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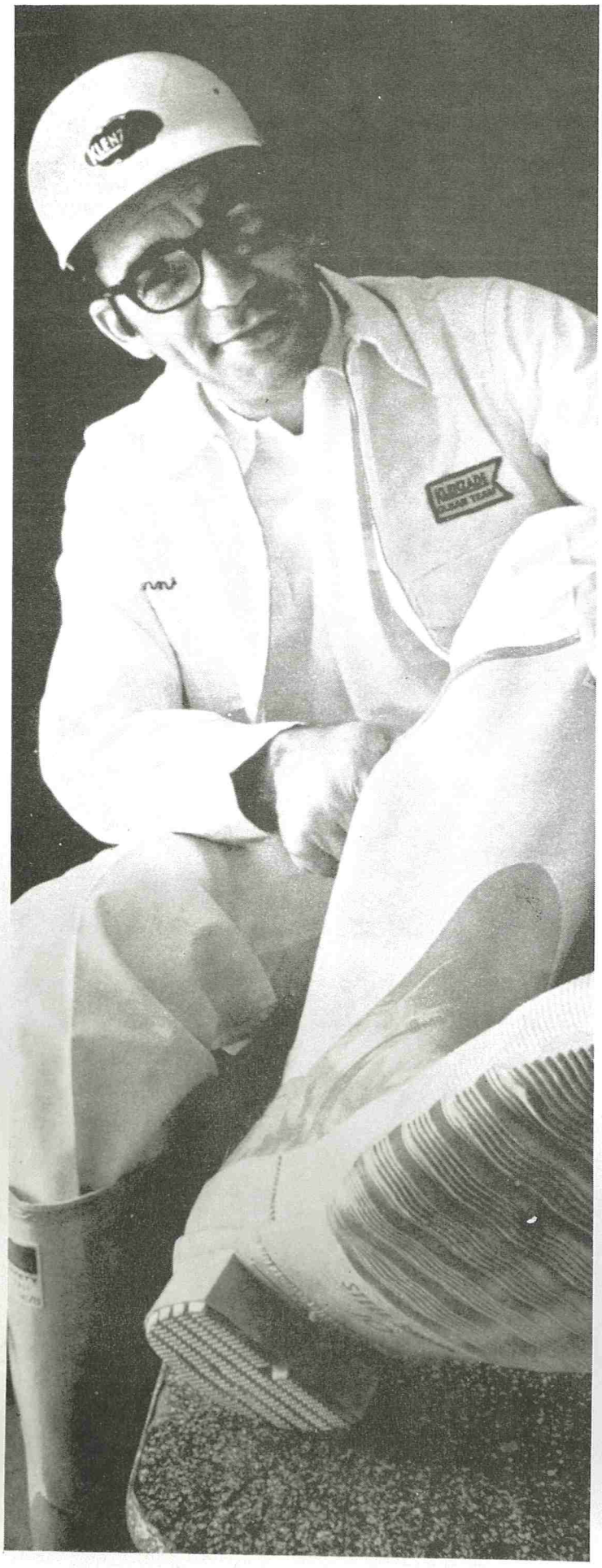

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Histamine and Tyramine Content of Meat Products

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(Received for publication September 19, 1974)

ABSTRACT

A survey was conducted to determine the histamine and tyramine contents of a variety of meat products. Histamine was found in all products at concentrations only slightly greater than one would expect from normal physiological amounts found in muscle. Semi-dry sausage products contained an average histamine concentration of 3.59 μg/g compared to an average of 2.87 μg/g in dry sausages. Country-cured hams averaged 1.69 μg histamine/g. Emulsion type products contained slightly less histamine than the fermented sausages. Braunschweiger, an exception, contained 3.6 μg histamine/g but would be expected to contain more histamine than other emulsion-type products because of its liver content. Data indicate that histamine is not formed to an appreciable extent in these meat products under normal processing conditions.

Detectable amounts of tyramine were found in 71% of the sausages and in 39% of semi-dry sausages. Tyramine was not detected in country-cured ham. Average tyramine concentrations were 244 and 85.8 μg/g in the dry and semi-dry sausages, respectively. The greatest tyramine concentration found in this study was 1237 μg/g in a Genoa salami. It is apparent that sufficient tyramine can occur in ripened sausages to be troublesome to tyramine-susceptible individuals.

Recent outbreaks of histamine food poisoning from consumption of commercially canned tuna fish (*1*) have again demonstrated the public health implications of biologically active amine formation in food. Build-up of amines such as histamine and tyramine with physiological activity is primarily due to microbial amino acid decarboxylation. Small amounts of histamine are normally found in a variety of food products including sauerkraut (*12*), wine (*13*), and cheese (*3, 7, 16, 17, 18, 20*). Although the toxicity of histamine to man is a controversial subject, ingestion of from 70-1000 mg histamine will usually cause clinical symptoms of intoxication (*9*). The small amounts normally found in lactic fermented or aged products are harmless to man, but problems arise when large numbers of histidine decarboxylating bacteria are present, which through improper handling or processing, are given the opportunity to produce appreciable amounts of histidine decarboxylase in the product. The probability of large amounts of histamine being formed in a food is greatly increased if much free histidine is present. This is particularly true for fish of the suborder *Scombroidei* (including tuna fish) that characteristically contain much free histidine (*11*).

Tyramine formation in foods has been extensively studied because of its relationship to illnesses noted in patients treated with monoamine oxidase (MAO) inhibitors which block the pathway for catabolism and inactivation of the amine after ingestion (*2*). Cheese has been reported to contain tyramine at concentrations up to 3.7 mg/g (*5*) and was the food initially associated with hypertensive disturbances noted in patients undergoing treatment with monoamine oxidase inhibitors (*2, 3*). Quantitative studies have shown tyramine to be present at variable concentrations in cheese (*14, 20*), wine (*13*), salted fish (*14*), meat extracts (*14*), and yeast extracts (*4, 14*).

Although little quantitative information is available concerning histamine and tyramine content of meat products, fermented sausage products have been shown to have potential for histamine development. Henry (*9*) reported that dry sausages should contain only 1 to 2 μg histamine/g based upon physiological muscle histamine levels, but sausages contaminated by undesirable microorganisms may easily reach histamine concentrations of 100 μg/g or greater during the early stages of the fermentation. Recently Dierick et al. (*6*) reported that the concentration of histamine and tyramine increased at least tenfold in experimental dry sausages with the rate of tyramine increase being maximal during the first 3 days of ripening.

Since there appears to be little information available on biologically active amine content of most meat products, the following study was completed to provide data on the presence of histamine and tyramine in a variety of meat products. Particular emphasis has been placed on obtaining data on dry and semi-dry sausages and on country-cured ham since amine build-up generally occurs in products undergoing lactic fermentation or long term aging.

MATERIALS AND METHODS

Source of meat products

All meat samples were purchased from retail stores except for a limited number of country-cured hams that were obtained directly from the processor. All samples were stored at −30 C until assayed. In most instances, histamine and tyramine analyses were done on adjacent portions of the same ham. Histamine and tyramine analyses of sausages were not always from the same sausage sample.

Tyramine extraction

Tyramine was extracted from sausage and ham samples using a modification of the method of Lovenburg and Engelman (*10*). Forty grams of the meat product were homogenized for 5 min with 80.0 ml of 0.1 N HCl in one pint Mason jars using a Sorvall Omni-Mixer homogenizer. The homogenate was then centrifuged at 4 C at 12,100 × *g* for 20 min. the fat layer was scraped off and the aqueous phase was transferred to a 125-ml Erlenmeyer flask and shaken for 10

min with 15 ml of n-heptane using a wrist action shaker. The mixture was transferred to a 50 ml centrifuge tube and centrifuged for 10 min at 12,000 × *g*. Twenty milliliters of the aqueous phase were adjusted to pH 10 with solid Na_2CO_3. The liquid was then saturated with NaCl (about 5 g), 15.0 ml of n-butanol were added, and the mixture was shaken for 10 min using a wrist action shaker. After centrifuging for 20 min at 12,100 × *g*, the butanol layer was decanted and used for amine quantitation.

Tyramine quantitation

Glass plates were coated with a 250-μ layer of silica gel (MN-Kieselgel G-HR 89, Brinkman Instruments, Westbury, New York). The layer was applied as a slurry consisting of 35 g of silica gel suspended in 70 ml of distilled water. Plates were dried for 2 h in an oven at 100 C and stored in a desiccator until used. Plates were reactivated at 100 C for 20 min immediately before use. The amine extracts (50 μl) were applied 2 cm from the base of plates with quantitative capillary pipets. The solvent system used consisted of $CH_3Cl:CH_3OH:NH_4OH$ (12:4:1).

After development, plates were air dried at 37 C for 24 h, sprayed with a 0.4% 7-chloro-4-nitrobenzofurazan (NBD-Cl) methanol solution, and held for 24 h at 25 C (*19*). The fluorescent amine spots were scraped from the plates and eluted from the silica gel with 3.0 ml of ethyl acetate. The silica gel-ethyl acetate mixture was agitated on a Genie Vortex mixer for four 30-sec intervals over a 10-min period and centrifuged for 15 min at 5000 × *g* to remove the suspended silica gel. Fluorescent measurements were determined on a Turner Model 430 Spectrofluorometer with an excitation wavelength of 464 nm and emission wavelength of 520 nm (*8*). These wavelengths gave the maximum sensitivity for the tyramine-NBD derivative. Amine contents were determined from the fluorescence of standards spotted on each plate, and the percent recovery (73 ± 9%) of the extraction method determined by extracting various samples of sausages containing known amounts of tyramine. Duplicate meat samples were extracted and quantitated.

Histamine analysis

Histamine was determined by the method of Shore (*15*). Five grams of the meat product were homogenized in 45 ml of 0.4 N perchloric acid using a Sorvall homogenizer. The homogenate was allowed to stand at room temperature for several minutes and then centrifuged. A 2-ml aliquot of the supernatant fluid was transferred to a centrifuge tube containing 5 ml of n-butanol, 0.25 ml 5 N NaOH, and 0.75 g NaCl. The tube was shaken for 5 min and centrifuged. To remove any free histidine, the butanol layer was transferred to a second tube containing 2.5 ml of NaCl-saturated 0.1 N NaOH, shaken for 1 min, and centrifuged. A 4-ml aliquot of the washed butanol extract was transferred to a third tube containing 2.5 ml of 0.1 N HCl and 7.5 ml of n-heptane. The tube was shaken for 1 min, centrifuged, and the organic phase removed by aspiration.

To 2 ml of the acid phase containing histamine was added 0.4 ml of 1 N NaOH followed by 0.1 ml of o-phthalaldehyde (OPT) (10 mg/ml in methanol). After 4 min at room temperature, 0.2 ml of 3 N HCl was added. The contents of the reaction tube were mixed using a Genie Vortex mixer following each addition.

The fluorescence at 450 nm resulting from activation at 360 nm was measured on a Turner Model 430 Spectrofluorometer. Histamine concentration was determined from a standard curve based on varyious concentrations of free histamine which were carried through the extraction procedure and OPT reaction. Reagent blanks were run to correct any native fluorescence.

RESULTS AND DISCUSSION

Table 1 shows the histamine contents of the meat products examined in this study. The highest histamine concentration was 7.81 μg/g found in a dry salami. The range in amount of histamine in all dry sausage samples was 0.74 – 7.81 μg/g. Semi-dry sausages ranged from 1.80 – 5.47 μg histamine/g and averaged slightly higher than the dry sausages (3.59 μg/g for semi-dry compared to 2.87 μg/g for the dry). Country-cured ham samples contained less histamine than the fermented sausage products. The greatest histamine concentration found in the country-cured hams was 2.66 μg/g and the average amount was 1.69μg/g.

TABLE 1. *Histamine Content of Various Meat Products*

Product		Histamine (μg/g) Range	Average
Fermented or aged products			
Dry sausage	(11)[a]	0.74-7.81	2.87
Semi-dry sausage	(11)	1.80-5.47	3.59
Country-cured ham	(27)	0.82-2.66	1.69
Fresh and cooked products			
Fresh ham	(3)	0.74-0.76	0.75
Cooked ham	(1)	0.55	0.55
Braunschweiger	(10)	1.52-4.69	3.60
Bologna	(10)	0.82-3.36	1.89
Weiners	(10)	0.66-3.63	1.75
Ground beef	(10	1.41-3.66	2.70

[a]Number of samples examined.

Three fresh ham samples and one fresh cooked ham were included in the study for comparison with the processed meat products. Histamine content of the fresh hams average 0.75 μg/g and the cooked ham contained 0.55 μg/g. These values agree well with published muscle histamine concentrations of 0.2–0.6 μg/g (*9*). Of the cooked, emulsion-type products, Braunschweigher contained higher histamine concentrations than did bologna or weiners. The average histamine concentration of Braunschweigher was 3.60 μg/g compared to an average of 1.89 μg/g and 1.75 μg/g in bologna and weiners, respectively. The Braunschweiger would be expected to have slightly more histamine than other emulsion products because liver has a higher physiological histamine concentration than muscle (1-30 μg/g) (*9*). Ground beef histamine concentrations ranged from 1.41–3.66 μg/g and averaged 2.7 μg/g.

The data just discussed indicate that histamine concentations in fermented or aged meat products and in cooked emulsion type products are only slightly greater than one would expect from amounts in normal physiological tissue. The amounts of histamine found in commercial meat samples are lower then values (approximately 60 μg/g dry matter) reported by Dierick et al. (*6*) in experimental dry sausages after a 36-day ripening period. Since ingestion of relatively large amounts (70 – 1000 mg) of histamine is required to produce moderate intoxication symptoms (*9*), the histamine present in products surveyed in this study hears no significance from a toxicity viewpoint. Furthermore, the absence of large amounts of histamine in any of the products examined seems to indicate that extensive histamine formation in red meat products would occur only through gross product mishandling.

The tyramine contents of dry and semi-dry sausages and country-cured hams are given in Table 2. Tyramine was found in 59.4% (22 tyramine positive of 37 sausages

TABLE 2. *Tyramine Contents of Various Sausage Products and Country-Cured Hams*

Product		Tyramine (μg/g) Range	Average
Country cured ham	(18)[a]	ND[b]	
Salami	(7)	ND- 56	8 (1)[c]
Thuringer cervelat	(2)	ND-162	
Lebanon bologna	(4)	ND-333	224.5(3)
Dry sausage			
Hard salami	(11	ND-392	210 (9)
Pepperoni	(5)	ND-195	39 (1)
Summer sausage	(1)	184	184
Farmer salami	(1)	314	314
Genoa salami	(5)	ND-1237	534 (4)
Smoked landjaeger	(1)	396	396

[a]Number of samples examined.
[b]ND = Not detectable at levels below 10 μg/g.
[c]Number of positive samples.

examined) of the dry and semi-dry sausages. Tyramine was not detected in any of the country-cured hams. Detectable amounts of tyramine were found in 70.9% of the dry sausages and in 38.5% of the semi-dry sausages. The average tyramine concentration of the dry sausages was 244 μg/g and 85.8 μg/g in the semi-dry sausages. The highest tyramine concentration (1237 μg/g) was present in a Genoa salami. There are several factors that could contribute to the wide range in amountof tyramine found in fermented sausages. The most important factors are the numbers of organisms present that can decarboxylate tyrosine, and the availability of free tyrosine. Dierick et al. (*6*) showed that there is generally an increase in free amino acids during sausage ripening. Variations in sausage processing may also account for differences in tyramine levels. Those processes that provide optimum conditions and sufficient time for growth of tyramine-forming organisms will cause production of more tyramine than when sausages are, for example, cooked immediately after the desired pH is attained.

The greater tyramine contents noted in dry sausages compared to semi-dry sausages could, except in those instances where the amount of tyramine was unusually high, result from differences in moisture content. Longer ripening times for dry sausage may also contribute to greater amounts of tyramine. The tyramine values reported here for ripened sausage products correspond closely to amounts of tyramine reported for other foods. For example, Voigt et al. (*20*) found average tyramine contents of 0.27 mg/g, 0.21 mg/g and 0.24 mg/g in extra-sharp, sharp, and medium Cheddar cheeses, respectively. Sen (*14*) reported tyramine concentrations from 5 μg/g to 2,170 μg/g in variety of cheeses. Sen (*14*) also found tyramine in amounts from 95 μg/g to 304 μg/g in meat extracts but only negligible amounts in various alcoholic beverages.

Judging from results of this survey, histamine is not formed extensively in processed meat products and potentially dangerous amounts are unlikely to occur. When one considers that Blackwell and Mabbitt (*3*) reported 0.5 mg tyramine/g in cheese to be a dangerous amount and 25 mg total tyramine intake to be a dangerous dose for patients receiving MAO inhibitors, dry sausages containing the higher tyramine concentrations would provide sufficient tyramine in moderate servings to produce pressor responses in tyramine susceptible individuals.

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Growth of *Salmonella heidelberg* at Room Temperature in Irradiated and Nonirradiated Potato Chip Dip

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ABSTRACT

Irradiated and nonirradiated cottage cheese-based potato chip dips were inoculated with low (10^1 cells/ml) and high numbers (10^6 cells/ml) of *Salmonella heidelberg*. At room temperature, both samples supported growth of *Salmonella*, but highest counts were found in the nonirradiated samples. *Salmonella* inoculated at low levels reached 10^6 cells/ml after 18 h but little change occurred with the high inoculum after 2.5 h. Growth of *Salmonella* was detected by both the conventional plate count and by a modified radiometric technique. Radiometric detection times were 2.5 and 18 h for samples inoculated with high and low numbers of *Salmonella* respectively, which corresponded to 10^6 cells/ml at the time of detection.

Salmonellae normally grow most rapidly at 37 C, however, growth is possible at much lower temperatures (*13*). This low temperature growth is influenced by conditions of the medium such as pH and salt concentrations (*1, 4, 14*) with most rapid growth being obtained under optimum conditions.

Growth of salmonellae in different foods is responsible for many cases of food poisoning every year (*2*) with great economic loss to processors and consumers. The fate of salmonellae in cultured milk and cheese products in the event of consumer contamination and subsequent temperature abuse is presently not clear. Potato chip dips prepared with dairy products are often allowed to remain at room temperature for substantial periods. These products can be contaminated either when prepared in the home or during consumption when pieces of food, often handled by many individuals, are placed into the dip. Therefore, this study was undertaken to investigate the growth of *Salmonella heidelberg* at low inoculum levels in a homemade cottage cheese-based potato chip dip (pH 5.3) subjected to temperature abuse. To assess growth of *Salmonella* without interference from the indigenous flora, portions of the dip in this investigation were subjected to pasteurizing doses of gamma radiation. Growth of *Salmonella* was detected by plate counts and by a modified radiometric technique. The latter has recently been demonstrated by several investigators to be faster, comparable in accuracy, and more sensitive than the former (*6, 7, 8, 16, 18*).

MATERIALS AND METHODS

Test organisms

Salmonella heidelberg, ATCC 8326, was used in these studies because it is among the serotypes most frequently isolated from human sources (*3*). This organism has been among the serotypes demonstrated in outbreaks of human illness attributed to consumption of contaminated raw milk (*5, 10, 11*), and dried milk products from contaminated dried milk plants (*12*).

Inoculation

The inoculum was prepared by adding 18-h brain heart infusion (BHI, Difco) broth cultures of the organism to 0.1% peptone water to obtain 55% transmittency at 660 nm in a Bausch and Lomb spectrophotometer. Appropriate serial decimal dilutions for inoculation were prepared in BHI broth. Irradiated and nonirradiated chip dip samples were equilibrated at room temperature for 4 h. Samples were inoculated with 0.1 ml of the appropriate dilutions of *S. heidelberg* in BHI broth. Both irradiated and nonirradiated control samples were prepared in the same manner except 0.1 ml of sterile BHI broth was added.

Sample preparation

One 8 oz. can of commercially canned minced clams plus juice was added to 16 fluid oz. of commercially prepared fresh small curd creamed cottage cheese. The mixture was blended at top speed for 2 min in a Waring Blendor. The final pH was 5.3. No pH changes occurred in any of the inoculated samples after incubation. To simulate home preparation as closely as possible, aseptic technique was followed only after blending of the ingredients. Both irradiated and nonirradiated samples were stored at 4 C for 18 h before use to reduce possible free radicals formed during radiation. Samples were then allowed to equilibrate at room temperature for 4 h before incubation.

Irradiation

Nine-milliliter aliquots of the samples were measured, using a 10-ml sterile plastic pipet with the tip removed, into sterile 50-ml serum vials and capped with sterile aluminum foil. Samples were irradiated in the College of Fisheries with 300 Krad of gamma radiation in the Cobalt 60 Mark II food irradiator at a dose rate of 160 Krad/h and a temperature of 24 C. Samples were irradiated to reduce or destroy the normal bacterial flora resulting from ingredients and handling contamination during preparation.

Enumeration

Salmonella counts were obtained by the spread plate method on brilliant green (BG) and salmonella shigella (SS) agars (Difco) using 0.1-ml volumes of the appropriate dilutions of the chip dip. Total counts of bacteria able to grow at pH 5.0 were obtained by the same method using Difco's tomato juice agar, special (TJ). Incubation was at 37 C for 2 days.

Radiometric procedure

Samples were inoculated with 0.1 ml uniformly labeled ^{14}C-glucose (Amersham-Searle, 280 mCi/mmole), 0.1 μCi/sample, containing 0.06 μg glucose. The same quantity of unlabeled glucose was added to samples counted by the spread plate technique. Samples were mixed by swirling for 1 min and incubated at room temperature. Three replicates were used for each treatment for each sampling time. One replicate was used as a blank and 2.0 ml of 1.0 N H_2SO_4 was added before adding the isotope. Each bottle (after addition of labeled glucose) was immediately capped with a serum cap pierced with a small plastic rod and cup assembly (Kontes Glass Co., Vineland, N.J.,; K-882320). Samples were

tested by the method of Harrison et al. (9) with incubation times of 2.5 to 18 h. All samples were counted in a Packard Tri-carb Scintillation Counter. Data were corrected for blank activity. Blanks consistently gave values of 38-45 counts per minute (CPM). Quenching was corrected by testing similarly quenched ^{14}C-glucose and $^{14}CO_2$ Standards. Recovery of $^{14}CO_2$ from the dip was complete. When 0.3 μCi of $Na_2\ ^{14}CO_3$ was added to each of five aliquots of dip and acidified as described, all of the label was recovered.

RESULTS AND DISCUSSION

Salmonella heidelberg inoculated at low levels can grow in a homemade cottage cheese based-clam potato chip dip, The organisms inoculated at 2.8×10^1 cells/ml were able to grow in the chip dip at room temperature during the 18-h test period (Table 1). Growth was obtained in both irradiated and nonirradiated samples with the highest level obtained in the nonirradiated samples. Counts increased from the inoculum level of 2.8×10^1 cells/ml on the irradiated samples to 2.2×10^4 and 2.7×10^4 cells/ml after 18 h as detected on brilliant green and salmonella shigella agars, respectively. During the same incubation period counts as measured with tomato juice agar increased to 6×10^4 cells/ml. These data indicate that better recovery of *Salmonella* was obtained on TJ agar with a pH of 5.0 than on the two selective agars used for *Salmonella.* No growth was obtained on the three media with the irradiated control showing the effectiveness of the irradiation. In addition, organisms growing on the three media from the irradiated inoculated samples were randomly selected and tested on triple sugar iron agar and shown to react as salmonellae.

Counts in the nonirradiated inoculated samples increased from the inoculum level of 2.8×10^1 to 5×10^5, 4×10^6 and 1.2×10^8 cells/ml on BG, SS, and TJ agars, respectively.

No growth was obtained when nonirradiated control samples were plated on BG and SS agars due to the inhibitory nature of these media. However, counts increased from 1×10^3 to 9.6×10^7 during 18 h of incubation when tested on TJ agar. The normal flora of a cottage cheese based chip dip would be composed predominately of lactic acid bacteria which were able to grow on the TJ agar at pH 5.0 and the organisms from the nonirradiated incoulated samples enumerated on TJ agar were a reflection of both and normal flora and the *Salmonella* which will grow at pH 5.0 (14).

Although samples were held at 4 C for 18 h and at room temperature for 4 h in an effort to reduce or eliminate inhibitory effects due to radiation, such as free radical formation, slightly lower counts were obtained on the irradiated than on the nonirradiated samples. Although this slight inhibition was evident, the irradiation was effective in reducing the competition from the normal bacterial flora on the samples.

With high levels of *S. heidelberg* inoculum (2.8×10^6 cells/ml), the changes in numbers of cells during 2.5 h of incubation were less than when low inoculum levels were used (Table 2). Results with both irradiated and nonirradiated controls were the same as already reported. Counts from an inoculum level of 2.8×10^6 cells/ml in irradiated chip dip decreased to 2.9×10^5 and 7.0×10^5/ml as measured on BG and SS agars respectively. The TJ agar counts increased from 2.8×10^6 to 4.0×10^6 cells/ml. Counts obtained from nonirradiated inoculated samples increased from 2.8×10^6 to 9.0×10^6 and 2.4×10^7 cells/ml on SS and TJ agars respectively. The irradiated inoculated sample showed a decrease in counts as measured on both BG and SS agars and only a very slight increase as measured on TJ agar. An increase in numbers of cells was obtained on both test media when nonirradiated chip dip was used.

Use of the radiometric technique for detection of *S. heidelberg* growth in this study was dependent on the initial inoculum and incubation time. The radiometric technique is a measure of the $^{14}CO_2$ produced by the total microflora present. At low inoculum levels, labeled $^{14}CO_2$ was detected in only nonirradiated samples after 18 h of incubation. The average $^{14}CO_2$ values were 1700 and 1570 CPM, respectively, for inoculated and control samples (Table 1), which corresponds to total counts of 1.2×10^8 and 9.7×10^7, respectively. Detection time was

TABLE 1. *Growth of* S. heidelberg *in chip dip at low inoculum level incubated at room temperature (21 C)*

Sample	Incubation time (h)	Average plate count/ml BG agar[a]	SS agar[b]	TJ agar[c]	CPM/ sample[d]
Irradiated Control	0-18	No growth in any sample			
Irradiated and inoculated with *S. heidelberg*	0	2.8×10^1	2.8×10^1	2.8×10^1	0
	4	3.0×10^2	2.0×10^2	1.0×10^3	0
	6	6.0×10^2	2.0×10^3	6.0×10^3	0
	18	2.2×10^4	2.7×10^4	6.0×10^4	0
Nonirradiated Control	0			1.0×10^3	0
	4	No growth		4.0×10^2	0
	6			1.6×10^4	0
	18			9.6×10^7	1570
Nonirradiated and inoculated with *S. heidelberg*	0	2.8×10^1	2.8×10^1	1.0×10^3	0
	4	—	5.0×10^3	1.0×10^3	0
	6	9.0×10^2	8.0×10^3	1.5×10^4	0
	18	5.0×10^5	4.0×10^6	1.2×10^8	1700

[a]Brilliant Green agar
[b]Salmonella Shigella agar
[c]Tomato juice agar
[d]Counts per minute/sample, represents the mean of duplicate samples corrected for blank activity

TABLE 2. *Growth of* S. heidelberg *in chip dip at high inoculum level incubated at room temperature (21 C)*

Sample	Incubation time (h)	Average plate count/ml BG agar[a]	SS agar[b]	TJ agar[c]	CPM/ sample[d]
Irradiated and inoculated with *S. heidelberg*	0	2.8×10^6	2.8×10^6	2.8×10^6	0
	2.5	2.0×10^5	7.0×10^5	4.0×10^6	59
Nonirradiated and inoculated with *S. heidelberg*	0	2.8×10^6	2.8×10^6	2.8×10^6	0
	2.5	—	9.0×10^6	2.4×10^7	101

[a]Brilliant Green agar
[b]Salmonella Shigella agar
[c]Tomato juice agar
[d]Counts per minute/sample, represents the mean of duplicate samples corrected for blank activity

found to be 2.5 h at a high inoculum level and corresponded to a minimum of 6×10^6 cells of *S. heidelberg*, a detection level in close agreement with that found by Previte (*16*) for *Salmonella typhimurium* in broth. More rapid detection times can sometimes be achieved by shaking samples during incubation and is recommended for future work.

Most cottage cheese manufacturing procedures will kill any salmonellae which may be present in milk used for cheese production or in nonfat dried milk used for fortification provided the cooking temperature is at least 51.7 C (*15*). *Salmonella heidelberg* can grow in a homemade cottage cheese based potato chip dip, pH 5.3, incubated at room temperature, and inoculated at low levels that might result from careless consumer handling. The number of cells can increase from levels of the borderline of detection to infective doses, of 3×10^5 to 5×10^7 (*17*), within a few hours, depending on the type and particular strain of *Salmonella*.

The radiometric technique has been shown to be applicable to estimation of numbers of bacteria in culture media with detection times directly related to the log of the initial inoculum (*18*). These studies suggest that radiometric techniques can be applied directly to detection of bacteria in dairy products, but only when high numbers of bacteria are present. This technique can, however, be used as a rapid method to determine the presence of heavy bacterial growth on a food under specific environmental conditions.

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A Research Note

Evaluation of an Automatic Gram-Staining Machine

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ABSTRACT

An automated staining machine—the Microstainer II—was evaluated for its effectiveness in the Gram staining of bacterial cultures. The conventional hand staining procedure was used as a comparison. Microscopic examination of stained slides revealed that both techniques gave correct Gram reaction of the bacterial cultures tested. The Microstainer II has an overall "efficiency factor" of 10 × compared to the conventional method.

To increase efficiency of laboratory analysis a few automated Gram-staining procedures and machines have been developed and tested (*1, 2, 4*). In 1973, Ryan et al. (*4*) reported a clinical laboratory evaluation of a commerically available automatic Gram-staining machine—the Microstainer I (TomTec Inc., Orange, Conn. 06477). Using *Staphylococcus aureus, Escherichia coli, Neisseria* sp., and sputum as test samples, they concluded that slides stained in the Microstainer were reproducible, accurate, and in most instances much clearer than those stained by hand. The purpose of this investigation was to evaluate a newer model of this machine (Microstainer II) using more species and strains of those organisms usually encountered in food microbiological analysis.

MATERIALS AND METHODS

Twenty-three named Gram-positive and 37 named Gram-negative organisms, and 24 unknown broth cultures (Table 1) were tested. Several loopsfull of cultures were placed on the glass slide for air drying and heat fixing. Duplicate sets of slides were made for each organism. One set of slides was stained by hand following the procedure recorded in *Manual of Clinical Microbiology* (*3*) and the other set was stained by the Microstainer II (marketed by Cooke Lab., Inc. Alexandria, VA 22314). The operation and mechanical aspects of the Microstainer were described in detail by the manufacturer and by Ryan et al. (*4*). Basically the machine has a reaction chamber in which up to 30 prepared slides can be stained at one time. Staining and washing times for each of the four reagents were previously determined and set on the digital timers on the Microstainer panel. When the stainer was activated, the reagents were sequentially introduced to the staining chamber, held in place for designated time intervals, and then drained back to the original container. A wash cycle between each reagent cycle was introduced to rid the slides of residual reagents. The time intervals for the four reagents and washing cycles used in this evaluation were as follows: crystal violet (30 sec), washing (50 sec), iodine (30 sec), washing (50 sec), alcohol (7 sec), washing (50 sec), safranin (60 sec), and final washing (50 sec). After staining, slides were automatically dried by hot air in the staining chamber. Slides were ready to be observed. The time sequence used in this study was determined by staining a series of slides containing pure and mixed cultures of *Escherichia coli* and *Staphylococcus aureus* by varying the reagent staining time. These time intervals were considered to be satisfactory for this study. Since the machine had the capacity of varying staining time between 1 to 999 sec, a wide range of time intervals could be used depending on needs of particular situations.

TABLE 1. *Bacterial cultures stained by the Microstainer and by the conventional method*

GRAM-POSITIVE BACTERIA[1]	
Bacillus cereus 43	*Sarcina lutea*
Bacillus danicus	*Staphylococcus aureus*
Bacillus megaterium 41	*Staphylococcus aureus* S-6
Bacillus polymyxa	*Staphylococcus aureus* 241b
Bacillus subtilis	*Staphylococcus aureus* 241c
Bacillus sulfidus	*Staphylococcus aureus* 241f
Clostridium acetobutylicum	*Staphylococcus aureus* 241g
Clostridium perfringens	*Streptococcus bovis*
Clostridium perfringens ATCC 32625	*Streptococcus faecalis*
Micrococcus flavus	*Streptococcus liquefaciens*
Micrococcus rhodochrous	*Streptococcus lactis*
Micrococcus varians	

GRAM-NEGATIVE BACTERIA	
Alcaligenes faecalis	*Pseudomonas aeruginosa* 3
Alcaligenes viscolactis	*Pseudomonas aeruginosa* 191a
Enterobacter aerogenes	*Pseudomonas aeruginosa* 191b
Enterobacter aerogenes 11a	*Pseudomonas fluorescens* 192a
Enterobacter aerogenes 11b	*Pseudomonas fluorescens* 192b
Enterobacter cloacae 1	*Pseudomonas putida*
Enterobacter cloacae 2	*Pseudomonas putida* 193
Enterobacter cloacae 3	*Salmonella cholerasuis*
Enterobacter cloacae 12	*Salmonella paratyphi* b
Escherichia coli	*Salmonella pullorum*
Escherichia coli 105	*Salmonella thompson*
Escherichia coli 106	*Salmonella typhimurium*
Klebsiella pneumoniae 1	*Salmonella typhosa*
Klebsiella pneumoniae 2	*Serratia marcescens*
Proteus vulgaris 186	*Shigella flexneri* 232
Proteus vulgaris 186a	*Shigella flexneri* 232a
Pseudomonas aeruginosa	*Shigella flexneri* 232d
Pseudomonas aeruginosa 1	*Shigella sonnei*
Pseudomonas aeruginosa 2	

UNKNOWN CULTURES[2]
11 Gram-positive Cocci
11 Gram-negative Rods
2 Gram-positive Rods

[1]All named cultures were obtained from the culture collection of the Pennsylvania State University except isolates of *Enterobacter cloacae, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* which were supplied by Dr. S. D. Kominos, Mercy Hospital, Pittsburgh, Pa.
[2]Unknown isolates were obtained from a variety of food commodities by students of a Food Microbiology course.

RESULTS AND DISCUSSION

Microscopic examination of slides under oil immersion revealed that both techniques gave correct Gram reaction of the respective named organisms. Of the 24 unknown cultures, 11 were Gram-positive cocci, 11 Gram-negative rods and 2 Gram-positive rods. Both methods gave the same results. Although Ryan et al. (*4*) graded the quality of the slides on a 4+ scale to compare the two methods, such a scale was not used in this study. For all practical purposes the two methods stained bacteria equally well. The amount of residual dyes on the slides was about the same in both methods.

It took about 6-7 h to carefully hand stain 90 slides individually with constant watching of the clock and changing reagents. For the same number of slides it took the Microstainer about 45 min. The only manual steps required were loading and unloading of slides and pressing the "start" button of the machine. When time, labor, and cleanliness were taken into consideration an arbitrary "efficiency factor" of 10 × was assigned for the Microstainer compared to hand staining.

The most time and energy consuming part of this study was the microscopic examination of all the slides using the oil immersion lens. It is apparent that a machine for the automatic read-out and print-out of Gram reaction of bacteria is needed to handle large numbers of slides. In conclusion, the Microstainer is a useful machine for Gram staining numerous slides. It should be helpful in large hospitals, public health laboratories, and quality control laboratories where numerous slides are processed daily. For smaller laboratories the conventional hand staining method remains more applicable.

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A Comparison of Phosphate Buffered and Distilled Water Dilution Blanks for the Standard Plate Count of Raw-Milk Bacteria[1]

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ABSTRACT

Raw milk samples were diluted with distilled water or distilled water with added phosphate buffer as recommended by *Standard Methods for the Examination of Dairy Products*. The standard plate counts were higher in diluent without phosphate buffer with both high and low count milk. The higher counts were significant when analyzed by a nonparametric sign test or a t-test of differences but were not significant with an analysis of variance technique. Reproducibility was not statistically different in the two diluents. It is suggested that the use of phosphate buffer for raw milk bacteria counts be discontinued until information showing definite advantages is provided.

Use of dilution water fortified with buffers was originally developed for studying biochemical oxygen demand in polluted waters that might not support adequate bacterial growth due to lack of necessary minerals or to high pH. Mohlman et al. (*7*) proposed a dilution water containing 500 ppm $NaHCO_3$ (bicarbonate water). Theriault et al. (*9*) compared distilled water, bicarbonate water, and phosphate buffer [1.25 ml per liter of stock solution of 34.0 g KH_2PO_4 in one liter of distilled water with the pH adjusted to 7.2 with 1 N NaOH—the same concentration now recommended by *Standard Methods* (*1*)]. These authors (*9*) concluded that phosphate buffer by itself was a suitable diluent for biochemical oxygen demand analyses of polluted waters.

Butterfield (*3*) extended the buffered dilution blank studies to the isolation of bacteria from the water of seven rivers or creeks. He found a high pH (7.6-8.2) in six of the waters after autoclaving and these remained high for 48 h. Distilled water also became alkaline but reverted to pH 7.2 in 48 h. Phosphate buffered distilled water with or without the added minerals remained at a constant pH (7.4). Bacterial counts were obtained in an unspecified agar incubated at 37 C. Highest counts were obtained using the above fortified phosphate buffer although phosphate buffer alone was nearly as good. Lowest counts were obtained with bicarbonate water with distilled water being somewhat better but not as good as phosphate alone.

As far as we know, the practice of using phosphate buffer for isolating bacteria from dairy products stems from the studies of the above workers on water-borne bacteria. The only reference to its use in *Standard Methods* is the paper by Butterfield (*3*). We undertook the study reported here because of the lack of data on the efficacy of phosphate buffer as a diluent for bacteria in dairy products.

MATERIALS AND METHODS

Methods advocated by *Standard Methods* (*1*) were followed with the exception of the distilled water [nonphosphate buffered (NPB)] series. This was a collaborative assay by the members of the Subcommittee for the Examination of Milk and Milk Products, Applied Laboratory Methods Committee, International Association of Milk, Food, and Environmental Sanitarians, Inc. Nine analysts secured their own raw milk samples (total of 82 for this study) which were from the states of Minnesota, Louisiana, Tennessee, North Carolina, Ohio, and Texas. Distilled water (NPB) with or without phosphate buffer (PB) at the concentration recommended by *Standard Methods* (*1*) and Theriault et al. (*9*) was used as the diluent. Duplicate petri-dishes were poured and in most instances replicate aliquots of raw milk were plated.

RESULTS

pH measurements

Distilled water pH values reported by the different laboratories ranged from 5.50 to 8.70 with 13 of 19 samples in the range 5.80 to 6.80. Freshly distilled water gave the highest values of 8.55 and 8.70; these, which were used in two days and one day, respectively, showed a drop in pH to 7.35 and 7.50, respectively. The other pH measurements reported were those of distilled water just before addition of milk or buffer. When buffer was added, the pH values reported were 7.05 in 14 cases;

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TABLE 1. *Effect of phosphate buffer in dilution water on plate counts of raw milk bacteria*

Investigator	Milk sample no.	Phosphate (PB) Rep. 1		Phosphate (PB) Rep. 2		No phosphate (NPB) Rep. 1		No phosphate (NPB) Rep. 2		Increase (+) or decrease (−) without phosphate[c]
A	1[a]	119[b]	124	146	158	179	168	198	194	+
	2	30.0	27.9	30.9	29.3	27.7	28.2	30.1	27.3	−
	3[a]	398	422	369	389	368	397	386	390	−
	4[a]	205	239	241	234	205	237	240	239	+
	5[a]	113	118	106	110	121	99	103	146	+
	6[a]	71	104	93	104	84	124	93	98	+
	7	13.1	8.4	12.5	11.7	10.7	12.2	14.7	14.6	+
	8[a]	350	319	337	291	314	277	298	267	−
	9	8.8	9.5	9.1	9.1	8.7	7.3	8.4	7.5	−
	10[a]	43	43	56	59	53	41	43	40	−
Arith. mean			139.03				139.98			+
Geom. mean[d]			4.326				4.337			+
B	11	4.2	4.8	4.2	4.5	4.1	5.0	4.0	3.0	−
	12	4.0	3.4	3.6	4.3	3.8	4.4	3.0	2.9	−
	13	6.8	7.6	7.4	8.0	4.7	4.4	6.7	8.1	−
	14[a]	62	57	55	61	62	61	54	40	−
	15	27	30	30	28	28	27	29	30	−
	16	3.9	2.8	3.6	4.2	3.2	3.7	2.0	3.1	−
	17[a]	69	77	72	64	69	57	70	71	−
	18[a]	60	53	50	51	67	72	75	79	+
Arith. mean			28.85				29.91			+
Geom. mean			2.720				2.658			−
C	19	21.0	20.6	21.8	22.9	21.6	19.5	24.4	23.0	+
	20	11.9	10.8	12.5	12.3	12.7	12.6	12.8	13.7	+
	21	15.2	13.8	15.9	17.7	16.3	17.2	17.4	17.9	+
	22	18.5	17.4	20.9	17.2	19.7	20.6	18.7	21.8	+
	23	10.1	9.6	10.3	9.7	8.7	11.2	11.1	9.9	+
	24	8.4	8.6	8.7	7.9	8.9	9.2	8.2	9.1	+
Arith. mean			14.32				15.26			+
Geom. mean			2.604				2.666			+
D	25	14.4	13.6			14.4	14.7			+
	26	6.2	7.2			7.3	7.4			+
	27	14.1	12.9			13.7	14.6			+
	28	3.0	3.9			2.8	2.9			−
	29	8.4	8.6			6.9	6.7			−
	30	7.1	5.8			5.2	4.3			−
	31	16.2	20.4			19.7	19.6			+
	32[a]	143	145			108	92			−
Arith. mean			26.86				21.26			−
Geom. mean			2.529				2.427			−
E	33[a]	104	110	106	115	120	111	123	98	+
	34[a]	210	242	270	265	274	302	232	237	+
	35	293	263	259	240	27.1	24.9	26.0	25.8	−
	36[a]	72	69	53	77	63	72	76	82	+
	37[a]	161	156	141	133	160	159	141	142	+
	38	8.3	11.0	9.2	10.3	9.9	9.5	10.5	8.3	−
	39	23.1	22.4	23.7	27.0	23.8	23.7	22.7	24.6	−
	40	8.4	7.5	9.4	6.7	9.8	8.1	8.3	9.7	+
	41	18.5	15.2	20.5	18.6	24.0	26.7	12.9	19.7	+
Arith. mean			99.41				102.28			+
Geom. mean			3.930				3.972			+
F	42	18.5	20.6	20.2	21.1	24.0	20.8	23.2	22.8	+
	43	28.7	26.4	25.2	28.5	29.1	24.2	24.7	24.9	−
	44[a]	77	71	71	60	56	79	79	66	−
	45[a]	57	61	57	54	51	60	64	61	+
	46	6.8	5.1	6.4	5.7	6.3	7.5	8.0	8.1	+
	47	6.7	6.6	6.4	6.9	6.5	7.2	9.4	7.0	+
	48	7.0	5.4	6.5	7.2	5.9	5.8	6.1	7.1	−
Arith. mean			27.64				28.38			+
Geom. mean			2.876				2.931			+
G	49	15.8	16.9	18.2	15.5	16.4	17.6	15.4	17.1	+
	50[a]	190	198	186	197	193	181	188	234	+
	51	14.0	15.1	18.0	18.7	16.3	16.6	17.1	17.2	+
	52	10.1	9.5	8.4	9.0	10.3	9.5	11.2	11.6	+
	53	9.5	11.2	10.5	10.0	9.0	9.5	7.8	8.6	−
	54	9.1	9.5	8.4	7.6	11.7	6.2	9.0	11.3	+
	55	3.4	3.2	2.3	2.7	3.2	3.2	3.6	3.3	+

TABLE 1. *Continued.*

Investigator	Milk sample no.	Phosphate (PB) Rep. 1		Phosphate (PB) Rep. 2		No phosphate (NPB) Rep. 1		No phosphate (NPB) Rep. 2		Increase (+) or decrease (—) without phosphate[c]
	56	6.3	5.3	6.4	4.7	6.2	5.7	5.0	5.1	—
	57	2.7	3.6	1.7	2.6	2.3	2.1	3.3	3.3	+
	58[a]	44	32	41	39	34	39	47	41	+
	59[a]	123	141	132	149	131	158	120	149	+
	60[a]	200	239	239	218	222	251	259	231	+
	61[a]	170	188	138	172	140	164	162	176	—
	62[a]	234	194	203	241	202	247	249	230	+
	63[a]	102	121	121	118	124	110	120	113	+
	64[a]	268	288	279	251	282	288	281	269	+
	65[a]	135	140	157	176	153	180	130	141	—
	66[a]	249	226	231	261	279	242	261	258	+
	67[a]	230	238	241	221	245	223	209	221	—
Arith. mean				108.56				111.48		+
Geom. mean				3.792				3.818		+
H	68	6.0	6.4			7.0	7.9			+
	69[a]	59	65			74	69			+
	70[a]	168	160			175	163			+
	71	5.4	6.2			5.6	6.4			+
	72	8.4	8.2			6.1	7.0			—
	73	10.6	9.4			8.9	10.0			—
	74	8.5	8.2			9.8	9.8			+
	75	6.6	7.1			7.9	6.8			+
	76	7.4	6.6			6.8	6.2			—
Arith. mean				30.94				32.62		+
Geom. mean				2.579				2.606		+
I	77	5.0	5.9			6.3	5.9			+
	78	7.0	6.4			6.8	6.7			+
	79	10.2	9.5			10.0	9.1			—
	80	9.3	8.7			10.0	10.1			+
	81	6.4	6.3			6.8	6.5			+
	82	7.0	6.5			6.4	5.9			—
Arith. mean				7.35				7.54		+
Geom. mean				1.972				1.998		+
All samples										
Arith. mean				54.00				54.64		+
Geom. mean				3.037				3.046		+

[a]These milk samples were "high count" with the 10^{-3} dilution used for enumeration.
[b]For actual counts per ml milk, multiply by 1000.
[c]Based on geometric means.
[d]Natural log.

7.10, 7.20, 7.35, and 7.5 for four other samples. The two highest were those having the highest initial pH values. Addition of 1 ml of milk to the unbuffered water brought the pH values to a range of 6.8 to 7.2. Addition of milk to the buffered dilution blanks resulted in a very slight drop in pH (about 0.1 unit) in most instances.

Analysis of paired differences

The original plate count values are shown in Table 1. The overall mean counts of NPB were very slightly greater than PB. Eight and seven of the nine analysts reported higher mean counts with NPB dilutions for arithmetic and geometric means, respectively. Of the 82 milk samples tested, the geometric means of plate counts in NPB dilution blanks were greater than PB in 50 cases. Using a nonparametric sign test for matched pairs (5), the differences between NPB as opposed to PB, as shown in Table 1, were found to be significant ($0.05 < P < 0.01$). A t-test of paired differences of the proportion $\frac{\text{NPB-PB}}{\text{PB}}$, transformed to $y = 2 \arcsin \sqrt{x}$ (for normalizing the distribution), also showed the two diluents to give significantly different means ($P < 0.05$).

Since a significant amount of milk phosphate could be carried over in the first dilution and less in the second, the possibility existed that high count milk, i.e. that with counts over 30,000/ml, would behave differently from lower count milk. Analyses of these milk samples showed NPB > PB in 20/30 high count and 30/52 low count samples. A Chi square test of goodness-of-fit, however, did not indicate these ratio differences to be significant.

Analysis of variance

An examination of the differences between plate counts of NPB and PB in Table 1 shows that analyst B found 7 of 8 samples with PB > NPB; although the overall arithmetic mean showed a reverse trend due to the 8th sample. Analyst C found all 6 samples with NPB > PB. The large discrepancy between these two analysts (the

TABLE 2. *Analysis of variance determinations for 82 milk samples*

Line	Source of variation	df	Sum of squares	Mean square	F	Significant with P<0.05	Significant with P<0.01
A	Analysts	8	366.226	45.7782	3.44	yes	yes
B	Samples/analysts	73	972.063	13.3159	914	yes	yes
C	Treatment (phosphate versus no phosphate)	1	0.020427	0.020427	1.40	no	no
D	Treatment × analysts	8	1.02921	0.128651	8.84	yes	yes
E	Treatment × samples/analysts	73	1.39636	0.019128	1.31	no	no
F	Between replicates/samples	164	2.38750	0.014558	1.63	yes	yes
G	Between petri dishes	328	2.93500	0.008948			
	Total	655	1346.06				

F-values were derived from the ratios of the mean squares of lines A/B, B/F, C/F, D/F, E/F, F/G.

TABLE 3. *Analysis of variance of variances between duplicate petri dishes for 82 milk samples*

Line	Source of variation	df	Sum of squares	Mean square	F-ratio	Significant with P<0.05	Significant with P<0.01
A	Analysts	8	6171.82	771.478	1.58	no	no
B	Samples/analysts	73	36144.2	495.126	1.40	yes	no
C	Treatment	1	50.5766	50.5766	0.54	no	no
D	Treatment × analysts	8	730.255	91.2819	0.22	no	no
E	Treatment × samples/analysts	73	31071.4	425.636	1.20	no	no
F	Between replicates/samples	166	58894.8	354.788			
	Total	327	133063.1418				

F-values were derived from the ratios of the mean squares of lines A/B, B/F, C/F, D/F, E/F.

other seven analysts showed a more random distribution of differences) indicated either chance differences or some personal, geographical, or other bias. The analysis of variance is designed to determine the true nature of such differences. The analysis of data of Table 1, transformed to natural logs, is shown in Table 2. There was, as expected, a highly significant difference between milk samples (line B). There was also a highly significant ($P < 0.01$) difference between analysts (line A) which might have been due to the particular samples analyzed. There was no significant difference between NPB and $\overline{PB}$ (line C). There was a highly significant interaction ($P < 0.01$) between treatments and analysts (line D). This probably accounts for the apparent significant difference obtained between NPB and PB with the nonparametric and t-tests when applied to the data of Table 1 (these tests do not detect interaction effects). There was no evidence that the higher means obtained using NPB were different from PB. The test for interaction between phosphate treatment and samples within individual analysts (line E) was not significant at the 5% level. Replicates of the same milk sample (line F) were significantly more variable than the variability between petri dishes of the same replicate (line G).

Test for reproducibility

A single degree-of-freedom variance between petri dishes was calculated for each replication of each sample. These differences were examined by analysis of variance. The results are summarized in Table 3. The only difference in reproducibility was in samples within analysts (line B) which showed a difference with $P < 0.05$ but not with $P < 0.01$. In other words variances between milk samples were different and were not associated with any particular analyst. It was interesting to note that in this study there was no difference in analyst reproducibility, although we had previously (*6*) found one analyst to have a significantly better reproducibility. This analyst did not participate in this current study.

DISCUSSION

The results we obtained, as reported here, do not indicate any advantage for using phosphate buffer in the dilution blanks. There was some evidence from the less powerful statistical methods used that the counts were higher without phosphate. It is possible, though, that where further dilutions are necessary, some advantage might accrue from the use of phosphate. Under most conditions, only one dilution of 10^{-2} is made for raw milk. Our results are based on this dilution.

Wagenaar and Jezeski (*10*) studied the survival of *Pseudomonas putrefaciens* in distilled water and in water buffered with gelatin phosphate (0.2% gelatin, 0.725% $NaH_2PO_4 \cdot H_2O$) and 0.37% Na_2HPO_4). They reported differences in survival of several strains in distilled water and in the case of the most resistant strain, found that gelatin phosphate greatly improved survival with phosphate being the active component. the best phosphate concentration was 2% with progressively less survival at 1 and 0.5%. *Standard Methods,* however, recommends a phosphate concentration of only about 0.04%. Atherton (*2*) found some evidence that increasing dilutions of 12-day stored pasteurized milk resulted in lack of growth of psychrotrophic bacteria presumably because of a need for phosphate (carried over by the milk at low dilutions) as a growth stimulant. He indicated, however, that in general the psychrotrophic bacteria were not noticeably affected by the lack of phosphate in the dilution water. Watrous (private communication, 1973)

indicated that the bacterial counts on stored processed dairy products such as pasteurized milk might be influenced by the presence or absence of phosphate. Other workers produced evidence that demineralized or distilled water decreases survival of pure cultures of *Escherichia coli* (*4*) and *Streptococcus faecalis* R (*8*).

It is apparent from our studies that the present use of phosphate in raw milk dilution blanks for the standard plate count is of no value in increasing the counts of bacteria or increasing reproducibility. It is possible that a dilution fluid similar in composition to the minerals of milk would be of more value for standard plate counts of the bacteria in dairy products. Peptone water should also be evaluated as a possible diluent for raw milk bacteria. Studies of such diluents are contemplated by our subcommittee.

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Starters and Bacteriophages in Lactic Acid Casein Manufacture

I. Mixed Strain Starters

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ABSTRACT

The bacteriological composition and acid producing activity of mixed strain starters used for commercial lactic acid casein manufacture were investigated. Rates of acid production by mixed starters from eight New Zealand casein factories were similar under standard laboratory conditions. *Streptococcus cremoris* was the predominant species comprising some 70-90% of total bacteria in all but one mixed casein starter. Most isolates in a random selection of 160 individual *S. cremoris* colonies took longer than the parent mixed starter to coagulate autoclaved skimmilk at 22 C. More than half required 2-3 days and some up to 7 days of incubation, in contrast to the 17-20 h required by the mixed starters. Only 20% of the *S. cremoris* isolates had the coagulation times and reached maximum cell densities characteristic of the parent mixed cultures. The slow-coagulating isolates grew to only low population densities in milk although acid production continued in stationary-phase cultures. The maximum cell density and rate of acid production could be increased by culturing together with a fast-coagulating strain, by addition of hydrolysed milk proteins or amino acids. Whey samples from the casein precipitation silos of eight commercial factories were examined for bacteriophage. Virulent phages were foundin all samples.

Lactic casein manufacture has been a significant portion of total dairy processing in New Zealand for nearly 20 years, about 40,000 tons being produced in the 1972/73 season. Manufacture commences with a large scale lactic acid fermentation of skimmilk to precipitate the casein. The resulting coagulum is heated and the curd, which then separates from the whey, is washed, dried, and ground to give powdered casein. The starters inoculated into the silos to initiate the fermentation have been selected arbitrarily. In most instances, mixed starters obtained from commercial supply houses as freeze-dried powders have proved to be adequate even though they were specifically formulated for cheesemaking. These starters generally comprise several strains of *Streptococcus cremoris, Streptococcus lactis, Streptococcus diacetilactis,* and *Leuconostoc* species although their exact composition is unknown and may vary on subculture (*13*).

The only requirement of starter bacteria in lactic caseinmaking is acid production. Instances of slow acid production by starters occur periodically in factories. However, unlike cheesemaking, slow acid production in caseinmaking rarely causes acute processing difficulties which jeopardize manufacture and total starter failure may be overcome by addition of mineral acid. Consequently, in most instances the reasons for the reduced starter activity are not determined. The variety of bacteria present in mixed starters has precluded evaluation of the significance of phage. When a defined multiple starter system was used in a commercial factory, virulent phages were found usually within a few days of introduction of the component organism (*17*). In addition, the starters appeared to be more vulnerable to phage attack during casein manufacture than in cheesemaking.

This study was undertaken to investigate the properties of mixed casein starters and to determine the reasons why most factories can use these starters for extended periods without obvious slow coagulation of the milk.

MATERIALS AND METHODS

Factory samples

Mixed starters were samples of freshly inoculated bulk starter milk. Samples were kept at 0 C for up to 24 h during transport to the Institute, then incubated at 22 C. After coagulation, cultures were transferred (1%) into autoclaved reconstituted skimmilk (RSM, 9.5% total solids), frozen using a solid CO_2-ethanol freezing mixture and stored at − 75 C. Inoculated cultures were thawed rapidly, incubated at 22 C overnight and subcultured (0.5% inoculum) daily in autoclaved RSM five times before use.

Whey samples were obtained after centrifugation (30,000 × *g* for 1 min) of coagulated milk from factory mother cultures, bulk starter vessels and casein precipitation silos.

Phage detection

Strains AM_2, R_6, Z_8 (*S. cremoris*), H_1 and ML_8 (*S. lactis*) were selected as potential indicators of phages in whey from casein factories. None of the five strains was sensitive to a common phage when tested against the phages of the Institute's collection. An additional 15 *S. cremoris* isolates derived from several mixed starters (*17*) were also tested to ensure that a wide range of organisms were investigated as possible hosts.

Samples of factory whey (0.05 ml) were added to culture tubes containing 10 ml autoclaved RSM. Each tube was inoculated (1%) with one of the 20 indicator cultures and incubated at 22 C overnight. This step was included to increase the titer of any phages present and reduce the likelihood of non-specific phage-associated lysin reactions (*10*). Whey preparations from these cultures were spotted undiluted and at decimal dilutions on lawns of the indicator organisms prepared by soft agar overlay on M16 agar plates containing 5 mM calcium borogluconate (*7*).

Coagulation time

The time (h) required by a starter (0.1% inoculum) to lower the pH of

RSM to 4.7 at 22 C was referred to as the coagulation time. Starters with coagulation times of less than 24 h (termed "fast-coagulating" starters) were grown in pasteurized (62.5 C for 30 min) RSM. All other isolates (termed "slow-coagulating" starters) were grown in autoclaved RSM. The cultures used for inoculation were grown in autoclaved RSM (0.1% inoculum, 22 C, 24 h) and determinations of coagulation times were done using RSM prepared from a standard batch of low-heat spray dried skimmilk powder.

Routine colony counts

The blender treatment described by Martley (*9*) reduced streptococcal chains in culture samples to uniform size, mainly diplococci. Samples were diluted in 1/10 strength M16 broth (*7*) and plated on an agar medium which differentiates *S. cremoris* from other streptococci (*12*). Plates were incubated at 30 C for 48 h aerobically, or in a 5% (v/v) CO_2-air mixture. In some experiments cultures were plated on milk agar (autoclaved RSM solidified with 1% Davis New Zealand agar).

Composition of mixed strain cultures

Freshly coagulated cultures of mixed starters were plated on the differential agar at dilutions giving about 50 colonies per plate. The proportion of *S. cremoris* organisms in a total of 500 colonies (about 10 plates) was determined for each starter. Individual *S. cremoris* colonies from each mixed starter were inoculated into autoclaved RSM. Subjectivity in selecting colonies was eliminated by plating at a dilution giving approximately 10 colonies per plate and isolating every visible *S. cremoris* colony on each plate. At least two plates were required for each mixed starter to obtain 20 *S. cremoris* colonies.

Differentiation of S. cremoris isolates

The individual components of cultures comprised of *S. cremoris* isolates 170 and 364 were distinguished by their consistently different response to incubation in CO_2-air mixtures when plated on differential agar (*12*). Isolate 170 failed to give visible colonies unless plates were incubated in an atmosphere enriched with CO_2 (*15*), whereas isolate 364 grew equally well in either normal or CO_2-enriched air. Colonies of *S. cremoris* isolates 186 and 366 showed consistent differences in both size and yellow color intensity when plated on differential agar and incubated in a normal atmosphere.

Materials

Trypticase (pancreatic digest of casein) and Lactalysate (pancreatic digest of lactalbumin) were products of Baltimore Biological Laboratories. Trypsin (type III, 2 × crystallized), glycine and the L-isomers of alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, theronine, tryptophan, tyrosine and valine were obtained from Sigma.

RESULTS

Use of mixed starters in casein factories

Important differences were found between starter propagation techniques used in casein factories and those employed in New Zealand cheese factories. In casein factories mother cultures were usually prepared by transferring coagulated culture (1-5%) into heat-treated (100 C for 1 h) skimmilk. Following incubation for 24 h at ambient temperature these cultures were inoculated (0.1-0.5%) into pasteurized skimmilk contained in bulk starter vessels. After coagulation at 22-26 C, bulk starter was inoculated into pasteurized skimmilk in the main coagulation silos (Table 1). Neither mother cultures nor bulk starters were prepared with aseptic precautions. No measures were taken to prevent airborne phage contamination and manipulations were carried out in the main factory. In contrast, starter cultures for cheesemaking are maintained aseptically and phage is excluded from both mother cultures and bulk starter cultures.

TABLE 1. *Mixed starter activity in commercial casein factories: summary of daily records over 90 consecutive days in the period November 1972-March 1973*

Factory	Starter inoculum (%)	Temperature at inoculation (C)	Mean processing pH ± S D[a]	Incubation required (h)
A	0.1 –0.5	22 – 24	4.59 ± 0.23	16 – 18
B	1.0	22 – 24	4.52 ± 0.06	10 – 14
C	0.02–0.2	22 – 24	4.56 ± 0.08	14 – 17
D	0.01–0.04	23 – 27	4.50 ± 0.10	18 – 20
E	0.2 –0.5	23 – 26	4.51 ± 0.09	16 – 17
F	0.05–0.1	26	4.42 ± 0.11	16 – 18
G	0.5 –1.5	23 – 25	4.33 ± 0.08	18 – 19

[a]Standard deviation.

The factors influencing rate of acid development in silo milk varied considerably between factories. Volume of starter inoculum and milk temperature were adjusted to give convenient coagulation times (Table 1). Starter activity was recorded over a 3-month period at eight factories (Table 1). Unacceptably slow coagulation occurred at two factories on a total of 8 days although coagulum formation in some silos was normal on these days. The slow coagulation was attributed, therefore, to inhibitory substances in the milk. There was a progressive decrease in rate of acid production by mixed starters in six of the eight casein factories. When this slowing of acid development caused inconvenience, replacement starters were prepared, usually from the freeze-dried powder used initially.

Phages in factory whey

Virulent phages, capable of producing plaques on lawns of sensitive hosts, were present in whey samples from all factories (Table 2). These phages also prevented

TABLE 2. *Presence of phages in whey from lactic casein factories*

Strain or isolate tested[a]	Factory							
	A	B	D	E	F	G	H	I
AM_2	–	+	–	–	–	+	++	+
H_1	–	+	++	+	++	++	+	++
ML_8	–	–	–	–	–	–	–	+
R_6	–	–	–	–	–	–	+	–
Z_8	–	+	–	–	–	–	–	–
114	–	–	–	–	+	–	–	–
134	–	+	+	–	–	+	+	–
158	++	–	–	–	+	–	–	–
168	–	+	+	+	–	+	+	+
170	–	+	+	–	–	+	+	+
172	–	–	–	–	–	–	+	+
184	–	–	–	–	–	–	–	–
186	–	+	–	+	–	+	+	–
188	–	+	+	++	–	–	++	++
224	–	–	–	–	–	++	–	–
240	–	–	–	–	–	–	++	–
242	–	–	++	–	–	–	++	–
266	–	–	–	–	–	–	–	–
272	–	–	–	–	–	–	++	–
290	–	++	+	–	–	–	++	–

[a]++ plaque formation.
+ zone of incomplete lysis.
– no visible reaction with undiluted whey.

coagulation of milk cultures of the appropriate indicator organism. Other phage reactions however, appeared as zones of incomplete lysis on lawns and acid production by the indicator strains was not eliminated entirely under simulated caseinmaking conditions. The phage reactions given by whey samples from the mother culture, bulk starter, and coagulation silos of a given factory were all similar.

Acid production by mixed starters

Mixed starters obtained from different factories had similar coagulation times under standard laboratory conditions (Table 3). The initial rate of acid production

TABLE 3. *Activity of* S. cremoris *isolates from mixed starters*

Factory	Mixed starter coagulation time (h)	Percent *S. cremoris*	Number of isolates and days at 22 C to coagulate milk					
			Days 1	2	3	4	5	7
B	17	92	9	6	1	2		2
D	17	86	2		18			
E	17	88	2	16	1	1		
F	19	36	11	2	3	2	2	
G	18	68	3	2	8	4	2	1
H	18	75	4	3	5	3	4	1
I	20	70	2	14	2		1	1
J	18	79	3		8	2	1	6

was similar to that observed with single strains but acid development in most mixed cultures consistently stopped at a higher pH value (4.5-4.6 rather than 4.2-4.3). Typical data are given in Fig. 1. The change in rate of acid

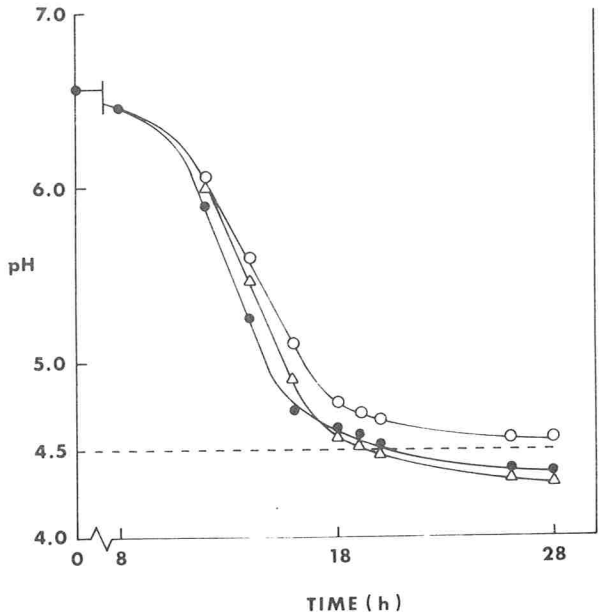

Figure 1. *Acid production by starters (0.1% inoculum of 24-h culture at 22 C) in pasteurized RSM at 22 C.* S. cremoris *242,* (△)*; mixed starter from factory E,* (O)*; and mixed starter E in pasteurized RSM containing 0.2% Trypticase,* (•).

production corresponding to that in fast-coagulating single strain cultures near pH 4.6, occurred in mixed starters near pH 4.7 (Fig. 1). Therefore in order that coagulation times recorded for mixed and single strain cultures reflected comparable stages in overall acid development, this higher pH value was adopted as the end-point in obtaining coagulation times of mixed starters.

In commercial practice, coagulation of the milk generally occurs several hours before the coagulum is "cooked" so that a reduction in the rate of acid production may not affect processing.

Composition of mixed starters

After five subcultures in the laboratory, coagulated mixed starter cultures contained about 5×10^8 colony forming units (cfu)/ml. In all but one of the mixed starters examined, 70-90% of the total bacteria were *S. cremoris* (Table 3). The remainder were identified only as acid producing streptococci.

Individual *S. cremoris* colonies were isolated and these required from 1 to 7 days to coagulate autoclaved skimmilk at 22 C (Table 3). Only 2-4 of the 20 colonies isolated from each of six mixed starters coagulated milk as rapidly as the corresponding parent culture, and less than half of the isolates from the other two mixed starters were as fast as the parent cultures. Most of the remaining isolates required 2-3 days incubation to coagulate milk, some even requiring up to 7 days.

It appeared therefore, that mixed starters were comprised largely of slow-coagulating *S. cremoris* organisms. The procedure of plating, selecting, and characterizing 20 colonies was repeated using nine single strain cultures known to have fast-coagulating times. All 180 isolates coagulated milk within 24 h at 22 C thus eliminating the possibility that slow-coagulating organisms were artifacts produced by plating on differential agar. In addition, colonies selected from milk agar plates of mixed starters B, D, and E (90% *S. cremoris*) gave a distribution of coagulation times similar to that found with the isolates from differential agar. The overall *S. cremoris* composition of mixed starters and range of activity of component organisms did not show large daily fluctuations because data similar to that recorded in Table 3 were obtained with bulk starter samples direct from factories and after subculturing six, seven and eight times.

Properties of slow-coagulating S. cremoris organisms

Growth and acid production at 22 C in autoclaved RSM cultures of a typical slow-coagulating *S. cremoris* isolate is shown in Fig. 2. The doubling time during logarithmic growth was about 1.8 h. The culture reached the stationary phase and a plate count of 7×10^7 cfu/ml after one day but acid production continued slowly until coagulation occurred after 6 days. This pattern of growth and acid production was characteristic of all slow-coagulating isolates examined and remained unchanged after prolonged subculture. A 100-fold increase in the inoculum (0.01 to 1%) caused no obvious change in the coagulation time of *S. cremoris* 366 and

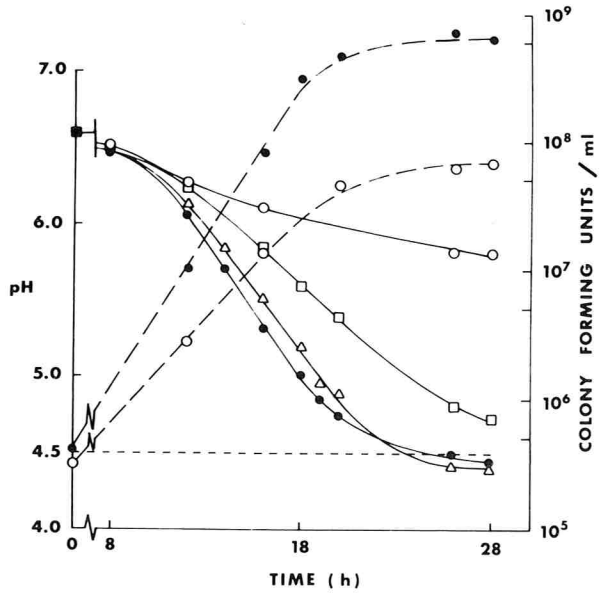

Figure 2. *Growth (broken lines) and acid production (unbroken lines) at 22 C in autoclaved RSM cultures (0.5% inoculum) of* S. cremoris *366. Control, O; plus 0.2% Trypticase, •; plus amino acids (0.5 mg/ml of each of 18 amino acids), △; plus 0.2 μg/ml tyrpsin, □.*

raising the incubation temperature from 22 C to 30 C decreased the coagulation time from 6 to 3 days.

The preponderance of slow-coagulating organisms in mixed starters indicated that growth stimulatory interactions between strains may occur in milk culture. Combinations of fast- and slow-coagulating isolates had coagulation times characteristic of fast-coagulating organisms (Table 4). Investigations of pairs of fast and slow isolates, which could be distinguished from each other in samples of combined cultures, showed that rapid coagulation was not solely due to growth and acid production by the fast component. From Table 4 it can be seen that in the presence of a fast strain, the slow-coagulating isolates multiplied in the same time to a population density 10 times greater than that reached when cultured individually. There was a corresponding increase in overall acid production and hence a shortened coagulation time. Various combinations of up to eight slow-coagulating isolates originating from different mixed starters were grown together in milk. All cultures had extended coagulation times which were similar to those of the individual components.

TABLE 4. *Stimulatory interactions at 22 C in pasteurized RSM between fast- and slow-coagulating* S. cremoris *isolates*

Isolate (% inoculum[a])		Incubation		cfu per ml x 10^{-7}	
Fast	Slow	(h)	pH	Fast	Slow
170(0.1)		17	4.7	68	
	364 (1.0)	15	6.1		4.6
170 (0.1) +	364 (1.0)	15	4.7	21	46
170 (0.01)		22	4.7	62	
	364 (0.1)	20	6.2		5.1
170 (0.01) +	364 (0.1)	20	4.7	11	45
186 (0.1)		19	4.7	59	
	366 (0.5)	18	6.2		6.2
186 (0.1) +	366 (0.5)	18	4.7	43	36

[a]Parent cultures: fast-coagulating isolate 6×10^8 cfu/ml
slow-coagulating isolate 1×10^8 cfu/ml.

In RSM containing 0.2% (w/v) Trypticase cultures of all 124 colonies isolated, which normally required 2-7 days to coagulate milk (Table 3), coagulated in less than 24 h at 22 C. Trypticase addition boosted cell densities as well as overall acid production to levels comparable with fast-coagulating organisms (Fig. 2). Addition of a mixture of 18 amino acids also shortened coagulation times. The 18 amino acids added individually had no apparent effect. The extent of stimulation obtained by supplementation with either Trypticase (0.2%, w/v) or amino acid mixture (0.5 mg/ml of each amino acid) was approximately equivalent (Fig. 2). Addition of larger amounts did not further enhance growth and acid production. Lactalysate supplementation (1.0%, w/v) of RSM gave greater stimulation of slow-coagulating isolates than observed with saturating levels of Trypticase or amino acid mixture. The coagulation time of *S. cremoris* 366 in RSM containing the optimum quantity of Lactalysate (1.0%, w/v) was 2 h less than the shortest period found with Trypticase addition. Addition of trypsin to pasteurized RSM also shortened coagulation times of slow organisms (Fig. 2). The optimum amount of trypsin was 0.2 μg/ml, concentrations above 0.5 μg/ml reducing the rate of acid production.

Enhancement of growth and acid development by Trypticase supplementation of RSM was not restricted to slow-coagulating isolates. Acid production by mixed starters increased and lower final pH values were reached (Fig. 1). In addition, there were 1-2 h reductions in the coagulation times of fast single strains.

DISCUSSION

Virulent phages were detected in whey samples from each of eight lactic casein factories located throughout the principal dairy manufacturing areas of New Zealand. Of the 20 potential indicator organisms used in the screening of whey samples, five strains (AM_2, H_1, ML_8, R_6 and Z_8) were representatives of different patterns of phage susceptibility amongst cheese starters. No phages in the Institute's collection attacked the remaining 15 test organisms which were recent isolates from mixed starter cultures (*17*). Therefore, when lytic reactions were observed on several of the diverse selection of indicator organisms, whey samples probably contained a number of different phages rather than a single phage type which attacked a broad range of hosts with varying efficiency. Crawford and Galloway (*2*) in a study of Scottish cheese factories found that phage was normally present in mixed starter cultures.

Phages are widespread in the factory environment and with no aseptic precautions in the starter handling procedures, cultures must repeatedly become contaminated by phages. Thomas and Lowrie (*17*) showed that regular addition of whey from previous manufacture to otherwise aseptically handled mother cultures enabled a

mixture of four *S. cremoris* isolates to be used daily for commercial caseinmaking over a complete dairying season without acid production failure due to phage. There is presumably a constant selection for organisms able to grow in the presence of phage. Continual phage contamination, causing the development of 'phage-tolerant' variants during growth of mother cultures and bulk starters, may be required for the prolonged use of mixed starters without obvious instances of slow coagulation in the casein precipitation silos.

Mixed starters for analysis of bacterial composition were obtained from casein factories as samples of freshly inoculated bulk starter milk. After coagulation, these cultures should consist of the organisms that grow actively during caseinmaking. These organisms were found to be predominantly *S. cremoris* even though the original powdered cultures initially contained several different species of bacteria. A similar dominance by *S. cremoris* was found in a European study of 70 mixed starters from cheese and butter factories (*8*). The *S. cremoris* component must be best able to survive phage attack and adapt to the rigorous conditions of culture used in factories.

Examination of the acid producing activity of individual *S. cremoris* isolates from the mixed casein starters showed that most were slow-coagulating organisms. About 80% of the randomly selected *S. cremoris* colonies had much longer coagulation times than the parent mixed starters. Garvie (*4*) mixed a slow variant of *S. cremoris* 924 with the fast parent strain in milk culture and found that a balance between the two cell types was established after several subcultures with the slow variant comprising about 95% of the population. This balance was independent of the initial ratio of fast and slow cells. Like the combination cultures of fast and slow variants of *S. cremoris* 924 the mixed casein starters had acid producing activities similar to those of their fast-coagulating minority components. Slow- and fast-coagulating isolates from casein starters had similar growth rates but, in milk, slow isolates reached only 10% of the normal maximum population density. Acid production continued in stationary-phase cultures until coagulation eventually occurred several days later. Therefore, slow coagulation resulted from growth limitation at low cell densities and not because of a reduced growth rate. Similar observations have been made with slow variants derived from *S. lactis* C2 (*1*).

Addition of amino acids or protein hydrolysate to milk increased the maximum cell density and rate of acid production of slow-coagulating isolates to levels characteristic of the fast-coagulating organisms and the parent mixed starters. This suggests a proteinase deficiency in slow-coagulating isolates from mixed casein starters. Milk contains small amounts of free amino acids and peptides (*3, 14*) which appear to permit only limited growth of these organisms. Accumulation of slow variants in milk cultures of lactic streptococci and their stimulation by addition of protein hydrolysates is well known (*1, 5, 6*). Slow variants appear to be deficient in a cell wall-bound proteinase due to loss of an extra-chromosomal element (*11*).

When paired in milk culture, both fast- and slow-coagulating organisms grew to similar population densities, suggesting that the fast organism supplied amino acids or peptides for growth of the proteinase-deficient organism. This interaction may be a consequence of proteinase localization near the surface of the bacterial cell wall (*16*), since the products released by this enzyme may diffuse into the surrounding medium. All mixed casein starters contained fast-coagulating *S. cremoris* organisms capable of stimulating growth of slow organisms. This stimulation may allow the dominance of mixed starters by organisms which, on isolation, grew to only limited population densities in milk.

Mixed casein starters usually stopped producing acid at pH 4.5-4.6, whereas typical single strain cultures fell to pH 4.2-4.3 under the same conditions. This difference has considerable practical significance since in lactic caseinmaking the higher pH values are preferred because excessively low coagulum pH has detrimental effects both in the processing and properties of the product. *S. lactis* cell wall proteinase activity declined rapidly as the assay pH was dropped below 6 (*16*) suggesting that the ability of fast-coagulating organisms to hydrolyze proteins in milk decreases as the pH falls. In mixed cultures, containing predominantly proteinase-deficient organisms, cell division may be prevented at higher pH values through nitrogen limitation thus accounting for the higher final pH of coagulated mixed cultures. Lower final pH values from addition of protein hydrolysate to these mixed starters supports this explanation. Thus a preponderance of proteinase-deficient organisms in mixed casein starters may fortuitously produce a coagulum near the preferred pH value for processing as well as minimal loss of casein yield from starter proteolysis.

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Starters and Bacteriophages in Lactic Acid Casein Manufacture

II. Development of a Controlled Starter System

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ABSTRACT

A controlled starter system was used for the first time in commercial lactic acid casein manufacture in New Zealand. Multiple starters of up to four components were constructed from 18 recently derived *Streptococcus cremoris* isolates which were not lysed by any of the phages in the collection of the New Zealand Dairy Research Institute. During the first season of casein manufacture, phages attacking 17 isolates were detected in the casein whey. Of these, 12 prevented adequate acid production by the appropriate host even at levels below 1 phage per 10 to 1000 ml in the milk before starter addition. In contrast, the first detected phages attacking the other five isolates did not significantly influence the rate of acid development; use of these starters continued until phages which eliminated acid production appeared. An alternative starter system based on the continuous selection of "phage-tolerant" cultures was investigated. Regular addition of whey, from previous manufacture, to the individual mother cultures of each component permitted long-term use of the multiple starter. This procedure of continued selection for phage-tolerant organisms has been used successfully for a complete season in a major casein factory.

Most of the casein manufactured in New Zealand is precipitated from pasteurized skimmilk by the lactic acid produced during growth of starter bacteria. Traditionally, mixed starters of unknown and variable bacteriological compositiion have been grown in the milk to produce the lactic acid (*10*). Until recently there has been little incentive for casein manufacturers to alter starter handling procedures, but the use of a controlled starter system now appears to offer advantages for the manufacture of new whey protein products as well as traditional lactic casein. For cheesemaking, carefully selected single strains have been used individually or in various combinations in starter rotations for many years (*3*). However, preliminary trials indicated that similar starter systems did not perform as satisfactorily in commercial casein factories as the somewhat crude methods already employed.

This paper describes construction of multiple starters and techniques necessary for their successful use in casein factories.

MATERIALS AND METHODS

Selection and characterization of isolates

Twenty mixed starters from the collection of the New Zealand Dairy Reserach Institute (NZDRI) known to contain strains of *Streptococcus cremoris, Streptococcus lactis, Streptococcus diacetilactis,* and *Leuconostoc* species were plated on agar media which allowed visual differentiation of *S. cremoris* from the other bacteria (*7, 9*). From each mixed starter, 20 *S. cremoris* colonies were inoculated into tubes of autoclaved reconstituted skimmilk (RSM, 9.5% solids) and incubated for 24 h at 30 C. Coagulated cultures were maintained by daily subculture (1% inoculum) in autoclaved RSM at 22 C.

Quantities of RSM (100 ml) were pasteurized (62.5 C for 30 min), cooled, inoculated from a culture of the test isolate (0.1 ml, 24-h culture at 22 C) and incubated at 22 C. The time (h) taken to reach pH 4.6 was recorded. This value was adopted as the coagulation time of the isolate. Repeated measurements on the same organism showed that 95% of determinations were within 1 h of the mean.

Twenty-eight phages of different host range were obtained from the collection of the NZDRI and diluted to about 10^8 plaque forming units (pfu) per milliliter. Samples (5 — 10 μl) were spotted on lawns of the test isolates prepared by soft agar overlay on M16 agar plates (*5*). Plates were incubated at 30 C for 18 h. The isolates showing no obvious phage reactions were retained. Different isolates were also distinguished from each other by their chain lengths and growth rates during culture in M16 broth.

Prophage inducibility of *S. cremoris* isolates was tested by treatment with mitomycin C as described by Lowrie (*4*). Dilutions of lysates were tested for phages by spotting on lawns of 20 potential indicator strains of lactic streptococci (*10*).

Examination of interactions between starter components

Lawns of each isolate were prepared from log-phase broth cultures. Each of these 18 cultures was then spotted onto the lawns. After incubation at 30 C plates were examined for evidence of any stimulatory or inhibitory interactions. In addition, coagulation times of paired strains in milk cultures (inocula 0.1% and 0.01%) were determined and compared with values for cultures of the individual isolates (inoculum 0.1%).

Starter handling at the commercial factory

Mother cultures maintained at 22 C by daily subculture in autoclaved RSM were replaced once a week from stocks subcultured weekly and held at 4 C. Bulk starters were grown from 0.1% inocula at 25 C in separate vessels (100 liters) containing heat-treated (90 C for 30 min) skimmilk. No precautions were taken to exclude phage from bulk starter vessels. After 16 h, equal volumes of each culture were combined in a mixing tank. Closed coagulation silos (27,000 liters) containing pasteurized skimmilk at 22-26 C were inoculated with 0.1-0.2% of this multiple starter. Following incubtion for 17-21 h the coagulum was "cooked" by heating to about 55 C. Up to 250,000 liters of skimmilk were processed daily. The plant including starter vessels was cleaned-in-place with hot NaOH and iodophor solutions.

Phage detection in casein whey

Whey samples from all coagulation silos were combined and tested daily for phage. Samples were centrifuged (30,000 × *g* for 1 min), filtered through a sterile membrane (0.45-μm pore size, Swinny filter,

Millipore Corp.) and spotted undiluted and at several dilutions on lawns of the components of the multiple starter. When lytic zones or plaques were observed the sensitive culture was withdrawn from the starter system and replaced by a culture which gave no obvious reaction with the phage.

Determination of "phage-sensitivity" of starters

Newly isolated phages were purified using standard procuedures. A measure of the sensitivity of a host organism to a specific phage was derived from the maximum initial phage level which allowed coagulation within 1 h of the time required by the control culture. Since plaque assays were often unreliable (see text), approximate phage levels in cultures and whey samples were determined by the dilution end-point method (*1*).

Development of "phage-tolerant" cultures

Autoclaved RSM cultures (inoculum 0.1%) were infected with 1-10 particles/ml of the appropriate phage isolated from the factory whey. Cultures were incubated at 22 C until coagulation occurred and then maintained by daily subculture at 22 C. The coagulation times of these cultures were determined at intervals for up to 30 subcultures and whey samples tested for the presence of phages.

RESULTS

Properties of S. cremoris isolates

Eighteen distinguishable isolates were obtained which had coagulation times of 16-20 h. Different isolates showed no evidence of any interactions when growing together either in milk or on agar plates. Treatment with mitomycin C induced complete lysis of isolates 186 and 240, suggesting that they may be lysogenic, but lysates gave no signs of lytic reactions when spotted on lawns prepared from a broad range of possible indicators.

Use of multiple starters for caseinmaking

Changes in proportions of component bacteria during growth of the multiple starter could not be determined since there were no techniques allowing adequate differentiation of *S. cremoris* isolates. A balance of the individual starters in the coagulation silos was ensured by growing cultures in separate bulk starter vessels. Although increasing the number of phage-unrelated organisms in the multiple starter reduces the effect when any one is eliminated by phage, operating more than four bulk starter vessels was impractical. Therefore multiple starters with four components were used. The effect on the coagulation time when component organisms were lysed by phage was assessed in the laboratory. Lysis of one starter made no detectable difference but the lysis of two or three staters together increased coagulation times by 1-2 h depending on the particular phage and its initial level.

Phages attacking 17 of the 18 different isolates appeared during the first season of manufacture. No phage was detected for isolate 114 which was used continuously in caseinmaking over two seasons. Usually a phage was isolated within one week of the starter being introduced although some organisms remained free of detectable phage for up to 9 weeks (Table 1). Derivatives of isolates 176 and 266 were obtained which were not lysed by their respective phages isolated from factory whey. These derivatives (318 and 320, respectively) were reintroduced but different phages which caused complete lysis were isolated within one week (Table 1). For 12 of the phages detected, addition of 1-10 particles to a freshly inoculated 100-ml culture of the appropriate host stopped acid production before the pH fell to 4.6. These host strains were classified as having a phage sensitivity of less than 10^{-1} phage/ml (Table 2).

TABLE 1. S. cremoris *isolates used in multiple starters and the days (in brackets) of use before detection of phage*

114 [a],	134 (51),	158 (40),	168 (63)
170 (5),	172 (2),	176 (5),	184 (29)
186 (17),	188 (6),	224 (5),	240 (22)
242 (3),	266 (2),	272 (5),	290 (8)
318 (3),	320 (6)		

[a]No phage detected.

TABLE 2. *"Phage-sensitivity" of* S. cremoris *isolates under typical caseinmaking conditions*

Group	Isolate	Phage-sensitivity[a]	Maximum titer recorded (phage/ml)
A[b]	134, 168, 172, 176, 184, 188, 266, 272, 318, 320	$<10^{-1}$	10^{10}–10^{11}
	242, 290	1–10	10^{10}–10^{11}
B[c]	158	10^{4} –10^{5}	10^{9} –10^{10}
	170	10^{1} –10^{2}	10^{6} –10^{7}
	186	10^{2} –10^{3}	10^{8} –10^{9}
	224	$>10^{5}$	10^{8} –10^{9}
	240	$>10^{6}$	10^{9} –10^{10}

[a]Maximum initial titer (phage/ml) allowing normal coagulation time (± 1 h).
[b]Acid production by group A strains eliminated by phage.
[c]Acid production by group B strains not eliminated by phage.

Despite the often rapid appearance of phages, the rate of acid production by these multiple starters was regular and the limited incidents of slow coagulation were not due to phage attack. A multiple starter consisting of isolates 114, 134, 158, and 240 gave regular acid production when used in small-scale casein manufacture at NZDRI. In contrast to the commerical factory, no phages were isolated from NZDRI casein whey during two seasons of manufacture thus emphasizing the importance of evaluating experimental starter systems in commercial factories where phages seem more likely to appear.

The first phages isolated for starters 158, 170, 186, 224, and 240 did not eliminate acid production under caseinmaking conditions and whey samples of high titer, determined by the dilution end-point method, gave zones of incomplete lysis when spotted on lawns. The coagulation times of the first three starters were unaffected until the initial titer of added phage reached a relatively high level (Table 2) while acid production by isolates 224 and 240 was not measurably influenced even when the phage present at inoculation exceeded 10^{5}/ml. Initial titers of 1/ml of the phages attacking isolates 158 and 240 yielded 10^{8} phage/ml in the coagulated culture

(inoculum 0.1% in pasteurized RSM at 22 C), a phage multiplication factor under simulated caseinmaking conditions of 10^8. In contrast the remaining 12 phages had multiplication factors of more than 10^{11}. The isolates with low phage multiplication factors were re-introduced into the multiple starter system at the casein factory even though phages were known to be present. However, phages which could eliminate acid production were isolated for these starters between 2 and 7 months after their re-introduction.

A similar progression of phages possessing successively higher multiplication rates was also found when the isolate *S. cremoris* 166 was introduced into cheese factories. The first phages isolated from the whey samples of eight cheese factories were used to determine the "phage-sensitivity" (the highest initial phage level permitting normal acid production) of isolate 166 under caseinmaking conditions. The sensitivity to three phages was less than 10^{-1} phage/ml. his level was more than 10^5 phage/ml for the other five phages.

Use of continuously selected "phage-tolerant" starters

Cultures tolerant to the phages which prevented adequate acid development were readily prepared in the laboratory for nine of the 12 isolates listed in group A (Table 2). After several subcultures the coagulation times reached constant values which were about 2 h greater than the parent cultures (Table 3). After subculturing 30 times, most cultures still contained the original phage (Table 3). Phage were not detected in some cultures when tested in either mid-logarithmic growth or after coagulation. Whey from these cultures was added to freshly inoculated cultures of the parent starter. After appropriate incubation, phage tests still gave negative results. Phage-tolerant cultures of isolates 134, 318, and 320 with a satisfactory rate of acid production could not be prepared.

TABLE 3. *Selectionof "phage-tolerant" cultures and the change of coagulation time with subculture*

Isolate	Number of subcultures	Coagulation time (h)[a] Phage-tolerant culture					Parent Culture
		1	3	7	11	30	
168		>24	19	21	19	20(+)[b]	18
172		>24	21	22	20	20(+)	19
176		>24	>24	>24	21	20(–)	18
184		>24	21	20	19	19(+)	20
188		>24	>24	21	20	21(–)	18
240		23	21	20	20	20(+)	20
242		22	23	23	22	22(+)	18
266		>24	21	22	20	19(+)	16
272		>24	22	23	22	21(–)	20
290		>24	22	21	20	21(+)	20

[a]Time (h) to reach pH 4.6 in pasteurized RSM at 22 C (inoculum 0.1%).
[b](+) Original phage present, (–) original phage not detectable.

Substantial changes in starter handling at the casein factory were made to capitalize on these findings. Filter sterilized whey, from previous manufacture, was added to each mother culture daily to continuously select for organisms able to produce acid in the presence of phages. In addition, the four mother cultures (selected from *S. cremoris* isolates 114, 158, 170, 186, and 224) were inoculated into a single bulk starter vessel. The coagulation times of starters subjected to this continued selection process were compared with the original parent cultures. Over 3 months the coagulation times had increased by about 3 h. Regular monthly replacement of factory cultures from frozen stocks of the original isolates limited this increase to less than 2 h. Raising the temperature of the skimmilk at inoculation compensated for this reduction in starter activity.

DISCUSSION

Most studies of lactic streptococci have concerned their use as starters in cheesemaking where the requirement for fast acid producers at temperatures of 30-40 C severely restricts the number of strains available. In casein manufacture the requirements of the culture are less rigorous and a much greater range of strains produce acid at an acceptable rate. Whitehead and Hunter (*11*) instituted rotations of phage-unrelated single strain starters for cheesemaking to prevent slow acid production caused by phage attack. The system depends on excluding phage from the bulk starter vessel and limiting the initial level of infection and rate of multiplicationin the cheese vat, principles of starter handling which have not changed to the present day (*3*). In caseinmaking, complete control of starters would require excluding phage from both the bulk starter vessel and the coagulation tanks but this is impractical under commercial conditions. Starters in casein manufacture are more vulnerable to phage attack than in cheesemaking. Whereas the rennet gel affords protection from phage attack in cheese manufacture (*6*), there is no rennet gel in lactic caseinmaking. In addition the cells undergo twice as many doublings during casein manufacture thereby allowing more cycles of phage replication and hence an increased likelihood of total lysis before sufficient acid has been produced.

A multiple starter system comprised of *S. cremoris* isolates with no known phages failed because phages capable of eliminating acid production appeared at high frequency. The origin of these phages is speculative. Some were presumably selected from the factory environment or the milk supply while others may have arisen as host range mutants or from host-controlled modifications. A multiple starter consisting of the isolates with apparently high phage-tolerance was tested. This initial phage-tolerance may have been due to poor phage absorption by the host or a low multiplication efficiency of the phage. The gradual appearance of more virulent phages capable of eliminating acid production also rendered this starter system impractical.

Reiter (*8*) suggested that the lysogenic strains present in a mixed culture obtained from a Scottish cheese factory conferred on the culture additional protection from phage attack thus permitting its use for long periods. Lysogeny appears to be relatively widespread

amongst the single strains isolated as cheese starters (*4*) and of the 18 casein starter isolates tested, two were inducible suggesting possible lysogenicity. However, phages appeared which eliminated acid production by these starters. Such results are not unexpected since it is well established that lysogenic immunity is confined to infection by the same or very closely related phages (*2*).

A system based on continuous selection of phage-tolerant starters was found to be the only practical means of using starters in a controlled manner for caseinmaking. Such cultures were produced readily in the laboratory by adding low levels of virulent phages to cultures of the respective hosts. In most instances cultures still contained the original phages after subculturing 30 times. These cultures presumably comprised the relatively stable balance of susceptible and resistant bacteria typical of classical pseudolysogeny (*2*). At the factory regular addition of whey, from previous manufacture, to mother cultures caused a continuous selection of phage tolerant starters. although the rates of acid production by the individual cultures decreased slightly, overall acid production by the multiple starter was kept constant for an entire manufacturing season by slight adjustment of manufacturing conditions and periodic renewal of cultures.

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A Research Note

Productivity of Boiled and Autoclaved Violet Red Bile Agar

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ABSTRACT

Violet Red Bile (VRB) Agar which was boiled had significantly greater productivity than did autoclaved VRB Agar when a 4-h *Escherichia coli* culture was enumerated. There was no significant difference between the boiled and autoclaved medium when a 16-h culture was examined.

The 13th Edition of *Standard Methods for the Examination of Dairy Products* (SMEDP) states in Chapter 4 that Violet Red Bile (VRB) Agar may be used after boiling for 2 min. It further states that VRB . . . . "can be autoclaved without serious impairment of productivity" (*1*).

Since the philosophy of SMEDP editors has been to recommend only one method rather than alternative procedures, and since the statement regarding preparation of VRB either by boiling or autoclaving is apparently without documentation, this study was undertaken to determine the preferable method.

MATERIALS AND METHODS

Culture

Escherichia coli ATCC 25922, used throughout the experiment, was maintained on Brain Heart Infusion slants and transferred weekly. For use, 9 ml of Nutrient Broth (Difco) was inoculated from the slant and incubated at 35 C for 20 h.

A low form culture flask containing 1 liter of Nutrient Broth, adjusted to 35 C, was then inoculated with 1 ml of the 20-h seed culture and rotated on a mechanical shaker at 100 rpm. Incubator facilities were not adequate to allow incubation of the low form flask at a temperature other than 35 C. Samples were taken after 4 and 16 h of incubation (predetermined to represent mid-log and mid-stationary phases of growth).

A known pure culture was used for this study rather than a natural source, such as raw milk, to make the procedure entirely reproduceable.

Media

VRB and Standard Plate Count (SPC) agars were both manufactured by Difco. VRB was prepared according to manufacturer's instructions and sterilized either by boiling for 2 min, as described in Chapter 4, 13th Edition SMEDP (*1*), or by autoclaving at 121 C for 15 min. SPC Agar was prepared according to manufacturer's instructions and autoclaved at 121 C for 15 min. All media were prepared fresh the day of use.

Procedure

The 4- and 16-h cultures were diluted to a working level in phosphate buffered dilution water prepared according to 13th Edition of SMEDP. The culture was plated in replicates of 20 plates each for VRB-boiled, VRB-autoclaved,a nd SPC agars. Plates were incubated at 32 C for 18-24 h and enumerated using a Quebec Darkfield Colony Counter. The plating scheme and statistical analysis were done as described in sections 4.86 and 4.87 of the 13th Edition of SMEDP.

RESULTS

The procedure was done on two separate occasions, Trial 1 and Trial 2, and results are presented in Tables 1 and 2. For each trial, the 4-h culture exhibited significantly greater yields on the VRB-boiled than on the VRB-autoclaved medium. The SPC Agar was included to give some indication of culture yield on a non-selective

TABLE 1. *Comparison of yield of VRB-boiled, VRB-autoclaved, and SPC agars when tested with* E. coli *ATCO 25922*

	4-h Culture				16-h Culture		
	VRB agar				VRB agar		
	Boiled	Autoclaved	SPC agar		Boiled	Autoclaved	SPC agar
Trial 1							
# of replicates	20	20	20		20	20	20
$\bar{X}$ plate count							
(× 10^6)	301.4	275.85	294.7	(× 10^8)	48.3	46.1	49.8
Range (× 10^6)	268-334	239-323	270-331	(× 10^8)	37-59	34-61	39.65
$\sim$	15.53	22.32	19.74		5.71	7.06	7.27
Trial 2							
# of replicates	20	20	20		20	20	20
$\bar{X}$ plate count							
(× 10^6)	303.4	270.9	309.6	(× 10^8)	49.9	47.2	58.9
Range (× 10^6)	253-334	227-298	285-357	(× 10^8)	37-63	37-59	41-66
δ	18.3	16.9	16.3		7.0	6.6	6.6

TABLE 2. *t-Values for differences in productivity between VRB-boiled. VRB-autoclaved, and SPC agars*

	Trial 1		Trial 2	
	4-h Culture	16-h Culture	4-h Culture	16-h Culture
VRB-boiled versus VRB-autoclaved	4.194[a]	2.03	5.83	1.26
SPC versus VRB-boiled	1.192	0.72	1.13	4.21
SPC versus VRB-autoclaved	2.82	1.64	7.37	5.65

[a]A t-value greater than 2.70 is significant.

medium. The VRB-boiled and SPC counts were comparable for both trials for the 4-h culture, but the difference in productivity between SPC and VRB-autoclaved agars was significant, indicating that the VRB-autoclaved medium is significantly more inhibitory to a young culture than either VRB-boiled or SPC agars.

For the 16 h mid-stationary culture, no significant differences in productivity were noted between VRB-autoclaved and VRB-boiled agars in either trial. In one trial, the SPC count was significantly greater when compared to results obtained with VRB when prepared either way, but the two VRB results were comparable.

Consequently, it appears that autoclaved VRB is significantly inhibitory to young, actively growing cultures, whereas there seems to be no effect on a more mature culture. Therefore, it is recommended that VRB agar be boiled for 2 min rather than autoclaved before use.

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Characterization of Waste Loads from Unit Processes of a Commercial Potato Canning Operation

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ABSTRACT

Characterization of waste loads from unit processes in a commercial potato canning operation revealed that the lye peeler and reel washer which removed the peel accounted for approximately 95% of the total load of total solids, suspended solids, volatile solids, COD, and BOD, and for 46% of the total wastewater flow. The total loads of suspended solids, COD, BOD, and wastewater from this relatively large plant were 65.2 lb/ton, 88.4 lb/ton, 54.7 lb/ton, and 2,950 gal/ton of raw product processed respectively. The BOD and wastewater loads from this plant were significantly less than those reported previously for a smaller operation. Results on unit waste loads serve to better define the standard raw waste load from potato canning as the sum of the unit processes.

Characterization studies of liquid wastes from potato processing generally report the load of suspended solids, biochemical oxygen demand (BOD), and wastewater for the composite effluent (*8,-10*). Studies by Weckel et al. (*11*) and the Environmental Protection Agency (EPA) (*5*) include additional data on waste loads from unit operations. Weckel et al. (*11*) found that lye blanching and abrasion peeling accounted for 86% of the total load of suspended solids, 98% of the BOD, 97% of the COD, and 82% of the wastewater flow (*11*). The effluent guidelines study conducted by EPA (*5*) includes data on wet caustic peeling of potatoes which should be applicable to canned as well as to frozen and dehydrated potatoes. Significant differences exist in the total BOD loads reported by Weckel et al. (*11*) and those reported by EPA (*5*) and others (*8-10*). The value of 186 lb BOD/ton obtained by Weckel et al. (*11*) is over three times as great as other reported BOD loads from potato processing. These differences in waste load per ton of raw product may be due to differences in the size of processing operation as noted by Bough (*1*) and Bough and Badenhop (*2*) for pimienton canning wastes. Since the results of Weckel et al. (*11*) are the only published detailed data known to this author on waste loads of unit processes in potato canning, clarification of these differences is important.

Previous reports have dealt with the characterization (*1, 2*) and treatment of vegetable canning wastes including the application of polymeric flocculating agents to canning wastes from pimientos (*3*). Emphasis was placed on segregation and separate treatment of concentrated unit processing wastes which contain a major portion of the total waste load. Such studies have been extended to potato wastes to determine the contribution of each unit effluent to the total waste load and to identify concentrated sources of wastes. The investigations included detailed data on waste loads from unit operations of a potato canning process substantially larger than that reported by Weckel et al. (*11*). The data are presented here to help clarify earlier differences.

EXPERIMENTAL

A flow diagram of the unit operations and effluents sampled in the commercial potato canning operation is shown in Fig. 1. The styles

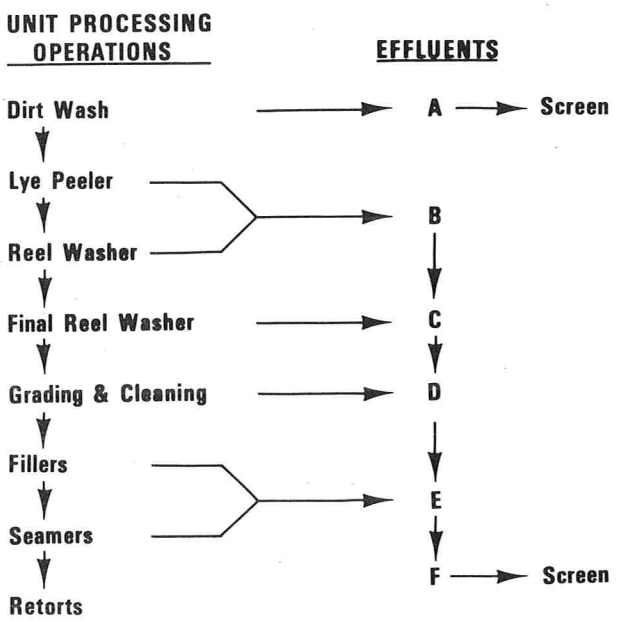

Figure 1. *Flow diagram of unit processing operations and effluents in a commercial potato cannery line*

canned at this plant were whole, diced, and sliced white potatoes. The effluent from the washing operation (A) was separate from the other unit operations. Potatoes were washed in a reel washer and conveyed by belt to a large immersion chamber for lye application. Most of the peel was removed by the abrasive action of the first reel washer. Lye which drained from the application chamber and the peel residue and effluent from the first reel washer entered a common floor drain (effluent B). The product was conveyed by belt through another reel washer (effluent C), over tables for hand grading and cleaning (effluent D), and into

Hand-Pack fillers. Effluents from fillers and can seamers entered a common floor drain (effluent E). Effluents B through E combined to form a composite flow (effluent F) which was sampled for composition, but the location of the only available sampling site under a dock precluded the determination of its flow rate before it was mixed with processing wastes from other vegetables. Thus, the total wastewater flow for potato canning was estimated by the summation of the wastewater flows from the unit processes.

The flow rate of effluent A was determined by measuring the rate of fill of a container of known volume. Effluents B, C, and D were measured with a trapezoidal weir as described previously (*1*). Effluent E was measured by a floating block method described by Mercer (*7*). The daily flow rates were divided by daily production rates to express flow as gallons of wastewater per ton of raw product.

Time-composite samples of each effluent were taken on different days by collecting 600 ml every 30 min over a 2-h period. Each sample was passed through a 20-mesh screen. Five different samples of each effluent were analyzed. Analyses were begun within 15 min of collection and were done in duplicate as previously described (*1*). Cooling water was discharged separately from processing wastes and was not included in this study.

Results obtained on concentrations of waste components, flow rates, and production rates were used to calculate waste loadings as pounds of waste component per ton of raw product.

RESULTS AND DISCUSSION

Concentrations of waste components in potato canning wastes as determined in this study are shown in Table 1. Effluent A from the reel washer used to remove soil and debris contained per liter, 682 mg fixed solids, 1,488 mg suspended solids, 1,443 mg COD, and 485 mg BOD. The most concentrated effluent issued from the lye chamber and the reel washer which removed the peel. It contained, per liter, 3,482 mg fixed solids, 5,504 mg suspended solids, 7,481 mg COD, and 4,648 mg BOD. Effluents C-E were much less concentrated, particularly the grading and cleaning washwater. These dilute wastewaters could perhaps by recycled in a countercurrent manner to the first two reel washers which remove soil and peel.

Effluent F was a composite of effluents B-E; effluent A from the dirt wash in the receiving shed did not combine with these wastes but was eventually mixed with wastes from other vegetables. The analytical results on effluent F are shown in Table 1 for comparison to the results obtained by summation of the unit waste loads in all the unit effluents. These total waste load figures are shown in the last line of Table 2. For comparison with Table 1 results, waste load data (lb/ton) are converted to concentration data (lb/gal) by dividing by wastewater flow data (gal/ton). The lb/gal expression of concentration is then converted by dividing by 8.345×10^{-6} into the more common mg/1 expression. For example, the total COD load shown in Table 2 is 88.4 lb/ton. When converted to mg/1, the apparent COD concentration in the total effluent was 3,590 mg/1. This value compares favorably with the analytical value of 2,780 ± 821 shown in Table 1 for the COD of the composite of effluents B-E. The calculated value for the BOD concentration in the final effluent was 2,222 mg/1 and the value shown in Table 1 for the composite of effluents B-E is 2,216 ± 339 mg/1. These comparisons indicate validity in determining total waste loads by summation of unit loadings as was done in this study.

While the composite effluent characterized in Table 1 does not contain the dirt wash effluent, it is still considerably more concentrated than the composite analyses reported by Weckel et al. (*11*). They reported the concentrations of total solids, suspended solids, COD, and BOD in wastewater from a potato canning operation to be, per liter, 607 mg, 32 mg, 280 mg, and 186 mg, respectively. Corresponding results in this study were,

TABLE 1. *Characterization of wastes from canning of potatoes*

Parameter	A Dirt Wash	B Lye Peeler & Reel Washer	C Final Reel Washer	D Grading & Cleaning	E Closing	F(B-E) Composite
Total Solids, mg/1	2,191 ± 382[a]	10,978 ± 570	404 ± 68	336 ± 81	449 ± 94	4,046 ± 478
Fixed Solids, mg/1	682 ± 296	3,482 ± 1,286	78 ± 19	103 ± 41	170 ± 60	1,014 ± 329
Volatile Solids, mg/1	1,518 ± 220	7,496 ± 518	321 ± 51	233 ± 56	280 ± 61	3,028 ± 150
Suspended Solids, mg/1	1,488 ± 421	5,504 ± 1,328	84 ± 24	22 ± 19	29 ± 28	1,856 ± 515
Dissolved Solids, mg/1	702 ± 153	4,963 ± 2,518	320 ± 72	311 ± 85	420 ± 105	2,188 ± 40
Settleable Solids, ml/1	26 ± 8	137 ± 3	3.3 ± 1.4	0.4 ± 0.5	0.2 ± 0.3	70
pH	6.6 ± 0.5	12.5 ± 0.3	6.8 ± 0.7	6.4 ± 0.6	6.1 ± 0.3	12.0 ± 0.2
Total Alkalinity, mg/1	36 ± 5	4,619 ± 1,219	40 ± 12	36 ± 7	36 ± 10	1,275 ± 293
COD, mg/1	1,443 ± 167	7,481 ± 932	205 ± 60	84 ± 26	110 ± 39	2,780 ± 821
BOD_5, mg/1	485 ± 181	4,648 ± 906	165 ± 32	82 ± 19	96 ± 27	2,216 ± 339

[a]The mean values and standard deviations shown were obtained by averaging the results from five time-composite samples of each unit effluent analyzed in duplicate.

TABLE 2. *The production of waste components per ton of raw potatoes canned*

Unit effluent	Total solids (lb/ton)	Suspended solids (lb/ton)	Volatile solids (lb/ton)	COD (lb/ton)	BOD (lb/ton)	Waste water (gal/ton)
A. Dirt wash	3.7	2.5	2.5	2.4	0.8	200
B. Lye peeler & reel washers	123.7	62.0	84.5	84.3	52.4	1350
C. Final reel washer	2.2	0.5	1.7	1.1	0.9	650
D. Grading & cleaning	1.1	0.1	0.7	0.3	0.3	375
E. Closing	1.4	0.1	0.9	0.3	0.3	375
Total, A-E	132.1	65.2	90.3	88.4	54.7	2950

per liter, 4,046 mg, 1,856 mg, 2,780 mg, and 2,216 mg. These differences are in part due to the larger volume of water usage (3,576 gal/ton) by the plant studied by Weckel et al. (*11*) in comparison to the plant characterized in this study which was 2,950 gal/ton as shown in Table 2.

The calculations shown in Table 2 indicate that approximately 95% of the total load of waste components was found in effluent B from the lye peeler and first reel washer. These wastes were contained in 1,350 gal of wastewater per ton of raw potatoes processed which accounted for 46% of the total wastewater load. These results are in close agreement with those of Weckel et al. who found 86-98% of the waste components were produced by lye blanching and abrasion peeling (*11*).

The recent EPA Effluent Guidelines survey on apples, citrus, and potatoes included data on unit processes for frozen and dehydrated potatoes (*5*). Even though the unit processes are different for canned potatoes, EPA's data on waste load from the wet caustic peeling operation should be similar to the same unit operation in potato canning. The reported loads for suspended solids, BOD, and wastewater were, per ton, 57.2 lb, 40.41 lb, and 719 gal, respectively (*5*). Thus, EPA's data on suspended solids and BOD from lye peeling are comparable to those shown in Table 2: 62.0 and 52.4 lb/ton, respectively. The wastewater load shown in Table 2 for the lye chamber and reel washers, 1350 gal/ton, is greater than the figure of 719 gal/ton given in the EPA report but less than the 2,935 gal/ton calculated from the data of Weckel et al. (*11*) for the sum of lye blanching, tumble peelers, and abrasion peeling or the 1,561 gal/ton given for lye blanchers and tumble peelers alone.

The total or composite waste load of BOD reported by Weckel et al. (*11*) of 186 lb/ton was greater than the 54.7 lb/ton shown in Table 2 or the values of 22.1 and 45.8 lb/ton given in the EPA report (*5*). These comparisons along with other literature data are shown in Table 3.

TABLE 3. *A comparison of composite waste loads reported for canned, frozen, and dehydrated potatoes*

Product	Susp. solids (lb/ton)	BOD (lb/ton)	Wastewater (gal/ton)	Reference
White potatoes	50	80	4,000	Rose et al. (*9*)
White potatoes	53	47	3,400	NCA (*8*)
Potatoes	52	50	3,070	Schmidt et al. (*10*)
Canned potatoes	32	186	3,576	Weckel et al. (*11*)
Frozen potatoes	38.8	45.8	2,710	EPA (*5*)
Dehydrated potatoes	17.2	22.1	2,100	EPA (*5*)
All product styles	31.8	36.2	2,460	EPA (*5*)
Canned potatoes	65.2	54.7	2,950	This study

The data reported by NCA (*8*), Rose et al. (*9*), and by Schmidt et al. (*10*) are in close agreement with the total loads, per ton, of 65.2 lb suspended solids, 54.7 lb BOD, and 2950 gal wastewater reported in the present study. Thus, the results reported here on unit operations help to define the standard raw waste load in terms of unit processes for potato canning operations that are typical of those surveyed by NCA, Rose et al., and Schmidt et al. (*8-10*).

The difference in the results of Weckel et al. (*11*) shown in Table 3 in comparison to this study may be due to the size of processing operation. It is noted that the production rate reported by Weckel et al. was approximately one-half of the average rate observed in the present study. In a previous study of pimiento wastes, a plant processing at approximately ⅓ the rate of another produced, per ton, 6.4 lb suspended solids, 66.5 lb BOD, 140.1 lb COD, and 6117 gal of wastewater (*2*). Corresponding values for the larger plant were 3.2, 35.4, 60.2, and 4840 (*1*). Thus, the waste load per ton of product was approximately twice as great from the smaller operation. In the case of the smaller potato canning operation characterized in the study of Weckel et al. (*11*), the BOD load was 186 lb/ton and the wastewater load was 3,576 gal/ton. Corresponding values in the present study were 54.7 lb/ton and 2,950 gal/ton, respectively. Thus, the larger plants were able to process with a greater efficiency for product packed and generated fewer wastes per ton of raw product than the smaller operations. Such "economies-of-scale" are important considerations in the design of processing plants and in the promulgation of effluent standards to regulate the discharge of wastes. The cost of waste treatment per ton of raw product will usually be higher for a small processor than for a larger plant because of proportionally greater generation of wastes and wastewater per ton.

The potato canning operation analyzed in this study employed a conventional "wet" caustic method for peeling which involved immersion in a hot lye bath and removal of the peel by the abrasive action of reel washers. Thus, lye and peel residue entered the wastewater stream. A new process, called "dry" caustic because the peel residue is removed by rubber rolls or disks, segregates peel wastes as a thick sludge and uses a minimum of wash water (*6*). Based on commercial scale comparisons, Cyr (*4*) found that "wet" peeling required about four times as much water, produced 4.5 times as much BOD per ton of raw potatoes, and 3.75 times as much dry solid material to be dewatered per ton as did "dry" caustic peeling. Thus, the plant surveyed here can consider a process change from "wet" to "dry" caustic peeling as a means of reducing wastes from potato processing. Such changes could become necessary as production volume expands to reduce the hydraulic and waste load on their biological treatment system.

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New Plastic Components

PLASTIC CONVEYOR COMPONENTS for material handling systems are detailed in a new catalog available from Norton Company's Plastic Component Operations. The use of self-lubricated engineering plastics in the manufacture of Norton conveyor components is cited as the reason for the products' low friction and excellent wear characteristics. In many applications the product may be operated without external lubrication.

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Exposure of Man to Mercury. A Review [1,2]

I. Environmental Contamination and Biochemical Relationships

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ABSTRACT

Mercury is naturally concentrated in geographical belts, but geological cycling has distributed the element in all strata of the earth. Natural concentrations of mercury are approximately 100 ppb in soil, 0.06 ppb in fresh water, 0.01-0.30 ppb in sea water, and 0.003-0.009 $\mu g/m^3$ in air. Concentrations vary, being highest near mineral deposits. The concentration of mercury in some areas has been significantly increased by human carelessness. An epidemic among Japanese fishing families, death of Swedish wildlife, and discovery of elevated mercury levels in American fish focused attention on this problem. The discovery that certain species are capable of methylating inorganic mercury indicates pollution with any chemical form of mercury is dangerous.

Alkylmercurials are the most dangerous form of mercury in the environment. Alkylmercurials are absorbed from the gastrointestinal tract, diffuse across the blood-brain carrier, and pass through the placental membrane in significantly higher proportions than other mercury compounds. The whole body half-life of methyl mercury in humans is 76 ± 3 days compared to half-lives of 37 ± 3 days for men and 48 ± 5 days for women observed for mercuric salts. Not readily broken down, sufficient concentrations of methyl mercury can cause irreversible damage to the central nervous system. Renal damage usually results from high levels of aryl- or alkoxyalkylmercurials and inorganic mercury; however, vapors of elemented mercury can damage the central nervous system. Organic mercury compounds cause chromosome changes, but the medical implications resulting from levels of mercury in food are unknown. The concentration of mercury in red blood cells and hair is indicative of the exposure to alkylmercurials. On a group basis, blood and urine concentrations of mercury may corrrelate with recent exposure to mercury.

INTRODUCTION AND HISTORICAL DEVELOPMENT

Even though mercury is a natural element, present since the formation of the earth and known to man since prehistoric times, a great concern over its toxicity has suddenly developed during the past 20 years. The first awareness of potential danger of mercury stems from an incident that occurred near Minamata Bay in Japan. In 1953, several families in the area suffered from a mysterious neurological illness which by late 1956 had reached epidemic proportions. Several departments in Kumamoto University investigated this disease which affected the peripheral nervous system, cerebellum, hearing, vision, and less frequently, pyramidal tracts. Progressive brain damage was indicated in servere cases. Forty-one of 111 reported cases were fatal, and most survivors were incapacitated to various degrees. In 1959, McAlpine and Araki (*159, 160*) reported that the cause of the disease was unknown, however, two facts had been established: first, that a relationship existed between fish consumption and occurrence of the disease, and second, that the fish responsible for the disease were caught in Minamata Bay. At that time, the causative agent appeared to be a toxic chemical which was added to the bay with the effluent from a nearby fertilizer plant. The authors stated that magnesium, selenium and thallium were the chemicals which had received the most attention, however, the similarities of symptoms to those noted by Hunter et al. (*111*) for methyl mercury poisoning had been recognized. In 1968, the Kumamoto University study group published a complete report on the disease expressing agreement that the toxic agent was methyl mercury. The mercury had drained into the bay with the effluent from an acetaldehyde plant, in which it was used as a catalyst, and was absorbed and concentrated in the tissues of fish and shellfish (*142*).

This somewhat detailed description of the incident serves to emphasize the limited knowledge of mercury which existed at the time, particularly with regard to the organic mercury compounds which have chemical and biological properties highly different from inorganic mercury compounds or elemental mercury. The danger of organic mercury became more evident in 1962 when the Minamata incident was repeated. In Niigata, Japan, along the lower Agano River, 26 cases and 5 deaths were documented officially as methyl mercury poisoning. The source was again fish and shellfish which concentrated mercury discharged by a chemical plant into the river (*113, 228*).

In the 1960s, Swedish scientists became concerned with environmental pollution by mercury when it was noted that wildlife contained lethal levels of mercury. Investigating the cause for decreases in wild bird populations, Johnels and Westermark (*123*) found the mercury level in feathers of museum specimens markedly increased after 1940. This increase coincided with the introduction of methyl mercury seed dressings and abruptly dropped after a ban was imposed on alkyl mercury seed dressings. Borg et al. (*25*) found lethal levels of mercury in a high percentage of animals found dead in the countryside in which poisoning was

[1] *Contribution from the College of Agricultural and Life Sciences.*
[2] *Part II of this review will appear in the next issue of this Journal. References cited in both parts are listed at the end of Part II.*

suspected. Included were pheasants, partridges, pigeons, finches, and corvine birds as well as their predators; eagles, hawks, falcons, owls, foxes, martens, and polecats. Westöö (*252*) stated that Westermark et al. (*247*) were the first to report that Swedish fish contained elevated levels of mercury which Westöö (*249*) identified as methyl mercury in 1966. In view of the Japanese experiences, the Swedish government adopted a limit of 1.0 ppm mercury in fish which were to be sold.

Canadian scientists became concerned over the widespread use of mercury in their country in the late 1960s, however, concern in North America finally reached a peak in 1970. At this time, Fimreite et al. (*70*) reported that fish taken from Lakes Erie and St. Clair contained significant quantities of mercury. In the following months, the FDA seized several lots of fish which contained high concentrations of mercury. Lakes which showed significant mercury pollution were closed to commercial and sport fishing, and reports of high levels of mercury in wildlife were published.

The public fear which was precipitated by headlines such as that in the *National Enquirer,* November 29, 1970, "Everyone in U.S. Is Being Poisoned by Mercury," have greatly hurt many areas of the food industry (*136*). At the time, many of the answers needed to reduce the near panic were not available. Numerous studies which have been conducted recently should aid in assessing the extent of the mercury problem. It is the intent of this review to present some of the information available, thereby arresting some unfounded fears and aid in identification of problems which exist. Topics to be covered are: (*a*) the natural levels of mercury; (*b*) man's uses of mercury and their impact on the environment; (*c*) the types of mercury compounds, their biotransformation and the differences in metabolic and toxicologic effect of the various forms; (*d*) the level of mercury in foods; (*e*) FDA regulations on mercury and regulations for other countries; and (*f*) methods of analysis for mercury.

NATURAL LEVELS OF MERCURY

The average concentration of mercury in the earth's crust is about 0.5 ppm, and it ranks 62nd from the top or 19th from the bottom on the list in abundance of elements in the earth (*240*). Most of the mercury is concentrated in geographical belts of the earth which are shown in Fig. 1. Mercury can exist in any of three oxidation states in nature: the metallic state $(Hg)^0$, the binuclear state $(Hg\text{-}Hg)^{+2}$ in which there is a covalent bond between the two atoms, or the mercuric state $(Hg)^{+2}$. The red mercuric sulfide, cinnabar, is the principal commercial source of mercury and it contains 86.2% mercury. Cinnabar is found in small veins or pockets in limestone, calcareous shales, sandstone, serpentine, chert, andesite, basalt, and rhyolite, most generally in areas of volcanic activity (*126*). The deposits are generally found within a few hundred feet of the surface.

Ehmann and Lovering (*61*) reported the mercury concentration of the earth's deep crust and upper mantel is 0.78 to 1.48 ppm. Day (*55*) stated that mercury reaches the surface by a process other than weathering. Since

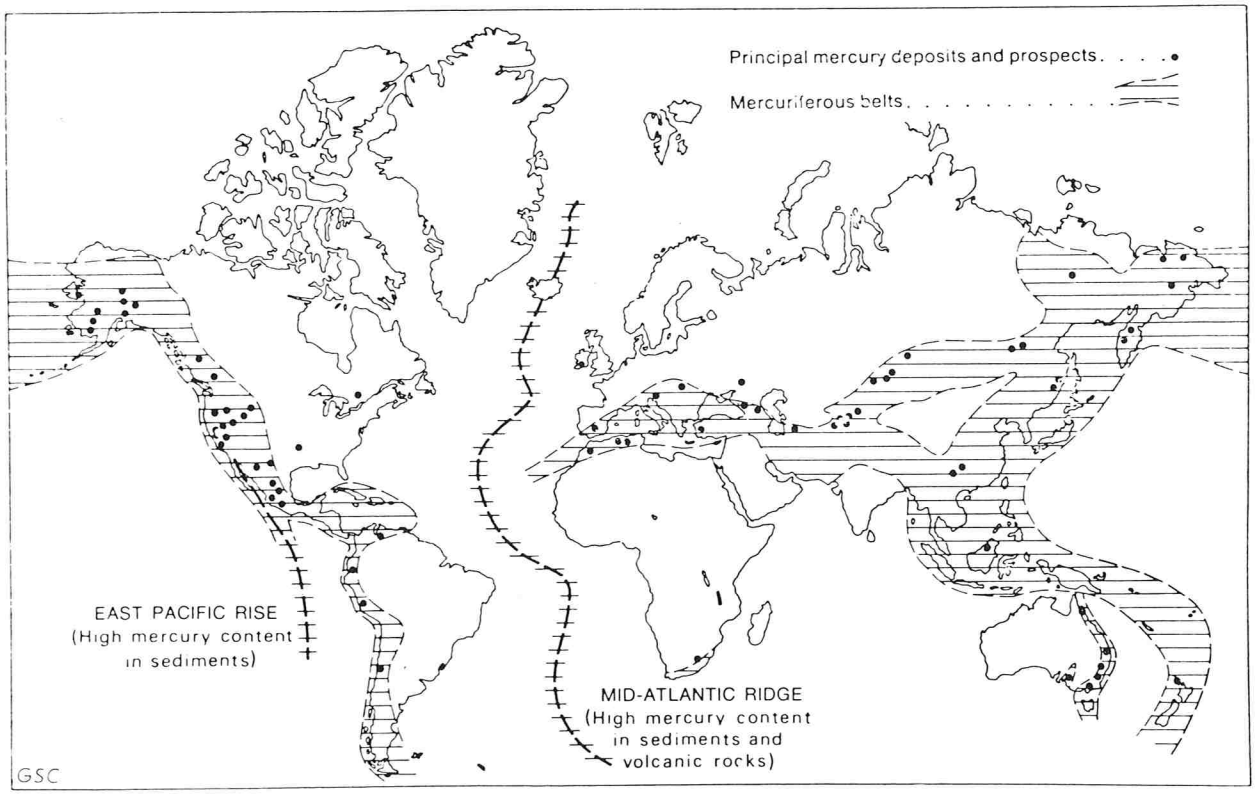

Figure 1. *Generalized map showing the the mercuriferous belts of the earth. From Jonasson and Boyle, reference* 125.

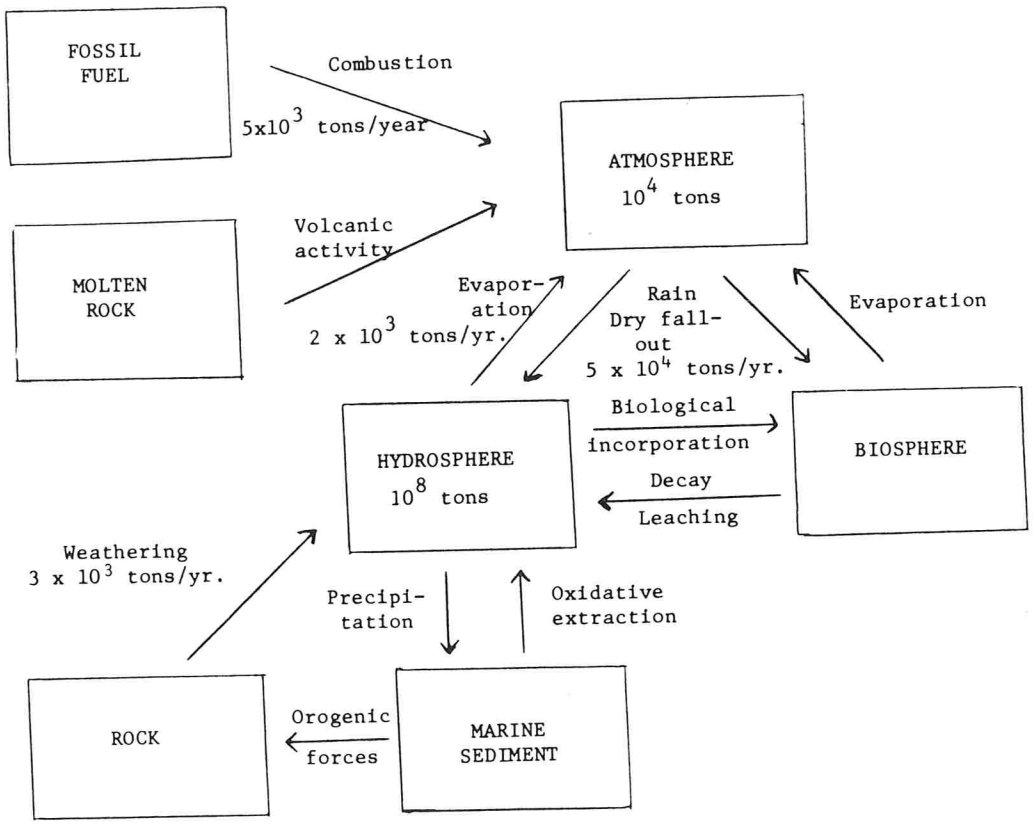

Figure 2. *Overall global cycling of mercury. From Peakall and Lovett, reference* 183. *Notes: (1) Input and output into the atmosphere do not balance as no figures appear to be available for evaporation from the biosphere or for volcanic activity. (2) Man's annual usage is* 10^4 *tons of which approximately half is recycled. (3) Data from references* 20, 27, 66, 121 *and* 253.

they are particularly mobile under magmatic and hydrothermal conditions, he theorized that mercury compounds are reduced by ferrous ions to metallic mercury. The highly volatile metal travels upward toward the surface where it is fixed by combination with sulfur. Volcanic activity releases mercury into the litosphere, atmosphere, and hydrosphere. The vapor equilibrium of mercury at room temperature is approximately 10 mg/m³ of air and this doubles with a 10-degree increase in temperature (*22*). Mercury vaporizes from the earth and water into the atmosphere where it is found both in the vapor form and as particles (*93*). Because of these unique properties, mercury is subject to a natural geological cycling which can be schematically shown (Fig. 2). Traces of mercury are therefore found over the entire earth.

Metallic mercury occurs in some ores (*126*); however, the concentration is not sufficient to be of commercial significance. Turekian and Wedepohl (*235*) reported the mercury concentration of sedimentary rocks ranged from 0.03 ppm in sandstone to 0.04 ppm in shales. In the igneous rock category, granites contain an average concentration of 0.08 ppm mercury (*235*). Joensuu (*121*) analyzed a number of coal samples for mercury and found concentrations ranging from 0.07 ppm to 33 ppm. In line with these values, Bailey et al. (*9*) reported crude petroleum samples collected from two wells contained 1.9-21.0 ppm mercury.

Determination of the natural concentration of mercury in soil is extremely difficult due to the possibilities of environmental pollution. A Geological Survey (*2*) estimated the background level at 0.1 ppm; however, other studies indicated lower background levels (*77, 132*). Goldwater (*93*) stated that higher concentrations of mercury would naturally occur in top soils rich in humus or near mercury ore deposits.

In the atmosphere, the concentration of mercury varies depending upon the locality. Because of its relatively high vapor pressure highest natural concentrations of mercury are found over cinnabar deposits. McCarthy et al. (*161*) found concentrations of 0.02-20 μg Hg/m³ air over ore deposits, whereas over non-mineralized land areas the concentrations range from 0.003-0.009 μg Hg/m³ air. Air over the ocean contains the lowest concentration of mercury: 0.0006-0.0007 μg/m³ was found by Williston (*253*). Precipitation by rain or snow reduces the mercury concentration in air to near zero, while other natural factors such as direction and speed of wind, temperature, and solar insulation change the level of mercury in the atmosphere (*253*).

Metallic mercury is relatively insoluble in water and because of the high density (13.546 at 20 C) (*240*), it collects in low spots in waterways. Ionic forms of mercury

are soluble in water, the degree depending on the acidity of the water and the presence of complexing ions. Wershaw (*246*) analyzed ground water samples from 73 areas of the United States and found 83% contained less than 1 ppb mercury, whereas only 2 samples had levels in excess of 5 ppb. These were in areas of mercury ore deposits and the concentration was a high as 80 ppb (*246*). Klein (*132*) analyzed 67 samples of water from ponds, lakes, and rivers in Northeastern United States and reported background levels were approximately 0.055 ppb when environmental pollution had not occurred. Joensuu (*121*) estimated the amount of mercury released into waterways by natural weathering. Basing his calculations on the amount of sodium and mercury present in the litosphere and the rate at which sodium is reached by weathering, Joensuu reported a maximum of 20.9×10^4 kg of mercury may be carried to sea each year. In sea water, Burton and Leatherland (*34*) found 0.014-0.021 ppb mercury in samples collected off the coast of England. Leatherland et al. (*144*) reported that in the Northeastern Atlantic, surface water contained 0.013-0.018 ppb mercury, the concentration decreasing with depth. Klein and Goldberg (*133*) found higher concentrations of mercury deeper off the coast of Japan. Values they reported were from 0.10 ppb at surface and 0.15-0.27 ppb at lower depths (*133*).

MAN'S USE OF MERCURY AND ENVIRONMENTAL CONTAMINATION

For centuries, man's use of mercury was limited to cosmetics and medicine. In 1557, Bartolome de Medina devised a process by which silver was recovered by amalgamation (*202*). Since that time, uses for mercury have increased and in 1971 over 10.5×10^6 kg of mercury were produced to meet demands (*36*). Increasing amounts of mercury are being recovered, repurified, and sold as secondary mercury. Many processes for reclaiming mercury from mercury boilers, electrical apparatus, dental amalgams, batteries, and sludge from processes using mercury as a catalyst have been developed (*126*). In 1971 over 5.8×10^5 kg of mercury on the U.S. market were secondary mercury (*36*). The difference between total mercury and secondary mercury sold is indicative of the losses of mercury which occur in the environment. Mercury pollution is unique from many other types of pollution in that the total earth concentration is not being changed; rather the problem occurs from redistribution of the mercury or changing it into a more toxic form. Thus, it is important to consider these aspects with regard to the production and uses of mercury to determine present day risks.

Figure 3. *Trends in production, consumption, and prices of mercury in the U.S. From references* 36 *and* 185.

The trends in production, consumption, and prices of mercury in the U.S. are shown in Figure 3.

Production

An extensive discussion of methods of producing mercury are beyond the scope of this review. Readers desiring further information on production of mercury should consult the publication by H. R. Jones (*126*), from which much of the following was taken. In the United States most mercury is extracted from the ore by a directly heated pyrometallurgical process. After crushing, ore is heated in retorts or furnaces to liberate the mercury as vapors. The vapors, after passing through dust collectors, are cooled by a condenser system and the liquid mercury is collected. Although the recovery from this process averages between 95-98%, only 1.24-12.4 g of mercury are obtained per kilogram of ore (*126*). The primary source of pollution in processing mercury is condenser stack emissions which range from 74.2-244 mg per kg of ore processed (*238*).

Uses

The largest users of mercury and trends in mercury consumption are shown in Table 1. The new public

TABLE 1. *Mercury consumed in the United States, by use (10^3Kg)*

Use	1955[1]	1965[2]	1968[3]	1970[3]	1972[3]
Agriculture[4]	255	107	118	62.4	63.3
Amalgamation	7.47	17.1	9.20	7.55	N/A
Catalysts	25.1	31.9	66.0	77.1	27.6
Dental preparations	70.3	119	106	78.8	103
Electrical apparatus	442	589	677	550	536
Electrolytic preparation of chlorine and caustic soda	107	302	602	517	397
General laboratory use	33.6	97.4	68.6	62.3	20.5
Industrial and control instruments	343	350	275	167	225
Paint					
Antifouling	25.0	8.79	13.5	6.83	1.10
Mildew-proofing	N/A	260	351	350	282
Paper and pulp manufacture	N/A	21.3	14.4	7.79	0.34
Pharmaceuticals	54.4	112	14.6	23.8	19.9
Other[5]	609	620	285	202	147
Total known uses	1971	2635	2600	2112	1823
Total unknown uses	—	—	—	7.82	0.76
Grand total[6]	1971	2635	2600	2119.82	1823.76

N/A values not available from these references.
[1]Data from reference *185* including portion listed as redistilled.
[2]Data from reference *65* including portion listed as redistilled.
[3]Data from reference *36*.
[4]Includes fungicides and bactericides for industrial purposes.
[5]Includes that portion listed as miscellaneous uses for redistilled mercury in references *185* and *65*.
[6]Discrepancies due to rounding off of above values.

awareness of mercury pollution has had some effects on the uses of mercury. Unfortunately, a more recent survey is not available.

The electrical industry, the largest consumer, utilizes over one-half of its consumption in the production of batteries (*97*). Other electrical products utilizing significant quantities of mercury are fluorescent lamps and high intensity discharge lamps, rectifiers, oscillators, and power control switches (*97*). Recycling of most of these items is impractical except for industries utilizing large quantities with volume turnovers. For this reason, much of the mercury is added to the environment with trash. Projections for mercury use in the year 2000 range from 8.6×10^5-13.8×10^5 kg; the broad range reflects both the continued demand for mercury batteries and a shift to the rechargeable nickel-cadmium batteries (*97*).

Use of mercury cells for production of chlorine gas accounted for over 23% of the mercury consumed in the U.S. in 1971 (*36*). The chlorine production process (*238*) involves a cell with two sections. The first section, the electrolyzer, uses mercury as a flowing cathode which flows co-currently with a salt solution such as sodium chloride. When a high current is applied between the carbon or metal anode and the mercury cathode, chlorine gas is formed at the anode and alkali amalgam at the cathode. The amalgam is separated and passed into the second section, the decomposer, where the amalgam becomes the anode to a short circuited graphite cathode. Then, the amalgam is converted back to mercury and alkali metal hydroxide with the production of hydrogen gas. Caustic soda produced by this process may contain up to 7 ppm mercury (*57*). The Environmental Protection Agency bulletin (*238*) identifies significant losses of mercury into the environment (up to 0.91 g Hg/kg of Cl_2) which may occur from chloralkali production but new processes have been developed which drastically reduce these emissions. An increasing percentage of chlorine also is being produced by the diaphragm process. Although the caustic produced by this process is of a lower grade, no mercury emissions are produced. In spite of improvements, pollution still is significant in chlor-alkali production. Cammaroto (*36*) indicated that 27.2% of the 8.45×10^9 kg of chlorine produced was from mercury cells and 153 mg of mercury were lost in the production of a kilogram of chlorine; therefore, over 4.25×10^5 kg of mercury were lost in the production of chlorine and alkali. Greenspoon (*97*) projected the need for 2.07×10^6 kg of mercury for chlor-alkali production in 2000. This could be excessive based on improved recovery and increased use of diaphragm cells.

The bactericidal and fungicidal properties of many mercury compounds have resulted in their use in paint and paper industries and in agriculture. The paint industry is the third largest user of mercury. Incorporation of phenyl mercury compounds into water-based paints inhibits mildew formation on painted surfaces as well as prolonging shelf life of paint in the can. At the Wisconsin Department of Natural Resources hearing one manufacturer reported that the amount of phenyl mercury added per gallon ranged from 0.45 g Hg for interior water-based paint to 3.41 g Hg for exterior alkyd based paint (*216*). Sibbett et al. (*206*) reported significant concentrations of mercury were found in the atmosphere of a room freshly painted with paint containing a mercurial fungicide. Goldberg and Shapero (*90*) reported the health hazard of such emissions were minimal. Marine paints contain mercurous compounds as antifouling agents which prevent slime buildup and marine growth on painted surfaces. Small concentrations of this mercury are released into the water (*102*). Extensive research is being conducted to find non-mercurial preservatives for paints, however non-mercurial compounds have not been as effective on the basis of shelf and surface life. The mercury requirements of the paint industry in the year of 2000 may reach $5.5\text{-}6.9 \times 10^5$ kg (*97*).

Slime formation in paper and pulp machinery results in production of inferior paper. Although highly effective, use of mercury-based slimicides has been largely discontinued in recent years as a result of the Federal Food and Drug Administration action (*35*) precluding the use for paper used in food packaging. Moreover, the concomitant development of other satisfactory slimicides has reduced this need. Replacement of mercury slimicides has aided in pollution control since approximately one-half of the mercury used passed out of the mill with the effluent whereas the other half bound to the paper fibers also polluted the environment at disposal (*35*). The pulping process utilizes large quantities of caustic soda and chlorine, both of which may contain mercury residues if produced by the mercury cell process.

Agricultural consumption of mercury is low in comparison to some other industries, however, special consideration is warranted because the mercury is applied directly to the environment. Mercurial compounds, which have a broad spectrum of fungal control, are unequaled by any other preparations used as seed dressings on barley, beans, corn, cotton, flax, millet, milo, oats, peanuts, peas, rice, rye, safflower, sorghum, soybeans, sugar beets, and wheat. Novick (*180*) listed the mercury-containing pesticides licensed by the Department of Agriculture in 1967 and also identified those subsequently restricted in use or prohibited. Worf (*255*) stated that use of these dressings had declined as a result of development of varieties of grain resistant to many fungal diseases. Also, environmental pollution by mercurial seed dressings may not be significant since common application rates would contribute only 1.8 g per hectare, which is much less than background levels. Sand et al. (*204*) reported that of 93 soil samples analyzed, the mean concentration of mercury was 114 ppb where mercury-treated seeds had not been used whereas a higher mean of 195 ppb mercury was found in soil where mercury-dressed seeds had been used.

Mercurial fungicides are used also as sprays on fruit trees and tomato plants before development of the fruit. In potato production, mercurial compounds have been used for both soil treatment of foliage sprays (*180*). Inoue and Aomine (*112*) reported that the absorption of phenyl mercury acetate by clay mineral soils was subject to the law of cation exchange. Absorption was greatly reduced at or greater than pH 7 as well as below pH 5 due to the reduction of phenylmercuric ions or the increase of hydronium ions. Montmorillonite gave the highest absorption rate followed by Allophane and Kaolinite types of soils.

The highest rates of application of mercurial fungicides most likely are for turf management. Eckert (*60*) stated that mercury fungicides have been used for years on golf courses. Non-mercurial fungicides are now replacing the mercury compounds to control summer diseases, but no effective alternatives have been found to control winter fungus diseases referred to collectively as snow mold. An inorganic mercurial fungicide containing 60% HgC1 and 30% $HgC1_2$ offers broad spectrum activity and residual effectiveness; phenylmercuric acetate is also used. Commonly applied only to the golf course greens, approximately 2.04×10^3 kg on an elemental basis of mercury compounds are applied annually to 182.1 hectares of greens in the State of Wisconsin (*60*). Of this, slightly over 2% was organic mercury and the remainder inorganic mercury compounds. Also, Novick (*180*) reported tha many commercial fertilizers, herbicides, and fungicides for use on home lawns contain mercury compounds.

General laboratory uses of mercury are extensive. Cooke and Beitel (*48*) stated that approximately 5.9×10^3 kg of mercury per year are lost by Canadian hospital laboratories through such uses as $HgC1_2$ for fixing tissue samples. Common uses of mercury compounds in food analysis laboratories given by Bradley (*28*) are mercuric chloride to preserve milk samples, mercuric sulfate or oxide in Kjeldahl nitrogen determinations, and mercuric sulfate in chemical oxygen demand determinations.

Medicinal uses of mercury are for pharmaceuticals, cosmetic preparations, and dental preparations. Mercury was once used to treat syphilis, as an aphrodisiac, and as a means of prolonging life (*57*). Use of organomercurial compounds as diuretics continues, but they are being replaced by non-mercurial compounds. Organomercurials under such trade names as Mercurochrome(R), Merthiolate(R), Mercresin(R), and Metaphen(R) are still very popular antiseptics. Mercurials are frequently used in sterilizing solutions for medical instruments and are often incorporated into pharmaceuticals and cosmetics to control microbial growth. It is interesting that in 1971, more mercury was used in dental preparations than in agriculture. Mercury is used in an amalgam with a silver-tin alloy and has proven more satisfactory with regard to relative permanence, compressive strength, abrasion resistance and ease of handling than other materials tested (*126*). With regard to pollution, dental preparations are trapped in the mouth of the patient; however, nearly all of the merucry utilized for other medical purposes eventually is added to the waterways through the sewer system.

Mercury is used for many other very diverse purposes, a number of which may contribute to envrionmental pollution. Bailey and Smith (*8*) estimate that there are almost 3,000 distinct applications of mercury. Uses of mercury may not be the only activity of man which contributes to environmental pollution by mercury. Joensuu (*121*) concluded that the burning of fossil fuels, which are relatively low in mercury content, released approximately 2.72×10^6 kg of mercury into the atmosphere annually due to the large quantities burned. Weiss et al. (*244*) analyzed permanent snow-fields in Greenland for mercury and thus were able to assess changes in atmospheric mercury concentrations over the past 2700 years. Using reliable techniques to date the various strata sampled, it was concluded that activities of man have had an impact on the environment. They estimated the major sources of atmospheric pollution would be: natural runoff into waterways, 3.8×10^6 kg per year; chloroalkali production, 3×10^6 kg per year, heating of limestone and shale containing mercury in the production of cement, 1×10^5 kg per year; burning fossil fuels, 1.6×10^6 kg per year; and from natural earth degassing, 2.5×10^7-1.5×10^8 kg per year. On the basis of these estimates, Weiss et al. concluded that alteration of terrestrial surfaces increases exposure of crust materials, and may be the greatest source of mercury pollution. These levels of mercury pollution, however, are too low to significantly affect the mercury content of mixed layers of the ocean. Miller et al. 164 gave support to this theory by their findings that museum specimens of tuna and swordfish dating back to 1878 had similar concentrations of mercury as those recently caught. Numerous other reports have concluded that ocean levels of mercury have not changed significantly.

Mercury pollution has resulted in elevated concentrations of the element in several isolated areas. Kitamura (*131*) reported that a maximum concentration of 2010 ppm mercury (wet weight) was found in sediment of Minamata Bay near the drainage channel of a chemical plant and the concentration dropped sharply as a function of the distance from the effluent outlet. Konrad (*135*) reported deposits of mercury in Wisconsin waterways: 684 ppm (dry weight) in bottom sediment just below the outfall of a chlorine-caustic soda plant with concentrations decreasing to 12 ppm one mile downstream; 11.5 ppm (dry weight) in bottom sediment below a city sewage treatment plant accepting waste from manufacturers of electrical batteries. Background levels ranged from 0.01-0.15 ppm Hg in bottom sediments. Numerous incidents of fish kills have been attributed to environmental contamination by mercury. Turney (*236*) reported an incident in Michigan in which the discharge of phenylmercuric acetate, used as a mildew inhibitor by

a laundry, was the apparent cause of a fish kill. Derrybury (*56*) reported that fish were killed in a Tennessee reservoir when steel drums which had contained phenylmercuric acetate were used on floating docks and houseboats. Johnels and Westermark (*123*) reported 5- to 10-fold increases in the mercury content of pike caught below pulp mills in Sweden when compared to the levels in pike caught upstream. Numerous other reports of localized pollution have been published.

BIOTRANSFORMATION

Dumping inorganic mercury, either in the elemental or ionic form, into waterways was considered a safe practice until recent years. It was believed that ionic mercury would readily bind to other inorganic ions, form precipitates and deposit as soil sediment, whereas, elemental mercury would remain inert and settle in low pockets in the waterway bed (*62*).

Japanese scientists had suspected biological methylation of inorganic mercury in Minamata Bay (*113*), but the theory was not pursued after methyl mercury was discovered in the effluent of the chemical plant. Later, Wood et al. (*254*) reported methyl mercury was produced enzymatically from inorganic mercury and extracts of methanogenic bacteria. Jensen and Jernelöv (*118*) reported organisms found in bottom sediments from fresh water aquaria had methylated inorganic mercury. Jernelöv (*120*) reported that mercury bound as the sulfide (HgS) was not methylated under anaerobic conditions, but aerobically, the sulfide was oxidized to the sulfate $(SO_4)^{-2}$ and methylation occurred. Wood et al. (*254*) reported that a nonenzymatic transfer of the methyl group from Co^{+++} to Hg^{++} may occur, the process being enhanced by anaerobic conditions and increasing concentrations of alkyl cobalamine synthesizing bacteria. Landner (*143*) described a process by which mercury was bound to homocysteine and methylated, thus forming a methyl mercury-homocysteine complex. Apparently, this later reaction is linked to the methionine biosynthesis pathway and could occur under aerobic or anaerobic conditions. Jernelöv (*120*) stated that methylation in lakes and rivers must be an aerobic process, since HgS was formed under anaerobic conditions and methylation would not occur. The pH of water also affects the methylation process; low pH favors the formation of monomethyl mercury, high pH favors the formation of dimethyl mercury (*120*).

The extent to which inorganic mercury is methylated in the sediment of our waterways has not been determined. Consideration must be given to this reaction for two reasons: first, methyl mercury shows a strong tendency to be absorbed by marine organisms and therefore represents a greater toxicological hazard than inorganic mercury in the food chain (*81*); secondly, transformation of divalentinorganic mercury to mono or dimethyl mercury can contribute to a release of mercury bound to organic sediment (*119*).

Synthesis of methyl mercury is not the only interconversion of mercury in nature. Oxidation, reduction, synthesis, and degradation of mercury compounds occur in nature. They cycle of mercury interconversions in nature in depicted in Fig. 4.

BIOCHEMISTRY AND TOXICOLOGY

The toxicological ramifications of mercury pollution

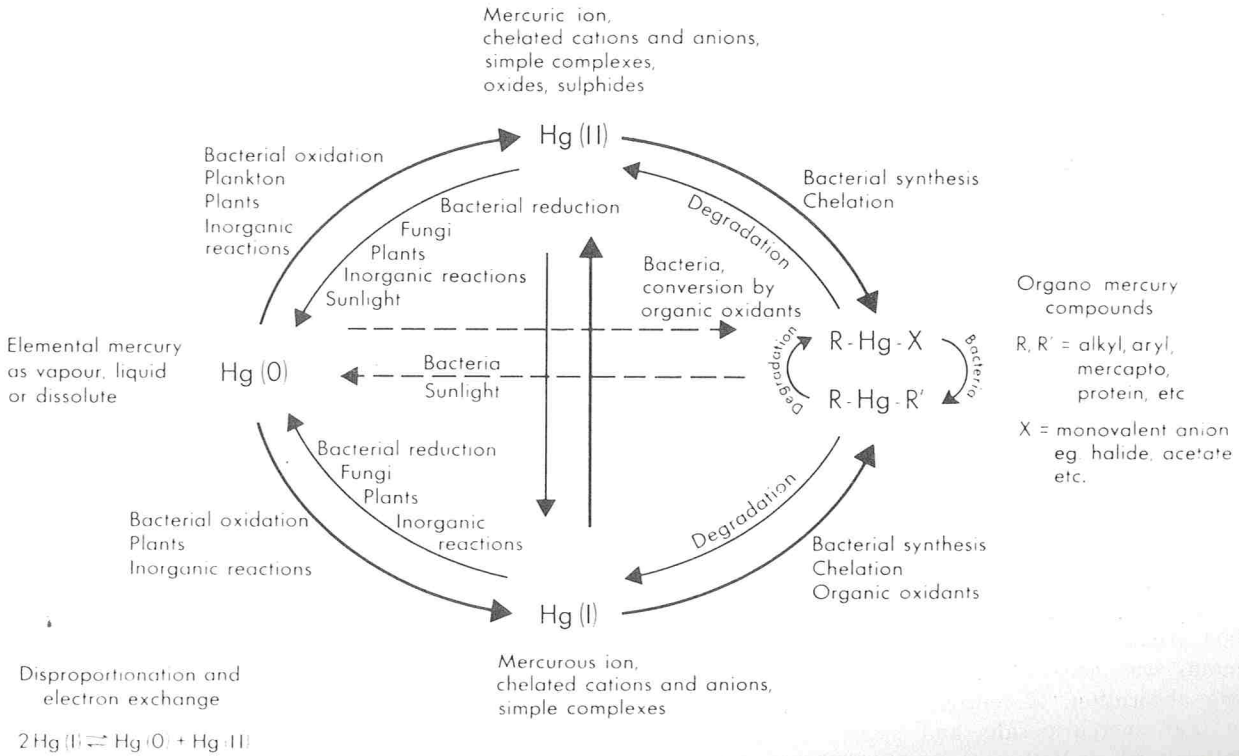

Figure 4. *Cycle of mercury interconversions in nature. From Jonasson and Boyle, reference* 125.

have been the subject of numerous investigations. Ancient authors such as Hippocrates, Pliny, Galen, and Avicenna reported mercury compounds were toxic, yet only recently the differences of the metabolism and toxicology of various mercury compounds have been understood. To discuss the biochemistry and toxicology of mercury, it is necessary to consider four classes of mercury compounds: elemental mercury, Hg^0; inorganic mercury salts, Hg^{+2} and Hg_2^{+2}; aryl mercurials, C_6H_5-Hg; alkoxyalkyl mercurials, R-O-Hg^+; and alkyl mercurials, R-Hg (R represents a short-length hydrocarbon chain). An understanding of the differences in the metabolism and toxicology of these four classes of mercury compounds is essential to evaluate the mercury residue problem.

Absorption

Mercury compounds may be absorbed by the body through the gastrointestinal tract, respiratory tract, or skin. The extent of absorption, however, was found to be highly dependent upon the form of mercury involved.

Elemental liquid mercury can be absorbed from the gastrointestinal tract. Bornmann et al. (*26*) observed a 10-fold increase in the concentration of mercury in blood and kidneys of rats that received metallic mercury orally. The extent of the absorption appears relatively low and the dangers of poisoning from liquid mercury by this route seem slight. Toxic signs were rarely noted in patients who received oral doses of 100 to 500 g of mercury in the treatment of bowel obstructions (*21*). Goldwater (*93*) stated that a person could probably swallow 454 g of liquid mercury with no adverse effects.

In the ionized form, mercury is absorbed somewhat more efficiently from the gastrointestinal tract; ingestion of mercury salts was chosen often as a method of suicide. In studies of humans who had consumed mercuric chloride and showed acute signs, it was estimated that 8% of the dose had been absorbed (*215*). Accurate evaluation of absorption rates is difficult following ingestion of acute doses: the corrosive effect of mercuric chloride undoubtedly changes the permeability of the gastrointestinal wall; vomiting is almost certain to occur within 1 h after mercuric chloride ingestion. Miettinen (*162*) and Rahola et al. (*194*) fed nontoxic doses of mercuric salt to 10 human volunteers and found only 85% of the dose excreted in the feces within 4 days. No differences were mentioned for excretion rates from patients who received the mercury in a liver paste mixture or in an ionic solution. In a study involving mice, Clarkson (*42*) concluded that an average of less than 2% of the daily intake of mercuric chloride was absorbed. Studies in which rats were dosed orally with mercuric acetate indicate that 20% of this compound was absorbed (*193*). The solubility of inorganic mercury compounds was generally believed the major factor affecting absorption. Mercurous compounds are less soluble than mercuric salts and apparently are less efficiently absorbed. Viola and Cassano (*239*) confirmed the poor absorption of mercurious compounds by autoradiographic studies.

Organic mercurials apparently are absorbed more rapidly from the gastrointestinal tract than are the inorganic salts. Data indicate that between 50-80% of an oral dose of phenyl mercury acetate is absorbed by rats (*193*).

Alkyl mercury compounds are absorbed in the gastrointestinal tract to an even greater extent than phenyl mercury compounds. Ekman et al. (*63*) and Falk et al. (*67*) fed low doses of [^{203}Hg]methyl mercury to human volunteers and reported almost complete absorption. Clarkson (*42*) reported an average of 98% of the methyl mercury was absorbed by rats fed contaminated diets.

Gastrointestinal absorption of mercury is of primary importance with regard to mercury residues in foods, however, harmful levels of mercury may be absorbed by laboratory personnel through the respiratory tract or through the skin.

The vapor state of elemental mercury is far more dangerous to man than the liquid state because of the higher absorption rate. Being relatively insoluble in water, vapors of metallic mercury are not absorbed by the mucous membranes, but pass further down the respiratory tract to the alveoli (*186*). Magos (*156*) reported that nearly 20% of mercury vapor injected intravenously was exhaled by rats within 30 sec. Kudsk (*138*) had human volunteers inhale concentrations of 50-350 μg mercury vapor/m^3 air and reported 71-88% absorption. Shepherd et al. (*205*) recorded approximately 10μg mercury/m^3 air exhaled by human volunteers who had inhaled a concentration of 200 μg/m^3. Methyl mercury compounds are also very volatile and readily absorbed in the respiratory tract. Mice, which Ostlund (*181*) exposed to vapors of radioactive dimethyl mercury for 45 sec, absorbed 50-80% of the total radioactivity. Since his primary concern was not the percentage of absorption, no indication was given about the extent of vaporization. Therefore, the 50-80% absorption may be lower than the actual amount possible.

Mercury compounds in the aerosal or dust form may also enter the respiratory tract. Although quantitative data on absorption of mercury compounds in these states are not available, it is likely that the extent of penetration is similar to that of other heavy metals. The task group on metal accumulation (*186*) described pulmonary absorption of metals and stated that the extent of absorption was dependent upon: size of particles, solubility of the compound, physical characteristics of the individual, route of inhalation, tidal volume, and respiratory rate.

Absorption of mercury compounds through the skin was studied by Friberg et al. (*83*) who reported that 6% of a 16 mg mercuric chloride/ml solution was absorbed through the skin of guinea pigs in 5 h. Silberberg et al. (*207*) were able to trace the penetration of mercuric ions by electron microscopy when a solution was applied to the

skin. Rates of absorption of methyl mercury compounds through the skin appear similar to those of mercuric chloride (*83, 175*).

Transport and transformation

Following absorption, mercury is transported by the blood system to various tissues of the body. Distribution of mercury between the components of blood is dependent upon the form in which the mercury exists.

Apparently, vapors of metallic mercury are absorbed preferentially by erythrocytes. Berlin et al. (*15*) exposed rabbits and monkeys to mercury vapor and found immediately after exposure 67-84% of the mercury in blood was associated with the cellular fraction as opposed to the plasma fraction.

In the mercuric form, more than half of the total concentration of mercury was found in the plasma. Berlin and Gibson (*16*) studied distribution of mercury in blood of rabbits receiving mercuric chloride by infusion. Considerable delay occurred between the time that serum levels of mercury were changed and equilibrium was established. They reported that 50% of the mercury in blood was bound to red corpuscles after equilibrium. In plasma most of the mercury was apparently bound to plasma proteins since no more than 1% of the mercury could be detected in the ultrafiltrate obtained when plasma was passed through a glomular membrane. Takeda et al. (*226*) reported that 4 days were required for equilibration of mercury between erythrocytes and plasma in rats given subcutaneous injections of mercuric solutions.

The aryl and alkyl mercurials are largely bound to the red blood cells. Berlin (*14*) infused phenylmercuric acetate and methyl mercury dicyandiamide into rabbits and found that about 10% of the phenylmercury and less than 20% of the methyl mercury was in the plasma. Less than 1% of the plasma mercury in either form was found in the ultrafiltrate fraction. When three human volunteers were given an oral dose of radioactive methyl mercury, radioactivity was detected in their blood within 15 min (*1*). The ratio of mercury in the erythrocytesto-plasma was 10:1 and remained constant for the 24 days following consumption. Results of numerous studies indicate that the erythrocyteto-plasma ratio is different for various species. Reported ranges are 300:1 in rats; 20:1 in man; and 10:1 in monkeys, cats, and mice (*44*). Suzuki et al. (*220*) reported that the blood cells collected from the umbilical cord contained a higher concentration of mercury than did maternal blood, whereas maternal plasma samples were higher in mercury than was plasma collected from the umbilical cord. The authors attributed these variations to the higher hemoglobin content of fetal blood and the higher concentration of thiol radicals in fetal hemoglobin.

Except for the loss of the anion fraction, most mercury compounds are absorbed into the circulatory system unchanged. Magos (*155*) reported that vapors of elemental mercury are rapidly oxidized to mercuric ions by blood in vitro. Kudsk reported that an enzyme system is likely involved in the oxidation, however, the exact process could not be determined (*139, 140*). Some reduction of mercuric ions to elemental mercury in the blood may also occur. Magos (*155*) observed that 0.5% of added mercuric chloride was volatized from blood samples. Clarkson and Rothstein (*46*) reported that following injection of mercuric chloride into rats about 4% of the amount excreted was exhaled.

Aryl and alkoxy mercury compounds are degraded to inorganic mercury in organisms. Miller et al. (*165*) found only 20% of the mercury in the liver and 10% of the mercury in the kidney was in the organic form 48 h after rats were injected with phenylmercuric acetate. Daniels and Gage (*52*) reported that when rats were administered [^{14}C]phenylmercuric acetate, about 85% of the radioactivity was detected in the urine within 96 h, whereas only 12% of the mercury was excreted in the urine and 50-60% was excreted in the feces. Indirectly, breakdown of aryl mercury compounds was also indicated by redistribution of mercury in the animal. As was previously indicated, organic mercury compounds were found in higher concentrations in red blood cells, whereas inorganic mercury ions were associated with the plasma fraction. Studies have shown that when phenylmercury compounds were administered, blood distribution initially resembled that expected of organic mercurials, but later, the distribution became similar to that of inorganic mercury (*175, 226*). Distribution of aryl and alkoxy mercury compounds in tissues and the excretion rates are further indicators of the relatively rapid breakdown of these compounds and will be discussed later.

Methyl mercury is apparently degraded to inorganic mercury in the body, however, the rate is considerably slower than noted for aryl or alkoxy mercurials. After administering methyl mercury to rats, 6-50% of the mercury excreted in the urine was inorganic mercury (*86, 178*). Methyl mercury apparently can be degraded to inorganic mercury in the liver and kidney of mice (*177*), and liver of rats (*178*). Six weeks after injection of methyl mercury in rats, the percentage of mercury remaining in the organic form was reported to be 90% in the liver, spleen, and blood; 75% in the plasma and brain; and 55% in the kidney (*86*). The relatively slow excretion rate and the constant distribution of methyl mercury among different organs are further indications of its relative stability (*175*).

Tissue distribution

The task group on metal accumulation discussed the factors which determine the distribution of metals among tissues (*186*). They indicated that most of the mercury absorbed by tissues is probably found in plasma. Mercury contained in erythrocytes most likely is unavailable for direct exchange with tissues, but is one of many pools of mercury in the body and is in continuous exchange with plasma. The task group stated that the exchange of mercury between plasma and tissues undoubtedly involves numerous complex factors, three of

which might be: (*a*) the fraction of mercury in the plasma which is in the diffusible form and the rate at which perfusion of tissue occurs; (*b*) permeability of the cell membrane to the form of mercury in plasma, and (*c*) availability and turnover rate of suitable membrane and intracellular ligands for binding mercury.

Clarkson (*44*) reviewed the binding of mercury compounds to the biological tissues and fluids. Studies covered in this review indicate that less than 1% of the mercury in plasma is in a diffusible form. Most evidence indicates that mercury is bound to thiol groups, however, ligand groups other than thiols also may be involved in binding mercury in tissues. A two-point attachment of mercury, one point through a thiol group and the other to a nonthiol ligand has been proposed for mercuric ions (*128*) and organomercurial molecules (*243*). A theoretical discussion of the transfer of mercury from blood to tissues was reviewed by Clarkson (*44*). The rate of equilibration between the nondiffusible bound mercury and the diffusible form will affect the tissue concentration. the availability of ligands and the strength of the mercury ligand bond on each side of the cell membrane will influence the concentration gradient which is established across the cell membrane. Clarkson indicated that certain sulfhydryl groups show higher affinities for mercury than others. Furthermore, some evidence indicates that cells may expend metabolic energy to concentrate mercury. The mechanism which selectively permits some forms of mercury to penetrate the blood-brain barrier and the placental wall has not been completely resolved (*44*).

The pattern of distribution of mercury among tissues varies depending on the form. Therefore, biotransformation of mercury to different forms results in a continuous change in distribution.

Inorganic mercury characterisitically shows a nonuniform distribution among body tissues. Berlin and Ullberg (*17*) injected mice with a solution of $^{203}HgCl_2$ and by autoradiographic techniques, observed the distribution over a 16-day period. Initially, the high mercury content of blood indicated intense concentrations in highly vascularized organs. In 24 h, mercury accumulated in the liver, spleen, bone marrow, and thymus, then decreased rapidly in the blood, liver, spleen, bone marrow and myocardium. However, in the brain, renal cortex, and testes, the decline in concentration was much slower. While mercury appeared in the placenta 24 h after injection, only traces of mercury were detected in the fetus. Throughout the entire study, the concentration of mercury was highest in the renal cortex. This agrees with data showing 85% of the mercury in rats located in the kidney 15 days after injection with mercuric chloride (*200*). Similar distributions were noted in guinea pigs by Nordberg and Serenius (*174*), rats by Swensson and Ulfvarson (*224*), and Friberg (*78*), and quail by Nishimura et al. (*173*). However in quail, a considerable portion of the mercury was found in the ova (*173*).

Distribution of elemental mercury, after inhaling vapors, was similar to that observed for inorganic mercury salts. Higher concentrations of mercury were found in the brain, blood, and myocardium than were noted with inorganic salts (*175*). Magos (*155, 156*) attributed this to a slight delay in oxidation of mercury vapor, permitting diffusion of vapors to occur across the blood-brain barrier. Berlin et al. (*15*) reported that the concentration of mercury in the brain of mice inhaling elemental mercury vapors was 10 times higher than that following an equivalent injection of mercuric salt. Similar values were noted in guinea pigs (*174*).

Phenylmercuric salts are distributed differently than inorganic mercury salts. Berlin and Ullberg (*18*) observed the following: phenylmercury was retained in the blood longer; larger portions were found in the liver and alimentary tact; more mercury was retained in the skeletal muscles; and accumulation in the kidney was slower than noted for inorganic mercury salts. Similar to mercuric chloride, high concentrations of mercury were found in the placenta, but only traces could be found in the fetus.

Takeda et al. (*226*) reported that distribution of phenylmercury in rats, initially after administration, was similar to that observed for alkyl mercury compounds. After 8 days, however, the distribution pattern more closely resembled that observed for mercuric chloride. The authors attributed the change in distribution pattern to the rapid breakdown of phenylmercury to inorganic mercury. Gage (*86*) reported similar results in rats injected with phenylmercury acetate. At the end of 6 weeks, the concentration of mercury in the kidney was 90 times higher than in the liver, spleen, brain, heart, and blood components. Ellis and Fang (*64*) studied distribution of phenylmercury and mercuric ions in rats for 120 h after a single oral dose. The concentration of mercury in organs of rats treated with phenylmercury was higher, reflecting a higher gastrointestinal absorption of the organic form. Highest concentrations of mercury were found in the kidneys for both mercury compounds. Blood levels were 30-40 times higher and liver 4-5 times higher in the phenylmercury treated rats than in those receiving mercuric chloride. Ulfvarson (*237*) noted that the pattern of distribution of mercury among tissues was dependent upon the dose of phenylmercury, indicating that a saturation phenomenon may occur.

Following the administration of alkyl mercury compounds, a relatively even and constant distribution of mercury among the organs was reported. Berlin and Ullberg (*19*) using single intravenous injections of [^{203}Hg]methyl mercury dicyandiamide in pregnant mice, studied distribution by autoradiography. One hour after injection, the highest concentration of mercury was found in blood; however, an accumulation was noted in the kidney, liver, pancreas, mucosa of the alimentary tract, and gallbladder. After 4 h, mercury was detected in the fetus, colon, and muscles, whereas the amount of

mercury in the liver was at its maximum. Twenty-four hours after injection, mercury was distributed uniformly through the body and fetus, except the central nervous system that contained a lesser amount and the bony skeleton in which mercury was not evident. During the following 15 days, the concentration of mercury decreased in all organs except the brain and spinal cord, where the concentration doubled. At day 16, the concentration of mercury in the brain was exceeded only by that in the renal cortex and colonic mucosa. Hirota (*109*), as reported by Kojima and Fujita (*134*), noted similar distribution of mercury in rats given a single intramuscular injection of methyl mercury, except that the concentration of mercury in the brain was always less than that found in liver, kidney, or blood within 40 days of injection. Maximum brain concentrations were reached at approximately 15 days.

Friberg (*78*) administered 10 daily injections of methyl mercury dicyandiamide and mercuric chloride to rats and studied the distribution. Blood, spleen, liver, and kidney of rats receiving methyl mercury contained more uniform concentrations of mercury, while the concentration of mercury in brain was 10 times higher than found in the rats receiving mercuric chloride. However, the liver of methyl mercury treated rats had a mercury concentration five times higher than the brain.

It became apparent that large differences exist between species with reference to concentration of methyl mercury across the blood-brain barrier. After equilibration, the ratio of concentration of mercury in blood-to-brain approached 20 for rats (*78, 86, 222*), .5-1.5 for mice (*177, 219*) and cats (*131*) and 6.5 for pigs (*191*). Nordberg and Skerfving (*175*) estimated the blood-to-brain ratios for mercury concentrations in man at 0.1-0.2.

It is not known whether such dramatic differences exist between species in the ability to transport methyl mercury across the placental membrane. Suzuki et al. (*220*) reported that higher concentrations of mercury were found in the blood cells of umbilical cord in humans than were present in the maternal blood cells. Matsumoto et al. (*158*) reported the presence of higher concentrations of mecury in brains of fetal rats than their mothers which had received methyl mercury chloride.

Takeda et al. (*226*) studied distribution of other alkyl mercury compounds in rats. Rats treated with ethyl and n-butyl mercury showed mercury distribution patterns similar to those noted for methyl mercury, however, increasing the chain length of the alkyl group resulted in a decrease in the brain-to-plasma ratio for mercury concentration. Platonow reported similar distribution for ethyl (*190*) and methyl mercury (*191*) given to pigs.

Ellis and Fang (*64*) studied the intracellular distribution of phenylmercury and mercuric ions in rat kidney and liver. Whereas concentration differences between the two organs were only slight, several differences were noted between the two forms of mercury. When the concentration of mercury in the cell fractions was based on moles of mercury per milligram of nitrogen, the differences were as follows: (*a*) binding of phenylmercury was greater than inorganic mercury in all fractions, nuclear, mitochondria, microsomal, and soluble, (*b*) the rate of accumulation was faster for phenylmercury in most fractions, while elimination was equally as fast, (*c*) the level of mecury in the fractions, particularly the nuclear and soluble, remained quite constant over the 120-h study period.

Norseth (*176*) reported intracellular distribution patterns for mercuric chloride, methyl mercury dicyandiamide, and methoxyethylmercury in rat liver. Concentration of mercury after injection of mercuric chloride increased during a 4-day period in the mitochondrial and lysosomal fractions, and remained relatively constant in the microsomal fraction. Of the three mercurials used, the smallest percentage of mercury in the mitrochondria occurred with methyl mercury, the largest with mercuric chloride. Approximately 25.9% of methyl mercury was associated with the microsomes, compared to 12.5% of mercuric chloride and 18.7% of methoxyethylmercury. Approximately 25% of mercuric chloride was associated with the lysosomes compared to 18% of methoxyethylmercury and 7% of methyl mercury. Concentration of mercury in the nuclear and soluble fractions was not determined.

Excretion of mercury

Inorganic mercury is excreted by the kidney, liver in bile, intestinal mucosa, sweat glands, and salivary glands with the major routes of excretion in urine and feces (*82*). Rothstein and Hayes (*200*) reported that for 5-7 days after rats were injected with $Hg(NO_3)_2$, a high concentration of mercury was found in the feces; however, after 15-30% of the dose had been excreted by this route, there was a sharp decrease in the mercury content of the feces. The remaining mercury was primarily excreted in urine. A total of 47-71% of the total dose was excreted by the two routes within 52 days. The change in the route of excretion apparently resulted from the change of tissue distribution of mercury. High fecal excretion occurred during the rapid decrease in all tissues with the exception of the kidney. By the 15th day, 86% of the remaining body burden of mercury was concentrated in the kidney, Prickett et al. (*193*) noted similar excretion patterns of mercury from rats injected with mercuric acetate. Miettinen (*162*) and Rahola et al. (*194*) gave an oral dose of $^{203}Hg^{+2}$ to 10 human volunteers and studied the retention and excretion. Eighty-five percent of the dose was found in the feces collected during the first 5 days. The biological half-life of the 15% of the remaining mercury after 5 days as determined by whole-body measurements, was 37 ± 3 days for men and 48 ± 5 days for women. These half-lives correlate with the biological half-life of mercury remaining after the first phase of excretion in the study reported by Rothstein and Hayes (*200*), and therefore, the excretion rates of inorganic mercury apparently are similar for man and rat.

Because of the varying turnover rate of inorganic mercury in different body tissues, it is generally believed that the danger of repeated doses of mercury may be underestimated using calculations based on whole body biological half-life of mercury. To evaluate the danger for critical organs, it is best to consider the half-life of mercury on the basis of body compartments. Cember (*38*) developed a theoretical four-compartment model consisting of: (*a*) a long term compartment, the kidney; (*b*) a short term compartment, the liver; (*c*) a tissue compartment, the rest of the body, and (*d*) an excretion reservoir. By assuming first order kinetics and using parameters established from studies of rats injected with mercuric nitrate, he solved different equations describing the turnover of mercury in each compartment. The model proved reasonably representative of the elimination rate observed in rats injected with mercuric chloride (*38*).

The elimination rate of mercury from the kidney is dependent upon the dose; excretion rates increased directly with dose. This phenomenon may be the result of an increase rate of turnover of kidney cells resulting from the toxic effect of mercury (*188*).

Hayes and Rothstein (*107*) reported that excretion of mercury by rats which had inhaled mercury vapors was very similar to that noted previously when solutions of mercuric salts were injected. High concentrations of mercury were initially present in the lung, but within 15 days, the level diminished to a level found in other tissues. Gage (*85*) studied the effect of continuous and intermittent exposure to mercury vapors on excretion rates in the rat. Whereas, continuous exposure resulted in the constant daily excretion, intermittent exposure produced fluctuating daily excretions with peak excretions occurring on days when exposure had not occurred. The authors concluded that a delay occurs between exposure and excretion, and urine levels of mercury do not readily diminish after exposure ceases.

Ellis and Fang (*64*) reported that the excretion rate for phenylmercuric acetate in rats was similar to that observed for mercuric acetate once maximum accumulation of mercury in the kidney had occurred. Gage (*86*) studied the forms of mercury in the excrement of rats receiving one injection of phenylmercury acetate subcutaneously. Percentages of organic mercury in the urine were about 100, slightly less than 50, and approximately 5% for the first, second, and third days, respectively. The feces contained a relatively low percentage of organic mercury, even on the first day.

Cember and Donagi (*39*) reported that elimination of phenylmercury with respect to route and rate is dependent upon the dose. Larger doses resulted in a higher proportion of the mercury being excreted in the feces and higher total excretion per day. Most studies indicated that 67% of the dose of phenylmercury excreted by rats is contained in the feces (*64, 86, 193, 226*). Correlation of the results of different reports is complicated by variations of dose, length of exposure, and time of sample collections.

Excretion of alkoxymercury appears similar to that of arylmercury compounds. Daniel et al. (*53*) thoroughly investigated the excretion of methoxyethylmercury in rats and showed rapid metabolism to ethylene and inorganic mercury with a half-life of 1 day. Excretion shortly after administration reflected a high concentration of organic mercury in the urine, however, after a few days urinary mercury was in the inorganic form. Bile samples collected contained a high proportion of organic mercury, however, feces contained only inorganic mercury. Because the feces content was less than expected on the basis of bilary excretions, resorption and metabolism must occur in the intestine.

Swensson and Ulfvarson (*223*) compared the excretion rates of four forms of mercury in Leghorn cocks. Ten days after injection, the percentages of the initial doses remaining in the body were 10% for methoxyethylmercury, 20% for phenylmercury, 40% for mercuric nitrate, and 80% for methyl mercury.

Little methyl mercury is excreted from the body by the lungs (*181*). The urine contained 20% of the mercury excreted by rats during the 10-day period following a single injected dose; however, less than 30% of the injected dose was excreted during this time period (*178*). In humans, urinary excretion seems even less significant. Aberg et al. (*1*) reported that less than 10% of the mercury excreted within 49 days was found in the urine Miettinen et al. (*163*) found less than 0.5% of the excretion during the first 10 days occurred in the urine, however, by day 100, the mercury content of urine was approximatley ¼ that of the feces. In both studies, the mercury content of urine increased during the sampling period (*1, 163*). The biological half-life of methyl mercury determined by whole body measurements of humans was 76 ± 3 days (*163*).

The major route of excretion of methyl mercury is the feces (*1, 14, 78, 86, 163, 178*). Norseth and Clarkson conducted in-depth studies of the intestinal excretion of methyl mercury. Approximately 50% of the mercury in the feces is in the inorganic form (*177, 178, 179*). Norseth and Clarkson (*179*) reported that samples collected from the upper part of the small intestine contained significantly lower relative amounts of inorganic mercury in rats. In this report, they indicated that total excretion of mercury per day in the bile greatly exceeded the fecal excretion, and that the bile is the major source of mercury in the small intestine. Approximately 77% of the mercury in the bile exists in the form of a methyl mercury cysteine complex which is rapidly absorbed in the intestine and none in excreted. Mercury which is excreted in feces reportedly arises from exfoliation of intestinal cells, pancreatic excretions, gastric content, plasma catabolism, and the non-cysteine bound mercury compounds in the bile. Biotransformation of protein-bound methyl mercury to inorganic mercury

apparently occurs in the cecum or colon, possibly by microbial action.

Accelerated excretion of methyl mercury has been accomplished by feeding rats reduced hair (*225*) and mice thiol containing resins (*47*). Both materials have a high capacity to bind mercury compounds, and because no absorption of resin or hair occurs from the gastrointestinal tract, absorption of the mercury is markedly decreased.

Takeda et al. (*226*) indicate that ethyl and n-butyl mercury demonstrated excretion patterns similar to methyl mercury. Dimethyl mercury, however, has a considerably different excretion pattern. Ostlund (*181*) reported that 80-90% of a dose of dimethyl mercury was exhaled by mice within 6 h, whereas the remainder was metabolized to monoethymercury.

Indices of exposure

Numerous attempts have been made to identify a body component which could easily be assayed for mercury concentration and would serve as a valid index of the extent of exposure. The major criterion for such an index would be that the mercury concentration of that component was proportional to the mercury content of the critical organs or tissues of the body. This relationship should hold during both the period of accumulation and the period following exposure. Special attention has been given to blood, urine, feces, and hair as indices of exposure to mercury.

Difficulty has been encountered in identifying an index of exposure for inorganic mercury, or those forms of mercury which are rapidly broken down in the body. As discussed earlier, tissue distribution of these mercurials changes with time. Highest concentrations of mercury accumulate in the kidney. The biological half-time of inorganic mercury in the kidney is longer than reported for other body organisms and tissues and this may account for the observed poor correlations of blood and kidney concentrations of mercury (*186*). Mercury in the urine reportedly correlates with the levels in blood, but is not necessarily indicative of the concentration in the kidney (*16, 213*). Goldwater (*91*) and Smith et al. (*213*) reported that a correlation between exposure to mercury vapors and blood and urine mercury levels exists on a group basis in spite of individual discrepancies. Brown and Kulkarni (*30*) received numerous reports showing the concentration of mercury in the urine and blood of factory workers exposed to mercury and others (*30, 79*) showed large daily fluctuations in mercury concentrations in urine. The international committee which met to establish the Maximum Allowable Concentration (MAC Committee) of mercury considered use of urine and blood as indices. The concentration of mercury in urine may aid in diagnosis of mercury poisoning, and indicate exposure to air concentrations of mercury when used on a group basis (*80*). Also a positive correlation between exposure and mercury levels in blood was probably on a group basis. Based on reports of distribution and excretion of inorganic mercury compounds, it seems reasonable to conclude that blood and urine levels of mercury would serve primarily as an indicator of recent exposure and not necessarily kidney concentrations (*82*).

Alkylmercury compounds are more uniformly distributed through the body than other mercury compounds and excretion rates of alkylmercury compounds from the various body tissues are relatively uniform. For these reasons, greater success has been obtained in locating indices of exposure for alkylmercury compounds.

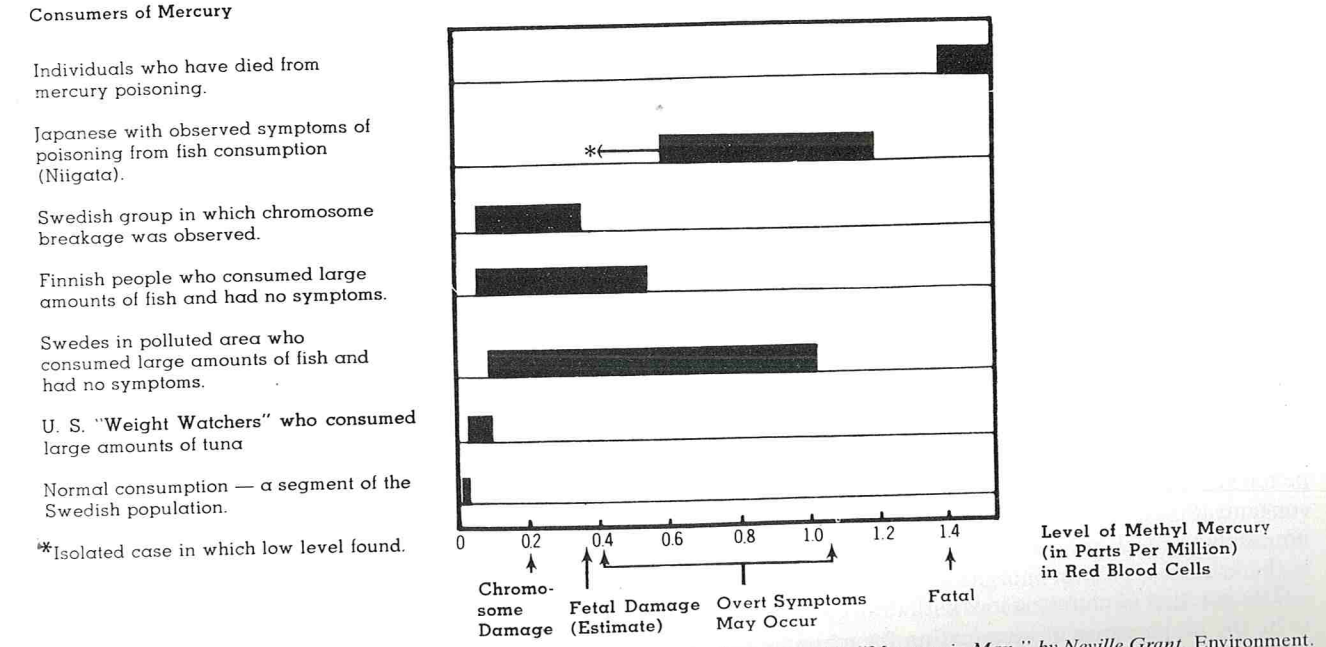

Figure 5. *Relation of methyl mercury levels in blood to physical hazard. Originally appeared in "Mercury in Man," by Neville Grant,* Environment. *Vol. 13, No. 4, pp. 2-15. Copyright © Committee for Environmental Information, 1971.*

The concentration of mercury in urine is of little value as an index of exposure to alkylmercury compounds. Lundgren and Swensson (*154*) studied workers exposed to alkylmercury compounds and reported that while under exposure, mercury was present in the urine, however, shortly after cessation of exposure, mercury could not be detected in the urine. Friberg et al. (*81*) and Kurland (*141*) reported that blood levels of mercury are a good indicator of the accumulation of alkyl mercury in the brain. Mercury concentration in red blood cells is probably most indicative of alkylmercury exposure; however, whole blood levels can be used if it is possible to exclude exposure to other forms of mercury (*186*). Figure 5 was presented by Grant (*96*) to summarize the relationship of methyl mercury levels in blood to physical hazard. A strong correlation between mercury levels of blood and hair during regular, continuous exposure was reported with hair levels approximately 300 times higher than blood levels (*81*). Work which was done in our laboratory showed a high correlation between methyl mercury content of diets and mercury levels in hair of guinea pigs. A correlation was also found between hair levels of mercury and mercury concentration of the optical cortex of the cerebrum. One advantage of hair as an index of exposure is that by clipping sections, it is possible to determine periods of previous exposure.

With reference to safe levels for indices of exposure, the MAC Committee (*80*) stated 10 μg Hg per 100 ml whole blood should not be exceeded. The Joint FAO/WHO Expert Committee on food additives (*69*) reported that the lowest mercury levels at the onset of neurological symptoms of mercury poisoning were 50 μg/g hair and 0.4 μg/g red blood cells.

Toxicology

Two concepts are of importance in the study of mercury toxicology: (*a*) the critical concentration, and (*b*) the critical organ. The critical concentration is reached when undesirable functional changes occur in the cell. The critical organ is that organ in which a critical concentration is first reached and may not necessarily be the organ which accumulates the greatest concentration (*186*).

Elemental mercury in the liquid state is not extremely toxic. As discussed previously, this is primarily a result of low absorption. The most danger of elemental mercury is encountered with the vapor state. Readily absorbed by the lungs, vapors of elemental mercury penetrate the blood-brain barrier. For vapors of elemental mecury, the critical organ may be either the central and peripheral nervous system or the kidney (*186*).

In the ionic form, inorganic mercury does not readily penetrate the blood-brain barrier, and thus the concentration of mercury in the central nervous system is not likely to reach the critical concentration. The kidney is the critical organ for inorganic mercury ions (*186*).

The reaction of mercuric ions with thiol groups appears to be the major cause of intoxication for mercury vapors or mercuric salts (*80*). Changes in membrane permeability, as well as interferences with enzyme activity, can occur as a result of the binding of mercuric ions to these ligands.

Rothstein (*199*) stated that almost every toxic action of mercurials is to some extent attributed to an interaction with sulfhydryl groups to form a complex known as a mercaptide. Because proteins contain sulfhydryl groups, they are the primary target for mercurial interaction and the consequent toxicological effects. Factors which may determine the probability of mercurial-sulfhydryl interaction are: (*a*) the presence of neighboring groups on the protein molecules which have an attractive or repulsive effect; (*b*) steric hindrance for mercurials with large organic portions; (*c*) protein configurations which may shield sulfhydryl groups and (*d*) properties of the medium among which would be pH and presence of anions. A thorough review of the effects of mercury on enzymes was presented by Webb (*241*).

The symptoms of poisoning by inorganic mercury were reported by Bidstrup (*21*). Acute doses cause gastroenteritis, abdominal pain, nausea, vomiting, and bloody diarrhea. Within a few days, gingivitis and stomatitis may develop, particularly if the patient maintains a low standard of oral hygiene. Excessive salivation, a metallic taste, swollen salivary glands, and ulcerated lips and cheeks may develop. Acute doses of mercury vapor are likely to cause a tightness in the chest making breathing difficult. Severe kidney injury leading to anuria and uremia are noted in acute causes of inorganic mercury poisoning. Tremors and delerium, resulting from damage to the central nervous system can result from exposure to acute or chronic doses of mercury vapors.

Numerous studies have been done to determine dose-response relationships, however, additional research is still needed in this area. The MAC Committee (*80*) relied heavily on the studies by Neal et al. (*169, 170*) to determine the safe levels of mercury vapor. These studies indicated that exposure to 0.1 mg mercury vapor/m^3 air would not cause poisoning. The MAC Committee (*80*) acknowledged that Russian studies had found typical symptoms of mercury poisoning in workers exposed to 0.1 to 0.3 mg mecury/m^3 air but concluded the symptoms were nonspecific.

The LD_{50} level of mercuric mercury received by intraperitoneal injection in mice is 7-13 mg/kg (*221*). Davies and Kennedy (*54*) reported that a subcutaneous injection of 0.75 mg of mercuric chloride/kg body weight caused kidney damage in rats, Fitzhugh et al. (*76*) gave oral doses of 40 and 160 ppm Hg^{++} to rats for 1 year and detected changes in the kidneys. The mercury concentrations in the kidneys were 16 and 49 μg/g wet weight, respectively (*76*).

Phenyl- and methoxyethylmercury compounds are readily degraded in the body, as discussed previously. Because of this decomposition, the toxicology of the aryl and alkoxy mercury compounds is similar to inorganic mercury. Little information is available on the toxicity of

these two classes of mercury compounds. For example, no serious toxic symptoms were reported for humans who consumed approximately 100 mg of phenylmercuric nitrate (*23, 242*). Fitzhugh et al. (*76*) fed diets containing 2.5 ppm phenylmercuric acetate to rats for 1 year and noticed only minor lesions in the kidney; however, pronounced kidney lesions occurred in rats fed a diet containing 10.0 ppm phenylmercury for 1 year. Tryphonas and Nielsen (*234*) found symptoms of poisoning including lesions of the gastrointestinal tract, kidney, and liver in pigs fed 2.28-4.56 mg Hg as phenylmercuric acetate/kg/day for 14-63 days. Goldwater (*94*) stated that 5 ppm phenylmercuric acetate in diets of rats resulted in senile changes in organs after 1 year; the changes rapidly regressed after feeding of mercury ceased.

Alkylmercury compounds have presented the greatest toxicological danger to man, particularly with reference to residue levels in foods. The problem encountered in Japan was discussed previously. In Iraq and Pakistan, hundreds of people became ill and many died when grain treated with methyl and ethyl mercury compounds was consumed, rather than being used for seed as was intended (*10, 103, 117*). In New Mexico, a family of seven consumed pork from pigs fed methyl mercury dicyandiamide (panogen) treated seed grain. Three children in the family evidenced severe brain damage (*51, 214*).

The critical organ for alkylmercury poisoning is the central nervous system (*186*). The MAC Committee (*80*) concluded from numerous studies that mercury concentrations of 10 μg/g in brain tissue were indicative of irreversible injury or death. Using a half-life of mercury in man of 70 days and a 15% distribution of the alkylmercury in the brain, the Committee concluded that an intake of 1 mg of alkylmercury per day would lead to toxic concentrations of mercury in the brain. The fetus can be of primary importance in cases of pregnancy. Several reports discussed earlier indicated that higher concentrations of mercury were found in fetal tissue in comparison to the concentrations found in the maternal tissues.

Tokuomi (*233*) thoroughly discussed the symptoms and signs of methyl mercury poisoning. A listing of the clinical features and frequency of their occurrence in 34 patients from the Minamata area is shown in Fig. 6. Harada (*104*) described the symptoms and signs of congenital methyl mercury poisoning which were presented in Table 2. Harada also reported that retardation of physical development, abnormal pneumoencephlograms (PEG) and abnormal electroencephlograms (EEG) were detected for patients who developed poisoning symptoms during early infancy. Takeuchi (*227*) stated that fetal methyl mercury poisoning could be distinguished from non-fetal poisoning because signs of underdeveloped and malformed central nervous systems were apparent in prenatal poisoning. Autopsies revealed a tendency for the lesions of the brain to be more localized in adults poisoned by methyl mercury, whereas, in the fetal and infantile brains, lesions are more diffuse (*227*). Atrophy of the brain with compensatory increases in fluid are macroscopically noticeable. In other parts of the body a reduction of red bone marrow, fatty degeneration of the liver, and erosion of the duodenum were reported (*227*). A discussion of microscopic changes in sections of the brain is included in this report by Takeuchi and will not be covered herein.

A latency period of one to several weeks occurs

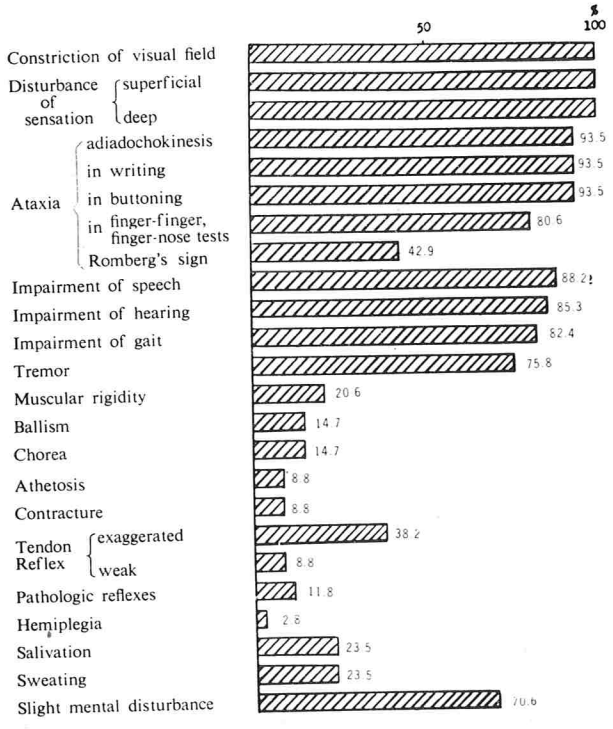

Figure 6. *Frequency of the occurrence of various symptoms and signs in Minamata disease. From Tokuomi, reference* 233.

TABLE 2. *Frequency at the first interview of occurrence of various symptoms and signs in minamata disease* (%)[1]

Symptoms	Congenital	Post-natal	
		Children	Adults
Mental disturbance	100	100	70.6
Disturbance in coordination	100	100	80-90
Disturbance in gait	100	100	82.3
Disturbance of speech	100	94.3	88.2
Disturbance of hearing	4.5	66.7	85.3
Constriction of visual field	?	100	100
Impairment of chewing and swallowing	100	88.9	94.1
Enhanced tendon reflex	81.8	72.2	38.2
Pathological reflexes	54.4	50	11.8
Involuntary movement	72.7	38.9	26.5-75.5
Primitive reflexes	72.7	0	0
Superficial sensation	*	*	100
Salivation	77.2	55.6	23.5
Forced laughing	27.2	28.6	

N.B. * : Not clear but all patients had sensation of pain.
[1]Reference *104*.

between administration of alkylmercury compounds and development of symptoms of poisoning (*178, 183*). Whereas, the latency period may represent the time required for breakdown of alkylmercury compounds and then the toxic action would result from the mercuric ion (*43, 44*); however, most data do not support this theory. Norseth and Clarkson (*178*) were unable to detect any significant breakdown of methyl mercury in the brain of rats during a 30-day period following intravenous injections. A more plausible explanation for the latency period is that alkylmercury compounds interfere with certain biochemical processes for which the effect is not immediately evident (*256*).

Pathological symptoms of alkylmercury poisoning have been documented to a greater extent than the biochemical effects which cause these symptoms. Many studies have indicated that methyl mercury may alter the protein synthesizing processes of the body. Yoshino, Mozai and Nakao (*256*) studied the incorporation of [U-^{14}C]leucine into brain proteins in brain cortical slices taken from rats which had been injected with methyl mercury thioacetamide. These authors reported a 43% reduction in protein synthesis in these brain slices obtained from animals which, with respect to neurological symptoms, were in the latent period of intoxication. Oxygen consumption and aerobic lactic acid formation by brain tissue slices decreased, however, not before the animal had developed neurological symptoms of methyl mercury poisoning. Likewise, succinic dehydrogenase activity of brain homogenates appeared normal until neurological symptoms developed and at this point there was a decrease in enzymatic activity. Brubaker et al. (*31*) injected labeled amino acids into rats which received injections of methyl mercury during the two preceding days. Twenty minutes after receiving the amino acids, the animals were sacrificed and the amount of labeled amino acids incorporated into proteins in liver was determined. Rats treated with methyl mercury incorporated more of the amino acids into protein than the untreated rats. In another paper (*153*), the 2-day treatment with methyl mercury was reported to proliferate the rough endoplasmic reticulum and decrease the amount of smooth endoplasmic reticulum of liver. A depression of the cytochrome P_{450}, cytochrome b_5, and aminopyrine dimethylase activity was noted in the liver microsomal fractions obtained from rats treated with methyl mercury. In contrast, Pekkanen and Salminen, 1972, (*184*) reported an increase in cytochrome P_{450} and NADPH-cytochrome C reductase activity in mice livers seven days after the animals were injected with methyl mercury nitrate.

In work done at the University of Wisconsin, effects of mercury compounds on RNA synthesis was studied. In rats given methyl mercury, a decrease in the RNA content was observed in spinal ganglion neurons and an increase in the RNA content of the anterior horn motorneurons of the spinal chord (*40*). Decreased synthesis of RNA by spinal ganglia after methyl mercury intoxication was confirmed in mice which were injected with [^{3}H]uridine (*41*). A significant decrease in the incorporation of the labeled uridine was noted (*41*).

An interesting, and possibly significant, discovery is the reported decrease in the toxic response noted when selenium was fed in a diet with methyl mercury. Iwata and Okamoto (*116*), as reported by Kojima and Fujita (*134*), found selenium reduced the toxic effect of methyl mercury. Ganther et al. (*87*) reported that rats receiving methyl mercury in the drinking water survived longer on basal diets supplemented with 0.5 ppm selenium than rats receiving the same diet without added selenium. These authors also found that Japanese quail fed 20 ppm methyl mercury in diets containing 17% tuna survived longer than quail fed the same level of methyl mercury in a corn-soya diet (*87, 88*). The selenium content of the diet containing tuna was 0.49 ppm. Potter and Matrone (*192*) verified the protective effect of selenite against the toxicity of methyl mercury and also mercuric chloride. They reported that selenite prevented the methyl mercury-induced reduction of liver size and the mercuric chloride-induced increase in liver size in rats. Selenite increased retention of mercury in the tissues regardless of the form of mercury fed. Ganther and Sunde (*88*) reported that their data indicated that tuna accumulate selenium concurrently with mercury in a 1:1 molar ratio. Whereas, the mechanism of protection by selenium is unknown, Ganther et al. stated that natural factors of protection such as with selenium should be considered when conducting toxicity studies (*88*).

Effects of mercury on chromosomes

Ramel (*196*) and Fiskesjö (*74*) reported that changes in chromosomes of onion were induced by alkyl-, alkoxyalkyl-, and arylmercury compounds. Changes reported were C-mitosis, polyploidy, and aneuploidy, with chromosome fragmentation occurring with phenyl and methyl mercury. Ramel (*198*) observed nondisjunction of the $\underline{X}$ chromosome in Drosophila eggs after treatment with organic mercury compounds. Fiskesjö (*75*) reported on in vitro studies with human leukocytes in which C-mitotic effects occurred in a medium containing methyl mercury at a concentration of $1\text{-}10 \times 10^{-5}$ M. Skerfving et al. (*209*) compared the incidence of chromosome breakage in lymphocyte cells with the concentration of mercury in the blood of persons eating varying quantities of fish. A statistically significant correlation was found between the number of lymphocyte cells with chromosome breaks or structural rearrangements and the mercury concentration of the red blood cells; however, no significant correlations existed between chromatid breaks, aneuploid or polyploid cells, or endore duplications and the mercury concentration of the red blood cells. Ramel (*197*) stated that the levels of mercury in food are sufficiently high to cause genetic effects in humans while the medical implications of these effects are unknown.

J. Milk Food Technol. Vol. 38, No. 5, Pages 301-303 (May, 1975)

Report of the Committee on Food Equipment Sanitary Standards, 1973-1974

The IAMFES Committee on Food Equipment Sanitary Standards, known hereafter as the Committee, is charged with the responsibility of cooperating with other interested health organizations and related industries in the formulation of sanitary standards and educational materials for the fabrication, installation, and operation of food equipment and to present to the membership those standards and educational materials which the Committee recommends be endorsed by the Association.

The purpose of this cooperative program is to aid industry in improving the design, construction, and installation of equipment so that it will lead to easy cleaning and proper functioning when placed into service in food establishments. It is the Committee's further purpose to cooperate with industry in the preparation of standards or guidelines which public health agencies will accept, thereby securing uniformity in the manufacture and nationwide acceptance of such equipment.

The following report will outline the Committee's activities during the past year in working with two health and industry organizations (National Sanitation Foundation's Joint Committee on Food Equipment Standards and the National Automatic Merchandising Association's Automatic Merchandising Health-Industry Council) and progress in meeting its purposes and objectives. It is expected these organizations will be the two groups that the Committee will work with during the coming year.

NATIONAL SANITATION FOUNDATION (NSF)

The Committee was represented at the 1974 meeting of the National Sanitation Foundation's Joint Committee on Food Equipment Standards, where action was taken on several proposals; and before the meeting, the Committee reviewed and submitted comments on each draft of these proposals. Since the meeting, the Committee has also reviewed and submitted comments on proposed changes to standards.

The Foundation staff is to be commended for setting aside the first half-day for a meeting of the public health representatives for them to review with the Foundation matters of mutual interest. This enabled the public health group to develop a productive and a uniform approach to agenda items and to expedite activities during the meeting of the Joint Committee.

Basic Criteria C-2 for special devices and equipment

The National Sanitation Foundation's criteria and standards to date have been limited for the most part to sanitary considerations. This position, in the opinion of a major restaurant chain sanitarian, has created problems for the users of NSF approved equipment, particularly in the area of durability of materials and finishes of food equipment. According to Foundation staff, it is extremely difficult and time consuming to test the durability of materials and finishes, because of the many variables in use and wear and the extent of preventive maintenance done on equipment. At the request of the Joint Committee, the NSF staff has made plans to develop a questionnaire and forward same to public health representatives throughout the country inquiring into the extent of the durability problem and soliciting recommendations for a workable solution.

During the 1973 meeting of the Joint Committee, the NSF staff was requested to determine the feasibility and advisability of developing a standard on backflow prevention equipment, such as vacuum breakers and other anti-siphon devices, for use with food service equipment. The Foundation staff advised the Joint Committee that, based upon a preliminary study during the past year, the development of such a standard is not considered feasible at this time. The staff further reported that this matter may be resolved through work of the ANSI-A-40 Committee, which has begun to develop a National Plumbing Code.

Standard No. 3-Commercial spray type dishwashing machines

The proposed revision to NSF Standard No. 3 was given a preliminary review for content only, since the Joint Committee members were not given sufficient time before the Joint Committee meeting to receive input from their respective Committees. This proposal along with suggestions offered by the Joint Committee is being referred back to the Standards Task Committee; and after the Task Committee has reviewed and accepted the proposal, it will be submitted to this Committee and others of the Joint Committee for final review, and comment or acceptance.

A major change to Standard No. 3, and one recommended by this Committee and accepted by the Joint Committee, provides that all final rinse spray arms (as well as wash water arms) shall be readily removable (without the use of any tools) for deliming and descaling and similar maintanance.

Standard No. 8-Commercial powered food preparation equipment

The proposed comprehensive revision to NSF Standard No. 8 was thoroughly reviewed by the Joint Committee. It was the consensus of the Joint Committee that some minor differences in construction and material requirements existed between the proposal and USDA requirements for meat and poultry processing equipment. Nevertheless, with the exception of resolving these differences, the proposed revision to Standard No. 8, as modified during the meeting, was accepted by the Joint Committee; copies of this revised Standard may be obtained from the Foundation.

One of the significant improvements made in the NSF Standards during this meeting of the Joint Committee was the deletion of Section 3.041 of NSF Standard No. 8, pertaining to specifications for soft solder. In addition to deleting any reference to soft solder in Standard No. 8, the Joint Committee also concurred that similar revisions would be made to C-2 and the other food equipment standards as they are reprinted.

Standard No. 18-Food and beverage dispensing equipment manual

At the recommendation of the Joint Committee, condiment dispensers will now be listed by NSF as non-refrigerated, mechanically refrigerated, or non-mechanically refrigerated; and any dispensers mechanically refrigerated shall be required to meet the performance requirements of NSF Standard No. 7-*Refrigerators and Storage Freezers.*

The guidelines developed by the NSF staff for cup stops on dispensing equipment were also reviewed by the Joint Committee and approved. These guidelines permit cup stops on beverage dispensers if the cup rim contact surface does not exceed ½" in width and if the activating motion of the dispensing lever is in the horizontal plane.

Standard No. 25-Vending machines for food and beverages

The continuing potential problem of carbonation backflow into copper lines or equipment of vending machines was again discussed by the Joint Committee. It was reported during the meeting that vending machines, with the exception of one manufacturer's equipment, had air gaps and no copper in the machine upstream of the carbonator. It was the consensus of the public health representatives of the Joint Committee that all copper tubing should be removed from post mix carbonation beverage machines. Consequently, the NSF staff was instructed to poll the members of the NSF Standard No. 25 Task Committee in regard to amending the Standard to permit only an air gap and to prohibit all copper tubing in such vending machines.

The subject of coin-operated office coffee machines was also reviewed, and the Foundation Staff reported that the Standard No. 25 Task Committee has met during the past year and recommended a

revision to NSF Standard No. 25 to include these machines. This Committee will be asked in the near future to review work of the Task Committee and to submit comments on the Task Committee's proposal.

Standard No. 29-Detergent and chemical feeders

The Joint Committee reviewed a proposal to amend Standard No. 29. to include a new Section 4.07 entitled "Performance Test" establishing specifications for testing Feeders. The proposal further included another new Section 4.08, entitled "Hydrostatic Tests for Feeders with Water Connectors." The Committee has studied these proposed revisions and, believing the criteria for the test as well as the test period to be inadequate, recommended that this proposal be re-submitted to the Standards Task Committee for further study.

Standard No. 33-Commercial cooking equipment exhaust systems

Standard No. 33 has provided reasonably adequate specifications for construction of open faced and slot-type hoods. However, Section 5.03, entitled "Other Types of Hoods" has not provided the same degree of specificity. This Committee has studied a proposal to delete the last phrase of this Section, which, in the opinion of some members of this Committee, would further weaken the Standard; it has submitted recommendations to the Foundation to review this portion of the Standard within the next year with the intent of providing adequate specificity for manufacturers for such equipment and for sanitarians who are charged with evaluating the adequacy of such equipment in the field.

Plans for future standards

The Joint Committee briefly reviewed the fourth draft of a proposed NSF Standard for supplemental flooring for food services, processing and transportation applications; this Committee has submitted its recommendation for this type of flooring to the Foundation for forwarding to the Standards Task Committee for Supplemental Flooring. It is anticipated that within the next year the Committee will have the opportunity to review another draft of this proposal.

The NSF staff reported that a proposed standard for Foam Cups and Containers was progressing satisfactorily. Phase I (development of testing equipment) has been completed and Phase II (testing of existing cups) is nearly completed. It is anticipated that this Committee will have an opportunity to review the the recommendations of the Task Committee within the next year.

At the recommendation of this Committee, the Joint Committee reviewed the need for sanitation requirements for drinking fountains. The Joint Committee agreed that the NSF should contact the American Refrigeration Institute regarding their requirements for drinking fountains, and explore the feasibility of developing an NSF Standard for Drinking Fountains. Currently, drinking fountains, in general, are difficult to clean and do not adequately protect the consumer from contamination and, as reported in the literature, provide only about the same degree of protection to the consumer as the common drinking cup.

NATIONAL AUTOMATIC MERCHANDISING ASSOCIATION

The National Automatic Merchandising Association's Automatic Merchandising Health-Industry Council (AMHIC) held its 19th annual meeting during October, 1973, and this Association and other public health organizations and the affected industries were represented and participated in AMHIC's discussion.

Carbonation backflow

The members of AMHIC, after several years of studying the potential carbonation backflow problem and reviewing reported outbreaks of copper poisoning due to carbonation backflow in vending marchines, agreed to a proposal to eliminate copper tubing in the internal carbonated water supply system downstream from the incoming water supply air gap of post mix beverage vending machines manufactured or re-manufactured on or after January 1, 1975. This significant step to protect the consumer was approved by AMHIC on the basis that application to re-manufactured machines needed further study and that application to newly manufactured post mix beverage vending machines would be contingent upon the availability of materials. This significant change was considered by industry and public health representatives of AMHIC as being a major step in safeguarding the health of the consumer.

Internal lighting

This Committee and others have recognized that vending machines frequently are located in areas without adequate lighting for cleaning the interior of the vending machines and felt that each vending machine should be equipped with lighting sufficiently adequate to enable the routeman to effectively service and clean the machine. Even though the merits of adequate lighting were discussed with all the members of AMHIC, no action was taken at this meeting, pending further study of this matter.

Icemakers for vending machines

The NAMA staff and members of AMHIC reported that some icemaker manufacturers have developed effective and efficient automated cleaning systems for their equipment being used in vending machines. Based upon the discussion, AMHIC recommended that NAMA initiate a study to determine the effectiveness of recently developed automatic icemaker cleaning systems as they relate to ice quality for the purpose of developing specifications for inclusion in the Evaluation Manual. A further recommendation was that a target date of July 1, 1974, be established for initiation of this project.

According to a recent report from the NAMA staff, a tentative protocol to do this study is being developed and selected manufacturers have been asked to participate in finalizing the proposed protocol. It is anticipated that the study would include a test machine that has been in operation for 50,000 vends or more, rather than using a new machine.

Organization plan and procedures

The AMHIC Organization Plan and Procedures was tentatively revised during the 1973 AMHIC Meeting, with final approval to be made later in the year. The proposed revisions concern voting procedures, membership, visitors and guests, and a few other minor changes. A copy of the revised Organization Plan and Procedures may be obtained from the National Automatic Merchandising Association.

Seal of approval program

After about 2 years of studying the advisability and feasibility of NAMA implementing a Seal of Approval Plan under the Vending Machine Evaluation Program and reviewing the pros and cons, the members of AMHIC accepted a recommendation by NAMA not to press for a Seal of Approval Program at this time. However, a sizeable number accepted NAMA's recommendation with the understanding that explorations on the feasibility and advisability of such an approval plan would be continued.

Administrative policies for vending machine evaluation program

The administrative policies for carrying out the Vending Machine Evaluation Program were amended to (1) require on and after January 1, 1974, the annual re-evaluation of all certified machines still in production; (2) require the first evaluation of vending machines of a new manufacturer applicant be conducted at the manufacturing plant or headquarters; (3) encourage evaluation agencies to make factory evaluations in all cases; and (4) require models made on a contract basis to be evaluated under each nameplate for which a Letter of Compliance is requested.

In addition, AMHIC requested NAMA to urge each manufacturer to post one or more model numbers on the machine front or interior, visible to inspection and vending operations personnel and that NAMA report the results to AMHIC during theyear with the goal of making such posting a mandatory requirement of Evaluation Manual.

Arthur J. Nolan Public Health Award

The AMHIC Organization Plan and Procedures was further amended to incorporate a suggestion of this Committee to specify that the Nolan Award Committee be composed of the immediate past three surviving award recipients of which the most recent recipient would serve as the Chairman of the Committee.

Standard for coin-operated special dispensers

According to the NAMA staff, some manufacturers of small office-type dispensing machines have requested NAMA evaluation under the Standard for Coin-Operated Special Dispensers of machines which utilize internal chocolate whipping devices. When AMHIC developed this Standard, the members, in general, had in mind using plain water and hot black coffee as acceptable liquid products to be dispensed through these machines. However, the Standard did not necessarily limit special dispensers to these two liquid products. In fact, AMHIC has used bacterial supportive criteria to include or exclude internal products and systems.

To assist in developing and assuring adequate safeguards, this Committee has submitted comments to the NAMA staff recommending that additional microbiological studies be made to determine the feasibility and acceptability of amending this Standard to permit the use of other food product systems, such as those for chocolate drinks, soups, tea, or carbonated beverages. Furthermore, AMHIC is currently exploring the advisability of submitting suggestions to FDA/PHS to encourage these regulatory agencies to recognize the specific aspects of the so-called mini-venders and to include these considerations in any proposed amendments to the USPHS Food and Beverage Vending Code.

Educational materials

With the assistance of this Committee and other members of the Automatic Merchandising Health Industry Council, the National Automatic Merchandising Association has revised two safety bulletins, entitled *Microwave Oven Safety* and *Copper Poisoning Prevention.* Both of these bulletins, which were published recently, provide much useful information to members of industry and regulatory agencies. The bulletin on *Copper Poisoning Prevention* has been developed and updated to aid in prevention of carbonation backflow into copper systems in those machines which were manufactured before January 1, 1975. It is believed that application of the principles and recommendations of this Bulletin can greatly reduce, if not prevent, carbonation backflow in vending machines being a source of foodborne illness due to copper poisoning. These bulletins may be obtained by members of public health agencies and organizations from the National Automatic Merchandising Association.

RECOMMENDATIONS

1. The Association reaffirms its support of the National Sanitation Foundation and the National Automatic Merchandising Association and continues to work with these two organizations in developing acceptable standards and educational materials for the food industry and public health.

2. The Association urges all sanitarians to obtain a complete set of the National Sanitation Foundation's Food Equipment Standards and Criteria and a copy of the National Automatic Merchandising Association—Automatic Merchandising Health-Industry Council's Vending Machine Evaluation Manual and related educational materials; to evaluate each piece of food equipment and vending machine in the field to determine compliance with the applicable sanitation guidelines (construction and installation specifications); and to let this Committee and the appropriate evaluation agency know of any manufacturer, installer, or operator failing to comply with these guidelines.

3. The Association urges all sanitarians and regulatory agencies to support the work of the Association's Committee, submit suggestions for developing new guidelines and for amending same, and subscribe, by law or administrative policy, to the principles represented by the Standards, Criteria, and Evaluation Manual for Food Equipment and Vending Machines.

This report of the Committee on Food Equipment Sanitary Standards is respectfully submitted by:

Karl K. Jones, Chairman, Purdue University, Student Hospital, West Lafayette, Indiana

Arthur L. Banks, Department of Health, Education, and Welfare, Brooklyn, New York

Robert R. Dalton, Michigan Department of Public Health, Lansing, Michigan

Carl Henderson, State Department of Health and Social Services, Santa Fe, New Mexico

Howard Hutchings, South Dakota Department of Health, Pierre, South Dakota

O. Donald Moore, Food & Drug Administration, Atlanta, Georgia

W. Joel Simpson, Pennsylvania Department of Environmental Resources, Harrisburg, Pennsylvania

Harold Wainess, Harold Wainess and Associates, Northfield, Illinois

PROGRAM

Sixty-Second Annual Meeting International Association of Milk, Food and Environmental Sanitarians, Inc.

In cooperation with
Ontario Milk and Food Sanitarians Assn. and
University of Guelph, Food Science Department
August 10-14, 1975

Royal York Hotel **Toronto, Ontario**

REGISTRATION

Sunday, August 10—1:00 p.m.-8:00 p.m.
Monday, August 11—8:00 a.m.-8:00 p.m.
Tuesday, August 12—8:00 a.m.-5:00 p.m.
Wednesday, August 13—8:00 a.m.-5:00 p.m.
Thursday, August 14—8:00 a.m.-12:00 Noon

REGISTRATION FEE—$10.00
Banquet—$15.00
Ladies Registration—$5.00
Students—No charge for Registration
National Mastitis Council Registration—$1.00

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L. O. Luedecke	Washington
Don Raffel	Wisconsin
Walter Wilson	Executive Board
Earl O. Wright	Executive Board

SUNDAY, AUGUST 10, 1975

1:00- 8:00 p.m.—Registration—Convention Floor Foyer
1:30- 5:30 p.m.—Executive Board — Confederation Room No. 3
6:00- 7:00 p.m.—Early Bird Reception—York Room
8:00-11:00 p.m.—Executive Board — Confederation Room No. 3

MONDAY, AUGUST 11, 1975

8:00 a.m.-8:00 p.m. Convention Floor Foyer

Special Meetings

9:00 a.m.-12:00 Noon—Executive Board—Confederation Room No. 3
1. Report on Local Arrangement
2. Report of Executive Secretary
3. Report of Sanitarians Joint Council

1:00- 3:00 p.m.—Affiliate Council—Manitoba Room
1. International Program
2. Discussion of Association Awards
3. Affiliate Input to Journal of Milk and Food Technology
4. Discussion of possible IAMFES and NEHA Merger
5. Future Meeting Location Recommendations
6. Election of Affiliate Council Officers
7. Other Pertinent Topics

1:30- 5:00 p.m.—Executive Board — Confederation Room No. 3
1. Report of Journal Management Committee
2. Regular Agenda
3. Committee Chairmen
4. Report of Affiliate Council
5. Meet with Past Presidents

1:30- 5:00 p.m.—Individual Committee Meetings
3:00- 5:00 p.m.—Farm Methods Committee—Tudor Room
6:00- 7:00 p.m.—Reception—Concert Hall

(Individual committee meetings are open to all registrants)

TUESDAY, AUGUST 12, 1975

8:00 a.m.-5:00 p.m.—REGISTRATION
Convention Floor Foyer
8:00 a.m.-9:00 a.m.—Executive Board
Confederation Room No. 3

Morning—General Session
Grand Ballroom

Harold E. Thompson—Presiding

9:30 a.m.—INVOCATION—William Harley
9:35 a.m.—ADDRESS OF WELCOME
David Crombie, Mayor of Toronto
Honorable William A. Stewart, Provincial Representative
10:15 a.m.—PRESIDENTIAL ADDRESS—Parnell Skulborstad
10:45 a.m.—A. B. Morrison, Ph.D.-Keynote Speaker
11:45 a.m.—Election Results, 1975

Afternoon—Milk Sanitation Section
Grand Ballroom

David Fry—Presiding

1:30 p.m.—DOOR PRIZE DRAWING
1:40 p.m.—BEHAVIOR OF ENTEROBACTER SPECIES IN SKIM MILK DURING FERMENTATION BY LACTIC ACID BACTERIA. J. L. Rutzinski and E. H. Marth.
2:00 p.m.—AN EXAMINATION OF METHODS FOR ASSESSING POST PASTEURIZATION CONTAMINATION. Guenter Blankenagel

2:40 p.m.—EFFECT OF MEDIUM, DILUTION FLUID AND INCUBATION TEMPERATURE ON ENUMERATION OF BACTERIA IN GRADE A AND MANUFACTURING GRADE MILK. Chamras Sanghirum and B. E. Langlois.

3:00 p.m.—DETERMINING SANITARY STATUS OF FARM MILK PIPELINES USING THE RINSE-FILTER PROCEDURE. R. W. Scroggins and R. T. Marshall.

3:20 p.m.—Milk Break

3:35 p.m.—PROPOSED NEW MICROBIOLOGICAL STANDARDS FOR CHEESE. D. L. Collins-Thompson

4:05 p.m.—NORTHEAST DAIRY PRACTICES COUNCIL. Richard P. March

4:45 p.m.—CORRELATION OF THE MILK QUALITY (MQG) WITH THE WISCONSIN MASTITIS TEST (WMT). R. Kevin Chumney and Dick H. Kleyn.

Afternoon—Food Sanitation Section
Ontario room

Henry Atherton—Presiding

1:30 p.m.—DOOR PRIZE DRAWING

1:40 p.m.—BACTERIOLOGICAL QUALITY OF HAMBURGER AT THE RETAIL LEVEL. C. L. Duitschaever.

2:00 p.m.—MICROBIOLOGICAL STANDARDS FOR MEAT IN CANADA. H. Pivnick.

3:30 p.m.—REMOVAL OF BISULFITE FROM SHRIMP BY HYPOCHLORITE RINSES. K. E. Weingartner, F. W. Knapp and J. A. Kroburger.

3:20 p.m.—Milk Break

3:35 p.m.—MICROBIOLOGY OF THE WILD RICE FERMENTATION. J. F. Frank, E. H. Marth, R. C. Lindsay, and D. B. Lund.

3:55 p.m.—NATIONAL SANITATION TRAINING PROGRAM. Richard J. Davies.

4:35 p.m.—POTENTIAL FOR AFLATOXIN PRODUCTION ON SMOKED MULLET. B. Y. Farhat and J. A. Koburger.

TUESDAY EVENING, AUGUST 12, 1975

7:30-9:30 p.m.—EVENING DISCUSSION GROUPS

7:30 p.m.—FOOD SANITATION-Algonquin Room
William F. Bower-Moderator
C. L. Duitschaever
H. Pivnich
David E. Hartley
Richard J. Davies

7:30 p.m.—MILK-Territories Room
Richard P. March-Moderator
E. L. Collins-Thompson
R. L. Richter
Robert Zall
H. E. Thompson

7:30 p.m.—ENVIRONMENTAL SANITATION-Manitoba Room
Walter Wilson-Moderator
J. B. Derbyshire
Larry J. Gordon
John B. Robinson

9:30-11:00 p.m.—Wine and Cheese Party

WEDNESDAY, AUGUST 13, 1975 - Grand Ballroom

8:00-5:00 p.m.—REGISTRATION-Convention Floor Foyer

Parnell J. Skulborstad—Presiding

8:30 a.m.—DOOR PRIZE DRAWING

8:45 a.m.—In Defense of Food Packaging-A. R. Chadsey

9:15 a.m.—Water and Waste Management-Everett M. Biggs

10:00 a.m.—Milk Break

10:15 a.m.—DOOR PRIZE DRAWING

10:20 a.m.—ANNUAL BUSINESS MEETING
1. Report of Executive Secretary
2. Report of Secretary-Treasurer
3. Committee Reports
4. 3-A Symbol Council Reports
5. Report of Resolutions Committee
6. Report of Affiliate Council
7. Old Business
8. New Business
9. Election of Officers

Ivan Parkin—Parlimentarian

Afternoon—Milk Sanitation Section
Grand Ballroom

William Harley—Presiding

1:30 p.m.—DOOR PRIZE DRAWING

1:40 p.m.—RECOVERY OF COMPOUNDS RESPONSIBLE FOR LIGHT-INDUCED FLAVOR IN MILK BY LYOPHILIZATION. J. D. White and A. H. Duthie.

2:00 p.m.—FEDERAL REGULATION FOR GRADE A MILK AND MILK PRODUCTS. H. E. Thompson.

2:40 p.m.—RESUMED FEEDING OF APPLE POMACE IN THE DAIRY REGIMEN. R. H. Walter.

3:00 p.m.—EFFICACY OF CHEMICAL CLEANERS AND SANITIZERS. J. K. Roh, R. L. Bradley, Jr. and D. B. Lund.

3:20 p.m.—Milk Break

3:35 p.m.—FIELD STUDIES OF BULK MILK TRANSPORT WASHING SYSTEMS. R. L. Richter.

4:05 p.m.—DETERGENTS AND SANITIZERS AS POSSIBLE CONTAMINANTS IN MILK. Robert R. Zall.

Afternoon—Food Industry Sanitation Section
Tudor Room

Phil Glass—Presiding

1:30 p.m.—DOOR PRIZE DRAWING
1:40 p.m.—HEAT INACTIVATION OF CONIDIA PRODUCED BY TOXIGENIC ASPERGILLI. M. P. Doyle and E. H. Marth.
2:00 p.m.—THE FEDERAL FOOD SERVICE PROGRAM. William F. Bower.
2:40 p.m.—THE NORMAL FLORA OF ROCK SHRIMP: SICYONIA BREVIROSTRIS. A. R. Norden and J. A. Koburger.
3:00 p.m.—MICROBIOLOGICAL EVALUATION OF RETAIL GROUND BEEF: CENTRALIZED VERSUS TRADITIONAL PREPARATION. J. G. Shoup, J. L. Oblinger and J. A. Koburger.
3:20 p.m.—Milk Break
3:35 p.m.—NUTRITION IN CANADA. Margaret Cheney.
4:05 p.m.—RECENT TRENDS IN FOOD AND BEVERAGE VENDING. David E. Hartley.
4:45 p.m.—FOOD POISONING OCCURRING IN CANADA DURING 1973. E. Todd.

Afternoon—Environmental Sanitation Section

George Hazlewood—Presiding

1:30 p.m.—DOOR PRIZE DRAWING
1:40 p.m.—INFLUENCE OF LOCATION AND SUBSTRATE ON LEVELS OF TRACE AND HEAVY METALS IN MUSHROOMS AND THEIR HEALTH SIGNIFICANCE. Amer El-Ahraf and Rudi Mattoni.
2:00 p.m.—INTEGRATING FOOD PRODUCTION AND PROCESSING INTO NATURE'S GEOCHEMICAL CYCLES. John B. Robinson
2:40 p.m.—SENSITIVITY OF GUINEA PIGS AND FETUSES TO DIETS CONTAINING ADDED METHYL MERCURY AND SELENIUM. A. G. Hugunin, R. L. Bradley, Jr. and W. E. Ribelin.
3:00 p.m.—PERSISTENCE OF ENTERIC ORGANISMS IN SOIL AND ON VEGETABLES SPRAY IRRIGATED WITH EFFLUENT AND SLUDGE. Joseph Lovett, Brenda Boutin and Jan M. Bisha.
3:20 p.m.—Milk Break
3:35 p.m.—FATE OF ANIMAL VIRUSES IN EFFLUENT FROM LIQUID FARM WASTES. J. B. Derbyshire.
4:05 p.m.—ORGANIZATION OF ENVIRONMENTAL PROGRAMS AT STATE LEVEL. Larry J. Gordon
VARIOUS ENVIRONMENTAL STRUCTURES IN CALIFORNIA HEALTH AGENCIES. Walter F. Wilson.
4:45 p.m.—SURVIVAL OF FOOT-AND-MOUTH DISEASE VIRUS IN CASEIN PRODUCE FROM INFECTED COW'S MILK. H. R. Cunliffe and J. H. Blackwell.

WEDNESDAY EVENING, AUGUST 13, 1975

6:00-7:00 p.m.—RECEPTION—Foyer and Room B
7:00 p.m.—ANNUAL AWARDS BANQUET—Ballroom
P. J. Skulborstad, President, Presiding
INVOCATION—Ivan E. Parkin
INTRODUCTIONS
PRESENTATION OF AWARDS
Walter F. Wilson, Chairman
1. Past Presidents Award
2. Citation Award
3. Honorary Life Membership
4. C. B. Shogren Memorial Award
5. Sanitarians Award
Sponsored by:
Klenzade Products, Inc.,
Division Economics Laboratories
Pennwalt Chemical, Inc.,
Diversey Corporation, Inc.
6. Industry and/or Education Award
Sponsored by:
Milking Machine
Manufacturers Council of the Farm and Industrial Equipment Institute

INSTALLATION OF OFFICERS
ENTERTAINMENT

THURSDAY, AUGUST 14, 1975

8:00 a.m.-12:00 Noon—Registration—NATIONAL MASTITIS COUNCIL
Convention Floor Foyer
7:30 a.m.—EXECUTIVE BOARD—Confederation Room #3—Breakfast Meeting

NATIONAL MASTITIS COUNCIL
1975 Regional Meeting
Roof Garden

PROGRAM

Dr. D. A. Barnum, Presiding

8:30 GREETINGS—N.M.C. President
8:35 WELCOME—Hon. W. A. Stewart, Minister of Agriculture and Food, Province of Ontario
THEME—Mastitis: The Picture in Ontario
8:50 *PERSPECTIVE*
(a) Ontario Milk Marketing System
George McLaughlin

9:05	(b) Role of the Ontario Ministry of Agriculture & Food Dr. J. A. Meiser
	GROUND COLOUR
9:15	(a) Ontario Central Milk Testing Laboratory Dr. J. A. Meiser
9:25	(b) Herd Milk Quality as affected by Mastitis Dr. J. A. Meiser
10:00	*BREAK*
10:15	*SOME ADDED COLOUR* Automated Cell Counting and More Dr. F. H. S. Newbould
10:45	*COMPLETING THE PICTURE* Eastern Ontario Milk Improvement Program. A total management concept. Dr. Geo. Fisher
11:15	*OVERPAINTING OR PICTURES TO COME* The future—Dr. F. C. Nelson,
12:00 Noon	*LUNCHEON* Break

Afternoon Session

Dr. K. A. McEwen, Presiding

1:30	The Veterinary Practitioner's Function: Convincing the farmer to help himself. Dr. R. K. Lothrop,
2:00	Mycoplasma Mastitis—Louise Ruhnke,
2:30	High Bacteria counts in farm bulk tanks—*Strep. agalactiae* the culprit. Dr. F. C. Nelson, Dr. G. Fisher Dr. T. Rothmel
3:00	*BREAK*
3:30	Mastitis Eradication—A Dream? Dr. F. H. S. Newbould
4:00	General Discussion
5:00	Adjourn
5:15	NMC Board Meeting—British Columbia Room

ENTERTAINMENT
MEN AND WOMEN

SUNDAY, AUGUST 10, 1975

6:00-7:00 p.m.—RECEPTION—York Room

MONDAY, AUGUST 11, 1975

6:00-7:00 p.m.—RECEPTION—Concert Hall

TUESDAY, AUGUST 12, 1975

9:30-11:00 p.m.—WINE AND CHEESE PARTY

WEDNESDAY, AUGUST 13, 1975

6:00-7:00 p.m.—RECEPTION—Foyer and Room B
7:00 p.m.—BANQUET & ENTERTAINMENT

LADIES HOSPITALITY ROOM

MONDAY-WEDNESDAY, AUGUST 11-13, 1975
9:00 a.m.-5 p.m.York Room

ENTERTAINMENT FOR THE LADIES

TUESDAY, AUGUST 12, 1975

9:30 a.m.	Coffee and pastries Information on Convention and Toronto. Introduction of Hostesses.
10:45 a.m.	Ladies meet at York St. entrance of Royal York Hotel for bus to the R.C.Y.C, (Royal Canadian Yacht Club), this club is one of the leading Yacht Clubs on the North American continent.
11:00 a.m.	Bus leaves hotel for R.C.Y.C. dock.
11:15 a.m.	R.C.Y.C. ferry leaves for this island club.
11:30 a.m.	R.C.Y.C. ferry arrives at island. Ladies are free to tour gardens and boat areas.
12:00 Noon	Luncheon. Sponsored by Ontario Ministry of Agriculture and Food
1:15 p.m.	Ladies leave club house for dock.
1:30 p.m.	Ferry returns ladies to mainland.
1:45 p.m.	Arrive at main land. Bus takes ladies to Ontario Place.
2:15 p.m.	Arrive at Ontario Place. Nominal admission charge. Ladies will have approximately one hour to tour on their own. A tour by a small train is available.
3:30 p.m.	Bus leaves for hotel.
4:00 p.m.	Arrival at hotel.

WEDNESDAY, AUGUST 13, 1975

9:00 a.m.	COFFEE AND PASTRIES
9:30 a.m.	DEPART FOR CITY HALL TOUR VIA THE SUBWAY
10:00 a.m.	TOUR OF CITY HALL—1-1½ hrs.
12:00 p.m.	LEAVE FOR LUNCH ED'S WAREHOUSE, 266 KING ST. WEST
12:30 p.m.	LUNCH, ED'S WAREHOUSE

Afternoon is free for shopping

Committee for Ladies

Mrs. J. W. Raithby, (Nancy)
49 Anglesey Blvd.
Islington, Ont.

Mrs. W. Kempa (Nada)
1058 Runningbrook Dr.
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Mrs. V. Jensen, (Joan)
100 Munro Blvd.,
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Association Affairs

The Iowa Affiliate Honors Seven at Their Annual Meeting

The annual meeting was held in Ames, Iowa at the Ramada Inn on March 24, 1975. This year a joint meeting was conducted with the Iowa Environmental Health Association. William Schlenker of Waterloo was awarded the DR. M. P. BAKER AWARD accompanied by a $50.00 savings bond for being the outstanding sanitarian of the year.

Mr. Schlenker graduated from Iowa State University with a degree in dairy industry in 1973. He has been involved in quality control in the Iowa dairy industry since his graduation. He is a charter member and past president of the Iowa Affiliate and presently is manager of American Milk Producers, Inc., Cedar Valley Division, Waterloo. Mr. Schlenker was cited for his outstanding leadership in the field of dairy sanitation.

President John Halbach awards William Schlenker the M. P. BAKER AWARD while Mrs. Schlenker looks on.

Six were awarded the twenty year award given by the Association. They were H. E. Hansen, Des Moines; Don Jaeger, Marshalltown; Grover Seeberger, Mason City; Wilbur Nielsen, Independence; Earl O. Wright, Ames and Harold Whitcomb, Sumner, Iowa.

TWENTY YEAR AWARD

Left to Right—E. Wright, H. Hansen, D. Jaeger, G. Seeberger and W. Nielsen.

The Iowa affiliate conducts two meetings a year. The next meeting of the affiliate will be held in November 1975.

Notice to Membership

Constitution Amendment

The Executive Board recommends that the Constitution be amended as follows:

ARTICLE IV — Section 1. Insert the words "and Secretary-Treasure" following the words "Second Vice-President" in line 4. Delete the "and" in lines 4 and 5. Add the words "and Second Vice-President" following the words "First Vice-President" in line 5. Delete the words "second Vice-President and" from lines 5 and 6.

ARTICLE VII—Section 1. Change the words "Secretary-Treasure" to the words "Executive Secretary."

By-Laws Amendment

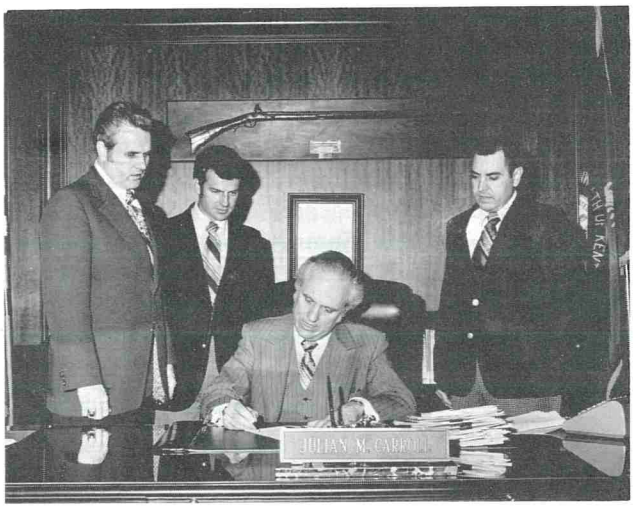
By-Laws amended as follows:

ARTICLE II — Section B. Change the wording of "Secretary-Treasure" to "Executive-Secretary." Delete the word "an" and replace with the word "the." Change the wording of "Executive-Secretary" to "Secretary-Treasure."

This is being done to bring the Constitution and By-Laws up to date. This will also provide for the Secretary-Treasure to advance on the Executive Board as the other board members have in the past. If this amendment is voted favorable, elections will be changed to electing only a Secretary-Treasure each year instead of a Second Vice-President and Secretary-Treasure. This will be presented to the membership for action at the annual meeting in August at the Royal York Hotel, Toronto, Ontario, Canada.

News and Events

Environmental Health Week



Left to Right—Bruce Lane, President-Elect; Dr. James Hartley, President; Governor Julian Carroll and Leon Townsend, Secretary-Treasurer.

At the request of the Kentucky Association of Milk, Food and Environmental Sanitarians Governor Julian M. Carroll proclaimed the week of February 23-March 1, 1975, "Environmental Health Week in Kentucky."

In connection with the proclamation the Kentucky Association of Milk, Food and Environmental Sanitarians, Inc. held its annual Educational Conference for Fieldmen and Sanitarians at Stouffer's Inn, Louisville, Kentucky.

Approximately 350 county and state sanitarians, administrators, health officers and milk and food industry fieldmen, plant managers, related service company personnel and university personnel were in attendance.

Customs Information

Tips for members and friends who will be attending the annual meeting in Toronto.

Carry with you the following:

1. Birth Certificate or a Naturalized Citizenship Certificate.
2. A brochure or program details about the 62nd Annual Meeting.
3. Local U.S. Customs Office should be contacted for latest information about the amount and type of duty free purchases that can be made in Canada.

In addition, for those travelling by air we are going to inform the Toronto International Airport about the details or our meeting in order that Customs clearance can be expedited.

Mr. Clarence Davis (left) and Miss Debbie Newslow proudly receive the prestigious NEIL C. ANGEVINE SUPERIOR QUALITY AWARD on behalf of T. G. Lee Foods, Orlando, Florida from Dr. C. Bronson Lane, Secretary of the American Cultured Dairy Products Institute (ACDPI). This annual award—initiated at the recent Kultures and Kurds Klinic in Garland, Texas—consists of an engraved plaque and revolving trophy which are awarded to the dairy plant with the highest cumulative score for all cultured products analyzed at the annual ACDPI training schools. This year's event drew 130 delegates from twenty-two states, Mexico, and Puerto Rico.

Certificates of merit for quality products were awarded to the following organizations: Anderson-Erickson, Des Moines, Iowa (lemon yogurt); Safeway Stores-Dairy Division, Garland, Texas (strawberry yogurt and all categories yogurt); Mid-America Dairymen, Minneapolis, Minnesota (plain yogurt); Kroger Co., Fort Worth, Texas (buttermilk); Smith Dairy Products Co., Orrville, Ohio (sour cream); Superior Dairies, Austin, Texas (cottage cheese).

Index To Advertisers

Classified Ads

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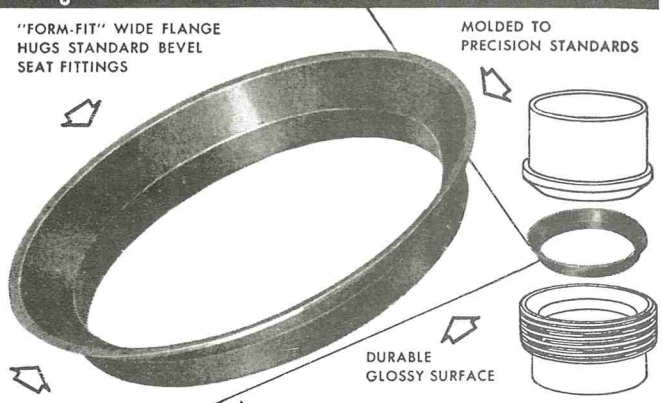

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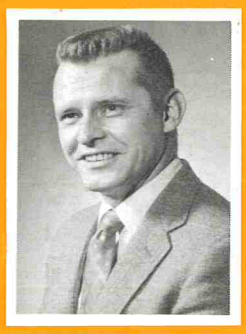
Dairy authorities speak out on better cow milking



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Milk quality: What a dairyman should look for in a milking system.

The basics of milking equipment stay the same regardless of herd size. In talking with dairymen, the question is often raised as to what type of milking system should be installed. The answer is that it should be a system which will produce minimum vacuum fluctuation at the teat end, fast milking, maximum cow comfort and high quality milk in the bulk tank. This is easier said than done! My first preference in milking systems is a low-line parlor, and my last preference is a high-line parlor, or around-the-barn pipeline. There is absolutely no excuse for the installation of a high-line parlor (milk transferred directly from the cow through the cluster and into an overhead line), both from the standpoint of vacuum fluctuation and milk quality.

The effect of high-lines on milk quality as measured by Acid Degree values (ADV) is presented in Table 1. ADV's measure free fatty acids in milk which are one of the causes of rancidity and increase with air agitation which occurs when milk is moved from the cow to an overhead milk line. Let me point out that I realize the practical consideration of around-the-barn pipelines in easing the milking chore, and the only way in which pipelines can be installed in most tie-stall barns.

I would hope that the day will come when low-line installations will be approved and practical in tie-stall barns. These may be at curb level or under the platform. However, when tie-stall barns are being constructed, they should have a slope of 1-1.5 inches/10 ft. toward the milk receiving area. This will allow the pipeline to be installed at both a lower level and at the same level for each stall. Weigh-jar parlors and bucket milkers fall in between low-line and high-line systems with regard to the criteria mentioned above. Short tube buckets or large capacity claws generally have less fluctuation than small claw, long tube bucket milkers.

Weigh-jars which are installed so that the milk entry into the jar is equal to or lower than the bottom of the claw, or lowest point of the connecting milk hose, will simulate a low-line. Most weigh-jar installations are set too high by either necessity when center weigh-jars are installed, or for the convenience of the operator. Anytime milk can be moved away from the claw by gravity, vacuum fluctuation is minimal, particularly when compared to lifting milk into a high-line.

Table 1 — Acid Degree Value Comparison between systems

	1968 - 1972		1972 - 1973	
System	No. Tested	Average ADV	No. Tested	Average ADV
Low-line parlor	20	0.84	6	0.88
Buckets	222	0.80	80	0.88
Weigh-Jar Parlor	78	0.74	28	0.93
High-line Parlor	46	0.99	18	1.07
Around-the-barn pipeline	180	1.11	100	1.25

Milk hoses on a low-line should be as short as possible. Remember, milk is lifted as solid column against a differential in pressure created by air entry into the claw. This causes a reduced vacuum at the teat end each time a column of milk moves into the milk line, and an increase to line vacuum when the milk clears the hose. It should be noted that a higher vacuum is necessary for a high-line system versus a low-line, to get the same job done due to milk lift. When overmilking occurs, a cow milked using a high-line system, is exposed to a higher vacuum.

Components in any milking system should include adequate claw capacity and narrow bore liners and shells. Inflations are the most important part of milking equipment and should be changed before reaching 1500 milkings and preferably at 1200 for moulded liners. Stretch liners should be changed around 900 milkings. If you notice a change in milking speed or cow comfort when new liners are installed, you waited too long.

Vacuum pump capacity for bucket units should be 4-6 cfm/unit, preferably the high figure. Pipeline systems are open systems and the capacity of the vacuum pump should be 8-10 cfm/unit (per New Zealand method). When using a vacuum recorder on a pipeline system with the vacuum measured at the teat end, the air reserve should be such that when a unit is dropped or kicked off by a cow, the system will have enough reserve so that a drop in vacuum is small, if any, on the other units.

Another area in which progress is being made, is in vacuum regulators. With sensitive regulators having fast response time, vacuum fluctuation can be minimized on the main vacuum and milk lines. Unstable vacuum in the main lines is magnified at the teat end. Adequate-sized vacuum and milk lines are assumed for the purposes of this discussion and recommendations can be obtained through the National Mastitis Council or the 3M Standards, available through your milking equipment dealer.

When looking at systems, two things should always be kept in mind: (1) buy equipment where quality equipment and *service* is the "by-word"; (2) when looking at a system, go to a dairyman already using it, and if possible, do some actual milking.