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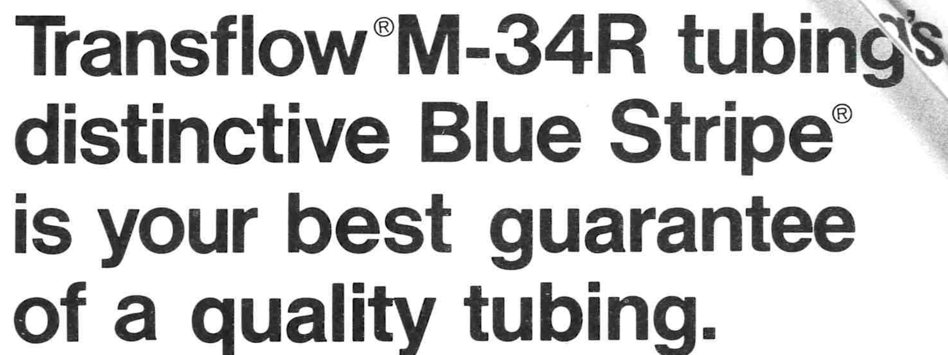

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Vol. 38 | April 1975 | No.4

Research Papers

Fate of Bacteria Exposed to Washing and Drying on Stainless Steel
R. B. Maxcy ---- 192

Influence of Selected Solutes on Thermally Induced Death and Injury of *Salmonella Typhimurium*
Amy C. Lee and J. M. Goepfert ---- 195

A Comparison of Cultural Methods used with Microcolony and Direct Fluorescent-Antibody Techniques to Detect *Salmonellae*
N. F. Insalata, W. G. Dunlap, and C. W. Mahnke ---- 201

A Vapor Pressure Osmometer for Determination of Added Water in Milk
K. Pensiripun, E. C. Campbell, and G. H. Richardson ---- 204

Effect of Antifungal Agents on the Carbohydrate Metabolism of *Kluyveromyces fragilis*
K. M. Shahani, M. G. Carrancedo, and A. Kilara ---- 208

Repair, Growth, and Enterotoxigenesis of *Staphylococcus aureus* S-6 Injured by Freeze-Drying
Daniel Y. C. Fung and Linda L. Vanden Bosch ---- 212

Extraction of Protein from Mechanically Disrupted Freeze-Dried Brewer's Yeasts
C. TU, C. Farnum, and J. Cleland ---- 219

Milk Quality in the New York Public School System
D. K. Bandler, R. O. Brown, and E. T. Wolff ---- 223

General Interest

Enrichment Serology and Fluorescent Antibody Procedures to Detect *Salmonellae* in Foods
John S. Hilker ---- 227

Treatment of Raw Milk Wastes by a Multi-Stage Biological System
John K. Sullins, William L. Arledge, and Eugene V. Ricci ---- 232

Grapes and Wine Technology: Grapes to Wine
A. M. Joe and K. M. Shahani ---- 237

E-3-A Sanitary Standards for Plate Type Heat Exchangers for Fluid Egg Products. Serial # E-1100 ---- 244

News and Events ---- 246

Association Affairs ---- 247

Index to Advertisers ---- 250

Classified Ad ---- 250

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Fate of Bacteria Exposed to Washing and Drying on Stainless Steel[1]

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(Received for publication July 31, 1974)

ABSTRACT

Washed equipment constitutes a unique, challenging environment for most microorganisms, survivors of which are contaminants for subsequent food processing. In this work, cells were treated by washing followed by drying on stainless steel test strips. Various components of cleaning cycles were studied to determine the relative destructive effects on representative organisms. Even the simplest sanitation processes, i.e. rinsing and drying, were destructive to cells of *Staphylococcus aureus, Pseudomonas fluorescens,* and *Escherichia coli.* Surfactants did not markedly alter the destructive forces of washing and drying. Formulated cleaner was even less destructive than dilute surfactant. Cells exposed to cleaning and drying processes were injured, and substantially reduced numbers were capable of recovery on selective media. Since there was not a great difference in the effects of the cleaning constituents on gram-positive and gram-negative bacteria, residues would not be expected to promote a selected microflora as has been indicated in previous work. Spores were resistant even after various shock treatments including heat.

Washing of equipment and its maintenance until used are challenges in all phases of the food industry. Washing removes most organisms and exposes those resisting removal to potentially injurious treatments. Furthermore, those cells remaining on the equipment may be in an unfavorable environment until subsequent food processing commences (*2, 4, 10*).

Most work on residual flora after cleaning has dealt with total numbers associated with harborages for partial protection of the cells (*9*). Thus cells may be subjected to sublethal effects of cleaning and sanitization, which produce injured cells that are sensitive to diluents and selective media (*3, 5, 6, 11-15*). Therefore, presence of injured cells may influence the whole basis of evaluating sanitation procedure, because microbial counts are a primary factor in evaluating results.

The purpose of this work was to study the fate of bacteria subjected to factors in common cleaning and storage processes and to determine the effectiveness of selective media in enumerating cells so treated.

METHODS

Cultures

Escherichia coli, Staphylococcus aureus, and *Pseudomonas fluorescens* were from the departmental culture collection. *Bacillus cereus* and *Bacillus licheniformis* were obtained from the Dairy Science Department, South Dakota State University, through the courtesy of Dr. J. H. Martin. Sporeformers were grown at 32 C in fluid thiogly collate medium with approximately .06 g calcium carbonate per 5 ml tube. Cultures were transferred daily to assure active growth. Spores were obtained by growing cultures on a starch-manganese sulfate nutrient agar and harvesting according to the method of Martin and Harper (*7*). The asporogenous bacteria were grown in nutrient broth at 32 C and harvested after 16 h.

Preparation of inocula

Vegetative cells were grown in 3 ml of nutrient broth and then diluted to a total volume of 30 ml with the test sanitation component being studied. The sample was then centrifuged for 10 min at 3000 × *g*. The supernatant fluid was decanted and the cells were suspended in 30 ml of fresh component being studied. The mixture was centrifuged, the supernatant fluid was decanted and the cells were resuspended in fresh sanitation component being studied. The process of centrifugation, decanting, and resuspending in fresh component was repeated. This procedure was considered "washing" for three times. Cells in the final suspension were determined following the plate count procedures in *Standard Methods* (*1*) and reported as numbers of washed cells.

Spores were obtained by growing bacteria on a solid medium and harvesting in the sanitation component being studied. Cells were centrifuged and resuspended for a washing process similar to the treatment of vegetative cells. Before washing, sporeforming cultures were heat shocked at 80 C for 10 min in various components of cleaning solutions as indicated in results.

Stainless steel surfaces

Pieces of 1-cm^2 stainless steel from commercial plates were cleaned, sterilized, and used as individual carriers for each test inoculum. The inoculum consisted of 0.01 ml of a washed suspension of cells as described above. The inoculum remained on top of the stainless steel pieces which were placed in sterile petri dishes, covered, and maintained at 23 C during the test period. The surviving cells were harvested by placing each stainless steel piece into an individual test tube containing 2.5 ml of phosphate buffer (*1*), except for one experimental series when sterile milk was used as indicated in the results section. Tubes were agitated to remove cells from the surface into the phosphate buffer. Proof of the effectiveness of the harvesting procedure has been given in an earlier publication (*2*). Numbers were determined using standard plating procedures (*1*) with plate count agar (Difco) and with selective media as indicated in the experimental results. Numbers reported represent the geometric mean of the number of trials indicated in the results.

RESULTS

Washing and drying vegetative cells

A culture of *S. aureus* was subjected to washing as described in the section "Preparation of inocula," followed by drying to simulate the washing and drying components of sanitation processes. A summary of results of 14 trials in duplicate is given in Table 1. Washing with

[1]*Published as Paper No. 3843, Journal Series, Nebraska Agricultural Experiment Station. Research reported under Project No. 16-8.*

TABLE 1. *Effect of cleaning and rinsing solutions on* Staphylococcus aureus

Treatment	Percent surviving		
	Water	Surfactant	Cleaner
None	100	100	100
Washing[1]	48	19	80
2 h drying	12	1.3	38
7 h drying	<1	<1	<1

[1]Washing was in the component of sanitation being studied.

water (distilled, tap, or sterile tap water gave similar results) reduced the numbers of cells to an average of 48% of the original culture. Cleaning solution was less destructive than water. Surfactant (anionic or nonionic) at a 0.1% concentration was more destructive in that only 19% of the cells survived the washing operation. After 2 h of drying in the presence of surfactant almost all cells were dead. There was less than 1% survivors of any of the treatments after 7 h drying.

P. fluorescens treated as described above was less tolerant of the adversities than was *S. aureus*. A summary of comparative results is given in Table 2. The

TABLE 2. *Effect of cleaning and rinsing solutions on* Pseudomonas fluorescens

Treatment	Percent surviving		
	Water	Surfactant	Cleaner
None	100	100	100
Washing[1]	7.5	9.1	17.5
2 h drying	2.0	8.9	8.9
7 h drying	<1	<1	<1

[1]Washing was in the component of sanitation being studied.

results were unexpected in two ways: (*a*) the high sensitivity of *P. fluorescens* to washing with water alone, since pseudomonads occur commonly in water with low solids concentration, and (*b*) the low resistance of *P. fluorescens* to surfactants, since it is highly tolerant of surfactants in a growth medium (*8*).

E. coli, when subjected to washing and drying processes described for *S. aureus* and *P. fluorescens,* showed resistance intermediate between the other two.

Heat treatments with subsequent washing and drying of spores

Cultures of *B. cereus* and *B. licheniformis* containing more than 50% of the population in the spore state were subjected to a heat treatment of 80 C for 10 min in various components of cleaning solutions. Inocula were then made onto test strips for further observations similar to those made with vegetative cells, e.g. *E. coli.* Results with the two species were similar and were combined for presentation in Table 3. Each value reported represents eight replications of duplicate tests. The menstrua for heat shocking, washing, and inoculation onto the squares for drying were constant throughout for each experiment. Phosphate buffer (*1*), acid, alkali, or surfactant had little effect on numbers of organisms recovered from test strips. Less than one-thousandth as many spores as vegetative cells were destroyed by the sanitation processes, heating, acid, alkali, or surfactant treatment. It is apparent that reasonably clean dry equipment would have spores as the predominant type of microbial contamination. Only harborages would contain a predominance of vegetative cells.

TABLE 3. *Survival of spores of* Bacillus cereus *and* Bacillus licheniformis *after subjection to 80 C for 10 min in various constituents of cleaning solutions*

Time on test squares	Percent surviving		
	Tap water	Cleaner	Surfactant
0	100	100	100
2 h	85	87	131
7 h	76	65	79
24 h	57	91	89

Washing treatments and recovery on selective media

Results presented in Tables 4 and 5 are a compilation of triplicate experiments with duplicate tests on each trial with either tap water or sterile tap water. Results with the two water sources were similar and were combined for simplicity of presentation.

Washing *E. coli* cells in water killed more than half the population (Table 4). Only an occasional cell survived 4 h

TABLE 4. *The effect of washing and drying of* Escherichia coli *cells on recovery with plate count agar and with violet red bile agar*

Treatment	Percent surviving	
	Plate count agar	Violet red bile agar
None	100	100
Washing[1]	38.5	37.5
1 h drying	34.3	29.2
2 h drying	29.4	22.1
4 h drying	<1	<1

[1]Washing was in the component of sanitation being studied.

on a stainless steel test piece. Interestingly, washing did not injure a great number of cells and hence they could be recovered on VRBA.

S. aureus was sensitive to washing in water in that 42.9% survived as measured by plate count agar and 37.7% as measured by Staphylococcus Medium No. 110 (Difco) (Table 5). There was a decrease in numbers asso-

TABLE 5. *The effect of washing and drying of* Staphylococcus aureus *cells on recovery with plate count agar and with S110 medium*

Treatment	Percent surviving	
	Plate count agar	Staphylococcus Medium No. 110
None	100	100
Washing[1]	42.9	37.7
1 h drying	26.9	24.3
2 h drying	17.2	11.9
4 h drying	<1	6.2
6 h drying	<1	<1

[1]Washing was in the component of sanitation being studied.

ciated with increased time on the stainless steel squares. Approximately 6 h was the limit of survival on the test strips.

Recovery of cells in milk

To determine the effect of exposure of washed drying cells to milk on the recoverability of *S. aureus* on Staphylococcus Medium No. 110, sterile milk was substituted for phosphate buffer in recovering cells from stainless steel test strips. Test strips were allowed to dry for approximately 4 h leaving visible liquid in which 1-5% of the cells of the original inoculum survived. Table 6 is a compilation of the data obtained with tap water, which had residual chlorine of 0.3 ppm, and with sterile tap water in which the chlorine had been dissipated. Three replications of duplicate trials with tap water and with sterile tap water showed that recovery in milk alleviated the limiting effects of the Staphylococcus Medium No. 110. Washing in tap water destroyed significantly more (P = 0.99) of the cells than did washing in sterile tap water.

TABLE 6 *Effect of washing with tap water or sterile tap water and subsequent recovery of* Staphylococcus aureus *in milk*

	Percent recovery			
	Plate count agar		Staphylococcus Medium No. 110	
Treatment	Tap water	Sterile tap water	Tap water	Sterile tap water
None	100	100	100	100
Washing only[1]	19	43	18	47
0 min drying	.1	2.6	0.1	4.3
30 min drying	>.1	4.0	>.1	5.1
1 h drying	>.1	3.0	>.1	3.7
2 h drying	>.1	1.8	>.1	2.4

[1]Washing was in the component of sanitation being studied.

DISCUSSION

Even the simplest sanitation processes are destructive to some bacterial cells. For example, tap water destroyed approximately 50% of the cells of the species tested, which is in agreement with the work of Straka and Stokes (*14*) who showed the sensitivity of cells to diluents in counting systems. Washed cells are extremely sensitive to drying. Very few vegetative cells survived 4 h, though there was visible liquid on the test strip most of the time. When drying was extended even a few hours beyond the disappearance of visible liquid, vegetative cells did not survive. Contaminants from external sources, such as air, therefore, would be expected to die off rapidly. These observations substantiate previous work (*10*) indicating that surviving organisms in sufficient numbers to be of significance in contaminating food of subsequent processing arise from harborages.

The extreme sensitivity of gram-negative bacteria to surfactants in washing and drying is surprising, as these organisms are able to grow in the presence of very high concentrations of surfactants or at low surface tension (*8*). These results support the logic of the practice of using surfactants in rinse solutions to aid drying and to reduce water spotting.

Spores in contrast to vegetative cells were extremely resistant to treatments common in sanitation processes. Spores would be expected, therefore, to account for most of the contamination on clean dry equipment. In practice, however, checks on sanitary practices indicate the presence of nonsporeforming organisms (*9*). The presence of harborages must be considered where there are organisms other than sporeformers (*10*).

In light of numerous reports showing the sensitivity of injured cells to selective media, cells exposed to multiple treatments in sanitation processes might be expected to be extremely sensitive to selective media. Results in this work, however, indicated cells to be only moderately sensitive to selective media. The injury phenomena as studied here would not be of major importance in evaluating sanitation procedures using common selective media. Furthermore, injured cells in a food such as milk would have an environment and nutrients so that they would recover sufficiently to regain resistance to selective media.

ACKNOWLEDGMENT

Technical assistance of Elva Steinbruegge is gratefully acknowledged.

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Influence of Selected Solutes on Thermally Induced Death and Injury of *Salmonella Typhimurium*

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ABSTRACT

A study was conducted to determine the influence of various solutes on heat-induced RNA breakdown, injury (as measured by inability to proliferate on salt containing media), and death of *Salmonella typhimurium*. Results showed that heating cells in phosphate buffer was more deleterious than heating in distilled water. Concentration of buffer was a significant factor in the effect of heat on the cells. Magnesium, spermine, and sucrose in the heating menstruum protected salmonellae from heat destruction. Conditions could be manipulated such that measurable RNA breakdown did not occur yet significant portions of the cell population were killed. Evidence accrued indicating that RNA breakdown was not the primary cause of cell death at 48 C.

Various solutes in the heating medium exert different effects on the heat resistance of bacteria, depending on the nature of the solute and its concentration. Although an increased heat resistance was observed when the water activity (a_w) of the heating menstruum was lowered, no direct correlation between a given a_w level and heat resistance was found (*4, 15, 26*). Rather, the chemical nature of the solute was the important factor in affecting heat resistance. For example, sucrose protected salmonellae from heat destruction far more efficiently than glycerol at given a_w levels down to 0.87 (*15*).

High concentrations of soluble carbohydrates generally increase the heat resistance of bacteria (*5*). Sucrose seems to be especially effective in protecting salmonellae from thermally induced death. Addition of this compound to phosphate buffer (*26*), egg yolk (*13*), or egg white (*14*) significantly increases the heat resistance of salmonellae. High concentrations of sorbitol, fructose, invert sugar, glycerol, and glucose have a moderately protective effect (*15*, J. M. Goepfert, personal communication), while low levels of glucose sensitize cells to heat (*26*). Similarly, low concentrations of mannitol, lactose, and ribose were reported to decrease the heat resistance of *Aerobacter aerogenes* (*36*). Yeast extract added to the heat resistance of *Escherichia coli* (*31*) and *Salmonella anatum* (*26*). Sterilized whole milk, trypticase soy broth, certain peptides and amino acids were also slightly protective to *S. anatum* (*26*).

Certain salts will profoundly affect the heat resistance of bacteria. Calhoun and Frazier (*9*) and Cotterill and Glauert (*13*) showed that addition of sodium chloride (NaC1) to the heating menstruum increased the heat resistance of *E. coli, Pseudomonas fluorescens,* and *Salmonella oranienberg.* They suggested that this protective effect could be due to the lowering of the a_w by the salt. Strange and Shon (*36*) studied the effects of potassium chloride (KC1) and NaC1 on the heat resistance of *A. aerogenes,* and found that in combination with elevated temperature both K^+ and Na^+ at concentrations above 0.1 M were lethal, with K^+ being more deleterious. Magnesium ions, and to a lesser extent, Mn^{2+} and Co^{2+} decreased the rate of death in distilled water at elevated temperatures. Moreover, addition of Mg^{2+} to a heating menstruum containing high ($\geqslant$0.1 M) concentrations of Na^+ or K^+ negated the death rate-enhancing activity of these ions.

Various types of lesions have been observed to occur when bacterial cells are subjected to heat. The cytoplasmic membrane is damaged, allowing the leakage of intracellular material (*1, 2, 17, 18, 20, 30, 32, 36*). Among the leakage products are amino acids, 260 nm-absorbing material, K^+, Mg^{2+}, Ca^{2+}, and phosphate ions. Ribosomal nucleic acid (RNA) is degraded (*3, 17, 29, 36, 39*) and strand scissions in deoxyribonucleic acid (DNA) have been reported to occur in certain instances (*16, 33, 44*). Often metabolic activities of heated cells are altered (*7, 38, 40*). Cells still viable but which have sustained lesions of these sorts are termed 'injured cells.' Most commonly, cell injury is measured by demonstrating that a portion of the viable population cannot grow on media containing either sodium chloride or certain bile salts (*6, 10, 11, 20*).

Although some work has been done on the effect of certain solutes on the heat resistance of bacteria and to demonstrate what lesions are induced by heat, there is little information on the mechanism by which these solutes exert their effect and the actual mechanism of death caused by heating. This study was undertaken to accumulate information about the solute effect on thermal injury and to investigate certain aspects of thermal destruction of salmonellae.

MATERIALS AND METHODS

Culture and growth conditions

S. typhimurium was obtained from the culture collection of the Food Research Institute. Stock cultures were maintained on nutrient agar (NA) slants. Working cultures were transferred daily in nutrient broth (NB) and incubated without agitation at 37 C.

Organisms used in the heating trials were grown in Trypticase Soy Broth (TSB). Test cultures were prepared by adding 0.5 ml of the working culture to 50 ml of TSB in 250-ml Erlenmeyer flasks. The cultures were incubated at 37 C for 16 h on a rotary shaker at 270 rpm.

Heating procedure

The cells from 50 ml of culture were harvested by centrifugation in a Sorvall RC2-B centrifuge at 10,400 × *g* at 0 - 2 C for 10 min. The supernatant fluid was decanted and unless otherwise indicated the cells were washed once with 50 ml of 0.1 M potassium phosphate buffer (pH 6.0). The concentration of cells in the heating trials ranged from $2\text{-}4 \times 10^9$/ml.

The heating vessel was a 200-ml three-necked distillation flask which was immersed in a water bath so that the level of water in the bath was 2.5 cm above the level of the test medium. The test solution was agitated throughout the heating experiment by a mechanical stirrer positioned through the center neck of the flask. A thermometer was mounted in one of the side arms to monitor the solution temperature. The remaining arm was used for introduction of the test organisms and for withdrawing samples during the experiment.

Assay for viability and injury

The assay system devised by Clark and Ordal (*10*) was used. One-milliliter samples were withdrawn from the heating vessel at intervals and were added to 9 ml of 0.1% peptone-distilled water. One-tenth-milliliter portions of the dilutions were surface-plated in duplicate on Trypticase Soy Agar containing 0.2% yeast extract (TSA-YE) and on Levine Eosine Methylene Blue Agar containing 2% NaC1 (EMB-NaC1). The TSA-YE count represented the total number of viable cells while the EMB-NaC1 count represented the number of uninjured cells. The difference between these two counts was taken to represent the number of injured cells.

Chemical analyses

Five-milliliter aliquots of heated cells were withdrawn at intervals during the heating period, cooled rapidly and centrifuged at 12,100 × *g* for 10 min. The supernatant fluid was decanted and the absorption at 260 nm was measured in a 1-cm curvette using a Beckman DB-G spectrophotometer, against a blank consisting of the uninoculated heating menstruum.

The cell pellet was resuspended in 5 ml 0.25 N perchloric acid ($HC1O_4$) for 30 min at 0 C to extract cold acid-soluble substances. The extracts were recovered by centrifugation for 10 min at 12,100 × g at 0-2 C, and were analyzed for 260 nm-absorbing material as described above.

The cell pellet was then extracted with 5 ml 0.5 N $HC1O_4$ for 25 min at 70 C. Ribonucleic acid (RNA) in the hot acid extracts was determined by the orcinol method (*41*) with reagent grade RNA (Nutritional Biochemicals Co.) as a standard. Deoxyribonucleic acid (DNA) was determined by Burton's modification of the diphenylamine reaction (*8*) using sperm DNA (Nutritional Biochemicals Co) as a standard. Protein was determined by the method of Lowry et al. (*22*) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Results obtained in a typical experiment employing 0.1 M potassium phosphate buffer (pH 6.0) as the heating menstruum are shown in Table 1. After 60 min of heating at 48 C, more than 90% of the cells could not grow on TSA-YE and greater than 90% of the survivors were unable to proliferate on EMB-NaC1. Forty-eight hours of incubation was necessary for colonies on EMB-NaC1 to attain a size comparable to those appearing on TSA-YE agar after 24 h. These findings are in accord with those reported earlier by Clark and Ordal (*10*). Heated suspensions of *Staphylococcus aureus* (*20*) and *Streptococcus faecalis* (*6, 11*) have also been observed to develop sensitivity to salt-containing media. These salt-sensitive cells recovered their tolerance to salt after being incubated under suitable conditions.

TABLE 1. *Effect of heating* S. typhimurium *in 0.1 M potassium phosphate buffer (pH 6.0) at 48 C as a function of time*

	Time (min)			
	0	10	30	60
TSA-YE count (cells/ml)	2.4×10^9	2.0×10^9	4.3×10^8	1.2×10^8
Death (%)	—	15	82	95
EMB-NaC1 (cells/ml)	2.2×10^9	1.4×10^9	6.0×10^7	2.0×10^6
Injury (%)	—	30	86	98
OD_{260} of leakage products	0.12	0.70	1.06	1.18
$OD_{260}$of cold acid extract	0.26	0.57	0.57	0.6
RNA content of cells (µg/ml)	125	85	70	65
Loss of RNA (%)	—	32	44	48
DNA content of cells (µg/ml)	46	46	44	45

Tomlins and Ordal (*39*) reported that 16s RNA was completely destroyed while 23s RNA was partially degraded when *S. typhimurium* was subjected to heating. This also occurred in the strain of *S. typhimurium* employed in the present study (Z. J. Ordal, personal communication).

In this study a rapid rate of RNA degradation was observed at the beginning (first 10 min) of the heating period. This degradation was accompanied by a rapid increase of 260 nm-absorbing material in the intracellular pool and a substantial release of 260 nm-absorbing material from the cells. The ratio of the absorption of 280 nm to that at 260 nm of the leakage material was always less than 0.5, indicating a high proportion of nucleic acid material. Determination of protein showed that only trace amounts of protein were present in the leakage products. No change in the DNA content of the heated cells could be detected, which implied that (*a*) the cells did not lyse and (*b*) that DNA was not appreciably degraded into fragments small enough to leak from the cells. RNA continued to be degraded and more 260 nm-absorbing material was released, although at a slower rate, throughout the remainder of the heating period. However, no further significant increase in the (cold acid-soluble) pool material was observed. A control cell suspension (maintained at 22 C for a like period) did not show loss of viability, increased sensitivity to EMB-NaC1, or breakdown of RNA. Thus, heating at 48 C in 0.1 M PO_4 buffer induced a degradation of RNA in *S. typhimurium* which in turn caused a rapid increase of 260 nm-absorbing material in the endogenous pool and the subsequent release of these substances into the environment, through a damaged cell membrane. These observations are in agreement with those of Strange and Shon (*36*), who suggested that death of *A. aerogenes* at 47 C was probably due to a sudden increase of RNA degradation products within the bacteria, which altered the normal metabolism of the cells.

Effect of different buffer concentrations

After measuring the effect of heating in 0.1 M PO_4 buffer an experiment to determine the effect of buffer concentration on the extent of cell injury and death was performed. Cells were heated in PO_4 buffer at concentrations ranging from 0.0 to 0.5 M (Table 2). As

TABLE 2. *Effect of different concentrations of potassium phosphate buffer (pH 6.0) on heat-induced death and injury of* S. typhimurium

	Concentration of buffer (M)	Time (min) 0	10	30	60
Death (%)	0	—	7	50	90
	0.001	—	5	73	91
	0.01	—	20	84	95
	0.1	—	12	77	93
	0.5	—	15	47	84
Injury (%)	0	—	17	69	52
	0.001	—	45	85	82
	0.01	—	34	50	95
	0.1	—	30	79	95
	0.5	—	46	59	60
OD_{260} of leakage products	0	0.18	0.55	0.81	0.96
	0.001	0.23	0.65	0.91	1.05
	0.01	0.19	0.82	1.20	1.32
	0.1	0.08	0.59	1.00	1.18
	0.5	0.09	0.14	0.26	0.56
OD_{260} of cold acid extract	0	0.16	0.27	0.28	0.26
	0.001	0.10	0.13	0.14	0.20
	0.01	0.16	0.17	0.20	0.22
	0.1	0.28	0.42	0.46	0.42
	0.5	0.31	0.41	0.64	0.81
Loss of RNA (%)	0	—	10	19	30
	0.001	—	21	31	32
	0.01	—	37	46	47
	0.1	—	36	44	46
	0.5	—	5	10	30

the concentration of PO_4 was increased from 0 to 0.01 M the extent of injury and RNA breakdown within the 60-min test period increased. Very little cell death and injury were observed in the initial portion (10 min) of the heating period when the rates of RNA breakdown and leakage were most rapid. This indicates that heat-induced RNA breakdown preceded death. The greatest degree of RNA degradation and leakage of 260 nm-absorbing material was obtained when the heating menstruum was 0.01 M buffer. In 0.001 M and 0.01 M buffer, there was very little increase in the amount of 260 nm-absorbing material in the pool, whereas in distilled water and 0.1 M buffer there was a rapid increase in pool material, after which the amount of 260 nm-absorbing material in the pool remained fairly constant. In 0.5 M buffer, a slower initial rate of RNA breakdown was observed and the rate increased after 30 min of heating. A higher proportion of the degraded RNA was retained in the endogenous pool at 0.5 M buffer, instead of being released to the environment. These data suggest that RNA breakdown and cell membrane damage are both influenced by buffer concentration but that these are independent effects each occurring maximally at different buffer concentrations.

Strange and Shon (*36*) observed that leakage of K^+ from *A. aerogenes* at 47 C was decreased by increasing the concentration of exogenous K^+. They postulated that maintenance of, and possibly addition to, the normal intracellular ionic concentration at this temperature might affect ribosomal stability through displacement of ribosomally bound Mg^{2+} by K^+. Increasing the exogenous K^+ concentration could progressively reduce leakage of this ion from the cells thus promoting competition with Mg^{2+} for binding sites on the ribosomes rendering the ribosomes unstable. Zitomer and Flaks (*45*) observed that K^+ favored the dissociation of the 70S ribosomal particle and lowered the critical number of Mg^{2+} ions associated with the ribosomes. This was attributed to a competition of K^+ with Mg^{2+} for particular binding sites on the ribosomes.

Three enzymes are known to be involved in RNA degradation. Ribonuclease 1 (RNase 1) is activated by ethylenediaminetetraacetic acid (EDTA) and phosphate and inhibited by Mg^{2+} (*42*). Polynucleotide phosphorylase requires phosphate and Mg^{2+} for activity (*43*), while phosphodiesterase, also known as ribonuclease II (RNase II), requires K^+ and Mg^{2+} and is stimulated by phosphate (*34, 35*). Since potassium phosphate buffer was used routinely in this study, and no Mg^{2+} ions were supplied in the heating menstruum it is likely that at least one of these enzymes (probably RNase I) is involved in the heat-induced degradation of RNA observed in this study. It was also observed that the degree of RNA degradation was less in 0.5 M buffer than in 0.01 M or 0.1 M buffer. A possible explanation for this is that the internal ionic concentration of the cells was altered to such a degree by the high potassium phosphate level that the intracellular conditons were not as conducive for ribonuclease activity. Higher concentrations of potassium phosphate (0.1 and 0.5 M) in the heating medium also resulted in the retention of more RNA degradation products in the intracellular pool, suggesting that the damaged permeability barrier was somehow stabilized by the presence of these concentrations of ions.

These results confirm that RNA is a very heat sensitive target. The data also suggest that if RNA breakdown is the cause of cell death a certain level of RNA breakdown must occur before the cells are unable to repair the lesions and become nonviable. It is also possible that RNA breakdown and cell death are not cause and effect and represent independent events in thermally treated cells.

Influence of washing on cell injury and death

Regardless of the concentration of buffer used in the heating medium, cells washed with distilled water before heating were more heat resistant at 48 C than cells washed with 0.1 M potassium phosphate buffer (pH 6.0). The extent of cell death and injury, RNA degradation, and leakage was considerably less in distilled water-washed cells than in buffer-washed cells. RNA was degraded more slowly, and the leakage of RNA breakdown products was more gradual in distilled water-washed cells in contrast to the rapid release of 260 nm-absorbing material which was characteristic of cells washed with and heated in buffer solutions.

One possible effect of washing bacterial cells with solutions containing K^+ or Na^+ would be the desorption of Mg^{2+} from the cells (*36*), thereby predisposing the cells to subsequent damage when they were later subjected to heat treatment.

Effect of Mg^{2+} ions on cell death and injury

Stange and Shon observed that addition of Mg^{2+} ions to the heating menstruum decreased the death rate and loss of RNA from *A. aerogenes* (*36*). To determine whether salmonellae were influenced in a similar fashion, *S. typhimurium* cells were heated in 0.1 M potassium phosphate buffer (pH 6.0) to which $MgSO_4$ was added at three different concentrations. (Table 3) The extent of

TABLE 3. *Effect of Mg^{2+} ions on heat-induced death and injury of* S. typhimurium

Concentration of Mg^{2+} in heating menstruum	OD_{260} of leakage products at		OD_{260} of cold acid extract at		% Loss		
(M)	0 min	60 min	0 min	60 min	of RNA[a]	% Death[a]	% Injury[a]
0	0.08	1.18	0.30	0.58	48	95	97
0.001	0.07	0.47	0.20	0.37	9	44	78
0.01	0.08	0.43	0.17	0.34	9	46	79
0.1	0.07	0.36	0.27	0.45	_[b]	53	39

[a]After 60 min of heating
[b]No measurable loss

cell death and injury dropped from 95% and 97% in the control (no $MgSO_4$ in the heating menstruum) to 44% and 78% when 0.001 M $MgSO_4$ was in the heating menstruum. A loss in RNA of only 9% was observed as compared to 48% in the control. The amounts of 260 nm-absorbing material in the pool and leakage products were correspondingly less than those in the control. When the Mg^{2+} ion concentration was increased to 0.01 M similar results were obtained. However, in the presence of 0.1 M $MgSO_4$ measurable RNA degradation did not occur, yet 53% of the cells died and 39% were injured during the 60-min heating period.

Magnesium ions have many functions in both metabolic activity and structural integrity of bacterial cells. The effects of magnesium on the heat stability of salmonellae is most likely due to several interactions of this ion. For example, magnesium ions stabilize ribosomes (*25*) as well as participate in maintaining the stability of the cell envelope of gram negatives. Moreover, low concentrations of magnesium inhibit RNase I activity in vitro (*42*) and this effect if it occurred in vivo would explain the lack of demonstrable breakdown of RNA during the heating of salmonellae in buffer containing magnesium at 0.1 M.

Although the precise role(s) of magnesium in protecting salmonellae from heat destruction has not been elucidated, these data have shed some light on the relationship between cell death, injury, and RNA breakdown. The observed absence of RNA degradation although 53% of the cells died and 39% were injured argues strongly that RNA degradation is not the primary cause of injury (as this is measured by salt sensitivity) or thermally induced death. Rather, it would appear that the injury and death caused by thermal treatment is more closely tied to lesions induced in the cell envelope of heated cells.

Effect of spermine tetrahydrochloride (ST) on cell death and injury

Certain polyamines have been shown to stabilize bacteria (*23, 24*) spheroplasts (*37*), protoplasts (*19*), ribosomes (*12*), and nucleic acids (*21, 28*) In light of our results with magnesium it was of interest to investigate the effect a polyamine had on the heat resistance of *S. typhimurium.*

A series of experiments employing spermine tetrachydrochloride (ST) were done and the results are shown in Table 4. Since it has been reported that the effects of polyamines are abolished in the presence of phosphate or NaC1 (*27*), distilled water and not buffer was used to make up the ST solutions. Significant protection from death was afforded by 0.0001 M ST in the heating menstruum. Forty-five percent of the cells were killed in 60 min at 48 C compared to 90% in the control (cells heated in distilled water). However, the extent of cell injury was greater in ST than in distilled water. RNA breakdown was considerably less in ST compared to the control. Incubating the cells in 0.0001 M ST for 15 min before heating decreased RNA degradation and the leakage of 260 nm-absorbing substances during subsequent heating. Prior incubation of cells in ST also resulted in membrane stabilization

TABLE 4. *Effect of spermine tetrahydrochloride (ST) on heat-induced death andinjury of* S. typhimurium

	OD_{260} of leakage products at		OD_{260} of cold acid extract at		% Loss of		
	0 min	60 min	0 min	60 min	RNA[a]	% Death[a]	% Injury[a]
Cells heated in 0.1 M potassium phosphate buffer	0.08	1.18	0.3	0.58	48	95	97
Cells heated in distilled water	0.08	0.96	0.16	0.26	31	90	52
Cells heated in 10^{-4} M ST	0.23	0.58	0.18	0.23	5	45	82
Cells heated in 10^{-4} M ST dissolved in 0.1 M potassium phosphate buffer (pH 6.0)	0.06	1.30	0.29	0.51	47	97	90
Cells suspended in 10^{-4} M ST for 15 min at 0 C before being heated in 0.1 M potassium phosphate buffer (pH 6.0)	0.11	0.63	0.17	0.41	25	87	43
Cells suspended in 10^{-4} M ST for 15 min at 0 C before being heated in distilled water	0.05	0.24	0.20	0.18	_[b]	57	68
Cells suspended in 0.1 M potassium phosphate buffer (pH 6.0) for 15 min at 0 C before being heated in 10^{-4} M ST	0.25	0.56	0.21	0.22	6	48	80

[a]After 60 min of heating
[b]No measurable loss

which was evidenced by a larger pool content of 260 nm-absorbing material compared to cells heated in distilled H_2O without prior exposure to ST. This occurred even though slightly more RNA degradation was observed in the pre-exposed cells.

Stabilization of protoplasts by polyamines is thought to result from an interaction between these positively charged molecules and negatively charged sites on the surface of the plasma membrane (*19, 24*). Polyamines stabilize ribosomes (*12*) and inhibit nucleic acid degradation (*21, 28*) in vivo by forming complexes with nucleic acids and thus preventing nuclease activity. From the results of this study, it appeared that when the cells were heated in ST, some molecules of the compound bound to the cell surface, enhancing membrane stability. Moreover, it is possible that some ST could have entered the cells and affected a stabilization of the ribosomes and nucleic acids, thus accounting for the reduced degree of RNA degradation. Cells pre-exposed to ST would bind ST and be protected in a fashion similar to those heated in ST. When pre-exposed cells are heated in phosphate buffer, however, ST molecules adsorbed on the cell surface or complexed with nucleic acids or ribosomes could be replaced or removed by the negatively charged phosphate (*19*). This would explain the greater RNA breakdown, and accumulation of 260 nm-absorbing material in the pool and subsequent leakage of these substances observed when the pre-exposed cells were heated in buffer. Incubation in buffer before heating in ST produced effects similar to those observed when cells were heated in ST without pre-exposure. It is also conceivable that ST is chelating PO_4^{3-} ions and inhibiting RNase I activity.

When the pre-exposed cells were heated in distilled water, no RNA breakdown was observed. Even though RNA was not being measurably degraded, some cells were still killed (57%). This provides further evidence that RNA degradation is not the primary cause of thermal death (at least under the heating conditions employed in this study.)

Effect of sucrose on cell death and injury

Various reports have shown that sucrose increases the heat resistance of bacteria (*1, 13, 15, 26*). *S. typhimurium* cells were heated in phosphate buffer containing sucrose at concentrations varying from 0-40% (w/v). There was an increasing degree of protection against death and injury as the sucrose concentration was increased incrementally to 30% (Table 5). Breakdown of RNA was also reduced in higher sucrose concentrations. Stabilization of the cell membrane by sucrose was suggested by the fact that significant portions of RNA degradation products were retained in the intracellular pool instead of being released from the cells when 20-40% sucrose was in the heating menstruum. If sucrose somehow blocked the entry of K^+ and/or phosphate ions into the cells, ribosome breakdown would be prevented and RNase 1 activity inhibited. In concentrated sucrose solutions the a_w of the heating menstruum would be reduced, which in turn would lower the a_w inside the cell perhaps rendering the intracellular conditions unfavorable for RNase activity. However, since glycerol and fructose solutions at the same a_w level as sucrose did not protect *S. montevideo* in the same fashion as sucrose (*15*), this suggests that a_w per se was not the controlling factor. By stabilizing the cell membrane, sucrose not only prevented leakage of RNA degradation products but other small molecules in the intracellular pool, such as metabolites, ATP, coenzymes, etc. This effect might possibly contribute to the increased heat resistance of *S. typhimurium* in sucrose solutions.

TABLE 5. *Effect of sucrose on heat-induced death and injury of* S. typhimurium

% Of sucrose (w/v) in heating menstruum	OD_{260} of leakage products at		OD_{260} of cold acid extract at		% Loss of RNA[a]	% Death[a]	% Injury[a]
	0 min	60 min	0 min	60 min			
0	0.08	1.18	0.30	0.58	48	95	97
10	0.07	1.00	0.30	0.77	50	97	92
20	0.07	0.54	0.29	0.88	33	70	75
30	0.05	0.33	0.30	0.52	12	45	72
40	0.05	0.31	0.29	0.53	10	45	73

[a]After 60 min of heating

Salmonellae do not metabolize sucrose and it has been assumed that this substance does not enter the cells. Experiments in this laboratory (unpublished data) have indicated that salmonellae can indeed take sucrose into the cell and thus protection conferred by binding to specific sensitive sites must be taken into account.

Although it was not possible to establish the exact mechanism of heat destruction of *S. typhimurium* in this study, evidence gathered indicated that degradation of RNA (at 48 C) was not the primary cause of heat induced death of this organism. It is also evident that events occurring during thermal treatment are quite complex and that environmental influences are extremely significant. It is also apparent that further understanding of these processes will only come about through extensive studies at the molecular level.

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A Comparison of Cultural Methods used with Microcolony and Direct Fluorescent-Antibody Techniques to Detect Salmonellae

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ABSTRACT

A comparison of various cultural methods for use with microcolony and direct fluorescent-antibody (FA) techniques to detect salmonella was conducted using 102 naturally contaminated samples. The methods were: (*a*) a 27-h microcolony technique, (*b*) a 51-h selenite-F FA microcolony technique, (*c*) a 51-h tetrathionate brilliant green FA microcolony technique, and (*d*) a 53-h FA technique from a broth enrichment. All were compared to an F.D.A. approved modification of the AOAC method. The 51-h FA microcolony technique, which employed tetrathionate brilliant green broth and brilliant green agar plates, produced 4.9% false-positives and no false-negatives, and produced the greatest frequency of salmonellae detection.

The success of a fluorescent-antibody (FA) technique in the detection of salmonellae depends heavily on the cultural procedures employed to increase the numbers of organisms. To facilitate reading of slides, a FA cultural method must (*a*) enhance growth of low numbers of salmonellae, (*b*) suppress growth of organisms other than salmonellae, and (*c*) minimize background fluorescence. Adaptation by Thomason (*4*) of the microcolony FA method developed by Chadwick and Abbott (*1*) offers a solution to these problems.

This study compares a 27-h FA microcolony culture technique, two 51-h FA microcolony techniques with different selective enrichment broths, and 53-h direct FA culturing technique which utilized a 5-h elective enrichment procedure. Two different plating media for developmemt of microcolonies are also evaluated. These comparisons were made to determine the best cultural combination which would satisfy the above three criteria.

MATERIALS AND METHODS

FA antisera

Bacto-FA Samonella Polyantisera (Lot RX 16214) was obtained from Difco Laboratories, Detroit, Michigan. The antiserum was rehydrated following the procedure of Insalata et al. (*3*).

Samples

In this study, 102 samples of naturally contaminated human food, animal feed, and raw materials were tested for the presence of salmonellae by utilizing various cultural combinations before FA slide readings (Fig. 1). The sample types tested were: bran products, chicken (frozen), chocolate chunks, chocolate powder, dried milk, egg albumin, eggs (frozen whole), fish meals, legs (frozen), lactalbumin, meat meals, rice products, soy flour, and wheat products. The samples were supplied by the Food and Drug Administration and other sources.

Methods

Pre-enrichment phase. While undertaking a 2-year research contract for the U.S.F.D.A. (CPF 69-33), to develop a fluorescent-antibody technique for detection of salmonellae in foods, the authors obtained approval of the project director to modify the standard AOAC salmonella method by using fluorescent-antibody salmonella (FAS) broth as a pre-enrichment phase. This approved modification was used in this work. Each 25-g samples was pre-enriched in 225 ml FAS broth. Blending of some samples in a Waring Blendor for 1 min at high speed was done to obtain a homogeneous suspension. The pH was adjusted to 6.8 to 7.2 when necessary. Samples were allowed to remain for 1 h at room temperature and then incubated for 23 h at 35 ± 2 C.

Cultural confirmation. To culturally confirm the presence of salmonella in the "elective" enrichment broths, a 3-mm loopful was streaked on selective media plates as outline in the AOAC method (*1*). For this work, as for that of Contract CPF 69-33, the "selective" phase was intended to decrease the numbers of non-salmonellae and the "elective" phase was intended as an additional time phase using a medium designed to increase the numbers of salmonellae organisms in the broth before F.A. staining of the organisms. All media used were Difco products except the triple sugar iron and lysine iron agar dehydrated media manufactured by Baltimore Biological Laboratories.

27-H microcolony technique. Methods described by Thomason (*4*) were implemented for development of microcolonies.

After incubation, several 3-mm loopfuls from the FAS pre-enrichment broth were placed on the surfaces of a brilliant green (BG) agar plate and a FAS (15 g Bacto-agar/liter added to FAS broth) plate to develop the microcolonies. Six samples were inoculated onto each plate (9 cm square). Two milliliter transfers to the two selective enrichments were also made for cultural confirmation at this stage.

After incubation of the BG agar plates and the FAS agar plates from 3 h at 35 ± 2 C, impressions of the microcolonies were made by firmly pressing an alcohol cleaned and dried microscope slide onto the agar, then lifting with forceps. The purpose was to impinge the organisms from the agar plates onto the slide for staining and microscopic examination.

Selective enrichments. Duplicate 2-ml aliquots from the FAS preenrichment broth were transferred to 18 ml Selenite-F (SF) broth and 18 ml tetrathionate broth with brilliant green (TBG). Incubation for both selective enrichments was 24 h at 35 ± 2 C.

51-H TBG microcolony and TBG cultural confirmations. After the 24-h incubation of the TBG broth, duplicate 3-mm loopfuls were inoculated onto the surfaces of a BG and FAS plate for development of 3-h microcolonies. At this time, the streaking of selective plates for the TBG cultural confirmation for salmonella was performed following AOAC methodology.

51-H SF microcolony and SF cultural confirmations. Several microcolonies were developed from the SF broth on BG and FAS

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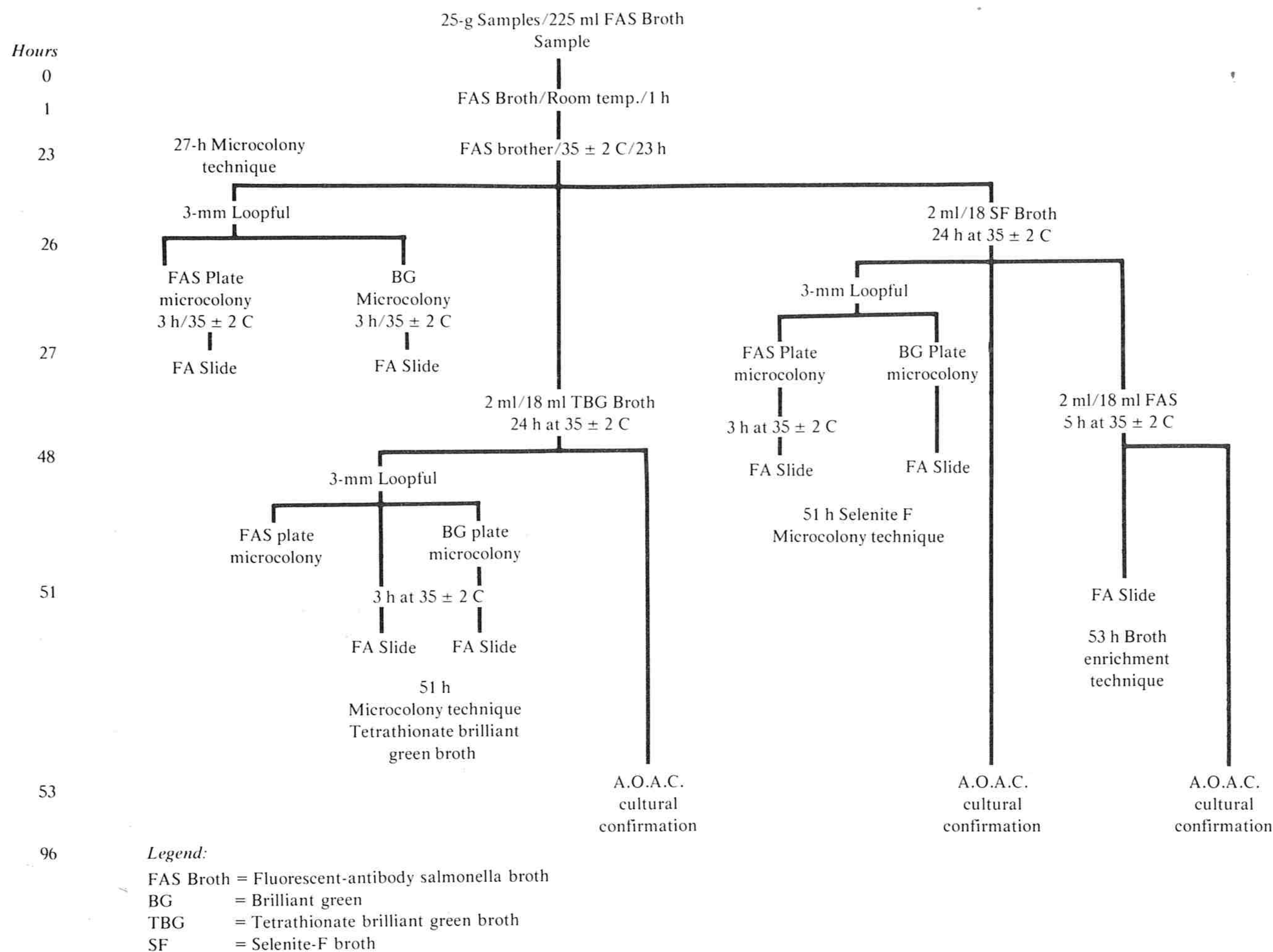

Figure 1. *Experimental design*

plates, as with the above TBG broth. A SF cultural confirmation was also done.

53-H broth enrichments and cultural confirmations. Two milliliters were withdrawn from the top third of the SF broth and transferred to 18 ml FAS broth for 5 h incubation at 35 ± 2 C. FA smears were then made on agar prepared slides (*3*). This broth enrichment was also culturally examined following AOAC methodology.

The procedures used for FA staining and microscopic examination was identical to those described by Insalata et al. (*3*).

RESULTS AND DISCUSSION

A total of 102 samples were examined. Table 1 lists the samples tested and the results for the three cultural methods used to confirm the presence of salmonellae. Salmonellae were recovered from 36 samples.

A chicken sample had been recorded as negative for *Salmonella* when tested by the SF and the 53-h cultural methods. However, this sample produced *Salmonella infantis* when the aliquot was restreaked from tryticase soy broth (T.S.B.).

A second isolate (*Salmonella kentucky*) was recovered from a sample of Canadian meat meal only after repreated restreaking from SF broth. The TBG and 53-h elective enrichment cultural techniques failed to detect salmonella. Additional culturing methods, beyond those routinely employed, were necessary to detect the salmonella organism in these instances.

Table 2 lists the FA slide results and the cultural confirmations for the 102 samples tested. The 51-h TBG microcolony with BG agar plates produced 4.9% false-positives and no false-negatives. The 51-h TBG microcolony with FAS agar plates produced 7.8% false-positives and no false-negatives.

The 51-h SF microcolony technique with BG agar plates and the 51-h SF microcolony technique with FAS agar plates yielded 9.8% and 10.8% false-positives respectively. Each technique produced 2.8% false-negatives.

The 53-h direct FA method utilizing a 5-h broth enrichment yielded 8.8% FA false-positives, and 2.8% FA false-negatives. In this procedure, SF was used as the

TABLE 1. *Comparison of results obtained for the three cultural methods tested*

Products	TBG CC +	TBG CC −	SF CC +	SF CC −	53-H CC +	53-H CC −
Bran product	0	3	0	3	0	3
Chicken (frozen)	1	1	0	2	0	2
Chocolate chunks	0	3	0	3	0	3
Chocolate powder	0	2	0	2	0	2
Dried milk	0	2	0	2	0	2
Egg albumin	0	4	0	4	0	4
Eggs (frozen whole)	0	5	0	5	0	5
Fish meals						
Baltimore	0	2	0	2	0	2
S	3	0	3	0	3	0
Frog legs (frozen)	0	3	0	3	0	3
Lactalbumin	2	0	2	0	2	0
Meat meals						
Beige	0	3	0	3	0	3
Black	6	1	6	1	6	1
Brown	4	1	4	1	4	1
Canada	3	2	4	1	3	2
Fuzz	5	0	5	0	5	0
K	3	0	3	0	3	0
Meat & liver	3	3	3	3	3	3
Red						
Sand & tan	0	3	0	3	0	3
Smoke	0	6	0	6	0	6
SR	3	1	3	1	3	1
Rice product	0	5	0	5	0	5
Soy flour	2	3	2	3	2	3
Wheat product	0	12	0	12	0	12
Totals	35	65	35	65	34	66

TBG/CC: Tetrathionate broth with brilliant green/Cultural confirmations.
SF/CC: Selenite F/Cultural confirmations.
53-H/CC: 53-H enrichment/Cultural confirmation.

selective enrichment before the 5-h broth enrichment.

On those occasions that the FA method yielded a false-positive, an attempt was made to isolate and identify the organism. Many of the FA false-positives were *Citrobacter* spp., *Enterobacter cloacae,* and *Escherichia* spp.

The FA microcolony techniques yielded slides with less sample debris, lower background fluorescence, and larger and brighter fluorescing salmonellae cells than did the slides made from the 53-h broth enrichment technique. The Microcolony slides developed from the BG selective agar plates contained fewer non-salmonellae than did the slides developed from the non-selective FAS agar plates. This is an advantage when observing slides for fluorescing cells.

Essentially, no difference was observed between TBG and SF for isolating salmonellae. However, the percentages of false-positives indicate that TBG (4.9% and 7.8%) and be more effective in restricting growth of competing non-salmonellae organisms than SF (9.8% and 10.8%).

These results confirm those reported by Thomason (*4*). The microcolony culturing technique, when combined with a FA staining method, yields an effective sample screening technique for the detection of salmonella.

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TABLE 2. *FA slide results and cultural confirmations (CC) for samples tested*[1]

FA Test		GA Agreement FA + CC +	GA Agreement FA − CC −	FA False + (FA+ CC −)	FA False − (FA− CC+)	Percent FA false − positives	Percent FA false − negatives
27-Hour microcolony with	BGA	34	59	7	2	6.8%	5.5%
	FAS	33	60	6	3	5.9%	8.3%
51-Hour TBG microcolony with	BGA	35	61	5	0	4.9%	0%
	FAS	35	58	8	0	7.8%	0%
51-Hour SF microcolony with	BGA	35	56	10	1	9.8%	2.8%
	FAS	35	55	11	1	10.8%	2.8%
53-Hour direct FA		34	57	9	1	8.8%	2.8%

[1]Abbreviations: CC +, a sample producing a salmonellae isolate by any one of the AOAC cultural trials; cc −, any samples not producing a salmonellae isolate by any one of the AOAC cultural trials; FA +, any smear showing rods of (*a*) proper morphology (with or without attached flagella) under darkfield and ultraviolet light, and (*b*) a 3+ or 4+ degree of fluorescence under ultraviolet light; FA −, any smear not conforming to the above; agreement occurs when a positive slide is confirmed by any oneof the AOAC cultural trials; the FA false-positive results when any slide positive could not be AOAC culturally confirmed; FA false-negative, any instance in which a slide was negative and any of the AOAC cultural trials yielded salmonellae.

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A Vapor Pressure Osmometer for Determination of Added Water in Milk

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ABSTRACT

A vapor pressure osmometer requiring a 5- to 7-microliter sample to saturate a 0.64 cm filter paper disc fixed a digital readout of milliosmolality in 110 sec. A coefficient of variability of 0.70 was obtained on a raw milk sample tested 25 times when an acetone impregnated tissue was used to clean the sample chamber between tests. Two hundred individual cow milk samples from 20 herds averaged 280.0 ± 3.0 milliosmols. Milk samples containing up to 25% added water were evaluated on both the vapor pressure osmometer and a thermistor cryoscope with a resultant correlation coefficient of 0.991. A collaborative study involving eight hospital and industry laboratories was conducted. When the results of two laboratories were discarded, due to instrument maintenance problems, there were no significant differences among the laboratories in their abilities to quantitate added water in milk.

Thermistor cryoscopes have provided the most practical approach to date to measure added water in milk (*1, 2*). Recently a vapor pressure osmometer has been adopted by the medical profession (*4*). The instrument measures dewpoint temperature depressions and might, therefore, be called a thermocouple hygrometer. However, the vapor pressure terminology was selected because the unit is used to evaluate vapor pressure, a colligative property, as predicted by temperature changes in the instrument chamber. The osmometer showed excellent agreement with serum samples when compared with a cryoscope (Kopp, J. B 1973. Osmolality Study. Wescor Inc., Logan, Utah).

Several advantages have been claimed which suggested the possible application of the instrument by the dairy industry and by food research laboratories for measuring water activity in intermediate moisture foods. This report summarizes the potential for vapor pressure osmometry for measuring added water in milk.

MATERIALS AND METHODS

Fresh, unpreserved milk samples collected for the Dairy Herd Improvement Association testing program were obtained from the Central Milk Testing Laboratory, Logan, Utah. Bulk tank milk samples were also obtained from the Utah State University dairy bulk tank and as received at the Cache Valley Dairy Association in Smithfield, Utah.

Cryoscope

An Advanced Instruments Inc. Laboratory Model thermistor cryoscope (Newton Highlands, Mass. 02161) was used for the comparative study. The manufacturer's instructions, and −0.422C and −0.621 C sodium chloride standard solutions were used.

Vapor Pressure Osmometer

A Wescor model 5100 LED panel meter vapor pressure osmometer was used in the comparative and collaborative studies in accordance with the manufacturer's instructions except where the acetone wash was incorporated as subsequently described. A reference solution of NaC1 at 290 milliosmolality was used for calibration.

Instrument operation

A filter paper disc 0.64 cm in diameter, was inserted into the milk sample using forceps. Milk was allowed to impregnate the disc through capillarity. The disc was completely saturated and excess solution was allowed to drain from the disc at the edge of the sample container before the disc was inserted into the instrument sample chamber.

The automatic time sequence was initiated upon sample insertion. The sample chamber was closed by turning a plastic knob at the top of the instrument. Figure 1 portrays a typical temperature cycle. Upon

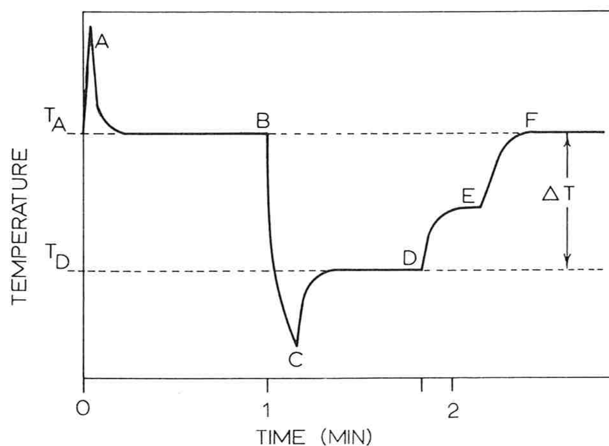

Figure 1. *Vapor pressure osmometer thermocouple junction temperature vs. time graph.* T_A *= ambient temperature,* T_D *= dew point temperature.* ΔT *= temperature difference used by instrument to determine osmolality. A =chamber sealing thermal transient. B = ambient temperature and pressure equilibrium, C = Peltier cooling step, D = dew point temperature equilibrium and completion of test cycle, E = wet bulb depression temperature following opening of the sample chamber and prior to complete drying of thermocouple. F = resotration of ambient temperature upon moisture evaporation from thermocouple.*

closing the chamber a high temperature transient (A) occurred due to developed chamber pressure. The sample chamber then returned rapidly to ambient instrument temperature T_A. The instrument automatically established a zero reference point during this period of thermal and vapor pressure equilibriation (B). A controlled direct

current was passed through the thermocouple junction causing cooling to a temperature below the dew point (C). The cooling interval varied with the osmolality of the sample. The electric current to the thermocouple was turned off, equilibrium was reached and the junction current ultimately correlated to the dew point temperature T_D. The final reading (D) on the instrument was fixed on the digital meter and an audible tone indicated the cycle was completed. Upon opening the chamber the T_A reading was restored following a lag [due to the wet bulb depression temperature (E)] until all the water had evaporated from the junction. When the chamber was opened, the water evaporated almost instantaneously and the thermocouple temperature quickly returned to T_A. The unit thus operated as a precision thermocouple hygrometer.

Percent added water was calculated from milliosmolal readings using the formula:

$$\% \text{water added} = \frac{R - S}{R} \times 100$$

—where R = milliosmolal of milk known to be free of added water and S = milliosmolal of sample.

RESULTS AND DISCUSSION

Repeatability of the osmometer indicated a coefficient of variability (CV) of 1.07% following 50 tests done on a single raw milk sample. Because some organic substances foul the thermocouple, it was suggested that the sample chamber pocket be washed between samples using an acetone soaked Kimwipe. The coefficient of variability was slightly improved when 25 replicate tests were done on a raw milk sample with acetone wash (Table 1). This value is comparable to that claimed in modern cryoscopy when a 0.2-ml sample is used.

TABLE 1. *Precision of the vapor pressure osmometer in determining osmolality of milk as affected by acetone wash of the sample chamber between tests*

	N	$\bar{X}$	S	CV
	(Replicates)	—(Milliosmolal)—		(%)
Without wash	25	283.9	2.16	0.76
With wash	25	284.6	1.98	0.70

Modern thermistor cryoscopes are claimed to have ± 2 milliosmolal precicision when measuring 0.2-ml samples and ± 1 milliosmolal precision when measuring 2-ml samples (Bulletin, Advanced Instruments Inc., Newton Highlands, Mass. 02161). This implies CV's of 0.69 and 0.34% on a 290 milliosmolal sample. Shipe's (*5*) data produced a mean CV of 0.41%. Thus instrument precision may be slightly inferior to cryoscopes when large samples are used, however, other advantages suggest applicability in spite of these differences.

Ten samples of raw milk from individual animals were obtained from 20 different herds on DHIA test. The mean and standard deviation were 280.1 ± 3 milliosmolal. This corresponded to a freezing point value of –0.521 ± 0.006 C. This is above the official upper limit of –0.525 C (*1*). If the freezing point is –0.525 C or below, milk may be presumed to be water-free or may be confirmed as water-free by aditional tests. Above this temperature, milk will be designated presumed to contain added water. Since the mean of the 200 individual cow milk samples was found above this value an explanation is in order. Henningson (*3*) listed several sources of variability which would cause areas to be without the expected range. A bias in instrument calibration might explain the lower mean value. As standard NaCl solution evaporates, the actual milliosmolal value will increase while the instrument calibration will remain at 290. Thus samples may have lower values than expected.

Authenticated samples, confirmed free of added water must be tested (*2*) before claims of low level water addition can be made. Cryoscope instrument procedures suggest that claims for added water should not be made if the instrument reads less than 2-3% added water (Instruction Manual, Advanced Instrument Inc., Newton, Highlands 02161). This suggests that more emphasis should be placed on the differential milliosmolal readings and that the base value will vary and must be checked before confirming low levels of water addition. If a consistent bias caused the low results in this study, the CV and the need for confirmatory testing suggests that instrument application is not adversely affected and that standard solution quality must be continually assured.

Known volumes of water were added to milk (V/V) and each sample was tested 10 times (Table 2). The mean difference among samples containing added water was +0.13% and the mean CV was 0.64%.

TABLE 2. *Percent water measured by a vapor pressure osmometer following additions of known quantities of water to a fresh raw milk sample; data represent means of 10 replicates*

Water added (%)	Osmometer reading ($\bar{X}$)	(S)	CV	Measured water added, $\bar{X}$ (%)
	—(Milliosmolal)—			
0	288.3	1.83	0.63	—
1	285.2	1.55	0.54	1.1
3	280.1	1.20	0.43	2.8
5	274.2	1.62	0.59	4.9
10	258.2	1.81	0.70	10.4
15	245.1	1.10	0.45	15.0
20	229.9	1.60	0.70	20.3
25	215.0	2.36	0.10	25.4
			$\bar{X}$ =0.64	

A comparative study between the vapor pressure osmometer and the thermistor cryoscope was conducted. Twenty samples with up to 25% added water were tested on both instruments (Table 3). The correlation coefficient was 0.991.

A collaborative study was done involving one technician in each of six different hospitals and the Wescor laboratory involving two different technicians and two separate instruments allowing the incorporation of eight collaborators. Each technician was instructed to test five samples of milk using a 290 milliosmolal standard solution and the same procedures involved in serum testing with the exception that acetone wash of the sample chamber was recommended between samples. Results are summarized in Table 4. The sample means

TABLE 3. *Correlation between a thermistor cryoscope and a vapor pressure osmometer following analysis of milk samples containing added water*

	Added water	
	Cryoscope	Osmometer
	(%)	(%)
	1.8	1.7
	2.0	2.1
	2.5	1.7
	2.5	2.5
	3.0	4.2
	3.0	2.9
	4.0	5.6
	5.0	6.0
	6.0	6.0
	6.0	7.9
	15.0	16.4
	15.9	15.6
	16.0	14.2
	16.0	17.5
	18.0	21.0
	19.0	20.4
	20.5	22.1
	21.0	20.0
	24.5	23.5
	25.0	26.0
$\overline{X}$	11.25	11.86
s	8.38	8.54
r	0.991	

closely approximated the true value except sample C was over estimated. This suggested a nonlinear problem at high concentrations of added water, however, this could not be confirmed in the comparative study or subsequent evaluations. There was a higher CV found at 25% in one instance (Table 2), however, the mean percentage of added water was only 0.4% high.

The CV was higher than anticipated in the collaborative study and an analysis of variance among all investigators indicated a highly significant difference. When the data were ranked as suggested by Youden (*6*), the data of investigators 6 and 7 were suggested for discard. This was confirmed by a least significant difference analysis. When these data were discarded, the CV's of the milk sample means dropped to 1.4 and to 5.8 and no significant differences existed among technicians (Table 5).

Upon investigation it was learned that technician 6

TABLE 5. *Analysis of variance—Excluding data from collaborators 6 & 7*

Source	df	MS	F
Laboratories	5	355.1	0.29ns
Samples	4	14704.5	12.21* *
Error	20	1204.3	

had not used the instrument for over one month and had failed to balance it before the collaborative study. In addition, technician 7 had experienced troubles and called the manufacturer to check the source. It was determined that the thermocouple was dirty. Proper instrument operation was restored following cleaning of the thermocouple. Thus discard of the data from the two collaborators appeared justified and the need for proper calibration and maintenance of the instrument was emphasized.

The record of instrument reliability has been very good. Of the approximate 200 units in the field, only one instrument developed a problem which required the replacement of a defective transistor, However, as experienced in the collaborative study, frequent thermocouple cleaning is essential to assure proper precision. Cleaning solution and procedures are provided with the instrument to assure proper maintenance of the delicate thermocouple.

Cryoscopy has required precooling in some instruments. We examined samples of milk taken immediately from the refrigerator and compared the results obtained with samples at room temperature and found no significant differences in instrument readings. Using sample volumes of only 5 to 7 liters in a large mass sample chamber assures rapid temperature equilibrium. The effect of acetone wash upon the sample chamber pocket did not appreciably change the sample chamber temperature. However, ambient temperature control is essential. Unless instrument temperature would be carefully maintained, field use of the vapor pressure osmometer would be limited and would require frequent calibration checks using the standard solution.

The vapor pressure osmometer has satisfactory precision and several advantages over the thermistor cryoscope. It has the potential for application in

TABLE 4. *Vapor pressure osmometer data on the five milk samples examined in the collaborative study*

Lab no.	Milk sample identity					Lab mean	Laboratory ranking					Total rank
	A	B	C	D	E		A	B	C	D	E	
	—(Milliosmolal)—											
1	265	250	209	280	272	255	4.5	5	6	4.5	6	26
2	265	255	205	278	273	255	4.5	2.5	7	6	5	25
3	268	255	228	287	278	263	3	2.5	3	3	3	14.5
4	255	244	223	277	274	254	6	6	4	7	4	27
5	253	242	238	270	269	257	8	7	2	4.5	7	28.5
6	281	276	245	293	291	277	1	1	1	1	1	5[1]
7	256	216	202	250	261	237	7	8	8	8	8	39[1]
8	277	253	211	289	279	262	2	4	5	2	2	15
Mean $\overline{X}$	265	249	220	279	275							
True Value	265	248	209	279	272							
s	10	17	16	13	9							
C.V.	4	7	7	5	3							

[1]Data with ranking totals below 9 and above 36 should be discarded (5% level) (*6*).

measurement of water activity in intermediate moisture foods. The authors recommend that vapor pressure osmometry be included as a standard method for the examination of dairy products. The industry should welcome the advantages and simplicity offered.

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Effect of Antifungal Agents on the Carbohydrate Metabolism of *Kluyveromyces fragilis*[1]

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ABSTRACT

Pimaricin and nystatin, two antifungal agents, inhibited endogenous respiration and aerobic and anaerobic utilization of sugars by *Kluyveromyces fragilis*. Three sugars, lactose, glucose, and galactose, were metabolized by a somewhat similar process as judged by the extent of inhibition caused by the antifungals, both aerobically and anaerobically. The degree of inhibition by the antifungals was more pronounced under anaerobic conditions. There existed a direct, but not proportional, relationship between concentration of antifungals and extent of inhibition. Nystatin impaired carbohydrate metabolism slightly more than did pimaricin. Also, *K. fragilis* contained lactase which was found to be a constitutive enzyme and was inhibited by the two antifungal agents.

Previous studies in this laboratory indicated that cottage cheese treated with two antifungal agents, pimaricin and nystatin, had better keeping quality than did untreated cheese (*7, 9*). The antifungal agents retarded spoilage caused by yeast and mold, but showed little or no beneficial effect against bacterial spoilage.

Several investigators have studied the effect of nystatin on carbohydrate metabolism of yeast (*1, 4, 6, 8, 11*). Lampen et al. (*4*) observed that nystatin inhibited growth of yeasts by affecting utilization of carbohydrate substrates. They also observed that at a pH below 7 nystatin was effective in inhibiting glycolysis and respiration of *Saccharomyces cerevisiae*. Marini et al. (*6*) reported this sensitivity to be a function of both pH and monovalent cations in the test system. It was also shown (*1*) that in *Candida stellatoidea* nystatin affected both the glycolytic cycle and the oxidative cycle of terminal respiration. Scholz et al. (*8*) demonstrated the differential sensitivity of various glycolytic enzymes of yeast to mycostatin both in vivo and in vitro.

Pimaricin resembles nystatin in that it is a polyene antifungal agent, but it is produced by a different species of *Streptomyces* (*10*). Relatively little is known about its mode of action. Hendricks and Berends (*3*) reported that as little pimaricin as 5 μg/ml significantly inhibited glucose fermentation by *S. cerevisiae*, but its respiration was affected only slightly.

Since *Kluyveromyces fragilis* is often encountered as a spoilage organism in dairy products, studies were made to determine the mode of action of nystatin and pimaricin on sugar metabolism of this yeast.

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MATERIALS AND METHODS

Culture and medium

The experimental strain of *K. fragilis* DDS 45, obtained from the Department of Botany, University of Nebraska, was cultivated in a medium consisting of Bacto-tryptone 3 g; malt extract 3 g; yeast extract 3 g; and carbohydrate 0.1 g in 100 ml distilled water. The medium without carbohydrate was sterilized by autoclaving at 115 C for 20 min. Glucose, galactose, and lactose were sterilized separately and added aseptically to the medium just before inoculation. The culture was grown in a stationary state at 30 C for 20 h. Resting cell suspensions of the yeast were prepared by centrifugation, washed twice with sterile ice-cold distilled water, and resuspended in 0.2 M phosphate buffer, pH 6.5.

Respiration studies

Respiration experiments were done by the conventional Warburg technique. For aerobic studies the system consisted of air as the gas phase. Each flask contained 1 ml of the resting cell suspension, 0.2 ml of 10% KOH in the center cup, and 0.3 ml of 0.1 M substrate, 0.1 ml of antifungal solution and distilled water to make up the final volume to 3 ml. For anaerobic studies the system was flushed with a mixture of nitrogen and carbon dioxide (95:5), and the rest of the system was the same as that described earlier except that KOH was omitted. For endogenous oxygen consumption no sugar was added. The temperature was maintained at 30 C for all experiments.

Solutions of pimaricin or nystatin were prepared in a 2% dimethyl sulfoxide-water mixture and added with the substrate to yield 5 or 20 μg of the antifungal agent per milliliter of the reaction mixture.

The substrate was placed in the side arm and was mixed with the contents of the main compartment of the flask after an initial equilibrium period of 10 min. The manometric readings were taken over a 60-min period.

Lactase assay

The enzyme lactase was estimated by the method of Caputto et al. (*2*) in which lactose hydrolysis was measured by formation of monosaccharides, as estimated with the modified Skinhoff reagent.

To estimate lactase, and triturated cells were prepared as follows: cells harvested from a 20-h broth culture of *K. fragilis* were washed twice with ice-cold distilled water and made into a fine paste by trituration with sand at 0 to 5 C with 0.2 M phosphate buffer, pH 6.8. The extract was centrifuged at 0 C and the clear supernatant fluid thus obtained was used for estimation of lactase. A unit of lactase is defined as the amount of enzyme which hydrolyzes one micromole of lactose per minute at 30 C and pH 6.8.

RESULTS

Aerobic utilization of sugars

Data presented in Table 1 reveal that as little as 5 μg of antifungals per milliliter significantly inhibited endogenous respiration as well as aerobic utilization of sugars by *K. fragilis*. Pimaricin inhibited endogenous respiration of cells by 30 to 63% and nystatin by 20.9 to 38.5%. Pimaricin inhibited aerobic utilization of lactose, glucose, and galactose by 68.0, 64.8, and 56.6%, respectively; whereas, nystatin showed a corresponding inhibition of 75.0, 72.4, and 71.0%, respectively. While only 20.9 to 46.3% of endogenous oxygen consumption

TABLE 1. *Effect of lower concentrations of pimaricin and nystatin (5.0 μg/ml) upon the endogenous and aerobic sugar metabolism of* K. fragilis[1]

Carbohydrates	Antib. conc. (μg/ml)	Oxygen uptake (μl) Endogenous	% Inhibition endogenous	With substrate	Due to substrate only	%Inhibition of substrate utilization
			Pimaricin			
Lactose	0	14.0		52.2	38.2	
	5	9.8	30.0	22.2	12.2	68.0
Glucose	0	15.8		67.6	51.8	
	5	9.3	41.0	27.5	18.2	64.8
Galactose	0	10.8		45.4	34.6	
	5	5.8	46.3	20.8	15.0	56.6
			Nystatin			
Lactose	0	8.6		35.5	26.9	
	5	6.8	20.9	13.5	6.7	75.0
Glucose	0	20.0		93.6	73.6	
	5	13.8	31.0	34.1	20.3	72.4
Galactose	0	21.0		67.0	46.0	
	5	15.0	38.5	28.3	13.3	71.1

[1]Each flask contained 3 ml of reaction mixture consisting of 1 ml of yeast cells (6.3 mg dry weight) in phosphate buffer pH 6.5, 0.3 ml of substrate (M/10), 0.2 ml of antibiotic or 0.2 ml of distilled water in control, 0.2 ml of 10% KOH in the center cup, and water to make final volume to 3 ml. For endogenous oxygen consumption, no sugar was added. The temperature was maintained at 30 C.

TABLE 2. *Effect of higher concentrations of pimaricin and nystatin (20.0 μ/ml) upon the endogenous and aerobic sugar metabolism of* K. fragilis[1]

Carbohydrates	Antib. conc. (μl/ml)	Oxygen uptake (μl) Endogenous	% Inhibition endogenous	With substrates	Due to substrate only	% Inhibition of substrate utilization
			Pimaricin			
Lactose	0	16.2		34.2	18.0	
	20	4.8	70.0	8.2	3.4	81.1
Glucose	0	17.2		53.1	35.9	
	20	5.2	63.3	12.9	7.7	78.5
Galactose	0	12.2		26.7	14.5	
	20	5.0	59.0	9.0	4.0	72.4
			Nystatin			
Lactose	0	15.0		39.7	24.7	
	20	6.9	54.0	8.6	1.7	93.1
Glucose	0	19.0		80.3	61.3	
	20	6.0	68.0	11.4	5.4	91.1
Galactose	0	23.0		42.9	19.9	
	20	12.0	47.8	10.2	1.8	90.9

[1]The system was the same as given in Table 1.

was inhibited by antifungal agents, 56.6 to 75% of the carbohydrate utilization by yeast cells was inhibited by the antifungals.

Data showing effects of higher concentrations of antifungals (20 μg/ml) upon endogenous respiration and aerobic utilizationof sugars are shown in Table 2. The antifungal agents inhibited respiration of *K. fragilis* cells by 47.8 to 70.0%. Pimaricin inhibited aerobic utilization of lactose, glucose, and galactose by 81.1, 78.5, and 72.4%, respectively; the corresponding inhibitions by nystatin were 93.1, 91.1, and 90.9%. In general, higher concentrations of the two antifungals inhibited both endogenous uptake and aerobic utilization of sugars more severely than did the lower concentrations (Table 1). However, an increase in the antifungal concentration did not manifest a propostional increase in the inhibition of oxygen uptake.

A comparison of the inhibitory effects of the two antifungals at different concentrations (Tables 1 and 2) revealed that nystatin impaired the carbohydrate metabolism of *K. fragilis* slightly more than did pimaricin.

Anaerobic utilization of sugars

The next phase of this study was to determine anaerobic utilization of lactose, glucose, and galactose by *K. fragilis* (Table 3). Five micrograms of pimaricin per milli liter inhibited anaerobic utilization of lactose, glucose, and galactose by 75.6, 76.2, and 74.5%, respectively, and nystatin inhibited carbohydrate

TABLE 3. *Effect of pimaricin and nystatin upon the anaerobic sugar metabolism of* K. fragilis[1]

Carbohydrates	Antib. conc. (μg/ml)	μl of CO_2 evolved Average	% Inhibition
		Pimaricin	
Lactose	0	18.9	
	5	4.6	75.6
Glucose	0	261.0	
	5	62.0	76.2
Galactose	0	16.6	
	5	2.7	74.5
		Nystatin	
Lactose	0	19.0	
	5	4.3	77.3
Glucose	0	221.0	
	5	30.0	86.4
Galactose	0	22.0	
	5	4.5	79.5

[1]The reaction system was prepared in the same manner as given in Tables 1 and 2, except that the flasks were flushed with nitrogen-carbon dioxide (95:5) gas mixture, and the KOH was omitted.

utilization by 77.3, 86.4, and 79.5% respectively. At the higher level of the antifungals (20 μg/ml), pimaricin inhibited lactose, glucose, and galactose metabolism by 84.7, 82.0, and 83.0% (Table 4). Corresponding inhibition by nystatin was 91.8, 84.1, and 87.3% respectively. Here again, nystatin suppressed anaerobic carbohydrate utilization slightly more than did pimaricin, under parallel conditions.

TABLE 4. *Effect of pimaricin and nystatin upon the anaerobic sugar metabolism of* K. fragilis[1]

Carbohydrates	Antib. conc. (μg/ml)	μl of CO_2 evolved Average	% Inhibition
	Pimaricin		
Lactose	0	51.0	
	20	7.8	84.7
Glucose	0	262.6	
	20	47.3	82.0
Galactose	0	29.3	
	20	4.8	83.6
	Nystatin		
Lactose	0	24.6	
	20	2.0	91.8
Glucose	0	257.6	
	20	15.0	94.1
Galactose	0	15.8	
	20	2.0	87.3

[1]The system was the same as given in Table 3.

Lactase

In general, the organism was able to utilize glucose more rapidly than galactose or lactose. Nevertheless, since the organism was able to utilize lactose, it was felt that the intact yeast cell might contain the enzyme lactase which initially hydrolyzes lactose into glucose and galactose.

Tests done with several separate preparations showed that the organism was able to hydrolyze lactose and possessed the enzyme lactase to the extent of 3 and 13 units with an average of 8 units per gram of dry cells (Table 5). The enzyme seemed to be constitutive rather than an adaptive, since yeast grown even on glucose or galactose showed the presence of this enzyme.

TABLE 5. *Presence of lactase in* K. fragilis[1]

Trial No.	Lactose hydrolyzed (mg/ml)	Enzyme concentration Unit/ml enz. extract	Enzyme concentration Unit/g dry cells
1	0.180	0.050	7.5
2	0.124	0.034	8.5
3	0.072	0.020	3.0
4	0.473	0.130	13.0
Average	0.212	0.058	8.0

[1]Reaction system: Each tube contained 0.2 ml of 0.1 M phosphate buffer pH 6.8, 0.2 ml of enzyme extract made up in pH 6.8 phosphate buffer (or 0.2 ml of water as blank control), 1.0 mg of lactose in 0.1 of water. The final volume was made up to 1.0 ml with water and incubated at 30 C for 10 min.

The effect of antifungals on lactase is presented in Table 6. At 5 μg/ml pimaricin and nystatin inhibited lactase activity by 32 and 76% respectively. At 20 μg/ml level, the antifungal agents inhibited lactase activity by 76 and 82%, respectively.

Conversion of milk sugar, lactose, into its constituent monosaccharides seems to be inhibited by the antifungal agents, thus impairing utilization of lactose by *K. fragilis* and also resulting in retardation of growth of yeast cells.

TABLE 6. *Effect of antibiotics upon lactase of* K. fragilis[1]

Antibiotics	Antib. conc. (μg/ml)	mg Lactose hydrolyzed/ml	% Inhibition
Control	0	0.473*	—
Pimaricin	5	0.320	32.35
Nystatin	5	0.112	76.32
Pimaricin	20	0.112	76.32
Nystatin	20	0.073	84.36

[1]Reaction system: Same as described in Table 5.
* From Trial 4 in Table 5.

DISCUSSION

Bradley (*1*) observed that nystatin at a concentration of 25 μg/ml or lower was fungistatic in nature. Results obtained in this study seem to be in general agreement with the observations of Lampen and Arnow (*5*) and Bradley (*1*). The fungistatic concentrations of nystatin inhibited endogenous respiration as well as the aerobic utilization of sugars by yeast and thus impaired reactions of general metabolic significance. Lampen et al. (*4*), using 5 μg of nystatin and 2.5 mg dry weight of *S. cerevisiae* cells, found that the aerobic and anaerobic utilization of glucose was inhibited by 62 to 82% and 74 to 96%, respectively, in the case of three different strains of the yeast. Bradley (*1*), on the other hand, using 25 μg of nystatin per milliliter observed that glucose utilization was inhibited to an extent of 25.0 and 97.7% under aerobic and anaerobic conditions, respectively, when 5 mg dry weight of *C. stellatoidea* cells were used. It has been shown (*1, 4*) that the normal fermentative and respiratory mechanisms of yeast were impaired by nystatin. This was further confirmed by Scholz et al. (*8*) and Marini et al. (*6*), who demonstrated that nystatin inhibited the enzymes of the glycolytic pathway.

The fact that under aerobic or anaerobic conditions the antifungals inhibited utilization of lactose, glucose, or galactose to almost the same extent, indicates that these sugars are metabolized by similar processes. The extent of inhibition by the antifungals was greater under anaerobic rather than under aerobic conditions. There was a direct but not propotional relationship between concentration of antifungals and percent inhibition of sugar metabolism. Under similar conditions, nystatin was slightly more inhibitory than pimaricin toward carbohydrate metabolism of *S. cerevisiae.*

The enzyme lactase, an integral part of the enzyme system involved in glycolysis, was present in the yeast. Further, the enzyme lactase was sensitive to the antifungal agents, indicating that the inhibitory effect of the antifungals toward sugar metabolism of *K. fragilis* was at the enzyme level. It is possible that pimaricin, like nystatin, partially blocked the glycolytic as well as the oxidative cycle of terminal respiration of *K. fragilis,* as has been shown for *C. stellatoidea* (*1*).

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Repair, Growth, and Enterotoxigenesis of *Staphylococcus aureus* S-6 Injured by Freeze-Drying

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ABSTRACT

Staphylococcus aureus S-6 cells in the mid-log growth phase rapidly (0.5-1 h) repaired freeze-drying injury when rehydrated at mesophilic temperatures (20-50 C), although these temperatures had a greater effect on subsequent growth of total survivors. At 15 C repair and growth processes were slow. At 10 C repair and growth did not occur. Enterotoxin B was released into the rehydration medium at about the time when survivors initiated growth but was difficult to demonstrate during the repair stage because of the short duration of this phase. Metabolic and macromolecular inhibitor studies on *S. aureus* S-6 cells injured by freeze-drying suggested that RNA synthesis was necessary as the first stage of repair.

Bacterial cell injury has been shown in cells exposed to heat (*7, 9, 14, 18, 22, 24, 27*), radiation (*1*), chemicals (*8, 19*), freezing and chilling (*10, 25*), and freeze-drying (*15, 16, 20, 21*). Ray and Speck (*17*) and Gomez et al. (*6*) recently provided detailed reviews on the subjects of freezing-injury and freeze-drying injury, respectively. Practical implications in detection and enumeration of injured cells were discussed by Speck (*23*). Sinskey and Silverman (*21*) postulated that sites of RNA synthesis and permeability alteration may be the most sensitive in freeze-drying injury of *Escherichia coli*. Ray et al. (*15*) reported that repair of freeze-drying injury of *Salmonella anatum* perhaps involved ATP synthesis for repair of damaged lipopolysaccharide portions of the cell wall.

Numerous reports have appeared concerning injury incurred by *Staphylococcus aureus* during heating (*9, 18, 22, 24*), but only limited data has been reported on injury to this organism by freeze-drying. In the present study, repair of freeze-drying injury in *S. aureus* was investigated in terms of the effects of rehydration temperature, release of enterotoxin B during the process, and effects of various inhibitors.

MATERIAL AND METHODS

Preparation of inoculum

S. aureus S-6 was obtained from M. S. Bergdoll (Food Research Institute, University of Wisconsin). This strain produces large amounts of enterotoxin B and small amounts of enterotoxin A. Stock cultures of the test organism were prepared by growing aerobically at 37 C in a medium containing 3% protein hydrolysate powder (Mead Johnson International, Evansville, Ind.), 3% N-Z amine NAK (Shaffield Chemical Co., Norwich, N.Y.), plus vitamins (PHP medium). When the cultures reached the stationary phase (approximately 500 Klett units; KU), 3-ml samples were aseptically transferred to sterile screw-cap test tubes, quick-frozen in a dry ice-acetone bath, and stored at − 15 C (*11*). When needed, cells were thawed, washed 3 × in sterile distilled water, and inoculated into 75 ml of PHP medium using a 0.1% inoculum. A nephelofiask (300 ml, Bellco Glass, Vineland, N.J.) equipped with a side arm was used for growth and turbidity measurement. The culture was then incubated at 40 C in a water bath shaker (Precision Scientific Co., Chicago, Ill.). When turbidity reached 70 KU (measured by Klett-Summerson colorimetry; 540 nm), after 3 to 4 h of incubation, the culture was freeze-dried as mid-log phase cells. In experiments with late-log phase cells, turbidity was allowed to increase to 400 KU before freeze-drying.

Freeze-drying procedure

Twenty-five milliliters of the culture were placed in a sterile 50-ml Erlenmeyer flask loosely fitted with a grooved rubber stopper. Quick freezing (ca 5 min) was achieved by placing the sample flask in a dry ice-ethanol slurry. Frozen cultures were placed in a VirTis model 10-MR-SA, chamber type lyophilizer (VirTis Company, Gardiner, N.Y.). Shelf refrigeration (− 40 C) was maintained until full vacuum (8 μm of Hg, measured by a VirTis McLeod Gauge) was achieved in the chamber, usually within 0.5 h. The shelf temperature was then elevated to 25 C. After 24 h, the samples flasks were stoppered under vacuum before removal from the chamber.

Rehydration procedure

The freeze-dried samples were rehydrated (within 1 week after lyophilization) with 25 ml of sterile distilled water adjusted to the desired test temperature under a bacteriological hood previously sterilized by ultraviolet irradiation. Time for rehydration and complete mixing of solution was less than 1 min. The zero-(0)-h rehydration data were obtained by removing 1 ml of the culture for viable cell count (*2*) and another 1 ml for enterotoxin B and α-hemolysin analysis. Samples were withdrawn at suitable intervals depending on the incubation temperature (ranging from 0.5 h to several days) to study repair, growth, and enterotoxigenesis of cells injured by freeze-drying.

Plating media and toxin analysis

The difference in colony forming units (CFU) obtained on plate count agar (PCA, Difco) and on PCA with 7.5% NaC1 added was used to evaluate the number of injured cells. In normal populations, the difference between these two counts was negligible while a 1- to 1.5-log difference existed for *S. aureus* S-6 cells injured by freeze-drying. Repair was indicated by an increase in the colony count obtained on PCA + 7.5% NaC1 agar while the count on PCA agar remained constant (*9*). Various concentrations (1 to 12%) of NaC1 in PCA had been previously tested in this laboratory (D. Y. C. Fung, unpublished data) for normal and injured cells and 7.5% NaC1 proved to be the most appropriate. A 7.5% NaC1 concentration is used in some commercial media, e.g. Staphylococcus medium #110 (Difco, BBL) and Mannitol salt agar (Difco, BBL) designed for isolation of *S. aureus* from foods and the environment. For enterotoxin B analysis, a 1-ml culture sample was centrifuged at 20,000 × *g* for 15 min to obtain cell-free supernatant fluid and toxin was assayed quantitatively by the capillary tube procedure of Fung and Wagner (*5*).

Effect of inhibitors

To investigate the mechanism of repair, a series of experiments were done using solutions of metabolic or macromolecular inhibitors as the rehydration medium. All compounds were dissolved in sterile distilled water and reported as final concentrations achieved in the rehydrated cultures. All experiments were done at 40 C using freeze-dried mid-log phase *S. aureus* S-6 cells. Compounds tested were: (*a*) cell wall inhibitors—bacitracin (75 μg/ml, Sigma); penicillin (2.5, 5, 50, and 100 μg/ml, Squibb and Sons, Inc.); (*b*) respiration inhibitors—2, 4-dinitrophenol (50 and 100 μg/ml, Fisher); NaN_3 (75 μg/ml, Difco); potassium cyanide (75 μg/ml, Fisher); (*c*) protein synthesis inhibitors—chloramphenicol (50 and 100 μg/ml, Calbiochem); kanamycin (75 μg/ml, Sigma); and (*d*) nucleic acid inhibitors—nalidixic acid (1, 5, and 10 μg/ml, Calbiochem); rifampicin (1, 0.01, 0.0075, and 0.005 μg/ml, Lepetit, Italy).

Other determinations

Release of α-hemolysin by *S. aureus* during the repair period was investigated using a microtitration procedure (D. Y. C. Fung and R. D. Miller, unpblished data) which determined α-hemolysin titer in cell-free supernatants using rabbit erythrocytes (5% suspension, BBL) as indicator. The total number of *S. aureus* S-6 cells during repair was monitored using a Petroff-Hauser counting chamber and phase-contrast microscopy (× 1,000).

RESULTS AND DISCUSSION

Data on growth, enterotoxin B and α-hemolysin production of uninjured *Staphylococcus aureus* S-6 in PHP medium incubated at 40 C are presented in Fig. 1. During the growth phase both PCA and PCA + 7.5% NaC1 media yielded approximately the same number of CFU/ml, which indicated that uninjured cells were not affected by 7.5% NaC1 in the plating medium. When

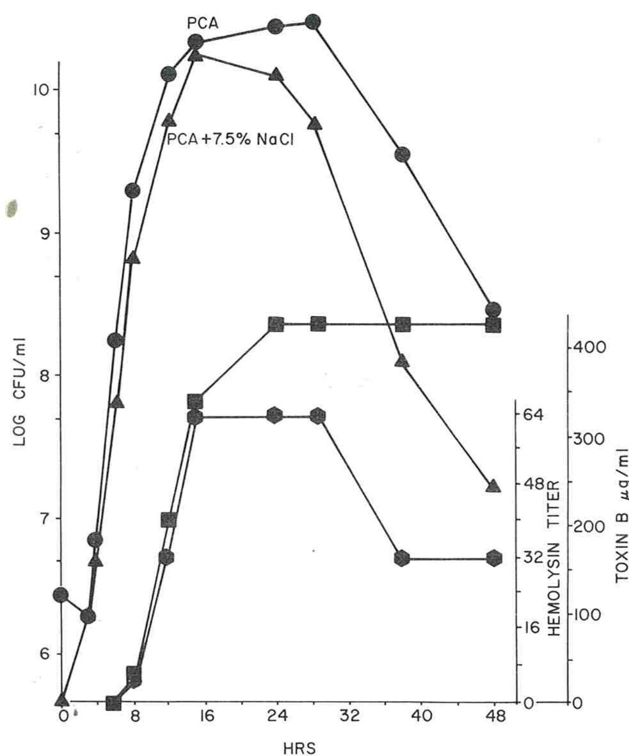

Figure 1. *Growth, enterotoxin B and α-hemolysin production of uninjured* S. aureus *S-6 in PHP medium.*
●—●, *PCA counts;* ▲—▲, *PCA + 7.5% NaC1 counts;* ■—■, *toxin Bμg/ml;* ⬢—⬢, *α-hemolysin titer.*

cells reached maximum stationary phase a population of injured cells appeared as indicated by the difference of CFU/ml on the two plating media. This difference increased as the population entered the death phase after 32 h of incubation. Enterotoxin B and α-hemolysin were actively released into the medium during the late log phase of growth. Enterotoxin B production reached a maximum level at 25 h and remained constant thereafter whereas the hemolytic titer reached a maximum at 16 h of incubation and was reduced to 32 after 40 h. Hemolysins are known to be unstable during prolonged incubation and aeration.

The phenomenon of repair of freeze-drying injured cells is illustrated in Fig. 2. At the time of rehydration of

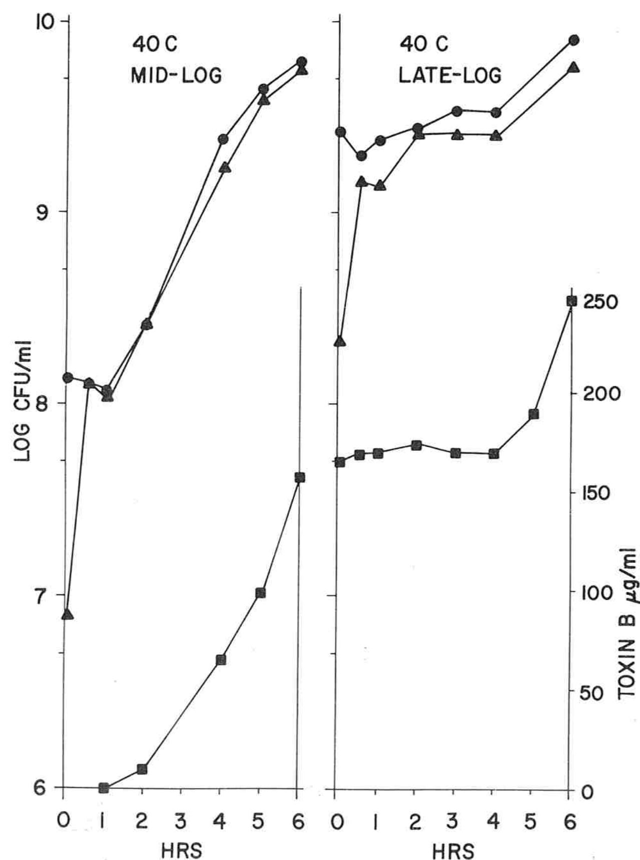

Figure 2. *Effects of 40 C rehydration temperature on repair, growth, and enterotoxin B production of* S. aureus *S-6 cells injured by freeze-drying. Mid-log cells and late-log cells were harvested for freeze-drying experiments.*
●—●, *PCA counts;* ▲—▲, *PCA + 7.5% NaC1 counts;* ■—■, *toxin Bμg/ml.*

lyophilized mid-log phase cells a large population of injured cells existed. However, after 0.5 h of incubation at 40 C the injured cells repaired rapidly. The total population (Petroff-Hauser count) of *S. aureus* S-6 cells remained constant during the repair stage and increased concomitantly with an increase in the viable count. After a short lag of 1 h survivors started to grow; enterotoxin B appeared in the medium. Appearance of extracellular enterotoxin at the time in initiation of growth was interpreted as the possible release of pre-formed toxin as

well as synthesis and release of new enterotoxin B during growth (R. D. Miller and D. Y. C. Fung, Abs. Ann. Meet Am. Soc. Micro. 1974:15).

Similarly, when lyophilized late-log phase cells were rehydrated a population of injured cells was observed which also completed repair in 0.5 h of incubation. However, there was a long lag (4 h) before these survivors initiated growth and, since the number of survivors at late-log phase was already large (ca. 3×10^9 cells/ml), only limited further growth occurred. It is difficult to ascertain from the data presented (Fig. 2) and from several repeated experiments (data not shown) whether extra enterotoxin B was released by the late-log phase cells during the repair stage. The amount of extracellular toxin remained relatively constant throughout the lag phase. Rapid release of toxin occurred when survivors started to grow further.

Repair of lyophilized mid-log phase cells rehydrated at 37 C and 45 C was rapid (0.5 h; Fig. 3). The survivors at 37 C incubation resumed growth with practically no lag phase (Fig. 3). This temperature (37 C) is usually regarded as optimal for growth (*26, 28*). Enterotoxin B was not detected until 2 h of incubation and the level increased rapidly parallel to the growth curve. When rehydrated at 45 C, there was a lag of about 3 h before survivors started to grow slowly. This temperature was considered to be near the maximal for growth and toxin production by *S. aureus* S-6 (*28*). Small amounts of toxin were present in the medium after 3 h of incubation and the rate of toxin release was slow compared to incubation at 37 C or 40 C.

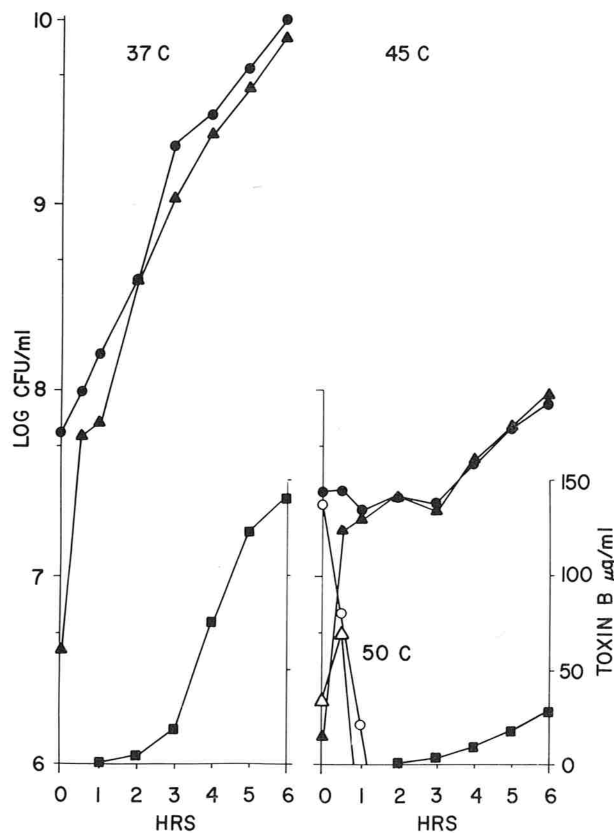

Figure 3. *Effects of 37, 45, and 50 C rehydration temperatures on repair, growth, and enterotoxin B production of* S. aureus *S-6 cells injured by freeze-drying.*
●—● *and* ○—○, *PCA counts;* ▲—▲ *and* △—△, *PCA + 7.5% NaCl counts;* ■—■, *toxin B µg/ml. The open symbols refer to 50 C data.*

Lyophilized cells died exponentially when rehydrated at 50 C (Fig. 3). It was observed that the injured cells first repaired (0.5 h) and then died along with the total surviving population which indicated that mild heat treatment (50 C) did not impair the repair process of *S. aureus* S-6.

At mesophilic temperatures (20 C to 30 C) of rehydration, the repair process was slower and incomplete compared to repair at higher temperatures. The 30-C rehydration data (Fig. 4) showed that 1 h was necessary for injured cells to repair and that an injured population existed even after initiation of growth of survivors. Enterotoxin B appeared in the medium 3 h after rehydration. At 20 C the time for repair was also 1 h; however, there was a long lag of 5 h before initiation of growth. The growth rate was also slow and enterotoxin B did not appear in the medium until after 24 h of incubation (data not shown). An injured population also existed even after prolonged incubation.

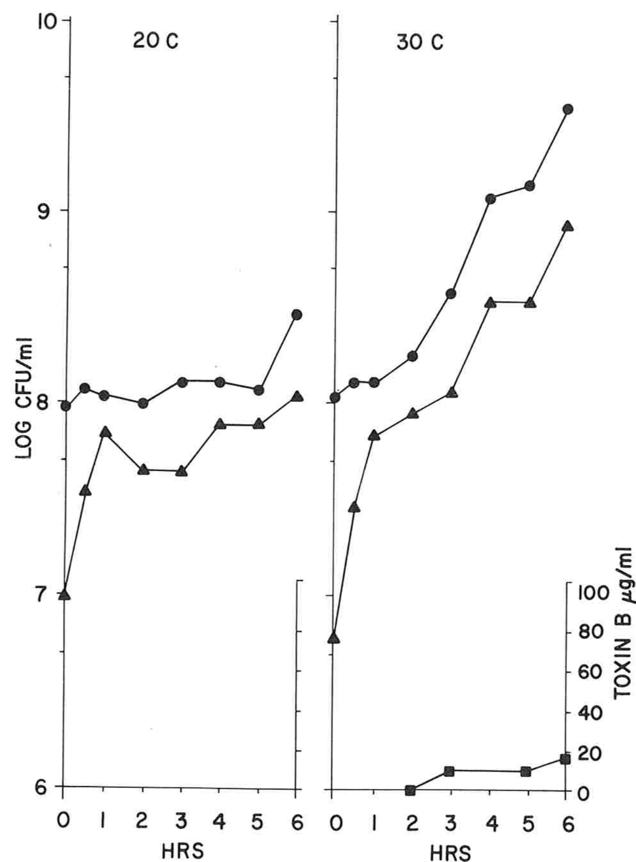

Figure 4. *Effects of 20 and 30 C rehydration temperatures on repair, growth, and enterotoxin B production of* S. aureus *S-6 cells injured by freeze-drying.*
●—●, *PCA counts;* ▲—▲ *PCA + 7.5% NaCl counts;* ■—■, *toxin B µg/ml.*

At the psychrotrophic rehydration temperature of 15 C, 12 h was needed to complete repair and 30 h elapsed before initiation of growth (Fig. 5). Cells resumed logarithmic growth extended over a 6-day period. Enterotoxin B was detected after 4 days of incubation. Total toxin increased to more than 20 μg/ml after 6 days of incubation. At the 10 C rehydration temperature a large population of injured cells existed due to the combined lyophilization injury and cold temperature injury. Repair of cells was negligible. Total survivors died slowly while the injured population remained relatively consistent. No enterotoxin B was detected during the entire experiment (Fig. 5).

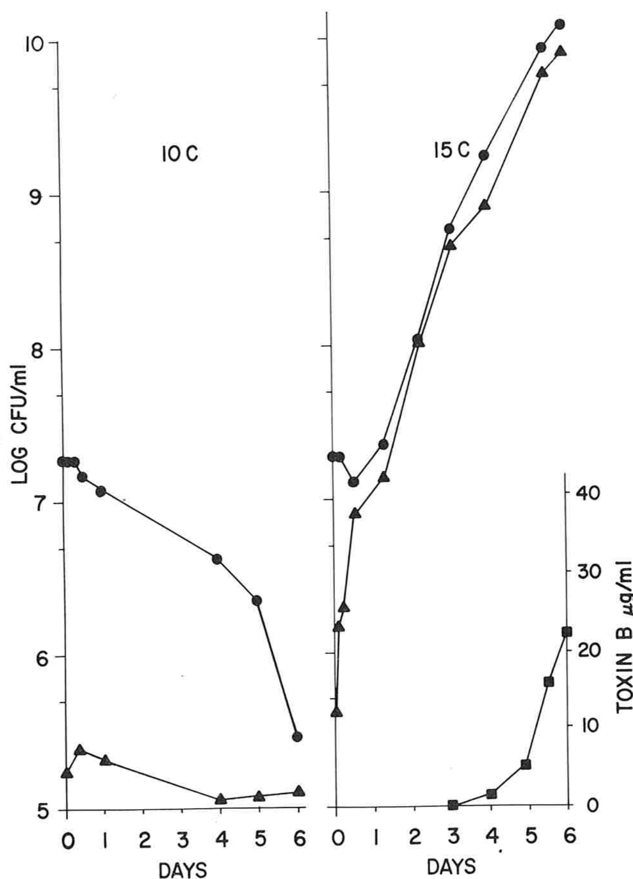

Figure 5. *Effects of 10 and 15 C rehydration temperature on repair, growth, and enterotoxin B production of* S. aureus *S-6 cells injured by freeze-drying.*
●—●, *PCA counts;* ▲—▲, *PCA + 7.5% NaCl count;* ■—■, *toxin B μg/ml.*

From these data it was concluded that mesophilic rehydration and incubation temperatures (20 C to 50 C) had less effect on repair of cells injured by freeze-drying than on subsequent growth of survivors. The repair process was completed between 0.5 and 1 h of incubation, while dramatic influence on subsequent growth or death rates by rehydration temperatures was observed. At psychrotrophic temperatures, injured cells repaired slowly and subsequent growth of survivors was also slow at 15 C; no repair or growth occurred at rehydration temperature of 10 C. The repair profiles for some of the mesophilic temperatures (20, 30, and 37 C) were in general agreement with similar studies on freeze-dried *S. anatum* by Ray et al. (*15*). However, at lower temperatures (10 C and 15 C), *S. aureus* cells injured by freeze-drying seemed to be much less able to repair and survive compared to *S. anatum* (*15*).

It was quite difficult to evaluate the release of enterotoxin B during the repair stage because in most instances the repair process was completed within a short time (0.5-1 h). Also, the single diffusion assay system (5) was not accurate enough to evaluate the release of small amounts (< 1 μg/ml) of enterotoxin B. A more sensitive system, such as the radioimmunoassay, would have been useful in studying the intracellular and extracellular enterotoxin B kinetics during the repair stage.

The effect of rehydration temperatures on rates and quantities of α-hemolysin production by freeze-dried cells resembled those of enterotoxin B production. α-Hemolysin first appeared in the medium after 2 h of incubation, similar to the time when enterotoxin B first appeared. These results suggested that release of α-hemolysin by freeze-dried *S. aureus* S-6 may be under a similar control mechanism as release of intracellular enterotoxin B.

The effects of antimicrobial agents, which either interfere with cellular functions or prevent synthesis of macromolecules, on repair of lyophilized cells are presented in Fig. 6 through Fig. 10. The cell wall inhibitors, bacitracin and penicillin, had no effect on the repair process (Fig. 6); although penicillin (2.5 μg/ml) killed the

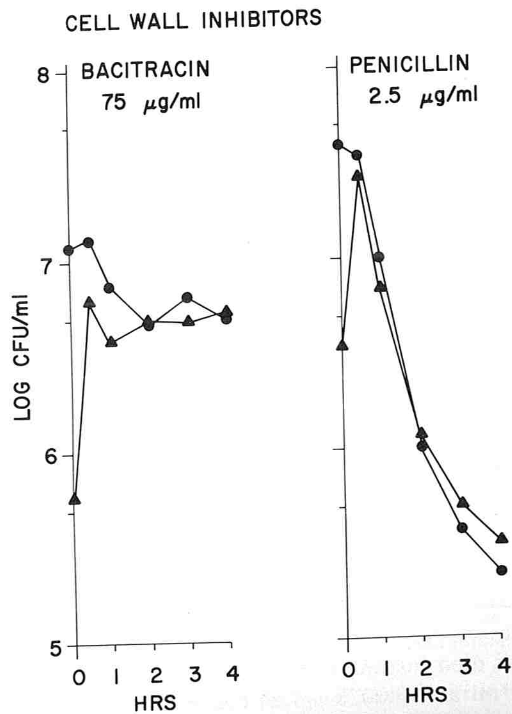

Figure 6. *Effect of cell wall inhibitors on repair of* S. aureus *S-6 cells injured by freeze-drying.*
●—●, *PCA counts;* ▲—▲, *PCA + 7.5% NaCl counts.*

total survivors rapidly. This curve resembled the repair and death curves of rehydrated cells at 50 C (Fig. 3). The data just described imply that in the nutritionally rich PHP medium damage to the cell wall by lyophilization did not affect the repair process. None of the three respiration inhibitors tested (2, 4-dinitrophenol, NaN_3, and KCN) had any effect on the repair process (Fig. 7).

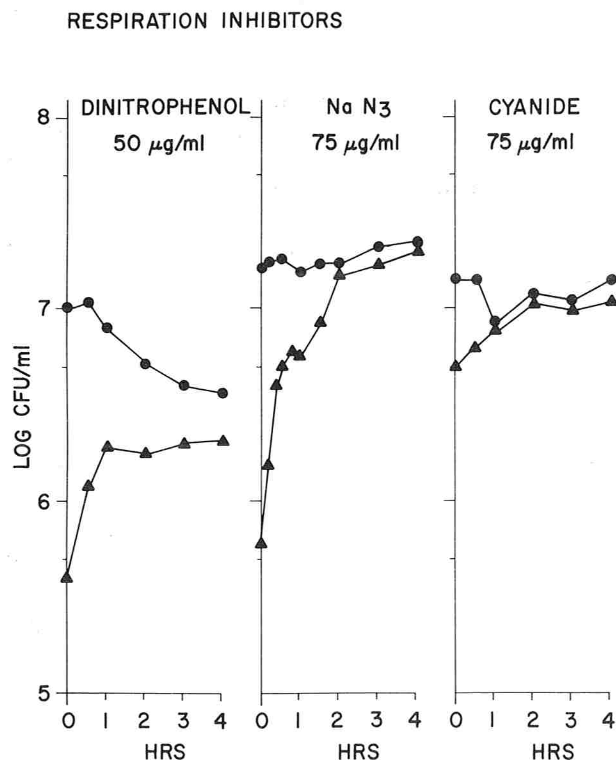

Figure 7. *Effect of respiration inhibitors on repair of* S. aureus *S-6 cells injured by freeze-drying.*
●—●, *PCA counts;* ▲—▲, *PCA + 7.5% NaCl counts.*

These results were not in agreement with those presented by Ray et al. (*15*) concerning the inhibitory effect of dinitrophenol and sodium cyanide on repair of freeze-dried *S. anatum*. Our data indicated that ATP synthesis through oxidative phosphorylation may not be necessary as the first stage of repair of freeze-dried *S. aureus* cells. Interference of protein synthesis using chloramphenicol and kanamycin (Fig. 8) also had no effect on the repair process of these cells.

Nalidixic acid was used as the inhibitor for DNA synthesis (Fig. 9). No inhibition of repair of freeze-drying injury was observed. In the presence of this compound only a small population of injured cells existed. The reason for this phenomenon is not known.

Lyophilized *S. aureus* S-6 cells were highly sensitive to rifampicin, which binds with DNA-dependent-RNA polymerase. At concentrations of 1 µg/ml the total survivors died instantaneously (data not shown) and, at 0.01 µg/ml and 0.0075 µg/ml concentration levels (Fig. 10), the total number of survivors as well as injured cells declined. The significant information obtained from the data shown was that the injured cells did not have the

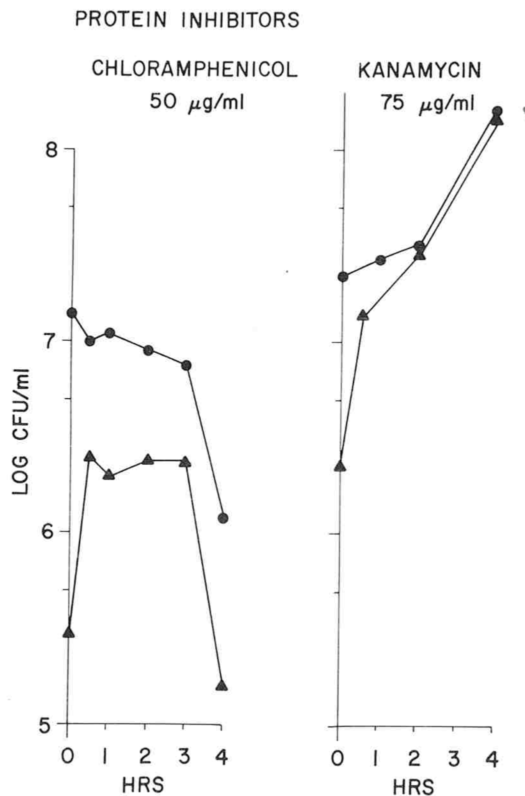

Figure 8. *Effect of protein inhibitors on repair of* S. aureus *S-6 cells injured by freeze-drying.*
●—●, *PCA counts;* ▲—▲, *PCA + 7.5% NaCl counts.*

ability to repair in the presence of rifampicin. As a comparison, cells injured by freeze-drying were able to repair in the presence of penicillin or at 50 C incubation (cf. Fig. 6 and Fig. 3) even though in both instances total survivors declined at approximately the same rate. These data imply that RNA synthesis was one of the more essential processes for repair of *S. aureus* cells injured by freeze-drying. Sinskey and Silverman (*21*), in identifying known events occurring in recovery of growth of freeze-dried *E. coli* cells, also cited RNA synthesis as the first event leading to repair and growth. Further lowering of the concentration of rifampicin (Fig. 10) to 0.005 µg/ml reduced the concentration to a level ineffective in suppressing sythesis of RNA of injured cells. The total surviving population remained relatively constant during the first 2 h and declined slowly while the injured cells repaired gradually until both curves reached approximately the same level.

Data from the effects of inhibitors on *S. aureus* S-6 cells injured by freeze-drying indicated that neither cell wall synthesis, cellular respiration apparatus, nor protein synthesis was directly involved in the recovery process. Leakage of RNA and not DNA was observed by Morichi in his study concerning metabolic injury in frozen *E. coli* (*12*). Our data also showed that DNA synthesis was not required for recovery of injured cells. However, resynthesis of RNA, which apparently leaked out of the cells during freeze-drying, was essential for the repair process. Such a conclusion was also made by Morichi

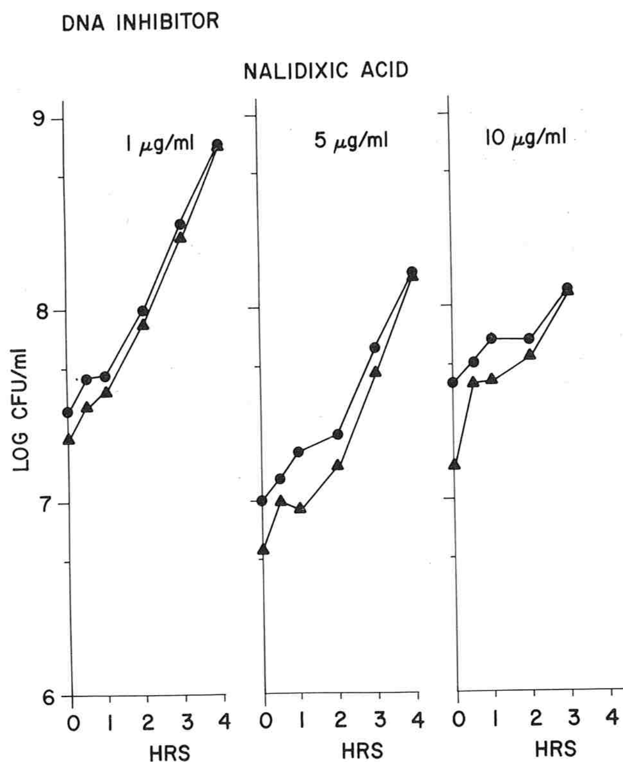

Figure 9. *Effect of DNA inhibitor on repair of* S. aureus *S-6 cells injured by freeze-drying.*
●—●, *PCA count;* ▲—▲, *PCA + 7.5% NaCl counts.*

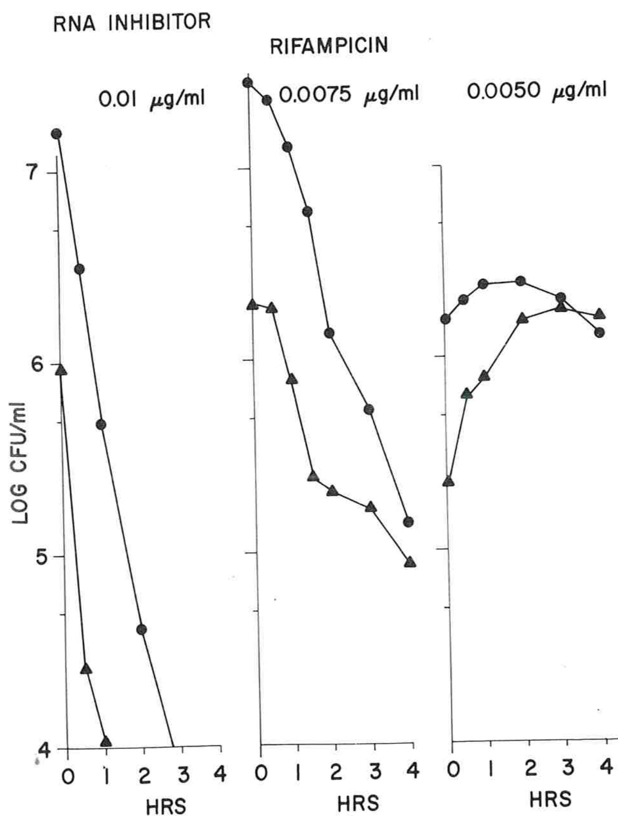

Figure 10. *Effect of RNA inhibitor on repair of* S. aureus *S-6 cells injured by freeze-drying.*
●—●, *PCA counts;* ▲—▲ *PCA + 7.5% NaCl counts.*

and Irie (*13*) on the repair of freeze injury of *Streptococcus faecalis* (*13*) and Clark et al. on the recovery of thermal injury of *S. faecalis* (*3*) as well as other similar studies (*21*).

Although data obtained in this study were compared with results from other freeze-drying injured studies, it should be noted that the latter data were collected with gram-negative cells.

In conclusion, repair of *S. aureus* cells injured by freeze-drying was not greatly affected by mesophilic rehydration temperatures although subsequent growth of survivors was influenced by rehydration and incubation temperatures. Enterotoxin B as well as *a*-hemolysin release from *S. aureus* cells injured by freeze-drying seemed to be related directly to growth of survivors; although leakage of small amounts of intracellular preformed enterotoxin B to the environment due to freeze-drying cannot be disregarded. Finally, RNA resynthesis seems to be the most critical event in the first stage of repair of *S. aureus* S-6 cells injured by freeze-drying.

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Extraction of Protein from Mechanically Disrupted Freeze-Dried Brewer's Yeasts

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ABSTRACT

Protein was extracted from mechanically disrupted brewer's yeasts with an alkaline aqueous solution. Treatment parameters such as pH, solvent-to-yeast ratio, temperature, and time were varied to establish optimum conditions for protein extraction. The pH was found to be the major single factor affecting yield of extractable protein as determined by the biuret method. Maximal and minimal protein solubilities were observed at pH 12 and 4.5, respectively. Under conditions for optimum extraction, the yield of extracted protein was 68.2%. Protein concentrates prepared from brewer's yeast under the selected extraction conditions were analyzed for their content of protein, ribonucleic acid, crude fat, ash, and essential amino acids.

A steady increase in the world's population and in many areas of unstable food production have created awareness of a potentially serious food shortage, the paucity of protein presenting the greatest problem. It would appear that one way of alleviating this problem would be to develop an unconventional protein source, such as microbial protein, for use as human food. Brewer's yeast, a by-product of the brewing industry, has long been recognized as an important source of protein. Dried brewer's yeast contains approximately 47 to 55% protein (*3, 13, 18*).

Studies have shown that microbial protein released from the cell wall is more digestible than protein contained within the cell (*12, 17*). An important merit of using cell-free yeast protein rather than whole yeast cells as a food ingredient is that the functional properties of yeast protein may be utilized to full advantage. In the intact yeast cell, functionality and availability of protein are limited by the physical barrier formed by the cell wall. Methods to extract protein from torula yeasts using a concentrated urea solution (*11*), sodium hydroxide solution or a combination of alkaline pre-treatment and concentrated urea solution (*12*) have been reported. These methods require either a long extraction time or concentrated extractants.

The present study was undertaken to extract protein from mechanically disrupted brewer's yeasts. The primary objective was to establish optimum conditions for protein extraction with an aqueous solution adjusted to various pH values with HC1 or NaOH.

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MATERIALS AND METHODS

Preparation of disrupted yeasts

Brewer's yeast slurry, kindly provided by Molson's Brewery Limited, Toronto, was centrifuged in an IEC centrifuge (Model B20) at 5000 rpm for 15 min at 0-4 C. The yeast paste was washed once with water and then centrifuged again before being debittered according to the method of Dwivedi and Gibson (*3*). The purpose of the debittering step was to remove the bitter substance contributed by hops to the yeast slurry so that this bitterness would not carry through to the final protein preparation. Following the debittering treatment and centrifugation, the yeast paste was washed three times with water and finally freeze dried. The freeze-dried yeasts were disrupted in a Tekmar analytical mill (Model A10, Tekmar Company, Cincinnati, Ohio) operated for 20 min and equipped with a water cooling system. The milling process was interupted at every 4-min interval to mix yeast cells to facilitate their uniform rupture. Samples of this disrupted yeast material were stained with a methylene blue solution and examined microscopically. Most cells were fractured under these conditions. This disrupted yeast material was stored in moisture-proof plastic bags in a freezer.

Protein extraction from disrupted yeasts

The parameters chosen for study to obtain optimal extraction of protein from ruptured yeasts were: (*a*) pH, (*b*) time, (*c*) solvent-to-yeast ratio, and (*d*) temperature. The general method employed to extract protein involved adjusting the pH of a slurry containing ruptured yeasts in water to the desired pH with either 1 N NaOH or 1 N HC1. Extraction was effected by gentle stirring using a stirrer (Fisher Thermis) for the desired time at the chosen temperature. Thereafter the insoluable residue was removed by centrifugation of 8000 rpm for 15 min. The residue was washed once with one quarter of the original volume of extractant (water adjusted to the extraction pH) and re-centrifuged. The supernatant fluid was added to the extract.

Preparation of yeast protein concentrate

The extract obtained under the selected conditions was adjusted to the determined pH of minumum protein solubility with 1 N HC1 or 1 N NaOH to precipitate protein. After centrifugation at 8000 rpm for 20 min, the precipitate was washed three times with water which had been adjusted to the protein precipitation pH. The washed precipitate was then freeze dried.

Methods of analysis

Moisture, crude fat, and ash contents were determined by the official methods of the A.O.A.C. (*1*). Protein content was determined by the biuret method as described by Herbert et al. (*7*). Acid hydrolysis of the samples for subsequent amino acid analysis was carried out following the Moore and Stein method (*14*). A Beckman amino acid analyzer (Model 121) was then used for amino acid determination. Ribonucleic acid (RNA) was determined by the method described by Herbert et al. (*7*). In this method RNA was extracted from samples with 0.5 N aqueous perchloric acid solution at 37 C for 2 h. The RNA content of the extract obtained was determined spectrophotometrically with orcinol reagent.

RESULTS AND DISCUSSION

The percentage of protein extracted from ruptured yeasts as a function of pH is given in Fig. 1. All the extraction parameters used in this determination were arbitrarily chosen. The solubility of yeast protein was apparently greater in the alkaline range, with 71.3% protein soluble at pH 12. Minimal protein solubility was observed at pH 4.5 with only 16% of protein extracted. Since maximal solubility of protein was achieved at pH 12, protein extraction was subsequently done at this pH.

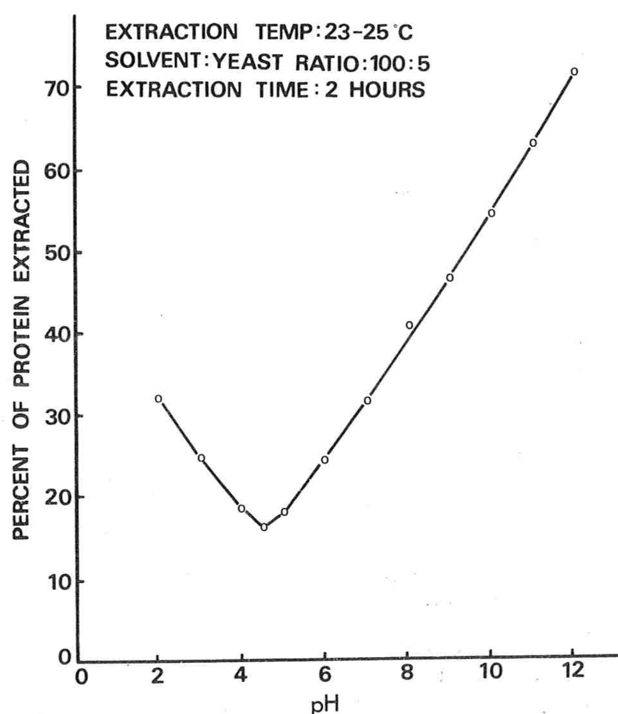

Figure 1. *Effect of pH on extraction of protein from mechanically disrupted, freeze-dried brewer's yeast.*

TABLE 1. *Effect of various parameters on the protein extraction yield at pH 12*

Temperature (C)	Solvent-to-yeast ratio	Extraction time (h)	Protein extraction yield (%)
23-25	100: 5.0	1	71.2
23-25	100: 5.0	2	71.3
23-25	100: 5.0	3	71.5
23-25	100: 5.0	1	71.2
23-25	100: 7.5	1	69.3
23-25	100:10.0	1	68.2
23-25	100:12.5	1	62.3
23-25	100:10.0	1	68.2
49-51	100:10.0	1	69.7
74-76	100:10.0	1	64.5

In Table 1 the effect of temperature, solvent-to-yeast ratio and extraction time on protein extraction yield at pH 12 is presented. Variation of extraction time between 1 and 3 h did not noticeably affect the yield of extracted protein; thus a 1-h extraction period was deemed suitable and adopted thereafter. Yield of extracted protein decreased with a decreasing solvent-to-yeast ratio. However, the appreciable drop in yield of extracted protein between the 100:10 and 100:12.5 ratios could be associated with the observed decrease in fluidity of yeast slurry at the 100:12.5 ratio. A solvent-to-yeast ratio of 100:10 was chosen because this ratio gave the best absolute yield of extractable protein from the amount of starting material used. Little difference was noted in yield of extracted protein between extraction at room temperature (23-25 C) and 49-51 C. By increasing the extraction temperature to 74-76 C a drop in yield of extracted protein resulted, perhaps because of increased protein denaturation or hydrolysis of the protein at the higher temperature. An extraction temperature of 23-25 C was thus chosen. Therefore, conditions selected for extraction of protein from disrupted yeasts were (*a*) an extraction pH of 12, (*b*) extraction time of 1 h, (*c*) a solvent-to-yeast ratio of 100:10, and (*d*) an extraction temperature of 23-25 C.

To compare the effect of extraction pH on amino acid content of protein preparations, a lower pH (pH 10) was also chosen to prepare protein concentrate. Effects of various parameters on yield of extracted protein from experiments done at pH 10 are summarized in Table 2.

TABLE 2. *Effect of various parameters on the protein extraction yield at pH 10*

Temperature (C)	Solvent-to-yeast ratio	Extraction time (h)	Protein extraction yield (%)
23-25	100: 5.0	1	54.0
23-25	100: 5.0	2	54.3
23-25	100:10.0	1	52.5
49-51	100:10.0	1	54.5

Conditions of temperature, time, and solvent-to-yeast ratio for protein extraction were the same as those established previously as optimum for pH 12.

The yield of extractable protein (68.2%) obtained at pH 12 in this study is comparable with or better than that obtained from torula yeasts by other workers using, in most instances, more extensive extraction procedures than those used in this study (*8, 11, 12*).

The minimum solubility of yeast protein in this study occurred at pH 4.5. Therefore, for preparation of protein concentrate, the extract obtained was adjusted to pH 4.5 to precipitate proteins. The yield of precipitable protein from the pH 12 and pH 10 extracts was 87.8% and 78.8%, respectively. Therefore 12.2% and 21.2% of the extracted proteins were not recovered from the pH 12 and pH 10 extracts, respectively. The protein concentrates prepared from extracts at both pH 10 and pH 12 were brownish. odorless, powdery materials with a faint taste characteristic of yeast. The pH 10 preparation was a slightly lighter in color than the pH 12 preparation.

Table 3 compares the composition of brewer's yeast with its protein concentrates. Both of the concentrates prepared contained a little over 80% protein. This indicates a substantial increase in protein content as compared with that of dried yeast (47%). The RNA content of both protein preparations was more or less the

TABLE 3. *Composition of the brewer's yeast and its protein concentrates*

	Yeast (%)	pH 10 Protein concentrate (%)	pH 12 Protein concentrate (%)
Protein	47.0	81.3	82.1
RNA	8.0	8.5	7.6
Moisture	5.8	4.2	4.1
Crude fat	1.8	0.6	0.7
Ash	4.8	1.8	2.5

same as that of the dried yeast, but, based on the total protein content, the amount of RNA was reduced markedly by about 45% in the pH 12 protein concentrate, and by about 40% in the pH 10 protein concentrate. However, the RNA content of yeast protein concentrates prepared in this work was still quite high and further reduction in RNA content would be desirable since Edozien et al. (*4*) have found that human consumption of high levels of nucleic acids may increase blood uric acid to an unsafe concentration. The crude fat content of both protein concentrates was low. This is undoubtedly an advantageous feature from the standpoint of stability of the products during storage.

TABLE 4. *Essential amino acid content of brewer's yeast, its protein concentrates and FAO reference protein (g/100 g protein)*

Amino acids	Brewer's yeast	pH 10 Protein concentrate	pH 12 protein concentrate	FAO[1] Reference protein
Arginine	6.1	5.8	5.6	—
Histidine	2.7	3.6	3.4	—
Isoleucine	4.9	4.9	4.5	4.2
Leucine	6.4	8.1	7.9	4.8
Lysine	8.4	8.5	8.3	4.2
Methionine	1.8	2.1	2.0	2.2
Phenylalanine	4.3	4.8	4.7	2.8
Threonine	4.7	4.4	4.3	2.8
Valine	5.8	5.7	5.6	4.2

[1]Food and Agriculture Organization of the United Nations, 1957 Protein Requirements. FAO Nutritional Studies, No. 16, pp. 52.

As shown in Table 4, the methionine content of the protein in both the pH 10 and pH 12 preparations increased slightly as compared with that of the dried yeast protein. However, the amount of this amino acid was still slightly below the amount specified by the FAO reference protein (*6*). The methionine deficiency in the protein of brewer's yeast has been reported by Lindan and Work (*9*), Stokes et al. (*16*) and Mojonnier et al. (*13*). On the contrary Block and Bolling (*2*) and Edwards et al. (*5*) have reported that the protein of brewer's yeast contained a sufficient amount of this amino acid to qualify as a good source of protein for human consumption. This discrepancy could be in part due to variations in analytical techniques, yeast strains, or growing conditions. In comparison with the FAO reference protein (*6*) the yeast protein concentrates obtained here contained other essential amino acids in very acceptable proportions. The high lysine content of the protein in both preparations was particularly outstanding. This characteristic makes the yeast protein concentrates ideal for complementing the protein of cereal products which are generally poor in lysine (*15*). With respect to the essential amino acid profile, very little difference was observed between the protein concentrate prepared at pH 10 and that prepared at pH 12. Apparently, a high pH such as pH 12 did not exert adverse effects on the essential amino acid content of the protein concentrate prepared under the protein extraction conditions used in this study.

CONCLUSIONS

The protein concentrate prepared at pH 12 could be a potentially promising material as a food ingredient. Extraction and recovery methods applied to protein in this study were also comparatively mild and simple. Since the device for rupturing yeast cells used in this work was not a highly efficient one, the protein extraction yield might be further improved if a more efficient cell disintegrator, such as Dyno-Mill disintegrator (*10*), was used.

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Report of the Membership Committee, 1973-1974

In 1973 we finished our first full year of activity. That year we added 315 new members. As of August, 1974 we added 384 new members. In 1973, with the guidance and hard work of Harold Barnum, the Inter-Mountain affiliate in Denver was reorganized. It held its first annual meeting that year. This year it held another successful meeting. While attendance at the second meeting was not as large as the first, more new IAMFES members were obtained.

In November of 1973 I met with Earl Wright in Oklahoma City and Tom Autry and Dave Cleveland took us to Stillwater to the University. We thought we would have a little time after the Dairy Technology Society meeting. We found they had two speakers scheduled, and we were both of them. I was not entirely happy about the results. We had an excellent meeting, it was well attended, there was lots of discussion and promises that they would organize an affiliate, elect officers, adopt a constitution and get the information to us. As of August, 1974 this has not been done. I do have a recent letter from Dave Cleveland telling me not to get discouraged. They had a few obstacles in the way which are now cleared up and we can look forward to an Oklahoma affiliate soon.

In January 1974 Walt Wilson, Earl Wright, and I met with the Dairy Industry Conference in Scottsdale, Arizona and at that meeting we did get an organization, election of officers, and they are now an active affiliate. We had hoped to make more progress in getting new members, but they expect to do better next year.

We also have a new affiliate in Canada, known as the Ontario Milk, Food, and Environmental Sanitarians Association, Eastern Division. They have filed an application, adopted a constitution, and elected officers. I am not sure at this time just how many members they have, but they will continue to get more. In addition to that, we have a very good opportunity to have another affiliate in Edmonton, Canada. I have had considerable corresponcence from there and they say they are having a meeting in February, 1975, which is a bad time for anyone to go to Edmonton, but they have promised that if some of us will attend they will guarantee a captive audience of at least 100, and out of that they are sure we can organize a substantial affiliate. I can assure you some one will be there.

I am a little disappointed about Mexico. I had made arrangements with Dr. Alberto Encinas to meet with Mr. Wilson, Mr. Wright, and myself in Scottsdale to discuss ways and means of organizing an affiliate in Mexico. I got there a day early to meet with him and he did not arrive. It was not until July, 1974 that I again heard from Dr. Encinas. This time he was very enthusiastic about organizing an affiliate. He is very capable of heading up an affiliate in Mexico. He at one time was Chief Health officer for the Republic. I have made many trips to Mexico and breaking appointments is an every day occurence. We do not want to get too discouraged.

So, in summary we have revived two inactive affiliates, obtained one new affiliate, and we are negotiating for two others. In 1974 we have a membership committee of 30, which is a gain of 7.

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Milk Quality in the New York Public School System

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ABSTRACT

A direct correlation is shown between the flavor and consumption of milk by school age children. Surveys at 693 schools in New York State show a high incidence of off-flavored milk, together with excessively high storage and serving temperatures in the schools. Complaints by school lunch managers and children should become cause for concern by milk producers, processors, and distributors. Research has also uncovered some basic problems in school milk sales—lack of information on the part of cafeteria workers, poor quality control by dairies, and questionable design of refrigeration equipment in the schools. In New York State, when school is in session, over 10% of the Class I milk sales are made to schools.

More than 5.5 billion half pints of milk are served to some 43 million U.S. school children each year for student consumption. During the school year this amounts to over 10% of the total Class I or fluid sales. On the national average this is only 0.7 of a half pint per pupil per day and in New York State just over 0.8 of a half pint. Under Federal lunch and milk programs, subsidized milk is provided either free or at a cost of less than five cents a carton. Under these economic conditions, children should be consuming milk at the rate of at least one half pint per pupil daily. Across the nation, if each child drank one half pint per day, it would mean a potential sales gain of 2.2 billion half pints of milk a year.

Better consumption is not achieved because many children say they dislike milk, especially at school. Some ask their parents not to buy the same brand of milk served at school. Nutritionists are concerned that negative attitudes established in early life become the eating patterns for the rest of life.

Industry leaders in New York State sensed this situation and provided a grant of $48,900 to the Department of Food Science at Cornell for study of the "Acceptability of School Milk." Funded by the New York State Milk Promotion Order, the study was to determine causes of student complaints and pinpoint problems that were limiting 100% acceptance of school milk. The project was started in February 1973.

MATERIALS AND METHODS

Since quality, temperature, and flavor of milk served in schools has never been studied or evaluated, the first activity was to measure these factors. This was done by 693 on-site visits to a random selection of the 5,500 school buildings in 734 New York State school districts. Before each visit, permission was secured from the school.

On-site visits involved completing a standard form showing milk temperatures, storage facility temperatures, serving temperatures, brand of milk and code date, and included the purchase of a sample for flavor evaulation. A short interview with the cafeteria manager was required to complete all of the information listed in the form (Fig. 1).

SCHOOL MILK

Date ______ Time ______ Cafeteria Manager ______

School ______ Address ______

Cold Storage: Walk in ( ) Refrigerator ( ) Chest ( ) Lowerator ( )
Temperatures, Ambient ______ Indicated ______ Unit Air (Walk-in) ______
Milk (overnight) ______/______ Milk (Fresh) ______/______

Product: Brand ______ Plant # ______ City ______
Code ______ Flavor Score ______ Defect ______
Code ______ Flavor Score ______ Defect ______
Code ______ Flavor Score ______ Defect ______

Service Area: Same Unit as Cold Storage Unit? Yes ( ) No ( )
Unit type ______ No. of Units ______
Other Unit type ______ No. of Units ______
(1) Unit Product Temp ______/______ Unit Air Temp. ______
(2) Unit Product Temp ______/______ Unit Air Temp. ______
(3) Unit Product Temp ______/______ Unit Air Temp. ______
Unrefrigerated Product Temp ______/______
Time units stocked ______ Time returned to storage ______

Delivery: Daily ( ) Mon ( ) Tues ( ) Wed ( ) Thurs ( ) Fri ( )
Delivery Time ______ Delivered to ______
Placed in cold unit by ______ Time ______
Delivery truck, refrigerated ( ) unrefrigerated ( ) don't know ( )

General: (1) Total students ______ Half Pints Daily ______
(2) Average Daily Milk Holdover ______ How much milk wasted ______
(3) Do children complain, yes ( ) no ( ) What do they say? ______
(4) Have children requested skim, yes ( ) no ( ) chocolate, yes ( ) no ( )
Why not available? ______
(5) Are there certain foods kids don't like milk with, yes, ( ) no ( ) What? ______
(6) Problems: Frozen ( ) Improper fill ( ) Flavor/odor ( ) Leaker ( )
Hard to open ( ) Dirty cartons ( )
(7) Type of container: Pure pac ( ) Sealright ( ) Am. Can ( ) Glass ( )
Other ( ) ______
(8) Cost to students, white ______ choc. ______ Cost to faculty, white ______ choc. ______
(9) Contract price, white ______ choc. ______ lo fat ______ skim ______
(10) Comments: ______

Name ______

Figure 1. *Standard form used to evaluate milk flavor and serving conditions in New York schools.*

Schools showing less than satisfactory results were revisited to confirm any serious deficiency.

School visits were made by the authors with the assistance of regional Agriculture and Markets Dairy Inspectors. Temperature checks were made with YSI Telethermometers[1] using surface and air probes. Ten YSI Model 43 Telethermometers were used. Flavor evaluations were made using the American Dairy Science scoring system as modified by the TriState Milk Flavor Program (*2*).

From February 1973 to June 1974, 3,069 half pint containers were checked for temperature and 1,626 pieces of refrigeration equipment were examined for proper operation. The authors evaluated 969 samples for flavor representing milk from 98 milk dealers.

RESULTS AND DISCUSSION

The intial interview with cafeteria managers yielded several complaints regarding milk served at their schools. The number and type of complaints are recorded in Table 1. Some cafeteria managers had more than one complaint; a few were completely satisfied and had none.

Leakers seemed to be the biggest complaint and were responsible for the unsightly and insanitary condition of refrigeration equipment. In many cases, milk cartons were inverted to facilitate the storage of more containers per milk case or lowerator basket. Certainly, many of the leakers we saw were due to poor quality control on the filling operation and were leaking from the bottom as well as the top. Frequently, it seemed that when containers were not "leakers" they were charred and hard to open. This, of course, is a major problem for elementary grade students.

TABLE 1. *Complaints from cafeteria managers on milk served in schools, February 1973 to June 1974, 693 schools*

Complaint	Number
Leakers	361
Improper fill	134
Charred/hard to open	104
Frozen milk	105
Off-flavor	102
Dirty cartons[a]	38
Dirty cases [a]	14

[a]Totals for 1974 only

"Improper fill" was partly due to the leaker problem and perhaps also to short fill. On questioning, managers had no real idea of how short the cartons were, if in fact they were underfilled. The shape of the "pure pac" type containers makes this difficult to determine without actual weighing.

Frozen milk was largely a self imposed defect. Some equipment, adjusted to maintain temperatures during the week, would freeze milk on weekends and holidays. In some instances, freezing occurred during delivery when milk was left on loading docks during cold weather.

One hundred two complaints of off-flavor were recorded. Most indicated that the milk was sour, but our evaluations showed less than 1% with that defect. It has also been our previous experience that when lay people complain of an off-flavor they cite the word "sour" for any and all off-flavors. A review of the flavor problem appears later in this report.

Dirty cartons and cases presented another sanitation problem for the schools. Dirty milk cases are a big complaint since female cafeteria employees must carry them against their uniforms to lift the 40- or 50-lb. weight. The lady with that duty often has a soiled outfit.

Most visits were made to coincide with lunch period to determine milk serving temperature. The authors selected 45 F (7.2 C) as the maximum acceptable temperature in reporting data. Table 2 shows that over a third of the milk was 45 F (7.2 C) or higher at serving time. In most instances, children took this milk directly from a refrigerated service device.

TABLE 2. *Temperature of milk on serving lines, 693 schools, February 1973 to June 1974*

	44 F or lower	45-49 F	50-59 F	59 F or higher	45 F and above
No. of serving lines	444	185	83	7	275
% Total serving lines	61.7	25.7	11.6	1.0	38.3

The type of refrigeration equipment did not seem to make a significant difference in serving temperature. Except for walk-in coolers (which are not a serving facility) all major types of equipment showed a 24 to 29% incidence of milk above 45 F (7.2 C) (Table 3). The lowerator, a cabinet that can hold several cases of milk which rise as the milk is removed from the top, has the best potential for maintaining temperatures. In most instances, the excessive temperatures were caused by overloading or improper adjustment of the thermostat.

TABLE 3. *Milk temperatures in different types of refrigeration equipment*

Type of unit	Total	40 F or lower	41 F to 44 F	45 F to 49 F	50 F or higher	Percent 45 F and above
Lowerator	307	122	111	48	26	24.1
Cold chest	211	98	55	35	23	27.5
Refrigerator	129	50	41	24	14	29.5
Walk-in	164	97	48	15	4	11.6

Where milk was placed on the serving line without benefit of cooling, the percentage of samples over 45 F (7.2 C) were in excess of 57% (Table 4). Fortunately, these instances were few and generally limited to an extra a la carte line in the cafeteria.

TABLE 4. *Temperature of milk served without benefit of refrigeration*

Total studied	44 F or lower	45-49 F	50 F or higher	Percent 45 F and above
54	23	18	13	57.4

Some excessive temperature problems are due to condition of the milk on delivery (Table 5). Inconclusive data indicate that almost 50% of the milk is coming into the schools at 45 F (7.2 C) or above. This is partly because half pint containers warm some 5 to 10 F on filling and sealing. These never completely cool in the cold storage room. (This research area is being investigated in more detail.)

TABLE 5. *Milk temperature at time of delivery*

Total studied	44 F or lower	45-49 F	50 F or higher	Percent 45 F and above
64	33	16	15	48.4

The significance of temperature on consumption is not as great as the authors had imagined. In most school districts there was only a 2% drop in consumption when the temperatures were above 45 F (7.2 C). This was somewhat more dramatic when the flavor was bad.

While the milk temperature in schools is an important consideration, the flavor was far more significant in relation to consumer acceptance. This was measured by comparing average daily attendance with average daily purchases of milk (Table 6). Where milk tasted good (38 to 40 score) the average consumption was 80 to 100%, averaging 90.4%. Where the flavor score was 37 or less, the average consumption was 60 to 70%, averaging 66%. In the Metropolitan area, where milk has a long history of rancid flavors, the average daily consumption was 57.5%, lowest in the state. This spread between good and poor tasting milk clearly shows that flavor is a major factor in the acceptance of white milk in schools.

TABLE 6. *Milk consumption in relation to flavor score, 693 schools, February 1973 to June 1974*

Flavor score	Percent of students drinking milk
38-40 (All schools)	90.4
37 or lower (Upstate)	66.0
37 or lower (Metro. N.Y.C.)	57.5

These data are confirmed when sales of chocolate milk are related to flavor quality of plain milk. Chocolate milk is available in some schools where it generally sells for an extra one cent per container. The authors found: (*a*) regardless of price, chocolate milk consumption is always equal to or greater than white milk; (*b*) when there is no price difference, about two chocolate are consumed for each white milk; and (*c*) where the white milk flavor is poor—preference for chocolate milk was no less than 3 to 1 and as high as 10 to 1. It is obvious that school children are making a natural selection when faced with off-flavored milk.

TABLE 7. *Flavor scores of 969 samples of milk served in 693 schools, February 1973 to June 1974*

Score	Category	No. of samples	% of Total samples
36	Poor	148	15.3
37	Fair	167	17.2
38	Fair	291	30.0
39	Good	305	31.5
40	Excellent	58	6.0

The flavor analysis of school milk is shown in Table 7. These figures are in line with survey results reported by Bandler and Barnard for the years 1970 to 1973 (*3*). The breakdown as shown in Table 8, sheds some light on the specific quality problems. Virtually all off-flavors were present in milk before it left the milk plant.

By far the most widespread objection is the cowy/barny/unclean off-flavor caused primarily by poor barn ventilation, dirty stables, and unclean conditions on the farm. This flavor is most easily detected because of the air space in the half pint container and perhaps the relatively warm temperatures. Children often drink right from the the container and put their noses directly into that headspace.

TABLE 8. *Flavor Analysis of 969 samples of New York state public school milk, February 1973 to June 1974*

Off-flavor	No. of samples	% of total
None	183	18.9
Flat	17	1.8
Cooked	28	2.9
Feed	142	14.7
Oxidized	20	2.1
Rancid	114	11.7
Salty	1	0.1
Cowy-barny/unclean	451	46.5
Malty-sour	10	1.0
Others	3	0.3
Total	969	100.0

Some of the unclean flavors resulted from low intensities of psychrotrophic bacterial spoilage and hydrolytic rancidity. Suspected samples are confirmed by direct microscopic examination and/or acid degree value test. However, the true cause of the unclean flavor is best determined by inspection of the farm supply, plant processing procedures, or the distribution system.

On the other hand, oxidized flavors were considerably less frequent in school milk. This results largely because of quick turnover of the milk. Very little milk is held more than 24 h. Consequently, both oxidized flavor and bacterial spoilage flavors were well under levels found in milk flavor surveys in other commercial channels. Feed, rancid, cooked, and "no criticism" were generally in line with previous findings.

As a matter of general interest, there appeared to be a trend in milk drinking habits of school children by age. In upstate areas where milk tended to be better, the average daily purchases of milk were fairly constant for all age groups (Table 9). In the Metropolitan New York City area, it appears that off-flavors are dramatically discouraging milk consumption in older children who have more opportunity to express a choice.

TABLE 9. *Percent of students drinking milk by age group in upstate and metropolitan New York city schools, February 1973 to June 1974*

Age group	Upstate	Metropolitan N.Y. City
Elementary schools	82.8	70.6
Jr. High schools	76.1	45.6
High schools	78.5	42.0

CONCLUSIONS

It is clear that milk going into schools is of about the same quality as that of the general supply. Even though it is available to students at a bargain price (the equivalentof 20 cents per quart) it is not even being consumed at the rate of one half-pint per pupil per day.

Generally, the problem is not being caused by schools.

Although many temperatures were higher than ideal, no significant decrease in consumption could be measured on the basis of temperature alone.

The major conclusion drawn is that the dairy industry is not exercising the quality assurance it should. Some containers and cases are dirty. Many continers leak or are hard to open. There may be a problem with proper fill, and milk is not being delivered at a cold enough temperature. Of greatest concern is the fact that the flavor is not consistently 39 score or better.

The flavor problem will only be corrected when a complete program of flavor control is instituted. Most plants continue to receive milk from producers without tasting each bulk tank load. Certainly half the flavor problems originate at the farm. Without a regular check on what is being received, there is little hope in changing the flavor picture.

School milk is at least 10% of the milk business. A case could be developed to say that it is even more important than that. Lifetime eating habits are established during the school years. Someone who doesn't like milk then is not likely to be a milk drinker as an adult.

Most dealers surveyed indicated that school milk was an important part of their business. However, this study showed that the type of quality control necessary to build sales and good will was lacking. There were exceptions, of course, but they are too few if school milk is, indeed, "the most important 10% of the milk business."

ACKNOWLEDGMENT

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Enrichment Serology and Fluorescent Antibody Procedures to Detect Salmonellae in Foods

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ABSTRACT

Methods outlined in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (BAM) and the Association of Official Analytical Chemists (A.O.A.C.) are accepted as standard procedures to which most accelerated procedures are compared. The enrichment serology (ES) and the fluorescent antibody (FA) procedures are reviewed as rapid procedures applicable to detection of *Salmonella* in animal feeds, condiments, foods, food by-products, and pharmaceuticals. Methodologies of the conventional cultural and accelerated procedures are presented along with evaluations of each procedure with known *Salmonella* serotypes.

The cultural method presently being used to detect *Salmonella* in foods is outlined in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (*18*). This method and the method outlined by the Association of Official Analytical Chemists (*10*) are accepted as the standard procedure to which accelerated procedures are compared.

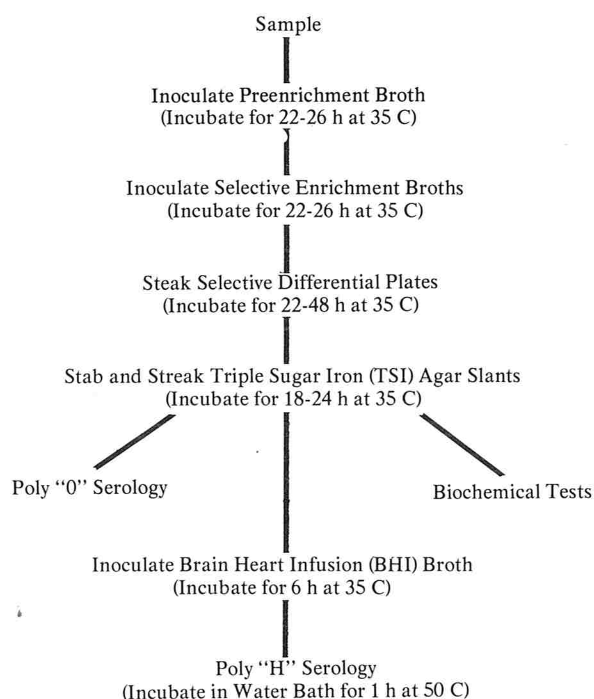

Figure 1. *Bacteriological Analytical Manual (BAM) Cultural methodology for* Salmonella detection.

Figure 1 illustrates the conventional cultural scheme for *Salmonella* detection. Samples can be preenriched in either Lactose Broth, Nutrient Broth, skim milk, or distilled water supplemented with 0.002% brilliant green dye. The choice of the preenrichment medium depends on controlling the pH value of the preenrichment culture between 4.5 and 9.0. If the pH value drifts outside of these limits, salmonellae will not be recovered. Samples to undergo preenrichment are incubated at 35 C for 22 to 26 h, and then transferred into selective enrichment broths of selenite cystine and tetrathionate media. Inoculated selective enrichment broths are incubated at 35 C for 22 to 26 h. Differential selective plates of brilliant green (BG), aged bismuth sulfite (BS), and *Samonella Shigella* (SS) agars are then streaked from each selective enrichment culture and incubated at 35 C. After 24 and 48 h plates are examined for typical salmonellae colonies. Typical colonies are picked at each examination and both stab-inoculated and streaked onto Triple Sugar Iron (TSI) and Lysine Iron Agar (LIA) slants. After incubation at 35 C for 18 to 24 h, biochemical as well as serological tests are done on cultures which have exhibited typical *Salmonella* responses.

Two rapid test procedures are considered in this presentation. These are the enrichment serology (ES) and the fluorescent antibody (FA) procedures.

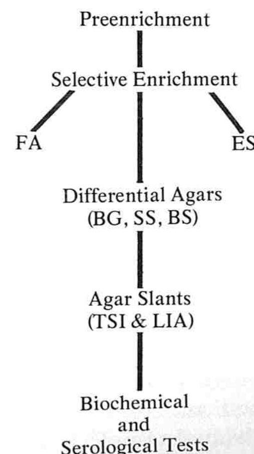

Figure 2. *Analytical scheme for the Fluorescent Antibody (FA), Enrichment Serology (ES), and the Cultural Procedure for the detection of* Salmonella.

Figure 2 illustrates the analytical scheme for the conventional cultural, enrichment serology, and fluorescent antibody procedures. It can be seen in this analytical scheme that the cultural procedure can be branched after the selective enrichment step into the FA and ES procedures. The media utilized for the preenrichment, and selective enrichment steps for the FA and ES procedures are identical to those utilized in the cultural procedure.

ENRICHMENT SEROLOGY

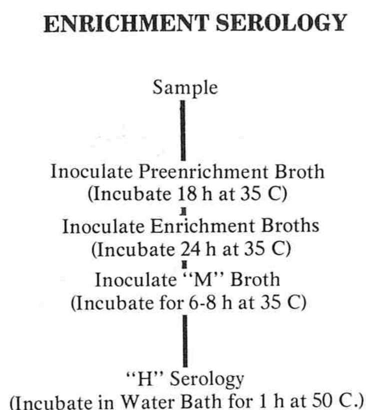

Figure 3. *Enrichment Serology Methodology for* Salmonella *detection.*

Figure 3 illustrates the ES procedure described by Sperber and Deibel in 1969 (*22*). For ES methodology, samples are preenriched for 18 to 24 h at 35 C. Following this incubation period, transfers are made into selective enrichment broths and incubated for 24 h. After this period has elapsed, 0.05-ml aliquots of each selective enrichment culture are inoculated into M broth (Difco). Specific and non-specific serological tests can then be done after 6 to 8 h of incubation. The pooled poly "H" antisera utilized in the serological portion of the ES procedure is composed of the Spicer-Edwards antisera set, Poly D, Poly F, and the Z_6 complex.

A typical positive ES response can be denoted by a dust-ball like agglutination or flocculation. This agglutination should not occur in a non-specific serological control.

Two studies comparing the cultural procedure and the ES procedure for detection of salmonellae have been published in 1969 by Sperber and Deibel (*22*), and Fantasia et al. (*2*). An excellent correlation was obtained between the cultural and ES procedures. Sperber and Deibel's study involved 105 samples representing 44 different materials. Samples included animal feeds, condiments, environmental samples from a food plant, and food products. Of the 105 samples analysed for the presence of salmonellae, 37 positives and 68 negatives were obtained by each procedure. Results obtained by the cultural method and the ES procedure were the same for each individual sample.

An additional comparison involving 689 samples of animal feeds, foods, and pharmaceutical products was conducted in 1969 by Fantasia et al. (*2*). In that study, 132 samples were salmonellae-positive, and 557 samples were negative. Two samples analysed by the ES procedure revealed opposite responses when compared to results obtained by the cultural method. One sample of fish meal revealed a negative ES detection which was culturally positive. That constituted a false-negative. One egg noodle sample in this study exhibited a positive ES reponse but a negative cultural result. That constituted a false-positive.

In addition to the false-negative response reported by Fantasia and his coworkers (*2*), Sperber and Deibel (*22*) reported that four serotypes of *Salmonella* were not detectable by the suggested pooled "H" antisera. The undetectable serotypes reported were *Salmonella agona* (Group B), *Salmonella pullorum* (Group D), *Salmonella gallinarum* (Group D), and *Salmonella quinhon* (Group X). It also follows that since the ES procedure depends on production of flaggelar "H" antigens, non-motile salmonellae variants will not be detected.

In spite of these shortcomings, comparisons of the ES and cultural procedures were encouraging. The ES procedure has met opposition for use as a screening procedure for accelerated detection of salmonellae. By definition, a screening procedure should reveal presumptive positives if salmonellae are present in a given sample with the same sensitivity and reliability as the cultural method. To meet this criterion, it is apparent that the sensitivity and reliability of the ES procedure had not been clearly established.

FLUORESCENT ANTIBODY PROCEDURE

The second accelerated method considered for the detection of salmonellae is the FA procedure which is shown in Fig. 4.

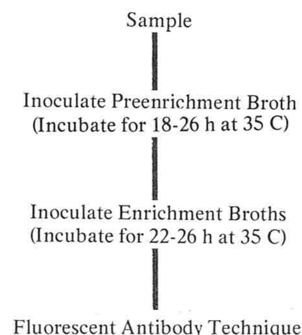

Figure 4. *Fluorescent antibody (FA) Methodology for* Salmonella *Detection*

The conventional FA methodology for detecting salmonellae is also similar to the cultural method. The same preenrichment broth and selective enrichment broths are employed. This technique branches from the cultural procedure after the selective enrichment step in a fashion similar to the ES procedure. For the FA technique, 0.001 ml of each 24-h old culture in selenite cystine and tetrathionate broths is placed on a clean microscope slide and allowed to air dry. Dried specimens

are then fixed by flooding the slide with a solution of ethyl alcohol-chloroform-formalin at concentrations of 110:60:30, respectively. After being fixed for 3 min smears are rinsed with 95% ethyl alcohol. The slide is allowed to air dry and then is stained.

The FA staining procedure is a matter of personal choice; a direct or indirect staining procedure can be employed. Indirect staining methods utilize the Spicer-Edwards pooled antisera diluted 1:500 and a counter stain of a fluorescein-conjugated 7S goat-anti-rabbit globulin as reported by Goepfert et al. in 1970 (*7*). The direct staining procedure employs a fluorescein-conjugated polyvalent anti-*Salmonella* globulin. It is prepared from motile organisms representative of somatic "O" groups A through S as reported by Insalata et al. (*12*); Markovits and Burboeck, (*16*); and Thomason and Wells (*23*). Slides are then washed two times in phosphate buffer, and finally with distilled water.

Smears which have been stained by either the direct or indirect method can be examined with a microscope equipped with an oil-immersion lens, dark-field condenser, and a mercury arc lamp as the light source. To attain the desired wave length of light at 435 nm, a BG 12 exciter filter is used along with heat-absorbing filters and barrier filters. An immuno-fluorescent microscope can also be used. The immuno-fluorescent microscope employs a 100-watt halogen light source in conjuction with a special fluorescein isothiocyanate interference filter.

A typical positive FA response is exhibited as an intense fluorescence, subjectively ascribed 3^+ to 4^+, based on a scale of 0 to 4^+. Weak fluorescent cells, that is 1^+ to 2^+ are considered negative for salmonellae by the FA procedure. An FA-positive response for salmonellae occurs when the cellular morphology is typical, that is a rod stained peripherally, with a 3^+ to 4^+ fluorescence.

The FA technique has been proposed by many investigators as a rapid, economical screening method to detect salmonellae. The application of this technique for examination of foods was first suggested by Arkhangel'ski and Kartoshova in 1962 (*1*). Two years later, Georgala and Boothroyd (*3*) employed the FA technique to examine raw meats for the presence of *Salmonella*. Their analysis indicated the potential of the FA method. Soon after the initial suggestion, the FA technique was found suitable for detecting *Salmonella* in egg products, by Silliker et al., 1966 (*21*); animal feeds and feed ingredients by Laramore and Moritz, 1969 (*15*); and other foods by Insalata et al. in 1967 (*13*).

Early investigations revealed that the FA procedure exhibited good correlations when compared with the cultural method. However, low incidences of FA-negatives, cultural-positives, and a relatively high incidence of FA-positives, cultural-negatives did occur. The FA-negative, cultural-positive results probably occurred because the FA *Salmonella* conjugate was incomplete. The high occurrence of FA-positives, cultural-negatives may have also been due to the conjugated antisera. Identical somatic "O" antigens can be found among several genera of the family *Enterobacteriaceae;* therefore, the preparation of a truly *Salmonella* specific conjugate is very unlikely as reported by Kampelmacher in 1959 (*14*). Investigators have also proposed that the FA technique be used as a screening procedure for detecting *Salmonella* organisms in meat, eggs, non-fat dry milk, and other foods. The respective investigators were Georgala and Boothroyd in 1965 (*4*); Silliker, et. al., 1966 (*21*); Reamer and Hargrove, 1972 (*19*); Reamer et al., 1969 (*20*); Harrington et al., 1970 (*8*); Insalata et. al., 1967 (*13*); Goepfert and Insalata, 1969 (*6*); and Insalata et al., 1972 (*12*).

A simplified fluorescent antibody system has also been reported by Markovits and Burboeck in 1971 (*16*). This system employs the immunofluorescent microscope previously described and a fluoro-kit manufactured by Clinical Sciences, Inc., Whippany, New Jersey. This system was also evaluated by Insalata, et al. in 1973 (*11*). No false-negatives occurred.

Presently, three reliable commercially prepared FA conjugates for *Salmonella* detection are available from either Difco, Clinical Sciences, Inc., or Sylvania. All three conjugated antisera covering somatic "O" groups A through S have been evaluated. These evaluations were conducted in 1973 and 1974 by Insalata et al. (*11*); Hilker and Solberg (*9*); Thomason and Hebert (*24*); and Mohr et al. (*17*). No false-negatives were experienced.

Comparison studies of both the ES and FA procedures have also been made using a cultural procedure as the reference standard on identical samples by Hilker and Solberg in 1973 (*9*), and Mohr et al. in 1974 (*17*). In both studies, the ES procedure exhibited a 2-3% incidence of false-negatives, i.e. ES-negative, cultural-positive. The conventional FA procedure, on the other hand, did not reveal any false-negatives. The FA procedure, then, appears to meet the criterion for screening food samples for detection of salmonellae.

Test responses to cultural, ES, and FA procedures with strains of *S. agona, S. quinhon, S. pullorum,* a non-motile *S. typhi,* and two strains of *S. gallinarum* are shown in Table 1. All of the test procedures detected *S. agona*. This result indicated that the suggested flagellar "H" antisera pool for the ES procedure does detect this organism, contrary to the publication of Sperber and Deibel in 1969 (*22*). *Salmonella agona* reacts with the "H" antisera of the "G" complex found in the Spicer-Edwards sera 1 and 3.

S. quinhon appeared positive in all of the test procedures except when the poly "H" antisera pool suggested for the ES procedure was allowed to react with growth material from BHI broth. The ES procedure would be expected to miss this strain because the specific flagellar antigen (Z_{44}) for the organism is not included in the original flagellar "H" antisera pool suggested for this procedure. Inspection of the serological results obtained on growth material from BHI and M broths indicates that the positive agglutination reaction was non-specific

TABLE 1. *Test reponses to the cultural, ES, and FA procedures challenged with known strains of* Salmonella

			salmonella Serotype					
			gallinarum (1)	*gallinarum* (2)	*agona*	*quinhon*	*pullorum*	*typhi*
ES	BHI broth	poly "H" antisera	–[a]	–	+[b]	–	–	–
		poly "H" antisera Z_{44}	–	–	+	+	–	–
	M broth	poly "H" antisera	–	–	+	+	–	–
		poly "H" antisera Z_{44}	–	–	+	+	–	–
FA	BHI	Difco's antisera	4+	4+	4+	4+	4+	4+
		CSI's antisera	4+	4+	4+	4+	4+	4+
	M	Difco's antisera	2+/3+	2+/3+	2+/3+	2+/3+	2+/3+	2+/3+
		CSI's antisera	2+/3+	2+/3+	2+/3+	2+/3+	2+/3+	2+/3+
BAM	BHI	BG	–	–	+	+	+	–
		SS	NG[c]	NG	+	+	+	+
		BS	NG	NG	+	+	+	+
		EMB	+	+	+	+	+	+
	M	BG	–	–	+	+	+	–
		SS	NG	NG	+	+	+	+
		BS	NG	NG	+	+	+	+
		EMB	+	+	+	+	+	+

[a]–: No typical response.
[b]+: Typical response.
[c]NG: No growth.

for cultural material grown in M broth and specific for growth material from BHI broth. This serotype was detectable using the modified antisera with Z_{44}, if it was grown in BHI broth. Apparently, the non-specific agglutination response that was experienced on growth material from the M broth culture may have been due to the formation of rough growth. This rough growth did not occur in BHI broth. Thus, use of a non-specific agglutination control in the ES procedure may yield false results for *S. quinhon* if it is used to negate a positive serological test.

The remaining serotypes of *Salmonella* were not detected using the suggested antisera pool for the ES procedure. *S. pullorum, S. gallinarum,* and a non-motile variant of *S. typhi* do not possess flagellar antigens. Therefore, negative responses to these serotypes would be expected when using the ES procedure.

The cultural procedure detected all of the known *Salmonella* serotypes. However, SS and BS agars were too toxic for *S. gallinarum,* and colony formation on BG agar was atypical. *S. gallinarum* formed green colored colonies on BG agar as opposed to typical fuchsia red colonies. Many investigators would have missed this serotype of *Salmonella,* unless EMB was employed as an additional medium to those already suggested for the selective differential plating step specified in the cultural procedure.

The FA procedure detected all of the known *Salmonella* serotypes using either Clinical Sciences's FA *Salmonella* conjugate or Difco's FA *Salmonella* poly antisera. *S. quinhon,* which is somatically identified as belonging to "O" Group X should have been missed by both Clinical Science's and Difco's FA antisera. These antisera consist of conjugated "O" groups A through S; however, due to crossing of related somatic antigens, the representative organism from Group X was detectable.

Also note that cells grown in BHI broth stained more intensely than those grown in M broth. This observation was in agreement with other experiences reported by

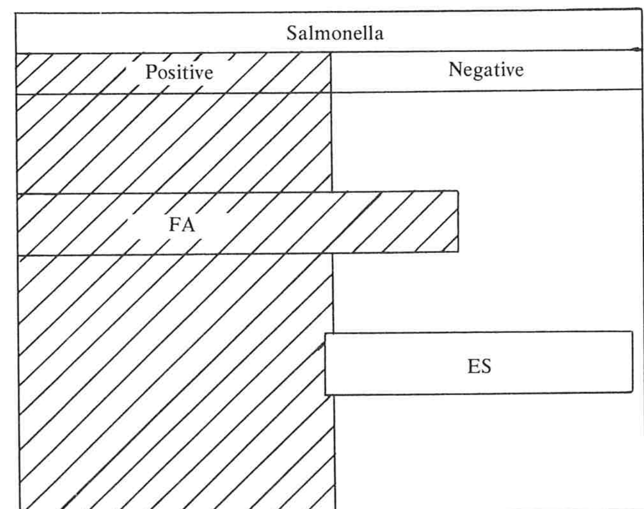

Figure 5. Salmonella *detection utilizing the FA and ES procedures—Zones of positive and negative responses utilizing the FA and ES procedures.*

Goepfert and Hicks in 1969 (5) FA staining of cell surfaces was found to be inhibited by mannose, which is present in M broth. Therefore, the FA procedure should not be utilized on bacterial growth material from M broth.

Figure 5 illustrates zones of positive and negative responses utilizing the FA and ES procedures. In summary, the FA technique is capable of detecting all of the *Salmonella*-positive samples, but also yields 5 to 27% incidence of presumptive positives (false-positives) in some samples even though *Salmonella* may not be present. The ES procedure is capable of detecting 97 to 99% of the *Salmonella*-positive samples and exhibits a 1 to 3% incidence of presumptive positives (false-positives) when *Salmonella* are not present.

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Treatment of Raw Milk Wastes by a Multi-Stage Biological System

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ABSTRACT

The Bristol, Virginia Division of Dairymen, Inc. is a storage and transfer operation for raw milk. As production increased and effluent quality regulations become more stringent, waste treatment progressed from land irrigation, to a two-stage biological system to the present four-stage biological system including: (*a*) full mixed anaerobic; (*b*) facultative-high rate trickling filter and non solids controlled aeration; (*c*) quiescent anaerobic; and (*d*) facultative-high rate trickling filter and activated sludge.

Treatment efficiencies were:

	Raw	*Treated*	*%Efficiency*
BOD_5	3920	73	98
COD	3300	40	99
TOC	1750	20	99
Total nitrogen	168	22	87
Total suspended solids	570	56	90
Oil and grease	1200	0	100

The Virginia Division of Dairymen, Inc. owns and operates a storage and transfer plant at Bristol, Virginia. Whole milk picked up at the producers is brought to the Bristol plant, stored temporarily, and subsequently shipped to customers again as whole milk.

Operation of the plant consists of two 8-h shifts from 7:00 a.m. to 11:00 p.m., 7 days a week. Production is approximately 800,000 lb. of milk per day.

All transportation of milk is accomplished by large stainless steel tank trucks. After each transaction, trucks are purged and cleaned at the Bristol plant. Waste from the cleaning operation is composed of whole milk, water, and sanitizing agents.

In the early days of the Dairymen, Inc. plant, wastes were disposed of by land irrigation in nearby fields. Increases in production, encroachment of population, and lack of suitable land for proper irrigation practices, precluded old methods and called for a new and improved waste treatment system.

THE ASSIGNMENT

The specific assignment by Dairymen, Inc. was to find a system so designed which would not only meet current regulations, but those anticipated within the next 10 years, or to be compatable with future requirements as specified by governmental regulations.

Existing governmental regulations emphasize BOD_5 and suspended solids. BOD_5 is mainly a measurement only of the soluble fraction of an organic waste. Milk is principally colloidal in nature and not immediately biodegradable by aerobic methods, since aerobic systems require that the food be in solution before it can be assimilated through bacterial cell walls. Future regulation can be expected to include the total oxidizable content of an effluent. This is measureable as TOD (total oxygen demand), UOD (ultimate oxygen demand), COD (chemical oxygen demand), or TOC (total organic carbon) which can be related to oxygen required for eventual oxidation.

Nitrogen is becoming recognized as the "limiting factor" or most critical nutrient in eutrophication of our surface waters. Emphasis is currently on the oxygen demand of ammonia with the requirement for conversion to nitrates. Both ammonia and nitrate can be considered nutrients with equal effects based upon nitrogen content.

TABLE 1. *Parameters and qualities used for establishing design criteria*

	Average	Maximum	Minimum
Flow, GPD	16,000	26,000	12,000
GPM	11	100	5
BOD_5, mg/l	4,700	8,000	2,000
COD, mg/l (Est.)	6,800	—	—
TOC, mg/l (Est.)	3,100	—	—
Total nitrogen, mg/l	170	—	—
Total suspended solids, mg/l	1,600	—	—
pH	6.8	8.8	2.0

Table 1 lists the raw waste parameters considered in developing design criteria for the new waste treatment plant. Although the prime criterion was removal of BOD_5, consideration of the slower oxidizable carbon fraction and nitrogen-based protein was given high priorities. Space limitations also called for a compact system.

All types of biological processes were thoroughly investigated with the following conclusions.

Activated sludge

This is one of the most common processes used in the milk industry and in other applications. Being short term and totally aerobic, it is highly effective only for

completely soluble organic wastes. A major portion of milk wastes are colloidal in nature and not immediately biodegradable. These solids, especially the fat fraction, cause serious bulking, high production of solids, and a general instability of operation.

Treatment of nitrogen-based organics is by cultivation of nitrifying bacteria which convert nitrogen compounds to nitrates. Removal of total nitrogen is accomplished by conversion to bacterial biomass and purging from the system. Conversion to free nitrogen is minimal or nil.

Anaerobic digesters

Enzymes are produced by anaerobic bacteria. As a result, the colloidal fraction of milk is hydrolyzed to lower molecular weight and soluble compounds. It is reported that the lactose fraction is broken down. Fats are hydrolyzed to biodegradable acids. Proteins are hydrolyzed to amino acids and ammonia and oxidized directly to free nitrogen.

Marked changes occur without actual purification or BOD_5 reduction. In fact, with very effective hydrolysis, BOD_5 can increase. Wastes are made more susceptible to biological oxidation.

Objections include; poor BOD_5 removal, odors, poor sludge settling characteristics, mixing problems, and required longer residence time.

Trickling filters

The facultative nature of the biomass is an asset in that hydrolysis is effected by the anaerobic portion immediately adjacent to the media. Reported activity in terms of BOD_5 is low, but as with anaerobic digestion, the colloidal or high molecular weight fraction is hydrolyzed to smaller and biodegradable compounds. With high hydrolyzing activity, BOD_5 can actually increase through a trickling filter.

With conventional stone media, plugging is a problem. Plugging is not a problem with plastic media having a completely vertical surface when used to treat high organic content wastes such as milk.

Aerated lagoons

Installation and operating costs are low. Highly trained operators are not required. Solids losses are high. Efficiencies are normally low. Odors are a problem unless the lagoon is fully mixed to prevent formation of sludge beds beyond the zone of mixing influence. Large land areas are high in cost.

In consideration of the four basic types of biological treatment, an aerated lagoon was precluded if for no other reason than land area requirements and related cost.

Activated sludge was required to achieve the low BOD_5 levels required, but hydrolysis of the milk colloids before use of activated sludge was considered paramount. Conversion of milk proteins to free nitrogen before activated sludge treatment was also stated as a requirement.

Some type of anaerobic treatment before the activated sludge stage was necessary for hydrolysis of milk colloids and conversion of proteins to free nitrogen. Reaction time was a major factor to be considered in treating the total volume.

The facultative feature of a trickling filter was considered to be a very desired asset for this system, effecting both hydrolysis, aerobic oxidation, and conversion of proteins directly to free nitrogen.

THE PROCESS

In the late 1950's, two Mead Corporation engineers, evaluating various types of biological treatment for difficult and highly concentrated wastes, developed a multi-staged biological approach (system) for which they were awarded to patent (*1*) that covered the mechanical process. This system is currently being marketed by the Aquatair Corporation, a subsidiary of the Mead Corporation of Dayton, Ohio.

The Aquatair flow pattern had the desired biological processes, but with hydrolysis of colloids and the low final BOD_5 requirements, it was felt that this could not be achieved with a single system. Rather than requiring 90% BOD_5 removal efficiencies, the requirement was 90% plus 90%, or 99%, if a total BOD of about 9000 mg/l is assumed.

The decision was made to place two Aquatair systems in series with some modifications to the basic flow pattern. Management of Dairymen, Inc. concurred with this approach. The Virginia Water Control Board gave a conditional approval to the design concept classifying it as experimental.

A description of individual process components follow.

Grit chamber and screens

In the cleaning of the tank trucks, dirt, gravel, and other external road contaminants enter the process sewer system. The grit chamber was installed to prevent these materials from entering the biological system.

Two-stage screens were installed to prevent rags, papers, plastic, and other non-bio-degradable debris from entering the biological system.

First stage anaerobic

Milk wastes are characterized by wide ranges of:

Flow. Although average flow is 16,000 GPD or 11 GPM, instantaneous flow rates can vary from 5 to 100 GPM. These variations are due to the batch type operations of cleaning tank trucks, transfer systems, and storage tanks. Flow rates vary widely during periods within a day and from day to day.

Organic loading. Milk content varies widely from the start of a wash through to the final rinse, from a measurable milk content through to clean water.

Sanitizing agents and detergents. Cleanliness is essential in a milk transfer and storage system. The primary sterilizing compounds are chlorine based, and the pH can vary over wide ranges from acid to alkaline.

These compounds are utilized to inhibit biological activity in the processing system and if allowed to enter

the biological system and pass through in the concentrations present in the process sewer, biological growth will be inhibited in the treatment plant.

The first stage anaerobic is agitated mechanically and is sized to attain an effective 1-day retention time. The last 4 ft of the anaerobic digester are baffled to create a quiescent zone for solids separation. Purposes for this unit process are to: (*a*) level out or equalize organic variations; (*b*) hydrolyze colloids, fats, and suspended solids fraction of milk; (*c*) dilute the sanitizing chemicals to a dilute and non toxic concentration, by full mixing; (*d*) hold coagulated and suspended solids in the digester to hydrolyze by the use of the quiescent zone before discharge; (*e*) effect a degree of anaerobic biological oxidation; and (*f*) hydrolyze biological biomass from the last stage aerobic system.

First stage facultative

This unit process is a combination non-solids controlled aerobic and a facultative high rate, high capacity trickling filter. Feed rate is high to prevent any possible plugging of the vertical surface plastic trickling filter media which could occur with low flow rates and a resultant thick build up of filter slime.

Purposes of this unit process are to: (*a*) hydrolyze colloids, fats, and suspended solids fraction of the milk which are carried through from the first stage anaerobic unit process in the facultative trickling filter; (*b*) effect a degree of anaerobic and aerobic biological oxidation in the trickling filter tower; and (*c*) effect a degree of aerobic biological oxidation in the diffused air aerated recirculation chamber. Air is provided by air injectors driven by diverting a portion of the recirculating pump flow from flow to the trickling filter. No attempt is made to control or clarify solids produced in the system.

Second stage anaerobic

The second stage anaerobic unit process is a duplicate of the first stage system. Purposes are to: (*a*) hydrolyze colloids, fats, and suspended solids fraction of the milk which have not been hydrolyzed in the preceding stages; (*b*) hydrolyze bacterial biomass from the preceding facultative stage; (*c*) hold coagulated and suspended solids in the digester by use of the quiescent zone before discharge; and (*d*) effect a degree of anaerobic biological oxidation.

Second stage facultative

This unit process is a combination solids controlled aerobic and a facultative high rate, high capacity trickling filter.

Purpose of this unit process are to: (*a*) hydrolyze any potential residual colloids, fats, and suspended solids fraction of the milk which are carried through from the second stage anaerobic digester in the facultative trickling filter; (*b*) effect a degree of hydrolysis of the biological biomass in the trickling filter which is produced in the solids controlled aerobic biological system; (*c*) aerobically biologically oxidize by use of the activated sludge process residual soluble organics from the preceding unit processes; and (*d*) effect an anaerobic and aerobic biological oxidation in the trickling filter tower.

Clarifier

This device is a conventional hopper bottom gravity and non-mechanical sludge removal clarifier. One submersible pump is used for sludge removal. A second is used for scum removal. Effluent is removed around the periphery of the clarifier. A scum baffle is provided to prevent discharge of floating solids.

The basic purpose of this unit process is to separate solids from the treated effluent.

The sludge pump operates on a timed cycle and normally recycles sludge back to the second stage facultative process. Solids concentration in the activated sludge process are controlled by manually operating the sludge pump to dischage either to the first anaerobic stage or to the sludge storage tank. The scum pump is controlled manually with discharge to either the first stage anaerobic or the sludge storage tank.

Chlorination

Gas chlorination is provided to control the bacterial population in the effluent.

Sludge storage tank

System design is to maximize hydrolysis and biological oxidation of milk solids and bacterial biomass produced in the biological system. Some refractory organic and inorganic solids will build up in the total system. These solids are purged from the system by diversion of solids from the secondary clarifier to the sludge storage tank.

OPERATION

As with most new systems or concepts, a debugging process became necessary. The screening system has proven less than satisfactory. Cigarette filters, plastic tables from sample bags, and other debris pass through the screens and have been responsible for plugging feed nozzles to the trickling filters and air aspirators. A different type of screening system is being considered.

The recirculation pumps on the second facultative stage were of insufficient capacity to supply flow to the trickling filter and air requirements for the activated sludge system. The air aspirator nozzle openings were too small and therefore easily plugged by debris. Pumps and air aspirators were increased in size.

Openings of the trickling filter feed nozzles were too small. They were easily plugged and velocity from the nozzles were too great to allow even distribution to the filter. Larger nozzles were added.

Overflow length of the secondary clarifier was too short, resulting in relatively poor solids separation. This was increased in length.

Hydrolysis of colloids, fats, and suspended solids has not been as total as desired. Initial design criteria were to attain complete hydrolysis before the second facultative stage. Refractive organic solids would be purged from the system from the secondary clarifier. With incomplete

hydrolysis, solids carry through to and affect operation of the activated sludge system.

For this system plans are to feed a broad spectrum enzyme into the system to improve the degree of hydrolysis. Experience with this system has dictated another approach to attain the degree of hydrolysis necessary to attain maximum levels of treatment. Biological hydrolysis is more effective under anaerobic conditions; in fact, anaerobic conditions are required with the concentrations experienced. Biological hydrolysis is a relatively slow reaction.

Installation of two parallel second stage anaerobic systems will meet the required criteria. One will be operated as a quiescent digester with effluent from the first facultative stage passing through and solids allowed to separate from the liquid. When solids build up to a predetermined level, flow will be diverted to the second parallel anaerobic system. The first will then be agitated and given time to effect the necessary hydrolysis. Operation of these systems will be rotated. Refractory solids will be purged from one of these rather than an external sludge holding tank. The flow pattern is illustrated in Figure 1.

TABLE 2. *Summary of treatment efficiencies*

Parameter	Raw	Treated	% Efficiency
BOD_5	3920	73	98
COD	3300	40	99
TOC	1750	20	99
Nitrogen			
Organic	162.0	17.5	
Ammonia	5.3	4.6	
Nitrite	0.5	0.1	
Nitrate	0.0	0.0	
Total	167.8	22.2	87
Oil and grease	1200	0.0	100
pH	6.6	7.5	
Total suspended solids	572	56	90

Table 2 summarizes efficiencies of the system over a 3-week period, from 19 June through 7 July 1974, with the second anaerobic stage operating without agitation. Individual test results are tabulated in Table 3. Testing

TABLE 3. *Tabulation of individual test results*

Date	Flow GPD	BOD_5 (mg/l) Raw	BOD_5 (mg/l) Treated	TSS (mg/l) Raw	TSS (mg/l) Treated	pH Raw	pH Treated
June 19, '74	15,400	3750	75	772	32	5.0	8.0
20	10,800	5600	25	624	32	6.8	8.3
21	15,400	5750	80	748	40	5.3	8.3
22	16,400	2000	100	720	63	6.8	6.8
23	10,100	2450	98	472	60	7.4	6.8
24	15,800	3100	96	472	52	6.9	7.6
25	19,500	—	88	—	40	—	7.9
26	14,500	3650	95	464	52	6.8	7.7
27	12,300	3450	76	424	70	6.9	7.9
28	13,700	4260	65	475	80	6.8	8.1
29	10,400	3780	74	460	72	7.1	8.0
30	11,300	5150	97	455	65	6.7	7.8
July 1, '74	16,800	4630	62	560	73	6.9	7.5
2	17,100	4100	58	675	70	6.8	7.6
3	15,500	3860	65	580	80	6.4	7.4
4	26,600	4160	57	695	59	6.0	7.5
5	13,200	4000	56	715	55	6.3	7.7
6	12,000	3650	75	690	42	6.3	7.6
7	15,400	3225	42	680	28	6.5	7.5

emphasis was on BOD_5, suspended solids, and pH. Correlation cannot be made directly on TOC, COD, nitrogen, protein, and milkfat with BOD_5 since all samples were not comparable. Data in Table 4 are results of tests made on equivalent samples.

TABLE 4. *Test data, 8 July 1974*

Parameter	Raw (mg/l)	Treated (mg/l)
BOD_5	2676	20
COD	3558	24
TOC	1888	5
Nitrogen		
Organic	118.7	8.7
Ammonia	5.3	3.8
Nitrite	0.3	0.2
Ntrate	0.0	0.0
Total	124.3	12.7
Oil and grease	1200	0.0

During the evaluation period, treated effluent was not chlorinated to prevent the effect of chlorination from clouding overall efficiency of the biological system. Some BOD_5 tests were also made on non-filtered and filtered treated effluent samples. BOD_5 contribution of

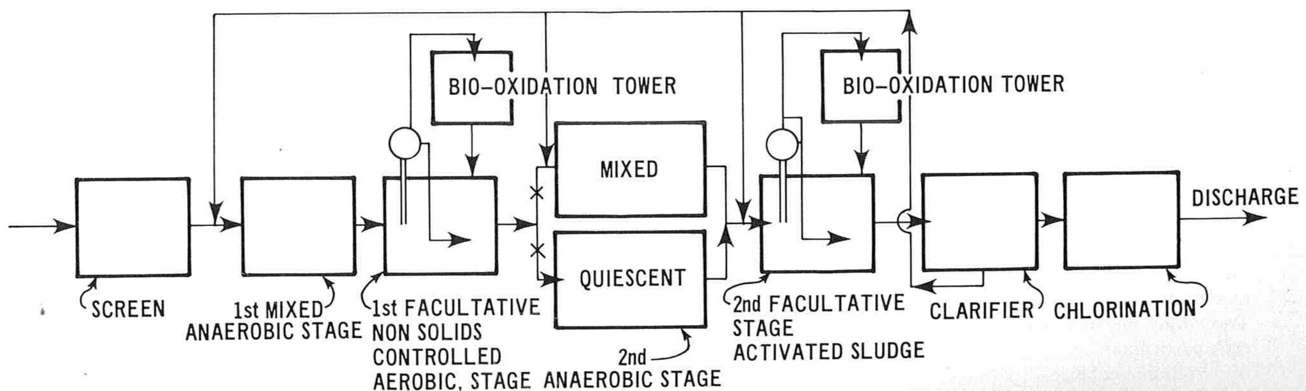

Figure 1. *Aquatair multi-staged biological treatment system for high concentrated organic wastes*

suspended solids was approximately one-half part per part of suspended solids.

Based on observations made during the 3-week, testing period it can be concluded that: (*a*) a multi-stage biolgical system is an effective method for achieving high degree of treatment for high concentration organic wastes; (*b*) emphasis on hydrolysis of high molecular weight organics is an effective method for treating total oxidizable organics; (*c*) hydrolysis of organic nitrogen compounds or proteins to amino acids and oxidizing anaerobically to free nitrogen is an effective method of nitrogen removal from waste effluents; and (*d*) chlorination and filtration is indicated to reduce this quality effluent to a refractory and stabilized level.

ACKNOWLEDGMENT

Presented at the 61st Annual Meeting of the International Association of Milk, Food, and Environmental Sanitarians, St. Petersburg, Florida, August 13, 1974.

REFERENCE

1. Sullins, J. K., and R. W. Self. Process and plant for treating sewage. U.S. Pat. No. 3, 261, 779.

The Sanitarians Joint-Council[a]

I. *A Joint-Council created*

There is hereby created an organization known as the Sanitarians' Joint Council which hereafter shall be referred to as the Council.

The Council as presently organized shall consist of representatives from the following organizations who shall be designated as participants:

Engineering and Sanitation Section of the American Public Health Association.

International Association of Milk, Food and Environmental Sanitarians, Inc.[b]

National Environmental Health Association[b]

National Society of Professional Sanitarians[b]

II. *Objectives and purposes*

The objectives and purposes of the Sanitarians' Joint Council shall be to consider ways and means of solving important problems of mutual interest to sanitarians' organizations which need unified action and which may be brought to the Council by any member.

Objectives and purposes of the Council shall also include:

A. Develop uniform definition for sanitarian.

B. Promotion of the professional status of the sanitarian.

C. Development of a sanitarian's specialty board.

D. Development of a recommended uniform law for registration of professional sanitarians.

E. Development and promotion of educational and other qualifications for sanitarians.

III. *Membership*

The Council shall consist of eight voting members and four alternate members.[b]

Two voting members and one alternate shall be appointed by each participant.

A. Voting members

Each organization shall appoint from its membership one voting member to serve on the Council for one year and one voting member to serve two years. Each year thereafter there shall be appointed a Voting Member to serve for a period of three years.

B. Alternate members

Each organization shall appoint an alternate member to serve for a period of three years.

IV. *The officers*

The Officers of the Council shall be the Chairman and the Secretary, elected annually from the Voting Members. The Chairmanship shall be rotated among the organizations represented on the Council.

The Chairman and the Secretary shall be from different organizations represented on the Council.

V. *Voting procedures*

In conducting the business of the Council, the voting member of each organization represented on the Council shall vote as a unit except in the case of the election of Council Officers.

Any proposal brought before the Council, with the exception of the election of officers, must be approved through unanimous vote of the voting members.

All proposals approved by the Council shall be submitted to each organization at its official business meeting. No proposal shall be put into effect by the Council without approval of the membership of each participant.

The Alternate Member shall have voting privileges only in the absence of a voting member at meetings of the Council.

The application of other participants shall be received by the Council and admission, shall be recommended by unanimous consent of the Council.

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[a] *This document creating a Sanitarian's Joint Council, outlining its objectives and purposes and prescribing certain rules and procedures, was adopted by the National Association of Sanitarians, the International Association of Milk and Food Sanitarians, and the American Public Health Association (Engineering and Sanitarian Section). It became effective on December 14, 1956.*

[b] *Amended June 26, 1974.*

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Grapes and Wine Technology: Grapes to Wine[1]

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(Received for publication November 18, 1974)

ABSTRACT

Wine making is an art which is developing more into a science. This paper concerns wine technology particularly as it relates to growing the grape to fermentation of grape juice, subsequent storage and aging of wine, and finally to its evaluation as a quality wine. Factors which affect growing of grapes, such as geographical location requirements, time of physiological maturity and subsequent harvest, are discussed. Classification of different vineyards according to the heat summation technique is described. During grape juice fermentation, the important role of sulfur dioxide, acidity, incubation temperature, and oxygen content are discussed as well as the relationship between sugar concentration and alcohol production. Type of storage and manner of aging the wine further play a very critical role in development of a quality wine. Finally, grading of wine is discussed with respect to the relative importance of individual characteristic qualities such as appearance, color, aroma and bouquet, vinegary taste, total acidity, sweetness, body, flavor, bitterness, and general quality. Ultimately, each bottle of wine must be judged on its own merits based on each consumer's attitude and experiences with wine drinking.

Evaluation of a quality wine can vary from one connoisseur to another. This is because appreciation of any wine is primarily due to a person's attitude and previous experiences with wine. In an attempt to clarify many of the factors involved in making a quality wine, this paper will present an overview of wine technology from growing of grapes to its final evaluation as a quality wine. Wine, as defined in this paper, is the product of the normal alcoholic fermentation of the juice of grapes.

GEOGRAPHY

Most vineyards are located near a large body of water such as an ocean, a river, or a lake. The water serves to store excess heat which may otherwise adversely affect growth or development of grapes. Climatic requirements include protection from high humidity to prevent mold growth and protection from severe wind to protect fruit damage. Grapes have a very extensive root system and as long as soil moisture throughout the root zone is kept above the permanent wilting percentage, grape maturation is not affected (*13*).

GRAPE COMPOSITION

At maturity, the grape skin contains most of the aroma, coloring, and flavoring constituents. When looking at a cross-section of the mature grape berry (Fig. 1), we find that the zone nearest the skin is lowest in acid,

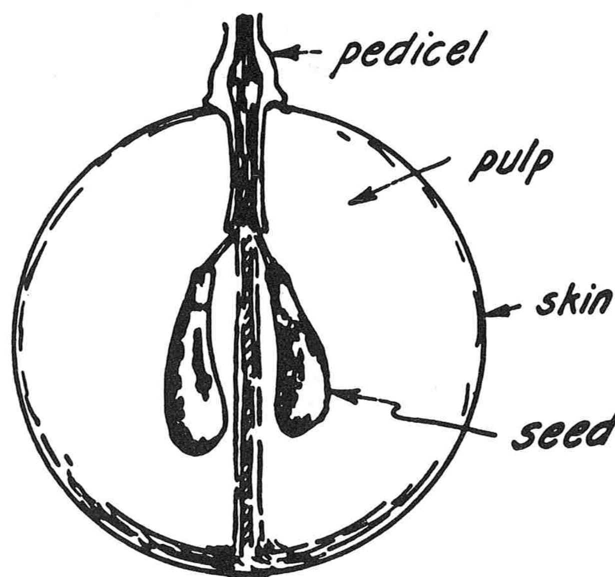

Figure 1. *Cross section of mature grape berry. Source: Amerine and Josyln (2).*

the intermediate zone is also low in acid, but highest in sugar, while the zone nearest the seed is highest in acid and lowest in sugar (*2*).

MATURITY AND HARVEST

The harvest date for grapes is not uniform each year. In general, if the growing season is too warm, grapes are harvested early; and if too cool, they are harvested later. The time of harvest for grapes depends on the use for which the grapes are intended. For example, sweet wines require a high sugar grape and relatively low acid content; whereas, dry wines require grapes which contain just barely enough sugar to produce the minimum alcohol content. Actually, according to the cumulative average temperature of that growing season, different vineyards are classified according to the type of wine for which the grapes are intended.

HEAT SUMMATION TECHNIQUE

Scientists at the University of California (*5*) have developed what is commonly known as the "Heat

[1] *Published with the approval of the Director as Paper No. 3899, Journal Series, Agricultural Experiment Station, Lincoln, Nebraska. Work was conducted under Research Project No. 16-26.*

Summation" technique to determine the total growing season for grapes for the manufacture of wine. This heat summation technique refers to the total length of the growing period that is above 50 F. The temperature summation during the period must be greater than or equal to 1600-1800 degree-days where the excess degrees above 50 F of the average temperature for that day equals the number of degrees for that day (*19*). On the basis of data collected for 12 years, Koblet and Zwicky (*16*) noted that the correlation between the season's temperature summation and the sugar content was greater than the correlation with the temperature summation of any month or with hours of sunshine. Furthermore, Winkler (*24*), according to the heat summation technique, has divided the state of California into five viticulture zones suitable for the growing of specific grapes (Fig. 2).

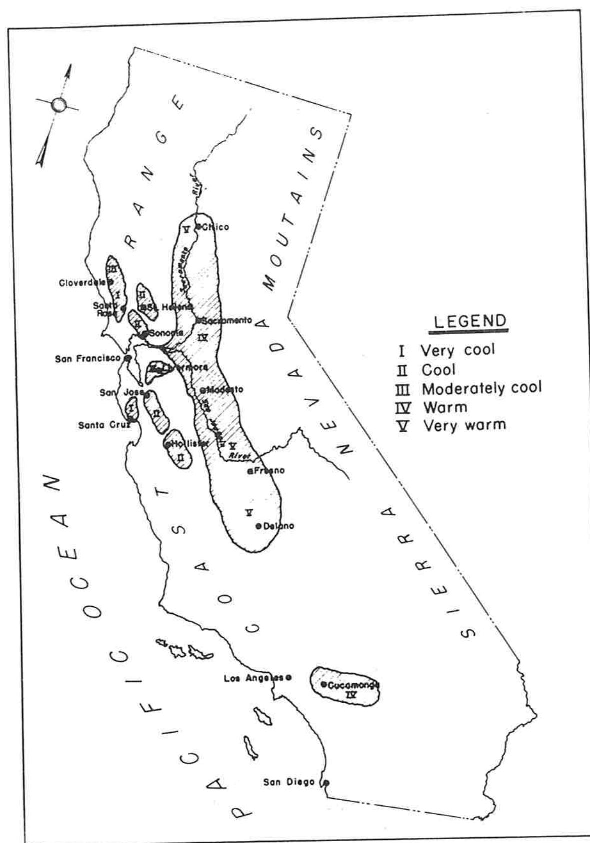

Figure 2. *Five viticulture zones in California. Source: Amerine and Singleton* (*4*).

Amerine and Singleton (*4*) reported that the same variety of grapes grown in region III will have more total acid when ripe than if it were grown in a warmer region, e.g. region V. This is because a higher proportion of the acid is metabolized by the grape under the warmer climatic conditions of region V. The ripening being faster in the warmer region, the fruit reaches a higher sugar content on the same date or reaches a given sugar content earlier. Also, they reported that a greater variety of pigments appear in the grapes grown in cooler regions than those grown in warmer regions. Therefore, the fruit from the cooler region tastes fruitier and is more tart, when compared to the fruit from the warmer region, which even though sweeter, will be more flat.

Generally, wines can be classified into two types: generic or varietal. "Generic" wines are defined as wines blended from several varieties of grapes. These wines have only the most general taste characteristics of the European wine types for which they are named. Examples of generic wines are burgundy, chianti, chablis, claret, rhine, and sauterne. Since generic wines are not standardized by U.S. laws, the wine producer usually blends for a specific constant taste year after year so that a similar name of wine will not necessarily taste the same. "Varietal" wines, on the other hand, obtain their flavor and name from the dominant grape variety which, by U.S. law, must account for at least 51% of the grapes used in their manufacture. In practice this percentage is much higher. Examples of varietal wines are Cabernet Sauvignon, Pinot Noir, Zinfandel, Pinot Chardonnay, Johanisberg Riesling, Chenin Blanc, and Sauvignon Blanc (*14*). In summary, Table 1 illustrates the recommended relationships between grape varieties, wine types, and the five grape-growing regions of California (*2*).

After deciding upon the harvest date, grapes are generally selectively and carefully picked by hand. This practice insures that grapes are brought to the winery uninjured and that the moldy clusters and rotten fruits are culled out and discarded.

PREPARATION OF MUST

After picking, grapes are transported to the winery as rapidly as possible where they are de-stemmed and crushed by machines. The stems are ground and returned to the soil as a mulch, and the mixture of the juice, skin, and seeds which is called "must" is then pumped into a fermentation vat. Here, 100 to 200 ppm sulfur dioxide in the form of either liquid sulfur dioxide or potassium or sodium bisulfite is added. The addition of sulfur dioxide serves (*a*) to control undesirable wild yeasts present in the waxy film on the grape skin and also the undesirable mold and bacteria present during fermentation, (*b*) to disrupt cells of the grape skin and thereby aid in release of the red pigments for red wine, (*c*) to inhibit browning enzymes of the grape since sulfurous acid denatures these enzymes and the Maillard reaction is prevented or retarded, (*d*) to act as an antioxidant by keeping the system under reduced conditions and thereby prevent reaction with the free oxygen present, and (*e*) to combine with the excess undesirable acetaldehydes formed furing fermentation and aging (*20, 21*). On the other hand, addition of too much sulfur dioxide will impart an objectionable and pungent burnt-sulfur off-flavor and -odor to the wine. Gruess (*9*) has reported that as little as 100 ppm of sulfur dioxide is sufficient to eliminate over 99.9% of the active cells of microorganisms from normal must. Wine yeasts, on the

TABLE 1. *Recommendations on grapes for the various types of wine and five grape-growing regions of California*[1]

Variety	Type of wine	Region of adaptation and quality of product				
		Region I	Region II	Region III	Region IV	Region V
White varieties						
Chardonnay	Varietal	Excellent	Good	Good	No	No
Chenin blanc	Varietal, dry or sweet	Possible	Standard	Standard	Standard	No
Emerald Riesling	Varietal, dry or sweet	No	No	Good	Good	Standard
Pinot blanc	Varietal	Good	Good	Possible	No	No
Sauvignon blanc	Varietal or sweet table in III	Good	Excellent	Good	No	No
White Riesling	Varietal	Excellent	Good	No	No	No
Red varieties						
Cabernet Sauvignon	Varietal	Excellent	Excellent	Good	No	No
Charignane	Generic	No	Possible	Standard	Standard	Possible
Grenache	Pink Varietal	Good	Good	Standard	No	No
Pinot noir	Varietal or pink	Excellent	Good	No	No	No
Ruby Cabernet	Varietal	No	Possible	Good	Good	Standard
Zinfandel	Varietal or pink	Good	Good	Standard	Possible	No

[1]Source: Amerine and Josyln (*2*).

other hand, when added at the right time after sulfiting are less sensitive to sulfur dioxide (*15*). In fact, Porchet (*18*) has demonstrated that wine yeasts can adapt themselves to sulfur dioxide and become comparatively resistant to it.

ROLE OF ACIDITY IN MUST

Optimally, must should be acidic with the pH adjusted below 3.6. The high acidity serves to prevent bacterial spoilage, inhibit autolysis of yeast cells, aid in extraction of skin pigments, and develop a better flavored wine. The optimum amount of acid is 0.7 to 0.9 g acid/100 ml must for dry wines and 0.6 g/100 ml must for sweet wines. Acid deficits are generally made up by addition of tartaric acid. An appreciable amount of added tartaric acid is insoluble in alcohol and will consequently precipitate out from wine as cream of tarter (bipotassium tartrate) during the later stages of fermentation and aging (*2*).

FERMENTATION

Next, the must is separated by pressing out the skin and seeds portion, called "pomace," which is discarded. The free-run juice is collected in a catch basin at the bottom of the press. This stage of pressing is important since pressing too early gives wine with low tannins and low color, whereas pressing too late results in wine high in tannins and with too much color.

If red table wines are being made, juice and pomace are sent together to tanks for a 4 to 14 day fermentation, then the pomace is pressed out. During fermentation, red wine gets its color from the pigment in the skins and its strong flavor and astringency from tannins and other substances in the skin and seeds (*7*). In the case of rosé wine production, the free-run juice is allowed to remain in contact with the pomace for only a 24-h period. On the other hand, for white wines, press juice should not be allowed to remain in contact with the pomace before fermentation (*8*). At the Robert Mondavi Winery in Oakville, California, white and rosé wines are fermented at temperatures no higher than 60 F for about 8 days; red wines are held in fermenters at temperatures up to 75 F for about 4 to 5 days (*10*).

The sulfured grape juice is pumped into the fermentation tank and inoculated with about 1% culture of a pure wine yeast, *Saccharomyces cerevisiae* var. *ellipsoideus*. After 12 h, the fermentation activity is high and a considerable amount of heat is released. Therefore, high-capacity cooling systems are utilized to remove excess heat produced and to slow down the rate of fermentation. The slow fermentation at lower temperatures produces more esters and a higher yield of alcohol along with a wine that is easier to clear and that is less susceptible to bacterial infection (*1*).

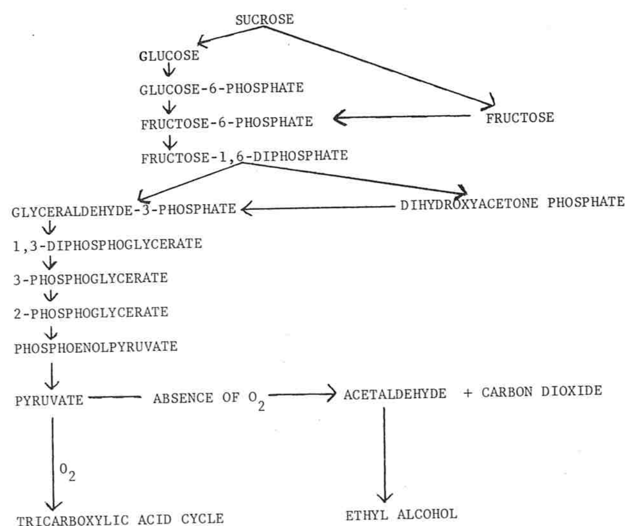

Figure 3. *Sugar metabolism and alcohol production by* Saccharomyces cerevisiae *var.* ellipsoideus.

Figure 3 illustrates the sugar-metabolism and alcohol production pathways that yeast cells follow during fermentation. As can be seen, a maximum yield of alcohol occurs in the absence of air. Excessive aeration allows yeasts to utilize the Tricarboxylic Acid Cycle for energy and cell multiplication, resulting in a flat oxidized wine of poor flavor. Therefore, effective fermentation requires an abundant amount of oxygen only initially to promote yeast multiplication. After the yeast has consumed most

of the dissolved oxygen, the anaerobic alcoholic fermentation can ensue.

On the basis of alcohol content, all wine is divided into two general classes. The table wines (also called "dinner," "dry," or "light" wines) contain not more than 14% alcohol by volume; the "aperitif" and "dessert" wines (sherry, port, muscatel) contain about 20%. The higher alcohol content is obtained by addition of grape brandy or alcohol. When added during the fermentation, grape brandy stops yeast growth resulting in a wine that has some of its sugar still present and which accounts for its sweetness (*1*).

Likewise, sugar itself above a certain concentration will also inhibit the percentage of sugar utilized by wine yeasts. Figure 4 shows how the percentage of glucose

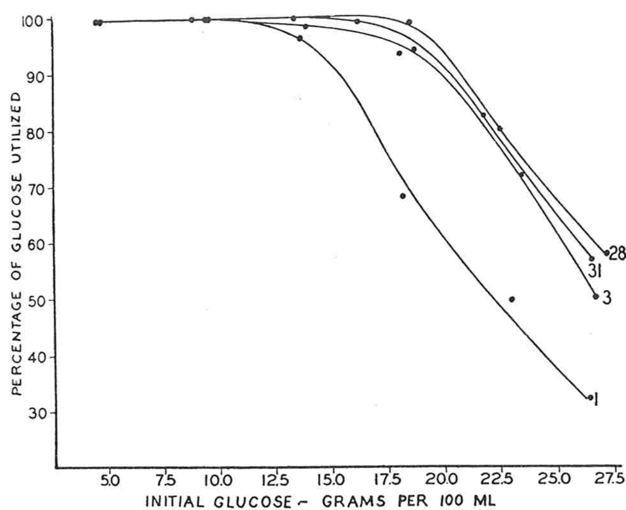

Figure 4. *The effect of initial glucose concentration upon percentage of glucose utilization by 4 strains (numbered 1, 3, 28 and 31) of* Sacchromyces cerevisiae *from the Seagram yeast stock culture collection. Source: Gray (12).*

utilization of four strains of *S. cerevisiae* from the Seagram yeast stock culture collection were inhibited by increasing concentrations of glucose (*12*). Gray (*12*) observed that this decrease in glucose utilization was in part due to plasmolysis of yeast cells. However, for selected wine yeasts, the optimum sugar concentration for maximum alcohol production is about 28% (*2*). Therefore, a particular strain of *S. cerevisiae* var. *ellipsoideus* is usually chosen for its ability to ferment rapidly at low temperatures and to yield a high percentage of alcohol per gram of sugar consumed. For 142 strains of *S. cerevisiae* var. *ellipsoideus*, Amerine and Kunkee (*3*) reported the minimum percentage of ethanol produced was 8.5 and the maximum 15.0.

STORAGE AND AGING

After the intitial fermentation stage, wine is transferred from fermentation vats into storage vats and tanks to complete the alcoholic fermentation and to undergo aging and stabilization. By allowing wine to stand, a major portion of theyeast cells and other fine suspended materials are collected at the bottom of the vat as sediment or "lees." The relatively clear wine must then be "racked" or carefully pumped or siphoned off without disturbing the lees. Completion of the first racking as soon as possible eliminates the possibility of introducing off flavors into the wine from autolyzed yeast cells that accumulate in the "lees" (*4*).

On the other hand, it is highly desirable to have a certain amount of yeast cell destruction during vat storage of wine. The presence of autolyzed yeasts, particularly in a wine of high acidity, stimulates growth of an added inoculum of *Lactobacillus* bacteria during this storage stage. The enzymes from these bacteria decarboxylate the wine's malic acid and convert it to lactic acid. This malolactic fermentation often referred to as the "secondary fermentation" mellows the high acid wine and results in a higher quality wine. An excellent comprehensive review is presented by Kunkee (*17*) on the history, occurrence, bacteriology, biochemistry, and control of the malolactic fermentation.

Another factor to be considered after the first racking has taken place, is to minimize contact between the wine and air; and to prevent excessive oxidation, browning, and growth of aerobic vinegar bacteria. This can be accomplished by keeping the container as full as possible and closed or by filling the headspace of partly full containers with nitrogen or carbon dioxide gas. However, a certain degree of wine oxidation is necessary for the manufacture of a quality wine. This can be accomplished by aging wine in wooden casks. Wooden containers serve the special purpose of allowing a very slow diffusion of oxygen through the wood pores (*4*). Aging itself is a complex process of oxidation, reduction, and esterification that results in formation of a desirable "bouquet" with elimination of the raw harsh simple flavor of new wine (*2*).

After aging in wooden containers, wine is ready to be bottled. Aging continues in the bottle. However, here, aging serves to eliminate the aerated odor wine acquired at the time of bottling, to reduce the wine's content of free sulfur dioxide, and to further improve its bouquet. As a general rule, a good red wine should be held in bottles for 5 to 10 years to reach peak maturity, while a white wine requires only 2 to 5 years (*1*).

SCORING OF WINE

Amerine and Singleton (*4*) have suggested a representative 20-points wine score-card for the systematic evaluation of wine (Table 2). In general, based on this scoring system, wines with a total rating of 17 to 20 points are considered outstanding; 13 to 16 points, commercially acceptable; and 9 to 12 points, commercially acceptable but with a noticeable defect. Out of 20 points, qualities like appearance, color, vinegary, total acidity, flavor, bitterness, and general quality, each account for 2 points, aroma and bouquet

TABLE 2. *Score card for wine grading*[1]

Characteristic	Points
APPEARANCE	2
Cloudy 0, clear 1, brilliant 2	
COLOR	2
Distinctly off 0, slightly off 1, correct 2	
AROMA AND BOUQUET	4
Vinous 1, distinct but not varietal 2, varietal 3	
Subtract 1 or 2 for off-odors, add 1 for bottle bouquet	
VINEGARY	2
Obvious 0, slight 1, none 2	
TOTAL ACIDITY	2
Distinctly low or high 0, slightly high or low 1, normal 2	
SWEETNESS	1
Too high or low 0, normal 1	
BODY	1
Too high or low, normal 1	
FLAVOR	2
Distinctly abnormal 0, slightly abnormal 1, normal 2	
BITTERNESS	2
Distinctly high 0, slightly high 1, normal 2	
GENERAL QUALITY	2
Lacking 0, slight 1, impressive 2	

[1]Source: Amerine and Singleton (*4*).

account for 4 points, and sweetness and body account for 1 point each.

Appearance is always the first noticeable quality of any wine. In this regard, "brilliant" is defined as having no visible suspended material in the wine, "clear" as very slightly hazy, and "cloudy" as very hazy with deposits. Unfortunately, American consumers consider the presence of sediment or development of haze during marketing or chilling of wine for table use as evidence of incipient spoilage. This is not always true since some delicately flavored, less stable old wines and even some young wines are still free of spoilage and are of acceptable flavor (*2*).

Therefore, if natural clarification does not occur during successive rackings, artificial methods called "fining" or inducement for clarification, must be used before bottling of the wine. Fining agents act by combining chemically with the colloidal particles in the wine, or by neutralizing electrical charges of the colloids inducing them to coalesce and form larger particles which settle out by gravity. During coalescence they also occlude. These particles can then be easily removed to obtain clear wine. Another more modern method involves use of sterile-pad millipore filters to obtain clear wine (*10, 23*).

TABLE 3. *Preferred color of different types of wines*[1]

Wine	Preferred color
WHITE TABLE	
Chardonnay	yellow to light gold
Sauvignon blanc	yellow to light gold
White Riesling	greenish yellow to yellow
SWEET TABLE	
Sauternes	light gold to gold
Tokay (Hungarian)	light gold to gold (no amber)
Rosé	pink (no amber or purple)
RED TABLE	
Pinot noir	low to medium red
Cabernet Sauvignon	medium red
Zinfandel	medium red
RED DESSERT	
Tawny port	amber-red
Ruby port	ruby red
WHITE DESSERT	
Muscatel	light amber-gold to gold
Tokay (California)	pink-amber
White port or angelica	medium yellow

[1]Source: Amerine and Singleton (*4*).

Color is the nest characteristic which is, of course, closely associated with the appearance of a quality wine. Wine makers around the world have arrived at a very general consensus as to what the color of certain types of wines should be (*11*). Table 3 shows the desirable ranges of color of different types of wines (*4*). Generally, white table wines should be free of brown color, since browning usually indicates excessive oxidation and the development of stale color, and young red table wines should have a full red color. However, slight browning is expected in old red wines (*4*). The particular pigment composition of the grape variety determines the color of grape juice. But the color of the subsequent wine is primarily due to the gradual disappearance of the anthocyanin pigments with the concomitant appearance of their modified polymeric forms. Thus, color of red wine after 5 to 10 years of storage is attributed primarily to these complexes (*11*).

Aroma and bouquet are considered to be the single most important quality factor of a wine. Aroma refers to the odors originating from the grape. Cabernet Sauvignon, Sauvignon vert, and Sémillon are examples of grapes with a slight aroma giving a distinctly flavored wine. Sauvignon blanc, Zinfandel, and Fresia are examples of grapes with a less distinct aroma yet still giving a recognizable wine. Finally, the Thompson seedless is an example of a grape without a distinct flavor yet giving a palatable but undistinguished wine (*2*).

On the other hand, the *bouquet* refers to the odors originating from alcoholic fermentation and aging. Fermentation odors are the background odors found in all wines. They are due to ethyl alcohol, higher alcohols, and small amounts of a large number of volatile components, especially esters. These compounds give wine its grapy and winy character. Of course, during aging, a gradual disappearance of fermentation odors occurs. Some undesirable odors are obtained from very late harvested grapes of low total acidity (rubber boot odor), from being bottled too long (corkiness), and from the presence of hydrogen sulfide and high volatile acidity (*4*).

Figure 5 is a gas chromatogram of some of the volatile flavor components of a wine in comparison to the relative response of these same components as measured by the human nose (*6*). From this experiment, Bayer (*6*) demonstrated that the height of the gas chromatographic peak produced by a wine constituent is not proportional to the quality or intensity of its odor; this is dependent upon the assumption that no synergistic or masking effects occur between odorous constituents. To approximately equate the smell response with the flame ionization response, odor thresholds of these components need to be determined or elucidated before peak heights or areas can be related to wine quality.

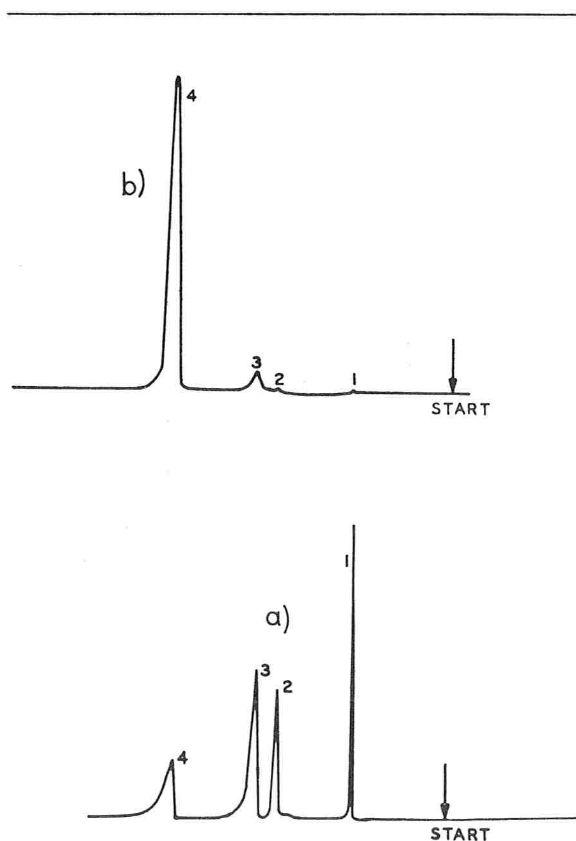

Figure 5. *Comparison of response of flame ionization detector (a) and response of smell (b) for (1) ethyl alcohol, (2) methyl acetate, (3) citral, (4) β-ionone. Relative response of nose (b) was calculated from minimal concentration which can be detected by smell. Source: Bayer (6).*

Another quality factor to be considered is the *"vinegary taste"* in wines. Highly undesirable, the vinegary taste is due to acetic acid spoilage caused by the presence of *Acetobacter aceti* or other acetic acid bacteria and oxygen in the wine.

Total acidity or the titratable acid content, calculated as tartaric acid, is another constituent which has to be present in the correct amount for wine to receive a high score-card rating. The range in mature grapes is 0.3 to 1.2% (pH 2.9-3.9). During grape ripening, there is a gradual decrease in titratable acidity. As indicated earlier, the correct acidity during fermentation and aging of the wine is very critical for production of a high quality wine. Thus, the fixed acid content of a wine, which is the difference between the titratable acid content and the volatile acid content (calculated as acetic acid), can help in differentiating certain wine types. For example, a dry table wine containing greater than 0.6% fixed acid will taste fresh and tart, whereas if it is less than 0.4%, the wine will taste flat and insipid. Likewise, sweet wines containing a high fixed acid content are considered to possess an undesirable sweet-sour taste (*2*).

The *sweetness* quality of a wine can vary widely, depending on the type of wine. The sweet taste in wines is primarily due to the presence of glucose and fructose in about equal proportions. The sugar threshold for the sweet taste in wine is somewhere between 0.75 and 1.5%. Interestingly enough, a wine with less than 1% sugar is usually noted by the wine judges as being without sweetness (*4*).

Another quality referred to as *body* is essentially a measure of the viscosity or alcohol content of the wine, or the degree of "wateriness" of the wine. A low alcohol wine normally has a low body or a "thin" mouth feel.

With regard to the *flavor* quality, fruitiness of a wine is in part due to the acid taste and to the presence of a small amount of leaf aldehyde or other fruity kinds of aromatic compounds. Tannins in small quantities impart desirable flavors to red table wines but are undesirable in white table wines.

Bitterness, a quality primarily due to the tannin content, is associated with the astringent taste of the wine. However, astringency itself refers more to the "feel" of the wine than to the bitter taste. For example, a "smooth" wine is one lacking in astringency and a "rough" one is considered to be too high in this character. Aging helps reduce the astringency by oxidation and by precipitating excess tannins. Quality wines, as a whole, do not contain sufficient tannins to give an objectionable astringent taste (*4*).

The last quality factor considered is the *general quality* of a wine. This can be looked upon as the "after-taste" and the "over-all" impression of the wine. An example of the after-taste would be an overall lack of extreme bitterness in the wine.

CONCLUSIONS

Quality wine making has always been an art which is rapidly developing more into a science. Yet, because all makers of quality wine understand that each year's wine will be slightly different from each succeeding year's depending on the climate that year, on how the grape juice was fermented and how adequately the subsequent wine was aged, they still sniff and taste repeatedly their wines before they will allow them to be sold.

But ultimately, each bottle of wine is judged solely on its own merits by wine connoisseurs and tyros alike. The wine connoisseur indeed looks for certain quality flaws in the wine based on his own past experiences, whereas the tyro will probably be more concerned simply with the taste and appearance in his evaluation of the wine. For example, in the United States, the natural tendency of the evolving wine drinker is to generally start sweet and then with more exposure to different kinds of wine progress to drier or less sweet wines (*22*).

Therefore, by presenting in this paper some of the more important factors involved in the manufacture and evaluation of a quality wine, it is hoped that the American consumer's understanding of wine quality will be expanded and thus enhance his overall appreciation of the different kinds of wines.

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Report of the Food Protection Committee, 1973-1974

The committee met on August 12, 1974 at the St. Petersburg Hilton Hotel. The following were present: Charles Felix, chairman; K. J. Baker, Howard Hutchings, Karl Jones, Harold McAvay, and Harold Wainess. The following items were considered.

1. *Food service manager certification programs*

Certification programs such as are in operation in the State of Ohio and in Washington, D.C. were reviewed by the committee. K. J. Baker, project officer for the FDA-funded Ohio program, reported that additional contracts have been let out this fiscal year to the states of Colorado and Virginia to determine whether and how the Ohio model will work under different circumstances. In discussing the need to develop suitable training programs favorable notice was given to a new course of instruction published by the National Institute for the Foodservice Industry entitled, *Applied Foodservice Sanitation.*

2. *Revision of the FDA/PHS Foodservice Ordinance and Code*

The anticipated publication in the *Federal Register* of the revised FDA/PHS model Foodservice Ordinance and Code has been delayed because of a change in format. The revision will appear in the *Federal Register,* in a matter of weeks, the committee was informed.

3. *Foodservice self inspection questionnaire*

The Committee discussed changes in the first draft of a questionaire on foodservice self-inspection programs prepared by an ad hoc subcommittee of the Food Protection Committee. A second draft will be prepared for the Committee's consideration and, if approved, will be sent to all 50 state agencies charged with foodservice sanitation as well as a number of the larger local departments. The questionnaire will attempt to delineate the "state of the art" of self-inspection as practiced, or not practiced, in the U.S. today. Results of the survey will serve as a resource for the development within IAMFES of a model for quality sanitation control in the foodservice industry.

Report of the Keep America Beautiful National Advisory Council, 1974

On December 4, 1973, the 20th Annual Meeting of Keep America Beautiful, Inc. was held in New York City. IAMFES is a member of the Keep America Beautiful (KAB) National Advisory Council which is made up of 102 national government, professional, and private organizations. I represented IAMFES at the National Advisory Council meeting as well as at a caucus of the Advisory Council a few months before.

I came away from those meetings with the impression that the KAB National Advisory Council represents a significant force in educating the public to a consciousness of personal responsibility in the national quest for quality of life. I further felt that IAMFES should be represented at the highest level as was true of most of the associations on the Advisory Council.

I recommend that either the President or the Executive Secretary of this Association attend the 1974 Annual Meeting of KAB to be held December 4 and 5 in New York City. This will enable him to assess the leadership potential IAMFES should exercise in the environmental activities of KAB and also the opportunities for useful contacts that the meeting provides. For example, there are 20 agencies of 9 federal government departments represented on the KAB Advisory Council, among them the National Oceanic and Atmospheric Administration, the U.S. Department of Commerce, the Secretariat of the U.S. Commission of UNESCO, the U.S. Department of State, the offices of Air Programs, Water Programs and Solid Waste Management Programs, the U.S. Environmental Protection Agency and many others with which the officers of this association have occasional or continual dealings. Those contacts could be strengthened by the personal involvement of the president or the executive secretary.

Respectfully submitted,
Charles Felix

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E-3-A SANITARY STANDARDS FOR PLATE TYPE HEAT EXCHANGERS FOR FLUID EGG PRODUCTS

Serial #E-1100

Formulated by
International Association of Milk, Food and Environmental Sanitarians
United States Public Health Service
United States Department of Agriculture
Poultry and Egg Institute of America
Dariy and Food Industries Supply Association

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

A.
SCOPE

A.1
These standards cover the sanitary aspects of plate type heat exchangers for liquid egg products.

A.2
In order to conform with these E-3-A Sanitary Standards, plate type heat exchangers shall comply with the following design, material, and fabrication criteria.

B.
DEFINITIONS

B.1
Product: Shall mean liquid egg products.

B.2
SURFACES

B.2.1
Product Contact Surfaces: Shall mean all surfaces which are exposed to the product.

B.2.2
Non-Product Contact Surfaces: Shall mean all other exposed surfaces.

C.
MATERIALS

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

C.1.1
Rubber and rubber-like materials may be used for gaskets. These materials shall comply with the applicable provisions of the "E-3-A Sanitary Standards for Multiple-Use Rubber and Rubber-Like Materials Used as Product Contact Surfaces in Egg Processing Equipment, Serial #E-1800."

C.1.2
Plastic materials may be used for gaskets. These materials shall comply with the applicable provisions of the "3-A Sanitary Standards for Multiple-Use Plastic Materials Used as Product Contact Surfaces for Dairy Equipment, Serial #2000," as amended.

C.2
All non-product contact surfaces shall be of corrosion-resistant material or material that is rendered corrosion-resistant. If coated, the coating used shall adhere. All non-product contact surfaces shall be relatively non-absorbent, durable and cleanable.

D.
FABRICATION

D.1
All product contact surfaces shall be at least as smooth as a No. 4 finish on stainless steel sheets. (See Appendix, Section F.)

D.2
All product contact surfaces shall be easily accessible for cleaning, either when in an assembled position or when removed. Removable parts shall be readily demountable. Heat transfer plates shall be readily removable from the press. Individual removable heat

[1]The data for this series are contained in the following reference: AISI Steel Products Manual, Stainless & Heat Resisting Steels, April 1963, Table 2-1, pp. 16-17. Available from: American Iron & Steel Institute, 1000-16th Street, N.W., Washington, D.C. 20036.

[2]Alloy Casting Institute Division, Steel Founders' Society of America, 21010 Center Ridge Road, Rocky River, OH 44116.

transfer plates shall be considered to comply with this requirement.

D.3
Internal angles of 135° or less on product contact surfaces shall have minimum radii of ¼-inch except where smaller radii are required for essential functional reasons. In no case shall such radii be less that 1/32 inch.

D.4
There shall be no threads on product contact surfaces.

D.5
Sanitary connections shall conform to the applicable provisions of the "E-3-A Sanitary Standards for Fittings Used on Egg and Egg Products Equipment and Used on Sanitary Lines Conducting Egg and Egg Products, Serial #E-0800."

D.6
Transfer plate gaskets shall be continuous and shall be bonded to the transfer plate in such a manner that the bond is continuous and mechanically sound, and so that in the environment of its intended use the gasket does not separate from the plate.

D.7
A leak protector groove of sufficient width to be readily cleanable and open to the atmosphere at both ends shall be provided to allow leakage past gaskets to drain to waste.

D.8
Presses (or frames) shall be provided with legs of sufficient length to give a clearance of at least 4 inches between the lowest part of the press and the floor. Legs shall have rounded ends with no exposed threads. If made of hollow stock they shall be effectively sealed.

D.9
Presses (or frames) shall be so constructed that when opened plates and/or terminal frames may be separated to provide a space for cleaning and inspection equal to the lesser of the width of one plate or 15 inches.

D.10
Non-product contact surfaces shall be free of pockets and crevices and be readily cleanable. Surfaces to be coated shall be effectively prepared for coating.

APPENDIX

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

F.
PRODUCT CONTACT SURFACE FINISH
Surface finish equivalent to 150 grit or better as obtained with silicon carbide, is considered in compliance with requirements in D.1 herein.

These standards shall become effective June 26, 1975.

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

News and Events

Columbus College Develops Health Science Program

Columbus College in Columbus, Georgia has developed an extensive health science program. They have nearly 500 students in six different allied health occupations. They have also developed a comprehensive continuing education program. The continuing education effort of Columbus College during 1974 involved nearly 1,500 people representing all the health professions, including the sanitarian. The continuing education activity is housed in a one and one-half million dollar facility dedicated in November of 1974.

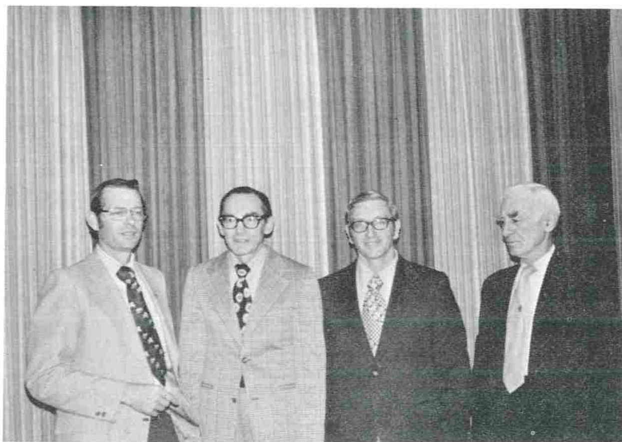
The academic programs of the college and the continuing education effort operate as a cohesive unit, thereby assuring an extremely high level of quality for our programs. They have recently concluded an agreement with the Center for Disease Control in Atlanta whereby they will be the sponsoring agent for offering continuing education unit (C.E.U.) credit for the various correspondence courses they offer. The courses, C.E.U. credit provided, and fees are as follows:

Course	*C.E.U. Credit*	*Cost*
Community Hygiene	3.2	15.00
Basic Mathematics for the Sanitarian	2.0	46.00
Communicable Disease Control	2.4	15.00
Vectorborne Disease Control	2.8	15.00
Waterborne Disease Control	2.8	15.00
Foodborne Disease Control	2.4	15.00
Community Health Analysis	3.2	15.00

Details of the operation of this program are available from:

Dr. Susan Peters
Health Homestudy Program
Community Services Division
Columbus College
Columbus, GA 31907

1975 Officers of the National Mastitis Council



Left to Right: Dr. W. Nelson Philpot, Mr. Burdet Heinemann, Dr. R. D. Nochrie and Dr. John Flake.

A very informative and well-rounded program was presented at the 1975 NMC Annual Meeting held in Minneapolis. Three-hundred and sixty members and guests were in attendance for all or part of the 2-day program which included research reports from two international authorities on mastitis control.

Mr. Burdet Heinemann, Mid-America Dairymen, Inc. of Springfield, Missouri was elected President. Dr. R. D. Mochrie, North Carolina State University, Raleigh was elected Vice President and Dr. John Flake of Washington, D.C. as Secretary-Treasure. Dr. W. Nelson Philpot, Louisiana State University is the immediate Past President.

The regional meeting of the Council is being planned for Toronto, Canada on August 14, 1975 in conjunction with the International Association of Milk, Food and Environmental Sanitarian Inc. annual meeting. The May 1975 issue of the Journal will contain the details of the program.

American Cultured Dairy Products Institute 1975 Conference Set

WASHINGTON, D.C., March 5, 1975—The Annual Conference of the American Cultured Dairy Products Institute will be held September 17-18, 1975, at Stouffer's Inn, Louisville, Kentucky.

This year's Conference will include general sessions as well as specific panel presentations relative to quality assurance and innovative manufacturing procedures for cottage cheese, sour cream, yogurt and other cultured dairy products.

The final program will be announced this summer and nonmembers of ACDPI should write the Institute office for further meeting information. The address is American Cultured Dairy Products Institute, 910-17th St., N.W., Washington, D.C. 20006.

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Association Affairs

Rocky Mountain Conference on Food Safety

The Rocky Mountain Affiliate, along with numerous other organizations and agencies, will again sponsor the Rocky Mountain Conference on Food Safety. The conference is scheduled at Colorado Women's College in Denver on May 1 and 2, 1975. The topics of discussion will be as listed:

1. New techniques in food preservation.
2. Thermoradiation treatment of foods.
3. Microwave ovens.
4. Resistant micro organisms in foods.
5. Insect and rodent control in the food industry.
6. Problems involved in long distance transportation of meat.
7. Certification of food service operators.
8. Colorado Occupational Health and Safety Act.
9. Animal health and food safety.
10. Deboning of meat.
11. Plant sanitation.
12. Antibiotics in foods.

Planning of the Annual Meeting

Left to Right: Wm. Kempa, Dr. Al Myhr, Cyril Duitschaever and Phil Glass.

The Ontario Milk and Food Sanitarians Association is busy planning the annual meeting for August 10-14 at the Hotel Royal York in Toronto.

The photo shows william Kempa, General Chairman of the Planning Committee, aided by Dr. Al Myhr, Co-Chairman; Cyril Duitschaever, Chairman of the Entertainment Committee and Phil Glass, Speakers Hospitality Planning Committee for the annual meeting.

Kentucky Educational Conference for Fieldmen and Sanitarians

The 1975 Educational Conference for Fieldmen and Sanitarians sponsored by the Kentucky Association of Milk, Food and Environmental Sanitarians, Inc. was held February 25 & 26, 1975, at Stouffer's Inn, Louisville, Kentucky.

Approximately 350 (city, county and state sanitarians, milk and food industry fieldmen and plant managers, related service company representatives and university personnel) were registered.

The program was separated into general sessions, food, environmental sanitation and milk sections. Seventeen subject topics were presented during the conference.

The following awards were given at the awards banquet by KAMFES:

Outstanding Sanitarian Award:
Donald L. Colgan
Fleming County Health Department
Flemingsburg, Kentucky

Outstanding Fieldman Award:
(plaque donated by Sep-ho Chemical Company)
Marion J. Smith
Hart County Creamery
Horse Cave, Kentucky

Outstanding Service Award:
W. Dale Scott
Farm Bureau Federation
Louisville, Kentucky

Honorary Memberships:
Harry A. Barry (retired)
Fulton County Health Department
Fulton, Kentucky

L. E. Mayhugh (retired)
Chappell Dairy
Campbellsville, Kentucky

William T. Vincent (retired)
Lexington-Fayette County Health Department
Lexington, Kentucky

A past president's plaque was also presented to Dr. James C. Hartley by incoming President, Bruce K. Lane.

Report of the Journal Management Committee, 1973-1974

Last year, we recommended that the duties and responsibilities of the Journal Management Committee be re-defined and spelled out in detail. A communication from the President suggested that the idea was a good one and that the Committee should proceed to do so.

We are not at all sure that the procedure for the Committee to define its own responsibilities is a good one as it means we are more or less writing our own rules as we see them, rather than proceeding on a set of guidelines laid down by the Board which is the group we should be serving. However, we have delineated some of the areas we feel should be covered by the Committee.

1. In the event of dispute over publication of a paper, the Committee should provide the editor with a review of the *Journal* policy with respect to suitability of the paper for appearance in the *Journal*. We understand, from the editor, that this is a very rare occurrence.
2. Make a continuing review and evaluation of the mechanical make-up of the *Journal*.
3. Make an effort to evaluate the membership's needs and desires about the *Journal* content.
4. Make recommendations on the material content of the *Journal* based on information resulting from the above evaluation.
5. The *Journal* tends to lack a specific editorial policy. The Committee could be of assistance in developing such a policy.

In addition, we have several recommendations for the Board's consideration. They are:

1. Some mechanism should be developed to elicit information from the various states relative to milk and food regulations, or other state activities, that may affect the various segments of the industry directly or indirectly.
2. We have repeatedly recommended that notice of our meeting should appear in trade publications. We are further suggesting that the Executive Secretary follow up and find out why such notices are not published, if the material is submitted in time.
3. We recommend that an interpretive review of each half-day session of papers be prepared for publication immediately after the meeting to provide a summary of pertinent points that were discussed. We believe this can be accomplished in two ways: (*a*) the session chairman could be charged with the responsibility of preparing such a review immediately after his session on the program; or (*b*) the Program Committee could obtain the services of university people or others in attendance at the meeting for the specific purpose of reviewing each session at the time they arrange the program. Possibly one individual could be added to the Journal Committee for the specific purpose of arranging for these reports. We are aware that abstracts are handed out at the meeting and published in the *Journal* at some later time, but, we believe that a general statement of the content of the half-day session may be helpful.
4. We strongly urge the Board to again consider sustaining memberships. We recommend that the names of sustaining members be published each month and that the name of a sustaining member who is also a holder of a 3-A Symbol appear with an asterisk or in boldface type.
5. We recommend the association prepare an outline of the many benefits that 3-A Symbol holders receive from the *Journal* such as: twice a year listing, the revision of the standards, and the publicity and advertising they can get from this routine activity. This outline should be prepared with the objective of approaching each symbol holder to consider some modest advertising in the *Journal* and/or becoming sustaining members.
6. We would like the Executive Secretary to furnish us with a break-down of the affiliation of subscribers to the *Journal*. Those people who buy the *Journal*, without becoming members, must do so for the *Journal* content. This break-down may provide us with an insight as to what a large number of people consider as desirable in our *Journal* as this is one area of our association that seems to be growing each year.
7. The Board should give some time to discussion of our *Journal's* name. Does the present name reflect the content of the *Journal?* Does it reflect the interest area of our members and subscribers? As the content of our *Journal* is generally associated with some aspect of sanitation, possibly "technology" is not the best name.

Respectfully submitted,
W. C. Lawton
Chairman

1975 Pennsylvania Dairy Fieldmen's Conference

The annual Pennsylvania Dairy Fieldmen's Conference will be held June 10 and 11, 1975. In addition, a program of interest to fieldmen is scheduled for the evening of June 9. The 1975 program will include talks on new concepts in farm dairy equipment, controlling milk temperature in bulk tanks, farm waste disposal, cleaning and sanitizing farm dairy equipment, and adulteration of milk with water.

A panel on dairy herd health problems by three outstanding veterinarians is programmed. Other topics will include a report of the 1975 Interstate Milk Shipper's Conference and changes in the Pasteurized Milk Ordinance of interest to fieldmen.

All meetings will be held at the J. O. Keller Conference Center on the campus of The Pennsylvania State University in State College, Pa. For further information contact:

Agricultural Conference Coordinator
410 J. O. Keller Building
University Park, Pa. 16802

Index To Advertisers

Babson Bros. ---------- Back Cover
Difco Laboratories ---------- 252
The Haynes Mfg. Company ---------- Inside Back Cover
National Sanitation Foundation ---------- 251
Norton Plastics ---------- 190
Partlow Corporation ---------- Inside Front Cover
Whitmire Research Laboratories, Inc. ---------- 247, 248

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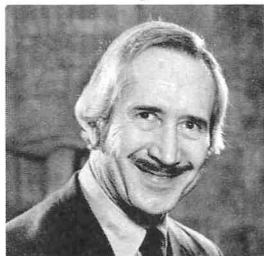
For Sale

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

This message, addressed to the public, is from the 1974 NSF Annual Report

BEYOND WHICH WE WILL NOT GO

Plain, ordinary non-political citizens have recently opened a new chapter in American history. They have caused the cancellation of multi-billion dollar projects including the Florida barge canal, the supersonic transport and two huge jetports because these ventures threatened the *environment.* That was before the oil embargo and the ensuing recession. Today, in a 180° reversal, many of these same citizens are less concerned about auto emissions than they are about sticker prices. They are likely to vote "No" on higher taxes to pay for better sewage treatment and they will be up in arms if ecologists propose any pollution control measures that eliminate local jobs.



At NSF we are not alarmed but we are concerned about these shifts in public sentiment. We have had decades of experience in trade-offs which put environmental quality on one side of the scales and socioeconomic values on the other. We have helped to establish more than 50 national standards for products that affect public health or environmental safety, and each of these NSF standards is based on consensus agreements between representatices of *government, industry* and the *consuming public.*

Out of this experience we have learned one great lesson: In every environmental situation there is a cutoff point beyond which no responsible citizen or agency should go. At this point, executives from industry refuse to give further consideration to profits. Public officials turn their backs on powerful special interests. Consumers stand ready to express themselves in no uncertain terms at ballot boxes and cash registers.

How do these diverse groups within our society reach a point of decision and a basis for agreement? They do it with facts that take the place of partisan rhetoric. They rely on the impartial, scientific findings of environmental laboratories such as we maintain at NSF in Ann Arbor. Parties to environmental standards arrive at pragmatic agreements on specifics such as parts per million, pounds per square inch or gallons per hour.

As a citizen, you are going to be called upon in the next few years to make judgments about the environment. You will have to weigh the cost of each to you in terms of expense, inconvenience or diminished freedoms. Many of your decisions may have little effect on your well being today but they may irreversibly affect the kind of world you pass on to your children or grandchildren. That is why *we cannot afford to forsake environmental quality even in a recession.*

As environmentalists who have close ties to public health agencies, industry and consumer organizations we urge you to take an active interest in environmental issues. Insist on fact finding and be guided in your judgment by demonstrable human costs as well as dollars.

Today our nation is faced with health care expenses that are soaring out of sight. A routine stay of seven days in a hospital can now exceed $1,300. One of the best and surest ways of bringing the cost of health care under control lies in the *prevention* of disease and injury.

A quality environment is the most basic defense of all.

President

NSF

National Sanitation Foundation—an independent non profit, non governmental organization dedicated to environmental quality. NSF Building, Ann Arbor, Mich. 48105. (313) 769-8010.

Government officials and professional workers concerned with public health and environmental quality are invited to write for the NSF Publications List. It is free. It lists all NSF standards and criteria as well as listings, reports and NSF literature.

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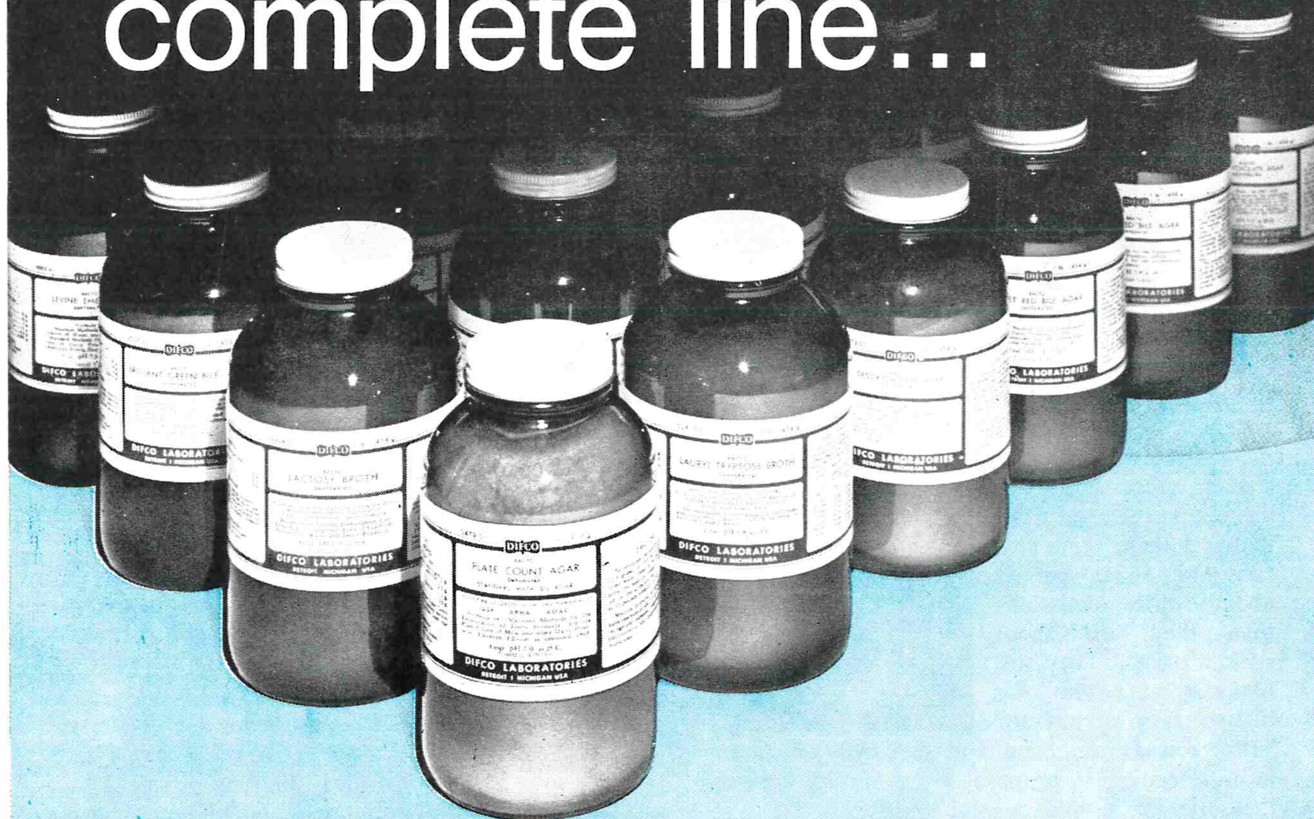

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Standard Methods for Examination of Water and Wastewater XIII 1971

AOAC
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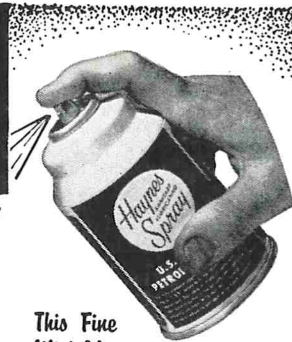

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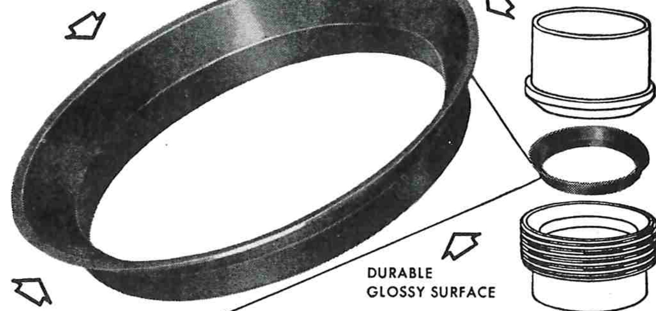

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