goal is would be something like: To return the most pounds of milk per cow per year and assure that we can raise every calf for the most dollar giving the dairyman the highest return on his investment of labor and time. A dairyman works very hard to make a living, and his returns should be as high as good planning and management can provide. We want to help him make money, and I don't want to see anyone go out of business because I haven't done everything in my power to help. We work closely with other people and organizations in an effort to help the dairyman. For instance, if the milking equipment is causing a problem we work with the dairy equipment dealer to correct the situation. No, it's not easy to coordinate a herd health program, but it can make those long hours in the milking parlor pay bigger dividends.