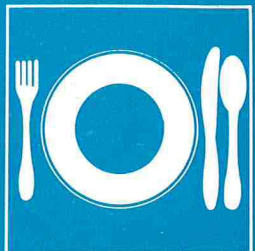
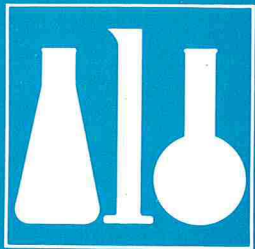


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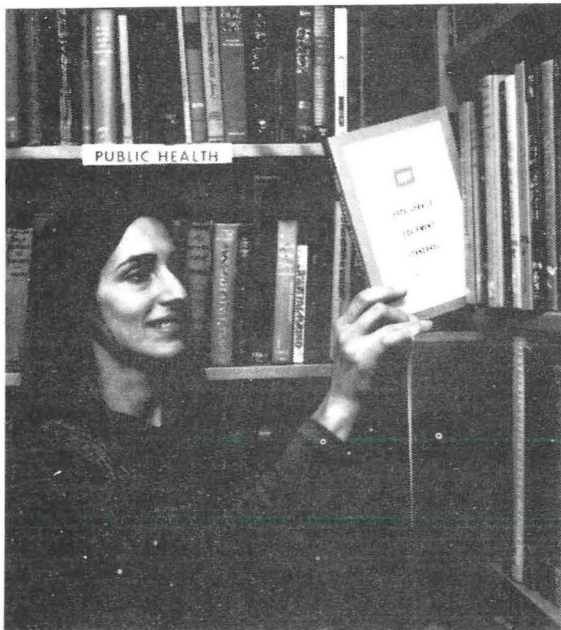


*Journal of Food Protection*TM

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National Mastitis Council
Annual Meeting
February 22, 23, 1977
Executive Inn
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NATIONAL MASTITIS COUNCIL ANNUAL MEETING

February 22-23, 1977

EXECUTIVE INN — LOUISVILLE, KENTUCKY

Everyone interested in prevention and control of bovine mastitis is cordially invited to attend the 16th Annual Meeting of the National Mastitis Council.

Vice President and Program Chairman James R. Welch has planned an outstanding program for this 16th Annual Meeting. Subject matter covers all segments of the mastitis problem, and this varied program will be of interest to veterinarians, researchers, extension dairy specialists, producers of products for treatment and control of mastitis, as well as dairy farmers.

Feature of the annual meeting will be program presentations by Dr. John Milne, National Dairy Laboratory, Ruakura Agricultural Centre, Hamilton, New Zealand. An international authority in the field of bovine mastitis, Dr. Milne will discuss natural defense mechanisms against mastitis which opens a different avenue of thought in the fight against this disease. Details of mastitis control in New Zealand will be discussed also.

Jack Linkletter, well known figure in sales promotion and consultant circles, who has wide experience in American agriculture, is the keynote speaker.

The role of milking machines in relation to the problem of mastitis will be handled by Steve Spencer, Extension Dairy Specialist, Penn State University. Dr. William Bickert, Michigan State University, will discuss automatic take-offs in relation to overmilking, and Dr. John H. Nicolai jr., Ellicott City, Maryland, will discuss vacuum level at the teat end.

Other subjects of vast interest include discussions of the new FDA Treatment Guidelines by Dr. Ann Holt, BVM, FDA; interaction of factors predisposing to mastitis by Dr. Paul D. Thompson, USDA, ARS; milk quality from the processor point of view by Don Gregg; and quality premium programs and the dairyman by Melvin Leppo.

A panel on prevention and control of udder infections will include a veterinarian — Dr. Jan H. Pol; a dairyman — David Thuemmel; and dairy cooperative representatives — Sid Beale and Lowell Allen. The Tuesday evening program chaired by NMC Research Committee Chairman Dr. James W. Smith will include panelists Dr. E. V. Caruolo — Antibiotic Residues; Dr. R. J. Eberhart — Coliform; Dr. W. Nelson Philpot — Teat Dips; Dr. C. C. Miller — Infusion Products.

Make your plans to attend this excellent meeting. It will start at 8:45 a. m. on February 22 and will adjourn at noon on February 23. Request advance registration form from the National Mastitis Council, 910 — 17th Street, NW, Washington, DC 20006.

Send request for room reservation directly to the Executive Inn, Watterson Expressway at Fairgrounds, Louisville, KY 40213. Ask for NMC special rate.

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Vol. 40

January, 1977

No. 1

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The Journal of Food Protection

Appearance of this first issue of the *Journal of Food Protection* marks the onset of a new era in publication of a scientific journal by the International Association of Milk, Food, and Environmental Sanitarians. The *Journal of Food Protection* has evolved from the *Journal of Milk and Food Technology* which, in turn, had evolved from the *Journal of Milk Technology*.

For purposes of this journal, "food protection" will be defined in its broad sense. Virtually everything that is done to a food from the time it is harvested or obtained from an animal (and sometimes even before it is harvested or while it is still "on the hoof") to the time it is consumed is done to protect the food. Handling and processing is done to protect food from microbial or other contamination, to protect food from microbial or other spoilage, to protect the nutritive and sensory qualities of food, and to protect the consumer from any illnesses that may be transmitted by food. Hence, papers on virtually all topics in food science, including the foodservice industry, will be considered for publication in the *Journal of Food Protection*.

Major emphases of the journal will be on: (a) cause and control of all forms (chemical, microbial, natural toxicants) of foodborne illness, (b) contamination (chemical, microbial, insects, rodents, etc.) and its control in raw foods and in foods during processing, distribution and preparing and serving to consumers, (c) causes of food spoilage and its control through processing (low temperatures, high temperatures, preservatives, drying, fermentation, etc.), (d) food quality and chemical, microbiological and physical methods to measure the various attributes of food quality, (e) the foodservice industry, and (f) wastes from the food industry and means to utilize or treat the wastes.

Review papers as well as reports of original research will be considered for publication. A well written, thorough, and interpretive review article is invaluable for many readers as a means to update their knowledge in a specialized area of food science. Consequently, review papers will receive the same careful attention as is given to research papers. Complete details about preparation of manuscripts will appear in the February issue of the journal.

ELMER H. MARTH

Editor

Journal of Food Protection

Effect of Low Temperatures on Growth of *Clostridium botulinum* Spores in Meat of the Blue Crab

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(Received for publication June 17, 1976)

ABSTRACT

The ability of unheated and heated spores of *Clostridium botulinum* types B, E, and F to grow and produce toxin in crabmeat from the blue crab at low temperatures was investigated. Sterilized crabmeat was seeded with 10^3 unheated spores/g or 10^4 heated spores/g and incubated anaerobically at 4, 8, 12, and 26 C. Broth cultures served as controls. Both unheated and heated spores of the three strains grew and produced toxin in crabmeat at 26 C in 3 and 6 days, respectively. In addition, unheated spores of the nonproteolytic type E strain grew and produced toxin in crabmeat at 12 C in 14 days. Neither heated spores of type E nor heated or unheated spores of types B and F grew in crabmeat at any refrigerated temperature within 180 days.

Crabmeat of the blue crab (*Callinectes sapidus*) is marketed as either a fresh or a pasteurized product. As a fresh product, the live crabs are either steamed in retorts at about 250 F or are boiled. The cooked meat is picked by hand into containers. The crabmeat is kept refrigerated for 7-14 days, after which its microflora render it unusable. The pasteurized product is similarly prepared but is then packed in hermetically sealed cans, which are heated in a water bath to an internal temperature of 180 F or over. This treatment destroys most of the spoilage microorganisms, thus extending the shelf life of the crabmeat to 6 months under adequate refrigeration (1, 5, 7).

Clostridium botulinum has been found both in the marine environments inhabited by the blue crab (2, 9, 10) and in the blue crab itself (6, 9, 11). Recently in our laboratories we have isolated *C. botulinum* types B and E from commercially packed fresh blue crabmeat and proteolytic type F from the pasteurized product (4).

Since nonproteolytic strains of *C. botulinum* are known to proliferate and produce toxin at temperatures as low as 3.3 C (2, 3, 5, 8), the purpose of this investigation was to determine whether unheated and/or heated spores of *C. botulinum* in crabmeat will grow and produce toxin at refrigeration temperatures.

MATERIALS AND METHODS

Cultures

The strains of *C. botulinum* used in these experiments were as follows. The nonproteolytic type E strain, G 21-5, was isolated in

our laboratory from gills of a blue crab from the James River. The proteolytic type B strain, 115B, was obtained from National Canners Association and the proteolytic type F strain, PC-F, was isolated in the FDA Atlanta District laboratories from commercially pasteurized crabmeat. The spore suspensions were grown in trypticase-peptone-glucose-yeast extract (TPGY) broth for 5 days at 35 C (proteolytic strains) and at 26 C (nonproteolytic strain). They were harvested by centrifugation, washed three times with sterile distilled water and resuspended in the water.

Sample preparation

One-pound portions of commercial fresh-picked crabmeat were autoclaved for 40 min in stainless steel containers and cooled in ice-water baths, and three 5-g portions from each container were tested for sterility. A spore suspension of the strain tested was mixed with the crabmeat, by means of large, sterile forks, to give a final concentration of 2×10^3 spores/g of crabmeat, and 10 g of seeded crabmeat was transferred into 16 x 125-mm sterile test tubes. Similar test tubes containing 10 ml of TPGY broth plus an inverted fermentation tube were inoculated with the same spore suspension to give the same concentration of spores. The following variations were introduced for experiments in which the crabmeat was heated after seeding with spores, to simulate pasteurization treatment. The inoculum level was raised in expectation of a drop during the heating process. The proteolytic strains of types B and F were mixed with the crabmeat to give a final concentration of 2×10^4 spores/g and the nonproteolytic type E strain to give a final concentration of 2×10^5 spores/g. After the tubes of crabmeat and TPGY broth were prepared as described above, they were placed in a 180 F water bath, monitored by copper-constantan thermocouples. The tubes with the proteolytic types B and F strains were kept in the water bath for 6 min after the crabmeat reached 180 F. The nonproteolytic, type E strain was kept in the water bath until the crabmeat reached 180 F and then immediately removed to an ice water bath. The inoculum of proteolytic types B and F strains did not diminish after 6 min at 180 F but the initial inoculum of 2×10^5 spores of the nonproteolytic type E strain dropped during heating to 2×10^3 spores/g of crabmeat and 2×10^4 spores/ml of broth.

Thirty tubes with crabmeat and 30 tubes with broth were placed in a 2-gallon Case anaerobic jar; two BBL GasPaks, an indicator, and a catalyst were placed inside, and the jar was closed tight. After 10-12 min at room temperature, during which anaerobic conditions were established (as evidenced by steaming of the jar walls, warming up of the jar top, and reduction of the indicator), the jar was placed in a carefully controlled incubator of the appropriate temperature. One jar was placed at 26 C as the positive control and one jar of each strain at 12, 8, and 4 C for the experimental temperatures.

To determine growth and toxin production, three tubes of each preparation were removed from the jar at various time intervals, the jar was immediately closed, its atmosphere was reduced as originally described, and the jar was returned to its appropriate temperature. One gram of crabmeat and 1 ml of culture from each tube were assayed for

growth by the three-tube MPN method. The remaining 9 g of crabmeat was extracted with 9 ml of gel-phosphate buffer and appropriate dilutions were injected into protected and unprotected mice. (Water was used for type E extraction, since it was subsequently trypsinized.) Dilutions of broth cultures were similarly injected into mice.

The experiment was designed for 180 days, the period of refrigerated storage of commercially pasteurized crabmeat.

RESULTS AND DISCUSSION

The experimental results are summarized in Tables 1 and 2, where G (growth) reflects a 2-3 log increase in the number of organisms and T the production of toxin.

The unheated spores of all three strains (Table 1) grew and produced toxin in broth as well as in crabmeat at 26 C in 3 days. In addition the nonproteolytic type E strain grew and produced toxin in broth at 12 and 8 C in 14 days, and at 4 C in 52 days. In crabmeat it grew and produced toxin at 12 C in 14 days, but not at any lower

temperature within 180 days. The proteolytic strains of types B and F, in addition to the 26-C positive control grew and produced toxin in broth only at 12 C in 110 days but not at any lower temperature or in crabmeat within 180 days.

In the heated series (Table 2) the spores of the nonproteolytic type E strain grew and produced toxin in broth at 26 C in 6 days, at 12 C in 20 days, and at 8 and 4 C in 30 days. In crabmeat they grew and produced toxin at 26 C in 6 days, but not at any lower temperature within 180 days. With type E only the inoculum of 2×10^3 spores/g of crabmeat decreased to the point at which, after 20 days, no viable organisms could be found in the crabmeat at any low temperature.

The heated spores of the proteolytic types B and F strains grew and produced toxin in broth at 26 C in 6 days and at 12 C in 60 days, but not at any lower

TABLE 1. Growth and toxin production of unheated *C. botulinum* spores in crabmeat and broth^a

Time (days)	26 C		12 C		8 C		4 C	
	Broth	Crabmeat	Broth	Crabmeat	Broth	Crabmeat	Broth	Crabmeat
Type B								
3	G, T	G, T	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—
52	—	—	—	—	—	—	—	—
110	—	—	G, T	—	—	—	—	—
Termination:	180 days							
Type E								
3	G, T	G, T	—	—	—	—	—	—
14	—	—	G, T	G, T	G, T	—	—	—
52	—	—	—	—	—	—	G, T	—
110	—	—	—	—	—	—	—	—
Termination:	180 days							
Type F								
3	G, T	G, T	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—
110	—	—	G, T	—	—	—	—	—
Termination:	180 days							

^aG = growth; T = toxin.

TABLE 2. Growth and toxin production of heated *C. Botulinum* spores in crabmeat and broth^a

Time (days)	26 C		12 C		8 C		4 C	
	Broth	Crabmeat	Broth	Crabmeat	Broth	Crabmeat	Broth	Crabmeat
Type B								
6	G, T	G, T	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—
30	—	—	—	—	—	—	—	—
60	—	—	G, T	—	—	—	—	—
Termination:	180 days							
Type E								
6	G, T	G, T	—	—	—	—	—	—
20	—	—	G, T	—	—	—	—	—
30	—	—	—	—	G, T	—	G, T	—
60	—	—	—	—	—	—	—	—
Termination:	180 days							
Type F								
6	G, T	G, T	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—
30	—	—	—	—	—	—	—	—
60	—	—	G, T	—	—	—	—	—
Termination:	180 days							

^aG = growth; T = toxin.

temperature within 180 days. In crabmeat they grew and produced toxin at 26 C in 6 days, but not at any lower temperature within 180 days. The inoculum remained unchanged in the crabmeat at 4 and 8 C throughout the experiment but at 12 C it dropped three logs after 150 days.

Heat resistance studies done in our laboratory (to be published) indicate that pasteurization of crabmeat, as practiced by the crabmeat industry, destroys spores of the nonproteolytic type E strains but does not affect spores of the proteolytic strains of types B and F. However, surviving spores of these strains do not grow in crabmeat under adequate refrigeration (4-8 C). On the basis of these experiments, storage of pasteurized crabmeat for a 6-months' period under strictly controlled refrigeration would not appear to be a public health hazard.

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Survival of *Vibrio parahaemolyticus* in Various Diluents

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ABSTRACT

Survival of *Vibrio parahaemolyticus* in various diluents was investigated. 100 mM potassium phosphate buffers (pH 6, 7, and 8) without added NaCl were extremely deleterious to *V. parahaemolyticus* 04:K11 in comparison to the same diluent with either 0.5, 3, or 6% NaCl. Maximum counts were obtained when phosphate buffers containing 3% NaCl were used as diluents. There were no significant differences among survivor counts of cells exposed for 20 min to 100 mM potassium phosphate-3% NaCl buffer at either pH 6, 7, or 8. Greater than 99.9% of the cell population was destroyed within 20 min in 0.1% peptone diluent without NaCl when compared to 0.1% peptone with either 0.5, 3, or 6% NaCl. Cell counts using 0.1% peptone diluents containing 3 and 6% NaCl were not significantly different; however, both were superior to 0.1% peptone containing 0.5% NaCl. Survival data for *V. parahaemolyticus* 04:K11 in distilled water containing 0, 0.5, 3, and 6% added salt were similar to data for the 0.1% peptone diluents. Culture age (4, 10, and 24 h) and time of exposure to the diluents also significantly affected survival of *V. parahaemolyticus* 04:K11 and 17802.

Studies on survival of bacteria in various diluents have raised questions concerning the suitability of dilution fluids such as water, saline, and phosphate buffer, commonly used in making bacterial counts. Straka and Stokes (8) and King and Hurst (5) have reported that loss of viability during dilution can be avoided by use of 0.1% peptone as a general purpose diluent. It is also particularly important in studies on bacterial injury that the diluent be optimal with regard to survival and that it does not serve as an added stress to injured cells.

Vibrio parahaemolyticus requires NaCl for growth and for viability (1, 2, 3). Growth of this microorganism has been observed at NaCl concentrations from 1 to 7% and at pH values from 5 to 11 (7). The osmotic fragility of this microorganism has been the basis of the recommendation of public health authorities of Japan that seafoods and utensils be thoroughly washed with tap water (9). Lee (6), reporting on a study involving three strains of *V. parahaemolyticus*, observed that 90% inactivation of cells (4-h culture) occurred within 4 min in distilled water, within 11 min in 0.2% peptone, and within 7 min in phosphate buffer.

The effect of various types of diluents on survival of different age cultures of *V. parahaemolyticus* was investigated in this study. The effect of length of time in diluent on cell survival was also examined.

MATERIALS AND METHODS

Stock slants of *V. parahaemolyticus* strains 04:K11 and ATCC 17802 were made on Trypticase Soy Agar (Baltimore Biological Laboratory) with an additional 2.5% NaCl (3 TSAS) and held at room temperature. "Working stock" cultures of the organisms were maintained in Trypticase Soy Broth (BBL) with an additional 2.5% NaCl (3 TSBS) held at room temperature. Stock slants were transferred at 4-week intervals and fresh "working stock" cultures for use in experiments were prepared at weekly intervals using inocula from the stock slants.

Cultures were prepared for use in experiments in the following manner: a transfer (0.04 ml) was made from the "working stock" culture to another tube of 3 TSBS which was incubated at 35 C for 12 h. This 12-h culture was then transferred (0.04 ml) to another tube of 3 TSBS which was incubated at 35 C for 3 h. The 3-h culture was used to inoculate (0.04 ml) the growth flask which contained 125 ml of 3 TSBS.

The temperature of the growth flask was held at 35 ± 0.05 C by means of a Haake Model E 52 constant temperature circulator mounted in an insulated water bath. Continuous mixing of the cultures was accomplished with a submersible magnetic stirrer (Cole-Parmer Instrument Co., Chicago, Ill.) placed in the water bath.

V. parahaemolyticus 04:K11 was grown as described above. One ml culture samples were removed from the growth flask at 4, 10, and 24 h and held 20 min in 99-ml dilution blanks. Separate experiments were done for each of the following sets of diluents: (a) 100 mM potassium phosphate buffer (pH 6.0) containing 0, 0.5, 3.0, and 6.0% NaCl; (b) 100 mM potassium phosphate buffer (pH 7.0) containing 0, 0.5, 3.0, and 6.0% NaCl; (c) 100 mM potassium phosphate buffer (pH 8.0) containing 0, 0.5, 3.0, and 6.0% NaCl; (d) 100 mM potassium phosphate-3% NaCl buffers, pH 6.0, 7.0, and 8.0; (e) 0.1% peptone containing 0, 0.5, 3.0, and 6.0% NaCl; and (f) distilled water containing 0, 0.5, 3.0, and 6.0% NaCl.

In the next phase of the diluent study, *V. parahaemolyticus* strains 04:K11 and ATCC 17802 were grown as just described. One-ml culture samples were removed from the growth flasks at 4, 10, and 24 h and held for 15, 30, 45, and 60 min in 99-ml dilution blanks containing the following diluents: (a) 100 mM potassium phosphate-3% NaCl buffer (pH 7.0); (b) 0.1% peptone-3% NaCl; and (c) distilled water-3% NaCl. The 4, 10, and 24-h cultures of 04:K11 and ATCC 17802 represented cells in the late logarithmic, stationary, and death phases of growth, respectively (B. S. Emswiler, Ph.D. thesis, VPI and SU, Blacksburg, Va. 24061).

The assay medium was 3 TSAS (tempered at 45 C). Pour plates were overlaid and incubated at 35 C for 48 h. All plate counts were done in triplicate. The data were subjected to statistical analysis using analysis of variance and Duncan's (4) multiple range test.

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RESULTS AND DISCUSSION

The effect of various concentrations of NaCl on survival of *V. parahaemolyticus* 04:K11 in 100 mM potassium phosphate buffer at pH 6.0, 7.0, and 8.0 after a 20-min exposure time are presented in Tables 1, 2, and 3. The phosphate buffer diluents (pH 6.0, 7.0, and 8.0) without

TABLE 1. Survival of *Vibrio parahaemolyticus* 04:K11 held for 20 min in 100 mM potassium phosphate buffer (pH 6.0) containing various concentrations of NaCl

Age of culture (h)	Diluent ^{1,2}			
	0% NaCl	0.5% NaCl	3.0% NaCl	6.0% NaCl
4	2.9 × 10 ⁴ a	7.4 × 10 ⁷ b	8.9 × 10 ⁷ b	8.2 × 10 ⁷ b
10	1.2 × 10 ⁸ a	3.9 × 10 ⁸ b	5.2 × 10 ⁸ c	4.5 × 10 ⁸ bc
24	7.6 × 10 ⁴ a	1.9 × 10 ⁸ b	2.1 × 10 ⁸ b	2.0 × 10 ⁶ b

¹Each value represents the number of cells per ml of original culture using the indicated diluent.

²Numbers in the same row having different lower case letters attached are significantly different at the level P ≤ 0.01.

TABLE 2. Survival of *Vibrio parahaemolyticus* 04:K11 held for 20 min in 100 mM potassium phosphate buffer (pH 7.0) containing various concentrations of NaCl

Age of culture (h)	Diluent ^{1,2}			
	0% NaCl	0.5% NaCl	3.0% NaCl	6.0% NaCl
4	1.2 × 10 ² a	7.3 × 10 ⁷ b	8.6 × 10 ⁷ c	3.0 × 10 ⁷ d
10	3.8 × 10 ⁷ a	6.2 × 10 ⁸ c	5.1 × 10 ⁸ bc	4.8 × 10 ⁸ b
24	3.8 × 10 ⁴ a	1.7 × 10 ⁸ b	1.9 × 10 ⁸ b	1.7 × 10 ⁸ b

¹Each value represents the number of cells per ml of original culture using the indicated diluent.

²Numbers in the same row having different lower case letters attached are significantly different at the level P ≤ 0.01.

TABLE 3. Survival of *Vibrio parahaemolyticus* 04:K11 held for 20 min in 100 mM potassium phosphate buffer (pH 8.0) containing various concentrations of NaCl

Age of culture (h)	Diluent ^{1,2}			
	0% NaCl	0.5% NaCl	3.0% NaCl	6.0% NaCl
4	3.1 × 10 ⁵ a	1.2 × 10 ⁸ b	1.2 × 10 ⁸ b	8.9 × 10 ⁷ c
10	8.2 × 10 ⁷ a	3.1 × 10 ⁸ b	3.0 × 10 ⁸ b	3.3 × 10 ⁸ c
24	5.2 × 10 ⁴ a	1.8 × 10 ⁸ b	1.9 × 10 ⁸ b	1.9 × 10 ⁸ b

¹Each value represents the number of cells per ml of original culture using the indicated diluent.

²Numbers in the same row having different lower case letters attached are significantly different at the level P ≤ 0.01.

added NaCl were extremely deleterious to *V. parahaemolyticus* 04:K11, causing population reductions of 90-99.9% within the 20-min exposure time in the diluent in comparison to the same diluents containing either 0.5, 3, or 6% NaCl. These results were not surprising since *V. parahaemolyticus* is a marine organism and requires NaCl for growth and viability (7). The younger cells (4-h cultures) in the logarithmic phase of growth were generally more susceptible to the absence of NaCl in the diluent than were cells in the stationary phase (10-h cultures) or the death phase (24-h cultures). There were some significant differences among survivor counts in 100 mM potassium phosphate buffer (pH 6.0, 7.0, and 8.0) containing 0.5, 3, and 6% NaCl. Overall, phosphate buffer diluents containing 3% NaCl resulted in the highest plate counts. The effect of different pH values on survival of *V. parahaemolyticus* 04:K11 in 100 mM potassium phosphate-3% NaCl buffer is presented in Table 4. There were no significant

differences among survivor counts of cells exposed for 20 min to 100 mM potassium phosphate-3% NaCl buffer at either pH 6.0, 7.0, or 8.0.

TABLE 4. Survival of *Vibrio parahaemolyticus* 04:K11 held for 20 min in 100 mM potassium phosphate-3% NaCl buffer at different pH values

Age of culture (h)	Diluent ^{1,2}		
	pH 6.0	pH 7.0	pH 8.0
4	2.2 × 10 ⁸ a	2.4 × 10 ⁸ a	2.5 × 10 ⁸ a
10	2.2 × 10 ⁸ a	2.0 × 10 ⁸ a	2.1 × 10 ⁸ a
24	9.9 × 10 ⁴ a	9.9 × 10 ⁴ a	1.1 × 10 ⁵ a

¹Each value represents the number of cells per ml of original culture using the indicated diluent.

²Numbers in the same row having different lower case letters attached are significantly different at the level P ≤ 0.01.

TABLE 5. Survival of *Vibrio parahaemolyticus* 04:K11 held for 20 min in 0.1% peptone diluent containing various concentrations of NaCl

Age of culture (h)	Diluent ^{1,2}			
	0% NaCl	0.5% NaCl	3.0% NaCl	6.0% NaCl
4	<10 ² a**	9.7 × 10 ⁷ b**	1.8 × 10 ⁸ c*	1.5 × 10 ⁸ c**
10	<10 ⁴ a*	2.4 × 10 ⁸ b*	2.9 × 10 ⁸ c*	2.5 × 10 ⁸ b*
24	<10 ³ a*	5.4 × 10 ⁵ b*	7.4 × 10 ⁵ c*	6.1 × 10 ⁵ bc*

¹Each value represents the number of cells per ml of original culture using the indicated diluent.

²Numbers in the same row having different lower case letters attached are significantly different at the levels *P ≤ 0.05 and **P ≤ 0.01.

Survival of *V. parahaemolyticus* 04:K11 in 0.1% peptone diluent containing various concentrations of NaCl is shown in Table 5. The viability of greater than 99% of the cells was lost within 20 min when dilutions were done with 0.1% peptone-0% NaCl in comparison to 0.1% peptone containing either 0.5, 3, or 6% NaCl. Survival of cultures of all ages was significantly greater in 0.1% peptone-3% NaCl than in 0.1% peptone-0.5% NaCl. In general, 0.1% peptone-3% NaCl diluent was also superior to 0.1% peptone-6% NaCl diluent.

TABLE 6. Survival of *Vibrio parahaemolyticus* 04:K11 held for 20 min in distilled water diluent containing various concentrations of NaCl

Age of culture (h)	Diluent ^{1,2}			
	0% NaCl	0.5% NaCl	3.0% NaCl	6.0% NaCl
4	<10 ² a	2.2 × 10 ⁸ b	2.5 × 10 ⁸ b	2.5 × 10 ⁸ b
10	<10 ² a	1.9 × 10 ⁸ b	2.6 × 10 ⁸ c	2.5 × 10 ⁸ c
24	<10 ² a	3.8 × 10 ⁴ b	5.8 × 10 ⁴ c	6.1 × 10 ⁴ c

¹Each value represents the number of cells per ml of original culture using the indicated diluent.

²Numbers in the same row having different lower case letters attached are significantly different at the level P ≤ 0.01.

Table 6 presents data on enumeration of *V. parahaemolyticus* 04:K11 using distilled water diluents containing various concentrations of NaCl. Bacterial destruction was rapid in distilled water containing no NaCl, with greater than 99% of the population being destroyed within the 20 min exposure time when compared to diluents containing 0.5, 3, or 6% NaCl. Lee (6) reported 90% inactivation of *V. parahaemolyticus* cells (4-h culture) within 4 min in distilled water. There were no significant differences between the number of survivors in 3% and 6% NaCl for all culture ages. Counts obtained using either 3 or 6% NaCl diluents were

significantly higher than with the 0.5% NaCl diluent for 10- and 24-h cultures.

Data from Tables 1-6 showed that 100 mM potassium phosphate-3% NaCl buffer (pH 7.0), 0.1% peptone-3% NaCl and distilled water-3% NaCl were apparently satisfactory diluents for *V. parahaemolyticus* 04:K11 within each experiment. The comparative effects of these three diluents on survival of *V. parahaemolyticus* 04:K11 and *V. parahaemolyticus* ATCC 17802, after exposure to the diluents for 15, 30, 45 and 60 min, are given in Tables 7 and 8. A holding time in the diluents of less than 15

TABLE 7. Survival of *Vibrio parahaemolyticus* 04:K11 in three diluents after different exposure times

Age of culture (h)	Exposure time (min)	Diluent ^{1,2}		
		100 mM phosphate-3% NaCl (pH 7.0)	0.1% peptone-3% NaCl	Distilled water-3% NaCl
4	15	2.3 × 10 ⁸ a	2.5 × 10 ⁸ b	1.9 × 10 ⁸ c
4	30	2.5 × 10 ⁸ a	3.1 × 10 ⁸ b	1.9 × 10 ⁸ c
4	45	2.5 × 10 ⁸ a	3.5 × 10 ⁸ b	1.7 × 10 ⁸ c
4	60	3.0 × 10 ⁸ a	4.7 × 10 ⁸ b	1.9 × 10 ⁸ c
10	15	3.8 × 10 ⁸ a	3.4 × 10 ⁸ a	3.6 × 10 ⁸ a
10	30	4.4 × 10 ⁸ a	4.1 × 10 ⁸ a	3.5 × 10 ⁸ a
10	45	4.2 × 10 ⁸ a	4.6 × 10 ⁸ a	3.5 × 10 ⁸ a
10	60	3.7 × 10 ⁸ a	3.6 × 10 ⁸ a	3.9 × 10 ⁸ a
24	15	6.0 × 10 ⁵ a	5.6 × 10 ⁵ a	5.7 × 10 ⁵ a
24	30	6.7 × 10 ⁵ a	6.1 × 10 ⁵ a	6.5 × 10 ⁵ a
24	45	5.6 × 10 ⁵ a	5.4 × 10 ⁵ a	6.0 × 10 ⁵ a
24	60	6.1 × 10 ⁵ a	6.2 × 10 ⁵ a	6.0 × 10 ⁵ a

¹Each value represents the number of cells per ml of original culture using the indicated diluent.

²Sets of numbers in the same row having different lower case letters attached are significantly different at the level of $P \leq 0.01$.

TABLE 8. Survival of *Vibrio parahaemolyticus* ATCC 17802 in three diluents after different exposure times

Age of culture (h)	Exposure time (min)	Diluent ^{1,2}		
		100 mM phosphate-3% NaCl (pH 7.0)	0.1% peptone-3% NaCl	Distilled water-3% NaCl
4	15	1.2 × 10 ⁷ a**	1.2 × 10 ⁷ b**	1.1 × 10 ⁷ c**
4	30	1.4 × 10 ⁷ a**	1.5 × 10 ⁷ b**	1.2 × 10 ⁷ c**
4	45	1.4 × 10 ⁷ a**	1.8 × 10 ⁷ b**	1.1 × 10 ⁷ c**
4	60	1.4 × 10 ⁷ a**	2.0 × 10 ⁷ b**	1.1 × 10 ⁷ c**
10	15	5.3 × 10 ⁸ a*	6.0 × 10 ⁸ b*	5.7 × 10 ⁸ a*
10	30	5.3 × 10 ⁸ a*	6.1 × 10 ⁸ b*	4.6 × 10 ⁸ a*
10	45	5.5 × 10 ⁸ a*	6.9 × 10 ⁸ b*	5.6 × 10 ⁸ a*
10	60	4.9 × 10 ⁸ a*	5.9 × 10 ⁸ b*	5.8 × 10 ⁸ a*
24	15	2.0 × 10 ⁶ a**	2.0 × 10 ⁶ a**	1.9 × 10 ⁶ a**
24	30	2.0 × 10 ⁶ a**	1.9 × 10 ⁶ a**	2.0 × 10 ⁶ a**
24	45	2.0 × 10 ⁶ a**	1.9 × 10 ⁶ a**	1.9 × 10 ⁶ a**
24	60	1.9 × 10 ⁶ a**	1.9 × 10 ⁶ a**	2.0 × 10 ⁶ a**

¹Each value represents the number of cells per ml of original culture using the indicated diluent.

²Sets of numbers in the same row having different lower case letters attached are significantly different at the levels * $P \leq 0.05$ and ** $P \leq 0.01$.

min before plating was not feasible due to the time required for making dilutions and plating all samples. The 4-h cultures of both strains 04:K11 and ATCC 17802

showed significant differences in numbers among the three diluents. Numbers from 0.1% peptone-3% NaCl were significantly higher than those from 100 mM phosphate-3% NaCl (pH 7.0) which were significantly greater than those from 3% NaCl-distilled water. The distilled water-3% NaCl offered the least protection to the cells in the logarithmic growth phase. It appeared that 0.1% peptone-3% NaCl afforded the 4-h cells an opportunity for multiplication since counts for both strains 04:K11 and ATCC 17802 gradually increased with time and were higher after 60 min than after 15 min exposure to the diluent. This increase in numbers in 0.1% peptone-3% NaCl is not unusual for this particular organism since *V. parahaemolyticus* is proteolytic and has a very short generation time. Survival of the 10- and 24-h cultures of strains 04:K11 and ATCC 17802 was not affected by type of diluent or time of exposure to diluents.

Data from this study indicate that the most satisfactory diluent for *V. parahaemolyticus* 04:K11 and ATCC 17802 is 100 mM phosphate-3% NaCl at pH 7.0. Furthermore, there is no apparent effect on survival of these strains of *V. parahaemolyticus* for exposure times in the diluent of up to 60 min. The variables of different strains, growth temperature, and growth medium composition need to be considered in future studies on selection of diluents for enumeration of *V. parahaemolyticus*.

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Reliability of Selective Media for Recovery of Staphylococci from Cheese

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ABSTRACT

The reliability of 12 selective media for recovery of four different strains of *Staphylococcus aureus* inoculated at manufacture into three cheese types was determined. Selective medium and time of ripening had a highly significant effect ($p < 0.001$) on reliability of the staphylococcal count. In addition, highly significant interaction effects were observed. The most reliable medium in the overall analysis was mannitol salt agar. However, this medium was not equally reliable at all times during ripening, and use of both mannitol salt agar and *Staphylococcus* medium no. 110 is recommended. The tellurite- and azide-based selective media were generally unsatisfactory, however tellurite glycine agar, Vogel Johnson (VJ) agar, and azide blood agar base were totally unreliable. In general, the salt-based selective media were most reliable. This applied also to the egg yolk media that use salt as the selective agent. Salt egg yolk agar and Colbeck's egg yolk medium generally gave higher recoveries of *S. aureus* than did Baird-Parker medium, Crisley et al. tellurite polymyxin egg yolk agar, and Hopton egg yolk azide agar, except in the unripened cheeses. The debilitating effect of cheese ripening on the staphylococcal cells was not eliminated by the egg yolk tellurite and azide media.

Cheese has been reported as a source of staphylococcal food poisoning when made from raw or pasteurized milk (1, 8, 9, 19). Minor and Marth (15) in their review of staphylococci in dairy foods cited outbreaks, surveys, and factors influencing growth of staphylococci in cheese. Growth of staphylococci in dairy foods is generally favored by low numbers of competitive organisms and is increasingly restricted by decreasing pH. Despite this, survival times of staphylococci in cheese can be considerable, up to 26 weeks in Cheddar cheese (20), making contaminated cheeses a potential source of staphylococcal food poisoning and of *S. aureus* in food handling and preparation.

Selective and differential media for isolation and enumeration of staphylococci have an inhibiting effect on growth of some of these cells (15). This was illustrated by Stiles and Clark (17) in a study of the reliability of selective media for enumeration of unheated and heated staphylococci. However, they reported that Baird-Parker's egg yolk tellurite glycine pyruvate agar (2), and also Crisley et al.'s tellurite polymyxin egg yolk agar (6) and Hopton's egg yolk azide agar (11) gave most reliable counts of *S. aureus* when compared to counts on nonselective media. Egg yolk added to the media appeared not only to serve as a diagnostic agent, but also

to provide protection for injured cells (17).

In the instance of dariworld cheese, staphylococci surviving cheese manufacture could well be heat injured, in other instances, the pH of the cheese during ripening could serve as a debilitating factor, limiting the ability of some of these cells to grow on selective media. The object of this study was to compare the reliability of selective media for recovery of staphylococci during the ripening of cheese.

MATERIALS AND METHODS

Test organisms

Coagulase-positive, enterotoxigenic *Staphylococcus aureus* strains MF31, 196E, 21B, and 2395 used in this study were obtained from the U.S. Public Health Service (Robt. A. Taft Sanitary Engineering Center, Cincinnati, Ohio).

Culturing

Test cultures were grown in Difco tryptic soy broth (TSB), in stationary culture at 37 C. A 24-h old culture of each strain was inoculated into 10 ml of TSB, frozen, and stored at -10 C. Cultures for inoculation of the cheeses were prepared by thawing and incubating frozen cultures at 37 C, and subculturing in TSB on two successive days.

Cheesemaking

Cheese types were selected that fell within the cheese maker's laboratory cheese making competence (10). Three of these cheese varieties were selected to give a range of fat, moisture, and pH values between varieties. The cheese varieties included Cheddar; caerphilly, a traditional Welsh cheese type, described by Lewis et al. (14); and dariworld, a cheese type developed by Irvine (13) in 1956. Manufacturing procedures for all cheeses were similar to those of Cheddar, until the latter stages of manufacture, when modifications were made that gave the varietal characteristics. Manufacturing procedures were described by Hinch (10). Cheeses were manufactured in three separate 50-gal. vats on eight separate occasions, allowing two replicates of each strain of *S. aureus* with each of the three cheese varieties. The strain of *S. aureus* to be used was randomly assigned to day of manufacture. *S. aureus* cultures in TSB were inoculated into the pasteurized cheese milk at the time of adding the lactic starter culture. Inocula were adjusted to give almost equivalent concentrations of staphylococci in cheese milk. The only variations from Hinch's description of the cheese making were: (a) cheeses were ripened at 13 instead of 10 C, to hasten the ripening process, and (b) instead of vacuum packaging in 'Cryovac,' the exterior of cheeses was dried after pressing and wax dipped (20), to facilitate sampling.

Sampling

The day after manufacture, cheeses were sampled by removing aseptically three 1.25-cm cores at three separate points on the cheese,

using a sterile cheese trier. Sample holes were filled immediately with cheese wax at 120 C. Sampling was repeated at 2-week intervals during the 10-week ripening period. The three sample cores were aseptically ground, and an 11-g sample weighed for blending. The remaining cheese sample was used to determine the pH.

Bacteriological

The 11-g sample was blended with 99 ml of sterile 0.1% peptone water in a sterile Waring Blendor jar for 2 min at high speed. Appropriate dilutions of the blended sample were prepared for plating, using 9 and 99 ml of sterile, 0.1% peptone water blanks, and 0.1-ml aliquots of the dilutions were surface streaked onto prepared media, as described by Stiles and Clark (17).

Media included the following nonselective control media: nutrient agar¹ (NUT), plate count agar¹ (PCA), and tryptic soy agar¹ (TSA); and the following selective media, recommended for isolation and enumeration of *S. aureus*: (a) *azide-based media*: azide blood agar base¹ (ABA) and egg yolk azide agar (EYAA) (11); (b) *salt-based media*: Chapman Stone medium¹ (CSM), mannitol salt agar¹ (MSA), Staphylococcus medium no. 110¹ (S110), Colbeck's egg yolk medium (CEY) (5) and salt egg yolk agar (SEA) (3); (c) *tellurite-based media*: tellurite glycine agar¹ (TGA), Vogel Johnson agar¹ (VJA), egg yolk tellurite glycine pyruvate agar (Baird-Parker's medium, ETGPA) (2), tellurite egg yolk agar (TEA) (12), and tellurite polymyxin egg yolk agar (TPEY) (6). The methods of media preparation were reported by Stiles and Clark (17).

Except for Chapman Stone medium, all plates were incubated at 37 C for 48 h before counting. Chapman Stone medium was incubated at 30 C for 72 h (7). Typical *S. aureus* colonies, and atypical colonies, were randomly selected throughout the study, especially from the nonselective agar media, and checked for coagulase production using defibrinated rabbit plasma.

Analyses

Mean staphylococcal counts per gram of cheese were calculated and log₁₀ transformed. These data were subjected to a complete factorial analysis of variance (two replicates × three cheese varieties × four strains *S. aureus* + 14 media × six sampling periods) using a computer program (21). Separate factorial analyses were also done for each sampling period. Differences between means were further studied using the Duncan's multiple range test in a computer program, based on the description of this test by Steel and Torrie (16).

RESULTS AND DISCUSSION

The moisture content and pH of cheeses made for use in this study are shown in Table 1. Generally, the

TABLE 1. Moisture content and pH of cheeses compared to results reported by Hinch (10)

	Cheese type		
	Cheddar	Caerphilly	Dariworld
Moisture (%)			
Mean	36.25	43.83	46.00
Range	35.30 - 38.01	41.17 - 45.76	43.22 - 47.73
Hinch	36.7	44.9	49.4
pH after pressing			
Mean	5.22	5.28	5.70
Range	5.10 - 5.30	5.10 - 5.35	5.60 - 5.80
pH during ripening			
Mean	4.90	4.80	5.30
Range	4.80 - 5.10	4.70 - 4.90	5.20 - 5.50
Hinch	5.00	5.15	5.10

proximate analyses of the cheeses manufactured for this study compared closely with those reported by Hinch (10). Dariworld cheeses had a lower moisture content, but higher pH than reported by Hinch. The pH of caerphilly cheeses was generally equivalent to, or slightly

less than that for the Cheddar cheeses. Although the pH values did not represent a wide range, a mean pH of 5.30 for the dariworld cheeses represented a marked difference from the caerphilly and Cheddar cheeses. The moisture contents, on the other hand, represented a reasonably wide range for hard to semi-hard cheese types. The object of providing different conditions for observing staphylococci during cheese ripening was achieved with these three cheese types.

The samples were taken after pressing, and at 2-week intervals during 10 weeks of ripening at 13 C. The mean staphylococcal count was calculated from the three plates for each medium, and log₁₀ transformed for analysis. Initial counts of *S. aureus* in caerphilly and Cheddar cheeses on nonselective media, immediately after pressing, were generally 10⁷ per gram, compared to 10⁵ per gram for dariworld cheese. During ripening, counts of *S. aureus* in caerphilly cheese decreased to approximately 10² to 10³ per gram, in fact, one log cycle for each 2 week period of ripening. Counts in Cheddar cheese decreased to 10⁴ to 10⁵ per gram. Counts of *S. aureus* in dariworld cheese increased to 10⁶ per gram during ripening, and at the end of the 10-week ripening period, counts ranged between 10⁴ and 10⁶ per gram. The relatively high heat treatment of dariworld cheese (up to 54.5 C for 3 to 5 min in 5% brine) probably accounted for this difference. Increases in the counts on this cheese might represent resuscitation and not growth of the cells (17).

Similar trends were also observed for the selective media, but the counts were generally lower than those on the nonselective media. As ripening progressed, the number of "contaminants" growing on the nonselective and selective media increased, making accurate colony counting more difficult, especially on the nonselective media. Possible variants of staphylococci were tested for coagulase production to determine whether they should be included in the count. The contaminants growing on Chapman Stone medium were excessive, obstructing the accurate counting of the staphylococcal colonies. This might be attributed to the lower incubation temperature and longer incubation time, which allowed time and opportunity for contaminants to grow. Chapman Stone medium was continued throughout the study, but the results were excluded from the statistical analysis, reducing the number of media to 14. As ripening progressed, the tellurite-based, egg yolk media, especially Baird-Parker's medium, allowed growth of increased numbers of pinpoint, shiny black colonies and atypical grey colonies. This made enumeration of typical colonies difficult, especially at reliable plate counts of more than 30 typical colonies per plate. Since the test cultures grew as medium sized, shiny black colonies on Baird-Parker's medium, only typical colonies that cleared the egg yolk (except for strain 196E, which did not clear egg yolk) were counted.

The results of the factorial analysis of the data are summarized in Table 2. The main factors affecting the

¹Prepared from Difco dehydrated media, all other media were prepared according to the directions contained in the references in the text.

counts were the bacteriological plating media and the time of ripening ($p < 0.001$). The strain of *S. aureus* also had an effect ($p < 0.05$) on the reliability of the count. Highly significant interaction effects ($p < 0.001$) were also observed, including media \times strains (MS), media \times cheese variety (MC), time of ripening \times cheese variety (WC), time of ripening \times media (WM) and time of ripening \times media \times cheese variety (WMC). Differences between replicates were also tested and found to be nonsignificant.

Although cheese variety had a nonsignificant effect on plate count in the overall study, analyses for the separate sampling periods gave significant cheese variety effects for sampling periods 1 (immediately after pressing) and 6 (after 10 weeks of ripening). Results of Duncan's multiple range test indicated that for sampling period 1 counts from dariworld cheese were significantly ($p < 0.001$) lower than for other cheese types, and for sampling period 6, counts from caerphilly cheese were also significantly ($p < 0.05$) lower. This confirmed that *S. aureus* cells were killed and/or injured during manufacture of dariworld cheese, resulting in lower counts immediately after cheesemaking, and that *S.*

aureus in caerphilly cheese died at a faster rate during ripening than in the other cheese types.

Time of ripening and cheese variety (WC) had a significant ($p < 0.001$) interaction effect on the staphylococcal counts. This interaction effect may be attributed to the different trends in the counts from caerphilly cheese compared to Cheddar and dariworld cheeses. The interaction effect from caerphilly cheese was most marked in the first four sampling periods, thereafter no interaction effect was observed.

Strain of *S. aureus* had a limited effect ($p < 0.05$) on the staphylococcal counts. The effect was caused by strains 196E and 2395 having counts significantly higher than strains MF31 and 21B. The results of the Duncan's multiple range test indicated that only for the initial sampling period ($p < 0.01$) and after 2 weeks of ripening ($p < 0.05$) could strain differences be detected. This result indicated that the strain effect might have been caused by the initial level of inoculation, or different effects of cheese manufacture on the cells. Strain differences were probably unimportant relative to other factors. This was further confirmed by the nonsignificant interaction between time of ripening and strain (WS).

TABLE 2. Summary of analysis of variance of factors affecting the reliability of staphylococcus media for enumerating *S. aureus* in cheese

Source of variation	Degrees of freedom	Mean squares	F Value	Probability
Cheese variety (C)	2	50.93	1.56	NS
Strain of <i>S. aureus</i> (S)	3	136.99	4.20	*
Interaction CS	6	14.91	0.46	NS
Error (a)	12	32.61		
Staphylococcal media (M)	13	35.18	54.18	***
Interaction MS	39	1.69	2.60	***
MC	26	2.07	3.18	***
MSC	78	0.55	0.78	NS
Error (b)	156	0.65		
Weeks of ripening (W)	5	331.31	190.85	***
Interaction WS	15	2.25	1.29	NS
WC	10	38.82	22.36	***
WSC	30	2.71	1.56	NS
Error (c)	60	1.74		
Interactions WM	65	1.78	5.63	***
WMC	130	0.52	1.64	***
WMS	195	0.39	1.23	*
WMSC	390	0.32	1.01	NS
Error (d)	780	0.32		

*Significant at the 5% level.

***Significant at the 0.01% level.

TABLE 3. Rank order of the mean staphylococcal counts on the bacteriological media and results of the Duncan's test for statistical difference at the 1% level of significance^a

Sampling period	Rank order of means (lowest 1 to highest 14)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	TGA	VJA	TPEY	ABA	SEA	PCA	S110	MSA	EYAA	TEA	NUT	CEY	ETGPA	TSA
2	VJA	TGA	TPEY	SEA	TEA	EYAA	CEY	S110	ABA	ETGPA	PCA	TSA	MSA	NUT
3	TPEY	VJA	TGA	CEY	ETGPA	ABA	MSA	TEA	EYAA	SEA	PCA	TSA	S110	NUT
4	TPEY	TGA	VJA	CEY	SEA	EYAA	ETGPA	TEA	MSA	ABA	PCA	TSA	S110	NUT
5	TGA	TPEY	VJA	ABA	EYAA	S110	TEA	ETGPA	CEY	MSA	PCA	TSA	SEA	NUT
6	TPEY	TGA	ETGPA	ABA	CEY	SEA	VJA	S110	TEA	MSA	PCA	TSA	EYAA	NUT
Overall	VJA	TPEY	TGA	EYAA	S110	ABA	ETGPA	TEA	CEY	SEA	PCA	TSA	MSA	NUT

-----Media not statistically significantly different from the tryptic soy agar (TSA) non-selective control medium.

.....Media not statistically significantly different from the nutrient agar, non-selective control medium.

^aSee text, Bacteriology, for interpretation of media codes.

Media for enumerating staphylococci had a marked effect on the apparent count ($p < 0.001$). This was observed for the overall analysis (Table 2) and for each sampling period during ripening. These results are summarized in Table 3. The results in Table 3 are based on Duncan's multiple range test. The media not significantly different from the two reference media, tryptic soy agar and nutrient agar, are indicated.

The three nonselective media were included in the study because of the difficulty anticipated with contaminating micro-organisms. Although tryptic soy agar was specifically prepared for the nonselective growth of staphylococci (15), tryptic soy agar only supported a higher count than nutrient agar in the initial sample. However, counts on these media were not significantly different, whereas counts on plate count agar were significantly lower than on either nutrient or tryptic soy agar. Either of the latter nonselective media may be used as the reference for comparison with the other media, however nutrient agar was selected for this study because it generally supported the highest staphylococcal counts.

In the overall analysis, only mannitol salt agar was not significantly different from either of the nonselective control media. In the separate analyses for each sampling period, however, mannitol salt agar was significantly different from the control media on two occasions, i.e. sampling periods 3 and 4, representing 4 and 6 weeks of ripening. Depending on the time of sampling, therefore, mannitol salt agar might or might not be the selective medium of choice. For the two sampling periods where mannitol salt agar was significantly different from the controls, Staphylococcus medium no. 110 was not significantly different. With the relatively high rank order of Colbeck egg yolk and salt egg yolk agars, especially in the overall study, the salt-based egg yolk selective media appeared more suited for enumerating staphylococci in these cheeses than other egg yolk-containing selective media. This was further confirmed by the data in Table 4.

The tellurite-based selective media included those without egg yolk (TGA and VJA) and with egg yolk

(ETGPA, TPEY, and TEA). For enumeration of unheated and heated cells of *S. aureus* (17), tellurite glycine and Vogel Johnson agars were satisfactory for enumerating unheated cells, whereas Baird-Parker's medium and Crisley et al.'s tellurite polymyxin egg yolk agar were satisfactory for enumeration of both unheated and heated cells. In contrast, results of this study indicate that both tellurite glycine and Vogel Johnson agars were totally unsatisfactory, and that none of the tellurite egg yolk media were entirely satisfactory for enumerating *S. aureus* in cheese. This is in contrast with the study of Takacs and Domjan (18) comparing Baird-Parker and Giolitti-Cantoni media for isolation and enumeration of *S. aureus* from soft cheeses. Although survival of staphylococci in soft and semi-hard cheeses would most probably be different, these authors found Baird-Parker medium reliable for enumeration of *S. aureus*, even after 28 days of storage of the soft cheese in 13% brine at 4 C. However, such conditions cannot be expected to simulate ripening of a semi-hard cheese, which seems to be one of the major factors influencing reliability of media in this study.

Tellurite polymyxin egg yolk agar was also unsatisfactory, supporting counts equivalent to tellurite glycine and Vogel Johnson agars, and always significantly different from the nonselective controls. Baird-Parker's medium was satisfactory for the initial sampling (immediately after cheese pressing), thereafter the reliability of Baird-Parker's medium declined with increasing time of ripening. Innes' tellurite egg yolk agar (12), though not satisfactory, could be considered equivalent to, if not better than Baird-Parker's medium for enumerating staphylococci in these cheeses.

The azide-based selective media were generally unsatisfactory. Egg yolk azide agar usually supported lower staphylococcal counts than azide blood agar base, except toward the end of the ripening period. By sampling period 6, egg yolk azide agar gave counts equivalent to, and not significantly different from, the nonselective controls. Despite this, these media must generally be considered unsatisfactory.

Interaction effects also influenced the reliability of the

TABLE 4. Percent recovery of all 4 strains of *S. aureus* from all 3 cheese varieties on selective and nonselective media compared to nutrient agar^a

Medium	Sampling period						Mean
	1	2	3	4	5	6	
MSA	85*	87*	14	8.7	22*	31*	41
SEA	65*	7.9	22	6.5	96*	7.1	34
CEY	141*	27	4.0	5.8	18	6.0	34
TEA	98*	9.1	16	7.7	18	27*	29
ETGPA	141*	42*	5.0	7.0	18	2.5	36
ABA	59*	36	13	12	8.7	3.1	22
S110	79*	30	98*	91*	17	15*	55
EYAA	85*	18	16	6.8	14	49*	31
TGA	32	6.2	2.5	1.4	1.3	2.5	7.6
TPEY	50	6.9	0.6	0.8	2.2	0.9	10
VJA	44	3.3	1.3	2.7	5.8	8.1	11
TSA	148*	87*	93*	69*	93*	48*	90*
PCA	68*	53*	30	21	47	38*	43

*Indicates media not significantly different from the nutrient agar reference medium (see Table 3).

^aSee text, Bacteriology, for interpretation of media codes.

staphylococcal counts. Interaction effect WC (time of ripening \times cheese variety) has been discussed, but media had highly significant interaction effects with strains (MS), cheese variety (MC), and time of ripening (MW) (see Table 2).

The source of the interaction MS (media \times strains) was principally strain MF31 which grew exceptionally well on Baird-Parker's medium, also strain 21B which responded differently to growth on many of the selective media, notably azide blood agar base and egg yolk azide agar, also mannitol salt agar, *Staphylococcus* medium no. 110, and tellurite polymyxin egg yolk agar. The source of the interaction MC could be attributed primarily to results from dariworld cheese. Staphylococci from dariworld cheese grew poorly on egg yolk azide agar and salt egg yolk agar, but grew more efficiently on mannitol salt agar, *Staphylococcus* medium no. 110, tellurite egg yolk agar, tellurite glycine, and Vogel Johnson agars than staphylococci from the other cheese types.

The MW (media \times time of ripening) interaction effect might be of even greater importance relative to selection of media for enumerating staphylococci in cheese. Interaction effects were greatest in the final sampling period (after 10 weeks of ripening), however significant interactions also occurred in the 4th and 5th sampling periods (6 and 8 weeks of ripening). As ripening progressed, growth response on the salt-based, selective media improved. This was most marked with mannitol salt agar and *Staphylococcus* medium no. 110. Other media also contributed to this interaction effect, especially the tellurite-based media, both with and without egg yolk.

In the previous study by Stiles and Clark (17), 70% recovery of the viable cells on a selective medium was taken as the criterion of acceptance for a selective medium. Percent recoveries for each medium at each sampling time are shown in Table 4. Since strain of *S. aureus* and cheese variety had a limited effect on the reliability of the count, counts were pooled for strain and cheese type. None of the selective media supported 70% recovery of the staphylococci when mean recovery percentages were calculated for all six sampling periods. However, *Staphylococcus* medium no. 110 and mannitol salt agar gave the best recovery levels. These were followed by Baird-Parker's medium, Colbeck egg yolk, salt egg yolk, egg yolk azide, and tellurite egg yolk agars. The latter are all egg yolk-containing media, which suggests that these media might be among those preferred for enumeration of *S. aureus* in cheese. However, the egg yolk-containing media, especially those using azide or tellurite as the selective agent, were most satisfactory for sampling period 1 (immediately after pressing), and recovery levels for the subsequent sampling periods were poor.

Detection of *S. aureus* in cheese might be done immediately after manufacture, but it is most likely to be done during or at the end of the ripening period. As such,

mannitol salt agar and *Staphylococcus* medium no. 110 would be the media of choice for enumerating *S. aureus* in cheese. It might be preferable to use both of these selective media, since recovery levels at each sampling period were markedly different. If an egg yolk medium is preferred, these data indicate that a salt-based, rather than an azide- or tellurite-based medium should be used. A disconcerting aspect of these data is the variation in the reliability of the selective media between sampling periods.

CONCLUSIONS

Reliability of selective media for enumerating staphylococci in cheese varied between media and with time of ripening. At some times, strain of *S. aureus* and cheese variety also influenced reliability of the staphylococcal count. Although mannitol salt agar was shown not to be significantly different from the nonselective reference media for the overall study, use of mannitol salt agar as the selective medium of choice cannot be recommended, because, at certain times during ripening, counts on mannitol salt agar were relatively unreliable. It appeared that mannitol salt agar and *Staphylococcus* medium no. 110 might be the most reliable combination of media for enumerating staphylococci in cheese.

Contrary to expectation, the tellurite egg yolk media were not generally satisfactory for enumerating staphylococci in cheese. If egg yolk media are preferred, either salt egg yolk agar or Colbeck egg yolk agar would be the media of choice. Unfortunately egg yolk was not added to *Staphylococcus* medium no. 110 in this study as recommended by Carter (4), and it might be of value to determine the reliability of this medium for staphylococci in cheese.

The results indicated the possibility that cells are being injured or debilitated in some way as cheese ripening progresses. Unlike sublethally heated staphylococci, egg yolk tellurite and azide media failed to give protection against the debilitating effect. This suggests a different type of injury than heat injury.

The drop in pH of 0.5 unit between the initial sampling and 2 weeks of ripening coincided with the marked change in reliability of the selective media. The pH has a debilitating effect on staphylococci but the role of pH in this study was not determined. None-the-less, the change in reliability of the selective media makes the time of testing cheese for *S. aureus* extremely important relative to choice of selective medium. For caerphilly and dariworld cheeses, where ripening is traditionally short, 2 and 2 to 4 weeks, respectively, the times for testing are limited. However, for Cheddar cheeses with a longer ripening period, time of testing becomes more important.

This study was undertaken with the view that, among the many selective media for staphylococci, one or more media would be reasonably or entirely satisfactory for enumerating staphylococci in cheese. Other media should be tested for their reliability, and if they are no

better than the wide range of media used in this study, modification of the media might be necessary to develop a satisfactory medium. The salt-based rather than the tellurite- or azide-based media are most likely to give favorable results.

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Production and Stability of Hemolysin, Phospholipase C, and Lethal Toxin of *Bacillus cereus* in Foods

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ABSTRACT

Production of hemolysin, phospholipase C, and (mouse) lethal toxin by two strains of *Bacillus cereus* in beef, pea, and banana was assayed. Both strains produced hemolysin, phospholipase C, and lethal toxin in beef, hemolysin and phospholipase C in pea, and only lethal toxin in banana, although growth was excellent in each food. In each instance the metabolites were produced at characteristic levels for the food in which the organism was grown and the time of incubation. Foods containing these products were subjected to heating, refrigeration, and freezing. At 65 C lethal toxin in beef was substantially inactivated in 3 min, but hemolysin and phospholipase C retained 50% of their activities after 30 min and about 13% after 120 min. Activity of the three substances in the different foods was decreased by approximately 50% at 4 C after 24 h, but in several instances was still substantially retained after 48 h. Freezing, holding at -37 C, and then thawing generally was less destructive of the activity of the three metabolites in the different foods although decreases of 50% or slightly more occurred in 48 h in most instances. Stability of the different metabolites was much the same with both strains of *B. cereus*. The influence of particular foods upon stability of hemolysin and phospholipase C was greater than upon lethal toxin.

Bacillus cereus has been incriminated in numerous outbreaks of food poisoning in Europe over the past 25 years; however, it is rarely reported as the cause of food-borne disease in the United States. Goepfert et al. (11), in their comprehensive review of *B. cereus* as a food poisoning organism, have suggested that this apparent lack of occurrence in the United States may be due to inadequate diagnosis of the causative agent and the similarity of symptoms and disease pattern with *Clostridium perfringens* food poisoning.

Experimental induction of *B. cereus* food poisoning in humans has given variable results even when large numbers of organisms have been administered to volunteers in food, as cultures, and as washed cell suspensions (7, 12, 13, 18). These and other reports indicate that conditions for production and stability of toxic factor(s) by *B. cereus* may be very fastidious. Lethality for mice has been easier to demonstrate (21, 22), but here too toxicity of *B. cereus* was variable and diminished in foods high in sugar and fat but increased after the organism was grown in vegetables.

While the specific agent(s) responsible for human food

poisoning remains to be proven, certain animals produce distinct pathological responses to products of *B. cereus* growth. McGaughey and Chu (16) reported production of phospholipase by *B. cereus*. Chu (6) observed that hemolytic and mouse lethal activities also were present in *B. cereus* filtrates. Initially it was suspected that these properties of *B. cereus* were functions of one bacterial product, however additional work (1, 2, 14, 17, 20) has since demonstrated that the phospholipolytic, hemolytic, and mouse lethal activities of *B. cereus* are associated with three distinct extracellular products. More recently Fluer and Ezepchuc (8) revealed that lethal toxin, devoid of hemolysin and phospholipase, produced a food poisoning syndrome in cats and was lethal for mice. It has also been shown that one or more substances produced by *B. cereus* other than the phospholipase or hemolysin can induce fluid accumulation in rabbit ileal loops (24), produce a necrotic reaction in guinea pig skin (9), and increase vascular permeability when injected intradermally into rabbits (10). The agent(s) responsible for these effects may or may not be identical to the mouse lethal toxin or be responsible for human food poisoning, as pointed out by the authors. Although these more recent studies indicate that *B. cereus* hemolysin and phospholipase are not required for pathologic responses in several animal species, their possible role as auxiliary virulence factors, as suggested earlier by Bonventre and Johnson (4), still remains. In this regard, increased vascular permeability in rabbits also was produced by unpurified and purified phospholipase of *B. cereus* in tests by Glatz et al. (10), although the effect was more transient than that produced by their hemolysin-free, phospholipase-free permeability factor.

While the relationships of the above to the human situation remain to be determined, little information on production and stability of hemolysin, phospholipase C, and lethal toxin of *B. cereus* in specific foods is available, although observations have been made in *B. cereus* culture filtrates and on partially purified preparations (2, 14, 15, 19, 23). This has been in part due to the greater difficulty of assaying for these entities in food than in culture filtrates.

The present study was initiated to gain information on production of hemolysin, phospholipase C, and lethal

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toxin by *B. cereus* in specific foods and to determine stability of these products when foods were refrigerated, frozen, or heated.

MATERIALS AND METHODS

Foods

The beef and pea products used in this study were commercial Gerber strained beef and beef broth (pH 6.3), and Gerber strained peas (pH 6.3). The banana product (pH 5.0) was prepared by slicing ripe bananas aseptically and blanching the slices at 100 C for 3 min. The slices were then pureed in a sterilized Waring Blender. None of these products exhibited microbial growth on nutrient agar at 30 C in 48 h.

Cultures

B. cereus strains 14579 and 7004 were obtained from the American Type Culture Collection. Strain 14579 was selected because it is the neotype strain of *B. cereus*. Strain 7004 was selected because it has been implicated in food poisoning and its enzymes have been studied by several researchers (15, 17, 23). Nutrient agar cultures were transferred to fresh beef infusion broth (5), incubated at 30 C in a rotary shaker for 24 h, subcultured and again incubated with shaking at 30 C for 16 h in fresh beef infusion broth, centrifuged from the broth and washed with physiological saline three times, and finally diluted with physiological saline to provide a standard inoculum in the log phase for inoculation of foods and production of enzyme activities.

Hemolysin, phospholipase C, and lethal toxin assay procedures

Hemolysin in filtered concentrates was assayed using the method of Johnson and Bonventre (14). One hemolysin unit was defined as the amount of hemolytic activity in the highest dilution of the original sample causing complete hemolysis at 37 C in 1 h in the test system.

Phospholipase C was assayed using the egg yolk medium of McGaughey and Chu (16). However, borate buffer (0.05 M, pH 7.4) with CaCl_2 (2.5 mM) was substituted for nutrient broth (14), and the egg yolk medium was filtered and then filter-sterilized by passage through a 0.22 μ -membrane. Serial dilutions of the concentrates were made with borate buffer (0.05 M, pH 7.4), and an equal volume of egg yolk medium was added to each tube. One phospholipase unit was defined as the amount of phospholipolytic activity in the highest dilution of the original sample causing turbidity at 37 C in 1 h in the test system.

Mouse lethality was used as an indicator of *B. cereus* lethal toxin. Twenty-gram male white mice (Strain Ha-ICR) were injected intraperitoneally with test sample extract serially diluted with sterile physiological saline. Mouse lethality by the intraperitoneal route has been demonstrated for *B. cereus* by others (3, 17). Four mice were used for each dilution and 0.5 ml of diluted sample was the standard amount injected. A unit of lethal toxin was defined as the amount of activity in the highest dilution of the original sample which killed at least 50% of the mice within 48 h. This relatively long period of observation was chosen because some of the mice injected with extracts of banana upon which *B. cereus* had grown required nearly 24 h to die. This was in contrast to mice injected with extract from beef after *B. cereus* growth, which usually succumbed within 6 h. In no instance did mice die from 0.5 ml of extract from control samples that had not been inoculated with *B. cereus*. It should be recognized that the mouse assay may not have measured lethal toxin only since no attempt was made to purify sample extracts with respect to this specific toxin before injection.

Production and recovery of hemolysin, phospholipase C, and lethal toxin from foods

Quantification of activities produced in beef, pea, and banana by *B. cereus* first required information on recovery of these substances from the foods. Recovery data were obtained by adding known quantities of hemolysin, phospholipase C, and lethal toxin (in the form of a sterile concentrated culture filtrate) to each food, removing a representative portion of the substances in the form of a food filtrate, and then concentrating the food filtrate for reassay of activity by the substances. Recovery data were then used to adjust measured activity values produced by *B. cereus* grown in the foods.

Production of substances for recovery studies. A loopful of actively growing *B. cereus* cells was transferred to 200 ml of fresh beef infusion broth prepared by the method of Burnett et al. (5) and incubated at 30 C for 24 h with rotary shaking at 120 rpm. After incubation the entire culture was cooled to 4 C and centrifuged at $862 \times g$ for 45 min to sediment the major portion of the cells. Remaining cells were removed by passing the supernatant fluid through a laboratory filtration apparatus (Millipore Sterifil System) with a 0.22- μ membrane. Sterile culture filtrates were concentrated by dialysis with cellulose tubing (Sargent-Welch, Springfield, NJ) against a 35:65 (vol/vol) polyethylene glycol 4000-water slurry for 24 h at 4 C. Hemolytic, phospholipolytic, and lethal activities were assayed by the methods described above.

Production of substances in food. Strained beef, pea, and banana were mixed with *B. cereus* standard inoculum to obtain an initial count of approximately 10^3 organisms/g. One hundred-gram quantities of food were then incubated at 30 C for 24 h and at 6 h intervals aliquots were removed for bacterial determinations by the aerobic plate count using nutrient agar and incubating at 30 C for 48 h. Cold borate buffer (0.05 M, pH 7.4) was then added to the remaining food sample and the mixture (2:1 (vol/wt) borate buffer-food) was agitated gently for 3 min. The resulting suspension was centrifuged at $862 \times g$ for 45 min. The supernatant fluid, commonly two-thirds of the total volume, was then filtered repeatedly through Whatman #1 paper until it passed easily. This was repeated using Whatman #42 and #50 paper to remove most of the suspended food particles. Remaining particles were further removed and the filtrate sterilized using the Millipore Sterifil System described earlier for culture filtrates. The sterile food filtrate was then concentrated and assayed for hemolysin, phospholipase C, and lethal toxin by the methods already described. Recoveries of hemolysin, phospholipase C, and lethal toxin added to beef, pea, and banana (in the form of assayed concentrated culture filtrate) were between 75 and 89% by these methods in repeated trials. When concentrated culture filtrate was stored at 4 C for a period equal to the time required for the detection process in food, and percent recoveries from food were adjusted for loss of activities in this control, the corrected recoveries from food were essentially 100% for each substance in all instances. In subsequent figures and tables corrected values for hemolysin, phospholipase C, and lethal toxin are reported as units of each substance/g of food.

Stability studies in refrigerated, frozen, and heated foods

Following the 24 h incubation for production of hemolysin, phospholipase C, and lethal toxin in foods inoculated with *B. cereus*, 100-g quantities of the foods were refrigerated at 4 C, frozen at -37 C in a chest freezer, and heated to 65 C in a water bath. After appropriate intervals, samples in flasks were rapidly brought to room temperature with running water, assayed as before, and values compared with those of the 24 h incubated foods not so treated.

RESULTS AND DISCUSSION

Hemolysin, phospholipase C and lethal toxin production in food

Inoculated food samples were analyzed at 6-h intervals for Strain 7004 in beef, pea, and banana. Strain 14579 was also studied in the three foods, but food was analyzed for activities only after 24 h.

Typical growth and production of hemolysin, phospholipase C, and lethal toxin in beef by Strain 7004 at 30 C is seen in Fig. 1. Beef supported growth of Strain 7004 well. A count of approximately 10^9 organisms/g was reached at 18 h and remained essentially constant to 24 h. Such high counts were repeatedly obtained with both strains of *B. cereus* in each of the foods studied. Hemolysin was present at a concentration of 0.5 unit/g at 6 h and increased to a maximum of 5.4 units/g at 24 h. Phospholipase C production steadily increased from 1

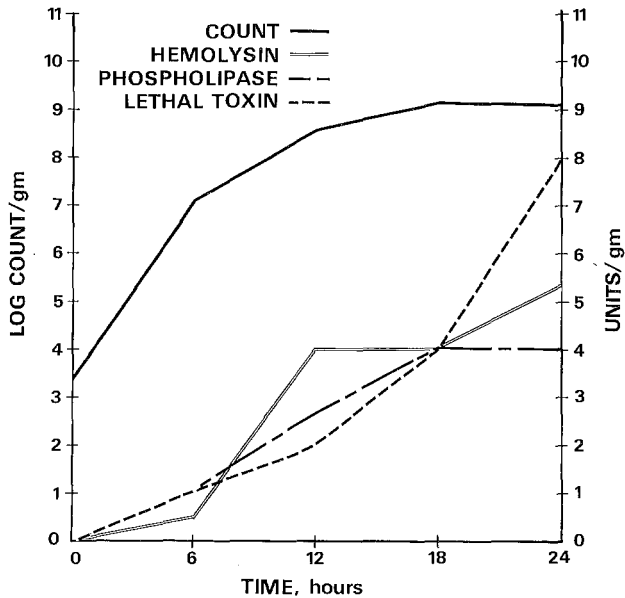


Figure 1. Growth of *B. cereus* Strain 7004 and production of hemolysin, phospholipase C, and lethal toxin in beef at 30 C.

unit/g at 6 h to a maximum of 4 units/g at 18 h and remained constant thereafter. Lethal toxin was produced in beef in larger amounts than in the other foods. One unit of lethal toxin/g was present at 6 h and the amount doubled every 6 h, reaching a maximum of 8 units/g at 24 h.

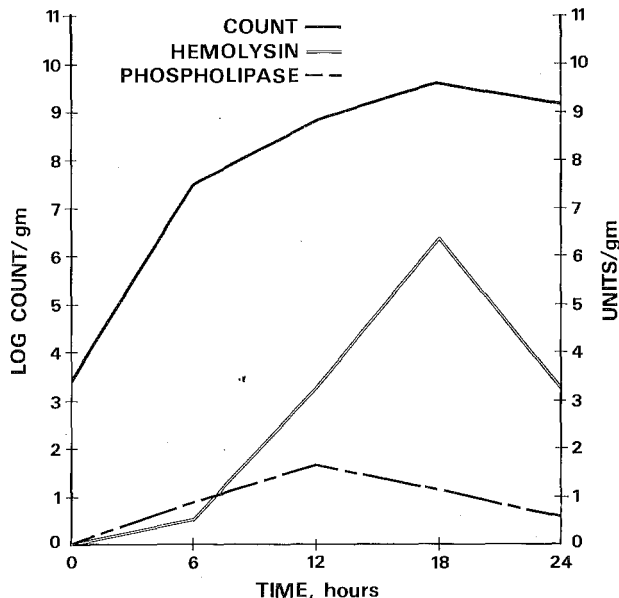


Figure 2. Growth of *B. cereus* Strain 7004 and production of hemolysin and phospholipase C in pea at 30 C.

Typical growth and production of hemolysin and phospholipase C by Strain 7004 in pea is seen in Fig. 2. Hemolysin activity of 0.4 unit/g was present at 6 h and reached nearly 6.4 units/g at 18 h. A sharp decline was noted after 18 h. Phospholipase C from Strain 7004 was produced in small amounts in pea, reaching a maximum of 1.6 units/g at 12 h and then declining. No assayable

quantity of lethal toxin was produced by Strain 7004 in pea. Since no lethality was observed, the experiment was repeated using food extract concentrated to a greater extent and lethality again was not present. The cause of this lack of production in pea is unknown. Perhaps pea is deficient in some nutrient required for lethal toxin production. The initial pH of the pea is probably not a factor in this instance since the pea had the same initial pH as the beef (pH 6.3) which supported toxin production.

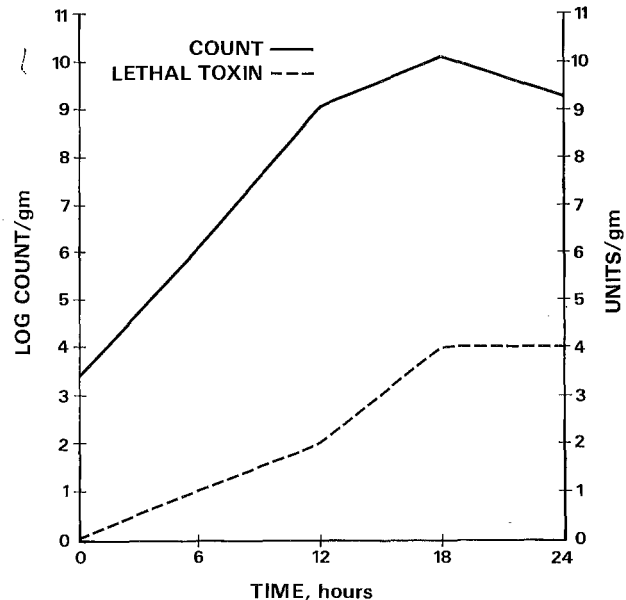


Figure 3. Growth of *B. cereus* Strain 7004 and production of lethal toxin in banana at 30 C.

Typical growth and production of lethal toxin in banana by Strain 7004 is seen in Fig. 3. No assayable quantity of hemolysin or phospholipase C was produced in banana by Strain 7004. The factors responsible for their lack of production are unknown. Possibly banana is deficient in some nutrient or a pH of 5.0 is too low for their production. Lethal toxin in banana reached 1 unit/g at 6 h and doubled by 12 h and again doubled by 18 h. Repetition of the experiment revealed lethal toxin to very much the same degree, and absence of hemolysin and phospholipase. A factor promoting additional investigation was the slow response of mice to injection with banana extracts. Some of the injected mice required nearly 24 h to succumb to the lethal toxin. This was in contrast to mice injected with extract from beef, which usually succumbed within 6 h. It was suspected that death of mice may have been caused by infection with *B. cereus* from improperly sterilized banana extract rather than from lethal toxin. This hypothesis was refuted when banana extract, reesterilized with 0.22 μ -membrane immediately before injection, repeatedly resulted in similar results. Nor were any mice adversely affected by injection with extract from banana that had not been inoculated with *B. cereus*. The cause of delayed death of mice is not known. It is possible that absence of

hemolysin and phospholipase C from banana could delay lethal response (compared to beef where they are present) if these enzymes were auxiliary virulence factors, but without additional data this is only conjecture.

Cell counts and amounts of hemolysin, phospholipase C, and lethal toxin produced by Strains 14579 and 7004 in beef, pea, and banana incubated 24 h are seen in Table 1. A comparison of the growth and activity data for

TABLE 1. Cell counts and levels of hemolysin, phospholipase C, and lethal toxin produced by *B. cereus* in foods at 30 C for 24 h

Food	Initial pH	Units/g food			Log count/g
		Hemolysin	Phospholipase	Lethal toxin	
<i>Strain 14579</i>					
Beef	6.3	8.8	3.2	4	9.06
Pea	6.3	3.8	0.8	<1	9.04
Banana	5.0	<0.1	<0.1	4	8.93
<i>Strain 7004</i>					
Beef	6.3	5.4	4.0	8	9.15
Pea	6.3	3.3	0.5	<1	9.20
Banana	5.0	<0.1	<0.1	4	9.30

the two strains illustrates several points. In beef, growth and production of phospholipase C by both strains was essentially equal, hemolysin production by Strain 14579 was slightly greater than by Strain 7004, and lethal toxin production by Strain 14579 was only half that by Strain 7004. In pea, phospholipase and hemolysin activities

produced by Strain 14579 were similar to the levels produced by Strain 7004, and neither strain produced an assayable quantity of lethal toxin. The production of lethal toxin by both strains in banana was identical, as was the absence of hemolysin and phospholipase.

Stability studies in refrigerated, frozen, and heated food

Stability data in the various foods held at 4 C are seen in Table 2. Cell counts decreased only very slightly in the 48-h storage period. With Strain 14579, hemolysin in beef lost 55% of its activity in 24 h and then remained stable through 48 h. Phospholipase C decreased 41% after 24 h and 57% after 48 h. Lethal toxin decreased at a rate similar to hemolysin. In pea, 80% of the hemolytic activity produced by this strain was lost in 48 h and phospholipase underwent a similar reduction. Lethal toxin in banana decreased 50% in 24 h and then remained constant through 48 h. A comparison of stabilities at 4 C for Strain 7004 reveals generally similar patterns. Such differences as appear between strains in specific foods or for a specific activity in more than one food are small.

Activity changes in the various foods held at -37 C and thawed are seen in Table 3. In most instances frozen storage up to 48 h followed by thawing was slightly less destructive than refrigerated storage of similar duration. Phospholipase produced in pea by both strains was

TABLE 2. Effects of refrigeration (4 C) on stability of hemolysin, phospholipase C, lethal toxin, and cell counts produced in foods by *B. cereus*

Food	Activities in foods ^a					
	Hours food at 4 C					
	0	24	48	0	24	48
	Strain 14579			Strain 7004		
<i>Beef</i>						
Hemolysin	5.8	2.6 (45)	2.6 (45)	4.9	2.5 (51)	1.8 (37)
Phospholipase	4.4	2.6 (59)	1.9 (43)	3.7	1.9 (51)	1.2 (32)
Lethal toxin	4	2 (50)	2 (50)	8	4 (50)	2 (25)
Log count/g	8.90	8.57	8.56	9.55	8.03	8.20
<i>Pea</i>						
Hemolysin	4.6	1.7 (37)	0.9 (20)	3.6	2.7 (75)	1.8 (50)
Phospholipase	1.7	0.9 (53)	0.4 (24)	1.3	0.9 (69)	0.7 (54)
Log count/g	8.94	9.19	8.79	9.98	9.76	9.41
<i>Banana</i>						
Lethal toxin	4	2 (50)	2 (50)	4	2 (50)	1 (25)
Log count/g	9.78	9.41	8.98	10.25	10.01	9.76

^aActivities of hemolysin, phospholipase and lethal toxin expressed as units/g food; numbers in parentheses represent percent remaining from zero hour values.

TABLE 3. Effects of freezing (-37 C) on stability of hemolysin, phospholipase C, lethal toxin, and cell counts produced in foods by *B. cereus*

Food	Activities in foods ^a					
	Hours food at -37 C					
	0	24	48	0	24	48
	Strain 14579			Strain 7004		
<i>Beef</i>						
Hemolysin	5.8	2.9 (50)	1.9 (33)	4.9	2.5 (51)	2.5 (51)
Phospholipase	4.4	4.4 (100)	2.6 (59)	3.7	2.5 (68)	1.2 (32)
Lethal toxin	4	4 (100)	2 (50)	8	8 (100)	4 (50)
Log count/g	8.90	8.69	8.60	9.55	8.76	8.58
<i>Pea</i>						
Hemolysin	4.6	3.4 (74)	2.1 (46)	3.6	3.6 (100)	2.7 (75)
Phospholipase	1.7	1.7 (100)	1.7 (100)	1.3	1.3 (100)	1.3 (100)
Log count/g	8.94	8.86	8.67	9.98	9.86	9.61
<i>Banana</i>						
Lethal toxin	4	4 (100)	2 (50)	4	4 (100)	2 (50)
Log count/g	9.78	9.71	9.37	10.25	10.01	9.68

^aSee footnote Table 2.

especially resistant to the freezing and thawing treatment, having lost no measurable activity. Hemolysin also lost less of its original activity in pea than in beef. Lethal toxin in beef and banana lost 50% of its initial activity after 48 h regardless of strain.

Heat stability data were obtained only in beef and results are given in Table 4. Stabilities of the three bacterial substances from Strain 14579 were virtually identical to those of Strain 7004. Lethal toxin was most rapidly inactivated at 65 C with only 13-25% remaining after 3 min and no assayable quantity detected after 30 min. It is likely that complete inactivation took place before 7 min, as was observed in additional trials. Hemolysin and phospholipase were very similar in their response to heat, and both lost essentially 30, 50, and 87% of their activities in 3, 30, and 120 min, respectively.

These results, in beef, are consistent with the heat stability of phospholipase C in water observed by Slein and Logan (23). The data also reveal phospholipase C stability in beef and pea at 4 C similar to that reported by Kleinman and Lands (15) in an aqueous suspension of low protein concentration. The current findings differ, however, from those of Ottolenghi (19), Bernheimer and Grushoff (2), and Johnson and Bonventre (14) with respect to hemolysin and phospholipase stabilities at elevated temperatures. These authors found hemolysin to be markedly less stable than phospholipase at 50 C or above in culture filtrates, whereas in the present study, hemolysin in beef possessed considerable stability to 65 C, which was equal to that of phospholipase for both strains of *B. cereus*. Further, Johnson and Bonventre (14) found lethal toxin more stable than hemolysin to heating at 45 C, whereas, in the present study at 65 C, lethal toxin in beef was far less stable than hemolysin. Heat stability of biological materials is affected by numerous variables that can exist in different media and this may explain the different findings in culture filtrates and beef cited above.

Since substantial losses of hemolysin, phospholipase, and lethal toxin occurred in foods of the present study in 48 h or less at 4 C and -37 C, it is likely that inactivations also take place rapidly at room temperature. If one or more of these products is indeed the cause of *B. cereus* food poisoning then their stabilities in different foods, as

well as their different levels of production, could help explain the variable food poisoning responses noted by other workers.

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TABLE 4. Effects of heat (65 C) on stability of hemolysin, phospholipase C, lethal toxin and cell counts produced in beef by *B. cereus*

	Activities in beef ^a				
	Minutes beef at 65 C				
	0	3	30	60	120
<i>Strain 14579</i>					
Hemolysin	4.8	3.2 (67)	2.4 (50)	1.2 (25)	0.8 (17)
Phospholipase	3.2	2.4 (75)	1.6 (50)	0.8 (25)	0.4 (13)
Lethal toxin	4	1 (25)	<1	<1	<1
Log count/g	9.39	8.74	6.24	6.33	6.17
<i>Strain 7004</i>					
Hemolysin	5.3	4.0 (75)	2.7 (51)	1 (19)	0.7 (13)
Phospholipase	4.0	2.7 (68)	2.0 (50)	1.3 (33)	0.5 (17)
Lethal toxin	8	1 (13)	<1	<1	<1
Log count/g	10.1	9.72	8.55	7.83	5.47

^aSee footnote Table 2.

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A Collaborative Study of the Delvotest-P Method to Detect Low Concentrations of Penicillin in Milk

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ABSTRACT

During a collaborative study at a large number of milk-receiving stations from a dairy industry in the Netherlands, in which penicillin was added in various concentrations of 0, 4, 5, 6, 7, 8 and 10 IU/l to milk samples, the Delvotest-P method gave reliable results in almost all instances. Of the 22 milk samples that were tested, both undiluted and diluted 1:1, by up to 17 control stations in seven experiments, a total of 518 readings were obtained. There were no negative readings among the 249 samples that contained 4 or more IU penicillin per liter and only one doubtful reading among 186 samples that were free of penicillin. In some instances the test was found a little more sensitive than one might conclude from the instructions.

In 1974 a new test method (Delvotest-P) for detection of antibiotic residues in milk appeared on the market. Our company was one of the first to introduce this method for screening of bulk milk at affiliated milk-receiving stations after the test proved to be suitable. The test has been described by Van Os et al. (1). In this article an indication is given of the limits within which penicillin concentrations in milk can be demonstrated. It was important to establish on a large scale how the presence

of a given penicillin concentration within the limits set would be judged by the various stations under field conditions.

For this purpose, seven experiments were done over a period of about 1 year with the co-operation of 17 milk-receiving stations within the company. The laboratory assistants at these stations had been instructed before in a short training program on use of the test method and had acquired sufficient experience to produce reliable readings. The results of this study are described herein.

MATERIALS AND METHODS

The Delvotest-P kits were obtained from the sales distributors in the Netherlands. Milk samples to which penicillin had been added in an amount unknown to the examiner, were sent in a deep-frozen state and under code number to the participating station with the request to analyze the milk with the Delvotest-P method and report the results obtained. Three different samples were tested in all instances; in one instance four different samples were tested (Nov. 22, 1974). In addition to the instructions in the brochure of the Delvotest-P, the following arrangement was made.

TABLE 1. Survey of results obtained with the Delvotest-P® method in 7 experiments

Experiment Date	I 1-10-74	II 20-10-74	III 22-11-74	IV 18-12-74	V 28-1-75	VI 23-6-75	VII 9-10-75
Number of control stations	6	12	10	12	14	13	17
Added penicillin (IU/l) ^a	0	10	8	8	7	0 ^b	6 ^b
Reading	+ ± -	+ ± -	+ ± -	+ ± -	+ ± -	+ ± -	+ ± -
Reading undiluted	0 0 6	12 0 0	10 0 0	12 0 0	12 2 0	0 0 12	13 3 0
Reading after 1:1 dilution	0 0 6	9 3 0	7 3 0	7 5 0	2 11 1	0 0 12	0 16 0
Added penicillin (IU/l)	5	0	0 ^c	4	0	6	0
Reading	+ ± -	+ ± -	+ ± -	+ ± -	+ ± -	+ ± -	+ ± -
Reading undiluted	5 1 0	0 0 12	0 1 19	8 4 0	0 0 14	8 5 0	0 0 17
Reading after 1:1 dilution	0 6 0	0 0 12	0 0 20	0 12 0	0 0 14	0 13 0	0 0 17
Added penicillin (IU/l)	10	5	4	0	10	10	10
Reading	+ ± -	+ ± -	+ ± -	+ ± -	+ ± -	+ ± -	+ ± -
Reading undiluted	6 0 0	10 2 0	8 2 0	0 0 12	14 0 0	13 0 0	16 0 0
Reading after 1:1 dilution	4 2 0	0 12 0	1 8 1	0 0 12	7 7 0	6 7 0	6 10 0

^aIU/l = 0.001 unit/ml.

^bOne station reported no result.

^cDouble series.

Each sample was to be tested as such (undiluted) and in a 1:1 dilution with distilled water. The test is read as negative (−, whole solid medium yellow), positive (+, whole solid medium purple) or doubtful (±, mixtures of both colours). A negative result implies a concentration of not more than 3 IU/1 of milk, a positive result a concentration of not less than 4 IU/1 of milk and doubtful results may be encountered at concentrations between 3 and 6 IU/1 of milk (3 IU/1 = 0.003 unit/ml).

RESULTS AND DISCUSSION

The results are summarized in Tables 1 and 2. Complete data are available from the author upon request.

TABLE 2. Results combined on the basis of penicillin concentrations

		Undiluted samples						
IU/1 ^a	0	4	5	6	7	8	10	
+	0	16	15	21	12	22	61	
±	1	6	3	8	2	0	0	
−	92	0	0	0	0	0	0	
		1:1 Diluted samples						
IU/1	0	2	2½	3	3½	4	5	
+	0	1	0	0	2	14	32	
±	0	20	18	29	11	8	29	
−	93	1	0	0	1	0	0	

^aIU/1 = 0.001 unit/ml.

Bearing in mind the large number of participants in this examination, Delvotest-P has proven to be suitable under field conditions in every respect. Among the samples to which no penicillin had been added, a slightly doubtful result was read only once (see below).

At concentrations which, according to the manufacturer's statement, should yield a positive result (≥ 6 IU/1) as well as at lower concentrations (≥ 4 IU/1) no negative readings were noted. The tendency to recognize concentrations below the limit indicated by the

manufacturer, may also be apparent from the large number of doubtful results at 2 and 2.5 IU/1, although in the interpretation of the results with these diluted samples, the results with nondiluted samples may have played a role. Incorrect readings were obtained at the concentrations 7 IU/1 ($2 \times \pm$) 2 IU/1 ($1 \times +$) and 3.5 IU/1 ($1 \times -$). It must be added here that at the stations where these results were obtained the result obtained with the other penicillin-containing sample on the same day, had like-wise been over- or under-estimated.

The percentages of correct readings at the various concentrations of penicillin in the non-diluted samples were:

Concentration (IU/1)	0	4	5	6	7	8
% Correct	99.5	100	100	100	85	100

In conclusion it may be said that with the Delvotest-P method reliable results were obtained. During the 2 years in which the method has been applied within the company no difficulties have been encountered. Moreover screening of incoming bulk milk with Delvotest-P has contributed to a further decrease in the number of antibiotic-positive samples; also the method has been increasingly found to be useful for examination of questionable milk samples on request of the farmer.

ACKNOWLEDGMENTS

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Influence of Pasteurization and Homogenization Treatments on Photocatalyzed Oxidation of Cream

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ABSTRACT

Heat treatment within the temperature range of 60 to 94 C promoted photocatalyzed lipid oxidation of cream. The sulfhydryl compounds cysteine hydrochloride, glutathione, thioglycolic acid, and mercaptoethanol at concentrations of 0.1 to 0.001% acted as antioxidants. Copper migrated from the serum phase to the fat globule surfaces when cream was heated and acted as an oxidation catalyst. The latter influence predominates over the antioxidant effect of the free sulfhydryl groups in heat treated cream. Homogenization of pasteurized cream inhibits lipid oxidation depending on the pressure used.

Much of the literature on lipid oxidation in dairy products relates to liquid milk and to ripened cream butter. Many factors including light, ascorbic acid, riboflavin, temperature treatment, and traces of copper and iron are known to influence the susceptibility of milk to oxidation. Studies on cream oxidation are few and seem, in the main, to confine attention to the effect of heat treatment on the general keeping quality and in some instances oxidative tendencies of the resultant butter.

In general, the earlier work relating heat treatment of cream to butter quality indicates that relatively high temperature treatment improved the keeping quality of butter and retarded oxidation. It was considered that the sulfhydryl (-SH) groups freed from the serum proteins acted as antioxidants. The widely held view that heat treatment is effective in preventing oxidized flavor through liberation of active -SH reducing groups was challenged by Aurand et al. (1) who found that addition of cysteine hydrochloride to milk did not inhibit development of oxidized flavor.

Smith and Dunkley (11) found that pasteurization of milk increased its susceptibility to oxidized flavor, a result, which they observed was consistent with commercial experience.

At least two changes which may influence lipid oxidation take place in milk during heating: (a) production of free sulfhydryl groups, and (b) migration of copper from the serum to the fat globule surfaces.

Copper plays a dominant role in oxidative changes in dairy products. Almost all of the copper in milk is bound to the protein and there is little if any present in the ionic form. The percentage of copper in milk which is

associated with fat globules is vitally important in lipid oxidation. King and Dunkley (4) attributed 10 to 35% of the copper of milk to the fat globule portion, while Samuelsson (7) found that 15 to 20% of the natural copper of milk is associated with the fat globules. Heat treatment causes migration of copper associated with milk serum to fat globule surfaces, and thus influences distribution of added copper among the different fractions of milk (8, 10).

Homogenization of milk is known to retard development of oxidized flavor. The influence of homogenization on the oxidative stability of cream has received little attention but it is generally assumed that the effect is the same as on milk.

The objects of this study were to determine the influence of different heat treatments on the photocatalyzed lipid oxidation of cream, to explain any trends observed and to establish the effect of homogenization and varying homogenizing pressures on oxidative changes in cream.

MATERIALS AND METHODS

Cream samples

Cream containing 35% fat was mechanically separated from well bulked milk which had been preheated to 40 C. Pasteurization was done either in a commercial HTST plant or in a laboratory pasteurizer.

The cream samples (100 ml) in 125-ml Pyrex containers were held in a cabinet at 5 C under a natural color fluorescent lighting strip which gave a light intensity, measured by an AVO light meter of 50 lumens/ft² on the cream samples. Peroxide value determinations were done in duplicate after predetermined holding times.

Separation of the fat of cream for peroxide determinations

The method of Stine et al. (13) was used. It employs a surface active agent (BDI reagent consisting of 30 g of Triton X-100 and 70 g of sodium tetrapyrophosphate made up to 1 liter with distilled water) combined with heat treatment to de-emulsify the fat globules followed by separation of the fat layer by centrifugation.

Peroxide test

Peroxide analyses were made by the ferric thiocyanate method of Loftus Hills and Thiel (5) as modified by Holloway (3). Quantities of 0.5 ml or of 0.1 ml of fat were used depending on the magnitude of the expected peroxide values. Peroxide values were expressed as m equiv. of oxygen per kg of fat.

Determination of copper concentration

Copper determinations were made using a Perkin-Elmer Atomic Absorption Spectrophotometer Model 103 fitted with a copper hollow

cathode lamp. The following instrument settings were used: Wavelength, 3247 Å; Oxidant, air at 45 psi pressure and a flow rate of 26.5 l/min; fuel, acetylene gas at 8 psi pressure and a flow rate of 3.5 l/min; lamp current, 8 ma.

A standard graph of copper concentration against transmittance readings was prepared each time the instrument was used by dilution of a stock solution of cupric chloride to give a range of copper concentrations. The instrument scale was adjusted to obtain a 100% reading for 1 ppm of copper.

The copper concentrations of cream sera were determined as follows: approximately 200 ml of each cream was centrifuged for 20 min at 3000 rpm. A 50-ml portion of the serum was collected using a syringe and 50 ml of 24% (wt/vol) trichloro-acetic acid added and the mixture shaken at 5-min intervals for 30 min to ensure complete precipitation of the protein. The precipitate was removed using a Whatman No. 42 filter paper.

Lanthanum oxide, 0.5 ml of 5% (wt/vol) solution was added as a suppressing agent and the filtrate concentrated about threefold by evaporation to obtain readings in the sensitive range of the atomic absorption spectrophotometer.

Homogenization of cream

Cream was heated at 72 C in a plate preheater and homogenized at different pressures in a Rannie single stage homogenizer. The samples were cooled to 5 C and placed in the display cabinet to catalyze oxidation.

Glassware

To avoid any possibility of copper contamination, all glassware, after washing with hot detergent solution, was steeped for 24 h in dilute nitric acid and then thoroughly rinsed with de-ionised water and dried.

RESULTS

Figure 1 shows the effect of heat treatment within the temperature range of 60 to 94 C on the peroxide values of cream held at 5 C under fluorescent light at an intensity of 50 lumens/ft².

The initial peroxide values of all samples after heat treatment and before exposure to light were practically identical. It is clear that as the treatment was raised from 60 to 94 C, the peroxide values, after exposure of the samples to light, increased. Differences in peroxide values are detectable after some hours and pronounced after 1, 2, and 4 days.

Double pasteurization of cream for buttermaking is a common practice in Ireland. Figure 2 shows that re-pasteurization of cream within the temperature range 60 to 94 C resulted in considerably increased peroxide values.

The control cream used in this experiment was pasteurized at 72 C for 15 sec and reached a higher peroxide value than the unpasteurized control used in the previous experiment.

When cream was heated, the copper concentration of the serum phase dropped. Figure 3 shows the percentage decrease in copper content in the cream serum.

Apparently copper migrates from the serum to the fat globule surfaces. At 66 C there was a reduction of about 5% of the serum copper while at 94 C for 15 sec the serum copper had diminished by 25%. Temperatures used for cream pasteurization usually fall within the range of 72 to 95 C and therefore within that where copper migration occurred.

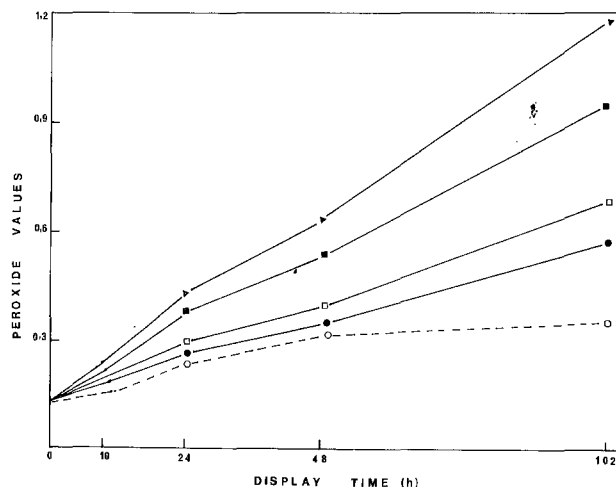


Figure 1. The effect of the temperature of pasteurization on the peroxide values of cream. Pasteurization temperatures: O—O Control (unpasteurized), ●—● 60 C for 15 sec, □—□ 70 C for 15 sec, ■—■ 82 C for 15 sec, and ▲—▲ 94 C for 15 sec.

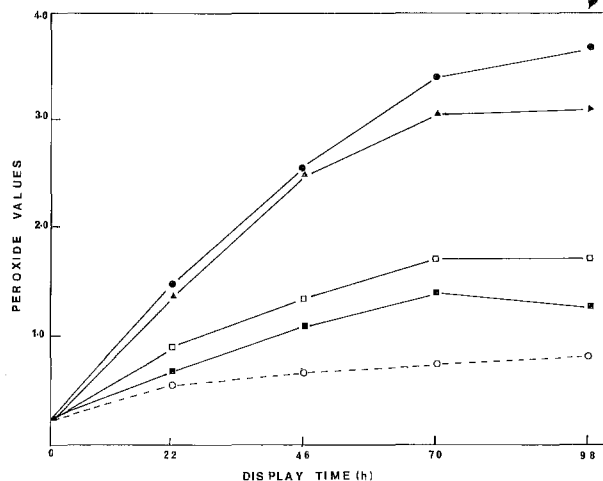


Figure 2. The influence of the temperature of repasteurization on the peroxide values of cream pasteurized at 72 C for 15 sec. O—O control pasteurized at 72 C for 15 sec, ■—■ repasteurized at 60 C for 15 sec, □—□ repasteurized at 70 C for 15 sec, ▲—▲ repasteurized at 82 C for 15 sec, and ●—● repasteurized at 94 C for 15 sec.

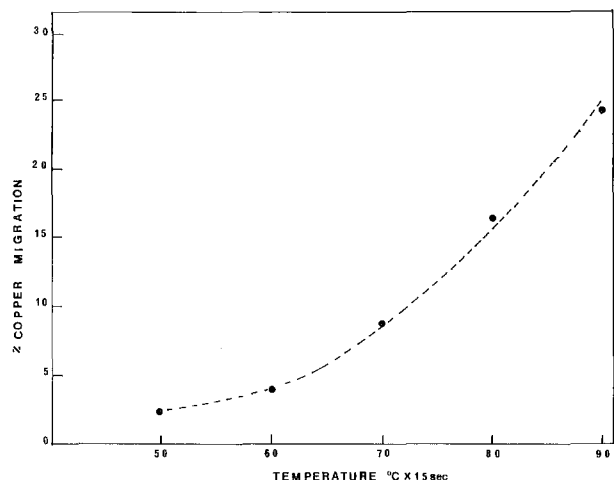


Figure 3. The influence of heat treatment on the migration of copper from the serum to the fat phase of cream.

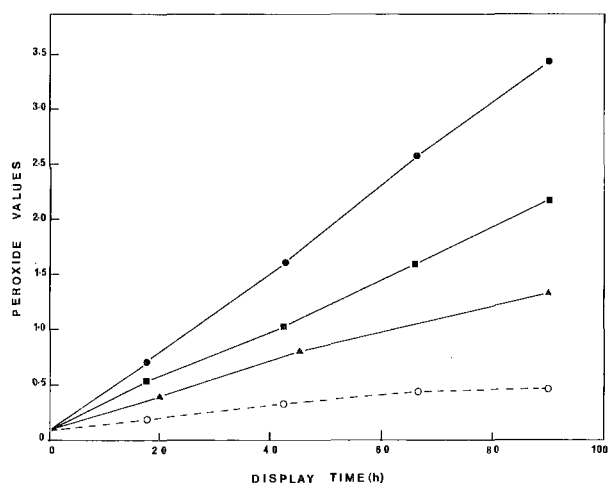


Figure 4. The influence of addition of EDTA on the peroxide values of cream. ○—○ raw cream, ●—● cream pasteurized at 82 C for 15 sec, ■—■ Cream pasteurized at 82 C + 0.05% EDTA, and ▲—▲ Cream pasteurized at 82 C + 0.2% EDTA.

Figure 4 shows the effect on peroxide values of addition of ethylenediaminetetraacetic acid (EDTA), a water soluble copper complexing agent, to cream before heat treatment. The EDTA (0.05 and 0.2%) was added 2 h before heat treatment and the sample well stirred at intervals to permit complexing of copper to take place. Addition of EDTA proved effective in retarding oxidation of heat treated cream. It would appear that the EDTA, by complexing copper, prevented its migration to the fat globule surfaces, and made it less available as an oxidation catalyst. In another experiment EDTA was added to cream after heat treatment. Its antioxidant influence was only very slight when compared with the effect shown in Fig. 4.

The influence of free sulfhydryl groups in photocatalyzed oxidation was studied by adding sulfhydryl containing compounds to pasteurized cream (72 C for 15 sec) and measuring the peroxide values after

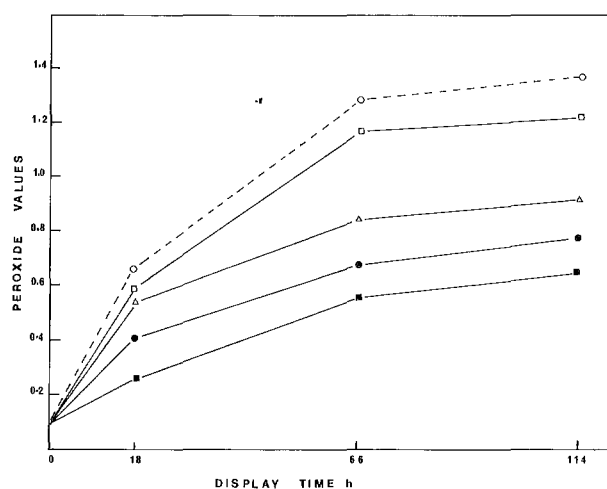


Figure 5. The influence of cysteine hydrochloride on the peroxide values of cream pasteurized at 72 C for 15 sec. ○—○ control, □—□ 0.01% cysteine hydrochloride, △—△ 0.05% cysteine hydrochloride, ●—● 0.10% cysteine hydrochloride, and ■—■ 1.0% cysteine hydrochloride.

different holding times. The following compounds were used: thioglycolic acid, cysteine hydrochloride, glutathione, and mercapto-ethanol. The control and test samples were pasteurized at 72 C for 15 sec and held in the display cabinet to render them more susceptible to oxidation and thus permitting easier detection of the effect of the added compounds.

Cysteine hydrochloride had a marked antioxidant effect over the concentration range of 0.01 to 1.0% (Fig. 5). Further experiments showed that cysteine hydrochloride had antioxidant activity at concentrations down to 0.001%. The compounds thioglycolic acid, glutathione, and mercapto-ethanol also inhibited photocatalyzed oxidation of cream when used at concentrations ranging from 0.001 to 0.1%.

Homogenization of cream inhibited photocatalyzed oxidation. The antioxidant influence of homogenization was apparent at pressures as low as 100 psi and increased with increasing pressures up to 2500 psi the relative benefit declining as the pressure was raised (Fig. 6).

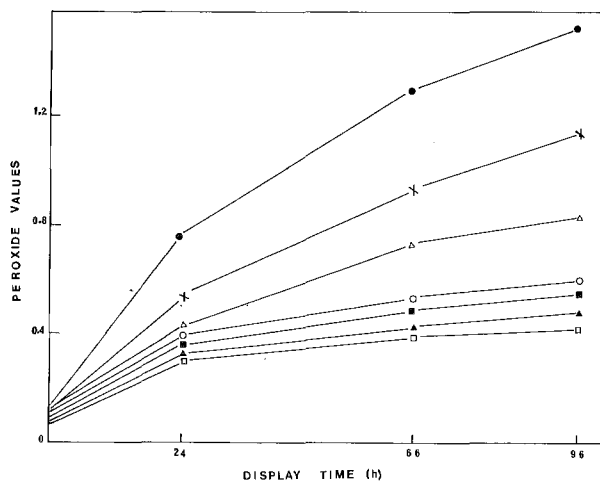


Figure 6. The effect of different pressures of homogenization on the peroxide values of cream. ●—● unhomogenized control, x—x 100 psi, △—△ 500 psi, ○—○ 750 psi, ■—■ 1,000 psi, ▲—▲ 1,500 psi, and □—□ 2,500 psi.

When the homogenized creams were subsequently heated to 80 C for 15 sec and then held under fluorescent light, the antioxidant influence of homogenization, though somewhat diminished, was still evident even at the lower pressures used.

DISCUSSION

Heat treatment of cream increased its propensity to photocatalyzed oxidation as measured by peroxide values. The higher the temperature up to 94 C for 15 sec, the greater the peroxide values after exposure to fluorescent light. This appears to conflict with the widely held opinion that liberation of free sulfhydryl groups when the serum proteins, β -lactoglobulin in particular, are denatured by heat confers antioxidant properties on many dairy products.

In a critical literature review, Wilkinson (13) noted that there was evidence both to suggest that free -SH

groups acted as catalysts in the oxidation of lecithin and certain fatty acids and also to support the view that -SH groups acted as antioxidants. He suggested that at low concentrations -SH groups have a pro-oxidant effect, whereas at high concentrations they have antioxidant properties. Aurand et al. (1) added various levels of cysteine hydrochloride to raw milk before heating and found no inhibitory effect on oxidized flavor development. The sulfhydryl compounds cysteine hydrochloride, glutathione, thioglycolic acid, and mercapto-ethanol when added to pasteurized cream in this study all showed definite antioxidant activity which increased with increasing concentration over the range of 0.001 to 0.1%.

Despite the antioxidant influence of sulfhydryl groups it is clear from this study that the net effect of heat treatment of cream is pro-oxidant. Smith and Dunkley (11) concluded that pasteurization increased the susceptibility of milk to oxidized flavor development. Samuelsson (9) noted that the TBA values of heated buttermilk increased with increase in heat treatment. In a previous study in this laboratory Phelan (6) showed that when the temperature of repasteurization of cream was raised from 80 to 105 C the TBA values, after holding, increased.

While relatively high treatment of cream leads to increased peroxide values, this pro-oxidant effect does not necessarily hold for other milk products. Thus we have found, in this laboratory, that high heat treatment of milk for drying gave lower peroxide values in both nitrogen and non-nitrogen packed full cream milk powder held at 10 C.

The antioxidant influence of free sulfhydryl groups released during heat treatment of cream is apparently overshadowed by another change which is decidedly pro-oxidant. Copper migrates from the serum phase to the surfaces of the fat globules where it acts as an oxidation catalyst (Fig. 3). If the migration of copper from the serum phase is inhibited by addition of certain chelating agents before heat treatment, the pro-oxidant influence is diminished.

When cream is heat treated before addition of a copper chelating agent, the role of the chelating agent in inhibiting oxidation is less. Presumably, the copper which had already migrated to the fat globule surfaces is not readily complexed by the chelating agent.

The view that singlet oxygen is involved in the primary initiation of lipid oxidation is gaining in favor. Singlet oxygen can be formed through photochemical reactions in the presence of a sensitizer. Light, particularly in the ultra violet region is a particularly effective initiator of oxidation when sensitizers such as copper and bound copper complexes are present. Heat treatment of cream

may perhaps alter the copper-protein complexes and thus increase their pro-oxidant effect.

Hill (2) has recently demonstrated the presence in milk of the antioxidant enzyme superoxide dismutase and has suggested that the small amounts of dismutase normally present in milk might play a part in the control of lipid oxidation. Although little is yet known about the role of superoxide dismutase in milkfat oxidation, the heat treatment given to cream in this study may have inactivated the enzyme thus allowing the superoxide anion and its products to catalyze oxidation.

The physical change in the fat phase brought about by homogenization inhibits development of oxidized flavor in pasteurized milk possibly through the formation of copper chelates (11). Likewise, homogenization of pasteurized cream (72 C for 15 sec) at pressures from 100 up to 2500 psi reduced the tendency of cream to photocatalyzed oxidation. The anti-oxidant effect, which increased with increasing pressures up to 1500 psi, was relatively greater at the lower pressures used.

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Influence of Feeding Dehydrated Poultry Waste on Composition and Organoleptic Quality of Milk¹

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ABSTRACT

A double reversal trial with three, 4-week periods was used to determine the effect on milk quality of feeding dehydrated poultry waste to lactating dairy cows. Fifteen cows were fed a control ration for 4 weeks (Period 1), followed by an experimental ration containing dehydrated poultry waste for 4 weeks (Period 2), and then by the control ration for 4 weeks (Period 3). Milk was collected daily and scored by five judges using the ADSA milk flavor scoring guide. Milk composition (fat, protein, lactose, and solids-not-fat) was determined on alternate days. During Periods 2 and 3 weekly composite samples were collected to determine concentrations of cadmium, copper, lead, zinc, and tocopherol. No significant differences were found in any of the milk quality factors studied.

Dehydrated poultry waste (DPW) represents a potentially valuable feed source for ruminants (5). Some reports (10, 12) have indicated that DPW can be added to the diet without adversely affecting production of a cow; however, this information is of little value unless data are available regarding the affects of DPW on flavor of milk and on some factors of public health concern. This study was undertaken to determine the affects of DPW on flavor of milk and on concentration of certain trace metals in the raw milk.

MATERIALS AND METHODS

Feed

The DPW was supplied by Protein Resources, North Hollywood, CA 91604. The DPW was prepared by heating poultry waste in a custom-made gas-fired dehydrator for approximately 30 min at 60 C. The typical composition of the DPW is given in Table 1. The lot of material used in this trial was tested by standard methods (1) and contained less than 1 of *E. coli* or salmonellae/g; the SPC was 30,000/g at the time of formulating. This lot of DPW was shipped to the feed mill at the California Polytechnic State University, San Luis Obispo, where it was mixed into the grain ration. Composition of the control concentrate ration and the experimental DPW concentration ration is given in Table 2. Composition of the experimental DPW ration was formulated to meet the nutritional balance in the control grain ration. Experimental and control grain rations were calculated to be isonitrogenous and isocaloric. Trace mineralized salt, dicalcium

TABLE 1. Typical analysis of dehydrated poultry waste (DPW)

Component	Amount
	(%)
Moisture	9.3
Crude protein (% N × 6.25)	24.1
Crude fiber	11.9
Crude fat (ether extract)	1.70
Ash	34.4
Calcium	9.1
Phosphorus	2.72
	(ppm)
Lead	7.0
Cadmium	1.3
Copper	51.1

phosphate, and limestone were removed from the experimental ration due to the mineral contributions of the DPW. The DPW constituted an average of 10% of the total grain ration and was readily accepted by cows.

Both control and experimental grain rations were fed based on production, in the barn, at the time of milking. Hay and corn silage were fed ad libitum outside the barn.

Experimental

The 15-cow herd consisted of nine Holsteins and six Jersey × Guernseys of mixed ages and lactation stages. Production in 305 days of lactation averaged 627 lb. of milkfat and 17,880 lb. of milk for the Holsteins, and 484 lb. of milkfat and 10,741 lb. of milk for the Jersey × Guernseys.

The 12-week trial consisted of three, 4-week periods. During periods 1 and 3, the herd was fed the control ration and during period 2 the herd was fed the experimental DPW ration.

At each milking, the total milk produced was collected in clean, sanitized, stainless steel cans from the pipeline milking system after passing through an in-line filter. Cans of milk were then promptly taken about one-half mile to the milk processing plant and quickly cooled in a jacketed milk vat to below 1 C. After collecting the PM and

TABLE 2. Composition of the grain rations

Feed ingredient	Control	Experimental
	(%)	(%)
Ground barley	29.7	27.7
Cotton seed meal	14.9	8.9
Urea	1.0	1.0
Dicalcium phosphate	1.1	
Limestone	0.4	
Trace mineral salt	0.5	
Beet pulp pellets	9.9	9.9
Rolled barley	41.6	41.6
DPW		9.9
Molasses	1.0	1.0

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AM milkings, representative samples were withdrawn from the tank for the organoleptic and chemical examinations. The remaining milk was pasteurized and stored at about 1 C until sufficient pasteurized milk had been collected to make Monterey Jack cheese.

Organoleptic evaluations

Raw milk samples were organoleptically evaluated daily by five trained judges using the ADSA milk flavor scoring guide. Judges made independent judgements and were not told of the nature of the trial, other than being informed that a new grain ration was being tried. Also, they were not informed as to when the new ration was being used.

Chemical analyses

On alternate days raw milk samples were collected and shipped to a commercial laboratory for duplicate analyses for milkfat, protein, lactose, and solids-not-fat by the Infrared Milk Analyzer (3).

A weekly composite raw milk sample was sent to the University of California, Davis, for determination of cadmium, copper, lead, zinc, and tocopherol. Procedures for samples collection to minimize contamination have been reported elsewhere (5).

Cadmium and lead were determined using the atomic absorption spectrophotometric procedure described by Bruhn and Franke (4). Copper was determined using the spectrophotometric procedure of King and Dunkley (8). Zinc was determined by the dry ashing-atomic absorption procedure proposed by the Analytical Methods Committee (2).

Tocopherol in milk was determined using the modified Roesse-Gottlieb extraction colorimetric procedure by Low and Dunkley (9). The fatty acid composition of the lipids extracted from the milk by a modified Roesse-Gottlieb procedure was determined by isothermal programmed gas chromatography of the methyl esters prepared by the procedure of Christopherson and Glass (6). An automated gas chromatograph equipped with a mini-computer was used to quantify the results (13).

Data were treated statistically by the methods proposed by Snedecor and Cochran (11).

RESULTS AND DISCUSSION

Results of the flavor evaluation by trained judges indicated that dietary DPW did not influence flavor of raw milk (Table 3). Extreme values of daily mean scores

TABLE 3. Variation of milk flavor scores during DPW trial

Measurement	Period	Treatment	High	Low	Mean
Flavor Score ^a	1	Control	39.3	38.5	38.8
	2	DPW	39.1	38.7	38.9
	3	Control	39.1	38.6	38.8

^aADSA scoring guide

together with the period mean of daily mean scores are shown. No significant differences between mean flavor scores during the three periods were found. All samples scored were criticized as having a slight feed flavor and no judge noted any defect other than feed. Milk of this flavor quality would be considered very satisfactory for bottling. Raw milk from the regular herd at California Polytechnic State University usually scored 38.5, based on previous findings of the University of California Cooperative Extension Milk Quality Program. Others (12) have reported a flavor score of 37.6 for milk from cows consuming DPW, but the control milk scored 37.5. Milk with this flavor score would be considered to have a pronounced feed flavor and under California conditions not acceptable for bottling directly. These authors used only one judge and evaluated samples for only 2 days;

whereas our study employed daily evaluations with five judges for over 12 weeks.

TABLE 4. Variation of trace metal and tocopherol concentrations in the raw milk

Variable	Period	Treatment	High	Low	Mean
Cadmium ($\mu\text{g}/\text{kg}$)	2	DPW	8.72	3.86	6.24
	3	Control	6.28	0.54	3.71 ^a
Copper ($\mu\text{g}/\text{kg}$)	2	DPW	30.8	28.8	29.8
	3	Control	35.8	33.9	34.8
Lead ($\mu\text{g}/\text{kg}$)	2	DPW	74.0	37.8	49.4
	3	Control	93.9	14.8	56.2
Zinc (mg/kg)	2	DPW	4.09	4.02	4.05
	3	Control	3.99	3.76	3.87
Tocopherol ($\mu\text{g}/\text{kg}$ lipid)	2	DPW	20.29	16.83	18.62
	3	Control	22.54	16.16	18.25

Results of analyses for cadmium, copper, lead, zinc, and tocopherol during Periods 2 and 3 are in Table 4. The extreme values found and the mean of all values are shown for each of the constituents. Comparison of the two periods using the student's t-test disclosed no significant treatment effects ($\alpha = .05$). Thus no influence of the DPW was noted. Cadmium concentrations for herd samples taken in a recent statewide survey (4) averaged 6 $\mu\text{g}/\text{kg}$, while lead concentrations averaged 90 $\mu\text{g}/\text{kg}$. Thus concentrations of cadmium and lead in this trial were in good agreement with what has been reported elsewhere in California and are within the USPHS permissible maximums for drinking water.

Similarly, concentrations of copper and tocopherol were well within the means reported statewide (5), and consequently feeding of DPW at the level used in these trials should cause no changes in the oxidative stability of cows' milk.

The zinc concentration in cows' milk is nutritionally important, since cows' milk provides the principal source of nutrition for infants. Our data (Table 4) show that the DPW ration did not affect concentration of zinc in the milk.

The trace mineral analysis was primarily a test for carryover of minerals from the DPW to the milk. Since only a very small proportion of metals ingested by a cow is transferred to milk, lack of an observed carryover could almost have been predicted.

Statistical comparisons of the gross composition

TABLE 5. Changes in gross composition of milk during DPW trial

Component ^a	Period	Treatment	High	Low	Mean
			(%)	(%)	(%)
Fat	1	Control	5.41	4.12	4.75
	2	DPW	5.53	4.45	4.82
	3	Control	5.00	3.72	4.47
Protein	1	Control	3.91	3.73	3.79
	2	DPW	3.82	3.57	3.74
	3	Control	3.80	3.49	3.70
Lactose	1	Control	4.67	4.28	4.46
	2	DPW	4.50	4.37	4.42
	3	Control	4.54	4.29	4.43
SNF	1	Control	9.01	8.61	8.78
	2	DPW	8.75	8.50	8.69
	3	Control	8.85	8.43	8.66

^aDetermined by an Infrared Milk Analyzer

(Table 5) of the milk produced in the three periods were made using the student's t-test. No significant treatment effects ($\alpha = .05$) were found.

TABLE 6. *Effect of treatment on fatty acid composition of milkfat*

Fatty acid	DPW	Control
	Period 2	Period 3
(g/100 g lipid ^a)		
Caproic (6:0)	2.51 ± 0.05 ^b	2.58 ± 0.04 ^b
Caprylic (8:0)	1.75 ± 0.01	1.82 ± 0.02
Capric (10:0)	4.42 ± 0.03	4.64 ± 0.10
Undecanoic (11:0)	0.43 ± 0.04	0.49 ± 0.02
Lauric (12:0)	5.20 ± 0.01	5.48 ± 0.21
Myristic (14:0)	13.60 ± 0.25	13.36 ± 0.25
Myristoleic (14:1)	2.31 ± 0.05	2.27 ± 0.06
Palmitic (16:0)	35.36 ± 0.30	33.94 ± 0.25
Palmitoleic (16:1)	3.98 ± 0.09	3.64 ± 0.13
Stearic (18:0)	8.02 ± 0.04	8.71 ± 0.14
Oleic (18:1)	18.88 ± 0.51	19.01 ± 0.65
Linoleic (18:2)	2.38 ± 0.03	2.79 ± 0.10
Linolenic (18:3)	0.98 ± 0.10	1.05 ± 0.04
Arachidonic (20:4)	0.21 ± 0.01	0.21 ± 0.01

^aExpressed as the methylester

^bMean ± Standard Deviation

Samples taken during the second and third periods were also examined for fatty acid composition of their triglycerides (Table 6). No differences were found between the two test periods. This test was included as a sensitive measurement of whether rumen activity was disturbed or modified by the DPW supplement.

The lack of any statistically significant treatment difference for the variables studied indicates that feeding of this lot of DPW to lactating dairy cows did not affect composition of milk or quality parameters that were measured. The inability of five experienced judges to detect any flavor differences indicates that there was no effect of dietary DPW on flavor of milk.

CONCLUSION

One trial with 15 cows and lasting 12 weeks is hardly sufficient to draw meaningful conclusions for the entire dairy industry. However, our objective was to determine whether any serious flavor and/or public health problems would result from freeing DPW to lactating

dairy cows. Our results indicate that none should be expected, and that further research at higher levels of DPW replacement with larger herds for longer periods is certainly justified.

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Estimating Somatic Cells in Milk Samples by the Membrane-Filter-DNA Procedure

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ABSTRACT

Further developments in the membrane-filter DNA procedure for determining the somatic cell content of milk samples are described. A statistical experiment has been done comparing the membrane-filter test to the direct microscopic somatic cell count. Under conditions of the assay a relationship of 0.093 absorbance unit per million cells was obtained at a wavelength of 490 nm. The membrane filter technique is more precise than the direct microscopic count and is not subject to technician bias. A method is described for standardizing the procedure between and within laboratories based on use of lyophilized bovine somatic cells trapped on membrane filters as a calibration device.

The somatic cell content of milk samples is a useful index of both udder health and milk quality. The most direct method of determining the somatic cell content of a milk sample is by microscopic counting procedures. Rather elaborate methods have been devised for conducting (6, 9) and standardizing the direct microscopic somatic cell count (DMSCC). There is, however, and inherently large variance associated with microscopic counting which ultimately determines the accuracy of the procedure in practical application. The DMSCC is further hampered because the determination involves a subjective judgement by the technician. Moreover, the time required for microscopic counting virtually precludes its use in large scale screening programs.

Other procedures can be used to count somatic cells in milk samples. Particle counters such as the Coulter counter have been adopted and more recently the Fossomatic® has been introduced which relies on a fluoro-optical counting procedure using a DNA-specific fluorescent dye (7). While these tests are accurate, and large numbers of samples can be processed in a work day, standardization continues to be a problem in addition to the initial high costs. These instruments also require relatively skilled technicians to operate and maintain them.

It was recognized that an accurate and simple test using preserved samples was required so that somatic cell

testing programs could be implemented more widely in the field. The membrane filter-DNA (MF-DNA) test was developed to meet this need and is a test that requires relatively simple equipment and which can be done on either preserved or unpreserved samples (4, 9). It is conceptually very simple and involves filtration of a detergent-dispersed sample of milk through a membrane filter with pores sufficiently small to trap somatic cells. A colorimetric procedure based on the reaction of indole with hydrolysed DNA (2, 5, 9) is then used to determine the DNA content which is directly related to the number of cells in a given volume of milk. Further development of the membrane filter-DNA procedure is described in this paper. The primary purpose of these experiments was to develop a statistically valid relationship between the MF-DNA procedure and the DMSCC. In addition, estimates of the variance, accuracy, and precision of the MF-DNA procedure were determined and compared to that of the DMSCC.

MATERIALS AND METHODS

DMSCC

Procedures used for microscopic counting of somatic cells were those described by the National Mastitis Council (3, 6, 8). A working factor of 5,900 was used in the strip counting procedure. The sum of the horizontal and vertical strip counts on each film were used as estimates.

MF-DNA

The MF-DNA procedure is diagramed schematically in Fig. 1. Two and a half milliliters of a milk sample are first mixed with 20 ml of a hot Triton X-100® (Sigma Chemical Company) EDTA diluent. The Triton X-100® (0.1% vol/vol) is a detergent that reduces the size of the fat globules and the trisodium EDTA (4.5 g/l) sequesters Ca²⁺ ions thus effectively reducing the size of casein micelles. The sample-diluent solution is then filtered through a cellulose acetate membrane filter of 3 to 5- μ pore size (Millipore Corporation Cat. #EMWPO2500, for this study, Lot #13157-7 filters were used) and somatic cells are left deposited on the filter. The membrane is then rinsed with 1 to 2 ml of a 0.9% NaCl solution to remove the last traces of detergent because the detergent itself reacts with the color reagent. The filters are then individually placed in 13 × 100-mm disposable glass test tubes and 5 ml of freshly prepared color reagent is added. The color reagent consists of one part 5 N HCl, two parts water, and one part 0.060% indole. The tubes are then incubated at 90 C for 25 min. Upon completion of the incubation, tubes are immediately cooled to ambient by immersion in a cold water

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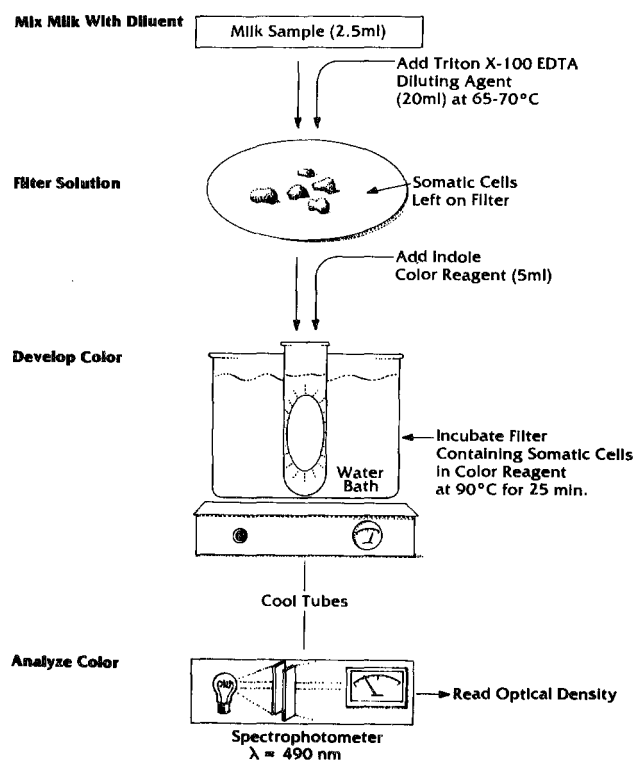


Figure 1. Schematic diagram of the membrane filter-DNA procedure.

bath. This will stop any further color development. After samples cool, the optical density of each solution is read in a 1-cm path length cell at $\lambda=490$ nm to determine intensity of color development.

Because the filter and the color reagent will each contribute to color development in addition to that caused by somatic cells, these contributions are subtracted by processing blank filters along with each incubation. A blank filter is one that undergoes identical processes of the technique except no milk is actually filtered (only diluent). The optical density of the blank is then subtracted from that of the corresponding filter which has cells trapped on it, to give the optical density caused by the cells-color reagent reaction alone. Four blank filters were incubated along with each rack of 72 tubes.

Equipment

The steps of the procedure just described can be carried out manually and the optical density measurement need not be made with a special instrument. However, to develop a procedure that is capable of great precision and is also able to handle a large number of samples in a day, an automatic mixing dilutor and a flow-through spectrophotometer were incorporated into the technique. The Digidil automatic dilutor-dispenser (Gilson Medical Electronics, Middleton, Wisconsin) is operated by thumb button on a hand-held probe, and it automatically mixes 2.5 ml of milk with 20 ml of hot diluent and dispenses the solution into a manifold holding 12 filters (Millipore Corp., Bedford, Mass. Cat. #XX2702550). Several such manifolds may be attached in series for processing large numbers of samples. The Digichrome flow-through spectrophotometer (Gilson Medical Electronics, Middleton, Wisconsin) is equipped with a digital readout, 1-cm path length cells, dual beam optics, and a 490-nm band-pass interference filter. The double beam optics contribute to stable readings and permit accurate measurements of low optical density values on a routine basis. Dual beam optics also permit automatic subtraction of

^aFor the statistical study, both blanks and regular filters were read referenced as water, and the blank values were manually averaged and subtracted to give the desired optical densities. This was done to determine the optical density of the individual blank filters and to also determine their variability.

^bBoth operators have had approximately 2 years of experience working with the DMSCC.

the blank solution by placing it in the reference cell^a. The solution in the sample cell is controlled by a hand-held probe with a thumb button, which when pressed, allows a new solution to be automatically drawn into the sample cell and its optical density measured. The Digichrome can be calibrated in either regular optical density units or in equivalent cell count concentration. Once the Digichrome has been calibrated to give cell concentrations directly, a CoCl_2 solution is used as a reference to check calibration on a daily basis.

Statistical design

The main purpose of this study was to determine the relationship between optical density measurements obtained with the MF-DNA technique and the somatic cell concentration as determined by the DMSCC. A secondary purpose was to examine the reproducibility of each method and to investigate each method's sensitivity to operator technique.

This study was designed to take identical samples and to use both techniques to determine either somatic cell concentration or optical density. The samples used were relatively fresh (24 to 28 h) refrigerated, unpreserved grade B bulk milk obtained from the Wisconsin Animal Health laboratories in Madison. They were selected to insure a wide distribution of cell concentrations which would normally be encountered in somatic cell screening programs. This range would typically vary from the lowest limits of detection to several million cells per milliliter. The experimental design of the study involved two operators actually doing the work (Operators A and B)^b. The study took place on two successive days, involving 36 different samples each day. For the total experiment three replicate DMSCC slides were made for each sample (half by Operator A and the other half by Operator B) and six MF-DNA assays were done on each sample, three by each operator. Later, over a period of several weeks, both operators read all the slides, thus giving each operator a total of three DMSCC replications for a specific sample. For a particular replicate set, both operators would do the MF-DNA technique and one would also prepare slides for the DMSCC. These operators would then switch roles, for the next replicate set. By having two people do the study, operator variability of each technique could be ascertained, and by doing replicates of both tests on each sample, method precision could be compared. A schematic diagram illustrating the position and function of each operator for day 1 is shown in Table 1. The design for

TABLE 1. Experimental design for correlation of the MF-DNA and DMSCC tests (day 1)

Replicate 1 (random sampling plan 1)	
Operator A	Operator B
MF-DNA method-36 samples	DMSCC method ^a -36 samples
DMSCC method-36 samples	MF-DNA method-36 samples
Replicate 2 (random sampling plan 2)	
DMSCC method ^a -36 samples	MF-DNA method-36 samples
MF-DNA method-36 samples	DMSCC method-36 samples
Replicate 3 (random sampling plan 3)	
MF-DNA method-36 samples	DMSCC method ^a -36 samples
DMSCC method-36 samples	MF-DNA method-36 samples

^aIndicates operator who actually made the slides for a particular replicate set.

the second day was identical to the first except that the operators switched their initial roles.

Samples were arranged on a table in a 6×6 matrix and the order of sampling was determined by a random number sequence generated before the experiment. Each replicate set had a different random sampling plan which was followed by both operators. All milk samples were coded as were all the DMSCC slides, so at no time did either operator know what sample was being measured. With the random sampling plans, the replicates were not taken in succession, or even at the same point within each replicate set. At the end of the study, 432 (72×6) estimates had been made for each procedure.

RESULTS

Filtration of milk samples

As originally described (4, 9) the procedure for

filtration of milk samples to trap somatic cells on membrane filters included the non-ionic detergent Triton X-100® to disperse the fat globules. Using this procedure we found that at times, 10% or more of the milk samples would plug the filter. When this occurs the sample is done again, usually using one-half the original volume of milk. Nevertheless, the plugging greatly inconveniences the technician doing the test. Attempts were made to alleviate these problems by use of other types of detergents with the idea that if dispersion were improved filtration would also improve. These attempts were unsuccessful and in general the detergents tested were less effective than the original Triton X-100. Ionic detergents which disrupted the nuclei of somatic cells causing DNA to form a gel caused all filters to plug. Filtration characteristics were improved considerably by using the chelating agent Na₃-EDTA (trisodium salt of ethylene diaminetetraacetic acid) to complex divalent ions in the milk sample and cause a dispersion of casein micelles. Filtration speed increased dramatically and the plugging frequency decreased to the 1 to 3% that we now normally observe.

Filterability of milk was tested with different filters having pore sizes from 1 to 5 μ since with smaller pore sizes filtration becomes impossible and with larger pores, cells or cell fragments might go through the filter. Placing this restriction greatly limits the types of filters available. Filters composed of cellulose acetate (2 and 3 μ), mixed cellulose esters (1 and 3 μ), polyvinyl chloride (2 and 3 μ), and Teflon® PTFE (3 μ) (all from Millipore Corporation, Bedford, Massachusetts) were found to be acceptable, in that milk samples could be filtered through them and were equivalent in this characteristic.

TABLE 2. Analysis of variance of experimental data comparing the MF-DNA and DMSCC tests

Source	DF	Sums of squares	Mean squares	Probability ^a
<i>DMSCC</i>				
Sample	62	4.329×10^{13}	6.983×10^{11}	< .01
Person	1	9.634×10^{12}	9.634×10^{11}	< .05
Sample \times Person	62	1.235×10^{12}	1.992×10^{10}	< .01
Trial	2	1.715×10^{11}	8.579×10^{10}	NS
Sample \times Trial	124	3.376×10^{12}	2.722×10^{10}	< .01
Person \times Trial	2	9.009×10^{10}	4.505×10^{10}	< .05
Sample \times Person \times Trial	124	9.742×10^{11}	7.857×10^9	< .01
<i>MF-DNA</i>				
Sample	62	2.053	3.312×10^{-2}	< .01
Person	1	3.597×10^{-3}	3.597×10^{-3}	NS
Sample \times Person	62	1.369×10^{-2}	2.209×10^{-4}	NS
Trial	2	4.129×10^{-3}	2.065×10^{-3}	NS
Sample \times Trial	124	4.953×10^{-2}	3.995×10^{-4}	< .01
Person \times Trial	2	3.796×10^{-3}	1.898×10^{-3}	< .01
Sample \times Person \times Trial	124	2.645×10^{-2}	2.133×10^{-4}	< .01
<i>Sample population properties</i>				
	DMSCC (n = 63)	MF-DNA (n = 63) optical density	Membrane filter blanks (n = 48) optical density	
\bar{x}	638,000	0.130	0.028	
σ	376,000	0.075	0.012	

^aProbability that the differences seen are due to chance alone.

Several other types were evaluated but a great number of samples plugged filters made from mixed cellulose esters, gave high blank values, and thus were unacceptable because of the error this introduces when subtracted from samples with small numbers of cells. The other three filter types gave approximately the same blank values as those reported below (see Table 2). The cost of the Teflon® filters is higher than that of the cellulose acetate and polyvinyl chloride such that cost becomes a limiting factor. The cellulose acetate filters, because of their pore size and microscopic morphology, are best suited to use in manifold arrangements.

The bubble point of the membrane filters becomes an important practical consideration when samples have different filtration rates. When filters with a pore size larger than 3 to 5 μ are used, the vacuum is reduced as soon as the first sample finishes filtering. With smaller pore sizes a pressure gradient can be maintained across a wet filter and all that is necessary is that a small amount of water be added to that filter with a squeeze bottle.

Filter morphology appears to be as important a determinant for milk filterability as pore size. The pore structure of cellulose acetate filters shows a definite

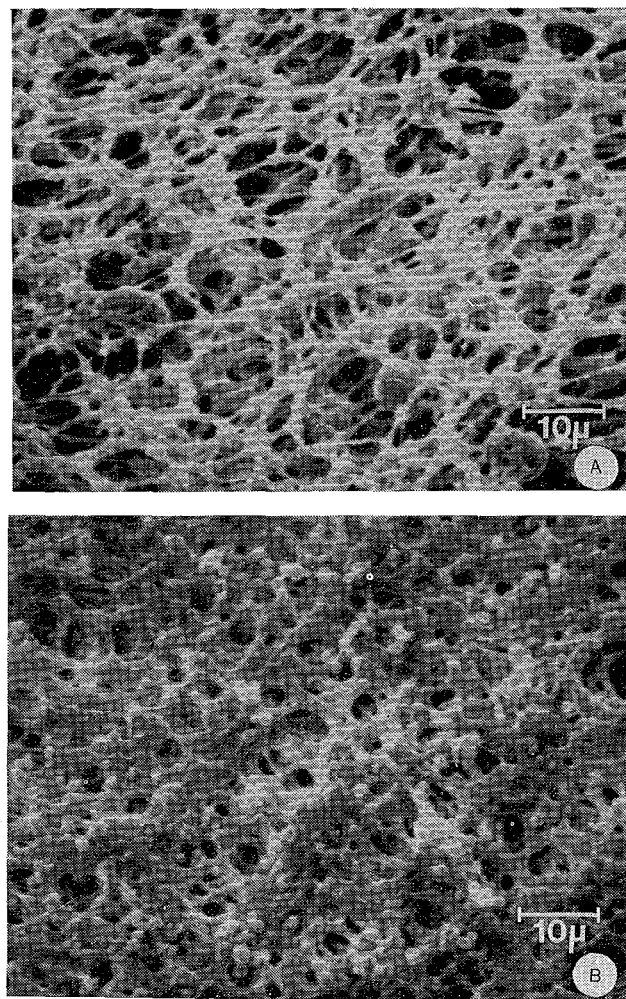


Figure 2. Scanning electron micrographs of cellulose acetate membranes (EMWP-02500) showing difference in morphology between the top face (a) and bottom face (b) of the filter.

polarity with one side having a much more open structure than the other (see Fig. 2). The best filtration characteristics are experienced with filtration occurring from the more open side of the membrane toward the more closed side. The structure might be thought of as being made of small funnels; the funnel must be in the proper orientation for filtration to occur. When used in the appropriate orientation (determined by the way they are packaged), plugging rates of 1 to 3% are routinely observed. The samples which plug the filters generally contain in excess of 1.5×10^6 cells per ml. Using 2.5 ml of milk, this is approximately what one might expect as a theoretical plugging threshold assuming a cell diameter of 10μ and a usable filter area of 2.8 cm^2 . Of the 432 filtering trials done in this study, less than 1% plugged.

Color development

The previous procedures for DNA determination using indole (2, 4, 5, 9) have placed the samples in boiling water baths for 10 min to hydrolyse the DNA and to allow the color reaction between the deoxyribose and indole to occur. In practice, in a laboratory doing many tests, it is difficult to maintain a boiling water bath because of evaporation and the associated humidity problems, especially in smaller laboratories. We have found that comparable results can be obtained using a standard, gabeled covered, laboratory water bath at a temperature of 90 C. The increase in time associated with this decrease in temperature is modest. Figure 3 shows the

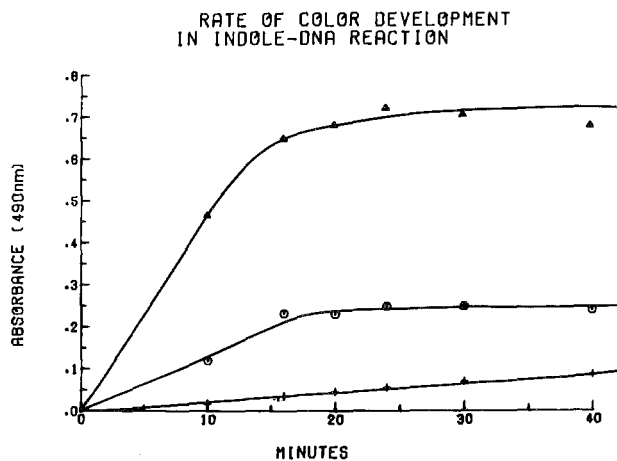


Figure 3. Rate of color development in the Indole-DNA reaction at 90 C; (+ - +) reagent blank, (O - O) 20 μ g DNA, (▲ - ▲) 60 μ g DNA.

rate of color development for two different concentrations of DNA. At 90 C the reaction is complete in 20 min as would be expected from general chemical principles where a 10-C increase in temperature would be expected to double the reaction time. However, to allow for a certain margin of error, it is recommended that color development be allowed to proceed for 25 min. As can be seen in the figure, the reagent blank value increases linearly with time giving the appearance that the reaction continues indefinitely (9) but when the blank value at any time is subtracted from the total color, the reaction resulting from DNA in the sample will remain

relatively constant after 20 min. Thus any reaction time in the range of 20 to 40 min might be used. However, the increase in the blank value decreases the accuracy of the test, so 25-min incubation is recommended.

Statistical evaluation

Figure 4 shows plots of the median values obtained by

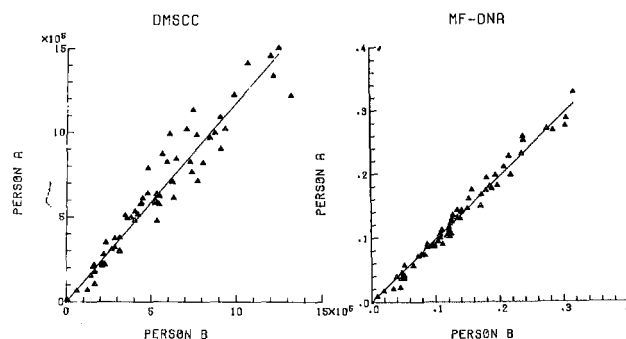


Figure 4. Comparison of operators doing the DMSCC and the MF-DNA procedures. The points represent the median (the middle one of three) estimates for the two operators for a given sample. The line is the least squares fit forced through zero. The units of the MF-DNA measurements are optical density at 490 nm.

Operator A versus those of Operator B for identical samples for both the MF-DNA and DMSCC method. Under ideal conditions Operators A and B will have made identical estimates, and thus the data points should fall on a line intercepting the origin with a slope of 1.0. True sample to sample differences can only move the points in a direction parallel to the 45° line. The scatter about the line is only due to variation in the ability of the technicians in making the estimate. The distance a point lies perpendicular (orthogonal) to the 45° line is all caused by variables other than true sample differences such as technician variability, environment factors, or equipment errors. Two things become apparent when the data are plotted in this manner. First, there is much greater scatter about the line for the DMSCC than for the MF-DNA procedure. Second, the slope of the least squared line (forced through zero) has nearly the theoretical slope of 1.0 for the MF-DNA procedure whereas the slope of the DMSCC data is approximately 1.2. The fact that the slope is greater than 1.0 indicates a bias on the part of Operator A with respect to Operator B. That is, Operator A consistently counted nearly 20% more cells than Operator B. This result was unexpected since both technicians were experienced with the DMSCC, and initially assured themselves that they were counting the same objects within a microscopic field as cells.

It was also noticed that in the DMSCC method, Operator A had greater variance in replicates than did Operator B. The range of the replicates was defined as the highest value minus the lowest value, and it was used as an estimate of the precision of each operator in doing the tests. The ranges of the estimates made by the two operators are arranged by different cell counts in Table 3. The data with DMSCC estimates greater than

TABLE 3. Ratio of ranges of somatic cell estimate by test^a

Samples	Number	R _i (DMSCC)	R _i (MF-DNA)
All Data	72	1.40	.983
<1,500,000	63	1.21	1.11
<1,000,000	52	1.17	1.25
<500,000	23	1.07	.968

^aRatio of the ranges of 3 estimates of Operator A to that of Operator B.

$$\frac{\sum \text{Ranges A}}{\sum \text{Ranges B}} = R$$

The Range is defined as the difference between the maximum and minimum estimate for a particular sample by an operator.

1,500,000 had a considerably greater variance than samples with fewer cells. Due to this greater variance it was felt that it might bias results and therefore all samples with an estimate greater than 1,500,000 were omitted. This decreased the number of samples analysed in the subsequent calculations from 72 to 63.

In developing the regression line relating the DMSCC to the MF-DNA optical density determinations, each of the operator's results were weighted with respect to their individual average variances to obtain a final weighted value for each sample. This was done for both techniques.

Since the two operators did not obtain identical results on identical samples (see Fig. 4), it was reasoned that an average value based on both operators' results would be closer to the truth than an average value from either operator alone. For a specific sample, a weighted average of Operator A's and Operator B's median estimates (middle of the three estimates), were computed for both the DMSCC and MF-DNA technique. To obtain the weighting values for each operator, it was assumed that the operator who was more precise (had a lower average range) was also more accurate. The weighting factors applied to the data were calculated as follows:

$$\frac{\text{average range for Operator A}}{\text{average range for Operator B}} = \frac{\beta}{\alpha} \quad [1]$$

where α is the weighting factor applied to the data from Operator A and β to that of Operator B. The weighting is directly proportional to the reciprocal of the average range; the data with greater average range (variance) receives less weight. The final weighted median ($\bar{M}_{A,B,n}$) for a specific sample (n) based on both A and B's median values was calculated according to Equation [2] and

$$\alpha \bar{m}_{A,n} + \beta \bar{m}_{B,n} = \bar{M}_{A,B,n} \quad [2]$$

was used for all samples for both techniques. It follows from equations [1] and [2] that if the average ranges for Operator A and B are equal, no weighting factors are applied. The weighted median values for the DMSCC were then plotted vs. the weighted median values for the MF-DNA method, and this is shown in Fig. 5.

From Fig. 5 it is seen that the relationship between the DMSCC and the optical density of the MF-DNA

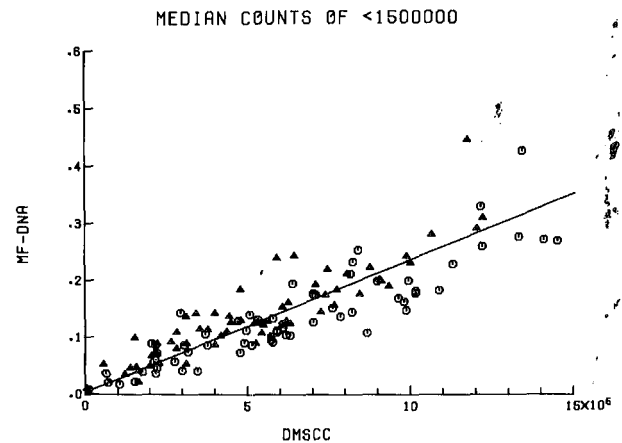


Figure 5. Correlation between the MF-DNA and DMSCC procedures. Each operator is represented by a different symbol. Data plotted are median values for the two operators doing both procedures. The line was computed using data weighted with respect to the average range of estimates of the operators as described in the text.

procedure is 0.232 optical density unit per 10^6 cells/ml (the optical density multiplied by 4.3×10^6 equals the cell concentration in millions per ml). Interpreted another way, this means for every 10^6 cells trapped on the filter, an optical density of 0.093 should develop. This latter calibration factor should be used if a volume other than 2.5 ml of milk is to be used.

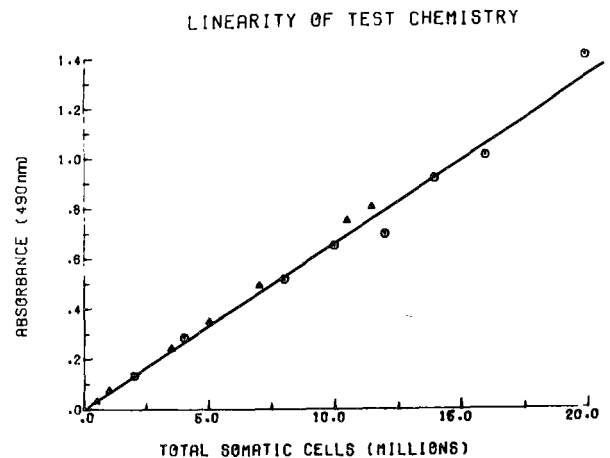


Figure 6. Linearity of the chemistry of the MF-DNA procedure. Data represent pooled results from two experiments: (\blacktriangle - \blacktriangle) serial dilution of a milk sample with homogenized milk; (O - O) serial dilution of isolated bovine white blood cells.

Figure 6 shows that the response of the chemistry in this test is linear to about 20 million total cells trapped on the filter. The scatter is lower in this instance than that in Fig. 5 because the estimates were made using serial dilutions of a stock cell suspensions. In practice it is not feasible to consider filtering samples with very high cell counts (it took nearly 1 h to filter the sample with 20 million total cells). As illustrated in Fig. 4, 7, and 8 the variance associated with the MF-DNA technique is less than that of the DMSCC at high cell counts and therefore the calibration factor can be used to estimate the number of cells on a filter regardless how large that number might be.

The coefficient of variation of the DMSCC procedure is plotted as a function of the mean estimate in Fig. 7.

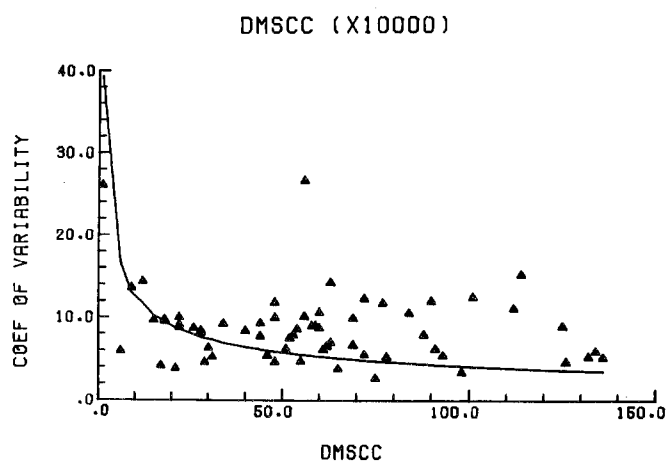


Figure 7. Coefficient of variability of the DMSCC in the experiment described. The plotted points are the observed coefficients of variability using the six individual estimates of the two operators; the line through the points was computed assuming a variance equal to the mean (i.e., poisson distribution).

Data from both operators were used and no weighting factors were applied. The line going through the data points was calculated assuming a Poisson distribution (7, 8), i.e. that the variance is equal to the mean. This should be compared to Figure 8a which shows the coefficient of variation of the MF-DNA procedure as a function of the mean estimate. The CV of estimates of greater than 0.040 optical density unit (200,000 cells/ml) is less than 10%. At a level of about 500,000 cells/ml it is about 5%. One of the things recognized as providing an important contribution to the accuracy of the test is the value of the filter blank and the variance associated with its estimate. Data shown in Figure 8b represent the true (computed) variance in the MF-DNA test. In other words, the variance in the individual estimate has been mathematically corrected for the variability in the filter blank value. This procedure allows estimation of the variance of the procedure under *ideal* conditions, and the points show considerably less scatter than in Fig. 8a.

The analyses of variance of the sets of data from the DMSCC and the MF-DNA procedure are shown in Table 2 along with characteristics of the sample population which were used in the analyses. Results with the DMSCC show that there was a significant difference between the persons doing the experiment as already pointed out in Fig. 4. It also shows that persons doing the trials obtained a consistent answer from one trial to another. Results using the MF-DNA procedure indicate that there was no significant difference between the two technicians, nor was there a significant difference between trials and there was no significant sample-person interaction.

Calibration and standardization of the MF-DNA test

The indole in the color reagent reacts with the

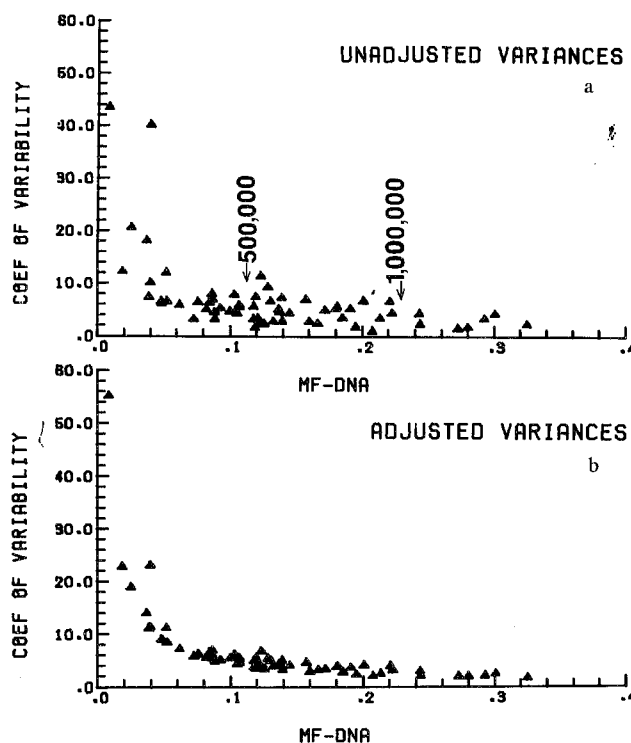


Figure 8. Coefficient of variability of the MF-DNA procedure as a function of the mean determination. Approximate cell counts are indicated by the arrows. In the upper panel the unadjusted variances were used (i.e., raw data). For the lower panel variances were adjusted to take into account variation in filter blank which we found. This was computed as follows:

$$S^2_{\text{Pooled}} = \frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{(n_1-1) + (n_2-1)}$$

$$N_{\text{Pooled}} = \frac{n_1 \times n_2}{n_1 + n_2}$$

$$CV_{\text{Pooled}} = \left(\sqrt{\frac{S^2_{\text{Pooled}}}{N_{\text{Pooled}}}} \right) \times 100$$

Where: n_1 = number of blank replicates.
 n_2 = number of sample replicates.

ABSORPTION SPECTRA OF COLORED COMPOUND FORMED FROM REACTION OF INDOLE WITH HYDROLYZED DNA

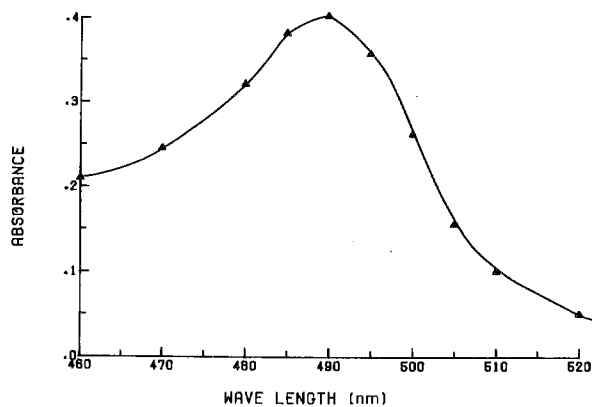


Figure 9. Absorption spectra of the colored compound formed from reaction of indole with hydrolyzed DNA. The spectrum was determined in a double beam spectrophotometer in a 1-cm square cuvette with a narrow slit setting on the monochromator. The narrow slit gives a band pass similar to the interference filter.

deoxyribose in the DNA hydrolysate to yield a product with a sharp absorbance maximum at 490 nm (see Fig. 9, Ref. 2). This sharp peak points up the critical importance of proper adjustment of the spectrophotometer if one with a monochromator instead of an interference filter is used. It was suggested (9) that deoxyribose be used to standardize the results obtained in different laboratories, but results were inconsistent when this was attempted. A different standardization procedure was therefore developed which involves use of lyophilized bovine somatic cells trapped on filters. Clarifier residue from a dairy plant is mixed with homogenized milk to prepare the standards. These standards also allow calibration of instruments with different optical configurations than the one which we use. Microscopic counts need not be done because once the calibration in optical density is made the appropriate calibration factor can be calculated from the relationship obtained in Fig. 5. It is recommended that two calibration standard filters be used each having a different count as this allows a more ready diagnosis of laboratory problems than with a single standard. It might be desirable to have even more standards but in studies to date this has not been found to be necessary.

DISCUSSION

In somatic cell regulatory programs and in programs monitoring the udder health of individual cows the accuracy and precision of a method determining the cell content ultimately determines its usefulness. Additional factors such as the maximum number of samples that can be processed in a certain time, ease and accuracy of calibration, cost of equipment, and skill required to do the test all are considerations. The test that best suits a particular situation usually represents a compromise between the factors just mentioned. Small laboratories can benefit from using procedures which do not require maintenance of expensive electronic equipment and from tests which can be done by relatively unskilled individuals.

Our results suggest that the MF-DNA procedure is not subject to the bias of the DMSCC and shows less variability than the DMSCC. By using large volumes of milk and automatic, highly accurate sampling equipment the sampling error becomes negligible. Various volumes of milk can be analyzed in the MF-DNA test. Thus, if increased precision of low counts is required larger volumes of milk can be assayed.

In laboratories which are currently using the test in screening programs our experience suggests that 500 samples can be tested by an individual technician in a normal working day (including reagent preparation, etc.). The automatic diluting and sampling equipment also greatly facilitates the test.

Several alternative procedures for handling samples might be used depending on the particular laboratory routine. For example, samples might be filtered over a

period of several days and the entrapped cells stored on the filters in a cool dry place and analyzed at a later time. We have not tested storage for longer than a week with unlyophilized material. The procedure can be used by laboratories doing a few tests daily or in those doing large numbers of samples on a sporadic basis. The volume of 500 samples per day would appear to be adequate for most dairy laboratories.

Bacteria which might contaminate samples also contain DNA but even relatively high bacteria counts should not contribute significantly to color development due to DNA in the sample. Values in the literature suggest that bacterial DNA content is 0.002-0.06 picogram per cell as compared to 6.0 picograms per cell for mammals (1). Thus, to give an equivalent color the bacterial count would have to be more than 100 times the somatic cell count.

One of the features of the MF-DNA test which is highly desirable from our point of view is the potential for standardization of the test within and between laboratories. This is made possible by use of somatic cells trapped on membrane filters. A second standardization is also possible because the colorimeter can be standardized independently of the MF-DNA test itself. The accuracy and other desirable features of this test should make it a useful alternative procedure for determining the somatic cell content of milk samples.

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A Research Note

Temperature Limits for Production of Aflatoxin by Twenty-five Isolates of *Aspergillus flavus* and *Aspergillus parasiticus*

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ABSTRACT

Aflatoxicogenic isolates of *Aspergillus* were tested for their aflatoxin production after 8 weeks of growth on wort agar medium at high ($41, 46 \pm 1$ C) and low ($2, 7 \pm 0.5$ C) temperatures. Controls were grown at approximately 22 C, a temperature known to be favorable for aflatoxin production. There were two replications of each treatment. All replicate cultures of the 25 isolates grown at 22 C were positive for aflatoxin. Aflatoxins were not detected in wort agar when cultures were incubated at other test temperatures. It appears that both *Aspergillus flavus* and *Aspergillus parasiticus* will not produce aflatoxins when grown at ≤ 7.5 C or at ≥ 40 C.

Our earlier investigations (6) demonstrated that production of aflatoxins, using only two *Aspergillus flavus* isolates, was limited by temperatures of ≤ 7.5 C or/and ≥ 40 C. Other investigators, in general working with one or a limited number of *A. flavus* or *A. parasiticus* isolates, have not conclusively demonstrated similar limiting temperatures. Rabie and Smalley (4) reported that small amounts of aflatoxin G₁ were produced after 12 days at 42 C; however, no B₁ was detected and there was no chemical or bioassay confirmation of the G₁ aflatoxin. Diener and Davis (1, 2) reported a minimal temperature for production in peanuts of 13 ± 1 C after 21 days at a relative humidity of 97-99%, although damaged kernels developed aflatoxin at 12 C. They also claimed a maximum temperature for aflatoxin production of 41.5 ± 1.5 C. This is in contrast to our studies (6) demonstrating increasing production at 13 C at 3, 6, and 12 weeks after inoculation. Sorenson et al. (10) studied production of aflatoxin in the temperature range of 8 to 37 C and reported that no aflatoxin was produced at 8 C. However, their tabulated data show that a small amount (less than 0.01 µg/g of rice) of aflatoxin B₁ and G₁ was recovered after 21 days at 8 C. Walbeek et al. (11) studied five strains of *A. flavus* at 7.5 and 10 C and reported aflatoxin production at both temperatures. Tempera-

tures negative for aflatoxin production were not determined. Schroeder and Hein (7) used four isolates in their investigation but studied aflatoxin production only at temperatures from 10 to 40 C and only for a 10-day period. They reported that small amounts of aflatoxin were produced at the two temperature extremes. More recently, studies by Shih and Marth (8, 9) appear to demonstrate production of small amounts of aflatoxins at 45 C. However, this seems questionable as there were no studies which would serve to eliminate the possibility that the inoculum contributed aflatoxins to the experimental flasks. This possibility is further re-enforced by their data showing no significant differences between treatments examined at 3, 5, and 7 days.^a In 1976, Northolt et al. (3) reported that aflatoxin B₁ was produced by *A. parasiticus* (NRRL-2999) at temperatures as low as 13 C and as high as 32 C.

On the assumption that different species and also strains of species, particularly from widely differing localities, may vary considerably in some of their physiological responses to environmental conditions, it appeared necessary to determine the limiting temperatures for aflatoxin production of a large number of isolates from various geographical and substrate sources.

MATERIALS AND METHODS

Twenty-five aflatoxin-producing *A. flavus*, *A. parasiticus*, or undetermined species of the *A. flavus* group (5) were selected. Isolates used in this study, from the Division of Microbiology's Culture Collection, were M-1, 3, 30, 52, 66, 112, 141, 185, 198, 201, 219, 224, 226, 260, 268, 270, 276, 320, 326, 332, 344, 346, 347, 363, and 892. They were isolated from samples of peanut, rice, seeds, walnut, corn, cottonseed, grass seed, ink, pond water, cocoa, and strawberry from various localities in the United States and Brazil.

To ascertain (a) what level of aflatoxin would be detected in extractions from flasks containing wort agar inoculated with spores of the highest aflatoxin-producing strain of *Aspergillus*, and (b) that the inoculum did not contribute any aflatoxin to the flasks after germination of spores and growth of *Aspergillus* for 20 h, a series of flasks was examined at intervals starting at 20 h after inoculation. At each extraction period three flasks were examined.

^aOne author (E.H.M.) agreed to the validity of these interpretations of their data (personal communication).

To determine aflatoxin production at the temperature extremes, spores of each isolate were inoculated into 50 ml of wort agar in each of ten 200-ml Erlenmeyer flasks. Approximately 20 h after inoculation, four flasks of each isolate were transferred to incubators (Precision Scientific Co., Chicago, Ill.) and held for 8 weeks at temperatures of 2 and of 7 ± 0.5 C; similarly, four flasks of each isolate were kept in Elconap Gravity Circulator Humidified Incubators (American Instrument Co., Silver Spring, Md.) at 41 and 46 ± 1 C. Two flasks of each isolate (controls) were kept at ambient laboratory temperature of about 22 C, which is within a favorable range for aflatoxin production.

Since no growth occurred at 2 and 46 C, cultures were extracted by flooding the surface of the agar with 50 ml of CHCl_3 , as were most of those grown at 7 C. Cultures grown at 41 C and at laboratory temperature (controls) were extracted with two 50-ml portions of CHCl_3 . Only cultures of isolate M-363 grew at 7 C. These were also extracted with a total of 100 ml of CHCl_3 because both flasks contained a white mycelial growth about 12 mm in diameter.

All CHCl_3 extracts were filtered through an S&S No. 588 filter paper, concentrated, and dried over steam. The presence or absence of any aflatoxins was determined by spotting samples onto thin-layer glass plates coated to about 0.25-mm thickness with Silica Gel G-HR. Authentic aflatoxins B_1 and G_1 were spotted as standards. Except for samples containing large quantities of aflatoxins, all dried extracts were diluted to only 0.5 ml with CHCl_3 for spotting.

Plates were developed with a solvent system of 7% methanol in CHCl_3 in an insulated chamber lined with blotting paper and were examined over long-wave ultraviolet light.

RESULTS AND DISCUSSION

In the experiment to ascertain what level of aflatoxin would be recovered, no aflatoxins were detected through 48 h. This demonstrated that, at 20 h after inoculation, there was no aflatoxin present in flasks subsequently grown at the various temperatures of the experiment. All three replicate flasks were positive for aflatoxins B_1 and G_1 when extracted 50 h after inoculation. The lowest detection level per 50 ml of wort agar for aflatoxin B_1 was 0.017 μg and for aflatoxin G_1 , 0.05 μg .

All replications of all 25 *A. flavus/parasiticus* isolates grown for 8 weeks at 2, 7, 41, and 46 C failed to produce any detectable aflatoxin. All replications of the 25 isolates grown at the control temperature of 22 C produced aflatoxin, ranging from a low production of 0.19 $\mu\text{g}/50$ ml wort agar (M-219) to a high production of 930 $\mu\text{g}/50$ ml wort agar (M-363), thereby demonstrating the ability of the isolates to produce aflatoxins when grown at favorable temperatures. All isolates kept at

41 C produced convoluted mycelial mats with sparse or no spore production. Growth of the controls was normal with abundant spore production.

It would appear from these and other experiments (1-4, 6-11) that neither *A. flavus* nor *A. parasiticus*, when grown on wort agar or various commodities, will produce aflatoxins either at ≤ 7.5 C or at ≥ 40 C. From this it seems probable that aflatoxin-free commodities stored at these temperatures should remain free of these toxic substances even when moisture conditions are favorable for growth of *A. flavus* and *A. parasiticus*.

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A Research Note

A Collaborative Study to Determine the Feasibility of Using 0.40-, 0.20-, 0.14-, and 0.10-Inch-Diameter Discs to Measure Sediment in Fluid Milk

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ABSTRACT

This collaborative study was done to determine whether laboratory personnel could successfully grade raw milk for sediment content using 0.40-, 0.20-, 0.14-, and 0.10-inch-diameter sediment discs. The 0.40-inch disc presently is accepted for grading sediment in mixed bulk milk samples. Technicians in 17 separate laboratories made 1,360 determinations (80 per laboratory) or 20 determinations for each size of sediment disc. Each laboratory graded the same set of samples. Samples were graded using photoprint standards that were prepared for this study. The laboratories were evaluated on the ability of laboratory technicians to grade the various sizes of sediment discs. Technicians in 13 of the 17 laboratories graded samples showing no significant differences in their ability to grade the various sized sediment discs. Four laboratories were not consistent in their ability to grade discs and showed significant differences in agreements with the previously determined sediment level as the diameter of the disc was reduced. Based on these results, most laboratories evaluated were capable of grading sediment discs with diameters of 0.40-, 0.20-, and 0.14-inch with consistency. With properly trained personnel in the laboratories, the study indicates the 0.10-inch diameter disc also can be used effectively for grading sediment in milk.

et al. (5) to determine the feasibility of using a 4-oz. sample of milk and 0.20-inch diameter disc to measure sediment in fluid milk. This study showed that the 4-oz. sample and the 0.20-inch diameter disc could serve for milk to be graded as accurately by laboratory technicians as when they used the 1-pint sample and the 0.40-inch diameter disc.

The purpose of this study was to determine the accuracy of using the following discs to measure sediment in fluid milk: (a) 1 pt sample and 0.40-inch diameter discs, (b) 4 oz sample and 0.20-inch diameter discs, (c) 2 oz sample and 0.14-inch diameter discs, and (d) 1 oz sample and 0.10-inch diameter discs.

The study was designed to measure the uniformity of sediment grade evaluation in each laboratory between the different sizes of sediment discs.

MATERIALS AND METHODS

Seventeen laboratories from throughout the United States were selected to participate in this study; one technician in each was designated to make the determinations. In nearly all of the participating laboratories, one of the authors demonstrated the procedure and preliminary grading of known samples using the new set of standard photographs. This new set of standard photos was developed with known amounts of sediment according to the fine sediment standard disc procedure (1). Twenty samples were collected from actual milk suppliers for each size of sample and sediment discs. This made a total of 80 samples. A panel of four professional graders graded the samples and all agreed on the grade for each sample. This set of 80 samples was then delivered to the 17 laboratories participating in the study. All laboratories received the same instructions for grading the samples.

Each technician in each laboratory graded the sediment discs into four classes as recommended by the U.S. Department of Agriculture, Agricultural Marketing Service (3). The 80 samples that were judged by the panel of experts fell into the following grades: (a) 12 received grade no. 1 (<0.5 mg), (b) 27 received grade no. 2 (<1.5 mg), (c) 29 received grade no. 3 (<2.5 mg), and (d) 12 received grade no. 4 (>2.5 mg).

RESULTS AND DISCUSSION

Laboratory technicians determined sediment grade by indicating whether the sample disc belonged in grade 1, 2, 3 or 4. The observation for this study was a visual grading of the sediment discs. Sample discs were number

Sediment testing is one of the measurements used to determine the quality of fluid milk. In 1954, Liska and Calbert (4) developed a sediment test method for bulk tank milk using a 1-pint mixed sample filtered through a 0.40-inch diameter disc. This method appeared in *Standard Methods for the Examination of Dairy Products* (2) and regulatory authorities considered it as an acceptable method for measuring sediment in a mixed sample of milk. However, 1-pint samples of milk from each producer are cumbersome for milk haulers to handle properly.

In 1974 a collaborative study was conducted by Wright

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coded with identical numbers being used between laboratories. Grading results of all the laboratories between the disc sizes are shown in Table 1.

TABLE 1. Comparison of 17 laboratories in grading 0.40-, 0.20-, 0.14- and 0.10-inch-diameter sediment discs showing agreement of technician with previously graded samples

	Disc sizes (in inches)			
	0.40	0.20	0.14	0.10
0 (agree)	252	200	226	179
± (disagree)	88	140	114	161
No. of samples	340	340	340	340

The success of the laboratories in grading sediment in milk using the various sized discs was evaluated using the Chi Square statistic with 3 degrees of freedom. Table 1 illustrates that in general the level of agreement of the laboratory technicians with the previously determined sediment level shows a significant change as the diameter of the disc was reduced. Evaluation of each laboratory's success indicated that four of the 17 laboratories showed a significant (<.01) change in agreement with the previously determined sediment level as the diameter of the disc was reduced. Of the remaining 13, six were very consistent as diameter changed while the remaining seven showed some disagreement, though not significant at the 5% level.

Table 2 shows that greatest disagreement between the

TABLE 2. The sum of the four laboratories that showed significant differences within their laboratory in grading 0.40-, 0.20-, 0.14- and 0.10-inch-diameter sediment discs

	Disc sizes (in inches)			
	0.40	0.20	0.14	0.10
0 (agree)	67	39	46	31
± (disagree)	13	41	34	49
No. of samples	80	80	80	80

discs occurred with the smaller diameter discs. This demonstrates the laboratory technicians' inability to grade the smaller sized discs in these four laboratories.

Upon further examination it was discovered that three of the four laboratories were grading with inexperienced graders. This may have been responsible for the inconsistency shown in Table 2.

In the group of 13 laboratories that did not show a significant difference, four showed a tendency of not being able to grade the smaller discs as accurately as the larger one; this is similar to the trend shown in Table 2.

In Table 3 comparisons show the tendency not to be as

TABLE 3. The four laboratories that did not show a significant difference but did show the tendency to be more inconsistent in grading the smaller sized sediment discs

	Disc sizes (in inches)			
	0.40	0.20	0.14	0.10
0 (agree)	68	49	55	49
± (disagree)	12	31	25	31
No. of samples	80	80	80	80

consistent with the 0.10- and 0.20-inch diameter discs as compared to the other two disc sizes. A comparison of data in Tables 2 and 3 shows that these laboratories were very accurate in grading the larger diameter disc

(0.40-inch). Their accuracy with that size emphasizes differences in their results when comparing the grading of the smaller sized discs.

The nine laboratories cited in Table 4 were very consistent in grading the 0.40-, 0.20-, and 0.14-inch diameter discs. The laboratories were less consistent in grading the 0.10-inch diameter disc even though they did not show a significant difference.

	Disc sizes (in inches)			
	0.40	0.20	0.14	0.10
0 (agree)	118	114	125	99
± (disagree)	62	66	55	81
No. of samples	180	180	180	180

consistent in grading the 0.40-, 0.20-, and 0.14-inch diameter discs. The laboratories were less consistent in grading the 0.10-inch diameter disc even though they did not show a significant difference.

An overall comparison shows that of the 1,360 samples graded, 857 (63%) were graded correctly and 503 (37%) were graded incorrectly.

SUMMARY

The results of this study indicate that most laboratories show no significant difference when measuring the consistency of response as sample size varies within each laboratory. Only four of 17 laboratories showed a significant difference in correct response as disc size varied within the laboratory.

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A Research Note

A Phage Typing System for Salmonellae: *Salmonella heidelberg*

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ABSTRACT

A system is described for phage of *Salmonella heidelberg*. The system was developed using a number of bacteriophages that were isolated from sewage.

Salmonellosis is a widespread disease affecting both man and animals. These microorganisms are one of the most common causes of food poisoning and constitute one of the major problems confronting world health officials. Fortunately it can be prevented if the organism, its origin, and mode of transmission are known.

Over 1600 serotypes of *Salmonella* have been identified to date and new isolates continue to enhance this already numerically impressive collection of microorganisms. The number of existing species in itself is not particularly disturbing for it can actually be epidemiologically useful. Many apparently unrelated outbreaks have been linked through identification of the organisms involved. However, if a particular species is fairly common it is difficult, if not impossible, to relate an isolate to a particular outbreak unless measures are first taken to characterize the "types" involved.

Serological methods exist for delineation of a number of bacterial isolates; however, the number of types distinguishable in this manner can be inadequate for epidemiological application.

In 1938 Craigie and Yen introduced a phage typing scheme for *Salmonella typhi* (4,5). Its success led to development and acceptance of the phage typing technique as a reliable laboratory procedure. Indeed *S. typhi*, *Salmonella typhimurium*, and *Salmonella paratyphi B* are now routinely typed in some Public Health Laboratories (1).

Phage typing schemes have been devised for a number of *Salmonella* serotypes. They include: *S. paratyphi A* (2), *Salmonella dublin* and *Salmonella enteritidis* (10), *Salmonella gallinarum* and *Salmonella pullorum* (11), *S. typhimurium* (3,9,16), *Salmonella thompson* (6), *Salmonella newport* (7, 12), *Salmonella anatum* (8), *Salmonella braenderup* (13) and *Salmonella blockley* (14). *Salmonella heidelberg* is commonly isolated from food products, and, is often accorded the dubious

distinction of being one of the 10 most frequently isolated serotypes. Given the significance and frequency of isolations, a phage set has been developed in our laboratory to characterize these microorganisms.

MATERIALS AND METHODS

The phages, in this study, were isolated from untreated sewage samples obtained from local treatment plants. Aliquots of sewage (100ml) were inoculated with a 1½-h-old nutrient broth culture of one of the biochemically and serologically confirmed *S. heidelberg* isolates collected for this investigation. The samples were incubated for 18 h at 37 C and passed through a 0.45-μ membrane filter. The filtrates were then assayed for presence of phage by plating them onto cultures initially used as inoculum. Phage isolates were purified by serial, single plaque passages and brought to titer using the procedure described by Swanstrom and Adams (15). Phages of sufficiently high titer were then diluted and tested against the *S. heidelberg* cultures in our collection. All of the phages employed in this study were used at a routine test dilution (RTD) of not less than 10⁻³. Phage isolates were selected and maintained for regular use if they were stable and potentially suitable for type differentiations.

Cultures to be typed were lightly inoculated into 3 ml of nutrient broth and incubated at 37 C for 1½ h or until turbidity was barely detectable. A small quantity of the broth culture was then flooded onto a nutrient agar plate, allowed to dry for approximately 15 min and spotted with drops of phage using a 1-ml syringe with a 26-gauge needle. The plates were incubated overnight at 37 C and read the following day. The cultures were examined with the aid of an ×10 aplanat hand lens and viewed through the bottom of the plate. Susceptibility of a phage was demonstrated by areas of clearing that ranged from isolated plaques to confluent lysis. Phage activity was recorded on the basis of the reactions described in Table 1.

TABLE 1. Method for recording degrees of lysis

CL	Confluent lysis
OL	Opaque lysis (opacity due to heavy secondary growth)
SCL	Semi-confluent lysis
<SCL	Less than semi-confluent lysis
+++	120 plaques
+++±	81-120 plaques
++	61-80 plaques
+±	41-60 plaques
+	21-40 plaques
±	6-20 plaques
-	0-5 plaques

RESULTS AND DISCUSSION

Seven phages were isolated. The lytic pattern of these phages is described in Table 2. Using these isolates, 180

TABLE 2. Reactions of type strains of *Salmonella heidelberg* with typing phages at routine test dilutions

Typing strains	Typing phages							Strains of <i>S. heidelberg</i>	
	1	2	3	4	5	6	7	No.	%
1	CL	—	CL	—	CL	<SCL	CL	3	1.6
2	SCL	CL	—	SCL	CL	CL	—	3	1.6
3	—	—	CL	—	SCL	+	CL	11	6.0
4	—	CL	—	CL	CL	CL	—	5	2.7
5	<SCL	—	—	—	CL	SCL	—	4	2.2
6	OL	—	+	SCL	CL	CL	—	2	1.1
7	OL	—	CL	—	SCL	++	CL	23	12.6
8	—	—	—	OL	OL	CL	—	2	1.1
9	—	—	++	—	CL	—	++	10	5.5
10	OL	—	—	—	SCL	—	—	13	7.1
11	OL	—	—	—	—	—	—	20	10.9
12	SCL	—	—	—	—	SCL	—	11	6.0
13	—	—	SCL	—	SCL	—	+	12	6.6
14	+++	CL	—	—	—	—	—	18	9.8
15	—	—	SCL	—	—	—	+++	14	7.7
16	OL	—	OL	—	OL	+++	—	12	6.6
17	—	—	CL	—	CL	+++	CL	11	6.0
18	—	—	—	—	CL	<SCL	—	1	0.6
19	OL	—	CL	—	++	—	CL	5	2.7

strains of *S. heidelberg* were classified into 19 distinct phage types. It is conceivable that more types exist and will be revealed as new cultures are examined. The characteristic pattern of the phage types established were readily reproducible reflecting the stability and practicality of the phages employed. Some of the phages were observed to affect both serologically related and dissimilar species of *Salmonella*. Efforts are now being made to utilize these phage isolates to characterize a number of other *Salmonella* serotypes.

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Diseases Transmitted by Foods Contaminated by Wastewater

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ABSTRACT

A historical and world-wide review of medical and engineering literature discloses that typhoid fever, infectious hepatitis, fascioliasis, and cholera are the diseases that have been most frequently transmitted by foods contaminated by sewage or irrigation water in agricultural or aquacultural practices. Wastewater-contaminated shellfish have resulted in 28 outbreaks of illness, watercress in 10, fish in three, and shrimp in one. Vegetables contaminated by night soil or raw or partially treated sewage were reported as vehicles in 21 outbreaks. Fruits were considered as vehicles in four outbreaks. For such outbreaks to occur, a complicated chain of events must occur for agents originally present in wastewater to survive natural destructive forces and wastewater-treatment processes or to multiply so that there are sufficient numbers to cause illness.

In a world of limited resources and expanding human populations, an ample and safe food is vital for populations to thrive. New food sources and improvements in agricultural and aquacultural methods must be investigated and utilized; wastes generated by increasing concentrations of people must be disposed of adequately. Recycling wastewater for watering and nourishing food crops or for growing fish contributes to both food production and waste disposal. But when wastewater is used to irrigate crops or to provide water in aquaculture, appropriate precautions must be taken to prevent the diseases that might otherwise be transmitted by wastewater-contaminated foods.

Foods can become contaminated during production either on farms or in watercourses, during processing in food processing plants, and during preparation in food service establishments and homes. The point at which contamination occurs will depend on the natural sources of a pathogen and on the opportunities for transfer at each stage of the food chain. Factors that contribute to reported foodborne disease outbreaks are reviewed (24).

Many of the pathogenic organisms that infect man reach him by being conveyed by more than one vehicle. For example, eggs of some parasitic organisms can appear in feces of infected persons, reach water in which they hatch into a form that infects a vector (as a snail), and, after a period of development metamorphose into a free-swimming form which penetrates tissues of fish or

water vegetables. Diseases caused by such parasites and features of their transmission are listed in Table 1. Few

TABLE 1. Parasitic diseases maintained by the cycle: Human feces to water or soil; water or soil to food; food for human consumption

Agent	Intermediate vehicle	Intermediate host	Food
<i>Clonorchis sinensis</i>	fresh water	snail	fish
<i>Diphyllobothrium latum</i>	fresh water	copepods	fish
<i>Echinostoma ilocanum</i>	fresh water	snail	snails, clams, limpets, fish, tadpoles
<i>Fasciola hepatica</i>	fresh water	snail	watercress
<i>F. gigantica</i>			water vegetables
<i>Fasciolopsis buski</i>	fresh water	snail	water vegetables
<i>Opisthorchis felineus</i>	fresh water	snail	fish
<i>Paragonimus westermani</i>	fresh water	snail	crabs, crayfish
<i>Taenia saginata</i>	soil (grass)		beef
<i>Taenia solium</i>	soil (grass)		pork

of these parasites are found in the United States, but they are prevalent in other regions of the world. Other parasites, such as those that cause hookworm infection, schistosomiasis, and leptospirosis, penetrate human skin in their infective stage and may be acquired by agricultural or aquacultural workers who work in wastewater-contaminated fields or ponds (180).

Most of organisms that are conveyed by wastewater-contaminated foods have less complicated transmission cycles. The organisms that have potential of transmission by such a route, and the diseases they cause, are listed in Table 2.

The following circumstances must occur for persons who ingest wastewater-contaminated foods to become ill. (a) The infectious agent must be present in citizens of a community or in animals on farms, or toxic agents must be used for industrial or agricultural purposes; and wastes from these sources must reach sewerage or

TABLE 2. Diseases and causative agents transmissible by food that has been contaminated by wastewater or by soil that contains fecal material

Disease	Agent
BACTERIA^a	
Arizona infection	<i>Arizona hinshawii</i>
<i>Bacillus cereus</i> gastroenteritis	<i>Bacillus cereus</i>
Cholera ^b	<i>Vibrio cholerae</i>
<i>Clostridium perfringens</i> gastroenteritis	<i>Clostridium perfringens</i>
Enteropathogenic <i>Escherichia coli</i> infection	<i>Escherichia coli</i> (certain toxigenic and invasive types)
Paratyphoid fever ^b	<i>Salmonella paratyphi</i> A <i>Salmonella paratyphi</i> B <i>Salmonella paratyphi</i> C <i>Salmonella sendai</i>
Salmonellosis ^b	<i>Salmonella</i> (over 1,500 serotypes)
Shigellosis ^b	<i>Shigella sonnei</i> <i>Shigella flexneri</i> <i>Shigella dysenteriae</i> <i>Shigella boydii</i> <i>Salmonella typhi</i>
Typhoid fever ^b	<i>Yersinia enterocolitica</i> <i>Yersinia pseudotuberculosis</i>
VIRUSES	
Adenovirus infection	Adenoviruses
Coxsackie infection	Coxsackie viruses
ECHO virus infection	ECHO viruses
Poliomyelitis	Polioviruses
Reovirus infection	Reoviruses
Viral hepatitis ^b	Hepatitis virus A
Winter vomiting disease	Norwalk agent
HELMINTHS	
Ascariasis ^b	<i>Ascaris lumbricoides</i>
Trichiniasis	<i>Trichuris trichiura</i>
PROTOZOA	
Amebiasis ^b	<i>Entamoeba histolytica</i>
Balantidiasis	<i>Balantidium coli</i>
Coccidiosis (Isospora infection)	<i>Isospora belli</i> , <i>I. hominis</i>
Dientamoeba infection	<i>Dientamoeba fragilis</i>
Giardiasis	<i>Giardia lamblia</i>

^aOther enteric bacteria which could conceivably be transmitted by foods but proof is inconclusive: *Streptococcus faecalis*, *S. faecium*, *Proteus* spp., *Providencia* spp., *Klebsiella* spp., *Citrobacter freundii*, *Enterobacter* spp., *Edwardsiella tarda*, *Aeromonas* spp., *Plesiomonas shigelloides*, *Pseudomonas aeruginosa*

^bReported outbreaks, see Table 8.

drainage systems. (b) The agents must survive and pass through all wastewater treatment processes to which they are exposed. (c) The waste-treatment effluent or water course receiving the effluent must be used as irrigation water for crops or as a growing environment for fish or watercrops or for washing or freshening harvested foods. Thus, the agents must survive in the receiving watercourse. (d) The agents must survive in the soil in which irrigated foods are grown. (e) The agents must contaminate a food product. (f) Then one of the following events must occur: (i) The agents must be present on the contaminated food in sufficient numbers to survive the remainder of the growing period, storage, and preparation and still cause illness. (ii) Bacteria on foods in insufficient numbers to cause illness must multiply and reach numbers that are necessary to cause illness. (iii) Bacteria, and perhaps other organisms, enter food preparation areas on raw foods, where they may be transferred to workers' hands or to equipment surfaces

which if inadequately washed will then contaminate other foods that they subsequently touch. (g) Sufficient quantities of the contaminated food that contain enough of the agent to exceed a person's susceptibility threshold must be ingested. Ingestion of foods contaminated to this level may result in sporadic cases of illness as well as epidemics. When insufficient numbers of pathogens to cause illness are ingested, the infected individuals may become carriers and subsequently contaminate other foods that they touch. Each step of this chain of events necessary for wastewater to contribute to foodborne outbreaks of human disease is reviewed based on information from engineering and medical literature.

PATHOGENS IN SEWAGE

Numerous investigators have isolated pathogens from sewage. These investigations have been reviewed by Rudolfs et al. (158, 159), Greenberg and Dean (74), Kollins (99), and Grabow (72). Animal wastes contain many of the same pathogens as well as other pathogens.

SURVIVAL IN WASTE—TREATMENT PROCESSES

Enteric pathogens survive some stages, and sometimes the entire process of wastewater treatment. Table 3 highlights the results of some typical investigations of the effect of various wastewater-treatment processes in removing or killing pathogens. Primary sedimentation usually removes less than 50% of coliform and pathogenic bacteria from sewage; it is relatively ineffective in removing viruses and protozoa. Activated sludge or trickling filter processes followed by secondary sedimentation usually remove over 90% of coliform or pathogenic bacteria remaining after primary sedimentation. Viruses can be significantly reduced by activated sludge but not by trickling filter processes. Sand filtration is required to remove amoebic cysts and *Ascaris* eggs. Anaerobic digestion reduces 90% of pathogenic bacteria from sludge but is less effective for destruction of *Ascaris* eggs. Hepatitis viruses, *Entamoeba histolytica* cysts, and tapeworm eggs withstand the chlorination treatment generally applied to waste-treatment effluent. Thus, many waste-treatment processes remove or kill 90% or more of the pathogens that are present in influent sewage, but some still remain in the effluent. If a million pathogens, for example, are present in influent sewage before exposure to processes which remove 90% of them, 100,000 survive; in the event of 99% destruction, 10,000 survive. Kampelmacher and van Noorle Janssen (92) found that 10^{10} salmonellae entered a trickling filter secondary treatment plant each hour and that 10^9 left the plant in the effluent each hour.

SURVIVAL IN RECEIVING WATERCOURSES AND USE OF WASTEWATER FOR IRRIGATION

Those organisms that survive wastewater treatment must also survive in receiving waters. Such survival has been reviewed by Rudolfs (158, 159), Dunlop et al (49), Clarke et al. (31), Kollins (19), and Grabow (72). Factors influencing survival in water include temperature, amount of light, flow rate, presence of oxygen, pH,

TABLE 3. *Survival of pathogens during various stages of wastewater treatment*

Pathogen	Process								Reference
	Settling	Activated sludge	Trickling filtration	Anaerobic digestion	Sand filtration	Drying	Stabilization pond	Disinfection	
<i>Salmonella typhi</i>		++							(26)
		++							(138)
		++							(156)
		++	++						(73)
	+++			+		-			(127)
				+			-		(68)
				++		+			(166)
									(123)
<i>Salmonella</i>	+	+					+/-		(122)
							+		(19)
								-	(34)
									(22)
<i>paratyphi</i>		++				+			(104)
				+		+			(26)
									(166)
<i>utrecht</i>	+++		++						(117)
<i>Shigella</i>		++	+++						(92)
<i>flexneri</i>							-		(26)
<i>Vibrio</i>		-							(34)
<i>cholerae</i>		++							(39)
<i>Streptococcus</i>			++		++				(26)
<i>faecalis</i>	++++		+++					++	(2)
Enteroviruses	+	+							(35)
	+	+							(20)
		++						+	(32)
Polioviruses		-							(108)
	+								(28)
	+		+			+			(93)
		+							(94)
									(109)
	++++	-	+	-				-	(178)
	+++	++						+	(95)
	++++	+++							(29)
									(96)
	++++	++							(153)
									(30)
			+++						(33)
							+		(112)
Coxsackie viruses	++++		+++					+/-	(66)
			+						(33)
								+++	(67)
		+							(109)
					+			+	(29)
	++++	-	+	-				+	(95)
	++++	++++	++++		++		+		(86)
			+++						(33)
ECHO viruses		+							(109)
Hepatitis virus A			+++						(33)
<i>Entamoeba histolytica</i>								+	(131)
	+							++++	(71)
	++++	++++	+++	-	-	+		++++	(40)
	+	+	+	+	-				(75)
<i>coli</i>	++								(104)
<i>Giardia lamblia</i>						+			(175)
<i>Ascaris lumbricoides</i>	+	+	+++	+	-	+			(104)
	+	+	+	+	-				(40)
	-			+					(75)
eggs	+++							+++	(147)
	+++	++	++	++++					(175)
<i>Trichuris trichiura</i>						+			(17)
Tapeworm eggs	+++	++							(104)
			++++	++				++++	(17)
<i>Taenia saginata</i>	+	+	+	+	-				(172)
	+++	++++	+++	++++	-				(75)
									(132)

- destruction; + survival; ++ over 90% removal; +++ over 50% removal; ++++ less than 50% removal

dilution, amount of dissolved material, and types of organisms in the water.

Irrigation water may be sprayed over crops, flow through channels in fields and seep to roots, or be flooded over the field. The numbers of pathogens applied to soil during irrigation can be of such magnitude that outbreaks can result. During an epidemiologic investigation, Renteln and Hinman (150) found that sewage plant effluent used to irrigate a field was channeled by gopher holes to a well pit and entered a community water supply through a defective well casing. Aerosols will be produced during spraying and pathogens trapped in the aerosols may be conveyed to locations other than the irrigated fields by wind and air currents.

SURVIVAL OF PATHOGENS IN SOIL

After wastewater or receiving water is used for irrigation, enteric pathogens must survive long enough to contaminate crops. Helminths not only survive in soil but must stay in soil for a period of several days to develop into an infective stage. Survival times for many enteric pathogens in soil are reviewed in Table 4. Factors that effect resistance include number and type of organism, type of soil (structure, moisture content, pH, amount of organic matter) temperature, amount of rainfall, amount of sunlight, protection provided by foilage, and competitive microbial flora. The range of survival times

TABLE 4. *Survival of enteric pathogens in soil*

Pathogen	Type soil	Survival (in days)	Reference
<i>Salmonella typhi</i>	moist sand	<12	(62)
	peat	<13	
	damp soil	35-74	
	soil surface (sunlight)	5-22	
	soil	70-80	(110)
	soil	35	(41)
	garden soil (in open)	32-58	(124)
	sandy soil	29-36	
	garden soil (hot house)	49-74	
	sandy soil (hot house)	53	
	moist neutral soil	70	(97)
	moist arid soil	<10-30	
	dry soil	<14	
	sand	<5	(13)
	muck	12	
	clay loam	12	
	sandy loam	5	
	adobe	21-105	(113)
	adobe-peat	28-100	
	loam	21-120	
sand	2-15		
peat	1-2		
loam sand	14-60		
<i>Salmonella</i>	soil and pasture	>200	(177)
	soil (raw sewage)	46	(145)
	soil	46-70	(15)
	sandy soil	35-84	(151)
	soil and pasture	>280	(111)
	soil	147-259	(168)
<i>Streptococcus faecalis</i>	sandy loam	28-77	(113)
	sand	33-35	
	loam	26-63	
	clay loam	33-49	
muck	40-77		
<i>Entamoeba histolytica</i>	moist soil	6-8	(14)
	irrigated soil	730-1035	(77)
<i>Ascaris ova</i>	soil	2190	(100)

suggests that pathogens introduced into a field by irrigation with wastewater would, despite considerable reduction in numbers, survive in the soil until harvest under some agricultural conditions. Pathogens in soil are more likely to contaminate foods if the soil is kept moist by intermittent application of irrigation water. Also, continuous application of wastewater to soil results in accumulation of pathogens in soil. Soil filters microorganisms and they often concentrate near areas where plants grow.

CONTAMINATION OF FOODS

Foods become contaminated from water during irrigation (flooding, spraying, or seepage), from soil when the plant grows, when vegetables or fruits fall on the ground, and when the crop is harvested. They also are contaminated by dust blowing over the crops or from workers, birds, and insects that convey organisms from irrigation water or soil to the foods. Organisms contaminating foods in one of these ways remain on the food surface until they succumb to dessication, exposure to sunlight, starvation, or action of other organisms. They do not penetrate into the vegetables or fruits unless their skin is broken. Survival times of many enteric pathogens on foods are reviewed in Table 5. These times suggest ample opportunity for crops that become contaminated during irrigation or during growth to remain contaminated until harvest. Shellfish and watercress trap and accumulate enteric pathogens; thus, they become particularly hazardous if grown in sewage-contaminated water. Foods sprayed with wastewater a short time before harvest could harbor pathogens on their surfaces.

Pathogens are infrequently isolated from foods in fields or after harvesting. When pathogens are found,

TABLE 5. *Survival of enteric pathogens on foods*

Pathogen	Vegetable	Survival (in days)	Reference
<i>Salmonella typhi</i>	vegetables (leaves and stems)	10-31	(41)
	radishes	28-53	(124)
<i>Salmonella</i>	lettuce	18-21	
	vegetables	40	(100)
	vegetables	28	(143)
	beet leaves	21	
	tomatoes (laboratory)	20	(160)
	tomatoes (field)	3	
	soil and potatoes	>40	(129)
	carrots	>10	
	cabbage and goose-berries	> 5	
	tomatoes	3	(58)
<i>Shigella sonnei</i> and <i>S. flexneri</i>	clams and shrimp	>60	(167)
	oysters	>40	
<i>S. sonnei</i>	tomato (surface)	2	(89)
	tomato (tissue)	10	
<i>Vibrio cholerae</i>	apple (skin)	8	
	vegetables	5	(70)
	dates	<1-3	
	shellfish	20-45	(142)
	vegetables	7	(59)
<i>Entamoeba histolytica</i>	vegetables (refrigerated)	<14	
	lettuce and tomatoes	< 3	(161)
<i>Ascaris ova</i>	lettuce and tomatoes	27-35	(162)

they are found in low percentage of samples and usually for only a short time after irrigation. Studies in which isolations were made are summarized in Table 6.

TABLE 6. Recovery of pathogens from foods contaminated by wastewater

Pathogen	Food	Source of contamination	Reference
<i>Salmonella typhi</i>	leaf tips	contaminated soil	(41)
	radishes	contaminated soil	(124)
	lettuce	contaminated soil	
<i>Salmonella</i> (other types)	celery	irrigation water	(50)
	vegetables	irrigation water	(48)
	fish	river water	(114)
	green onions	irrigation water	(65)
<i>Salmonella</i> , <i>Shigella</i> other bacterial pathogens (serologic evidence)	white perch	river water near heavily populated areas	(88)
	fish	river water	(69)
Enteropatho- genic <i>Escherichia coli</i>	fish	river water	(69)
Enteroviruses (Polio, ECHO, Reo, Coxsackie)	vegetables	irrigation water	(10)
	oysters	sewage-contaminated sea water	(125)
<i>Ascaris ova</i>	lettuce	irrigation water	(175)
Helminth ova	cabbage	irrigation water	
	vegetables	irrigation water	(173)
	cucumbers	irrigation water	
	tomatoes	irrigation water	
	carrots	irrigation water	

SURVIVAL, CROSS-CONTAMINATION, AND MULTIPLICATION

Foodborne disease organisms must survive processing and preparation steps and bacteria usually have to multiply to reach infective levels. If wastewater-contaminated fruits, vegetables, or seafoods are cooked so that the contaminated portions reach 165 F (or even lower temperatures for sufficient time), vegetative bacteria (but not spores), protozoa, helminthic eggs, and most viruses will be killed. All foods, however, are not cooked before serving, and the temperatures reached during cooking may be too low to kill pathogens.

Contaminated raw foods bring pathogens into food preparation environments. These organisms are killed in foods that are thoroughly cooked, but before cooking they can contaminate the hands of any worker who touches them, or they can contaminate equipment that they contact. Such cross-contamination is commonly reported in outbreaks of salmonellosis; the initial source is usually raw meat or poultry. Some food service workers and homemakers are aware of this danger, but they are unaware that wastewater-contaminated vegetables present the same hazard. Citizens in the United States assume that the fruits, vegetables, and shellfish they eat are free from fecal contamination. But this is true only if wastewater has not contaminated crops or the waters in which shellfish grow.

Contaminated foods which are to be eaten raw or used as a raw ingredient in a salad can support growth of foodborne disease bacteria under the following set of conditions: foods must contain sufficient moisture and

essential nutrients to support bacterial growth; foods must be held within a temperature range that permits the contaminating bacteria to multiply, usually near the organism's optimal temperature for growth; and foods must be held at such temperatures for sufficient time for enough organisms or toxins to be produced to cause illness in those who ingest the contaminated food.

NUMBERS OF PATHOGENS REQUIRED TO CAUSE ILLNESS

Ingestion of contaminated food does not always result in illness. The pathogens must be swallowed in sufficient quantity to exceed a person's threshold of susceptibility if illness is to result. Human volunteer feeding studies have indicated susceptibility threshold levels of various enteric pathogens. Table 7 reviews such studies. Conceivably, wastewater-contaminated food could harbor 10 *Shigella dysenteriae* 1, 180 *Shigella flexneri* 2a, or 1,000 *Vibrio cholerae* biotype ogawa (numbers found to cause illness in adult volunteers) when they reach the consumer. Thus, ingestion of foods contaminated at these levels could result in illness. Such foods could also convey 1 *Entamoeba coli*, 10 *Giardia lamblia*, and 1,000 *V. cholerae* biotype inaba, which if ingested could result in infection and carrier status. Ten thousand *Salmonella typhi* and *V. cholerae* biotype inaba could, conceivably, be present on foods recently fertilized with night soil, human manure, or raw sewage; and ingestion of this amount of these pathogens could cause illness. Certain waterborne outbreaks of these diseases and other diseases, such as salmonellosis, suggest that even lower levels of organisms than those indicated by volunteer feeding studies cause illness. As few as 15,000 *Salmonella cubana* caused death in infants who were given contaminated carmine dye for diagnostic purposes (103). All volunteer studies cited were conducted on health adults. Infants, elderly persons, malnourished persons, and persons with concomitant illness would be more susceptible—perhaps a one or more log reduction in dosage could result in illness of such persons. Most of the other organisms in which human feeding tests have been done, as well as some of the organisms just cited, usually require time for multiplication before the large numbers (100,000 or more) necessary to cause illness would be generated. Thus, it would be unlikely for an infective dose of salmonellae to be on lettuce or other raw vegetables, but it is possible for the same foods to contain an infective dose of shigellae, *Ascaris*, or *E. histolytica*. One saving feature is that shigellae and *E. histolytica* do not survive long in the competitive environment which occurs in wastewater, soil, or foods. Salmonellae and many of the other bacteria which have been mentioned would become problems if contaminated foods were allowed to stand at room temperatures or refrigerated in large masses.

OUTBREAKS

On some occasions all circumstances described have occurred and outbreaks of human illness have resulted.

TABLE 7. *Clinical response of adult humans to various challenge doses of enteric pathogens*

Organism (strain)	Challenge dose											Reference	
	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸	10 ⁹	10 ¹⁰	10 ¹¹		
<i>Shigella dysenteriae</i>													
(1) (A-1)		+		++									(105)
(1) (M 131)	+	++	+++	++++									(105)
<i>Shigella flexneri</i> (w)									+++				(164)
(2a)				+	+++	++++	+++	++++					(51)
(2a)		+	+++	+++	+++								(54)
<i>Vibrio cholerae</i>													
inaba 569B (unbuffered)				-		-	-	++	++	-	++		(84)
inaba 569B (+NaHCO ₃)	-		(+)	+++	+++ ^a	+++ ^a		+++ ^a					(84)
ogawa (+NaHCO ₃)			++			++ ^a							(84)
<i>Salmonella typhi</i>													
(Quailes) vi			-		++		++	++++	++++				(82)
(Zermat) vi				+++									(82)
(Ty2V) vi						++							(82)
(O-901)								++					(82)
(Ty2W)								+					(82)
(Quailes)					+		++		++++				(83)
(Quailes)					++								(52)
<i>Salmonella newport</i>					+	++							(120)
<i>Salmonella bareilly</i>					+	++							(120)
<i>Salmonella anatum</i>													
(I)				-	+								(118)
(II)				-	-	-	+						(118)
(III)				-	-	++	+	+					(118)
*						++	+	+					(119)
<i>Salmonella</i>													
<i>meleagridis</i> (I)				-	-	-	++						(118)
(II)							++						(118)
(III)						+	++						(118)
*							+++	+++					(119)
<i>Salmonella derby</i>							++						(120)
<i>Salmonella</i>													
<i>pullorum</i> (I)				-	-	-	-	-	-	++++			(121)
(II)				-	-	-	-	-	-	++++			(121)
(III)				-	-	-	-	-	-	++++			(121)
(IV)				-	-	-	-	-	-	++			(121)
<i>Escherichia coli</i>													
(O111:B4)						+++		+++	+++				(60)
(O55:B4)								+++	+++	+++			(91)
(O6:H16)								++	++	++			(53)
(O124:K72:H-)								+		(+)			(53)
(O143:K?H-)				(+)		(+)		+++					(53)
(O144:K?:H-)				-		+		+++					(53)
(O148:H28)								+		++++			(53)
<i>Streptococcus</i>													
<i>faecalis</i> var. <i>liquefaciens</i>									+	++			(162)
<i>Clostridium</i>													
<i>perfringens</i> type A (Heat- resistant)								++	+++				(43)
<i>Clostridium</i>													
<i>perfringens</i> type A (Heat sensitive)									++++				(80)
<i>Entamoeba coli</i>	(++)	(++)	-	(++++)									(148)
<i>Giardia lamblia</i>	(++++)	(++)		(++++)	(++++)	(++++)							(149)
Norwalk agent ^b													
**1		++											
2		+++											
3		+											(46)
Hepatitis virus A ^b	+++												(81)
(fecal filtrates)	++												(130)

-- = 0, + = 1-25, ++ = 26-50, +++ = 51-75, ++++ = 76-100 % of volunteers developing illness

*Refeeding trials of volunteers who months before became infected by the same strain

**1, 2, 3 refers to serial passages of stool filtrates

(+) Infections without illness

^aCholera-like diarrhea

^bFecal filtrates

TABLE 8. *Outbreaks associated with foods contaminated by sewage or wastewater*

Disease	Source of contamination	Food	Reference
Typhoid fever	Sewage-polluted waters	oysters	(5)
Typhoid fever	Sewage sludge fertilizer and irrigation	celery	(128)
Typhoid fever	Sewage-contaminated watercress beds	watercress	(176)
Typhoid fever	Sewage-polluted water	shellfish	(133)
Typhoid fever	Sewage-polluted water ^a	oysters, raw vegetables and fruits	(106)
Typhoid fever	Human manure	rhubarb	(141)
Typhoid fever	Privy-polluted water-cress beds	watercress	(139)
Typhoid fever	Sewage irrigation	vegetables, blackberries	(7)
Typhoid fever	Sewage irrigation	raw vegetables	(6)
Typhoid fever	Sewage-polluted water	oysters	(25)
Typhoid fever	Sewage-polluted water ^a	oysters	(107)
Typhoid fever	Sewage-polluted water ^a	oysters	(107)
Typhoid fever, gastroenteritis	Fecal-polluted (privy and boats) water	oysters	(146)
Amebiasis	Sewage irrigation	vegetables	(4)
Taeniasis	Sewage irrigation	beef	(137)
Typhoid fever	Sewage-polluted wastes (privies and raw sewage)	oysters	(134)
Typhoid fever, paratyphoid fever	Secondary sewage treatment (activated sludge) ^b plant effluent irrigation and wash water	vegetables	(45)
Typhoid fever	Sewage	clams	(79)
Shigellosis	Irrigation water (Primary treatment plant effluent) ^c	cabbage	(64)
Amebiasis	Night soil	vegetables	(1)
Typhoid fever	Sewage-polluted water-cress beds	watercress	(42)
Ascariasis	Sewage spray irrigation	vegetables	(12)
Typhoid fever	Sewage manure	vegetables	(77)
Typhoid fever	Night soil	vegetables	(174)
Ascariasis	Night soil	vegetables	(3)
Typhoid fever	Sewage irrigation	apples	(154)
Salmonellosis	Sewage irrigation and sludge	vegetables	(101)
Typhoid fever	Human fecal material as manure	endive	(78)
Typhoid fever	Human fecal material as manure	raw salad	(78)
Typhoid fever	Night soil	vegetables	(100)
Ascariasis	Night soil	vegetables	(100)
Ascariasis	Night soil	vegetables	(126)
Hookworm infection	Sewage farming	vegetables	(140)
Viral hepatitis	Sewage-polluted water	oysters	(155)
Typhoid fever	Sewage ^a	vegetables, fruits, shellfish	(37)
Diphyllobothriasis	Sewage-polluted water	fish	(165)
Fascioliasis	Animal feces ^a	watercress	(57)
Viral hepatitis	Sewage-polluted water by primary treatment effluent, raw sewage, and septic tank discharges ^c	clams	(152)
Typhoid fever	Sewage-polluted water ^a	oysters	(21)
Viral hepatitis	Sewage-polluted water (overtaxed treatment plant effluents)	clams	(49)
Viral hepatitis	Sewage-polluted water (treatment plant by-passed)	oysters	(115)
Viral hepatitis	Sewage-polluted water	oysters	(169)
Viral hepatitis	Sewage-polluted water	oysters	(170)
Fascioliasis	Sewage-polluted water ^a	watercress	(135)
Cholera	Sewage-polluted water	shrimp	(90)
Fascioliasis	Sewage-polluted water ^a	watercress	(38)
Salmonellosis	Dead animal in polluted water from which cows drank	raw milk	(61)
Viral hepatitis	Sewage-polluted water	oysters	(170)
Viral hepatitis	Sewage-polluted water	clams	(170)
Taeniasis	Human feces contaminated trench silo	rare beef	(27)
Minamata disease (organic mercury poisoning)	Industrial waste	fish, shellfish	(136)
Salmonellosis	Sewage-polluted water by untreated sewage (culture positive)	whitefish	(102)
Fascioliasis	Animal feces contaminated watercress bed	watercress	(63)
Viral hepatitis, gastroenteritis	Sewage-polluted water by cesspool	clams	(8)
Gastroenteritis	Sewage-polluted water ^a	clams	(44)
Salmonellosis (animal infection)	Animal dung slurry used to irrigate pastures	grass	(44)
Viral hepatitis	Sewage-polluted water ^a	clams	(87)
Fascioliasis	Animal feces contaminated water used for watercress beds	watercress	(157)
			(9)

TABLE 8. *Continued.*

Cysticercosis (animal infection)	Sewage-contaminated irrigation water used for cattle watering	water	(23)
Ouch-ouch disease (cadmium poisoning)	Mining waste used to flood rice fields	rice	(56) (98)
Fascioliasis	Animal feces contaminated watercress bed	watercress	(76)
Viral hepatitis	Sewage-polluted water	clams	(171)
Cholera	Sewage-polluted water ^a	shellfish	(55)
Viral hepatitis	Septic tank effluent contaminated water ^c	watercress	(85)
Salmonellosis (animal infection, possible human cases)	Human sewage flowing over grazing land	grass	(18)
Viral hepatitis	Sewage-polluted water ^a	clams	(16)
Viral hepatitis	Sewage-polluted water ^a	oysters	(116) (144)
Cholera	Sewage-polluted water for growing and freshening	mussels	(179) (11)
Cholera El Tor	Night soil, raw sewage irrigation	vegetables	(180) (36)

^aImplied^bSecondary treated sewage^cPrimary treated sewage

Such outbreaks and source of contamination and vehicle for each are listed in Table 8. This table, which is obviously biased by incompleteness, shows that foods grown in water have been vehicles frequently. Shellfish which were contaminated in their growing area or during bloating after harvesting were vehicles in 28 outbreaks; watercress was the vehicle in 10 outbreaks; fish were vehicles in three outbreaks, and shrimp was the vehicle in one outbreak. Vegetables contaminated by night soil, human manure, or raw or partially treated sewage were reported as vehicles in 21 outbreaks. Fruits were considered vehicles in only four outbreaks. Before 1960, typhoid fever led the list of foodborne disease outbreaks attributed to wastewater contamination. The foods incriminated in these outbreaks were found to have been grossly contaminated with night soil or raw sewage. Outbreaks of infectious hepatitis are now reported most frequently, followed in frequency by outbreaks of fascioliasis and cholera. These findings reflect, in part, improvement in epidemiologic technique.

The epidemiologic evidence presented in many of the reports would not stand up to critical evaluation. On the other hand, a number of the investigators proved their hypotheses with epidemiologic and laboratory evidence. There are enough of these investigations to indicate that wastewater from mining, industrial, agricultural, community, and household sources have contaminated foods that, when eaten raw, have resulted in outbreaks of foodborne illness. These outbreaks will continue to occur sporadically if raw or partially treated wastewater is discharged into watercourses which are used for irrigation or aquaculture, and the contaminating pathogens survive in the wastewater, in soil, and then on contaminated foods in sufficient quantities.

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Hormonal Substances in Human Milk, Cow's Milk and Dairy Products

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ABSTRACT

Hormone-like substances are normal constituents of cow's and human milk. Progesterone has been quantified in cow's milk and dairy products with butter containing a high value of 300 ng/g. Estrogenic substances have been detected in cow's and human milk but literature values are sparse or tend to vary widely possibly because quantitative methods available lacked specificity and sensitivity. Pregnanediol is rather unique to human milk and its presence in elevated levels has been associated with prolonged neonatal jaundice of nursing infants. The concentration of this steroid in affected mother's milk appears to be in the range of 150-450 ng/ml. 17-Ketosteroids may be present in cow's and human milk but here again limited literature values vary widely. Corticoids have been reported in cow's milk at a concentration of 3.1-3.7 ng/ml. Thyroxine and triiodothyronine are present in human milk but practically undetectable in cow's milk. Prostaglandins (PGF_{2a}) have been determined to be present in cow's milk at concentrations between 0.1 to 0.4 ng/ml.

The steroid hormones as elaborated by the endocrine system occur in relatively small concentrations in body fluids. In fact, concentrations of these substances are so low in body fluids that only recently has it been possible to routinely quantify them. Use of microtechniques employing thin-layer chromatography, column chromatography, gas-liquid chromatography, radioisotopes, competitive binding assays, and radioimmunoassays has made it possible to estimate nanogram and picogram quantities of steroid hormones in one milliliter of milk.

The primary impetus behind measuring various steroid hormones in milk resides in their diagnostic value to veterinarians and in aiding the husbandryman to manage reproduction. For example, maintenance of elevated concentrations of progesterone in milk 19 or more days after breeding is being recommended as early evidence of pregnancy.

Since the dairy cow is usually pregnant 180 to 220 days at the end of her lactation period, market milk contains mixtures of milk from pregnant and nonpregnant cows. Thus, it is only natural that small amounts of steroid hormones associated with the reproductive cycle are present. By the same token, human milk also contains many of the same steroid hormones.

It has been only within the past few years that the steroid hormone content of dairy products has been

measured with a view toward the public health implications. Most analyses indicate that these hormones are present in the nanogram or picogram per milliliter level and, as such, should not constitute a public health threat. Thus development of ever increasing sensitive assays for these substances demonstrates their presence in natural foods such as cow's milk and mother's milk and, in effect negates the argument for zero tolerance.

This report is not an exhaustive review of the literature but is intended to give an overview of the subject. It's likely that peptide hormones would be digested in the alimentary tract, at least in adults. Thus, for the most part, peptide hormones are not included in this review. However, prolactin in milk has been studied in some detail (31) and has been quantified using radioimmunoassay techniques (29). Individual samples of cow's milk vary widely in prolactin concentration but usually range from 5 to 200 ng/ml with an average prolactin concentration of about 50 ng/ml (29). Quantities of steroid hormones present in human milk, cow's milk, and dairy products will be discussed. Hormones which have been measured in these foods include progesterone, estrogens, pregnanediols, and 17-ketosteroids. In addition, miscellaneous hormones such as the corticoids and thyroxine will be covered briefly.

PROGESTERONE

Presence of progesterone in cow's milk during pregnancy has been proven by isolation and characterization using gas-liquid chromatography coupled with mass spectrometry (12). There has been considerable disagreement and confusion, however, on the concentration of progesterone in cow's milk. Reasons for the disparate analyses have been attributed to possible existence of progesterone metabolites which interfere with assay procedures, differences in assay techniques among laboratories, sample deterioration with storage, nonspecificity of antisera used in radioimmunoassay procedures, and time at which milk samples were taken. A major reason for differences in progesterone concentrations in milk appears to be the sampling procedure. It is well known that the amount of fat in milk samples is dependent on the time during the

milking process at which a sample is obtained (49). Also there is a positive correlation between the fat content of the milk sample and the progesterone concentration (21, 23, 37). Thus, from a consumer's point of view, only pooled milk samples from complete milkings are relevant with regard to progesterone concentration. Samples from partial milkings would give a biased concentration of progesterone. Consequently, only those papers in the literature that clearly indicated the analysis for progesterone represented whole milk samples or pooled whole milk samples will be considered in this report.

In 1956, Pigato and Guzzonato (41) attempted to quantify progesterone metabolites in the morning milk of five cows pregnant from 3 to 7 months. Whole milk samples were hydrolyzed with acid and the conjugated and non-conjugated hormones were extracted into benzene. Estrogens were separated from androgenic 17-ketosteroids and 20-ketosteroids (progesterone metabolites) by solvent fractionation. The 17-ketosteroids were separated from the 20-ketosteroids using the Girard T reagent. The 20-ketosteroids were quantified using the colorimetric reaction of Zimmermann (57). Levels of 20-ketosteroids ranged from 40 $\mu\text{g}/\text{ml}$ for the cows pregnant 3 months to 96 $\mu\text{g}/\text{ml}$ for the cows pregnant 7 months. As we shall see, these values for 20-ketosteroids are approximately one thousand-fold greater than those reported subsequently for progesterone in milk. It seems likely that the nonspecific solvent fractionation and colorimetric procedures could include adventitious ketonic materials thus yielding high values.

Laing and Heap (27) determined the progesterone levels in samples taken from the whole volume of milk obtained at afternoon milking of a herd of British Friesian cows. Milk samples were analyzed for progesterone by a competitive protein binding assay. The reliability of the method was comparable with that obtained for the assay of plasma progesterone. Of 16 cows in the herd known *not* to be pregnant but still lactating the average progesterone concentration was 5.10 ng/ml with a range of 1.30 to 15.89 ng/ml. In 17 animals known from later clinical examination to be pregnant the average progesterone concentration was 19.69 ng/ml with a range of 7.1 to 35.6 ng/ml. Samples were analyzed over the 19th to 215th day of pregnancy and there was a tendency for the progesterone concentration to decrease in the latter stages of pregnancy. However, subsequent research from the English group has consistently indicated lower levels of progesterone in the milk of pregnant cows (10,20). A partial explanation might lie in the sampling techniques used in these later studies whereby initial portions of the milking appeared to be taken resulting in lower fat and progesterone concentrations. On the other hand, Heap et al. (20) reported a serious interference in the radioimmunoassay technique used to quantify the progesterone. They observed that milk samples analyzed by a highly specific antiserum gave results consistently

lower than those measured with a less specific antiserum. They suggested that the competitive protein binding assay and the radioimmunoassay using the less specific antiserum detected another and, as yet, unidentified compound(s) present in milk at an average concentration of about 1-2 ng/ml.

Nuti et al. (37) analyzed the pooled milk of 4 cows in mid-diestrus (days 11-14) using 2 radioimmunoassay procedures employing two different antisera. The mean concentrations of progesterone varied from 7.43 ± 0.95 to 12.30 ± 2.42 ng/ml depending upon the method.

Subsequently, this same research group (15) analyzed the progesterone concentrations in pooled milk from the bulk tank of a dairy farm over a 12-month period and a variety of dairy products from four markets. A radioimmunoassay procedure was used with minimal cross-reaction with other steroids. The antigen used for immunization and as a standard was a pure, synthetic progesterone. Validity of the assay was proven (16) as indicated by parallelism among unknowns, whether milk or plasma, and between unknowns and the standard. Furthermore, validity of the assay procedure was confirmed using gas-liquid chromatography (37).

The results of these analyses are listed in Table 1. The

TABLE 1. Concentration of progesterone in dairy products^a

Product	No. of samples	Fat %	Progesterone concentration (ng/ml)	
			Mean	SE
<i>Unprocessed products</i>				
Whole milk	12		11.3	± 0.6
Skim milk	12		4.6	± 0.4
Cream	12		58.7	± 5.3
<i>Processed products</i>				
Whole milk	4	3.5	9.5	± 0.5
Skim milk	4	0.01	2.1	± 0.6
2% Milk	4	2.0	5.8	± 0.4
Buttermilk	4	2.0	4.7	± 0.8
Half-and-half	4	13.0	27.0	± 2.5
Cream	4	35.0	72.7	± 5.8
Butter	4	79.6	132.9	± 5.1

^aFrom Ginther et al. (15).

mean progesterone concentrations varied from 2.1 ng/ml in skim milk to 133 ng/ml in butter. The progesterone concentrations tended to correlate with the fat content of the dairy product with butter being the highest in progesterone.

Hoffman et al. (22) also determined the concentration of progesterone in milk products using a radioimmunoassay procedure. The samples were obtained directly from the dairy or from the market. The results of these analyses are listed in Table 2. In general, there seems to be fairly good agreement between the results in Tables 1 and 2. However, the concentration of progesterone in the butter sample in Table 2 is substantially higher than in butter in Table 1.

Progesterone given by mouth to humans is much less effective compared to that administered by injection (17). This would imply that the progesterone is poorly absorbed from the gastrointestinal tract or that it is substantially modified biochemically after absorption.

TABLE 2. Concentration of progesterone in various dairy products^a

Product	Fat (%)	Progesterone concentration (ng/ml or g)
Whole milk	3.5	12.50
Skim milk	ca. 0.1	1.40
Cream	ca. 32.0	43.00
Buttermilk	ca. 1.0	6.50
UHT whole milk	3.5	11.75
Low fat UHT milk	1.5	6.00
Low fat sour milk	1.5	4.20
Unsweetened condensed milk	10.0	12.25
Butter	82.0	300.00
Nonfat dry milk	1.5	17.10
Whole milk powder	25.0	98.40

^aFrom Hoffman et al. (22).

Relatively large oral doses of progesterone are essential to evoke a contraceptive response in human females. Pincus (42) administered 300 mg/day by mouth over the menstrual cycle and observed an inhibition of ovulation in 30 of 50 women. Analysis of urine samples collected on days 19 to 21 of the cycle indicated that approximately 5% of the ingested progesterone was excreted in the urine as pregnanediol. This would suggest that the oral progesterone was poorly absorbed. However, one must realize that absorbed progesterone could also have been excreted as metabolites other than pregnanediol.

Stone and Kupperman (50) gave 1,000 mg of progesterone daily to each of 13 patients for 10 to 12 days, starting with the 8th day of the cycle. Of 16 cycles studied, inhibition of ovulation was evident in only six cycles. Thus, even when massive doses of progesterone are administered orally, the biological response is far from universal. In view of the large oral doses of progesterone necessary to effect a contraceptive response in human females, it is clear that the nanogram levels of progesterone found in dairy products would not have a significant biological effect.

ESTROGENS

Bioassay procedures that detect total estrogenic activity have been used to derive a semiquantitative indication of the presence of estrogenic materials in milk and milk-products (36, 55). Munch (36) was able to detect only estrogenic activity but no androgenic activity in milk. However, Vogt et al. (55) observed an increase in uterus weights of juvenile mice fed low levels of dried whole milk powder but a reversion in the weights of uteri from juvenile mice fed high levels of whole milk powder. They could not confirm the uterotrophic activity of whole milk powder by feeding skimmilk powder or butter.

Turner (54) made an early attempt to quantify the estrogenic activity in dried whole milk and colostrum using a bioassay procedure. In each assay, 10 ovariectomized mice were fed dried whole milk for 10 days and the uterus wt/body wt % was determined. A standard curve for estrogenic activity was prepared by feeding graded amounts of diethyl stilbestrol added to a mixture of commercial dried skimmilk and butter. This diet would, of course, include any endogenous estrogens in the skimmilk-butter feed. From the standard curve,

estrogenic activity was reported as diethyl stilbestrol equivalents.

The uterus wt/body wt % values for mice fed mouse chow compared to those fed dried whole milk were 0.04 and 0.05%, respectively. Using diethyl stilbestrol as the standard, Turner was unable to detect 90 ng or less of estrogenic activity per gram of dried whole milk. However, there is a discrepancy in the standard curve calculation which resulted in an overestimation of diethyl stilbestrol equivalents by a factor of 10. Thus, the above value should be 9 ng or less of estrogenic activity per gram of dried whole milk. All values reported subsequently will be corrected.

Estrogenic activity was not detectable in milk of 11 nonpregnant cows. Subsequently, Turner assayed the milk of 32 pregnant cows. Of 10 cows pregnant from 27 to 97 days, only a single cow produced milk which stimulated uterine weight in excess of the control range. Of 11 cows pregnant from 104 to 193 days, three cows produced milk estimated to contain 14 to 18 ng of diethyl stilbestrol equivalent/g of dried milk. Since milk is 88% water, this would amount to about 1.7 to 2.2 ng/ml of fluid milk. Of 11 cows pregnant 200 to 268 days the average uterine wt/body wt % was 0.09. Milk from one cow contained approximately 35 ng/g dried milk (4.2 ng/ml); whereas, the milk of three cows in this group gave a response equivalent to about 18 ng/g dried milk (2.2 ng/ml). Thus the milk sample with the highest activity in all of Turner's studies had a diethyl stilbestrol equivalent of 4.2 ng/ml.

Turner also assayed 18 samples of milk collected at monthly intervals. The average uterine wt/body wt % over the 18 months was 0.06. However, when cows were on pasture there was evidence of estrogenic activity in the milk. For example, in August and September this amounted to about 13.5 ng/g dried milk (1.6 ng/ml of milk). It is known that uterotrophic substances are produced by plants (5).

Pope and Roy (43) were unable to detect estrogenic activity in normal milk using a mice bioassay. They could detect <1 ng of 17 β -estradiol per ml of milk.

Monval-Gerondeau et al. (35) estimated the estrogens in cow's milk using a double isotopic labeling procedure. Only those estrogens occurring in the milk fat were quantified. The sensitivity of the method was about 12 pg/ml of milk for estrone, 10 pg/ml for 17 β -estradiol and 16 pg/ml for estriol. The reproducibility of the method was very good. Table 3 lists the concentration of estrogens found in samples of commercial milk products. The highest total level of estrogens occurred in the September milk samples containing 114 pg/ml.

Lunaas (28) analyzed milk from one nonpregnant cow over 4 days for conjugated and non-conjugated estrone and estradiol. Selective extraction procedures were used to separate conjugated from non-conjugated estrogens. Hormones were quantified using a spectrofluorometric procedure which is both highly sensitive and nonspecific. The free estrone concentration in milk was 20-40 pg/ml;

TABLE 3. Estrogens in commercial milk samples^a

Product	Fat % (g/100 ml)	No. of deter- minations	Estrone	17- β Estradiol (pg/ml)	17- α Estradiol	Estriol
Pasteurized commercial milk (March)	3.4	10	31 (23-39)	10 (3-17)	—	37 (26-46)
Pasteurized commercial milk (Sept.)	3.4	4	78 (68-87)	30 (28-33)	6.3 (6.2-6.4)	—
Partially skimmed powder	1.8	4	28 (19-33)	< 5 (0-4)	7 (5-10)	65 (53-84)

^aFrom Monval-Gerondeau et al. (35).

whereas the conjugated estrone ranged from <20-40 pg/ml. The free estradiol ranged from 60 to 200 pg/ml and the conjugated estradiol varied from 120 to 160 pg/ml. Thus the total estrogen in milk from a nonpregnant cow was <220-440 pg/ml.

Recently, Monk et al. (33) estimated the free estrone and estradiol in the total milk from one quarter using radioimmunoassay techniques. The whole milk was extracted with ether and the extract was used for quantification of the steroids. Recovery of added free estrogens to the milk was very good when corrected for procedural losses. Table 4 lists the concentrations of free

TABLE 4. Average quantities of estrone and estradiol in milk during pregnancy^a

Stage of pregnancy (days)	No. cows	Estrone	Estradiol (Average \pm SE)	Sum
55-81	5	57 \pm 20	85 \pm 9 (pg/ml)	142 \pm 20
107-145	4	35 \pm 13	52 \pm 14	87 \pm 22
205-209	4	97 \pm 21	49 \pm 21	146 \pm 27

^aFrom Monk et al. (33).

estrogens found in the milk of cows at various stages of pregnancy. The total free estrogens in milk ranged from 87-146 pg/ml which is about the same level found by Monval-Gerondeau et al. (35). Lunaas (28), however, demonstrated about half of the total estrogens in milk were present in conjugated forms. At this point, considering the highest estrogen levels found by Turner (54) and the lowest concentrations observed by Monval-Gerondeau et al. (35), the estrogen values for cow's milk range from 4200 pg/ml to 78 pg/ml or about a 60-fold variation in concentration. However, other workers have observed significantly higher concentrations of estrogens in cow's milk. In 1956, Guzzonato and Pigato (18) attempted to quantify the estrogens in the milk of five cows pregnant from 3 to 7 months. Whole morning milk was hydrolyzed with acid and the conjugated and non-conjugated estrogens were isolated by solvent fractionation. However, acid hydrolysis is known to destroy some estrogenic substances (1). Moreover, the extraneous materials extracted after acid hydrolysis of milk is extensive. The estrogens were estimated using the Kober colorimetric reaction (25) which is relatively insensitive and highly nonspecific. Total estrogen values ranged from 25 to 40 μ g/ml for animals pregnant 3 months and 7 months, respectively. These levels of

estrogen are orders of magnitude greater than those reported in the most recent literature. It is likely that the nonspecific fractionation and colorimetric procedures used in this study would include extraneous materials leading to high values.

Chicchini (7) determined 17 β -estradiol and estrone in the milk of six nymphomaniac and six normal cows during the estrus cycle. He apparently sampled the entire milking in each instance. However, the method of Ittrich (24) was used to quantify the estrogens. In this method, the fat phase is discarded and the conjugates in the aqueous phase are hydrolyzed by hydrochloric acid with possible destruction of some estrogens. This is followed by various partitioning steps between organic solvent and alkaline aqueous phases. The estrogen mixture is then subjected to chromatography on Al₂O₃ using organic solvents to develop the column. This procedure separates 17 β -estradiol, estrone, and estriol which are then quantified using a modification of the Kober (25) reaction utilizing strong sulfuric acid and a substituted phenol to form the final color complex which is estimated colorimetrically. It's doubtful this reaction would be very specific, and the specificity of the method would depend in part on the fractionation procedure. The ranges of estrogens found in the milk of six normal cows by Chicchini (7) were as follows: at estrus, 0.25-0.80 μ g/100 ml 17 β -estradiol and 1.85-4.63 μ g/100 ml of estrone; at mid-estrus 0.27-0.48 μ g/100 ml 17 β -estradiol and 0.92-1.08 μ g/100 ml of estrone. Thus the estrogen values varied between 2.5 and 8 ng/ml for 17 β -estradiol and 9.2 and 46.3 ng/ml for estrone. These values are substantially higher than those of previous investigators.

Subsequently, Chicchini (8) determined the estrogen content of milk from nine Friesian cows on each of the first 7 months of pregnancy. Here again the methods of Ittrich (24) and Kober (25) were used to quantify the hormones. The average total estrogen contents were 12, 16, 24, 55, 101, 236, and 494 ng/ml. These values are an order of magnitude higher than those previously reported (7) and considerably higher than the results of other investigators.

Pascoli (40) reported even higher values for the total estrogens in milk from several cows. Estrogens were measured colorimetrically using the Kober (25) reaction. Values for total estrogen ranged from 2.95 to 3.70 μ g/ml of milk. Since these levels are several orders of magnitude higher than values generally reported by other workers, they are of doubtful validity. In view of the wide variation in estrogenic activity reported in cow's milk, a careful, detailed study of estrogen levels in cow's milk and dairy products is called for.

To put the estrogen content of cow's milk into a better perspective, we might consider the estrogens evident in human milk. In 1953, Rossi (45) used a rather nonspecific solvent partitioning fractionation technique to isolate the total estrogens in postpartum human milk. Total estrogenic content of the morning and evening milk as estimated by the Kober (25) reaction averaged 1.8

and 0.9 $\mu\text{g}/\text{ml}$ in 11 women with a male fetus and 1.1 and 0.6 $\mu\text{g}/\text{ml}$ in nine women with a female fetus, respectively. Sas et al. (46) determined the steroid content of pooled samples of milk from 10 healthy women on the 2nd to 10th day and on the 15th, 20th, and 25th day postpartum. Estrogens were determined by the colorimetric method of Ittrich (24). Total estrogen (estriol, estrone, estradiol) values corrected for procedural losses are given in Table 5. The estrogen content of human milk over the first few weeks postpartum is substantially higher than those values for cow's milk reported by most other investigators. Sas et al. (46) estimated the daily amounts of estrogen consumed in the mother's milk by the nursing infants. These values are also in Table 5. As indicated in Table 5, the concentration of estrogens in milk postpartum goes through a maximum at about 5 days then declines to about the levels found in cow's milk at 25 days. Comparison of the levels of estrogens in human milk to those in cow's milk suggests that a nursing infant would receive more estrogens over the first month from human milk than cow's milk. However, much more detailed and extensive analyses will be necessary to firmly establish this. The reported values for estrogens in milk that were derived using nonspecific fractionations and colorimetric techniques such as the Kober reaction are of doubtful validity. The estrogenic content of milk reported in this older literature appears excessively high when compared to more recent studies involving more specific assay procedures. Furthermore, the chemistry of the Kober reaction is exceedingly complex, and Oliver et al. (38) have emphasized the need for strict controls over the purity of the reagents, the reaction vessels, the heating temperature, and the necessity for a knowledge of the history of the reaction mixtures. Since the specificity of these colorimetric techniques depends, in part, on prior fractionation procedures, it is not unreasonable to suspect that plant phenolics derived from the cow's diet, for example, may be contributing to the high estrogenic values. In any event, further careful, detailed studies on quantifying estrogenic activity in human and cow's milk are needed.

Concern for the infant-nursing mothers taking contraceptive steroids has been expressed. Molen et al. (32) have calculated that a child consuming 600 ml of breast milk from a mother receiving 5 mg 17 α -ethynyl-4-estren-17- β -01 and 150 μg 17 α -ethynyl-1,3,5(10)-estra-1,3,5-triene-3,17 β diol 3 methyl ether; 17 α -ethynyl estradiol 3-methyl ether would get about 8 μg of steroid + metabolites per day.

TABLE 5. Estrogen content of mother's milk and daily estrogen dose to the nursing infant^a

	Days post-partum											
	2	3	4	5	6	7	8	9	10	15	20	25
Total estrogen (corrected) (ng/ml)	241	403	465	843	336	595	312	351	186	140	57	15
Daily consumption of milk (ml)	50	160	320	350	450	480	500	530	560	600	600	600
Daily dose of estrogen ($\mu\text{g}/\text{day}$)	12	65	149	249	151	250	156	136	104	84	34	9

^aFrom Sas et al. (46).

PREGNANEDIOL

Pregnane-3 α , 20 β -diol represents a rather unique situation with regard to infants nursing on mother's milk. Apparently, this steroid does not exist to any appreciable extent in cow's milk but does appear in mother's milk. The presence of this steroid in human milk has been associated with the occurrence of neonatal unconjugated hyperbilirubinemia or neonatal jaundice.

In 1963, Arias et al. (3) first reported that breast milk of mothers nursing newborns with prolonged unconjugated hyperbilirubinemia strongly inhibited the formation of o-amino-phenol glucuronide and of bilirubin glucuronide in vitro. Thus the steroid interfered with normal excretion of bilirubin. The inhibitory activity was negligible in milk of randomly examined mothers. These same investigators (2, 4, 14) stated subsequently that this inhibitory activity was not present in cow's milk and that pregnane 3 α , 20 β -diol was present in inhibitory but not in noninhibitory human milk. Pregnane-3 α , 20 β -diol competitively inhibits glucuronyl transferase activity in vitro. Furthermore the unconjugated hyperbilirubinemia could be produced in very young, full term infants by feeding pregnane 3 α , 20 β -diol at levels of approximately 1 mg/day, which is the quantity the above authors estimated to be secreted per day in breast milk. This type of neonatal jaundice is rapidly reversible and immediately disappears when the affected infant is placed on a formula of cow's milk (56).

The incidence of breast milk jaundice has been estimated to vary between 1 in 200 to 1 in 500 breast fed infants (56). Severe hyperbilirubinemia in the neonatal period can result in neurotoxicity known as kernicterus which results in irreversible brain damage (56).

Severi et al. (48) estimated the concentration of pregnane-3 α , 20 β -diol using thin layer techniques for separation followed by colorimetric analysis. Krauer-Mayer et al. (26) claimed this method of analysis had a sensitivity of 0.05 μg and practically absolute specificity. Concentrations of pregnane-3 α , 20 β -diol in human milk samples implicated in neonatal jaundice were estimated at 150 to 450 ng/ml by Severi et al. (48) and at 200 to 420 ng/ml by Krauer-Mayer et al. (26). In the latter analyses, the 420-ng/ml value was observed in breast milk 42 days postpartum.

Fontaine et al. (13) reported on four cases of breast milk jaundice resulting from milk containing 75 to 385 ng of pregnane-3 α , 20 β -diol per ml. Using a gas-liquid chromatographic method, Tanaka (53) determined

concentrations of this steroid to be 2.38 to 0.4 $\mu\text{g}/\text{ml}$ of breast milk depending upon the time postpartum. Sevegli and Battista (47) reported on 11 cases of neonatal jaundice caused by pregnanediol in mother's milk. However, the levels of pregnanediol (0.4-2.2 mg/ml) in the breast milk reported by these workers is orders of magnitude higher than that reported by other workers in the field.

Apparently breast milk implicated in neonatal jaundice contains elevated levels of pregnane-3 α , 20 β -diol for extended periods, whereas the steroid level of milk from normal women decreases rapidly from 350 ng/ml on the second day postpartum to undetectable levels on the third day postpartum (36). The view that pregnane-3 α , 20 β -diol in breast milk is responsible for breast milk jaundice is not universally held, however (46).

17-KETOSTEROIDS

In an early paper on 17-ketosteroids in postpartum human milk, Rossi (44) used solvent fractionation to obtain the androgens which were quantified with the colorimetric reaction of Zimmermann (57). By this method the average 17-ketosteroid content of the morning and the evening milk was 103 and 66 $\mu\text{g}/\text{ml}$ in 11 women with male fetuses and 146 and 97 $\mu\text{g}/\text{ml}$ in nine women with female fetuses, respectively. Sas et al. (46) found lower levels of 17-ketosteroids in pooled milk from normal, healthy lactating women over the first 25 days postpartum. The 17-ketosteroids studied included dehydroepiandrosterone, androsterone, androstenedione, androstandion, and chlor-dehydroepiandrosterone. Extremely high levels of these steroids were reported in human milk. Uncorrected total 17-ketosteroids ranged from 12.52 $\mu\text{g}/\text{ml}$ at day 2 postpartum to a high of 35.60 $\mu\text{g}/\text{ml}$ at day 4 postpartum to a low of 0.4 $\mu\text{g}/\text{ml}$ at day 25 postpartum. However, Darling and Harkness (11) suggest that contaminants might be responsible for the high values of Sas et al. (46).

Pascoli (40), using a colorimetric technique, reported extremely high values for the 17-ketosteroids of cow's milk. The concentration of these steroids varied from 66.4 to 83.4 $\mu\text{g}/\text{ml}$ of milk. Subsequently, Pigato and Guzzonato (41), using relatively nonspecific procedures of fractionation and colorimetric analysis (41), estimated the 17-ketosteroids in milk of five cows pregnant from 3 to 7 months. Values varied from a high of 45 $\mu\text{g}/\text{ml}$ to a low of 36 $\mu\text{g}/\text{ml}$ and decreased as pregnancy progresses. These enormous amounts of 17-ketosteroids in cow's milk were not confirmed by Darling et al. (10) using more sensitive and sophisticated techniques. Darling et al. (10) surveyed the steroids in cow's milk by oxidizing the steroid mixture to steroid ketones which were separated and estimated using gas-liquid chromatography. The 5 α -androstane 3, 17 dione obtained from the milk of four pregnant cows varied from 0.7 \pm 0.4 ng/ml to 5.0 \pm 2.2 ng/ml over 136 days of pregnancy. Darling et al. (10) suggested that the precursors for the above oxidized steroid were in part androsterone and epiandrosterone. Thus the concentrations of 17-

ketosteroids in cow's milk appear to be relatively low.

MISCELLANEOUS HORMONES

Paape et al. (39) reported that the levels of corticoid in cow's milk ranged from 3.1-3.7 ng/ml.

Strbak et al. (51) determined the amount of thyroxine, which was concentrated in the lipid phase, in human milk using a protein displacement method. For five determinations on human milk the levels were 46 \pm 8 ng/ml. In a second series of analyses, the values ranged from 50 to 72 ng/ml of milk. There was no evidence of thyroxine in cow's milk. Subsequently, Strbak et al. (52) used a competitive binding assay with a high specificity for thyroxine to analyze human milk. These workers examined 94 samples of human milk from 45 lactating mothers from the 3rd day up to the 9th week after delivery. Immediately after initiation of lactation the thyroxine content of the milk was only 13 \pm 3 ng/ml. During subsequent days of lactation the levels of thyroxine in milk increased. Analysis of cow's milk formulas showed very small or a doubtful content of thyroxine. Montalvo et al. (34) examined human breast milk and colostrum from four healthy euthyroid mothers for thyroxine and triiodothyronine. Milk was obtained on day one, 1 week and 1 month postpartum. Colostrum contained a mean of 6.5 \pm 2 ng/ml of thyroxine and a mean of 0.38 \pm 0.09 ng/ml of triiodothyronine (T_3). At 1 week postpartum the mean thyroxine level was 96 \pm 4 ng/ml whereas the mean T_3 value was 1.13 \pm 0.25 ng/ml. One month postpartum the levels of thyroxine had decreased to 20.7 \pm 6 ng/ml and those of T_3 to 0.99 \pm 0.06 ng/ml. Thus thyroxine and T_3 are secreted in significant amounts in human milk but hardly at all in cow's milk.

Because of the possibility that certain prostaglandins ($\text{PGF}_2\alpha$) might be used to regulate the estrous cycle of cattle and sheep to allow insemination at preset times, considerable interest has arisen in measuring prostaglandin F (PGF) in milk before and after administration of these compounds. Manns (30), using a radioimmunoassay procedure, measured PGF in milk and blood of four cows for periods of 30 min before and 7 h after intramuscular injection of 30 mg of $\text{PGF}_2\alpha$. Six $\text{PGF}_2\alpha$ and four control (vehicle only) injections were performed on the four cows. Maximal concentration of PGF in milk (.91 \pm .12 ng/ml) occurred at 1 h after injection and declined to preinjection concentrations of .2 to .4 ng/ml at 7 h after injection. Hansel et al. (19) have carried out an experiment with six Holstein cows in which $\text{PGF}_2\alpha$ was measured in milk obtained at two regular milkings just before $\text{PGF}_2\alpha$ administration and in milk obtained at eight successive milkings after $\text{PGF}_2\alpha$ administration. Twice daily milkings were made at 0800 and 1800. The $\text{PGF}_2\alpha$ was administered into the uterus at 5- or 10-mg doses. The concentration of PGF before administration of the $\text{PGF}_2\alpha$ was approximately .14-.15 ng/ml milk. After administration of the $\text{PGF}_2\alpha$, concentrations of PGF varied between .17 to .23 ng/ml milk over the next seven milkings. On the eighth milking

after administration, the PGF concentration in milk had fallen to pre-administration levels to .12 ng/ml. The concentrations of the metabolites of PGF_{2a} in milk have not been measured yet.

If one assumes an average concentration of PGF in milk of 0.2 ng/ml and that it is concentrated in the fat phase, then one can readily calculate an approximate concentration in butter as 5 ng/g. To put this level in perspective, intraamniotic instillation of 40 mg of PGF_{2a} is used to induce abortion in humans (17). Manns (30) points out that the quantities of PGF_{2a} in milk from treated or untreated cows is insignificant relative to those required orally to cause abortion in humans.

Apparently, exogenous PGF_{2a} can affect steroid metabolism. However, Hansel et al. (19a) found no evidence for elevated concentrations of testosterone in milk of PGF_{2a}-treated cows. Mean concentrations of testosterone in milk for three cows with active corpora lutea for the 10 milkings ranged from .45 to .71 ng/ml. On the other hand, in the work of Hansel et al. (19), milk progesterone concentrations for the three cows with functional corpora lutea were high (7.6 to 22.5 ng/ml) in the samples collected before treatment and declined to a concentration of less than 3.0 ng/ml by 72 h after treatment. Progesterone concentrations in milk in the remaining three cows rose from a mean of 4.3 ng/ml before treatment to a mean of 13.1 ng/ml. These animals were at days 2 to 5 of their estrous cycles when treated, and development of corpora lutea proceeds in a normal way when animals are treated with PGF_{2a} at this stage of the cycle.

Brewington et al. (6) failed to detect the conjugates of acne-causing hormones (glucuronides of testosterone, androsterone, or estradiol) in cow's milk at concentrations of < 10-15 µg per liter.

CONCLUSIONS

The analytical procedures for progesterone in milk based on radioimmunoassay appear to yield reliable results. The levels of progesterone found in dairy products are biologically insignificant when compared to the massive oral doses of progesterone needed to exert a biological effect in humans. There is a great need to obtain reliable, quantitative data on estrogens in cow's and human milk. Older values based on colorimetric techniques are extremely high when compared to recent data predicated on more specific techniques. Pregnenediol in human milk has been associated with the occurrence of neonatal jaundice of nursing infants. Since this steroid is essentially absent in cow's milk, the jaundiced condition can be alleviated by substituting cow's milk for human milk. Disparate analytical results for 17-ketosteroids in milk point out the need for further, careful measurement of those steroids in human and cow's milk. Limited analyses indicate very low levels of corticoids, thyroxine, triiodothyronine, and prostaglandins (PGF_{2a}) in cow's milk. Since milk is a very complex biological fluid reflecting metabolic processes, it

is likely that other hormones and hormone metabolites will be found there.

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Toxicological Considerations in the Selection of Flexible Packaging Materials for Foodstuffs

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ABSTRACT

High molecular weight polymers are the "backbone" of the flexible packaging industry, although a variety of papers, fabrics, and metallic foils are also employed. Almost without exception, high molecular weight polymers are physiologically inert and, therefore, pose no toxicological problems in themselves. However, monomers and low molecular weight fractions that may be incorporated in certain commercial polymers can be a source of concern, as can be other low molecular weight chemicals purposely added to commercial polymers, in some instances, to modify their processing or functioning characteristics. Conditions of use must also be considered when employing polymeric films (with or without additives) as the food contact components of packages. A polymer may be completely nontoxic and an adequate barrier to other package constituents when used to contain foodstuffs if the resultant package is subsequently stored at room temperature, under refrigeration, or frozen. However, when used as the inner ply of a boil-in pouch, a retortable pouch, or an oven bag, it must be determined that it is not subject to some degree of thermal breakdown or allows permeation of adhesive or ink components not approved for direct contact with food. Finally, if there is a requirement to sterilize packages before filling, as in aseptic packaging, the effect of the sterilizing medium on the packaging material must be fully understood.

Flexible Packaging is a broad field. It embraces many package forms including overwraps, bags, tubes, pouches, tetrahedrons, and an infinite variety of thermoformed shapes.

Materials employed in this field encompass a diversity of web-formed products including almost all species of commercially available thermoplastic polymers, papers, and metallic foils. In producing packages these web-form materials may be used individually or in combinations — as in laminations. In addition, a wide range of adhesive are used in producing some of the laminations and in assembling some of the package forms. Further, since decoration and identification of packages are major considerations, this necessitates use of numerous and varied ink formulations because of the many different types of surfaces they must be applied to, the various printing methods employed in the industry, and the special performance characteristics sought.

All-in-all the possible permutations and combinations are exceedingly vast, so that it might seem that determining the toxicological suitability of each particular flexible package for each particular foodstuff and type of service would be a major challenge, but this

problem becomes manageable because most foodstuffs fall into a very limited number of categories insofar as their action as extractants are concerned, and because, in the great majority of packages, only the food contacting ply could possibly be a source of contamination.

CLASSES OF FLEXIBLE PACKAGES

Flexible packages are of two general classes as far as the nature of the food contacting ply is concerned. One class comprises all those packages fabricated from a web stock by heat-sealing and includes pouches, most transparent bags, tetrahedrons, thermoformed packages, tubes, and most overwraps, in all of which the food contacting ply is formed from a thermoplastic polymer. The second class of flexible packages is made up of those that are assembled by some sort of adhesive and includes envelopes and so-called specialty bags such as those used for packaging coffee, rice, flour, sugar, spices, dessert powders, pet foods, etc. The food contacting ply in this second class of packages may be either a thermoplastic polymer or some variety of paper such as natural or bleached kraft, glassine, or parchment. Accordingly, in most instances, the packager of a particular food product is concerned with the toxicological aspects of a single thermoplastic polymer or a single type of paper, and any additives which the film or paper manufacturer has added during his processing.

Because various classes of paper, glassines, and parchments have been used in the packaging of foodstuffs for generations and since the primary constituent of these webs is cellulose, which is present in many foodstuffs themselves, it was quite reasonably assumed that, except possibly for some of the newer additives, there were no toxicological hazards involved when this type of web was used in the food contacting portion of any package.

Even before the Food Additives Amendment of 1958 was promulgated a number of the thermoplastic films had also been used in food packaging for 20 or more years and this same line of reasoning was applied here, also, with the result that A. J. Lehman of the FDA in 1956 published in the Quarterly Bulletin of the Association of Food & Drug Officials of the United States (Volume 20, page 159) a list of those polymers acceptable for use in

food contact application. This list of "prior sanctioned polymers" remained unquestioned until quite recently, and even now, with one dramatic exception, there is no reason to doubt its wisdom. However, all those polymers commercialized a few years before Lehman's list or since, have been required to have their safe use proven by animal feeding testing.

The first of the "new" polymers to have its safe use as a food contacting ply in food packages established was polypropylene, followed by polyethylene, and a host of other polymers, altogether more widely used today than the prior sanctioned materials. As a generality, it can be stated that all the extensive animal feeding tests that have led to the approved use of "new" polymers demonstrate that very high molecular weight compounds are unaffected by the digestive process, are unabsorbed, pass through the system unmodified and accordingly are completely non-toxic. Consequently, no reason has been found for apprehension concerning the use of "pure" high molecular weight polymers in food contact applications.

MINOR COMPONENTS ARE OF CONCERN

However, films produced solely from "pure" high molecular weight polymers are a rarity, and it is those minor components of food packaging films which are not high molecular weight polymers that must be of concern to the toxicologist and the food packer. Such minor, lower molecular weight components of food packaging films fall into three categories: (a) additives intentionally added to the polymer to either aid in its conversion to a film or to beneficially modify the physical characteristics of the film produced; (b) low-molecular weight raw materials or by-products of the polymer manufacturing process which may unintentionally be present in the finished product; and (c) lower molecular weight materials not actually present in the food packing film as originally produced but which are introduced into the film (and eventually the foodstuff packages) from an adjacent non-food contacting ply, such as an adhesive, overlacquer or decorative ink, by the extractive action of the foodstuff under certain very special conditions.

Intentional additives

The additives which are intentionally added to polymers to aid in their conversion to film-form or to modify the film's physical properties include anti-block compounds such as ultra fine silica, slip agents as fatty acid amides, thermal stabilizers such as BHT, plasticizers, release agents, and combustion inhibitors (which latter are rarely, if ever, used in food packaging applications).

Since no additive can be used in any food contacting film unless FDA approved, and since the thorough toxicological testing of such specific compounds before their intentional addition is relatively simple, their presence in food contacting films is of little concern to the food packer once he has been assured by his supplier that those additives present are on the approved list and

are within the generally very low concentration allowed.

In this regard, however, the food packer should always be sure his packaging material supplier knows the end use involved. There are many films and laminates generically similar to the food packaging films and laminates, but which are intended for industrial applications. Many of these require the presence of types and/or quantities of additives which are completely inappropriate to food packaging applications from a toxicological point of view. For example, lead compounds are commonly employed to stabilize certain vinyl compositions used in industrial applications, and most combustion-resistant plasticizers are highly toxic, particularly at the concentrations required for their effective use.

Unintentional additives

The second group of "minor components" of polymeric films has the potential of being the most troublesome, since they are not intentionally added and may be difficult to detect, isolate, and identify. The fact that there have been so few problems with low molecular weight raw material and manufacturing by-products in commercial films is a tribute to both the polymer manufacturers and the FDA's rigorous requirements relative to new polymer approval as well as the quality regulations enforced on approved products.

The subtleness of the problems possible with this group of unintentional additives is well illustrated by polyvinyl chloride which has been used in food contact applications for approximately 40 years and enjoyed "prior sanctioned" approval, but which only very recently was demonstrated to frequently contain small quantities of vinyl chloride monomer, which compound was, at about the same time, demonstrated to be a carcinogen. This new knowledge has resulted in a tightening of manufacturing controls and specific FDA regulations which assure that present food contacting films based on vinyl chloride are toxicologically safe, beyond question.

Naturally the polyvinyl chloride experience prompted a reexamination of all the "prior sanctioned" polymers and particularly polyvinylidene chloride (Saran), but to date these recently aroused suspicions have been found to be baseless. However, as a result of this one experience, one can anticipate even more critical examination of any new polymers of the future before their food use approval.

Extracted by foods

The third group of low molecular weight components referred to earlier are compounds not present in the food-contacting polymeric films as produced, but in some packaging situations can be caused to migrate from an adjacent ply or coating into the film and then into the packaged foodstuff by the extractive action of the food. Naturally the possibility of such a sequence of events demands that the next adjacent ply or coating have low molecular weight components, that they be soluble in the

food-contracting ply, and that the foodstuff have the capability of acting as an extractant under the condition of packaging, processing, and/or storage.

The just described combination of circumstances would be rare, but the simultaneous occurrence is enhanced when the next adjacent "ply" is a two-component (i.e., reactive) adhesive, the foodstuff is composed of high proportion of liquid, and the completed package is exposed to an elevated temperature for an appreciable time. This combination does result when stews and similar semi-fluid entrees are retorted (usually at 250 F), since all currently offered retort pouches do employ a polyurethane adhesive between the high temperature resistant sealing ply and the central, oxygen and moisture impermeable foil ply.

However, even in this extreme situation and using exaggerated processing conditions, it has been found that the quantities of unreacted adhesive components that are extracted into food simulants are very low — well under the 50 ppm which have been allowable for can coatings, for example. Nevertheless, the FDA is currently adopting more stringent standards than have previously been felt to be necessary. As a result that agency is requiring animal feeding tests to definitely establish the non-toxic effects of the extracted adhesive components (epoxy and polyester pre-polymers) before approving this relatively new package form. The fact that well over a billion units of retort pouch packaged food have been consumed by humans in Japan, without any problems, is not deemed to have any pertinence.

This new degree of caution on the part of the FDA is undoubtedly due to several facts such as the unexpected vinyl chloride monomer situation previously described; the pressure of some consumer activist groups that over-react to such terms as "additive," "artificial," and "chemicals;" and the fact that analytical techniques have progressively improved in sensitivity and accuracy to the point that 50 ppm is no longer accepted as a "practically zero" extraction level, the "practical zero" now being something like 50 ppb.

With regard to the statement concerning certain consumer activist groups, it is apparent that much of their membership is made up of persons blissfully unaware that all foodstuffs are composed entirely of "chemicals" and that some of these natural chemicals such as oxalic acid, arsenic salts, selenium salts, and some vitamins are quite toxic in rather moderate quantities. In fact, if the 50 ppb limitation on known toxics were enforced against foodstuffs themselves rather than on the packaging material many grains, fruits, and vegetables that have been part of the human diet for many generations would be forbidden and we would all be doomed to die from a diet deficient in some essential vitamins and minerals.

COMPOUNDS INTRODUCED BY INTERMEDIATE PROCESS

Up to this point we have only considered the possible toxic contamination of foodstuffs by low molecular

weight components of the flexible packaging material which the packers purchase from their supplier, but it should be noted that it is at least conceivable that low molecular weight compounds could be introduced into a packaging material after fabrication by some intermediate process before actual filling and sealing. For example, should the packer be using an aseptic filling and sealing process, the packaging material must be pre-sterilized and this operation could introduce compounds that would subsequently be absorbed into the food.

In the better known practice of aseptically packaging into cans, the empty cans are usually pre-sterilized in-line by steam. In this instance, as long as the interior can coating is not subject to any thermal breakdown by the high pressure steam temperature, there is no possibility of introducing a toxic substance. However, when aseptically filling flexible packages, pre-sterilization of the food contacting surfaces with steam would be unusual. Chemical or radiation pre-sterilization are the preferred methods and in both techniques the possibility of introducing a toxic substance does exist.

There are two quite different procedures followed when aseptically filling flexible packages. In one the slightly over-length pouches are first fully sealed, then sterilized (either long before filling or in-line; as desired), then filled near one end in a sterile environment by an oversize "hypodermic needle." A second end seal is made below the point of needle entry just before the needle is withdrawn and, finally, the extra end tab is trimmed off. This is a truly aseptic system, but can be used only for packaging of flowable products—either liquid or finely powdered.

The second aseptic procedure used in flexible packaging could more accurately be called "ultra-clean" rather than aseptic, since the film or lamination is sterilized, in-line, on form, fill and seal equipment by passing the web, as it is unwound from the roll, through a sterilizing bath (usually a peroxide solution). In this system the sterilizing bath and a subsequent drying station are all contained in a sealed chamber along with the form, fill, and seal equipment, making any sort of statistically valid, periodic testing of the packing material for sterility before filling most difficult, if not impossible.

In this "ultra-clean" process just described, even if one can assume that commercial sterility is invariably attained, there is still the possibility of incomplete removal of the sterilizing medium from the web before package formation and filling. Accordingly, in addition to selecting a sterilizing medium which will have no chemical effect on the sealing ply, one must seek a sterilizing medium of very low toxicity, if possible. The only alternate to the latter would seem to be a rigorous sampling of filled units for analysis to assure that any incorporated sterilizing medium was not above a previously established safe level.

Toxicological considerations for the first described,

truly aseptic, filling system are both simpler and more complex. They are simpler in that effectiveness of sterilizing the empty containers is readily determined, and in that once the compatibility of the package structure and the sterilizing method have been established, one does not have to be concerned further with the possibility that any toxic sterilizing medium or a by-product thereof is present in the package at the time of filling. On the other hand, a degree of complexity is introduced because of the several methods that can be used to sterilize the sealed, but empty pouches. Depending on the composition of the fully sealed pouches they may be sterilized thermally, by means of ethylene oxide, or, preferably, by irradiation.

If the pouch structure is physically capable of withstanding retorting conditions it may be so sterilized if a very small amount of moisture is introduced into each pouch just before sealing. But if this option is exercised one must determine that extraction through the sealing ply of any low molecular weight components of adhesives is within tolerable limits, which would be anticipated to be so because of the small quantity of "extractant" employed.

Ethylene oxide sterilization is a possibility for most pouch structures which do not contain a foil ply. However, it must be clearly established whether or not all ethylene oxide is removed from the pouch interiors in the normal sterilizer cycle. If not, it must be determined how many hours such pouches must be retained unfilled to allow complete diffusion of the gas. Otherwise any residual ethylene oxide would be absorbed into the foodstuff and at least hydrolyzed to ethylene glycol. In some instances this might be oxidized to oxalic acid. Neither of these chemicals could be classified as highly toxic, but both are undesirable and probably

unacceptable in more than trace quantities.

Ethylene oxide sterilization of pouches having chlorine-containing plies such as polyvinyl chloride is definitely to be avoided since interaction between the gas and the film can produce ethylene chlorhydrin, which is definitely toxic and unacceptable in any food product.

All pouch structures are susceptible to sterilization by irradiation using either gamma ray or electron beam. The latter alternate is much to be preferred from both economic and operational safety standpoints. Further, excessive gamma radiation can cause breakdown of some polymers thereby generating low molecular weight components that could be extracted into foodstuffs. However, pre-fabricated and boxed pouches can rapidly and inexpensively be sterilized by the electron beam technique without any damage to the structure or the fear of generating any extractable, low molecular weight compounds.

IN SUMMARY

In summary, while toxicological conditions can be encountered when employing flexible packaging for foodstuffs, this is highly unlikely because of the physiologically inert nature of high molecular weight polymers, papers, and those metallic foils employed in the industry. This minimal risk can be further reduced to essentially zero if the food packer makes sure his packaging material supplier fully understands the nature of the product to be packaged and any unusual processing or handling conditions to which the package will be exposed.

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A Field Topic

Know the Score in Your Food Markets

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ABSTRACT

To close the quality control circle, suppliers of perishable foods to markets must be rated. Once or twice a year quality control records of these processors should be surveyed to see that proper records, controls, and bacterial counts are available. Inspections should be made to see that suppliers have the necessary facilities kept in correct condition. When food markets have inspection forms and gained knowledge to use these materials, they are well on the way to know the score in their store. It is then up to management to educate and motivate all employees in the importance of excellent quality control standards. These standards must be set high and must be checked honestly and regularly. Finally, market management should keep a file of inspection records and take pride in improving scores.

Food markets must provide consumers with more than attractive packages and surroundings. Products sold inside these stores must have been prepared with a knowledge of, and compliance with good sanitation procedures. These sanitation standards must be worthy of the customers' trust.

Food chains and associations must recognize that the individual store is too often the weakest link in the sanitation chain of food handling, and they must work to educate and strengthen this final guardian of quality foods in proper food protection.

The United States Department of Agriculture Seal, USDA, on meat means little if such meat is cut with dirty knives, on an unclean block, placed in greasy lugs, and then ground in a grinder that has not been regularly washed. United States Public Health-approved milk can spoil if this market does not store

and display such milk under proper refrigeration.

The best prepared food held under ideal conditions can develop flavor problems if a program of rotation is not instituted and some idea of shelf-life of various food products is not understood at the store level.

In trade magazines we see articles with the titles such as "New Sanitation Regs.—Look Tough." We see articles that describe how few hamburger samples examined in state regulatory laboratories meet bacteriological standards. We see articles by food associations that often criticize new inspection requirements and food quality standards as being impractical or as showing too little knowledge of market operations.

Often food stores do not seem to be concerned with quality standards of foods that they prepare until regulatory agencies provide and begin to enforce such standards. Too frequently management and department heads have failed to spend the necessary time on grading or establishing market sanitation guide lines. In many instances failure of any of the stores' meat, dairy, or delicatessen departments to meet regulatory microbial or sanitation standards seem to bring a cry on the part of food markets for relaxing such standards or in transferring blame to their suppliers. Regulatory inspections made of food stores are often criticized for not being fair. Field sampling of foods is said to be left to individual inspectors who inspect markets in a convenient location and fail to check and sample foods and stores that are away from

main travel areas. Others complain that nearby competitors were not being rated.

On the other side, inspection departments of regulatory agencies often feel that markets consider fines imposed or the inconvenience of attending hearings on violation of sanitation practices as just a cost of doing business. In many instances more effort is spent on getting to know inspection personnel and discussion mutual problems than on reestablishing a mutually designed and effective quality control program in the market.

The individual market must not wait for public opinion to force it to improve its sanitation practices. If a serious food poisoning problem or any violation in the food processing area of a market is released to the news media, corrective action is swift—but at a tremendous cost. Ask the cranberry, dairy, mushroom, and fish industries what this lack of public confidence means.

Markets and food associations must accept the challenge of improving the food that is processed. They must accept the quality control responsibility that is necessary when the stores' products and especially when the markets' labels are offered to the public.

Markets should work toward a goal of self-regulation within a framework of mutual cooperation between regulatory, health, and educational agencies in formulating a sanitation program. It is time for food stores to act and not wait to react to other interested parties in the food field.

QUALITY CONTROL SCORING

It is in the perishable food preparation area of the market that we should begin our quality control scoring. A scoring form should rate and list requirements that are uniform for each of the perishable food areas; sanitation requirements for the meat departments are equally important in the produce and delicatessen sections of the store.

The model sanitation score forms that follow were made after checking

numerous food industry requirements as well as consulting and observing food processing in many markets.

Items that are frequently marked

on the scoring form include the following.

1. *C. Separated facilities for potential hazardous food and materials.* (a) Glass bottles should never be stored on open shelves

TABLE 1. *Scoring form for perishable food handling areas*

Preparation areas	Meat	Produce	Deli
Store _____ Date _____			
Inspector _____			
1. CONSTRUCTION			
A. Floors, storage racks, shelves; impervious, good repair, floor graded and drained _____			
B. Walls-Ceilings: smooth, tight, good repair, proper finish _____			
C. Separated and protected facilities for potential hazardous foods and materials _____			
D. Adequate light properly protected _____			
E. Adequate space _____			
F. Utensils and equipment corrosive resistant, smooth and good repair _____			
2. CLEANLINESS			
A. Floors _____			
B. Walls and ceilings _____			
C. Attached fans and fixtures _____			
D. Storage racks _____			
E. Coolers _____			
3. FOOD PROCESS			
A. Movement: cooler to display case _____			
B. Protection _____			
C. Rotation _____			
4. WASH FACILITIES			
A. Sink compartments _____ Size _____			
B. Adequate hot water _____			
C. Suitable detergents and sanitizers _____			
D. Cleaning utensils _____			
5. EQUIPMENT STORAGE			
A. Clean surface _____			
B. Stored to drain and dry _____			
C. Single service items stored properly _____			
6. WASTE DISPOSAL			
A. Surface drainage of sewage prevented _____			
B. Trash and scraps covered _____			
7. WATER SUPPLY			
A. Safe source properly developed and protected _____			
8. PERSONNEL			
A. Hand wash sink with soap dispenser, individual towels, separate from equipment sink, available and used _____			
B. Clean outer garments, hats and hair nets _____			
C. Eating, Smoking, Clothing; in designated areas _____			
9. TOILET			
A. Properly located, clean, self-closing doors, vented, lighted, towels, soap dispenser, covered trash, proper fixtures, hand wash sign _____			
10. RODENTS AND INSECTS			
A. Outer openings-screened, tight doors opening outward _____			
B. Rooms free of insects and rodents _____			
C. Back room neat and clean, doors closed _____			
11. VEHICLES			
A. Deliveries made in clean properly constructed vehicles-refrigerated for perishable foods _____			

over sinks or above food preparation areas. (b) Lights over foods and food handling areas must be shielded. (c) Pesticides and medicinals should be stored away from food areas. (d) Returned and rejected perishable foods such as meats should be stored in closed plastic containers away from fresh food. (e) Iced poultry should be stored in a separate well-drained trough. (f) Raw meat and raw fish should be stored separate from cooked meats and salads in the delicatessen display case.

1. *F. Utensils and equipment corrosive resistant.* (a) Plastic and stainless steel are preferred over aluminum for food platters, lugs, etc. (b) Plastic cutting blocks and plastic knife handles can be cleaned and sanitized more effectively than a wood surface.

2. *C. Attached fans and fixtures.* Meat track switch handles, door latches, vents, and air-conditioning fans must be regularly cleaned.

3. *A. Movement.* Often food is brought into the preparation rooms from coolers and is cut, blended, wrapped, and left in trays or storage racks for long periods before being placed in the refrigerated display counters. The processing room is not cold enough to prevent bacterial growth.

3. *B. Protection.* (a) Food must be protected from contamination at all times. It must be properly covered, wrapped, and stored. (b) Beef can not be cut on equipment used for raw poultry unless equipment is properly cleaned and sanitized between use.

3. *C. Rotation.* All perishable food should be dated. This date should be the final date when such food is offered for sale. This shelf-life date must be realistic. To do this, flavor and bacterial tests should be available both for date of packaging as well as for the expiration date of such food products. Too often the expiration date is picked with commercial consideration, rather than with quality record. Proper rotation of perishable food must be maintained for a dated food program to be effective.

4. *D. Cleaning utensils.* (a) Cloth towels or sponges should not be used in cleaning and drying food handling equipment. (b) Cleaning brushes should be clean and properly stored.

5. *B. Equipment storage.* Equipment should not be stored nestled unless completely drained and dry.

5. *C. Single service items.* These should be stored in a closed container or else stored inverted.

7. *A. Water supply.* When private water supplies are used in the market, yearly water purity tests must be taken.

8. *C. Personnel.* Eating, smoking, and clothing storage must be separate from food processing departments.

9. *Toilet.* A food handler must have toilet facilities that are clean and meet proper sanitary requirements. It can not be stressed enough that this area must be in complete compliance. It is unfortunate that this is the most frequently violated major item on the score sheet.

10. *C. The back room.* This section in a market must be neat and clean with food products stored properly off the floor. Poor housekeeping is found in many back rooms. These rooms must be considered as an important area where proper food handling methods must be maintained.

11. *Vehicles.* Stores must insist that their food deliveries arrive in proper vehicles.

TABLE 2. *End of operation inspection*

While it is important to make an inspection during the food processing, it is equally important to make an inspection when the food handling equipment is expected to be properly cleaned and stored.

All food handling equipment and food contact surfaces must be inspected: Tables, grinders, knives, saws, etc. are examined and any equipment found dirty are listed under department at fault.

The addition of bacteria from dirty food handling equipment affects adversely both the keeping quality and the flavor of the prepared foods.

DIRTY FOOD EQUIPMENT

Meats _____

 Produce _____

 Deli _____

 Comment _____

TEMPERATURE CONTROL

Once a perishable food product has been produced under proper sanitary condition, it is important to maintain quality and prevent spoilage of these foods. To do this, proper temperature control must be observed. Coolers and display cases for fresh meat, dairy, and delicatessen products must be kept at temperatures between 35 and 40 F.

Food preparation areas, such as for meats, should be kept at below 50 F. But in all perishable food areas, food must move rapidly from cooler to display case. Frozen foods must be maintained at 0 F or below.

Temperature control must also take into account temperature fluctuations. Even if no health or bacterial problem is caused by such temperature variations, serious flavor problems may develop in foods. Temperature fluctuation can cause sandiness in ice cream, can cause development of rancidity in milk, and can bring about excessive bleeding in meats.

Every store manager should know

maximum temperatures changes in each food section. This includes time and temperature checks on the defrost cycle. To do this, it is necessary to have the tools of the trade; an accurate, easy to read thermometer in each refrigerated cooler and display case, plus a check thermometer in each store.

It is important to know where to take correct product temperatures. A thermometer with its bulb placed in an air duct does not represent the temperature of food in the refrigerated case. Because of the many ambients, such as the temperature of the store, air currents, position of display lights, etc., a correct product temperature should be taken between two food packages in the center of the display cases.

Perishable foods must not be displayed above the load limit line in the case and should not be stored so as to block circulation of cold air in the case.

In the delicatessen, it is important to keep hot products, such as barbecue chickens, in a closed display case with a temperature maintained above 140 F.

Finally, proper temperature controls will increase the shelf-life of perishable foods. At 40 F milk should keep for 10 days. At 45 F, this same milk will keep for only 5 days. Temperatures in different locations of the store should be recorded on a form such as in Table 3.

TABLE 3. *Temperatures in the store*

1. Store _____
 2. Meat cooler _____
 3. Produce cooler _____
 4. Deli refrigeration _____
 5. Dairy cooler _____
 6. Produce displays _____
 7. Meat cases _____
 8. Dairy cases _____
 9. Cold cuts _____
 10. Deli, cold _____
 11. Deli, hot _____
 12. Bakery cases _____
 13. Preparation areas _____
 14. Frozen food cooler _____
 15. Frozen food cases _____
 A. Ice cream _____
 B. Juices _____
 C. Vegetables _____
 D. Prepared foods _____
 E. Fish _____
 F. Poultry _____
 G. Pastries _____
 H. Misc. _____

STORE APPEARANCE

Certainly markets must continue to be efficient, bright, and attractive. A quality control program must include what the customer sees; the store's shine. While this is often the area where emphasis in cleaning is placed, it is important to check each store to see that management knows what appeals to the customer. This again can be done best with a inspection form (Table 4).

TABLE 4. *Store appearance*

1. Parking lot _____
 2. Sidewalk and entrance _____
 3. Floors _____
 4. Walls and ceiling _____
 5. Lighting _____
 6. Shelves and racks _____
 7. Personnel appearance _____
 8. Preparation areas _____
 9. Check-out counters _____
 10. Aisles _____
 11. Display cases _____
 12. Merchandise _____
 A. Cleanliness _____
 B. Condition _____
 C. Neatness _____
 13. Surroundings _____
 A. Trash area _____
 B. Delivery entrance _____
 C. Drainage _____

The rating must begin where the customer enters the store's property—at the parking lot. Include the condition and cleanliness of the sidewalk and the store entrance, cover employee appearance, and place special notice on areas that patrons stop for longer periods such as food preparation departments and check-out counters.

The finish on floors, walls, ceilings, shelves, and display cases should be bright, smooth, and clean. Aisles should be free of clutter. Merchandise should be stored neatly, in good condition, and free of dust and dirt. Surroundings of the store must be properly drained and kept picked up.

It is the store's appearance that the customer compares with that of other competing markets. The psychological feeling of cleanliness and the market's arrangement can readily relate to the consumer and equal repeat sales. It can prove valuable for an inspector to compare and rank a market with its nearby competitor.

LABORATORY CONTROL

With attractive surroundings, efficient services, proper temperature controls, and food handling equipment that looks, smells, feels, and is clean, the market is well on its way to a high level of customer acceptance and customer satisfaction. However, there is another field of quality testing that the market should consider—laboratory control.

A market can never rely totally on bacterial counts for its quality control program. Many perishable foods have no bacterial standards. Certain foods that are frozen, have added inhibitors, or pH values away from neutral can result in lower bacterial counts after a period of storage than they were on date of preparation.

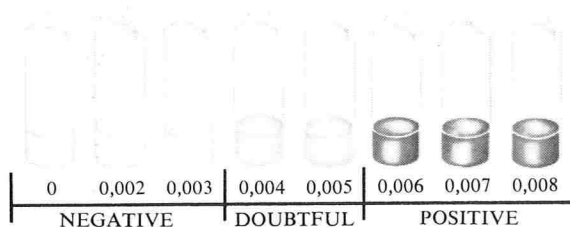
There are two classes of bacterial counts. (a) Quantative standards or guidelines such as results of the Standard Plate Count. These are bacterial standards or guidelines that authorities have set at values that they feel the particular food can meet with proper attention given to cleaning, sanitizing, housekeeping methods, and temperature control. (b) Quality standards or guidelines such as counts of *Escherichia coli*. These are bacteria that authorities feel either should not be present in the product tested or should be present in very small numbers. These bacteria should be destroyed by proper processing methods and adequate sanitation should keep them out of processed foods. Presence of these bacteria indicates post-processing contamination and a possible health threat.

Swabs taken of "cleaned" food handling equipment can be tested by aerobic plate count method as well as by the coliform method to check the efficiency of cleaning, rinsing, and sanitizing procedures.

Of raw meat products hamburger can be tested by the aerobic plate count to check on proper quality control in the meat department. Foods such as milk and custards that are ready to be consumed can be checked with both a coliform test and the aerobic plate count.

These tests are inexpensive and together give a good indication of the quality of the foods tested. With this limited but valuable testing program, a meaningful start can be made to improving and protecting foods being offered to customers.

Simple!

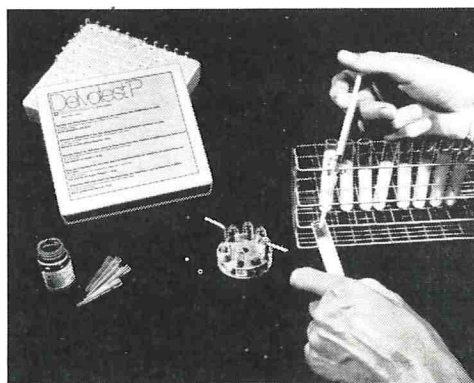


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It's sensitive. Penicillin concentrations of 0.003 I.U. or less per ml. of milk always yield a negative test result (entirely yellow), while levels of 0.006 I.U. or higher give a positive result (entirely purple). In-between concentrations give yellow-purple and purple colorations.

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Calendar of Events

January 28, 1977. EDUCATIONAL SEMINAR. Sheraton-Oak Cliff Hotel, Dallas, Texas. Sponsored by the North Texas Association of Milk, Food and Environmental Sanitarians. Open to the public. Fee: \$3.00. For further information contact Brenda Dawson, NTAMFES Secretary-Treasurer, (214) 226-1544.

February 7-9, 1977. STATISTICAL QUALITY CONTROL SHORT COURSES FOR THE FOOD PROCESSING INDUSTRY. Methods and techniques, applications and decision making. Mini Center, University of California, Davis. Registration fee: \$115. For further information contact: Robert C. Pearl, Food Science & Technology Department, University of California, Davis, CA 95616. (916) 752-0980 or 752-6021.

February 9-10, 1977. DAIRY INDUSTRY CONFERENCE. Center for Tomorrow, Ohio State University, Columbus, Ohio. For information contact: John Lindamood, Department of Food Science and Nutrition, 2121 Fyffe Road, Ohio State University, Columbus, OH 43210.

February 13-16, 1977. INTERNATIONAL EXPOSITION FOR FOOD PROCESSORS. Superdome, New Orleans, Louisiana. Sponsored by the Food Processing Machinery and Supplies Association, 7758 Wisconsin Ave., Washington, D.C. 20014.

February 15-16, 1977. DAIRY MARKETING FORUM. Sponsored by the University of Illinois. Ramada Inn Convention Center, Champaign, IL. Fee: \$20.

March 3-5, 1977. BAKING INDUSTRY SANITATION STANDARDS COMMITTEE MEETING. Hyatt Regency Chicago Hotel, Chicago, IL.

March 16, 1977. NRA ALLIED MEMBER SEMINAR. O'Hare Marriott Hotel, Chicago, IL.

March 21-25, 1977. MIDWEST WORKSHOP IN MILK AND FOOD SANITATION. Center for Tomorrow, Ohio State University,

News and Events

Columbus, Ohio. For information contact: John Lindamood, Department of Food Science and Nutrition, 2121 Fyffe Road, Ohio State University, Columbus, OH 43210.

March 28-April 1, 1977. SHORT COURSE ON MAXIMIZING CONSUMER SATISFACTION BY COMPUTERIZED FOOD MANAGEMENT AND DIET CONTROL. For food service and dietary executives. For information contact: Dr. Joseph L. Balintfy, College of Business and Management, University of Maryland, College Park, MD 20742.

March 29-31, 1977. WESTERN FOOD INDUSTRY CONFERENCE. Freeborn Hall, University of California at Davis. Registration fee: \$18.00. For information contact: Robert C. Pearl, Food Science & Technology Department, University of California, Davis, CA 95616 (916) 752-0980.

April 5-7, 1977. NATIONAL CONTROLLED ATMOSPHERE RESEARCH CONFERENCE. Kellogg Center for Continuing Education, Michigan State University, East Lansing, Michigan. For further information contact: D. H. Dewey, Department of Horticulture, Michigan State University, East Lansing, MI 48824, or W. G. Chace, U.S. Department of Agriculture, ARS, Beltsville, MD 20705.

April 20-22, 1977. DAIRY AND FOOD INDUSTRIES SUPPLY ASSOCIATION 58TH ANNUAL MEETING. Beach Club Hotel, Naples, Florida. For information contact: DFISA, 5530 Wisconsin Ave., Washington, D.C. 20015 (301) 652-4420.

May 10-12, 1977. SECOND INTERNATIONAL POWDER & BULK SOLIDS HANDLING & PROCESSING SHOW. O'Hare International Trade & Exposition Center and the Regency O'Hare, Rosemont, Illinois. For information contact: Aaron Kozlov, Industrial & Scientific Conference Management, Inc., 222 West Adams St., Chicago, IL 60606 (312) 263-4866.

May 21-25, 1977. NRA RESTAURANT HOTEL - MOTEL SHOW. McCormick Place, Chicago, IL.

June 5-8, 1977. 37TH ANNUAL MEETING AND FOOD EXPO, INSTITUTE OF FOOD TECHNOLOGISTS. Philadelphia Civic Center, Philadelphia, Pennsylvania. More information is available from Dan Weber, Director of Convention Services, Institute of Food Technologists, 221 N. LaSalle St., Chicago, IL 60601.

June 9-11, 1977. INTERNATIONAL SYMPOSIUM ON SALMONELLA IN POULTRY AND PROSPECTS FOR CONTROL. University of Guelph, Guelph, Ontario, Canada. For further information contact: Office of Continuing Education, Johnston Hall, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

June 13-15, 1977. ANNUAL CONFERENCE, NATIONAL ASSOCIATION OF COLLEGES AND TEACHERS OF AGRICULTURE. The Pennsylvania State University, University Park, Pennsylvania. For further information contact: Dr. Robert E. Swope, 217 Agricultural Administration Building, University Park, PA 16802.

October 5-7, 1977. SOUTHEASTERN NATIONAL FOOD-SERVICE SHOW. Atlanta Marriott Hotel, Atlanta, GA.

Indian Dairy Science Journal

The Indian Dairy Science Association has announced the publication of a new quarterly, the *Indian Journal of Dairy Science*. The journal's content will include original research articles, review articles and bibliographies pertaining to dairy science. Annual subscription rate is \$10.00 (U.S.), with issues appearing in March, June, September and December. Inquiries should be sent to Dr. V. K. N. Nambudripad, National Dairy Research Institute, Karnal 132001, Haryana, INDIA.

New Paperboard Trays Developed by International Paper for Foods Cooked in Conventional or Microwave Ovens

International Paper Company has introduced a unique line of paperboard tray products which now makes it possible for food processors to use one economical "convenience" package for foods that are recooked in a conventional or a microwave oven.

The new Pressware™ dual oven trays enable processors to package and store their foods in the same tray that the consumer takes home, pops into the oven, and then even uses to serve the heated product. And it doesn't matter whether the food is cooked in a conventional or a microwave oven; the tray is designed for either form of cooking.

The specially-treated Pressware

tray products can be used for a wide range of foods, including entrees, casseroles, pizzas, pies, and other bakery items. In fact, several food companies already are testing the products for cakes, vegetable casseroles, and pies.

Pressware tray products are available in both circular and rectangular shapes, and have an aesthetically appealing design which makes them more attractive when used in serving situations.

The unique trays also reduce cooking time which saves energy for consumers. And, when the meal is completed, the trays are readily disposable.

Tjepkema Named by Beatrice Foods



Dr. Roy Tjepkema

Dr. Roy Tjepkema has been named director and general manager of the Beatrice Foods Co. Research Center and Quality Control Department, it has been announced by Wallace N. Rasmussen, Beatrice Foods president and chief executive officer.

Dr. Tjepkema, who has served as director of research and product development for Peter Eckrich &

Sons, Inc., Division of Beatrice Foods in Ft. Wayne, Ind., for the past few years, succeeds Carl F. Obenauf, named manager of Beatrice Foods Co. plant in Beloit, Wis.

Prior to joining Eckrich in 1968 as a product development administrator, Dr. Tjepkema was involved in nutritional product development in Ohio.

He received his bachelor's degree in dairy and food industries at the University of Wisconsin and, after also earning his master's degree there, completed work on his doctorate in food science and industries at Wisconsin in 1967.

IFT 1977 Annual Meeting in Philadelphia

The 1977 annual meeting of the Institute of Food Technologists and its associated "FOOD EXPO" will be held in Philadelphia, June 5 through 8, 1977.

The technical sessions and the exposition will be held in the Philadelphia Civic Center.

More than 9000 members and friends are expected to attend, to inspect the new offerings of some 375

exhibitors and to hear more than 400 technical papers and symposia. More information is available, from Dan Weber, Director of Convention Services, Institute of Food Technologists, 221 N. LaSalle St., Chicago, IL 60601.

The IFT is a 15,000-member professional scientific society, representing all disciplines in food science and technology.

Western Food Industry Conference

The 6th Annual Western Food Industry Conference will be held during March 29-31, 1977 at the University of California, Davis campus. The Northern and Southern California Institute of Food Technologists sections, California Dairy Industries Association, American Oil Chemists Society, and University of California at Davis are co-sponsoring this Conference. The Conference is designed to update and to further the technical and scientific expertise of food processors. The Conference sessions will cover topics related to: "Directions in Public Policies," "Advances in Fats and Oils," "Marketing New Products," "Dairy Processing and Products," "Water and Energy Conservation," "Nutrition and Food Safety," "Food Processing and Packaging Developments," "Factors Influencing Food Stability," and "Technical Management Philosophies." For a program and registration form, contact Mr. Robert C. Pearl, Extension Food Technologist, Food Science & Technology Dept., University of California, Davis, CA 95616; telephone number (916) 752-0980. The registration fee is \$18.00 per person.

Annual Meetings of American Meat Institute

Dates and locations of the next four American Meat Institute annual meetings have been set. They are: November 5-8, 1977, McCormick Place/Conrad Hilton, Chicago

November 11-14, 1978, McCormick Place/Palmer House, Chicago

November 3-6, 1979, McCormick Place/Conrad Hilton, Chicago

November 8-11, 1980, McCormick Place/Conrad Hilton, Chicago

For additional information, contact Charles Yuska, American Meat Institute, P.O. Box 3556, Washington, D.C. 20007, telephone (703) 841-1030.

Virtis Develops New Lyophilizer



The Virtis Company, Inc., Gardiner, N.Y. has developed a new 8-liter, mid-size lyophilizer for use in research and the routine production of control sera.

The new Unitrap II freeze-dryer offers increased capacity and many conveniences. A visible condenser permits direct observation of ice build-up throughout processing.

The caster-mounted 29-inch steel cabinet houses a Copelmatic compressor and a spring suspended, shock-mounted, Hyvac 7 vacuum pump or a direct drive vacuum pump. Hyvac 7 is designed to pull 5-millitorr with gas ballast fully open. Thermocouple vacuum gauge and electric condenser readouts are standard.

For further information on the Unitrap II or any other freeze-dryer, write the Virtis Co., Inc., Route 208, Gardiner, N.Y. 12525, U.S.A.

Association Affairs

California Affiliate Holds Joint Meeting



Mr. Dick Ayers, (left) President-elect of the California Association of Dairy and Milk Sanitarians, presents a plaque to the retiring President, Harold Heiskell. Mr. George M. Mardikin, banquet speaker, looks on (far right).

The 58th Annual Meeting of the California Association of Dairy and Milk Sanitarians was held jointly with the California Dairy Industry Association, California Fieldmen's Advisory and Bureau of Milk and Dairy Foods Control, California Department of Food and Agriculture on October 18, 19 and 20 at Burlingame, California.

This was the first time a joint effort had been held in California. The object of the joint session was to bring these organizations together and to continue the possibility of joint sessions in the future. The meeting was a success and the organizations agreed to continue to hold their annual session together.

The program was divided into three areas: The Dairy and Milk Sanitarians Association planned the first day's meeting, the second day was planned by the California Dairy Industry Association and Milk Plant Fieldmen, while the third day was conducted as a refresher course by the Bureau of Milk and Food Control of the California Department of Agriculture.

The annual banquet was held Tuesday evening with an outstanding program presented by Mr. Mardikian, an authority on world food conditions. He has been an adviser to five Presidents on world food conditions and feeding of the United States Army.

Affiliate Meetings

- CONNECTICUT — January, 1977. Hartford area.
- FLORIDA — March 15-17, 1977. Kayhler Plaza, Orlando.
- KENTUCKY — February 22-23, 1977. Stouffer's inn, Louisville.
- MINNESOTA—April 19 and April 21, 1977. Outstate educational meetings at Alexandria and Albert Lea.
- ONTARIO—March, 1977. Holiday Inn, Highway #427, Etobioke.
- PENNSYLVANIA — June 13-15, 1977. State College.
- VIRGINIA — March 8-9, 1977. Donaldson Brown Center, VPI & SU, Blacksburg.

Association Affairs

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For Sale

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

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Future IAMFES Annual Meetings

August 14-18, 1977 ----- Sioux City, Iowa
 August 6-10, 1978 ----- Kansas City, Missouri
 August 12-16, 1979 ----- Orlando, Florida

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ELIASON CORPORATION is an independent manufacturer of Sanitary, Rustproofed and Fireproofed STEEL modular WALK-IN COOLERS, FREEZERS and ACCESSORIES for SANITARY off-floor, "FOOD STORAGE and SERVICING of perishables" through SAFE access easy latching and patented Easy Swing DOORS. ELIASON ECONO-COVERS are famous around the World and "patented to conserve energy as NIGHT COVERS on Self Service OPEN FRONT and OPEN TOP" Refrigerator and Freezer FOOD DISPLAY CASES, nationally and internationally!

ELIASON dependability and performance in SANITARY FOOD PROTECTION SAFETY is an acknowledged ELIASON asset amongst even anticompetitive association member foundation group's coercive PUBLIC RELATIONS sales seals and low minimum none performance certifications "that limit performance, upgradings, better ideas and ingenuity". Many so called "voluntary" industrial standards, for ALL to COMPLY with instead of DEFY in favor of "Competitive Benefits" were a basis for the SENATE's Subcommittee on Antitrust and Monopoly PUBLIC HEARINGS March 1975 Plans "to make Lawful Competition, Legal again", in this REPUBLIC!

ELIASON, at a National Sanitarian annual seminar in Nashville, last June, released FREE to USERS or SANITARIANS it's authored, published, copyrighted "MANUFACTURERS warranty/guarantee, of Sanitary, Safety and Performance SPECS Plan": to provide FULLY VOLUNTARY, but signed commitments that their own FOOD, HEALTH and SAFETY related, EQUIPMENT, PRODUCTS and SERVICES will fulfill the LAWS and valid codes and regulations! This uncomplicated program is expected to obsolete or outlaw minimum labeled "irregulatory enforcements", in every imaginable field of commerce. And, as a "freebee" costing nil for usage puts Buyers and Professionally Accountable Sanitarians "in lawful driver's" SEATS!

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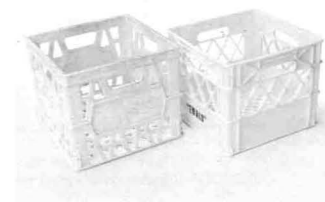
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Remember, every sanitation program involves choices. There is the choice between the cost of energy and the cost of chemicals. And between labor and new cleaning procedures. The point is, you should have the opportunity to

make those choices. Klenzade is ready to help with some of the most knowledgeable personnel and specialized laboratories in the detergent industry...

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—Samples of your water are analyzed by sophisticated atomic absorption tests to identify minerals and other substances present. **Organic Biochemical**

Research Lab—Here's where Klenzade defoamers, wetting agents and water conditioners were developed. Where new molecules were actually synthesized. **Product Engineering Electrical**

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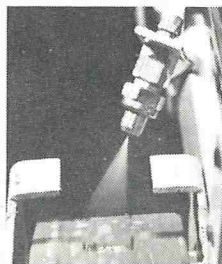
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—Among our staff is an associate referee of A.O.A.C. (Assn. of Official Analytical Chemists) which establishes standards for measuring the effectiveness of sanitizers/disinfectants against viruses.

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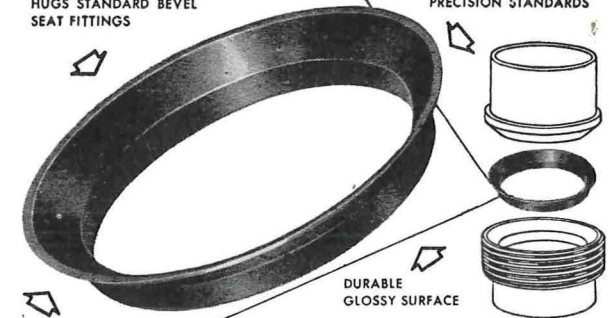
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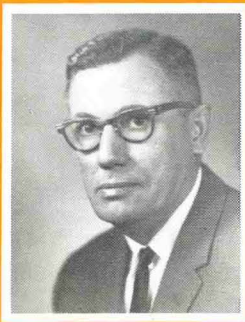
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Ralph Bonewitz, Extension Specialist
Department of Dairy Science
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What questions should a dairyman ask before expanding or modernizing?

Here are some of the most basic questions I believe dairymen should ask themselves before they begin a program of modernization or expansion:

What about my future in dairying? Will the changes I make be profitable in the long run? Will the changes be feasible in the short run?

Long run profitability refers to a period of eight to ten years or more. It is usually studied through the use of budgets. In the budgeting process, capital expenditures are prorated over the life of the assets by arbitrary depreciation methods.

Short run feasibility refers to the income-generating ability of a business in a short period of time. It is usually studied through the use of a projected cash flow.

Watch your cash flow

Projected cash inflow and outflow during the period are compared, reflecting payment requirements from credit agencies as well as all normal expenditures. Some business changes are capable of being profitable in the long run *but they are not capable of meeting short run demands for cash, particularly when payment requirements for capital expenditures are concentrated in the early years of an investment.*

Management must expand as herds get larger. More failures are due to lack of management than any other one thing. Hours spent thinking and getting facts will pay off and the manager or owner must assume this responsibility. A manager must have the ability to get the right things done at the right time.

Here are some more questions:

Will my expansion or modernization plan improve the chances and ease of producing a higher quality

product? Will it increase the ease of the key jobs associated with dairying? Will it increase the efficiency of labor, investment and/or equipment? Will the modernized set-up provide the minimum movement of men, animals and material? Can this modern dairy farm compete with nonfarm occupations for good labor or manpower?

Do you as a manager or owner have a concern for production, people, quality milk and a desire to make the dairy more profitable? Will you develop a good milking program? One that obtains high production per cow, controls mastitis, produces clean excellent-flavored milk and uses a minimum amount of labor?

Check this planning list

Tomorrow's profitable dairy farm can be expected to be a planned operation. Here are some of the things you will want to consider during the planning phase: 1) Size of herd to be accommodated; 2) Dairy breed; 3) Climate conditions; 4) Topography of farmstead, slope, drainage and exposure; 5) Size and productivity of the farm; 6) Feeding program as related to feed storage and equipment for feeding; 7) Existing buildings and equipment that will fit into long range plans; 8) Availability of labor; 9) Capital available for investment; 10) Sanitary regulations or codes or present and potential milk markets; 11) Personal preference of the owner; 12) Water and air pollution regulations.

Planning with pencil and paper prior to the start of construction permits you to analyze combinations of equipment and building arrangements without any expenditure. Visit as many farms as possible and incorporate the good points seen at these farms into your facilities. Use university people and private consulting people.

Unless a dairyman regularly tests production, a pipeline milker can be the best friend that a cull cow ever had. The cull cow strolls in with the good producers, gives only enough milk to color the line, eats almost as much feed as the best cows, and then goes her merry way. A dairyman needs profitable production from every cow. You will call the correct signals and build a profit-winning dairy team if you use production records.

Feeding practices important

Constant attention to feeding practices, breeding for production and herd health during expansion are very important. A well designed, properly installed milking system is essential to proper milking. You can't afford an inadequate or poorly maintained milking system.

Productivity must be the key

The productivity of a resource depends on the amount and kind of other resources with which it is combined. As an example, when capital is substituted for labor, it requires fewer hours of labor per cow. However, unless production increases or more cows can be handled, the machinery and equipment cost will offset the savings in labor. Productivity of a resource then depends on the kind and quality of the resource with which it is combined. Modern technology is closely related then to the substitution of capital for labor and land. It increases the demands on management, emphasizes financial management and increases technical requirements of hired labor.

Finally, all of this modernization should help make dairying more pleasant, as well as more productive for you and your family.

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