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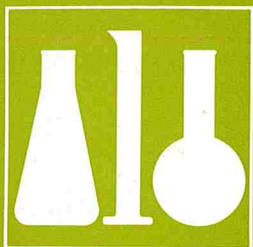
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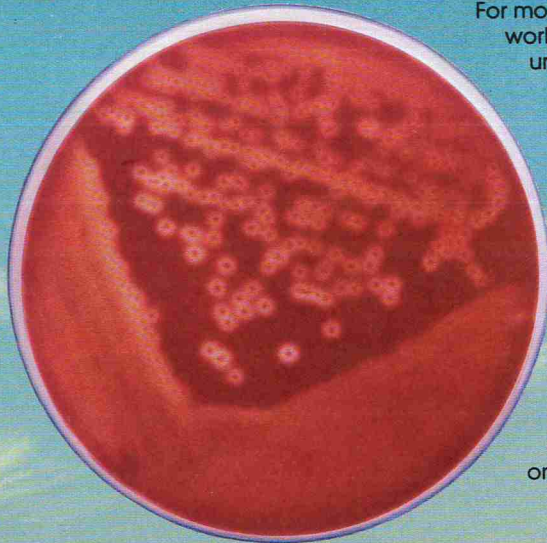
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Book Review

Large Dairy Herd Management C. J. Wilcox and H. H. Van Horn. University Presses of Florida, Gainesville, Florida 32603. 1978. 1046 p., illus. $29.95.

Large Dairy Herd Management contains proceedings of the Large Dairy Herd Management Symposium held at the University of Florida, Gainesville, in January 1976.

This book is the first authoritative work on management of industrialized dairy herds. More than 70 nationally recognized authors contributed chapters on the basic aspects of dairy husbandry with special emphasis on genetics, reproduction and nutrition. Information presented ranges from the latest basic research to detailed practical techniques. Additional sections examine the unique economic structure of modern dairy herds including accounting practices, financial management, budgeting, taxation and estate planning.

This volume will be of great interest to owners and managers of large dairy herds as well as to teachers in agriculture education and scientists.

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Effects of Salts, Sugars, and Salt-Sugar Combinations on Growth and Sporulation of an Isolate of *Eurotium rubrum* from Pancake Syrup

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ABSTRACT

An osmoduric-saccharophilic fungus, identified as a strain of *Eurotium rubrum* Konig, Speikermann and Bremer and isolated from a bottle of syrup, showed optimum growth (i.e., increase in colony diameter) on Sabouraud's agar amended with 60% (w/v) sucrose (calculated $a_w = 0.964$) and still grew near optimally at 110% ($a_w = 0.927$). On glucose, fructose or arabinose, optimum growth occurred at 40% (w/v) ($a_w = 0.962, 0.962,$ and 0.954, respectively), but glucose supported better growth than did fructose or arabinose. In the presence of glycerol, optimum growth (i.e., increase in dry weight of mycelium) occurred at a 10% (v/v) concentration ($a_w = 0.972$) and no growth occurred above 35% ($a_w = 0.878$). In general, growth was better with 12-C > 6-C > 5-C > 3-C compounds. The fungus did not grow on concentrations of inorganic salts above 30%; growth on salts was best with (on a w/v basis) 10% KCl ($a_w = 0.957$), 5% NaCl ($a_w = 0.972$) or 10% CaCl$_2$ ($a_w = 0.965$). In the absence of either organic or inorganic solutes, there was essentially no growth. When sucrose and either KCl or NaCl were added together, growth was greater on a salt/sugar mixture than on the same concentration of salt alone, and, at equivalent calculated osmotic pressures and a_w, sucrose alone supported better growth than did any salt/sugar mixture. These data indicate that the fungus has a requirement for, and a tolerance to, high solute concentrations. At equivalent osmotic pressures and a_w, however, sugars supported greater growth than did inorganic salts.

Osmoduric microbes are capable of growing in environments containing concentrations of solutes inhibitory to most microorganisms, and some osmophilic microbes have a requirement for high concentrations of either salt or sugar for growth. Most microorganisms, however, are inhibited by high concentrations of salt (10 to 15%) and/or sugar (50 to 70%) (*11*). Growth and survival of many fungal species in environments containing high concentrations of salt or sugar result, in part, from their resistance to the high osmotic pressures and low water activity (a_w) that inhibit most other microorganisms.

Species of the genus *Aspergillus* are among the more osmoduric fungi, and they are usually capable of growing on media containing high concentrations of salt or sugar. Members of the *Aspergillus glaucus* group, especially, are also noted for their osmophilic properties, and they not only require high solute concentrations but can usually tolerate up to either 20% NaCl or 80% sucrose (*4*). These fungi are often found in environments with high osmotic pressure and low a_w, such as jams, syrups and cured meats (*14*).

The fungus studied in this investigation has been identified as a strain of *Eurotium rubrum* Konig, Speikermann and Bremer (D.I. Fennell and R. D. Goos, personal communication). *E. rubrum* was formerly classified as *Aspergillus rubrum*, a member of the *A. glaucus* group (*14*). The fungus, which was found in a bottle of commercial syrup, was studied to determine its response to various solutes.

METHODS

Isolation of the fungus

The fungus was isolated, after 3 to 4 weeks of incubation on Sabouraud's agar (Difco), from a commercial bottle of syrup containing sugar syrup (82.9%), corn syrup (13.7%), maple sugar syrup (3.0%), potassium sorbate (0.05%), caramel color, vanilla, maltol and artificial flavor.

Media

Sabouraud's agar (Difco) and Sabouraud's broth (Difco) were supplemented with various concentrations of either sucrose, glucose, fructose, arabinose, glycerol, NaCl, KCl, CaCl$_2$, or with mixtures of sucrose and either NaCl or KCl.

Procedures

Agar studies. Plugs were cut, with a sterile cork borer having a 9-mm diameter, from a lawn of the fungus on agar amended with 60% sucrose. The plugs were removed with a sterile spatula, and one plug was inverted in the center of each control (medium only) or experimental (amended with a solute) petri plate. The plates were incubated at 25 ± 2 C, and observations and measurements of the diameter of the colonies were made daily, in four different directions, to the nearest mm. In addition, the degree of sporulation was estimated by the size of the sporulating area and the intensity of the color produced by the spores. All experiments were done in triplicate, and most experiments were repeated several times.

Broth studies. A 0.1-ml spore suspension in sterile saline solution (0.85% NaCl), made by scraping spores from a lawn of the fungus on agar amended with 60% sucrose, was transferred to broth amended with various concentrations of the solutes. The flasks were incubated in a temperature-controlled shaker bath (25 ± 2 C, 100 oscillations/min) for 7 days, and the fungal growth was collected on a tared filter membrane (Millipore, 0.45 μm), which was dried at 105 ± 2 C for 24 h and weighed to the nearest 0.1 mg.

All data were analyzed statistically and expressed as the mean ± standard error.

Calculation of osmotic pressure and a_w.

Theoretical osmotic pressures were calculated using the formula, $\pi = inRT/V$, where π is the osmotic pressure in atmospheres, i is the

van't Hoff factor, n is the number of moles of solute, R is the universal gas constant, T is the absolute temperature, and V is the volume in liters (8).

Water activity values for the various concentrations of individual solutes were obtained from standard tables (12,13) or, where not available, were approximated using the formula, $a_w = P/P_0 = n_2/n_1 + n_2$, where P and P_0 are the vapor pressures of the solution and the solvent, respectively, and n_1 and n_2 are the number of moles of solute and solvent, respectively (13). The a_w of the salt/sugar mixtures was computed by subtracting the sum of the differences between 1.000 and the a_w of each salt and sugar concentration from 1.000. The contribution of the Sabouraud's media to the a_w was not included, as both control and experimental systems contained these media.

Scanning electron microscopy

A 5-day-old culture was fixed in 3% glutaraldehyde in cacodylic acid buffer (pH 6.8) at 4 C for 72 h. After fixation, plugs were cut from the fungal lawn and dehydrated in a graded ethanol series from 50 to 100%, dried by the critical point method (2), and coated with carbon and gold/palladium (60%/40%) to an average thickness of 15 nm. The plugs were mounted on specimen stubs and viewed with a scanning electron microscope (Advanced Metals Research Model 1000) at a beam specimen angle of 45° and an accelerating voltage of 20 Kv.

RESULTS AND DISCUSSION

The fungus grew well only in the presence of carbohydrates and/or inorganic salts. In the absence of any solutes, other than those contained in the Sabouraud's media, there was essentially no growth (e.g., 3 to 4 weeks of incubation were needed just to detect growth in the initial isolation of the fungus on Sabouraud's agar). At equivalent osmotic pressures or a_w, the carbohydrates supported better growth than did the inorganic salts.

Growth of the fungus was best in the presence of sucrose, with optimum growth, both in broth and on agar, occurring at a concentration of 60% (w/v) ($a_w = 0.964$). Growth at concentrations above and below this optimum decreased rapidly in broth but decreased only slightly on agar (Fig. 1). This difference may have reflected the two methods of measurements: growth on agar was measured by an increase in the diameter of the colonies, which did not necessarily indicate an increase in biomass, as did the dry weight measurements from the studies in broth, but only in the spread of hyphae. However, the general agreement between growth on agar (inoculum was mycelial plugs) and in broth (inoculum was spores) indicated that equilibration between the plugs and the agars on which the plugs were placed was not responsible for the results obtained.

Sporulation patterns on agar followed the growth patterns, with the optimum in sporulation occurring also at 60% sucrose and with marked decreases in sporulation above and below this optimum.

Growth on glucose was slightly better than on fructose, although the a_w of both was the same, and maximum growth and sporulation occurred at 40% (w/v) ($a_w = 0.962$) for both sugars (Fig. 1). *Aspergillus umbrosus*, another member of the *A. glaucus* group, also grew best in the presence of 20 to 40% glucose (3). The similarities between growth on fructose and glucose were not
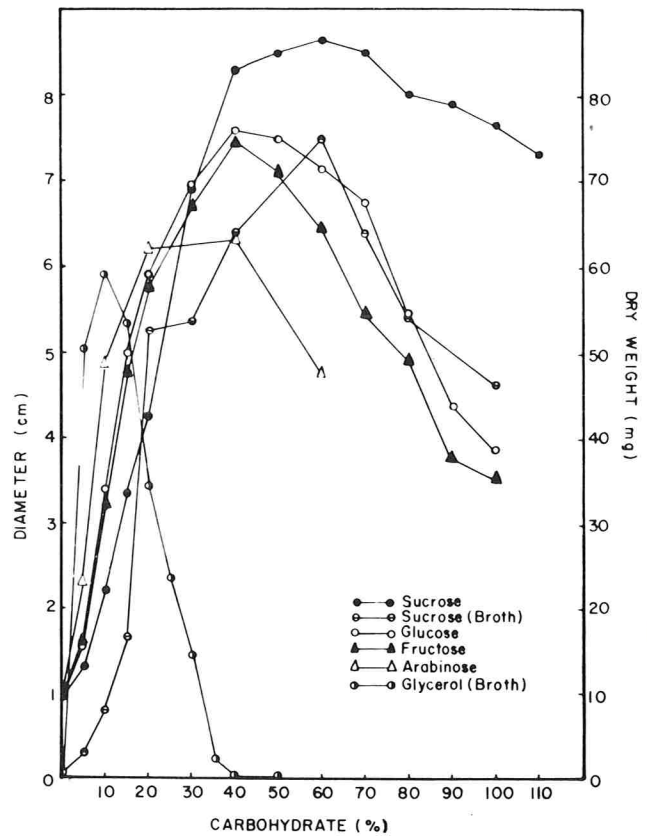
Figure 1. *Growth of* Eurotium rubrum *after 7 days in the presence of various carbohydrates. All concentrations are in % w/v, except glycerol which is in % v/v. The standard error of the mean for the various points ranged between 0.019-0.155. Dry weight measurements made only in sucrose or glycerol broths.*

unusual, as fructose appears to be equivalent to glucose as a substrate for growth of many fungi (5).

E. rubrum grew better in the presence of arabinose than with sucrose, glucose, or fructose at equivalent concentrations up to 20% (w/v); above 20%, however, growth was better in the presence of the other sugars. Maximum growth and sporulation of the fungus on arabinose occurred between 20 and 40% (Fig. 1). The lesser growth with concentrations of arabinose above 20% was not due to the a_w, inasmuch as growth on 80% sucrose ($a_w = 0.950$) or on 50% glucose or fructose ($a_w = 0.952$) was significantly greater than on 40% arabinose ($a_w = 0.954$).

Reductions in growth with concentrations of arabinose, glucose, fructose and sucrose above an optimum concentration apparently occur with numerous fungi (5). The inhibitory effects of the extremely high sugar concentrations may have resulted from (a) some factor (e.g., number of enzyme molecules available, level of phosphate, permeation rate of the sugar) limiting the amount and rate of carbohydrate utilization, with the residual carbohydrate exerting an inhibitory a_w or osmotic pressure, or (b) the initial osmotic pressure of the external cell environment may have been above, and the a_w below, the optimum required for maximum growth, and normal metabolism was inhibited.

Glycerol, in contrast to the saccharides studied, supported maximum growth at a concentration of 10% (v/v) ($a_w = 0.972$) and was inhibitory above 15% ($a_w = 0.956$) (Fig. 1). This inhibition may have been related, in part, to the high viscosity of broths amended with glycerol, which, in turn, influenced the rate of gas exchange. As the concentration of glycerol increased, the viscosity increased, and the rate of gas exchange probably decreased. Inhibition by concentrations of glycerol above 15% was apparently not a result of the a_w, as growth in the presence of 70% glucose ($a_w = 0.934$) or 110% sucrose ($a_w = 0.927$) was substantially better than in the presence of 20% glycerol ($a_w = 0.939$).

The reduction in growth as the length of the carbon chain of the solutes decreased was also apparently not a result of decreases in a_w, as, at equivalent a_w, 12-C > 6-C > 5-C > 3-C in supporting growth of the fungus.

Optimum growth occurred on NaCl, KCl, or $CaCl_2$ at lower concentrations than on the organic solutes, and there was little growth and sporulation above or below the optimum (Fig. 2). KCl and $CaCl_2$ enhanced growth at concentrations between 5 and 15% (w/v), but these salts became inhibitory at concentrations above 15%. However, growth was significantly better in the presence of KCl than of $CaCl_2$, even though the reduction in a_w caused by KCl ($a_w = 0.979$ to 0.914, between 5 and 20%) was slightly greater than that caused by $CaCl_2$ ($a_w = 0.983$ to 0.918, between 5 and 20%). Maximum
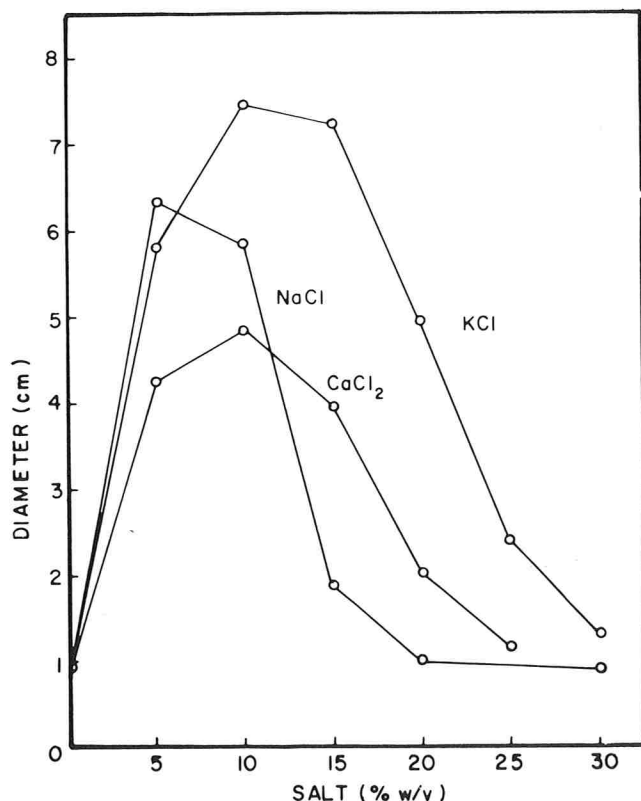
growth and sporulation on agar amended with either KCl or $CaCl_2$ occurred at a concentration of 10% ($a_w = 0.957$ and 0.965, respectively). The inhibition with concentration of KCl above 15% was similar to that observed with other members of the A. glaucus group (6), and that with $CaCl_2$ was similar to that reported with Eremascus albus and presumed to be the result of increased osmotic pressure (10). NaCl enhanced growth at low concentrations (5 to 10%; $a_w = 0.972$ to 0.942) but was inhibitory at concentrations above 10%. Maximum growth and sporulation in the presence of NaCl occurred at a concentration of 5% (Fig. 2).

The differential effects of the various salts and sugars on growth were not the result of differences in the pH of the amended agars, which ranged from pH 4.13 to 5.65, inasmuch as growth and sporulation of the fungus were not statistically different between pH 5.0 and 7.0, and decreased by only 6% at pH 4, 8, or 9.

In the presence of combinations of sucrose and either KCl or NaCl, maximum growth was obtained with 40% sucrose and 5% KCl (although growth was almost as good with 20 or 60% sucrose and 5% KCl) and with 20% sucrose and 5% NaCl (although growth was almost as good with 40 or 60% sucrose and 5% NaCl) (Table 1). At concentrations of salt above 20%, there was essentially no growth, regardless of the sucrose concentration. Mixtures with KCl supported better growth than did those with NaCl. At equivalent a_w, growth was better with sucrose alone than with combinations of sucrose and salt, but the combinations supported better growth than did the salts alone at comparable a_w. Furthermore, growth was significantly better on all mixtures containing sucrose plus 5% NaCl or KCl than on the same concentration of salt alone, even though the a_w of the mixtures was considerably lower. This effect was most pronounced with NaCl.

When agar was amended with sugars or salts to give equivalent calculated osmotic pressures, optimum growth occurred between 50 and 60 atmos, depending on the solute used. The sequence of the amount of growth with the different solutes was sucrose > glucose > fructose > KCl > NaCl > $CaCl_2$ (Fig. 3).

Inhibition of growth with salt concentrations above 10 to 15% was probably related, in part, to the low a_w (< 0.920) and to the absence of a sufficient concentration of carbohydrate. The saccharophilic nature of the fungus was demonstrated by the significantly better growth, at equivalent a_w and osmotic pressures, on carbohydrates than on salts alone and on mixtures of sucrose and either KCl or NaCl than on either salt alone.

The inability of the fungus to grow well on salt concentrations above 20%, regardless of the sucrose concentration, and the observation that, at equivalent a_w and osmotic pressures, growth was better on sucrose than on combinations of sucrose and salt, indicated that the inhibitory action of high salt concentrations was not totally counter-acted by sucrose. Although the fungus was more tolerant of low a_w in the presence of high

Figure 2. Growth of Eurotium rubrum after 7 days in the presence of various inorganic salts. The standard error of the mean for the various points ranged between 0.014-0.271.

TABLE 1. *Growth of* Eurotium rubrum *after 7 days in the presence of various concentrations of sucrose, salt, or sucrose/salt mixtures.*

Sucrose concentration (% w/v)	NaCl concentration (% w/v)	KCl concentration (% w/v)	Water activity (a_w)	Osmotic pressure (atmos)	Growth (cm)
5	0	0	0.997	3.57	$1.32 \pm .051$[a]
0	5	0	0.972	41.80	$6.33 \pm .211$
0	0	5	0.979	32.77	$5.80 \pm .094$
10	0	0	0.995	7.14	$2.19 \pm .034$
0	10	0	0.942	83.60	$5.85 \pm .114$
0	0	10	0.957	65.55	$7.45 \pm .075$
15	0	0	0.992	10.71	$3.33 \pm .066$
0	15	0	0.910	125.40	$1.86 \pm .085$
0	0	15	0.936	98.32	$7.24 \pm .271$
20	0	0	0.989	14.28	$4.23 \pm .149$
0	20	0	0.876	167.20	$0.99 \pm .023$
0	0	20	0.914	131.10	$4.93 \pm .096$
20	5	0	0.961	56.08	$6.95 \pm .050$
20	0	5	0.967	47.05	$7.52 \pm .027$
20	10	0	0.930	97.88	$6.01 \pm .048$
20	0	10	0.945	79.83	$7.05 \pm .050$
20	15	0	0.898	139.68	$3.42 \pm .037$
20	0	15	0.924	112.68	$6.18 \pm .080$
20	20	0	0.864	181.48	$1.08 \pm .039$
20	0	20	0.902	145.38	$4.60 \pm .065$
30	0	0	0.983	21.42	$6.91 \pm .092$
0	30	0	0.802	250.80	$0.90 \pm .0$
0	0	30	0.870	196.65	$1.29 \pm .053$
40	0	0	0.977	28.56	$8.31 \pm .047$
40	5	0	0.949	70.36	$7.15 \pm .065$
40	0	5	0.956	61.33	$7.76 \pm .178$
40	10	0	0.919	112.16	$5.07 \pm .064$
40	0	10	0.936	94.11	$7.08 \pm .045$
40	15	0	0.887	153.76	$2.54 \pm .015$
40	0	15	0.913	126.88	$5.65 \pm .040$
40	20	0	0.853	195.76	$1.13 \pm .043$
40	0	20	0.891	159.66	$4.49 \pm .050$
50	0	0	0.971	35.70	$8.50 \pm .0$
60	0	0	0.964	42.84	$8.66 \pm .0$
60	5	0	0.936	84.64	$6.85 \pm .050$
60	0	5	0.943	75.61	$7.47 \pm .074$
60	10	0	0.906	126.44	$3.89 \pm .022$
60	0	10	0.921	108.39	$6.07 \pm .067$
60	15	0	0.874	168.24	$2.01 \pm .023$
60	0	15	0.900	141.16	$4.56 \pm .072$
60	20	0	0.850	210.04	$0.90 \pm .0$
60	0	20	0.878	173.94	$2.93 \pm .064$
70	0	0	0.957	49.98	$8.50 \pm .0$
80	0	0	0.950	57.12	$8.00 \pm .150$
90	0	0	0.942	64.12	$8.00 \pm .100$
100	0	0	0.935	71.40	$7.64 \pm .131$
110	0	0	0.927	78.54	$7.31 \pm .114$

[a]Mean diameter growth $\pm$ S.E.M.

carbohydrate concentrations, the a_w of mixtures containing salt concentrations above 20% was less than 0.900 and probably below the tolerance level of the fungus, regardless of the carbohydrate concentration.

With all solutes, the appearance of spores was delayed as the solute concentration increased. The color of the sporulation areas on agar containing sugars or low concentrations of NaCl or KCl (up to 10%) was yellow-orange, but, above 10% NaCl or KCl, the sporulation areas were primarily blue-green. Microscopic examination (1000 ×) revealed that the color change corresponded to a change in the predominant reproductive structures of the fungus. The yellow-orange color was due to production of cleistothecia and ascospores, whereas, with higher concentrations of NaCl or KCl, sexual reproduction was inhibited and production of conidia, which were blue-green, was predominant. These light microscopic observations were confirmed by scanning electron microscopic examination, which showed the characteristic asexual and sexual reproductive structures of the genus *Eurotium* (7). Conidial heads, containing elliptical and spiny conidiospores, and cleistothecia exhibiting a mosaic walled structure and bivalved ascospores with a ridge around the central groove, were observed (Fig. 4 to 7). Cleistothecia were predominant in the yellow-orange sporulation areas, whereas essentially only conidia were present in the blue-green areas.

The fungus studied was tolerant of extremely high osmotic pressures (computed to be as high as 110 to 115 atmos) and low a_w (computed to be as low as 0.900) produced by the presence of sugars and inorganic salts. However, at equivalent calculated osmotic pressures and
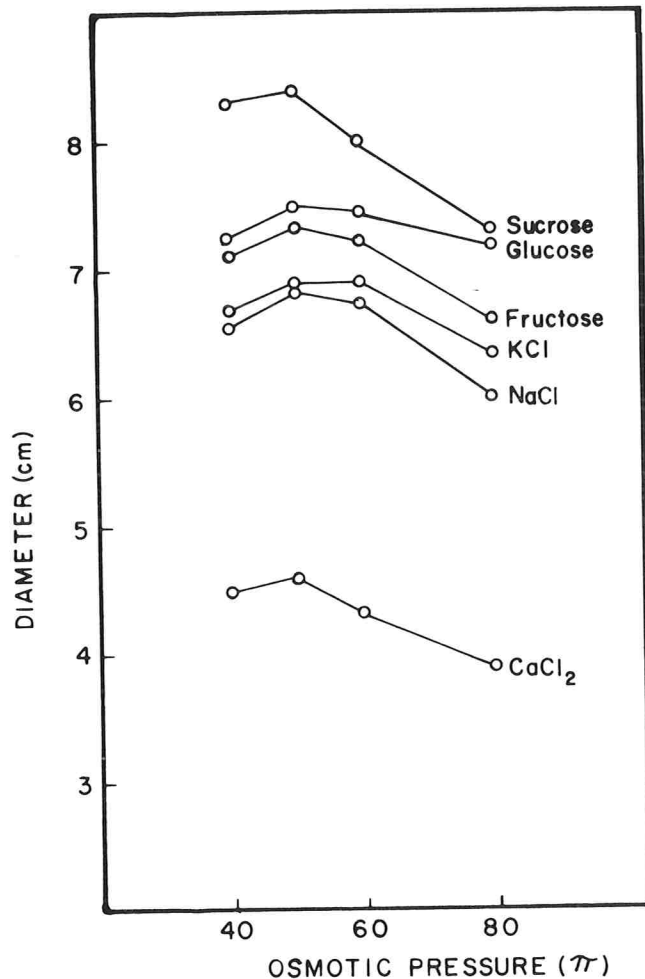
Figure 3.   *Growth of* Eurotium rubrum *after 7 days in the presence of various carbohydrates and salts at concentrations to give equivalent computed osmotic pressures (in atmospheres). The standard error of the mean for the various points ranged between 0.026-0.144.*

Figure 4.   (Top) *Scanning electron micrograph of conidiophore with many elliptical, spiny conidiospores.* × *4,000.*

Figure 5.   (*Bottom*) *Scanning electron micrograph of mycelium exhibiting many mosaic walled cleistothecia.* × *180.*

a_w, sugars, at osmotic pressures below 115 atmos and at a_w above 0.900, supported greater growth than did inorganic salts. This indicated that neither the a_w nor the osmotic pressure alone affected growth of *E. rubrum* but that the type of solute was also important, in agreement with results obtained with other fungi (*1,9*). Whether the tolerance of this fungus is to low a_w (i.e., the ability to generate sufficient energy to utilize bound water) or to high osmotic pressure (i.e., to withstand the exit of water from the internal to the external cell environment: plasmolysis) cannot be determined from this study. Nevertheless, this tolerance of low a_w and/or high osmotic pressure and the requirement for high sugar concentrations apparently enable this fungus to survive and grow in environments (e.g., syrup) that are too harsh for most other microorganisms.

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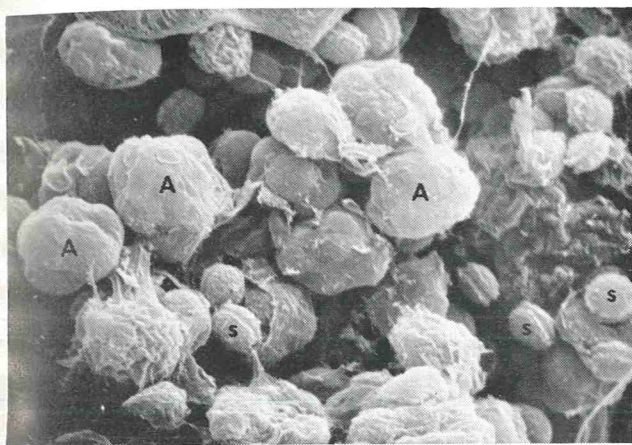
Figure 6. *Scanning electron micrograph of ruptured cleistothecium showing many asci (A) and ascospores (S).* × *4,000.*
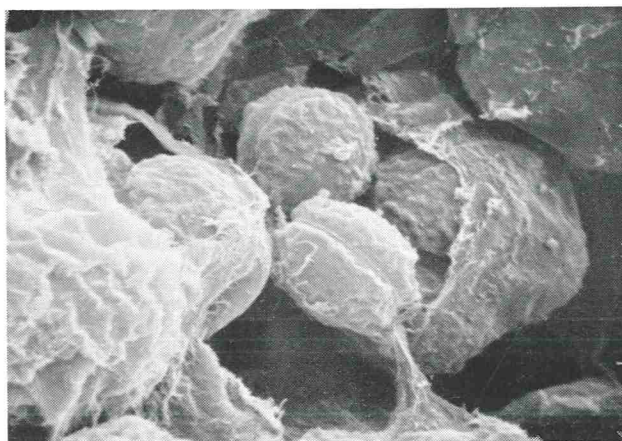
Figure 7. *Scanning electron micrograph of ruptured ascus releasing bivalved ascospores.* × *14,000.*

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Coliforms and *Enterobacteriaceae* Isolates from Selected Foods

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ABSTRACT

We obtained coliform counts and *Enterobacteriaceae* counts using violet red bile agar (VRB) and VRB + 1% glucose (VRBG), respectively, of samples of five food products. From each set of VRB and VRBG plates, 28 to 40 "typical" colonies were randomly selected and identified by use of the R-B Enteric Differential System. A pure culture of each isolate was also subjected to the sequential tests for gas production in LST and BGLB broths (confirmed coliforms) and in EC broth at 45.5 C (fecal coliforms). IMViC reaction patterns of EC-positive cultures were also determined. Approximately 80% of the VRB isolates from broiler skin and from mechanically deboned poultry meat (MDPM) met all the criteria for fecal coliforms, whereas only 62.5% and 36.5%, respectively, of the VRBG isolates from these two products met these criteria. Fewer than 10% of the VRB and VRBG isolates from chicken pot pie, ground beef, or pork sausage produced gas in LST broth. The percentages of fecal coliforms and *Escherichia coli* (Type I or II) among the 179 VRB isolates were 34.1 and 33.5, respectively. Corresponding percentages for the 193 VRBG isolates were 20.7 and 19.7. *E. coli* was the predominant species isolated on both media from broiler skin and MDPM. *Enterobacter agglomerans* was the principal species isolated from chicken pot pie and pork sausage; *Serratia liquefaciens* predominated in ground beef.

Use of an *Enterobacteriaceae* count in place of the traditional coliform or "coli-aerogenes" count is gaining favor in a number of European research laboratories as an indicator of the "sanitary" or "hygienic" quality of raw foods (*11,12,15,19*). The solid medium recommended for recovery and enumeration of this family of bacteria in a food sample is violet red bile agar (VRB) to which 1% glucose (VRBG) is added (*16*). Information concerning the types of bacteria that form "typical" coliform colonies on VRB, as well as the relation of the "coliform count" obtained with this medium to the number of confirmed coliforms, fecal coliforms, and/or *Escherichia coli* in a food product has been reported (*5,7-10,18,21*). A review of the literature, however, failed to reveal corresponding information relating to VRBG and the *Enterobacteriaceae* count.

Obviously, a great number of factors will influence a decision to substitute an *Enterobacteriaceae* (VRBG) count for a coliform (VRB) count in the routine examination of foods. One of the most important factors, particularly when the purpose of the examination is to estimate the degree of fecal contamination in raw foods, is the relation of numbers of fecal coliforms or *E. coli* to each count. Information concerning the relative proportions of other types of bacteria on both VRB and VRBG

media from the same food products also should contribute to a better understanding of the significance of both these indicator groups. With the above considerations in mind, we undertook this study.

EXPERIMENTAL PROCEDURES

Two samples of each of five foods purchased at a local supermarket were used as sources for the isolates for this study. The food items were broiler carcasses, frozen chicken pot pie, frozen mechanically deboned poultry (chicken) meat (MDPM), ground beef, and pork sausage.

Fifty grams of each sample and 450 ml of 0.1% peptone were homogenized in a Waring Blendor for 1 min. For the chicken sample, skin only was used. An aerobic plate count (APC), by use of duplicate pour plates of standard methods agar (BBL) incubated at 20 C for 72 h, was obtained for each sample.

To estimate the coliform count, appropriate aliquots of the serial dilutions were pour-plated with VRB agar (Difco). The overlayed plates were incubated at 35 C for 24 h. To estimate the *Enterobacteriaceae* count, the same procedure was used except that aliquots were pour-plated with VRBG (*5*).

On both media, all obviously typical or characteristic colonies, i.e., red or purple colonies with diameters > 0.5 mm surrounded by a zone of precipitated bile, were counted. However, because of the reports by Hartman (*10*) and Jones et al. (*13*) that colony size alone is not an adequate criterion for selecting coliform colonies on VRB, colonies judged to be "borderline" also were counted. All counts were reported as logarithmic averages and expressed as number of bacteria per gram.

By use of a numerical grid (*2*) and a table of random numbers (*20*), 20 colonies (or as many as could be obtained up to 20) were selected from both the VRB and the VRBG plates for each of the two samples of food. Each colony was inoculated into each of the four tubes of media that comprise the "R-B Enteric Differential System" (Diagnostics Research, Inc). All four tubes of media were successively inoculated without retouching the colony. After 24 h of incubation at 35 C, the results of the biochemical tests obtained from the four tubes were recorded and entered into the Enteric Analyzer (Diagnostic Research, Inc.) to identify the organism to species. Each isolate was also confirmed to be oxidase-negative by Kovac's oxidase test (*13*). Isolates that were not readily identified by this system were subjected to additional conventional tests (*3*). In this study, species names are in accordance with the nomenclature and classification recommended by Ewing (*4*).

A loopful of the culture of each isolate from Tube #1 of the R-B system was streaked onto plates of brain heart infusion agar (BHI) and incubated at 35 C for 24 h. Purity of the culture was confirmed visually and by microscopic examination of a gram-stained smear. If not pure, isolated colonies were once again picked and inoculated into the R-B system and purity was determined again. A colony from each pure culture was then picked and streaked onto the surface of a BHI agar slant, incubated for 24 h at 35 C and then maintained at 4 C. A loopful of a 24-h BHI broth culture, prepared from the stock culture, was used to test for production of gas in lauryl sulfate tryptose broth (LST) after 24 and 48 h of incubation at 35-37 C. Growth from LST-positive (48 h) tubes was transferred to tubes of brilliant green lactose bile (BGLB) broth and incubated for 24 and 48 h at 35-37 C. Growth from

BGLB-positive (48 h) tubes was transferred to tubes of EC broth and incubated in a 45.5 ± 0.5 C water bath for 48 h. Isolates of EC-positive cultures showing a + + − − or a − + − − IMViC reaction pattern were judged to be *E. coli* (6).

RESULTS AND DISCUSSION

The coliform and *Enterobacteriaceae* counts and the aerobic plate counts for the food products examined are given in Table 1. Bacterial counts obtained by using VRB did not differ significantly (p < .01) from those using VRBG. Large numbers of both indicator groups, on the order of logs 4.4-5.5/g, were found in the ground beef and pork sausage samples. These latter samples also exhibited rather large APC values.

TABLE 1. *Aerobes (APC), coliforms (VRB), and* Enterobacteriaceae *(VRBG) in food products sampled (logarithms of number per gram sample).*

Product	Sample No.	APC	VRB	VRBG
Broiler skin	1	5.8	2.0	2.0
	2	5.8	1.8	2.1
Chicken pot pie	1	3.8	2.6	2.2
	2	3.7	1.6	2.3
MDPM	1	5.1	2.6	2.6
	2	5.1	2.7	2.6
Ground beef	1	7.3	4.6	4.7
	2	7.6	4.7	4.5
Pork sausage	1	7.9	4.4	4.5
	2	8.8	4.5	5.5

Results of the sequential gasometric coliform tests of the VRB and VRBG isolates from each of the food products obtained after 48 h of incubation at the appropriate temperature are presented in Table 2. All 33 of the broiler skin and 35 of the 40 (88%) MDPM isolates from VRB produced gas in LST; when they were subcultured from this broth they also produced gas in BGLB. Only very small percentages of the VRB isolates from the chicken pot pie, ground beef and pork sausage, however, produced gas in LST or BGLB broths, indicating that relatively large numbers of non-coliforms present in these products had grown on the VRB plates. Approximately 80% of the VRB broiler skin and 90% of the MDPM isolates that were positive for both LST and BGLB produced gas in EC broth at 45.5 C and were therefore classified as fecal coliforms. None of the chicken pot pie or ground beef isolates and only three of the pork sausage cultures met the criteria for fecal coliforms.

A similar pattern was observed with the VRBG isolates although the percentages of confirmed and fecal coliforms were lower, e.g., 61 (34.1%) of the 179 VRB isolates were EC-positive whereas only 40 (20.7%) of the 193 VRBG isolates produced gas in this broth. Thus, despite the similarity in counts obtained, there were apparently quantitative and/or qualitative differences in the microflora that formed colonies on these two media.

An analysis of the distribution of the IMViC types among the EC-positive isolates (Table 3) revealed that all but one of the VRB and two of the VRBG isolates were *E. coli* type I or II.

Table 4 shows the frequency distribution of species of

TABLE 2. *Numbers of coliform (VRB) and* Enterobacteriaceae *(VRBG) isolates that produced gas in LST, BGLB and EC broths.*

Product	Medium	No. of isolates	No. positive		
			LST	BGLB	EC
Broiler skin	VRB	33	33	33	26
	VRBG	40	38	37	25
Chicken pot pie	VRB	28	1	1	0
	VRBG	40	2	2	0
MDPM	VRB	40	35	35	32
	VRBG	39	19	19	14
Ground beef	VRB	39	5	4	0
	VRBG	40	0	0	0
Pork sausage	VRB	39	3	3	3
	VRBG	34	4	1	1
Total (%)	VRB	179 (100)	77 (43)	76 (43)	61 (34)
Total (%)	VRBG	193 (100)	63 (33)	59 (30)	40 (21)

TABLE 3. *IMViC types among EC-positive isolates from VRB and VRBG media.*

Isolates	VRB	VRBG
Total no. of isolates	179	193
No. EC-positive	61 (34.1%)[a]	40 (20.7%)
No. IMViC type		
E. coli	60 (33.5%)	38 (19.7%)
+ + − − (type I)	58	35
− + − − (type II)	2	3
Other		
+ + + +	0	1
+ + − +	0	1
+ − + −	1	0

[a]Numbers in parentheses are percents of total numbers of isolates.

Enterobacteriaceae, as identified by the R-B system, among the VRB and VRBG isolates. The 118 isolates (75 of the VRB and 43 of the VRBG) identified as *E. coli* by the R-B system included 91 of the 98 (93%) identified as *E. coli* type I or II by the IMViC system (Table 3). The remaining seven were identified by the R-B system as follows: from VRB one *Serratia liquefaciens*; and from VRBG two *Enterobacter agglomerans*; two *S. liquefaciens*; one *Klebsiella ozaenae*; and one *Enterobacter aerogenes*. The three non-*E. coli* fecal coliforms were identified as *S. liquefaciens* (+ + + +), *Citrobacter freundii* (+ + − +), and *Proteus mirabilis* (+ − + −).

On both media, the predominant non-*E. coli* species were *E. agglomerans*, (which constituted the largest percentage of the chicken pot pie and pork sausage isolates), and *S. liquefaciens*, (the most common isolate found in the ground beef samples). The proportions of total *E. agglomerans* isolated from the two media were approximately equal (37% on VRB; 36% on VRBG). *S. liquefaciens*, however, constituted a lower percentage of the VRB (14%) than the VRBG (36%) isolates. One hundred thirty of 135 isolates identified as *E. agglomerans* and 87 of 94 identified as *S. liquefaciens* were unable to produce gas in LST. The ability of these species to produce acid from glucose could account for the formation of characteristic colonies on VRBG media. The formation of "typical" colonies by these species on VRB media, however, cannot be explained unless, as suggested by Ray and Speck (17), carbohydrates in the food from which isolations are made, were fermented

producing sufficient acid to give rise to the characteristic colonies. Small amounts of other substances such as yeast extract in VRB media might also serve this function.

Possible reasons for the greater number of isolates identified as *E. coli* by R-B, than by the EC and IMViC tests include (*a*) the narrow "colimetric" definition of this species, which excludes strains that may be lactose-negative, anaerogenic, or sensitive to elevated temperatures or exhibit variant IMViC patterns, and/or (*b*) incorrect identification of species by the R-B system, i.e., disagreement with the identity as might have been determined by conventional single-tube biochemical testing. This latter possibility, of course, would also apply to the identification of the non-*E. coli* species as well, particularly to members of the *Klebsiella-Enterobacter-Serratia* group (*1*).

Our results support Hartman's conclusion (*10*) that the percentages of confirmed coliforms among VRB isolates vary markedly with the food products being examined and indicate that this conclusion also applies to VRBG isolates. Our data also suggest that, in many instances, the use of an *Enterobacteriaceae* count, instead of a coliform count, as an index of sanitary quality of raw foods could actually be counterproductive, for the *Enterobacteriaceae* count includes greater proportions of non-coliform species, thus resulting in lower proportions of fecal coliforms and *E. coli*. It follows that knowledge concerning the composition of the *Enterobacteriaceae* flora of a food product at various stages of processing and distribution is essential to evaluate the significance of either count.

ACKNOWLEDGMENTS

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TABLE 4. *Frequency distribution of* Enterobacteriaceae *identified by the R-B system in VRB and VRBG isolates from the same food samples.*

Product	No. of isolates	Escherichia coli	Enterobacter agglomerans	Serratia liquefaciens	Citrobacter freundii	Klebsiella ozaenae	Klebsiella pneumoniae	Serratia marcescens	Proteus mirabilis	Enterobacter cloacae	Enterobacter aerogenes	Enterobacter hafniae	Arizonae hinshawii	Salmonella sp.
VRB Isolates														
Broiler Skin	33	30	0	1	0	0	0	0	1	0	0	0	1	0
Chicken Pot Pie	28	9	23	3	0	0	0	1	0	0	0	0	0	0
MDPM	40	32	2	2	3	0	1	0	0	0	0	0	0	0
Ground Beef	39	9	10	16	1	2	0	0	0	0	1	0	0	0
Pork Sausage	39	3	32	3	0	1	0	0	0	0	0	0	0	0
TOTAL	179	75	67	25	4	3	1	1	1	0	1	0	1	0
VRBG Isolates														
Broiler Skin	40	27	3	6	0	1	1	1	0	0	1	0	0	0
Chicken Pot Pie	40	0	37	0	1	0	0	0	0	1	1	0	0	0
MDPM	39	15	11	11	2	0	0	0	0	0	0	0	0	0
Ground Beef	40	0	1	35	0	0	0	0	0	0	1	2	0	1
Pork Sausage	34	1	16	17	0	0	0	0	0	0	0	0	0	0
TOTAL	193	43	68	69	3	1	1	1	0	1	3	2	0	1
GRAND TOTAL	372	118	135	94	7	4	2	2	1	1	4	2	1	1

Comparison of Two Methods and Improvements for Colorimetric Determination of Nitrite in Cod Roe

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ABSTRACT

Attempts were made to develop a sensitive and reproducible method to determine nitrite in cod roe. Two diazotation-coupling reaction methods were considered; (a) the method defined by the Ministry of Health and Welfare of Japan (Method 1) and (b) the reference method of ISO (Method 2). Since the nitrite content in cod roe was much less than in meat products, Method 2 was modified to make it suitable for microanalysis at 1 ppm level as NO_2. Modifications included reducing volumes of color-development solutions and making changes in the color development process, thus making the color intensity four times as great as before. Carrying out corrections with both reagent and water blanks made the effect of the blank on measured values negligible. Recoveries of nitrite at 20- and 2-ppm levels were 94.7 and 88.1%, respectively, reproducibility being ± 7.9%, as the coefficient of variation. The obtained values by the modified method were, on the average, higher than those of the original method by 37.1%. Nitrite contents obtained by Method 1 were lower than those by the original Method 2. These low values might be attributed to loss of nitrite during extraction from the sample without pH adjustment, since the measured value showed a remarkable increase by addition of alkaline solution before extraction. Nitrite contents in imported cod roe were within the range 0.16-1.03 ppm expressed as NO_2.

Mentaiko is a kind of salted cod roe usually colored with new coccine or other red dye and students often eat it along with rice for their lunch. It is a product of Hakata and Kita-kyusyu districts of Japan and salted cod roe used as the raw material is imported from South Korea; the quantity of salted marine products that passed through the harbors of Shimonoseki and Moji during 1977 amounted to 1,296.3 tons. In Japan, use of nitrite (sodium nitrite) as a food additive for color fixative and for other purposes is limited. Concerning fish roe, it is used only with salmon roe (sujiko and ikura) with the residual level of 5 ppm as NO_2; its use on cod roe is strictly prohibited. It is well known, however, that cod roe contains small amount of nitrite as its own component.

The objectives of this work were to modify an existing nitrite method to use for determination of small amounts of naturally-occurring nitrite in cod roe, to show the validity of this method through recovery tests, to compare this method with the prescribed method now in use, and to demonstrate the usefulness of the method by use on cod roe samples.

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MATERIALS AND METHODS

Methods

It is prescribed in the Sanitary Inspection Guide (*1*) that the nitrite content in salmon and cod roe should be measured by use of both uranyl acetate and zinc acetate as precipitants. The former substance, however, is designated as one of the nuclear fuels and one must apply to the Director-General of the Science and Technics Agency for sanction every time he wishes to obtain it from a supplier. Harada (*2*) made it clear that mercuric chloride is an excellent precipitant applicable to roe, but use of this substance is not recommended because of its toxicity.

Accordingly, it was undertaken by us to apply the already established methods to determine nitrite in meat products for determination of the micro-quantity of nitrite in cod roe. Two methods are now available for this purpose; one is the analytical method for food additives in foodstuff authorized by the Food Chemistry Division of the Ministry of Health and Welfare of Japan (*3*), while the other is the international standard method established by ISO (International Standardization Organization) and adopted in our laboratory for inspection of imported meat products (*4*). Both methods are based on colorimetry of nitrite by the diazotation-coupling reaction though the former uses zinc sulfate-sodium hydroxide while the latter employs the Carrez reagent as the precipitant. The details of the procedure are as follows.

Method 1 (method of Ministry of Health and Welfare)

Reagents. 0.5 N NaOH, 12% $ZnSO_4 \cdot 7H_2O$ solution, 10% ammonium acetate buffer (dissolve 100 g of guaranteed ammonium acetate in 900 ml of water, adjust pH to 9.0 with 10% ammonia water, and make to 1,000 ml with water), 1% ammonium acetate buffer (dilute buffer) (dilute 10% ammonium acetate buffer to one tenth concentration and readjust the pH to 9.0 with 10% ammonia water), sulfanilamide solution [dissolve 0.5 g of guaranteed sulfanilamide in 100 ml of hydrochloric acid (1 + 1), stable for 4 weeks], naphtylethylenediamine solution (dissolve 0.12 g of N-(1-naphtyl)-ethylenediamine (guaranteed) in 100 ml of water, filter if necessary; keep in a refrigerater in a brown bottle; stable for 2 weeks), nitrite-nitrogen standard solution (weigh accurately 0.493 g of sodium nitrite previously dried for 24 h over sulfuric acid in a desiccator, dissolve in sterile water to make 1,000 ml; standard stock solution).

Take 10 ml of the standard stock solution, add water to make 100 ml, then take 2 ml from the 100 ml solution, add 10 ml of 10% sodium acetate solution and water to make 100 ml, and use thus prepared solution as the nitrite-nitrogen standard solution (prepare freshly before use). One ml of nitrite-nitrogen standard solution = 0.2 μg NO_2-N.

Preparation of sample solution. Weigh 10.0 g of sample cut in pieces, add adequate volume of water at about 80 C and homogenize. Pour the contents into a volumetric flask of 200 ml, wash the contents several times with warm water and add the washings to the same flask. Add 10 ml of 0.5 N NaOH, shake well, then add 10 ml of 12% $ZnSO_4 \cdot 7H_2O$ solution, shake well and then heat for 20 min in a water bath at 80 C, shaking occasionally. Cool to room temperature in cold water, add 20 ml of 10% ammonium acetate buffer, make to 200 ml with water, mix the contents well, filter through a dry filter paper for quantitative analysis (Toyo Filter Paper No.5C) into a ground stoppered conical

flask, discard the first 10 ml of filtrate and use the obtained clear filtrate as a sample solution. Carry out the procedure by use of 10 ml of water instead of sample to prepare a blank solution.

Determination. Place each 20 ml of sample solution and blank solution into separate volumetric flasks (25 ml) a, b, respectively; to each flask add 1 ml of sulfanilamide solution, mix, add 1 ml of naphtylethylenediamine solution, make to 25 ml with water, mix the contents well and let color develop. Place the same quantity of sample solution into another volumetric flask (25 ml) c, add 1 ml of dilute HCl (1 + 1) and water to make 25 ml, mix the contents well and measure the optical densities of contents of flasks a, b and c, after 20 min, at the wavelength of 540 nm using water as a reference. Aa: O.D. of flask a, Ab: O.D. of flask b, and Ac: O.D. of flask c.

Substract the sum of Ab and Ac from Aa, read the corresponding concentration from the calibration curve, and calculate the nitrite content in the sample (C_{NO_2}) by the following formula.

$$C_{NO_2} \text{ (ppm)} = 3.28 \text{ A}$$

where A is the nitrite-nitrogen content in 20 ml of sample solution

Preparation of calibration curve. Place 0, 2, 5, 10, 15 and 20 ml of nitrite-nitrogen standard solutions into separate volumetric flasks of 25 ml, to each flask add dilute buffer to make to about 20 ml, add 1 ml of sulfanilamide solution, mix, add 1 ml of naphtylethylenediamine solution and water to make 25 ml, and mix the contents well. Place 20 ml of diluted buffer into another volumetric flask of 25 ml, then proceed the same as for the standard solution to prepare the blank solution. After 20 min, measure the optical density of each solution at the wavelength of 540 nm using this blank solution as a reference. Prepare a calibration curve.

Method 2 (reference method of ISO)

Reagents. Reagent I (dissolve 106 g of potassium ferrocyanide trihydrate [$K_4Fe(CN)_6 \cdot 3H_2O$] in water and dilute to 1,000 ml), Reagent II (dissolve 220 g of zinc acetate dihydrate [$Zn(CH_3COO)_2 \cdot 2H_2O$] and 30 ml of glacial acetic acid in water and dilute to 1,000 ml), borax solution, saturated (dissolve 50 g of sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) in 1,000 ml of tepid water and cool to room temperature), sodium nitrite standard solution (dissolve 0.150 g of sodium nitrite ($NaNO_2$) in water and dilute to 1,000 ml in a volumetric flask, pipette 10 ml of the solution into a 1,000-ml volumetric flask and dilute to the mark; 1 ml of this solution contains 1.0 μg of NO_2 per ml; the standard solution shall be made on the day of use), color development solution I (dissolve, by heating on a water bath, 2 g of sulfanilamide ($NH_2C_6H_4SO_2NH_2$) in 800 ml of cold water, filter, if necessary, and add 100 ml of HCl, while stirring, dilute to 1,000 ml with water), color development solution II (dissolve 0.1 g of N-1-naphtylethylene diamine dihydrochloride ($C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$) in water, dilute to 100 ml with water), and color development solution III (dilute 445 ml of hydrochloric acid to 1,000 ml with water.

Store the color development solutions in well-stoppered brown bottles. They shall be kept in a refrigerator for not longer than 1 week.

Procedure. Proceed from a representative sample of at least 200 g. Weigh, to the nearest 0.001 g, about 10 g of homogenized sample. Transfer the test portion quantitatively into a 300-ml conical flask, and add successively 5 ml of saturated borax solution and 100 ml of water at a temperature not below 70 C. Heat the flask for 15 min on a boiling water bath and shake repeatedly. Allow the flask and its contents to cool to room temperature and add successively 2 ml of reagent I and 2 ml of reagent II. Mix thoroughly after each addition. Transfer the contents to a 200-ml volumetric flask. Allow the flask to stand for 30 min at room temperature. Dilute to the mark with water. Mix the contents of the flask thoroughly and filter through a fluted filter paper.

Pipette a portion of the filtrate, but not more than 25 ml into a 100-ml volumetric flask and add water to obtain a volume of about 60 ml. Add 10 ml of color development solution I, followed by 6 ml of color development solution III; mix and leave the solution for 5 min at room temperature in the dark. Add 2 ml of color development solution II, mix and leave the solution for 3 min at room temperature in the dark. Dilute to the mark with water. Measure the absorbance of the solution in a 1-cm cell at a wavelength of 538 nm.

Pipette respectively into nine 100-ml volumetric flasks 0, 2, 4, 6, 8, 10, 12, 16 and 20 ml of sodium nitrite standard solution and add water to obtain a volume of about 60 ml. Proceed as described above, starting from "add 10 ml of color development solution I...". Draw the calibration curve by plotting the measured absorbances against the concentrations, in μg of NO_2 per 100 ml, of the solutions.

Calculate the nitrite content of the sample, expressed as milligrams of NO_2 per kg, using the formula

$$NO_2 = C \times \frac{200}{m \times V}$$

where

m is the mass, in grams, of the test portion;

V is the volume, in milliliters, of the portion of the filtrate taken for the determination; and

C is the concentration of nitrite (NO_2) in μg per 100 ml, read from the calibration curve.

The difference between the results of two determinations carried out simultaneously or in rapid succession, by the same analyst, shall not be greater than 10% of the assayed nitrite content. Report the result to the nearest 1 mg per kg of the product.

RESULTS AND DISCUSSION

Preparation of calibration curve in lower concentrations of nitrite

In this section, investigations were carried out by use of Method 2. Since the nitrite content of cod roe is, on the whole, much lower compared with that in meat products, the calibration curve has to be prepared at lower concentrations. Besides, it was undertaken to reduce the volume of color developing solution from 100 to 25 ml to increase the color intensity. The modified process of Method 2 is expressed in Fig. 1. The calibration curves obtained are as shown in Fig. 2. Good linearity was obtained by the modified procedure as well as the original procedure, and from the results it is evident that each 1 ml addition of color development solutions I, II and III was enough for determination of less than 5 μg of nitrite. We found it was not necessary to leave the solution for 5 min at room temperature in the dark after addition of color development solutions I and III.

Since only 1 ml each of sulfanilamide and naphtylethylenediamine solutions are used in Method 1, it is impossible to reduce the final volume of the procedure as was done in Method 2.
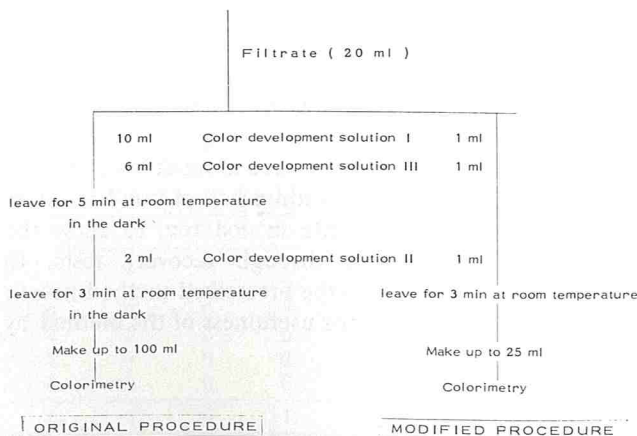
Figure 1.   *Partial modification of Method 2.*
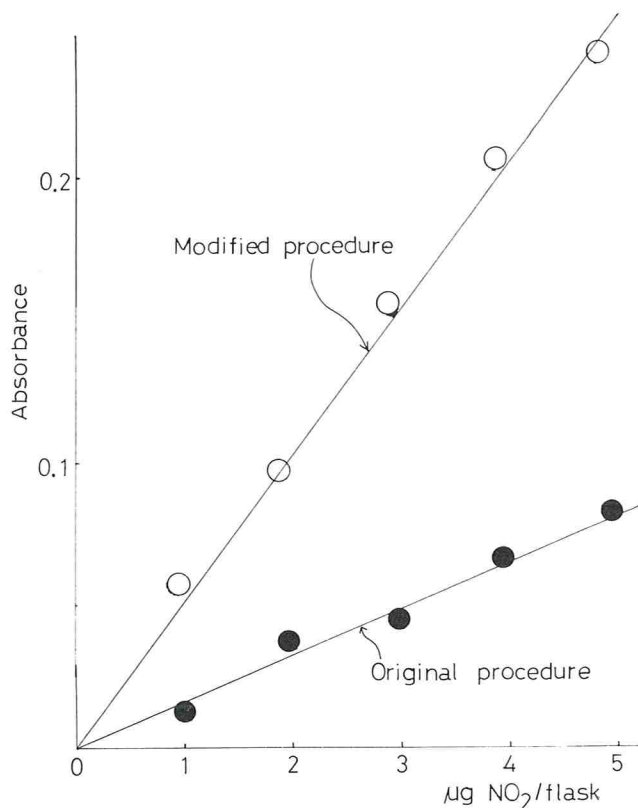
Figure 2. *Calibration curves of nitrites by Method 2.*

Problems with blank values

Three kinds of blank values were measured in the original and modified procedures of Method 2 to compare them to each other. *Absolute blank*: No nitrite-nitrogen standard solution was used in preparation of calibration curve. *Blank 1*: 10 ml of water was used instead of the sample. This value is used to know the degree of color development by reagents other than nitrite. *Blank 2*: The same quantity of water was used instead of color development solutions I and II. This value serves to show the degree of coloration of the sample.

If cod roe is naturally or artificially colored, the blank value 2 will not be negligible. Values of blanks 1 and 2 measured against the absolute blank are as shown in Table 1. Sometimes seemingly high values for nitrite in cod roe were obtained by use of the original procedure. Such a phenomenon, presumably, was partly due to the disturbance by blank values. Contrary to this, the effect

of blank values was below 0.1 ppm as NO_2 in the modified procedure. Reproducibility of determined nitrite contents through the full process of the original and modified procedures of Method 2, expressed as coefficients of variation were 5.2 and 2.9%, respectively.

Recovery of nitrite

Tests were done with Method 2. Two ml of stock nitrite solution (0.1 mg NO_2/ml) or 2 ml of nitrite standard solution was used and both the original and modified procedures were followed. Next the same quantity of nitrite was added to 10 g of cod roe (corresponding to 20 and 2 ppm, respectively) and the spiked samples were subjected to the two analyses. Results are summarized in Table 2. Recoveries were satisfactory for both original and improved procedures either with or without cod roe. At the added level of 2 ppm, however, recovery of the original procedure was low compared with that of the modified procedure, the obtained values being scattered. It was proved that the modified Method 2 was particularly adequate for determination of a micro-quantity of nitrite in cod roe.

TABLE 2. *Recovery of added nitrite by Method 2.*

	Without cod roe		With cod roe	
Added level (ppm No_2) Method 2	20	2	20	2
Recovery (%)				
Original method	99.6	77.5	93.8	73.5
Modified method	98.9	91.5	94.7	88.1

Inspection on imported cod roe

The original and modified Method 2 were applied to 12 samples of imported cod roe; results are in Table 3. Seemingly high values were obtained by use of the modified procedure. No reliable data have been published up to now in Japan on a survey of the natural nitrite content in cod roe.

Comparison of Method 2 versus Method 1

Since Method 1 is presently established as the analytical method for nitrite by the Ministry of Health and Welfare of Japan, a comparison of Method 2 with Method 1 has to be done. Preliminary examination of several samples of cod roe revealed that the values obtained by Method 1 were, on the average, 48.4% lower than those of the original Method 2. Furthermore, recovery of nitrite added at the level of 5 ppm of NO_2 by Method 1 was as low as 58.0%. Since it is agreed that in

TABLE 1. *Comparison of blank values in the original and modified Method 2.*

	Original procedure		Modified procedure	
	O.D. at 538 nm	Equivalent to NO_2 ppm	O.D. at 538 nm	Equivalent to NO_2 ppm
Blank 1[a]	0.004	0.33	0.003	0.06
Blank 2				
Sample No. 1	0.001	0.08	0.001	0.02
Sample No. 2	0.000	0.00	0.003	0.06
Sample No. 3	0.002	0.16	0.005	0.10
Sample No. 4	0.005	0.40	0.005	0.10
Sample No. 5	0.003	0.24	0.002	0.04

[a]Mean value of five trials.

TABLE 3. *Comparison of measured values of nitrite contents in imported cod roe by Method 2.*

| Sample | Nitrite content (NO_2 ppm) | |
	(1) Original procedure	(2) Modified procedure
No.1	0.64	0.73
" 2	0.09	0.16
" 3	0.18	0.37
" 4	0.18	0.26
" 5	0.24	0.39
" 6	0.30	0.37
" 7	0.67	1.03
" 8	0.64	0.69
" 9	0.43	0.61
" 10	0.40	0.80
" 11	0.60	0.60
" 12	0.50	0.50
Mean value	0.41	0.54
[(b)/(a)] × 100	100	131.7

Method 1 the final volume of color developing solution, that started from 20 ml of filtrate, is to be made to 25 ml, it is impossible to reduce any more the final volume of color developing solution. In Method 2, it is described that sample is to be heated at not below 70 C after the pH of the solution has been made alkaline by addition of saturated, borax solution. The test sample is homogenized in Method 1 with hot water at about 80 C before addition of the alkaline solution (0.5 N NaOH), and it is presumed that homogenizing at a slightly acidic pH might lead to a partial decomposition of nitrite. Accordingly, it was undertaken to modify the preparation process of the sample solution in Method 1 to that shown in Fig. 3. In the modified procedure, the sample is heated after addition of the alkaline solution. Results are as shown in Table 4. It became clear that the values for nitrite by Method 1 were doubled by this modification, indicating that a considerable amount of nitrite was lost in the original procedure during heating at 80 C. The obtained values, however, were a little lower than those of the modified Method 2.

Method finally established

Taking into account reproducibility and other factors, we finally decided to choose the modified Method 2. The finally adopted method is as follows:

As for the reagents and solutions used, refer to Method 2. Proceed as described in Method 2 to obtain clear filtrate.

Pipette 20 ml of the filtrate into a 25-ml volumetric flask, add each 1 ml of color development solutions I, III and II in turn, mixing the contents well after each addition. Leave the solution for 3 min at room temperature and then dilute to the mark with water. Measure the absorbance (A) of the solution in a 1-cm cell at a wavelength of 538 nm against a reagent blank prepared in the same manner starting from 10 ml of water. Prepare sample blank from 10 g of the same sample without addition of color development solutions and measure the color intensity at the wavelength of 538 nm
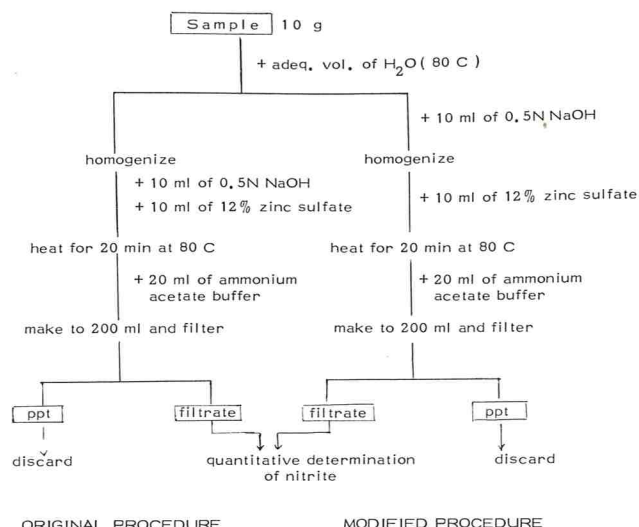
Figure 3. *Partial modification of Method 1.*

TABLE 4. *Comparison of original and modified procedures of Method 1 and Method 2.*

| Sample | Nitrite content (NO_2 ppm) | | | |
| | Method 1 | | Method 2 | |
	Original procedure	Modified procedure	Original procedure	Modified procedure
No. 1	0.27	0.33	0.46	0.47
" 2	0.45	0.99	0.67	1.03
" 3	0.24	0.59	0.43	0.61
Index	100	193.8	162.5	219.8

against a water blank prepared starting with 10 ml of water in the same manner without addition of color development solutions (B). Subtract B from A.

Pipette, respectively, into nine 25-ml volumetric flasks 0, 2, 4, 6, 8, 10, 12, 16 and 20 ml of nitrite standard solution and add water to obtain a volume of about 20 ml. Proceed as described in the measurement of absorbance (A) starting from "add each 1 ml of color development solution I, II and III...", and read the absorbance of each flask against the first solution into which no nitrite was pipetted. Draw the calibration curve by plotting the measured absorbances against the concentrations, in μg of NO_2 per 25 ml of the solution. Calculate the nitrite content of the sample by use of the calibration curve.

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Influence of Exercise and Stage of Lactation on the Milk Somatic Cell Response of Normal Quarters to Adrenocorticotrophin

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ABSTRACT

Intramuscular injections of 200 IU of adrenocorticotrophin (ACTH) for 4 consecutive days failed to elicit detectable increases in the somatic cell content of milk from normal quarters despite large and sustained increases in the concentration of circulating leukocytes. Neither exercise nor stage of lactation had any detectable effect on the milk somatic cell response to ACTH. During midlactation (86 days in milk, 26 kg daily production) milk somatic cell counts for uninfected and *Corynebacterium bovis*-infected quarters averaged 28 and 77×10^3 cells/ml of milk, respectively, and during late lactation (302 days in milk, 5-kg daily production), 28 and 58×10^3 cells/ml of milk. The data suggest that neither ACTH nor late lactation will increase the concentration of milk somatic cells in quarters free from mastitis pathogens. These results support the use of the somatic cell concentration of milk as a criterion on which to assess milk quality.

The basic defense response to irritation of the mammary gland is an increase in milk somatic cells, primarily leukocytes (*19*). By far the most common cause of irritation is bacterial infection (*24*). Because "milk" has been legally defined as the "lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows" (*1*), an excessive somatic cell concentration in the milk has been construed as adulteration. The United States Public Health Service has established 1,500,000 somatic cells/ml as the maximum acceptable concentration in mixed herd milk (*2*). However, the International Dairy Federation (IDF) has established 500,000 cells/ml as the upper limit for normal foremilk from an udder quarter (*3*).

The usefulness of milk somatic cells as an index of milk quality has been questioned because factors other than intramammary infection have been alleged to cause an increase in the somatic cell content of milk (*23,30*). However, more recent studies have shown that neither stress of confinement (*4*), estrus (*10*), high ambient temperature (*21*), nor hormones [corticosteroids and adrenocorticotrophin (ACTH)] associated with stress (*16,18,20*) increased the concentration of somatic cells in milk. In a review by Giesecke and Van den Heever (*9*) of factors contributing to elevated milk somatic cell counts, these authors proposed that the failure to detect elevations in milk somatic cells following injection of corticosteroids or ACTH was attributed to a lack of exercise by cows during administration of these

hormones. As a result, cows were not allowed to satisfy the requirements of the General Adaptation Syndrome (GAS) of Selye (*25*). According to this theory, the elimination of metabolically highly active cells of the udder epithelium seems to be an essential part of the GAS, and for this to occur, cows which are under the influence of ACTH and corticosteroids should be allowed to exercise for the appropriate dissipation of energy and shedding of epithelial cells into milk.

The common assumption is that the concentration of somatic cells in milk increases during late lactation (*6,7*). and that stress imposed during late lactation will increase milk somatic cells additionally (*22*). Recently Smith (*26*) reported increased milk somatic cell count following four daily intramuscular injections of 200 IU ACTH into cows during late lactation, average 5-kg daily milk production and with no evidence of intramammary infection, but not during midlactation.

The objectives of the present study were to determine if the concentration of milk somatic cells would increase in cows following injection of ACTH and allowed free access to exercise, thereby satisfying the requirement of GAS (experiment 1) and to determine if ACTH administered during late lactation would increase the concentration of milk somatic cells (experiment 2).

MATERIALS AND METHODS

Experiment I. Milk somatic cell response to ACTH in cows maintained under loose housing conditions

Ten Holstein cows averaging 176 days in milk and 21-kg daily production were selected. The cows were maintained in a three-sided free stall unit with access to an outside feed bunk where they were group fed ad libitum. Cows were uninfected in all four quarters as determined by twice weekly cultures on blood agar of aseptically collected foremilk samples.

The cows were subjected to three treatment periods: (*a*) 4-day control, (*b*) 5-day injection [four daily intramuscular injections (IM) of 200 units ACTH (Porcine ACTH, Sigma Chemical Company, St. Louis, MO) in 3 ml of saline solution, 1 day for carryover], and (*c*) 4-day control. The ACTH was suspended in 0.85 percent sterile saline solution, and 3 ml were injected intramuscularly.

Milk (100 ml) was collected from the weigh jar at each milking. Duplicate milk films were prepared and processed for the Direct Microscopic Somatic Cell Count (DMSCC) (*14*) and stained with pyronin Y-methyl green stain as previously described (*17*), but with a 20-min differentiation period in the first N-butanol wash. In addition, the Wisconsin Mastitis Test (WMT) (*28*) was conducted on all milk samples.

Results were analyzed using a least-squares anaylsis of variance. The model contained the effects for error, periods and the interaction of cows with periods.

Experiment II. Effect of stage of lactation on the milk somatic cell response to ACTH

Eleven Holstein-Friesian cows averaging 86 days in milk and 26-kg daily production were selected for testing at midlactation. The cows were divided randomly into two groups and placed in controlled environment chambers at 21 C and approximately 60% relative humidity. After a 2-week acclimation period, each group was subjected to five treatment periods: Group I. (a) 4-day control; (b) 5-day injection [four daily intramuscular injections of 200 units ACTH (Porcine ACTH, Sigma Chemical Company, St. Louis, MO.) in 3 ml of saline, 1 day for carry over]; (c) 5-day control; (d) 5-day injection (4 daily intramuscular injections of 3 ml of saline, 1 day for carry over); and (e) 5-day control. In Group II, the ACTH and saline treatment periods were reversed. For testing at late lactation (302 days in milk and 5 kg daily) the cows were reassigned at random into two groups and treated as before.

The cows were allowed 1 h of exercise before milking with a quarter milking machine (DeLaval Separator Company, Poughkeepsie, NY.) at 0930 h (morning milking) and at 2230 h (evening milking). Access to the chambers was restricted to the experimenter and the milker.

Quarter foremilk samples for diagnostic bacteriology were collected aseptically twice weekly before the 0930 milking, beginning 2 weeks before the experiment and continuing until the end of lactation. Quarters were considered infected if the same bacterium was detected in two consecutive samples in cultural examination. All cows were free of clinical mastitis.

At every milking during the midlactation and late lactation treatment periods, duplicate milk films were prepared from well-mixed milk of each udder quarter. Films were processed for the DMSCC.

Mammary vein blood samples were collected at 0800 h during treatment periods 1, 3, and 5 and at 0800 h and 2100 h during periods 2 and 4. Leukocyte counts were made with an electronic cell counter (Model B Coulter Counter, Coulter Electronics Co., Inc., Hialeah, FL.); a 100-μm aperture tube and a threshold setting of 9 were used. Blood smears for differential leukocyte counts were stained with Wright's stain (Fisher Scientific Company, Silver Spring, MD.).

Midlactation and late lactation results were analyzed separately; a least-squares analysis of variance was used. The model for DMSCC and milk yield contained the effects for cow-quarter combinations, periods, AM versus PM milking differences and the interactions of cow-quarters with periods and with AM-PM differences. Cow-quarters were considered random effects. Because milk from *Corynebacterium bovis* infected quarters has a higher concentration of somatic cells than milk from uninfected quarters (17), a condition that may influence the DMSCC response to stage of lactation or to ACTH, they were analyzed separately within each stage of lactation. The model for total and differential circulating leukocytes contained the effects for cows, periods, and AM versus PM milking differences and the interactions of

cows with periods and with AM-PM differences. Cows were considered random, and period and AM versus PM differences were tested against their corresponding interactions with cows in those instances where these interactions were significant. Pooling for *C. bovis*-infected and uninfected cows was based on the view that *C. bovis* is not a mastitis pathogen (5) and that the circulating leukocyte response to ACTH for *C. bovis*-infected and for uninfected cows is similar (20).

RESULTS

Experiment I. Milk somatic cell response to ACTH in cows maintained under loose housing conditions

There was a slight but nonsignificant (P > 0.05) increase in DMSCC during the 5-day ACTH injection period (Table 1). Wisconsin Mastitis Test results remained unchanged (P > 0.05) throughout the three experimental periods. Total daily milk yields decreased significantly (P < 0.01) during the ACTH injection period and tended to increase during the post ACTH injection control period. The concentration of total circulating leukocytes averaged 8,085 cells/cmm during the 4-day control period, increased (P < 0.01) to 19,420 cells/cmm during the ACTH injection period and then returned to preinjection concentration during the following 4-day control period.

Experiment II. Effect of stage of lactation on the milk somatic cell response to ACTH

Cultural examination of the aseptically-collected midlactation foremilk samples indicated that all cows were free of intramammary mastitis pathogens, that 32 quarters cultured positive for *C. bovis*, and that 11 quarters were bacteria-free. During late lactation, one cow was dropped from the experiment because of an injury sustained during the interim period. Sixteen quarters cultured positive for *C. bovis*, and 23 quarters were bacteria-free.

Circulating leukocytes (Table 2) increased in concentration (P < 0.01) during the injection of ACTH in midlactation and late lactation. During midlactation, four daily injections of 200 IU of ACTH increased the concentration of circulating leukocytes 60% for cows in group I and 34% for cows in group II. During late lactation, the concentration of circulating leukocytes increased 23% in group I and 25% in group II. Circulating leukocytes returned to preinjection levels during the 5-day control period. No leukocyte response was observed during the saline solution injection period.

The response in the percentage of circulating lymphocytes and neutrophils was similar for both groups

TABLE 1. *Changes in milk somatic cells, WMT score, circulating leukocytes and milk yield following injection of ACTH (Experiment 1).*[a]

Parameter measured	Periods			Significance level[c]	Coefficient of variation (%)
	4-Day control	5-Day injection[b]	4-Day control		
Somatic cells					
× 10³/ml milk	50	100	70	NS	157
WMT Score (mm)	4.0	3.9	3.8	NS	34
Daily milk yield (kg)	20.8	17.8	18.2	**	22
Total circulating leukocytes					
× 10³/mm³ blood	8.0	19.4	7.5	**	20

[a]Each value represents the mean of 10 cows.
[b]Four daily intramuscular injections of 200 IU ACTH plus 1 day for carryover effects.
[c]Significant F ratios between experimental periods: NS not significant (P > 0.05), ** P < 0.01.

during midlactation and late lactation (Table 2). During the ACTH-injection periods, the percentage of circulating lymphocytes decreased (P < 0.01) whereas neutrophils increased (P < 0.01). ACTH injections were also accompanied by a significant decrease (P < 0.01) in circulating eosinophils.

The DMSCC response to ACTH is shown in Table 3. During the ACTH injection period, the decrease in the concentration of milk somatic cells was significant (P < 0.01) for uninfected quarters in Group I only. Midlactation DMSCC (Table 2) for uninfected and *C.*

bovis infected quarters for the five periods averaged 28 and 77 × 10³ somatic cells/ml milk, respectively.

Midlactation, daily quarter milk yield (Table 4) for uninfected and *C. bovis*-infected quarters for the five periods averaged 5.9 and 6.5 kg, respectively. During late lactation, daily quarter milk yield averaged 1.1 and 1.3 kg. During midlactation, the ACTH injections significantly (P < 0.01) depressed milk per quarter an average of 0.75 kg when compared with that of the preinjection control period. During late lactation, ACTH significantly (P < 0.01) depressed milk yield for *C. bovis-*

TABLE 2. *Mean total and differential circulating leukocyte response to ACTH for cows in midlactation and in late lactation.*

Group	4-Day control	5-day injection	5-day control	5-day injection	5-day control	Significance level[a]	Coefficient of variation (%)
			Periods				
			Midlactation				
I (N = 5 cows)		ACTH[b]		Saline[c]			
Leukocytes/mm³	7,110	11,360	7,030	7,260	7,120	**	25
Lymphocytes, %	51	40	50	48	56	**	17
Neutrophils, %	38	54	37	41	31	**	23
Monocytes, %	4	3	6	5	5	**	52
Eosinophils, %	7	3	6	5	8	**	56
II (N = 6 cows)		Saline		ACTH			
Leukocytes/mm³	7,890	7,950	7,200	9,660	6,640	**	21
Lymphocytes, %	41	47	45	36	42	**	16
Neutrophils, %	44	42	40	54	45	**	18
Monocytes, %	5	3	5	5	5	*	70
Eosinophils, %	9	7	8	4	6	**	54
			Late lactation				
I (N = 4 cows)		ACTH		Saline			
Leukocytes/mm³	8,070	9,920	7,230	8,000	8,120	**	19
Lymphocytes, %	58	41	50	47	53	**	17
Neutrophils, %	28	49	34	32	34	**	38
Monocytes, %	5	4	3	3	3	NS	70
Eosinophils, %	8	6	12	17	10	**	38
II (N = 6 cows)		Saline		ACTH			
Leukocytes/mm³	7,418	7,496	7,263	9,114	6,596	**	17
Lymphocytes, %	55	56	54	43	54	**	15
Neutrophils, %	32	33	35	50	36	**	27
Monocytes, %	4	3	4	3	3	NS	74
Eosinophils, %	9	7	7	4	7	**	51

[a]Significant F ratios between experimental periods: NS not significant (P > 0.05), *P < 0.05, **P < 0.01.
[b]Four daily intramuscular injections of 200 I.U. ACTH plus 1 day for carryover effects.
[c]Four daily intramuscular injections of saline solutions plus 1 day for carryover effects.

TABLE 3. *Mean milk somatic cell response to ACTH for cows in midlactation and in late lactation (data are expressed as cells × 10³/ml milk).*

Group	4-day control	5-day injection	5-day control	5-day injection	5-day control	Significance level[a]	Coefficient of variation
			Periods				
			Midlactation				
I (N = 5 cows)		ACTH[b]		Saline[c]			
Uninfected quarters (N = 6)	45a	23b	52a	47a	34c	**	106
Corynebacterium bovis- infected quarters (N = 14)	92	67	131	127	85	NS	92
II (N = 6 cows)[d]		Saline		ACTH			
Uninfected quarters (N = 5)	23	16	14	14	13	NS	87
Corynebacterium bovis- infected quarters (N = 18)	41	48	93	42	45	NS	233
			Late lactation				
I (N = 4 cows)[d]		ACTH		Saline			
Uninfected quarters (N = 15)	48	49	36	42	45	NS	50
II (N = 6 cows)		Saline		ACTH			
Uninfected quarters (N = 8)	10	14	14	13	14	NS	65
Corynebacterium bovis - infected quarters (N = 16)	47	57	62	66	60	NS	40

[a]Significant F ratios between experimental periods: NS not significant P < 0.05, **P < 0.01. Means with common superscripts were not significantly different (P > 0.05; Duncan's multiple-range test) from each other within the rows.
[b]Four daily intramuscular injections of 200 I.U. ACTH plus 1 day for carryover effects.
[c]Four daily intramuscular injections of saline solution plus 1 day for carryover effects.
[d]One cow in this group had a "blind" quarter.

infected quarters in group II.

For the entire study, DMSCCs averaged slightly higher during the morning milking than during the evening (53 vs 41 × 10³ cells/ml milk) (Table 5). However, this effect was significant (P < 0.01) only for uninfected quarters in group I. Milk yield was lower (P < 0.01) during the morning than during the evening milking. For the entire study, quarter milk yield averaged 1.8 kg during the morning and 2.2 kg during the evening.

DISCUSSION

The significant increase in circulating leukocytes after administration of ACTH coincides with results from previous reports (16,18,20). We have shown (16) that the concentration of circulating leukocytes increases 2 h

following intravenous injection of 200 IU ACTH and remains elevated for 24 h. Thus, ample circulating leukocytes were available for migration into milk during both experiments 1 and 2.

That the circulating leukocyte response to ACTH during late lactation was approximately 45% less than the response observed during midlactation agrees with previously reported observations (16). In this earlier study, the circulating leukocyte response to 250 IU ACTH was 35% less for cows in the 41st to 42nd week of lactation than for cows in the 20th week of lactation. The possible physiological significance of this finding could be that this reduced capacity to mobilize circulating leukocytes during late lactation could be a factor contributing to the high incidence of new intramammary infections during the first few weeks after cessation of

TABLE 4. *Effects of 4 daily intramuscular injections of 200 I.U. Adrenocorticotrophic hormone (ACTH) on daily quarter milk yield (data are expressed as kilograms of milk).*

			Periods				
Group	4-day control	5-day injection	5-day control	5-day injection	5-day control	Significance level[a]	Coefficient of variation
			Midlactation				
I (N = 5 cows)		ACTH[b]		Saline[c]			
Uninfected quarters (N = 6)	7.3a	5.9b	6.4c	6.2c	6.2c	**	7.9
Corynebacterium bovis - infected quarters (N = 14)	6.6a	6.2b	6.3b	6.2b	6.5a	**	7.1
II (N = 6 cows)[d]		Saline		ACTH			
Uninfected quarters (N = 5)	5.4	5.4	5.4	5.2	5.4	NS	11.1
Corynebacterium bovis - infected quarters (N = 18)	7.1a	7.0a	6.7b	6.1c	6.6b	**	9.5
			Late lactation				
I (N = 4 cows)[d]		ACTH		Saline			
Uninfected quarters (N = 15)	1.1	0.9	1.0	1.0	1.0	NS	17.0
II (N = 6 cows)		Saline		ACTH			
Uninfected quarters (N = 8)	1.1	1.1	1.2	1.1	1.2	NS	14.6
Corynebacterium bovis - infected quarters (N = 16)	1.5a	1.4b	1.3b	1.1c	1.1c	**	13.3

[a]Significant F ratios between experimental periods: NS not significant (P > 0.05), **P < 0.01. Means with common superscripts were not significantly different (P > 0.05; Duncan's multiple-range test) from each other within the rows.
[b]Four daily injections of ACTH plus 1 day for carryover effects.
[c]Four daily injections of isotonic saline solution plus 1 day for carryover effects.
[d]One cow in this group had a "blind" quarter.

TABLE 5. *Variation in milk somatic cell content and milk yield between morning and evening quarter samplings.*

	Somatic cells × 10³/ml		Significance level[a]	Quarter milk yield, kg		Significance level
Group	Morning[b]	Evening[c]		Morning[b]	Evening[c]	
			Midlactation			
I (N = 5 cows)						
Uninfected quarters (N = 6)	53	28	**	2.8	3.4	**
Corynebacterium bovis - infected quarters (N = 14)	119	83	NS	2.9	3.5	**
II (N = 6 cows)[d]						
Uninfected quarters (N = 5)	20	12	NS	2.4	2.9	**
Corynebacterium bovis - infected quarters (N = 18)	64	48	NS	3.0	3.7	**
			Late lactation			
I (N = 4 cows)[d]						
Uninfected quarters (N = 15)	46	41	NS	0.4	0.6	**
II (N = 6 cows)						
Uninfected quarters (N = 8)	12	12	NS	0.5	0.6	**
Corynebacterium bovis - infected quarters (N = 16)	57	60	NS	0.6	0.7	**
Mean	53	41	NS	1.8	2.2	**

[a]Significant F ratios between morning and evening sampling times: NS not significant (P > 0.05, **P < 0.01.
[b]Milking following 11 hr interval.
[c]Milking following 13 hr interval.
[d]One cow in this group had a "blind" quarter.

milking or drying off.

The popular belief (22) that high blood leukocyte values induced by ACTH result in high milk somatic cell counts was not verified by the results of the present study. This finding discounts ACTH as a predisposing factor to high milk somatic cell counts and supports results obtained in other studies (16,18,20,21).

Importantly, the results from experiment 1 of this study suggest that exercise by cows while under the influence of ACTH had no detectable effect on promoting the shedding of cells into milk. This observation tends to discount the role played by GAS, as proposed by Giesecke and Van den Heever (9), on increasing the concentration of somatic cells in milk of cows under stress. On the contrary, our results support results of others (27,29) who reported that corticosteroids have the effect of maintaining mammary epithelial cell numbers during late lactation, and that corticosteroids will prevent, not enhance, the shedding of epithelial cells into milk. Furthermore, recent findings indicate that epithelial cells are not shed into milk to any great extent and what was once traditionally thought to be epithelial cells are in reality large monocytes which contain phagocytized milkfat globules (11,12,13).

The milk somatic cell counts obtained from uninfected and C. bovis-infected quarters in the present study are in good agreement with concentrations reported for normal milk (24). That the concentration of milk somatic cells was not higher in late lactation than in midlactation agrees with the findings of Duitschaever and Ashton (8). In this latter study, weekly bucket milk samples from 11 cows throughout lactation failed to show a significant increase in milk somatic cell concentration with advancing lactation in the absence of infection. Furthermore, in a study involving 824 cows, Natzke et al. (15) reported that cows uninfected in all four quarters also failed to show a rise in somatic cell concentration with increasing stage of lactation.

The stable milk somatic cell concentration during the large decrease in milk yield in late lactation suggests that the cell content of milk from noninfected quarters is unrelated to milk yield. This conclusion is further supported by the fact that the concentrations of somatic cells in milk for morning and evening milkings rarely differed significantly despite significant fluctuations in milk yield. Natzke et al. (15) also suggested this same conclusion, through the stability in cell concentration during the (assumed) decrease in secretory volume over lactation.

CONCLUSIONS

Neither ACTH, stage of lactation, milk yield, nor milking time had any appreciable effect on the concentration of somatic cells in milk from noninfected quarters. Furthermore, allowing cows access to exercise while under the influence of ACTH, to allow for the appropriate dissipation of energy and assumed release of epithelial cells into milk, did not result in elevated milk somatic cell counts.

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Bacterial Flora of Fish from Tropical Sea Water

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ABSTRACT

Five lots of fish were examined microbiologically for aerobic plate count at 10 and 30 C, coliform and enterococcus counts, presence of coagulase-positive *Staphylococcus aureus* and genera of bacteria appearing on the aerobic plates. After initial analyses, fish were stored in ice, held there for a week and then aerobic plate counts and isolation of colonies for identification as to genus were done again. Aerobic plate counts ranged from 10^4 to 10^7 per gram of skin at the beginning of storage in ice and increased to 10^6 to 10^9 per gram at the end of 7 days of storage. Coliforms and enterococci were found in fresh fish but no *S. aureus* was detected. Gram-negative bacteria accounted for about half of the bacteria on fresh fish, predominated as spoilage advanced and eventually formed the major part of the flora. *Pseudomonas, Moraxella* and *Alcaligenes* were the principal genera of gram-negative bacteria at the end of storage.

Currently processing of fish is a small industry in Central America and particularly in Guatemala. Two major benefits that could result from enlarging this industry are: (*a*) providing products for export to help improve the economy of the country and region, and (*b*) providing more fish for people in this region.

Most of the seafood currently processed in Guatemala is exported to highly developed countries. Hence modern facilities are available to harvest and process shrimp, the high-priced seafood which is exported (5). Exporting fishery products can cause problems when consuming countries impose quality standards that must be met even though the technical expertise in the producing country may not be adequate.

Consumption of fish by Central Americans is limited because fresh fish are not readily available since adequate systems for preservation and distribution are lacking (4,5). Even if such facilities were introduced, their cost might result in increased prices for fish which could not be paid by most residents.

The INCAP/INCNND Nutritional Survey (10) of 1971-1972 revealed that the dietary problems of people in Central America are an inadequate intake of high-quality protein; a marginally adequate intake of calories; a widespread deficiency in intake of vitamin A, riboflavin and thiamin; and seasonally inadequate intake of vitamin C. Consumption of more fish could help to minimize some of these deficiencies since fish is a good source of nutrients such as high-quality protein (fish muscle has 17% or more protein) and minerals such as Ca, Fe and P (8).

The microbial flora of fish and methods of handling, to a large extent, determine the shelf-life of fish when distributed in the fresh state. Microbiological and chemical spoilage of fish from temperate or cold water have been studied extensively. Most attempts to introduce modern techniques for preservation of fish in the tropics have been based on results of such studies (12).

Research on spoilage of fish from tropical waters has usually been done with fish stored in ice. These fish, when in ice, seem to have a longer potential shelf-life than do fish from cold water (2). The idea persists that enzymes of fish, rather than bacteria, are of major importance in spoilage of fish from tropical waters. Of course, the reverse is true for fish from temperate or cold water.

This investigation was done to learn more about the microbial flora of fish from tropical waters before and after the fish were stored in ice. Such information may be useful in developing suitable distribution systems in Central America. The information also can be compared with that already available for other fish so that differences, if they exist, can be established.

MATERIALS AND METHODS

Collection of samples

One of the two fishing companies that operate on the south coast of Guatemala agreed to cooperate in collecting samples of fish caught during the normal shrimp fishing operation. Fish were not gutted on the fishing vessel and were kept in ice until they reached the laboratory. About 24 h elapsed between catching at sea and initial analyses of fish.

Aerobic plate counts

Aerobic Plate Count (APC) were made using Plate Count Agar (PCA, Difco Laboratories). Incubation times were 48 h at 30 C for counts of mesophilic flora and 7 days at 10 C for psychrotrophic flora. Plate Count Agar was prepared according to the manufacturer's directions but 75% of the distilled water was replaced with formulated sea water (11).

Dilutions were made with distilled water containing 2.5% NaCl. Standard dilution bottles (9) were used. A sterile surgical blade was used to cut 1 g of unscaled skin from one side of each fish. The area represented by the 1 g of skin was similar for the different fish that were tested. The skin was put into a sterile metal blender jar with 99 ml of diluent. The blender was operated at low speed for 3 min.

Indicator organisms

All fish caught in the study came from the open sea between 2 and 6 miles off the coast. Although there are no estuarine waters in the area, coliform and enterococcus counts were made to determine if possible fecal contamination was present from the discharge of several rivers along the coast.

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For coliform counts, the 5-tube most probable number (MPN) technique and lauryl tryptose' broth (Difco) were used. Tubes were inoculated with a suspension of gills and intestines prepared by weighing 11 g of gills and intestines into a sterile metal blender jar and adding 99 ml of diluent.

Other dilutions were made as needed to inoculate the lauryl tryptose broth. Incubation was at 30 C for 48 h with reading of tubes at 24-h intervals. A loopful of material from tubes showing fermentation of lactose was transferred into EC medium (Difco) which was then incubated at 44.4 ± 0.2 C for 24 h (15).

This gave results for fecal coliforms and further confirmation for presence of *Escherichia coli* was done by streaking plates of eosin methylene blue agar (BBL) with material from positive tubes of EC medium. Colonies with a characteristic metallic sheen were isolated and the IMViC tests were done to identify *E. coli* types I and II.

Because of differences of opinion on what are the best indicator organisms to monitor sanitation in fish products, enterococcus counts were also made, using a MPN technique with azide dextrose broth (Difco), modified by adding 0.003% bromothymol blue. Suspensions (1:10) of gills and intestines served as inocula and tubes were incubated at 30 C for 48 h. Transfers were made from positive tubes into ethyl violet broth (BBL). Further confirmation that isolates were group D enterotococci was done by growing the isolates at 10 and 45 C; growth at both temperatures and a gram stain showing gram-positive streptococci demonstrated that the isolates were enterococci.

Presence of coagulase-positive *Staphylococcus aureus* was determined using Vogel and Johnson medium (19) (BBL) for isolation of possible *S. aureus* cultures. Plates were inoculated by streaking with the previously mentioned suspension of gills and intestines. Typical brilliant black colonies were picked to test for gram reaction and coagulase reaction.

Isolation of cultures

After counting colonies on APC plates, six to eight colonies on each plate were selected randomly and inoculated onto slants of the PCA sea water agar; isolates from plates incubated at 10 C were again incubated at 10 C and isolates from plates incubated at 30 C were incubated at 32 C. After growth, isolates were stored at 10 C until they were identified.

Storage of fish in ice

After doing initial counts, the same fish were gutted and washed with tap water as would have been done by the vessel crew or plant personnel. Using ice made from laboratory tap water (0.2 to 0.4 ppm of residual chlorine), all fish of a given lot were stored in an ice chest made of polyurethane foam. The chest was perforated at the bottom to assure that water dripped out of the container. For this work, there was no interest in having fish suspended in an ice water-nutrient mixture that could support excessive bacterial growth.

The ratio of fish to ice was 2:1 by weight in two lots and 1:1 in the three other lots. During storage, the ice chest was checked every 24 h for ice level and if there was not enough, more ice was added.

After 7 days of storage, the APC counts of fish were repeated so changes in numbers and types of bacteria could be determined. The same techniques described earlier for counting and making isolations were used.

Identification of isolates

To identify the genus of isolates, the following tests were used: gram stain, oxidase test, pigment production, motility tests, flagella stain, sugar fermentation tests, litmus milk reaction, aerobic vs. anaerobic growth in sugar media, and growth in neutral and acid peptone broth. Methods followed were those of the Society of American Bacteriologists (18), of Gibbs and Skinner (6), Gibbs and Shapton (7), and Sharf (16). Classification was based on descriptions in the eighth edition of *Bergey's Manual* (1).

RESULTS AND DISCUSSION

Most work on the microbial flora of fish has been done in experiments on a single species. Even when working with only one species, variations in the microbial flora have been found during different periods of the year (13).

This work was done using several species of fish mainly because the source of the samples was a local fishing company that caught shrimp. The fish used in this study were caught with the shrimp. At the beginning of the work the aim was to use only one or two species; however, it was found to be a difficult task since every time fish were delivered to port different species of fish were included in the samples provided by the boat crew. Consequently, several species of fish were used for our tests. Each batch of fish collected in a trip to the fishing company plant and transported to the laboratory for analyses will be designated as a "lot." The identity of fish and the number tested is given in Table 1.

TABLE 1. *Species of fish involved in the study.*

Name	Number of units
Salmonete (*Upeneus grandisguanus*)	5
Curvina (*Cynoscion phoxacephalus*)	3
Guabina (*Stellifer pizarroensis - Gobiomorus maculatus*)	5
Lenguado (*Solea vulgaris*)	1
Guapote (*Cichlasoma managuense*)	3
Barbuda (*Rhombus loevis*)	1
Pompano (*Carangidae* sp.)	1
Cachaco[a]	1

[a]Not identified.

According to Thatcher and Clark (19), when fish has more than 10^6 microorganisms per g or cm^2, it is considered to have a very short potential shelf-life or may even to be at incipient spoilage. Results in Table 2 indicate that most fish from lots 1 and 2 had 10^6 or more microorganisms per g when they arrived at the laboratory. However the samples did not have any signs of spoilage such as off-odors or a soft texture.

After 7 days in ice (fish:ice, 1:1) there was no appreciable change in the microbial load; fish at the end of storage exhibited no evidence of spoilage.

Results of the APC at 30 and 10 C were similar. According to Thatcher and Clark (19), when the mesophilic count (30 C) is appreciably greater than the psychrotrophic (10 C) count, there might be contamination from sources other than the natural habitat of the fish, at least for fish from temperate water.

To accelerate spoilage, lots 3, 4 and 5 were stored with less ice (fish:ice, 2:1) than the other lots. Microbial populations that initially were generally less than 10^6 microorganisms per g had increased to 10^7, 10^8 and even 10^9/g after 7 days. These fish exhibited organoleptic evidence of spoilage. There was no marked difference in the APC at 30 or 10 C, suggesting that strict mesophiles did not form a major part of the flora. Instead of differences in APC at 30 or 10 C, a more direct way to detect extraneous contamination might be to determine presence of indicator organisms such as fecal coliforms, enterococci and *S. aureus*, none of which are considered to be a normal part of the flora of fresh fish. Numbers greater than 10 fecal coliforms/g and 10^2 *S. aureus*/g are

considered to be the result of gross mishandling of the fish (14).

Fecal coliforms were recovered from fish in all lots but one; however, only five fish had more than 10/g. Smaller numbers were detected in some fish of lots 2, 3 and 4 and none were found in lot 5 (Table 3).

Results of enterococcus counts did not correlate well with those of tests for fecal coliforms. A test for enterococci is considered more useful than one for coliforms for testing frozen fish since enterococci are less susceptible to inactivation by freezing than are coliforms. Only three fish had enterococcus counts greater than 10^2/g. No samples yielded *S. aureus*.

Based on results of APC and tests for indicator organisms, lots 1 and 2 seemed more contaminated than the others. The first samples were probably subjected to more handling by the vessel crew than was desired; this did not happen with lots 3, 4 and 5 because instructions were given to the people at the plant to avoid any unnecessary handling.

The main part of this study was to identify the principal genera of bacteria present on recently-caught fish and to determine how this population changed during storage of fish in ice. Lots 1 and 2 were kept in ice (fish:ice, 1:1) as recommended for tropical countries by FAO (3), whereas lots 3, 4 and 5 were stored using half the recommended amount of ice (fish:ice, 2:1).

Table 4 lists the number of isolates in different genera recovered from fish skin. At initial testing, the largest number of gram-positive isolates were micrococci. When we compare the number of micrococci isolated from plates from initial testing and incubated at 10 or 30 C with the number obtained under the same conditions after 7 days of storage of fish in ice, a marked reduction in number of micrococci had occurred.

If we consider the gram-negative bacteria, *Moraxella* and *Alcaligenes* initially accounted for 65% of all gram-negative isolates and *Pseudomonas,* commonly considered as the most important genus in cold-storage of fish, constituted only 12%.

TABLE 2. *Aerobic plate counts (APC) per gram of fish skin at the beginning and end of 7 days of storage in ice.*

Lot No.	Kind of fish	Ratio of fish to ice	Initial		After 7 days	
			APC-30 C	APC-10 C	APC-30 C	APC-10 C
1	Curvina	1:1	5.4×10^7	1.8×10^7	1.2×10^8	1.2×10^8
	Guabina	1:1	3.4×10^7	2.9×10^7	8.5×10^7	1.3×10^8
	Salmonete	1:1	3.0×10^7	2.8×10^7	4.7×10^6	4.2×10^6
	Lenguado	1:1	1.2×10^7	4.0×10^7	1.7×10^8	9.5×10^7
2	Salmonete	1:1	9.9×10^5	8.0×10^5	1.7×10^7	7.6×10^6
	Curvina	1:1	7.8×10^5	8.3×10^5	9.2×10^6	1.9×10^7
	Guabina I	1:1	8.0×10^6	2.2×10^6	1.3×10^7	1.2×10^7
	Guabina II	1:1	1.3×10^6	1.6×10^6	1.2×10^7	1.3×10^7
3	Guabina I	2:1	1.6×10^5	1.0×10^5	6.4×10^8	9.0×10^8
	Guabina II	2:1	1.3×10^6	8.6×10^5	8.8×10^8	1.1×10^9
	Salmonete I	2:1	2.5×10^4	4.5×10^4	3.8×10^7	2.1×10^7
	Salmonete II	2:1	3.5×10^4	2.0×10^4	1.7×10^7	2.1×10^7
4	Guapote	2:1	7.0×10^4	1.0×10^4	1.6×10^9	4.0×10^8
	Barbuda	2:1	6.5×10^4	5.2×10^5	1.0×10^9	4.0×10^8
	Cachaco	2:1	8.0×10^5	1.6×10^4	8.9×10^7	6.9×10^7
	Pompano	2:1	5.5×10^4	2.1×10^4	1.6×10^9	4.0×10^8
5	Curvina	2:1	1.0×10^5	1.0×10^3	1.4×10^8	1.4×10^8
	Salmonete	2:1	1.0×10^5	2.6×10^4	5.6×10^6	8.7×10^7
	Guapote I	2:1	1.0×10^5	5.2×10^5	1.5×10^8	2.6×10^8
	Guapote II	2:1	1.0×10^5	3.0×10^4	1.6×10^8	1.5×10^8

TABLE 3. *Most probable number of indicator organisms per gram at the beginning of storage in ice.*

Lot No.	Sample	Enterococci	Fecal coliforms	Escherichia coli I	Escherichia coli II
1	Salmonete	240	23	18	4
	Guabina	0	26	24	2
	Curvina	23	20	15	3
	Lenguado	3.6	0	—	—
2	Salmonete	0	0	0	0
	Curvina	0	9	9	0
	Guabina I	2400	2400	—	—
	Guabina II	4	4	2	1
3	Guabina I	11	4	3	0
	Guabina II	0	0	0	0
	Salmonete I	0	0	0	0
	Salmonete II	0	4	3	1
4	Guapote	11	11	11	0
	Barbuda	7	0	0	0
	Cachaco	7	3	3	0
	Pompano	3	0	0	0
5	Curvina	0	0	0	0
	Salmonete	0	0	0	0
	Guapote I	460	0	0	0
	Guapote II	23	0	0	0

A comparison of frequency at which micrococci and *Pseudomonas, Moraxella* and *Alcaligenes* were isolated after 7 days, shows that an increase in the number of *Pseudomonas* occurred at the expense of other species. For example, the frequency of isolation of micrococci was reduced, that of *Moraxella-Alcaligenes* decreased from an original 65 to 34% of the gram-negative isolates and that of *Pseudomonas* increased from 12 to 49% of the gram-negative group.

Since bacteria in the genus *Pseudomonas* often are the principal spoilage agents of fish (*14*), we were interested in how these bacteria grew on fish from tropical waters during storage in ice.

Figures 1 and 2 give results obtained when adequate and inadequate preservation with ice was used. There was a larger proportion of *Pseudomonas* in the flora when preservation was inadequate rather than adequate; however, this increase in proportion of *Pseudomonas* does not by itself explain the spoilage of lots 3, 4 and 5 after storage in ice. These three lots of fish also had greatest numbers of microorganisms per gram of skin. Thus *Pseudomonas* not only constituted a larger

Figure 2. *Frequency in percentage of the principal bacterial groups in lots 3, 4 and 5 (inadequate preservation) before and after storage in ice.*

proportion of the flora after than before storage but also was present in larger numbers in poorly iced than in properly iced fish. This could explain why fish in lots 3, 4 and 5 were spoiled at the end of the test. Also, other gram-negative microorganisms play a role in spoiling of fish and there were more gram-negative organisms in these same lots of fish than in the other lots. These organisms also could have contributed to spoilage of these fish.

Results of this study agree with findings of other authors (*17*) in relation to proportions of different genera in the flora of fish skin before and after storage in ice. However, total numbers of microorganisms found in this study were larger than those reported by other authors who studied tropical fish, possible because our samples were more nearly representative of commercial practice than were samples in some other tests. Our samples initially had APC values large enough to suggest that spoilage was imminent, but we detected no signs of spoilage. There may be a relation between the kind of fish and the microbial load that the fish can support without being spoiled. This needs to be investigated.

Figure 1. *Frequency in percentage of the principal bacterial groups in lots 1 and 2 (well-preserved) before and after storage in ice.*

TABLE 4. *Number of isolates in different genera that were recovered from fish skin before and after storage of fish in ice.*

Incubation tempera-ture/time	Time	Mic.[a]	Staph.	Strep.	Arthr.	Lactob.	Leucono.	Bac.	Coryne.	Morax.	Flavob.	Enterob.	Acinet.	Alc.	Pseudo.
10 C	0 day	39	2	2	4	0	0	2	3	12	1	0	0	24	4
	7 days	9	2	0	1	1	0	0	0	9	0	6	1	11	20
30 C	0 day	30	9	2	1	0	1	0	0	7	4	11	3	16	7
	7 days	8	4	1	3	7	0	0	0	20	0	12	2	4	42
0-day	All iso-lates	69	11	4	5	0	1	2	3	19	5	11	4	40	11
7 days	All iso-lates	17	6	1	4	8	0	0	0	29	0	18	3	15	62

[a]Abbreviations:

Micrococci--Mic.	Coryneforms--Coryne.
Staphylococci--Staph.	*Moraxella*--Morax.
Streptococci--Strep.	*Flavobacterium*--Flavob.
Arthrobacter--Arthr.	Enterobacteria--Enterob.
Lactobacilli-Lactob.	*Acinetobacter*--Acinet.
Leuconostoc--Leucono.	*Alcaligenes*--Alc.
Bacillus--Bac.	*Pseudomonas*--Pseudo.

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Coming Events, con't. from p. 772

Dec. 3-8, 10-15--DAIRY DAYS. Sponsored by University of Nebraska-Lincoln Institute of Agriculture and Natural Resources, Nebraska State Dept. of Agriculture, and Nebraska milk marketing outlets. Cooperating organizations are Nebraska Veterinary Medical Association and Dairy Women of Nebraska. Two programs, one week apart, will be held in the following locations: Columbus, NB---Dec. 3, 10; Beemer, NB---4, 11; Hartington, NB---5, 12; O'Neill, NB---6, 13; Ravenna, NB---7, 14; Beatrice, NB---8, 15. Contact: Nebraska Dairy Women, plant fieldmen, or county extension agents for advance registration.

Jan. 14-15--NSF SEMINARS, Los Angeles, CA. See Feb. 11-12 NSF entry. Contact: Education Service, National Sanitation Foundation, P.O. Box 1468, Ann Arbor, MI 48106, 313-769-8010.

Feb. 11-12--NSF SEMINARS, Kansas City, MO. First day seminar covers sanitation aspects of design and construction of foodservice equipment. Second seminar covers specific facility plan preparation and review. Participants may attend either one or both seminars. Contact: Education Service, National Sanitation Foundation, P.O. Box 1468, Ann Arbor, MI 48106, 313-769-8010.

Mar. 26, 1980--ONTARIO FOOD PROTECTION ASSOCIATION, Annual Meeting. Holiday Inn, 970 Dixon Road, Toronto.

Comparing Three Methods for Counting Raw-Milk Pour Plates [1]

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ABSTRACT

Six hundred Grade A raw milk samples were plated on Standard Methods agar and the pour-plate counts compared using three methods. Counts of each sample were determined after incubation at 26, 30 and 32 C for 72 h with an automatic colony counter (ACC), Quebec colony counter and hand tally (HT) and Quebec colony counter and electronic probe with digital register (DT). No significant differences were found between the counts obtained with the HT and the DT for any of the three incubation temperatures. The ACC counts were lower (P < .01) than the manual counts at each temperature. The ACC counts were .39, .35 and .31 log counts lower than the manual count at 26, 30 and 32 C, respectively. The ACC counts at 72 h were higher (P < .01) than the ACC counts at 48 h for all temperatures. The difference was about .41 log counts. The two manual counts were totally correlated, while the correlation between the ACC counts and the manual counts was .97. Regression equations were formulated for predicting the 72 h HT count from either the 48 or 72 h ACC count.

Instruments have been developed which automatically count and record the number of colonies on agar plates (2,3,8,9). Automatic colony counters (ACC) were developed to provide an accurate method of counting colonies in agar plates with a significant saving in time and labor. Apparently, most ACC can tolerate variations in agar color, agar depth, optical density, etc. with little detectable effect on counting efficiency (4,9).

A few studies have evaluated the acceptability of using the ACC for counting agar plates prepared from raw milk or food samples, or have compared the ACC results with those obtained using conventional counting methods (2,3,4,8,9,11).

The objective of this study was to compare the results obtained when raw-milk pour plates were counted using three counting methods, the ACC and two manual counting procedures. In addition, the ACC was used to determine differences in counts of agar plates incubated at three temperatures and counted after 48 and 72 h.

MATERIALS AND METHODS

Samples

Six hundred Grade A raw milk samples were analyzed during a 10-month period. Samples were collected by the milk truck driver from individual farm bulk tanks after addition of the fourth milking and just before emptying the tank. Samples were collected in Whirl Pak bags, refrigerated during transportation to the laboratory and held below 4 C until analyzed.

Plating procedures

Counts were determined on Standard Methods agar (BBL) using the pour plate method (5). Triplicate plates were prepared per dilution plated and one plate of each dilution was randomly selected and incubated at 26, 30 or 32 C for 72 ± 2 h.

Counting procedures

After incubation, the same person counted each plate using the three counting procedures described below. The automatic colony counter (ACC) used was the Fisher Bacterial Colony counter (Model 480) with a 11.5-cm Hitachi TV monitor. The counter was operated according to instructions furnished by the manufacturer. Based on preliminary studies using Standard Methods agar, the compensation setting was adjusted to give a 33% increase in the scanned plate count. This setting was found to give the maximum counts that were compatible with manual counts. Colonies were counted to a minimum resolution of 0.3 mm and a minimum diameter of 0.2 mm. Before the plates were counted, the bottoms were cleaned with a Kimwipe tissue soaked in 95% alcohol to remove markings, dirt and fingerprints. After each count, plates were rotated approximately 120° to obtain a different counting position, which resulted in three counts per plate. Each plate was counted using the ACC after 48 and 72 h of incubation.

The second counting method (hand tally) used a Quebec Colony Counter and a hand tally to register the count after 72 h of incubation (5).

The third counting method (digital touch) is based on the principle that when a grounded probe and counting probe both touch a conductive surface, a circuit is completed and a count is registered. The plate was placed on a Quebec Colony Counter, the lid removed and the grounded probe of the AO Automatic Register, Model QR-1 (American Optical Co.) adjusted to permit the arm to make contact with the agar medium. A second probe (counting probe) containing a needle point was moved from colony to colony and automatically registered a count each time the counting probe touched the agar surface. Plates were counted after 72 h of incubation.

Analysis of data

The three colony counts obtained per plate per incubation condition with the ACC were averaged arithmetically and one value used for the analysis. The Statistical Analysis System (SAS) programs were used to analyze the data (1). Duncan's multiple range test was used to determine if the mean counts obtained with the three counting methods differed significantly from each other.

RESULTS AND DISCUSSION

The Standard Plate Counts of the raw milk samples ranged from 20×10^1 to 71×10^7 per ml. The mean logarithmic aerobic counts obtained with the three temperatures are given in Table 1. The differences between the counts obtained with the two manual counting methods (Hand Tally and Digital Touch) were not significant for any of the incubation temperatures. The automatic colony counter (ACC) counts were lower

TABLE 1. *Effect of counting method on the mean count of raw milk samples after incubation for 72 hours at 26, 30 and 32 C.*

Counting method	Incubation temperature (C)			Mean count[a]
	26	30	32	
	(Log$_{10}$/ml[b])			
ACC[c]	5.73[d]	5.62[d]	5.57[d]	5.64[d]
Hand tally	6.04[e]	5.97[e]	5.96[e]	5.99[e]
Digital touch	6.03[e]	5.97[e]	5.96[e]	5.99[e]

[a]Means of 1794 counts.
[b]Each value represents means of 598 counts.
[c]ACC = automatic colony counter.
[de]Means in columns with different superscripts differ significantly (P < .01).

(P < .01) than the manual counts at each temperature. The differences between the ACC and manual counts decreased with a decrease in incubation temperature. The ACC counts were .39, .35, and .31 log counts lower than the manual counts at 26, 30 and 32 C, respectively.

Several factors seem to be responsible for the differences between ACC and manual counts. Colonies with diameters less than 0.2 mm could not be counted by the ACC used in the study. In addition, colonies not separated by a distance of 0.3 mm were not recognized as individual colonies. The smaller differences in counts at the lower incubation temperatures indicate that a greater percentage of the microflora was able to grow and form colonies countable by the ACC and/or that the colonies formed were larger than at the higher temperature.

When the ACC is used to count pour plates, the portion of the petri dish that extends beyond the inner periphery of the stacking ring located on the bottom of the dish is screened off. The area beyond the stacking ring represents 18 to 20% of the total area of the petri dish. To compensate for the colonies located in this area and not counted directly by the ACC, the counter can be adjusted to automatically compensate for up to 33% of the total area lost because of the stacking ring. In this study, the colonies outside the ACC-viewed area were estimated by using a compensation setting of 33%. This compensation apparently was not sufficient to increase the ACC counts to correspond with the manual counts. Most of the colonies not counted by the ACC would be counted by manual counting methods. Many colonies excluded by the ACC tend to be common in aerobic pour plates prepared from raw milk, especially if incubated at 32 C.

Other investigators have reported differences between ACC and manual counts for raw milk samples to be less than those obtained in this study (2,3,8,11). Plates in other studies were manually screened, before counting, to exclude plates with spreaders and/or with colonies smaller than the resolution of the ACC. Plates were not screened in this study and all values obtained were included in the analysis. While most researchers have reported manual counts to be higher than those of the ACC, some have reported higher counts for ACC (2,11). Differences in results seem to be due to the extent that plates were screened before counting, differences in compensation setting, the resolution capacities of the counters and variations inherent in individual counters.

The mean aerobic count obtained with the Automatic Colony Counter for raw milk samples after 48 and 72 h of incubation at three temperatures are given in Table 2. Manual counts were not made at 48 h because the digital touch method destroyed the plates and prevented them from being incubated an additional 24 h. The mean ACC counts were higher (P < .01) after incubation for 72 h than after 48 h at all temperatures. The differences between the counts obtained at 48 and 72 h were similar for the three temperatures (about .41 log counts).

TABLE 2. *Effect of incubation time on mean counts of raw milk samples obtained with automatic colony counter for plates incubated at three temperatures.*

Incubation time (h)	Incubation temperature (C)			Mean count[a]
	26	30	32	
	(Log$_{10}$/ml[b])			
48	5.32[c]	5.20[c]	5.17[c]	5.23[c]
72	5.73[d]	5.62[d]	5.57[d]	5.64[d]

[a]Means of 1792 counts.
[b]Each value represents means of 597 counts.
[cd]Means in column with different superscripts differ significantly (P < .01).

The higher ACC counts obtained at 72 h were, in part, due to increases in colony size as a result of the longer incubation time. The larger colonies resulted in higher counts because the ACC could detect only those colonies with diameters greater than 0.2 mm as well as the tendency for the ACC to count the larger colonies more than once. The additional incubation time resulted in an increase in the number of colonies with a diameter greater than 0.2 mm. Other studies have shown counts to be higher after 72 h than after 48 h (6,7,12).

Correlation coefficients between the three counting methods combining the results for all incubation temperatures are given in Table 3. The two manual counting methods were totally correlated. The next highest correlation was between the ACC counts and the manual counts after 72 h of incubation (.97). A 0.85 correlation was obtained between the 48 and 72-h ACC counts. The lowest correlation (0.70) was between the ACC count at 48 h and the manual counts at 72 h.

Regression equations which can be used to predict the Hand Tally count at 72 h using either the 48 or 72-h ACC counts are given in Table 4. The regression equation using the 72-h counts is similar to that reported by LaGrange et al. (8). Similar values have been reported by other investigators (2,9), except with a negative intercept. These investigators either manually screened plates or used spread plates of pure cultures.

TABLE 3. *Correlation coefficients between three methods used to count raw milk samples.*

Counting methods	Digital method (72 h)	ACC[a] (48 h[b])	ACC (72 h)
Hand tally (72 h)	1.00	.70	.97
Digital method (72 h)	—	.70	.97
ACC (48 h)	.70	—	.85
ACC (72 h)	.97	.85	—

[a]ACC = automatic colony counter.
[b]Incubation time.

TABLE 4. *Regression equations predicting the 72-hour hand tally count from the 48-hour and 72-hour automatic colony counts for raw milk samples.*

Log_{10} 72 h hand tally count = 1.94 + 0.77 ($\log_{10}$ 48 h ACC[a] count)
Log_{10} 72 h hand tally count = 0.34 + 1.00 ($\log_{10}$ 72 h ACC count)

[a]ACC = automatic colony counter.

The results of this study indicate that Automatic Colony Counters are suitable for counting pour plates from raw milk samples. The ACC seems to be more suitable for use in routine quality control than for use in regulatory work.

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Pennsylvania Association Accepts Abnormal Milk Policy

A "Recommended Abnormal Milk Policy" was prepared by a committee of the Pennsylvania Dairy Sanitarians Association and accepted late last year by the Executive Committee of that association.

Since that time over 450 copies of the policy have been distributed throughout Pennsylvania.

For additional information on the policy, contact: William J. Killough, Secretary-treasurer, Pennsylvania Dairy Sanitarians Association, RD1, Box 393, Conestoga, PA 17516.

Pennsylvania Dairy Sanitarians Association Recommended Abnormal Milk Policy

The purpose of this policy is to assist dairymen to produce more milk of good quality. It should not be misunderstood as recommending changes in present regulatory standards.

Pennsylvania dairymen shall be notified of all abnormal milk test results. Dairymen shall be informed that a recheck shall be conducted within 14 days of the first abnormal milk count of 750,000 Somatic Cells per ml** or greater. The second consecutive count of 750,000 Somatic Cells per ml or greater will be immediately followed by a farm visit by a Pennsylvania Approved Inspector. During this visit the following evaluations shall be made:

1. Herd Survey Sheet shall be completed (NDPC Guideline #18, page 77c).
2. An evaluation of the milking equipment shall be made.
3. An evaluation of the milking procedure shall be made.
4. A cow slide screening test may be conducted.
5. A discussion of the above evaluations shall be held with the dairymen as they relate to abnormal milk and Somatic Cells.
6. Recommendations as to corrective action to be taken shall be made.

If the Pennsylvania Approved Inspector determines that a milking machine dealer's evaluation is needed for the milking system, forms shall be left with the dairyman for the dealer to complete. The dealer shall be contacted by the dairyman. A copy of the completed form will be returned to the Pennsylvania Approved Inspector.

If a veterinarian is needed to evaluate the herd health, forms shall be left with the dairyman for the veterinarian to complete. The veterinarian shall be contacted by the dairyman. A leucocyte level screening test (CMT or Somatic Cell Count) shall be conducted by the veterinarian. A copy of the completed form will be returned to the Pennsylvania Approved Inspector.

The purpose of this policy is to identify problem dairies before they reach leucocyte levels that require regulatory action or levels that may cause severe hardships to dairymen through loss of production and/or dairy animals. The purpose is *not* to reduce the regulatory levels of Somatic Cells.

**Equivalent levels may be determined by laboratory curve comparing WMT with DM-SCC, where applicable.

Abnormal Milk Committee
Franklin R. Balliet, Chairman
Ogden C. Bacon
Dr. R. J. Eberhart
Al Gottfried
Elwood S. Hench
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Dr. Lawrence Hutchinson
J. Gene Lauver
Stephen B. Spencer
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Osten elected President of Dairy Division of NASDA

Orlowe M. Osten, Director, Dairy Industries Division, Minnesota Department of Agriculture, was elected President of the Dairy Division of the National Association of State Departments of Agriculture (NASDA) at the 21st Annual Meeting held in Branson, Missouri, July 23-26.

Other officers elected to lead the dairy organization, which includes membership in 41 states, were: President-Elect - Jess D. Sturm, Wyoming Dept. of Agriculture; Vice President - Jay Boosinger, Florida Dept. of Agriculture; Secy/Treas. - Alfred R. Place, New York Dept. of Agriculture.

Organoleptic Examination of Pears from Trees Infused with Oxytetracycline to Remit Symptoms of Pear Decline

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ABSTRACT

One year after trees had been infused post-harvest with oxytetracycline (OTC) to remit symptoms of pear decline, pears were harvested and tasted. In four sessions, the panel of tasters discerned between pears from trees treated with OTC and pears from untreated diseased trees. In three of the sessions, the fruit from treated trees was preferred over fruit from untreated diseased trees.

Pear decline, presumably caused by mycoplasmalike organisms (*2*), has been reported since 1948 in western North America (*5*), and more recently in Connecticut (*6, 7*). In California, infusion of trees with oxytetracycline caused remission of the symptoms of pear decline (*8*). Similar results have been obtained in Connecticut with OTC increasing number and size of fruit and vigor of trees (*6, 7*).

When an antibiotic is used to control diseases of plants or animals, the marketable product must not contain antibiotic in excess of legal limits, and the treatment should not produce a deleterious flavor in the product. In this report we describe experiments to determine if the flavor of pears from trees treated with oxytetracycline could be discerned from pears from untreated trees with symptoms of pear decline.

MATERIALS AND METHODS

Treatment of trees and fruit harvest

Trees (*Pyrus communis* L. cultivar Bartlett) with symptoms of pear decline in two orchards in Connecticut were used for this study. After harvest in the fall of 1976 and 1977, twenty 12-year-old trees in orchard A were infused with 0.1 g of active ingredient (a.i.) oxytetracycline (OTC) (Terramycin Tree Injection Formula, Pfizer, Inc., New York, NY 10017), and 20 other trees with 1.0 g of a.i. OTC per tree in the fall of 1977. After harvest in 1977, 32 8-year-old trees in orchard B were infused with 0.5 g or 2.0 g of a.i. OTC per tree (*4*). Untreated trees with symptoms of pear decline were controls in each orchard.

Pears from orchards A and B were harvested on September 5 and 6, 1978, respectively, and stored at 3 to 4 C for 4 days before testing. The harvest dates coincided with commercial picking in these orchards.

Samples and preparation

Pears from each orchard were harvested separately. A random selection was made from each tree, and about 45 kg of the selected pears were pooled for each treatment. In this way, six pooled samples of fruit were formed that consisted of fruit from control trees and fruit from trees infused with high (1.0 or 2.0 g of OTC/tree) or low (0.1 or 0.5 g of OTC/tree) levels of OTC in each orchard. All fruit was

examined microbiologically by a technique that could detect 0.16 µg of OTC/g tissue (*10*). No inhibitory activity was detected.

For taste-testing each day, about 10 kg of pears were randomly selected from each of the 45-kg pooled samples. Two to three hours before testing the fruit was peeled and cored. Pears were either blended into sauce or cut into 2-3-cm cubes and refrigerated until used. The pooled samples were tested once prepared as sauce and once as cubes.

Taste panel

The panel consisted of volunteers from the Experiment Station staff. Fruit was tasted on four consecutive days, but only samples from one orchard (either sauces or cubes) were tasted on any one day. The number of tasters varied from 20 to 24 each day with a ratio of female to male of 1:1.8.

Taste testing

Either 25 g of sauce or 3 to 4 cubes were presented in plastic cups. Panel members were given a spoon for the sauce or toothpicks for the cubes. Water was available to rinse the mouth. Only two randomly numbered samples were presented to each panelist at a time, and the panelists were required to taste the samples in the order in which they were presented. Panelists did not know how many treatments were involved.

Panelists were asked to record on a questionnaire which of each pair they preferred. They were also asked to judge their preference for the chosen sample as slightly, moderately, very much, or extremely more desirable than the sample not chosen, but they also had the option to check that they had no preference between the two samples. These ratings were later assigned numerical values for analysis (*11*). Such hedonic scale testing and the questionnaire have been described (*1,3,9,11*). Each day, panelists randomly were given 12 pairs of samples (from two treatments and the control) to provide a basis for statistical analysis and to preclude differences caused by the order of sample presentation. Obviously, in six of the paired tests both samples were the same.

Statistical analysis

Statistical analysis of dissimilar pairs of samples was made by the method of Scheffé (*11*) and for similar samples as described by Amerine et al. (*1*). Hedonic scale testing does not measure acceptance but rather preference of one sample over another. Acceptance can only be predicted (*9*).

RESULTS

Analysis of paired similar samples

Data obtained from the six identical pairs presented each day were used to test the reliability of the panelists, i.e., whether they could discern that two samples were the same. Chi-square analysis indicated that on 3 of the 4 days panelists did not prefer one sample over the other more than by chance alone. On day 4 of tasting, however, there was a significant difference ($p \leqslant .05$) in preference for the fruit from only one of the six paired tests. The

chi-square analysis demonstrated the validity of using the statistical methods for paired dissimilar samples (*11*).

Analysis of sauce: fruit from orchard A

The main effects are significant, i.e., differences existed in taste preference between different treatments. There was a significant difference between pears from untreated trees and pears from trees treated with 0.1 g of OTC ($p \leqslant .01$), between pears from untreated trees and those treated with 1.0 g of OTC ($p \leqslant .01$), and between pears from trees treated with 0.1 g and 1.0 g of OTC ($p \leqslant .05$) (Table 1).

The order of taste preference of pears from trees treated with OTC is shown in Table 2. The fruit from the 0.1-g OTC treatment was preferred the most frequently, followed by fruit from the 1.0-g OTC treatment, and lastly, fruit from the untreated trees.

TABLE 2. *Taste preference of pears from trees treated with oxytetracycline (OTC).*

Taste prefer-ence order	Orchard A		Orchard B	
	Sauce	Cubes	Sauce	Cubes
1	0.1[1]a	1.0a	0.5a	none a
2	1.0a	0.1a	2.0a	2.0b
3	none b	none b	none b	0.5b

[1]OTC treatment levels in any column followed by the same letter do not differ significantly at $p \leqslant .01$.

Analysis of sauce: fruit from orchard B

The main effects were significant and there were differences in preference between pears from different treatments. There was a significant difference ($p \leqslant .01$) between pears from the untreated trees and pears from trees treated with either 0.5 or 2.0 g of OTC. There was not a significant difference between the fruit from the 0.5-g OTC treatment and those treated with 2.0 g of OTC (Table 1).

The order of taste preference for sauce made from pears from trees treated with OTC is shown in Table 2. Sauce from fruit from the 0.5-g OTC treatment was preferred over sauce from fruit from untreated trees more frequently than the fruit from the 2.0-g OTC treatment over the fruit from untreated trees. However, both comparisons were significant ($p \leqslant .01$). Although

the difference between pears from the 0.5-g and 2.0-g OTC treatments was not significant, the order of preference was for sauce made from fruit from the 0.5-g OTC treatment over that from the 2.0-g treatment.

Analysis of cubes: fruit from orchard A

The main effects were significant and there were differences in the preference for pears from different treatments. There was a significant difference between fruit from untreated trees and that from trees treated with 0.1 g of OTC ($p \leqslant .01$), and between fruit from untreated trees and that from trees treated with 1.0 g of OTC ($p \leqslant .01$) (Table 1). There was no significant difference between fruit from trees treated with 0.1 g and 1.0 g of OTC.

The order of taste preference (Table 2) shows that fruit from trees treated with 1.0 g of OTC was preferred over fruit from trees treated with 0.1 g of OTC and from untreated trees. Also, the fruit from trees treated with 1.0 g of OTC was preferred over the fruit from untreated trees more than the fruit from trees treated with 0.1 g of OTC was over the fruit from untreated trees. This preference order is different from that found with sauce from the same pears, but still pears from treated trees were preferred over pears from untreated trees.

Analysis of cubes: fruit from orchard B

The main effects were significant and there were differences in the preference between pears from different treatments. There was a significant difference ($p \leqslant .01$) between fruit from untreated trees and that from trees treated with 0.5 g of OTC, and between the fruit from untreated trees and that from trees treated with 2.0 g of OTC (Table 1). There was not a significant difference between fruit from trees treated with 0.5 g and 2.0 g of OTC.

The order of preference (Table 2) shows that fruit from untreated trees was preferred over that from trees treated with either 0.5 or 2.0 g of OTC. This order of preference is the opposite of that in the three other analyses. Although the last difference was not significant, the order of preference was for fruit from trees treated with 2.0 g of OTC over fruit from trees treated with 0.5 g of OTC.

TABLE 1. *Significance between taste preferences of pears obtained from trees treated with oxytetracycline (OTC) and untreated trees.*

Sample	Significance value[1]		Treatments[2]			Differences		
	$Y_{.05}$	$Y_{.01}$	$a\,1$	$a\,2$	$a\,3$	$a\,1 - a\,2$	$a\,1 - a\,3$	$a\,2 - a\,3$
Sauce: orchard A	.406	.507	−1.189	.841	.348	−2.030**[3]	1.537**	.493*
Sauce: orchard B	.457	.571	−.514	.438	.076	−.952**	.590**	.362
Cubes: orchard A	.485	.610	−.767	.250	.517	−1.017**	1.284**	.267
Cubes: orchard B	.465	.581	.841	−.198	−.643	1.039**	1.484**	.445

[1]Differences between treatments must differ by more than this value to be significant at $p \leqslant .05$ or $p \leqslant .01$ as indicated.
[2]According to Scheffé (*11*) there is a parameter, *a*, for each treatment such that the difference in parameters for any two treatments is equal to the average preference of one treatment over the other and that the sum of *a* over all the treatments is zero. Thus, in the analyses described, a_1 refers to the parameter associated with pears from untreated trees, and a_2 and a_3 refer to OTC treatments of 0.1 and 1.0 g per tree in orchard A, and 0.5 and 2.0 g OTC per tree in orchard B.
[3]Values significant at $p \leqslant .05$ (*) or at $\leqslant .01$ (**).

DISCUSSION

Oxytetracycline treatment of trees with pear decline did not adversely affect consumer preference for fruit from these trees. In fact, in three of four taste sessions, panelists showed a preference for pears harvested from trees treated with OTC to alleviate the symptoms of pear decline. In one session, cubes of fruit from untreated trees were preferred over those from treated trees (see analysis of cubes from fruit from orchard B). Fruit texture may have been a factor in the preference for these cubes since during the picking and preparation of the fruit from orchard B it was obvious by texture and color that the fruit from treated trees was not as ripe as the fruit from untreated trees. When sauce was prepared from this fruit, texture was not a factor, and then fruit from treated trees was preferred.

All control trees had symptoms of pear decline. It would have been appropriate to test pears from healthy trees but since so many of the trees in the two orchards were affected (33% and 56% in orchards A and B, respectively), we could not reliably select disease-free trees to make such a test.

Some phytopathogenic disorders do impart an off-flavor to fruit (12). However, all of the fruit from the two orchards was marketable, including fruit from trees with pear decline.

Treatment of trees with OTC causes remission of the symptoms of pear decline and increases the number of fruit per tree, fruit size and tree vigor (6, 7). Now we have shown that fruit from trees treated with OTC to remit symptoms of pear decline could be discerned organoleptically from fruit from untreated diseased trees in all taste sessions. By the method of collection, preparation and presentation of samples, our results show that in three of the four taste sessions the fruit from treated diseased trees was preferred over fruit from untreated diseased trees.

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Comparative Study of Micro-ID, Minitek and Conventional Methods with *Enterobacteriaceae* Freshly Isolated from Foods

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ABSTRACT

A total of 400 *Enterobacteriaceae* isolates freshly taken from broiler carcasses, ground beef, pork sausage, raw shrimp, pre-wrapped sandwiches, raw carrots, lettuce and fresh strawberries was inoculated into the 15 biochemical tests of the Micro-ID (4-h) system and into the 15 corresponding tests in the Minitek (24-h) and conventional systems. For each food there were 750 biochemical test comparisons (50 isolates × 15 tests). The overall agreement between Micro-ID and conventional tests was 96.8%, whereas the agreement between Minitek and conventional tests was 93.6%. Three laboratory technicians who independently recorded results of 6000 biochemical tests from each of the three systems were in complete agreement for 99.3%, 98.9% and 99.7% of the Micro-ID, Minitek and conventional tests, respectively. Thus results obtained with the miniaturized systems were as easy to read and interpret as conventional tests in tubes. The most frequently encountered *Enterobacteriaceae* from these foods were *Escherichia coli* (broiler carcasses, pork sausage), *Enterobacter agglomerans* (carrots, lettuce, shrimp, strawberries), *Enterobacter cloacae* (pre-wrapped sandwiches), and *Serratia liquefaciens* (ground beef).

Micro-ID and Minitek are two miniaturized test kits for identification of *Enterobacteriaceae*. Micro-ID (The General Diagnostics Division of Warner-Lambert Company, Morris Plains, New Jersey) is a recently introduced self-contained identification unit containing reagent-impregnated paper discs for 15 biochemical tests requiring only 4 h for differentiation of *Enterobacteriaceae*. Micro-ID is an extension of Pathotec (General Diagnostics), which consists of individual reagent-impregnated paper strips requiring only 4 h of incubation. At this time only a few reports have been published on the Micro-ID system (*1,11,12,28*) with clinical organisms and none involving food isolates. Several clinical studies have shown that the Pathotec strips are highly accurate, quick and versatile (*2,4,5,14, 20,24,26*).

The Minitek system (BBL, Division of Becton, Dickinson and Company, Cockeysville, Maryland) is a miniaturized test kit also utilizing reagent-impregnated paper discs requiring 24 h of incubation that has been used in clinical laboratories for about 4 years. Researchers have also reported that the Minitek system is highly accurate and versatile for the biochemical classification of clinically important *Enterobacteriaceae* (*3,6,10,15,16,18,22,25*).

The objectives of this study were (*a*) to compare Micro-ID and Minitek to the corresponding conventional tube tests for obtaining biochemical information on *Enterobacteriaceae* freshly isolated from various foods, and (*b*) to determine if the results obtained from each of the systems are easy to read and interpret by different laboratory technicians.

EXPERIMENTAL PROCEDURES

Samples of freshly processed broiler carcasses, raw carrots, ground beef, lettuce, pork sausage, pre-wrapped sandwiches (cheeseburgers), raw shrimp and fresh strawberries were procured from local processing plants or retail outlets. For all the foods, except broiler carcasses, 50 g were weighed and blended for 1 min with 450 ml of 0.1% peptone water in a Waring Blendor. Broiler carcasses were individually placed in plastic bags and vigorously shaken for 1 min with 300 ml of 0.1% peptone water. Serial dilutions were made from the blended food material and broiler carcass rinse fluid and plated on Violet Red Bile Agar (Difco) containing 1% glucose (*21*). The plates were then overlayed with the same agar and incubated for 24 h at 35-37 C. After incubation, a numerical grid (*9*) and a table of random numbers (*27*) were used to select 50 isolates from each of the eight foods. The 400 isolates were streaked on Brain Heart Infusion (BHI) Agar (Difco) plates to determine purity. Each purified isolate was then inoculated into (*a*) the Micro-ID system, (*b*) the 15 corresponding Minitek discs and (*c*) the 15 corresponding conventional tests.

The Micro-ID system consists of a molded styrene tray containing 15 reaction chambers contain a single combination substrate/detection contain a substrate disc and a detection disc. The remaining 10 reaction chambers contain a single, combination substrate/detection disc. Except for the Voges-Proskauer test, each disc contains all the reagents required to perform the indicated biochemical test. For the Voges-Proskauer test, approximately 0.1 ml of 20% KOH must be added to the inoculation well after incubation. The surface of the styrene tray is covered with clear polypropylene tape to contain the organisms suspensions in the chambers during incubation but allows complete visibility. The hinged cover opens to provide easy access to the inoculation ports. Each Micro-ID unit comes individually sealed in a moisture-proof foil packet containing a desiccant. The Micro-ID system is comprised of 15 tests, which are shown in Table 1.

For inoculation of Micro-ID, in accordance with the manufacturer's instructions, a sufficient amount of each organism from a BHI agar slant incubated for 24 h at 37 C was emulsified in 3.5 ml of physiological saline solution to result in a suspension having a turbidity equal to or greater than a McFarland No. 0.5 turbidity standard. Then 0.2 ml of this suspension was pipetted into each of the 15 inoculation wells. The Micro-ID units were incubated for 4 h at 37 C, held in an upright position by a support rack supplied by the manufacturer.

The Minitek system consists of a small plastic plate containing 12 wells and a plastic top. Paper discs impregnated with individual substrates (obtained from the manufacturer) were placed into each well of the plates. For each isolate, bacterial growth from a BHI agar slant incubated for 24 h at 37 C was put into a 1.0-ml vial of Minitek inoculum broth. This broth contains trypticase peptone, L-tryptophan,

TABLE 1. *Biochemical tests used in this study.*

Test sequence	Biochemical test	Abbreviation
1	Voges Proskauer (acetoin production)	VP
2	Nitrate reductase activity	N
3	Phenylalanine deaminase activity	PD
4	Hydrogen sulfide production	H_2S
5	Indole production	I
6	Ornithine decarboxylase activity	OD
7	Lysine decarboxylase activity	LD
8	Malonate utilization	M
9	Urease activity	U
10	Esculin hydrolysis	E
11	o-Nitrophenyl-β-D-galactosidase activity	ONPG
12	Arabinose (acid production)	ARAB
13	Adonitol (acid production)	ADON
14	Inositol (acid production)	INOS
15	Sorbitol (acid production)	SORB

N-2-hydroxyethylpiperazine-N'-3-ethanesulfonic acid (HEPES) buffer and purified water. The paper discs were inoculated with 0.05 ml of the broth suspension dispensed from a pipettor with disposable tips. The Minitek discs used in this study corresponded to the 15 tests of the Micro-ID system (Table 1). Following inoculation, the hydrogen sulfide, indole, ornithine decarboxylase, lysine decarboxylase, urease, arabinose, adonitol, inositol and sorbitol discs were overlaid with 5 drops of sterile mineral oil. The plates were put in a humidor (available from the manufacturer) and incubated for 24 h at 37 C.

The conventional tests corresponding to the 15 tests of the Micro-ID system (Table 1) were carried out according to the procedures of Edwards and Ewing (13). After the appropriate periods of incubation, results from the Micro-ID, Minitek, and conventional tests were read and recorded independently by the same three laboratory technicians.

RESULTS AND DISCUSSION

The overall agreement of Micro-ID and Minitek systems with conventional tube tests with *Enterobacteriaceae* freshly isolated from eight different food sources is shown in Table 2. There were 750 comparisons (50 isolates × 15 biochemical tests) for each food. The overall agreement between results of Micro-ID and conventional biochemical tests was 96.8%; the highest agreement with results of conventional tests was with isolates from non-meat foods (carrots, lettuce and strawberries). We consider these results with the Micro-ID excellent, especially when one considers that this system only required 4 h of incubation, whereas several of the corresponding conventional tests required many days of incubation. The overall agreement between Minitek and conventional biochemical tests was 93.6%, which we believe would also be considered excellent by most laboratories. Although Minitek results agreed with

these of conventional tests for isolates from carrots and lettuce only 88 and 89%, respectively, the value for the other six foods were greater than 93%.

The agreement with conventional values for each of the 15 Micro-ID tests, (Table 3), except for inositol (89.8%) and sorbitol (85.0%), was very good (94.5-100%).

In a study of isolates from clinical sources, Aldridge et al. (1) reported values similar to ours for percentage agreement between Micro-ID and conventional tests. Although they did not show results for inositol and sorbitol, they reported that these two tests resulted in the highest incidence of unusual colors that make distinguishing a positive from a negative reaction difficult.

TABLE 3. *Agreement of individual tests in the Micro-ID and Minitek systems with the corresponding conventional tests.*

Test[a] (N = 400)	Reactions by conventional tests		Overall percent agreement with conventional tests	
	Positive	Negative	Micro-ID	Minitek
VP	278	122	97.8	97.0
N	398	2	99.8	99.5
PD	2	398	100.0	100.0
H_2S	3	397	100.0	99.0
I	88	312	99.3	99.0
OD	163	237	94.5	89.8
LD	140	260	97.8	95.8
M	244	156	98.8	93.3
U	40	360	98.5	97.8
E	303	97	95.0	93.8
ONPG	389	11	99.0	98.3
ARAB	342	58	99.3	99.8
ADON	71	329	97.3	96.5
INOS	175	225	89.8	78.0
SORB	323	77	85.0	67.0

[a]See Table 1 for abbreviations. Fifty isolates for eight foods were examined for each test.

The correlation with results of conventional tests for each of the 15 Minitek biochemical discs was very good (93.3-100%) (Table 3) for all tests except inositol (78%), ornithine decarboxylase (89.8%) and sorbitol (67%). Our results with inositol are similar to those reported by Hanson et al. (17) who found a 74.1% correlation between results of Minitek and conventional tests with 325 strains of *Enterobacteriaceae*. However, Hansen et al. (16) observed much greater agreement between Minitek and conventional tests for inositol (95.6%) and sorbitol (99%). Others (17,18) have reported some difficulties with the Minitek H_2S discs, but we did not encounter any problems with this test. In fact, we found that in 396 of 400 observations the H_2S discs corresponded to the conventional result.

TABLE 2. *Overall agreement of Micro-ID and Minitek with conventional tests using* Enterobacteriaceae *isolated from food.*

Food source (N = 750)[a]	Micro-ID			Minitek		
	No. agree	No. disagree	Percent agreement	No. agree	No. disagree	Percent agreement
Broiler carcasses	720	30	96.0	721	29	96.1
Carrots	728	22	97.1	663	87	88.4
Ground beef	727	23	96.9	716	34	95.5
Lettuce	740	10	98.7	669	81	89.2
Pork sausage	722	28	96.3	698	52	93.1
Pre-wrapped sandwiches	723	27	96.4	724	26	96.5
Shrimp	716	34	95.5	721	29	96.1
Strawberries	730	20	97.3	705	45	94.0
Total	5806	194		5617	383	
Average			96.8			93.6

[a]Fifteen tests were done on each of 50 isolates from each food.

False-negative reactions accounted for approximately 60% of the discrepant results for both Micro-ID and Minitek (Table 4). Differences in incubation time may have contributed to most of these false-negatives. The false-positive reactions are probably, in part, due to differences in substrate formulation between the conventional and the two miniaturized systems. In the miniaturized systems, carbohydrate to peptone ratios are greatly increased because of the need to generate a reaction within several hours rather than days. Research at General Diagnostics has shown, for example, that much higher percentages of *Escherichia coli* and *Enterobacter agglomerans* exhibit a positive sorbitol reaction with Micro-ID than with the conventional test (*19*). This finding agrees with our results.

TABLE 4. *Number of discrepant biochemical reactions with Micro-ID and Minitek tests.*

	Micro-ID		Minitek	
Test[a] (N = 400)	False-positive	False-negative	False-positive	False-negative
VP	3	6	7	5
N		1		2
PD				
H₂S			4	
I		3		4
OD		22	17	24
LD	1	8	2	15
M		5	4	23
U	1	5	9	
E	4	16	9	16
ONPG	2	2	7	
ARAB		3	1	
ADON	3	8	10	4
INOS	7	34	83	5
SORB	58	2	6	126
Total	79	115	159	224

[a]See Table 1 for abbreviations.

An important consideration in the use of rapid tests is whether the results are clear-cut and easy to read and interpret. Three laboratory technicians who independently evaluated and recorded each of the 18,000 test reactions were in complete agreement for 99.3% of the Micro-ID, 98.9% of the Minitek, and 99.7% of the conventional tests (Table 5). Thus the reactions of the biochemical test with both miniaturized kits were no more difficult to interpret than the conventional tubed tests.

Because Micro-ID is a new system, we decided to investigate the variation between two lots of these units. Half of the isolates from each food were tested using one lot and the other half with the second lot. Our findings (Table 6) showed practically no difference between the lots, suggesting that variation between lots has been minimized by the manufacturer of this system.

Table 7 lists the most frequently encountered species of the *Enterobacteriaceae* family from each of the foods as identified by Micro-ID. Each culture was identified by coding results of the 15 Micro-ID tests into a five digit octal number, as per the manufacturer's instructions, and then referring to a Micro-ID identification manual to determine the species corresponding to that code. In a

TABLE 5. *Agreement of three laboratory technicians in interpreting results of Micro-ID, Minitek, and conventional systems[a].*

Test	Micro-ID	Minitek	Conventional
VP	393/400	397/400	400/400
N	397/400	396/400	400/400
PD	399/400	400/400	400/400
H₂S	400/400	395/400	400/400
I	398/400	397/400	395/400
OD	399/400	398/400	399/400
LD	396/400	389/400	397/400
M	398/400	391/400	398/400
U	399/400	392/400	399/400
E	397/400	395/400	397/400
ONPG	395/400	393/400	399/400
ARAB	399/400	399/400	400/400
ADON	396/400	400/400	399/400
INOS	389/400	396/400	398/400
SORB	391/400	396/400	398/400
Total	5946/6000	5934/6000	5979/6000
	(99.3%)	(98.9%)	(99.7%)

[a]Values are the number of times the three laboratory technicians were in complete agreement/number of tests.

TABLE 6. *Comparison of two lots of Micro-ID.*

	Lot number	
Food source	OA580	OA505
Broiler carcasses	357/375[a]	363/375
Carrots	361/375	367/375
Ground beef	357/375	370/375
Lettuce	366/375	374/375
Pork sausage	368/375	354/375
Pre-wrapped sandwiches	373/375	350/375
Shrimp	347/375	369/375
Strawberries	370/375	360/375
Total	2899/3000	2907/3000
	(96.6%)	(96.9%)

[a]Number of agreements to conventional tests/number of observations.

previous study in our laboratory with other miniaturized systems (*9*), the organisms of the *Klebsiella-Enterobacter-Serratia* (KES) group isolated from foods were difficult to classify accurately. However, in this study, even though members of this group were frequently encountered, the correlation of both the Micro-ID and Minitek systems to conventional systems was very good and most encouraging.

High correlation to conventional tests (reported in this study) plus savings in cost (*8,23*), labor (*15*), space (*16*), and time (*1,7,16,17*) all indicate that miniaturized systems can serve as practical alternatives to conventional methodology for the biochemical characterization of foodborne *Enterobacteriaceae*.

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TABLE 7. Enterobacteriaceae *isolated from each food as identified by Micro-ID.*

Source	Predominant organisms	Other organisms isolated
Broiler carcasses	*Escherichia coli* (47)[a]	*Citrobacter freundii* (2), *Enterobacter cloacae* (1)
Carrots	*Enterobacter agglomerans* (39)	*Enterobacter cloacae* (4), *Serratia rubidaea* (7)
Ground beef	*Serratia liquefaciens* (37)	*Enterobacter aerogenes* (1), *Enterobacter agglomerans* (8), *Enterobacter hafniae* (1), *Yersinia enterocolitica* (3)
Lettuce	*Enterobacter agglomerans* (50)	None
Pork sausage	*Escherichia coli* (31)	*Enterobacter agglomerans* (18), *Serratia rubidaea* (1)
Pre-wrapped sandwiches	*Enterobacter cloacae* (26)	*Citrobacter diversus* (2), *Enterobacter aerogenes* (1), *Enterobacter hafniae* (2), *Escherichia coli* (1), *Klebsiella ozaenae* (1), *Klebsiella pneuomoniae* (12), *Serratia liquefaciens* (5)
Shrimp	*Enterobacter agglomerans* (21)	*Citrobacter diversus* (1), *Citrobacter freundii* (3), *Enterobacter cloacae* (6), *Klebsiella ozaenae* (1), *Klebsiella pneumoniae* (1), *Klebsiella rhinoschleromatis* (2), *Proteus vulgaris* (2), *Serratia liquefaciens* (11), *Serratia rubidaea* (2)
Strawberries	*Enterobacter agglomerans* (50)	None

[a]Numbers in parentheses indicate the number of each type of organism found in 50 isolates of each food source.

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Botulism Control by Nitrite and Sorbate in Cured Meats: A Review [1]

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ABSTRACT

Nitrite plays a major role in the botulinal safety of cured meat products. When used at appropriate levels, it retards *Clostridium botulinum* growth and delays production of its deadly neurotoxin. Even though the incidence of botulinal spores in meat is very low, factors such as the tonnage of cured meats consumed, the potential for mishandling such products, and the nature of the disease necessitate the use of nitrite or other equally effective compound(s) for extra safety. Residual nitrite and nitrosamine levels in cured meat products have been decreasing in recent years through control and research conducted by the meat industry and related institutions. Such levels are minimal compared to total nitrate and nitrite amounts ingested or formed in the human body. Sorbate, especially in combination with nitrite at concentrations adequate only for cured meat color and flavor development, is at least as effective as currently used nitrite levels in delaying *C. botulinum* growth and toxin production. The mechanism(s) through which nitrite and/or sorbate perform their actions upon *C. botulinum* remains to be found. A review of the pertinent research completed to date should be helpful in the search for the mechanism(s).

Nitrites, of one form or another, accidentally or intentionally, have been used in meat curing and preservation for many centuries. Such use has been approved and regulated by Federal agencies for over 50 years. During the current decade, meat curing with nitrite has become a controversial issue due to reports showing that nitrite added to certain meats serves as a precursor of carcinogenic nitrosamines. In recent months, several news releases and an unpublished report implicated nitrite as a cancer causing agent in experimental animals (*278*).

Nitrite addition to meat is responsible for the traditional and distinct color and flavor of products such as ham, bacon, bologna and frankfurters. Another major function of nitrite in meat curing is its effect on retarding *Clostridium botulinum* growth and toxin production. The excellent botulism safety record of commercially processed cured meat products is attributed in part to inclusion of nitrite in the formulation.

Obviously, the nitrite issue is an important one, and any action on the matter without careful consideration of all the aspects involved would possibly have major health, economic and social impacts. In its final report to the Secretary of Agriculture, the Expert Panel on Nitrites, Nitrates and Nitrosamines recommended that alternate preservatives with the potential to replace nitrite in cured meats should be evaluated. One such preservative, potassium sorbate, has already been proposed for partial replacement of nitrite in bacon production. Assuming that no data disqualifying the formulation are submitted, product preserved in this way could be in regular manufacture within a year.

Considering the above, a comprehensive examination of botulism, cured meats, nitrite and sorbate and the relationships among these seemed appropriate and timely. Some aspects were examined only briefly, primarily to provide background information and knowledge to better understand the subject. Other aspects were more extensively reviewed and hopefully this will help us to understand and possibly explain the effects reported and the mechanisms involved.

BOTULISM

Historical

Botulism is a rare but very often fatal neuroparalytic disease affecting man and animals. It is caused by ingestion of a heat-labile protein neurotoxin elaborated by the vegetative cells of the microorganism *C. botulinum* (*49,237,266*). The disease can also be caused by wound infection (*162,187*). Botulism as a foodborne disease has been recognized for over 1000 years. Dollman (*59*) has summarized the historical documentation of botulism, and Dickson (*55*) gives an excellent review of the early history of the disease.

The incidence of the disease is world-wide. It can result from the consumption of a variety of foods, including canned meats, vegetables, fruits and fish, and it affects a large variety of animals, including fish, birds, and most mammals (*266*). Botulism was first recognized in Europe as a disease caused by the consumption of sausage products. The term "botulism" was derived from the Latin word *botulus*, meaning sausage. Some medical workers with classic Greek background called the disease "allantiasis," from the Greek word *allantika* meaning sausage (*237*). This term is still being used for botulism in Greece. In recent years, plant rather than animal products have been implicated with the disease, and the historical derivation of the word botulism has lost its significance.

The organism

Van Ermengen (287) in 1897, was the first to isolate and describe in causative organism of botulism from an outbreak in Holland resulting from consumption of raw salted pork. He named the microorganism *Bacillus botulinus* and gave all the basic and essential facts about botulism: (*a*) it is an intoxication, not an infection; (*b*) a specific bacterium produces toxin in a food; (*c*) the toxin is ingested with the food and it is not inactivated by the regular digestive process; (*d*) it is relatively resistant to mild chemical agents and susceptible to heat; (*e*) it is inhibited by high salt concentrations; and (*f*) not all animal species are susceptible to the disease (237).

In *Bergey's Manual of Determinative Bacteriology*, the causative organism of botulism belongs to the genus *Clostridium*, which together with the genus *Bacillus*, is classified in the family *Bacillaceae* (49). The genus *Clostridium* includes several pathogenic species which produce a variety of toxins. They are very strict anaerobes, catalase-negative, gram-positive rods, producing heat-resistant spores. The species *C. botulinum*, with the outstanding characteristic of elaborating toxins, is divided into seven types, A through G, according to the serological specificity of the toxins (237). Types A, B, E and F produce human botulism, while types C and D are implicated with animal botulism (172,237,266). Type G was isolated only recently from soil samples in Argentina (89), and no clinical cases have been reported.

Types A and B were the first to be isolated and identified (31,32). Type C was first discovered in 1922, simultaneously in the United States and Australia (21,227,228). Type D was isolated from the carcass of a cow in South Africa, and it was designated as such by Meyer and Gunnison (164). Type E was encountered in the Ukraine and Germany in 1936 and 1937 (113), while Moller and Scheibel (169) were the first to recognize type F in Denmark. Another type, producing a toxin with serological characteristics of both A and F strains, was isolated by Gimenez and Ciccarelli (88) and it was designated as A_f.

The different types of the species *C. botulinum* have been classified into four distinct groups, based on their cultural and serological characteristics (237,266). The similarities among these groups are that they are all clostridia and produce toxin with similar pharmacological action. Group (I) includes the strains previously known as "ovolytic," which are all the proteolytic type A strains and the proteolytic strains of types B and F. Group (II) includes all type E strains and the non-proteolytic strains of types B and F. Group (III) includes the type C strains and the strains of type D. Group (IV) includes the proteolytic but nonsaccharolytic type G strains.

The growth requirements of the organism include several amino acids, growth factors and inorganic salts. It can best grow in rich organic media, such as meat, and without competition it can even grow in relatively poor media such as vegetables, fruits, and moist straw (172,237). The minimum pH for growth is 4.6 and the maximum 8.3 (172,237,266). The minimum temperature for growth and toxin production is 3.3 C for some type B and E strains and 10 C for type A strains, and the maximum is 55 C. The optimum temperature for growth of type B and F strains is 37 C and of type E strains 30 C (172,209).

A water activity (a_w) of 0.975 (5% NaCl) inhibits growth of type E strains, while types A and B can grow in up to 10% NaCl or up to 50% sugar concentrations and at an a_w value of 0.935 (172,266). Combinations of unfavorable pH, temperature and solute concentration are more restrictive to growth than is each variable alone (176). Although *C. botulinum* is a strict anaerobe, growth and toxin formation can take place in the presence of oxygen given a low enough oxidation-reduction potential, as in the interior of cooked sausages (172). In general, the vegetative cells have the heat resistance of mesophilic bacteria, being killed in a few seconds at temperatures above 60 C, while the heat resistance of the spores is high and varies with the type. The heat resistance of spores decreases with type, diminishing from A to E. Generally the nonproteolytic (Group II) strains are of lower heat resistance than the proteolytic (Group I) strains (69). Spores of type F strains have a heat resistance similar to that of type A and B spores. The $D_{121\,C}$ value for type A spores is 0.232 min, which determines the *botulinum cook* or process of 12D equivalent to an $F_{121\,C}$ or F_0 value of 2.78 min (172,266). The spores of some strains are among the most resistant bacterial spores encountered, surviving more than 30 years in a fluid medium (118).

The toxins

Vegetative cells of different *C. botulinum* types can synthesize during growth eight types of neurotoxins, based on their serological activity (237). The toxins are released mainly from the cells during autolysis or cell degeneration, after growth has reached a maximum (172,266). They are simple proteins, composed of 21 amino acids with a molecular weight of 900,000, and appear to consist of an acid-labile autoxigenic (Ea) and a non-toxigenic (Eb) component. The Eb component protects the Ea component from the acidic gastric juice during ingestion (172,237). A unique characteristic of the botulinal neurotoxins is their extreme toxicity. The estimated human lethal dose is about 5×10^{-8} g, or 1 g of toxin could kill more than 500 million people if properly diluted (266).

Purified toxins can be inactivated in a few minutes at 50 C at pH 6.9, while they are stable for months in phosphate buffer (0.5 M, pH 6.2-6.8) or in NaCl solution (0.1 M, pH 7.2). Higher temperatures are necessary to inactivate the toxins in food systems. Types A and B can be inactivated in 6 min at 80 C and 90 min at 65 C. More details about toxin stability are given by Riemann (199). The activity of toxin produced by type E strains is increased by trypsin, while types A and B are believed to produce their own proteolytic enzymes (237,266). After entering the circulatory system, the toxin molecules are

bound specifically to the neuromuscular junctions, where they act by blocking the presynaptic release of acetylcholine from cholinergic junctions, thus blocking normal voluntary muscle movement.

The disease and its symptoms

The symptoms of botulism are described in medical literature dating back to the 1700's. The usual incubation period is 18-36 h and the onset of symptoms may occur as soon as a few hours or as late as 8 days after the contaminated food has been ingested (35,266). The earlier the onset of symptoms, the higher the toxin concentration and the higher the probability of fatality.

Most common symptoms and percentage of their occurrence are: blurred vision, diplopia, photophobia (90.4%); dysphagia (76.0%); generalized weakness (57.7%); nausea and/or vomiting (55.8%); disphonia (54.8%); dizziness or vertigo (30.8%); abdominal pain, cramps, fullness (20.2%); and, diarrhea (15.4%). Other less common symptoms include: urinary retention or incontinence, sore throat, constipation and paresthesias (35). Most common signs of botulism and percentage of their occurrence are: respiratory impairment (73.1%); specific muscle weakness or paralysis (46.2%); eye muscle involvement, including ptosis (44.2%); dry throat, mouth or tongue (21.2%) and dilated, fixed pupils (15.4%). Other less common signs are: ataxia, postural hypertension, nystagmus and somnolence (35). In general, botulism toxin causes muscle paralysis, starting with the eyes and the face and progressing down to the chest and finally to the arms and legs. Eventually, when the toxin concentration is high, the diaphragm and chest muscles become paralyzed and death may occur due to asphyxiation. Disease therapy includes treatment of the symptoms associated with respiratory impairment and use of therapeutic antitoxin (266).

Incidence

In the United States, botulism began receiving increased attention during World War I when its occurrence appeared to increase. An explanation for that increase could be the rise in population in the area west of the Mississippi River, where *C. botulinum* type A is very common in the soil. Other reasons for the higher incidence of the disease recorded after World War I might very well be the increase in the amount of home canning as well as the better understanding and identification of the disease and its symptoms (237). From 1899 through 1973 a total of 688 outbreaks occurred in the United States (35). According to the Center for Disease Control (CDC), for the same periods (1899-1973) the number of cases was 1,784 with 978 reported deaths. For the first 50-year period the case fatality rate was above 60%, but since about 1950 it has shown a gradual decrease with a 23.5% rate for the period 1970-1973. This decline can probably be attributed to improvements in supportive care and intensive respiratory care (35,266). Of the 688 foodborne botulism outbreaks reported (1899-1973), 23.1% were

caused by type A toxin, 6.3% by type B, 3.2% by type E, 0.1% by type F, and in 67.3% the type was not determined (35).

A distinctive geographical distribution of botulinal toxin types exists in the United States. Ninety percent of the recorded outbreaks of type A occurred in states west of the Mississippi River and 67% of the type B outbreaks were recorded in Eastern States. Type E outbreaks have been mostly reported from Alaska and the Great Lakes area (35). This regional distribution of outbreaks is in agreement with results of soil surveys showing a predominance of type A spores in samples from the West and of type B spores in samples from the Northeast and Central states (163). Burke (32) reported that nine of the 14 strains isolated on the Pacific coast were of type A and all nine strains isolated on the East coast were of type B. Spores of type E strains have been isolated from the marine life and sediments of the Pacific Northwest and the Great Lakes (24,69).

Of the 688 recorded outbreaks, 72% have been caused by home-processed foods, 9% by commercially processed foods and 19% by unknown foods (35,266). Vegetables, fish, fruit and condiments have been the most common vehicles of botulism intoxication, while meat and milk products have caused relatively few outbreaks (Table 1). Type E botulism was found to be important in 1963 when 22 cases were reported (216,217). Most type E outbreaks have been associated with consumption of fish or fish products. Types A and B are the major causes of botulism in heat processed foods, because the high heat resistance of their spores permits survival in underprocessed foods.

TABLE 1. *Botulism outbreaks.*[1]

Food Processing Type	Period			% of total
	1899-1949	1950-1973	1899-1973	
Home processed	382	113	495	72.0
Commercially processed	48	14	62	9.0
Unknown	47	84	131	19.0
Food Product[2]				
Vegetables	—	—	150	68.5
Fish and Fish Products	—	—	29	13.2
Condiments	—	—	20	9.2
Meats	—	—	11	5.0
Other	—	—	9	4.1

[1]Calculated from data given in (35).
[2]Only from outbreaks in which the toxin type was determined.

Methods to prevent botulism include: Destruction of the causative organism through sufficient heat processing (sterilization) or irradiation, mild thermal processing of the food combined with addition of appropriate food preservatives to retard growth of the heat-injured spores, product dehydration or solute addition to decrease the water activity to inhibitory levels, acidification, and refrigeration or freezing (49,266).

Besides the references mentioned, more information on all aspects of botulism can be found in the Proceedings of the First United States/Japan Conference on Toxic Microorganisms (115). Schantz and Sugiyama (223) reviewed several aspects of botulism such as history, epidemiology, diagnosis etc. They (223) also give more detailed information about the toxins and aspects associated with them. Hobbs (117) gives an excellent review of type E botulism in fish products, including a summary on botulism in general.

SODIUM NITRITE IN CURED MEATS

Historical

Use of nitrite in meat curing is lost through eons of time. Experience and scientific knowledge have indicated that when nitrite is added to meats, it performs the following functions: (a) it produces the characteristic cured meat color and affects flavor; (b) it has antioxidant activities which prevent the "warmed-over" flavor; (c) it retards C. botulinum growth and toxin production, which can occur if the product is mishandled and temperature-abused and (d) it may have, along with salt, a positive effect on product texture, which is not clearly defined. A short review follows of the early events that led to regular use of nitrite in meat curing, as well as the major highlights which have marked that use through the years.

Preservation of meat with salt preceded the intentional use of nitrate and nitrite by many centuries. Binkerd and Kolari (22) gave an excellent review of the history and use of nitrate and nitrite in meat curing, indicating that cooked meats and fish were preserved in sesame oil jars as early as 3000 BC in Mesopotamia. The Council for Agricultural Science and Technology (48) gave an extensive report on use of nitrite in meat curing, its effects on botulism control and human health and the possible consequences of unavailability of nitrite for meat preservation.

It is believed that meat preservation was first practiced in the saline deserts of Hither Asia and in coastal areas (22,220). During Homer's time (900 BC) the preservation of meat with salt and smoke was well established, and later it was transferred to the Romans. In ancient Greece, salt was manufactured in "salt gardens" and used freely in the preservation of fish (134).

During the late Roman times it was noticed that red patches were formed on the surface of meat preserved with salt. Desert salts contain nitrate and borax impurities (220). The thought that the reddening effect noticed was due to nitrate impurities of the salt led to deliberate addition of nitrate to the meat to achieve color uniformity (49). The first form of nitrate to be used was saltpeter or nitre [Ca(NO$_3$)$_2$], which was formed by nitrifying bacteria and found on the walls of caves and stables (134). As time passed, use of nitrate became regular, curing techniques were developed, the importance of nitrite over that of nitrate was realized, nitrite's effects and functions were determined, and curing of meat became a science. Finally, secondary effects of using nitrite were studied and these led to the current public interest and controversies.

In the National Provisioner Handbook of 1894, a pickle formula was given consisting of 7.66 lb. of salt, 2 lb. of sugar, and 0.33 lb. (3333 $\mu g/g$) of saltpeter (22). A saltpeter level of 5,000 $\mu g/g$ for preserving and giving juiciness and flavor to bacon was common practice during that time. The transformation of meat curing from an art to a science started toward the end of the 19th century when chemists became employed by the meat industry. Development of curing methods such as dry cure, wet and pickle cure combinations, and pumping occurred during this time. Another achievement of this period was the recognition that nitrite was formed through microbial action on nitrate, and that nitrite instead of nitrate was responsible for cured meat color development (22). The first report of nitrite existence in cured meats was by Polenske (195) in 1891, who concluded that it was derived through bacterial reduction of nitrate. The effect of nitrite, and not nitrate, in producing cured meat color was reported by Lehman (153) and Kisskalt (144) in 1899. Haldane (108) in 1901 demonstrated nitrosohemoglobin formation by addition of nitrite to hemoglobin and transformation of the compound to nitrosohemochrome, which is the cured meat color. This pioneer scientific work was confirmed by Hoagland (116) in 1908, who also explained the microbial and enzymatic reduction of nitrate to nitrite, nitrous acid and nitric oxide.

On May 1, 1908 the Bureau of Animal Industry of the United States Department of Agriculture (USDA) permitted the addition of saltpeter to meat or meat food products (275). On January 19, 1923 the Bureau of Animal Industry (USDA) gave permission for experimentation on the direct use of nitrite in meat products. A series of experiments were undertaken by Kerr et al. (143) to determine the practicability, as well as the advantages and disadvantages, of the direct use of nitrite in meat curing. The aspect of human safety was given primary consideration. That extensive and pioneer work led to the following conclusions: (a) sodium or potassium nitrate could be successfully replaced by sodium nitrite in the curing of meat; (b) a quantity of 1/4 to 1 oz of sodium nitrite per 100 lb. of meat (156-625 $\mu g/g$) was sufficient for color fixation, depending on the meat and the curing process employed; (c) the levels of sodium nitrite necessary for meat curing were not higher than the nitrite levels found in meats cured with nitrates, and unconverted nitrate was avoided; (d) the curing period could be shortened by the direct use of nitrite; (e) the quality and wholesomeness of meats cured with sodium nitrite were not inferior compared to meat cured with nitrates and (f) as a result of these findings, the USDA authorized use of sodium nitrite in meat curing in Federally inspected plants in 1925. The authorization (October 19, 1925) among others, stated that "the finished product shall not contain sodium nitrite in

excess of 200 $\mu g/g$'' (22).

In the 1920's, interest in the antibacterial effects of nitrite started to develop due to its preservative action in raw cured meat (74,154,256,257,258). Later, Tarr (260,261,262) reported that 0.02% (200 $\mu g/g$) nitrite at pH 6.0 inhibited several genera of bacteria such as *Achromobacter, Aerobacter, Escherichia, Flavobacterium, Micrococcus,* and *Pseudomonas.* Brooks et al. (28) in 1940 reported that nitrite reduced microbial growth on the surface of bacon.

Barnes and Magee (19) in 1954 discovered the hepatotoxic properties of nitrosamines, and Magee and Barnes (157) in 1956 discovered the carcinogenic properties of nitrosamines. A rare liver disease occurred in ruminants and mink in Norway during the 1957-1962 period. Studies to determine the cause of the disease showed that it was related to feeding animals a herring meal that was preserved with sodium nitrite. N-nitrosodimethylamine was isolated and characterized as the toxic substance present in the feed (49). The suggestion was made that nitrosamines might also occur in human food if their precursors (amines and nitrite) are naturally present or added. Nitrosamines were found in nitrite-treated fish by Sen et al. (223) in 1970, and in various processed meats by Fazio et al. (76) in 1971. Since then, nitrosamines have been found in fried bacon and in some instances in severely fried country-style ham. Lijinsky and Epstein (155) in 1970 made one of the earliest public calls for reduction or elimination of nitrite from the human diet.

As a result of all these events, some questions were raised about the importance of nitrite and nitrate in relation to botulinal protection and nitrosamine formation in cured meat products. Late in 1969 a government-industry working group was organized to study the safety of nitrite and nitrate in foods (3). A combined effort was undertaken in 1972 by the USDA, the Food and Drug Administration (FDA) and the American Meat Institute (AMI) to study the roles of nitrite and nitrate in processed cured meats. Major interest was given to factors associated with *C. botulinum* toxicity and nitrosamine formation, and five cured meat product categories were examined. The cured meat products studied were: canned cured meats, smoked comminuted sausage, bacon, fermented sausage and dry cured primal cuts. The objectives of the studies, as listed by Bard (18), were: (a) to determine whether or not approved levels of sodium nitrite reduced the risk of *C. botulinum* toxin production, (b) to evaluate whether detectable levels of nitrosamines were formed when nitrite was used at approved levels, (c) to decide whether regulations governing use of sodium nitrite could be adjusted or modified in such a way that the benefits of such use would be retained while the risks would be minimized or eliminated and (d) to determine whether or not use of nitrate in conjunction with nitrite was of any benefit.

The experimental work was conducted by researchers of the USDA, FDA and the meat industry, and in some instances the Food Research Institute (FRI) of the University of Wisconsin was also involved. That extensive research concluded that: (a) sodium nitrite, when used at approved levels, reduced the risk of botulinal toxicity in cured meat products; (b) nitrosamine (nitrosopyrrolidine) was mostly found in crisp-fried bacon at parts per billion (ppb) levels; (c) lowering the nitrite and increasing the ascorbate or isoascorbate levels in bacon decreased nitrosopyrrolidine occurrence in the fried product and (d) sodium nitrate did not produce any apparent effects in controlling *C. botulinum.*

During the current decade the interest in nitrite and its effects on cured meat safety and human health has been increasing, and it has reached the point of being a controversy. Research on its role related to *C. botulinum* safety, nitrosamine formation, and possible reduction or total replacement has been intensified. In 1973, the Secretary of Agriculture established an Expert Panel on Nitrites, Nitrates, and Nitrosamines for a 2-year period, to examine the role of nitrite and nitrate in cured meats and their public health significance as related to botulism and nitrosamines. In July 1974 the Panel issued three recommendations (3): (a) use of nitrate be discontinued in all meat and poultry products, except dry-cured products and fermented sausages; (b) the nitrite level permitted for curing of meat should be limited to 156 $\mu g/g$ in all cured meat products, except bacon and dry-cured meats for which more research was required and (c) the permitted residual nitrite level should be reduced from 200 to 100 $\mu g/g$ in cooked sausage products, 125 $\mu g/g$ in canned and pickle-cured products and 50 $\mu g/g$ in canned, cured sterile products.

Data gathered during that period (1972-1974) indicated that nitrosopyrrolidine was rather consistently found in crisp-fried bacon. The importance of the matter as related to the Delaney clause led the Secretary of Agriculture to decide against incorporating the Panel recommendations into the regulations and to extend the charter of the Panel for an additional 2 years to further examine the issues and consider new scientific data. Later the Panel was enlarged, and its interests and expertise were broadened. Based on data from the industry and other institutions, the Panel issued its final report in September, 1977. The recommendations included lowering the ingoing nitrite levels in most products and eliminating them from all infant, junior, and sterile-processed products. The recommendations of the Panel appear in Table 2 taken from (3). In some instances the Panel did not make any recommendations due to insufficient data or requested more information before it could make its final recommendations.

The Panel also recommended the evaluation of alternate preservatives with the potential to replace or reduce nitrite in cured meats, and if the testing were adequate and positive the USDA should approve such alternates. On April 27, 1978 a petition was filed with the USDA to allow the addition of potassium sorbate to bacon in conjunction with 40 μg of nitrite/g. The petition

stated that it allowed a reduction of nitrite in bacon along with a reduced potential for nitrosamine formation on frying, that the bacon produced in this manner was essentially of the same color and flavor as bacon presently available, that based on experimental data the antibotulinal protection in such bacon was at least equivalent to present commercial products and that mold inhibition during aerobic storage was improved (6). On May 15, 1978 the USDA, after considering the above petition, proposed that bacon in the future be produced with 40 μg of nitrite/g of product and 0.26% (wt/wt) potassium sorbate (277). This proposal is to become effective within 1 year unless data are submitted to show inadequate botulism protection, or nitrosamine formation at levels detectable by presently available techniques.

A review of nitrite and its effects in cured meat products follows. Some aspects are only briefly examined, while others, related to botulism, are more extensively reviewed. Finally a summary of sorbate, its effects on foods in general, and in cured meats in particular with regard to botulism and nitrite is given.

Nitrite effects on color, flavor, rancidity

As previously stated, the effect of nitrate and subsequently nitrite on the cured meat color development was noticed many years ago. Haldane's work (108) demonstrated the mechanism of cured meat color formation through nitrite and hemoglobin. Kerr et al. (143) reported that as little as 20 μg of residual nitrite/g was sufficient for an acceptable color and flavor in hams.

In recent years, several reports show the effect of nitrite on the color of cured meat products. An unpleasant gray color in unsmoked frankfurters prepared without nitrite in the cure was reported by Wasserman and Talley (293). Hustad et al. (123) reported that cured meat color was absent from nitrite-free wieners, while no color differences were noticed in wieners formulated with 50, 100, 150 μg of nitrite/g. In a bacon study (114), product formulated without nitrite received the lowest color ratings, while bacon produced with 15 μg of nitrite/g was rated slightly higher. All bacon treatments made with 30-170 μg of

nitrite/g were bright pink and retained their color during storage. Improved color and organoleptic properties were reported by Kemp et al. (142) in packaged slices of dry-cured hams containing nitrate and/or nitrite. The effect of nitrate and nitrite on the color and flavor of country-style hams was studied by Eakes et al. (67). They reported that curing with nitrate and/or nitrite gave a more acceptable color than did curing with sucrose and salt only. Eakes and Blumer (66) found that 70 μg of nitrate and/or nitrite/g gave an acceptable and adequate color to pork loins and country-style hams.

It has been shown that nitrite concentrations considerably lower than those used in practice, will provide the characteristic cured meat color (159). The main portion of the total nitrite (156 μg/g) added to cured meat products is used for control of C. botulinum, and only a small fraction (about 25 μg/g) is needed for development of the characteristic color and flavor of the products. Hustad et al. (123) reported that as little as 25-50 μg of sodium nitrite/g was adequate to give the typical color and flavor in wieners. Theoretically, only 3 μg of sodium nitrite/g is needed to give the characteristic cured meat color (220). This nitrite concentration allows for only 50% conversion of myoglobin to nitric oxide myoglobin. To attain a stable color, and since nitrite is also used in other reactions, at least 25 μg/g is necessary, assuming that the distribution is adequate. However, under commercial conditons, a level of up to 75 μg/g could be needed (220). Incomplete color formation was attributed by Kerr et al. (143) to insufficient nitrite penetration into the meat, and to an unusually low hemoglobin concentration. Ingram (125) states that for 30 years it has been known that as little as 5 μg of nitrite/g is adequate to give a satisfactory cured color for a limited time, and that concentrations up to 20 μg/g are necessary to provide adequate color stability. However, detailed experimentation to confirm the above is lacking.

Brooks et al. (28) in 1940 repeated Haldane's (108) conclusions that cured meat color is related to nitrosohemoglobin formation through the reaction of nitrite with hemoglobin found in muscle tissue and blood. The role of nitrite in formation of cured meat color has been well established, and the chemistry of the

TABLE 2.   *Sodium nitrite, sodium nitrate, sodium ascorbate/isoascorbate usage and residual sodium nitrite in various cured meat products* (3).

| | Canned, cured — | | Comm. sterile | Bacon | Cooked sausage | Other pickle cured | Dry cured cuts | Fermented sausage | Infant, junior, toddler foods |
	Perishable	Shelf-stable							
Ingoing sodium nitrite (target level)[2]	156 ppm	156 ppm	50 ppm	120 ppm	100 ppm	156 ppm	100 ppm	100 ppm	0[1]
Ingoing sodium nitrate (target level)[2]	0	0	0	0	0	0	300 ppm[3]	0[3]	0[1]
Ingoing sodium ascorbate or isoascorbate (target level)[2]	550 ppm[7]	550 ppm[7]	—[6]	550 ppm	550 ppm[7]	550 ppm[7]	—[6]	—[6]	0[1]
Minimum residual sodium nitrite at time of manufacture*	125 ppm	125 ppm	—[4]	80 ppm	—[5]	125 ppm	—[6]	—[6]	0[1]

*USDA will set maximum residual sodium nitrite levels at appropriate time after manufacture if it is deemed necessary for protection of public health.
[1]In the meat portion of any infant, junior or toddler food product.
[2]In the proposed rulemaking, USDA will present acceptable ranges around target level.
[3]The manufacturer may present data to USDA showing need for more sodium nitrate and safety of requested levels from nitrosamine formation in product.
[4]USDA to set level in proposal, but less than 50 ppm.
[5]USDA to set level in proposal, but less than 100 ppm.
[6]Insufficient data available at this time to make any recommendation.
[7]Insufficient evidence presented to the Panel, although indications are that its role as a blocking agent would be similar to that in bacon.

curing process has been reviewed (11,13,17,61,84,168, 259). Fox (84) gave a good report on the chemistry of meat pigments and indicated that nitrite was responsible for development of cured meat color through its reduction to nitric oxide and reaction with the meat pigment myoglobin. The compound formed through the reaction of nitric oxide and myoglobin is called nitroso-myoglobin or nitric oxide myoglobin and is relatively unstable. The protein moiety of the compound is denatured by heat during thermal processing and a much more stable compound, the cured meat pigment nitrosohemochrome, is formed. However, it is unstable in the presence of light.

Brooks et al. (28) were the first to describe the relationship of nitrite to cured meat flavor. Bailey and Swain (14) reviewed the very limited studies and reported on the interaction of nitrite and meat constituents that influence flavor. Ingram (125) reported that the nature of the effects of nitrite on cured meat flavor was still unknown. He (125) also reported that about 50 μg of nitrite/g was necessary for flavor development, but satisfactory quantitative evidence for this is lacking. Most studies reported on the effect of nitrite on cured meat flavor are either on processing and sensory evaluation of different products, or on chemical analyses of the reactions between nitrite and the meat components. Wasserman (288) gave a review of these studies, but the information available was insufficient for a complete discussion of the role of nitrite on cured flavor development. Cho and Bratzler (39) on pork longissimus dorsi muscle and Wasserman and Talley (293) on frankfurters, performed the first rigorous tests on the role of nitrite in cured meat flavor formation, indicating that nitrite-containing products showed a more intense cured flavor.

Considerably less than 200 μg of nitrite/g is required to provide the characteristic cured meat flavor (171). Hustad et al. (123) reported that the flavor of wieners made with nitrite was judged significantly higher than the flavor of wieners formulated without nitrite. The acceptance and stability of bacon were affected by added nitrite level (114). Bacon made without nitrite had lower initial flavor scores and the flavor was lost more rapidly than bacon formulated with nitrite. Kueper and Trelease (147) reported that nitrite dramatically improved the flavor and appearance of fermented sausage. An increase in bacon and a decrease in "porky" flavor were reported by Mottram and Rhodes (171) with increasing nitrite levels. The effects of nitrate and nitrite levels on thuringer sausage were studied by Dethmers et al. (52). Addition of 50 μg of nitrite/g was necessary for the development of typical thuringer flavor and appearance. A higher nitrite concentration (100 μg/g or more) gave higher flavor and appearance ratings. However, Wasserman and Kimoto (291) reported that the flavor of laboratory-produced bacon without nitrite was rated comparable to that of a popular national brand.

Information on rancidity retardation by nitrite in cured meats is given by Cross and Ziegler (50) and Watts

(294). Herring (114) found that off-flavors were higher and increased more rapidly in bacon without or with only 15 μg of nitrite/g, while the rates of off-flavor development and the decrease in acceptance scores were lowest in bacon produced with 170 μg of nitrite/g.

BOTULISM, NITRITE, CURED MEATS

The most important nitrite effect in cured meat products is its inhibitory action against C. botulinum growth and toxin production. Extensive research has been conducted in relation to the role of nitrite in controlling growth of spore-forming anaerobic bacteria. To retard bacterial growth, nitrite concentrations higher than those needed for color and flavor development are necessary. The botulinal safety of commercially processed cured meat products through the years has been attributed to the action of nitrite, as well as other factors such as low spore incidence, heat process, pH, refrigeration etc.

Nitrite levels

An excellent review of the interaction between nitrite levels and clostridia was presented to the USDA Expert Panel on Nitrites, Nitrates and Nitrosamines by Foster and Duncan (81) in 1974. Generally the effect of nitrite in controlling C. botulinum growth and toxin formation increases with increasing nitrite concentrations. This has been known since the early stages of nitrite use when it was observed that higher initial nitrite concentrations extended the shelf-life of the products.

Greenberg (98) and Johnston et al. (137) indicated that the initial nitrite concentration·was more critical in controlling botulinal growth than the residual nitrite concentration. This has recently been challenged and it will be discussed in more detail later in the text.

Grever (103) reported that to guarantee complete prevention of clostridial growth, 100 or 200 μg of nitrite/g should be added, depending on the heat treatment, and the pH should be lower than 6.2. This, however, can be questioned since several other factors can be involved and change the conditions. Christiansen et al. (40,41) determined that increased levels of formulated nitrite decreased the probability of botulinal toxin formation in canned, comminuted cured meat and in bacon inoculated via pickle or after slicing. Similar results were reported with wieners (25,123). Bowen et al. (25) found that nitrite levels above 50 μg/g inhibited toxin formation. Hustad et al. (123) reported that without nitrite, toxicity was detected after 14 days at 27C, whereas with 50 μg of nitrite/g one toxic sample was detected after 56 days, and with higher nitrite concentrations no toxic samples were found.

pH effects

The average pH of most cured meat products falls within the range of 5.6 to 6.6 (150). Dozier (60) in 1924 examined the pH growth range of 37 strains of vegetative inocula and 19 strains of spore inocula in phosphate buffered double strength veal infusion-2% Difco

peptone. The results revealed that the pH growth range for the vegetative inocula was 4.89 to 8.89 and for the spore inocula 5.01 to 8.89. The limiting pH for growth of *C. botulinum* spores type A and B is 4.6 to 5.0 *(128,266)*. Townsend et al. *(274)* studied the effect of pH on growth of *C. botulinum* in a variety of foods and found that pH 4.7 was the lowest level at which germination and growth could be expected. It was also determined that some food products were less favorable for germination than others, and that type A strains were equally or more tolerant to acid than type B strains as far as toxin production and growth were concerned.

Nitrite has been shown to be more effective with decreasing pH in controlling *C. botulinum* growth. Grindley *(104)* was the first to observe this nitrite effect under acid conditions and suggested that this phenomenon could be associated with the presence of nitrous acid. Tarr *(260,261,262)* confirmed Grindley's *(104)* conclusions, showing that the preservative action of nitrite in fish was increased by acidification. He also showed that the inhibitory effect against several species of bacteria increased markedly at pH levels below 6.0. The data indicated that at pH 6.0, 0.02% (200 µg/g) sodium nitrite entirely inhibited or strongly retarded bacterial growth, while at pH 7.0 very little or no inhibition was observed. That the nitrite effect in controlling bacterial growth increases at lower pH levels has also been shown by Castellani and Niven *(34)*, Silliker et al. *(236)*, and Roberts and Ingram *(210)*. A ten-fold increase in the inhibitory effect of nitrite from pH 7.0 to 6.0 was reported by Roberts and Ingram *(210)*. This ten-fold change with drop of one unit in pH was also demonstrated by Castellani and Niven *(34)* with *Staphylococcus aureus*; Eddy and Ingram *(68)* with *Bacillus* species; and Perigo et al. *(184)* with *Clostridium sporogenes* strain Putrefactive Anaerobe 3679 (PA 3679). Shank et al. *(234)* reported that undissociated nitrous acid was the molecular species active for inhibition of *C. botulinum* spores, and consequently maximum protection was afforded at lower pH levels. They *(234)* also showed that as the pH approached 5.5, the nitrite effect started to diminish as it reacted with constituents of the medium.

In one study of actual meat systems undertaken by the USDA, FDA and AMI, Christiansen et al. *(42)* investigated the effects of nitrite, dextrose and starter culture on growth of *C. botulinum* in a summer-style sausage. The data showed that dextrose in the formulation helped decrease the pH during storage and that a level of 50 µg of nitrite/g was sufficient to inhibit growth and toxin production. When the product was formulated without dextrose, the pH remained at its initial level of 5.63 and 150 µg of nitrite/g was insufficient to prevent toxin production. In formulations without nitrite, fewer of the samples containing starter culture became toxic compared to those containing only dextrose even though the pH was the same 1 week after storage. Since the pH of meat cannot be easily lowered due to its high buffering capacity *(49)*, it is fortunate that the pH of cured meat products is low enough (5.6-6.6) for nitrite to be effective in retarding *C. botulinum* growth.

Spore incidence in meats and inoculation levels

Raw material contamination and the inoculum size used in botulism studies can greatly influence the rate of *C. botulinum* growth and toxin production. The inoculum levels employed can override the effects of the inhibiting agents used, or they can interact with them and accordingly affect the safety of the products. The natural spore inocula found in raw meats are also of importance because knowledge of their size can help decide what inoculation levels would be logical in studies. Knowledge of the levels of natural contamination would also be helpful in judging the practical significance of botulism studies and how they can relate to real life.

Generally the anaerobic spore contamination of raw meats is considered to be low and the botulinal contamination even lower. Ayres *(12)* determined the number of putrefactive anaerobic spores in canner-grade beef to be in the range of 0.007 to 0.06 per gram, with a maximum of 1.4 spores per gram. In freshly ground trimmings, the maximum anaerobic spore load was found to be 42, while the aerobic spores ranged from 1 to 19,000 per gram. In another study, Steinkraus and Ayres *(245)* found the usual number of putrefactive anaerobic spores in fresh pork trimmings to be less than 0.18 per gram. In cured pork trimmings and cured pork luncheon meat the average spore load was less than 1 per gram, while the maximum spore count found in any sample tested was 51 per gram. A variety of meat products was surveyed by Greenberg et al. *(101)* in the United States and Canada. Of the 2,358 samples tested, only one botulism-positive was detected. The incidence determined by the above studies is significantly lower than what subsequent studies have shown, and use of a selective medium in the isolation procedure could be responsible for that *(150)*. Pivnick et al. *(190)* stated that it is probable that some cured meats are safe because of the scarcity of *C. botulinum* in the raw products. Insalata et al. *(129)* examined 100 samples of vacuum-packaged meat and some cheese products and found only one type B-positive frankfurter sample. Of 372 meats surveyed by Abrahamson and Riemann *(1)*, only six cooked ham samples were positive. When the sample size was increased (75 g), 19 of 26 samples showed toxin production.

United States meats and meat products surveys for *C. botulinum* incidence were discussed by Roberts and Smart *(214)* and indicated only an occasional incidence of one cell per 0.6-3 kg of pork. A United Kingdom study of experimentally-produced bacon from commercial bacon pigs showed that 36 of the 297 samples tested contained *C. botulinum* types A and B *(214)*. The sample weight ranged from 25 to 100 g. The above study was followed by a more systematic one *(215)* which demonstrated that 30 of the 684 bacon samples tested

contained type A, B or C toxin. Roberts and Smart (215) concluded that the incidence of *C. botulinum* in meat is relatively low, with the possible exception of bacon. Of 75 luncheon meats and 17 sausage samples tested, only one positive sample (type B) was found in a luncheon meat (249).

It is expected that with improved methodology and increased sample size, the determined incidence will be higher. Lechowich et al. (150) indicated that the occurrence of spores is a function of geographical location and food product under consideration. They (150) also stated that some important factors to be considered when evaluating spore incidence studies include sample size, isolation and growth media and incubation temperatures. The above authors (150) concluded that, although the data available were relatively inadequate and the procedures and sample sizes varied from one study to the next, an approximation could be made that the average incidence was one *C. botulinum* spore per 1-7 lb. of product. This appears to be a low incidence, but as Lechowich et al. (150) stated, the potential for botulism is quite apparent when the tonnage of products manufactured and consumed is considered.

Inoculation studies have demonstrated that increases in the spore inoculum level can override the inhibitory effect of nitrite and allow toxin production (25,40,41,123, 190,193,244). Some reports indicate that the commonly employed heat processes ($F_0 = 0.05$-0.6 min) and curing salts concentrations (78-156 μg of nitrite/g/2.0-2.5% sodium chloride) would be inadequate in controlling botulism toxicity if the spore inoculum level was in the range of 100 to 10,000 per gram of meat. Ingram (125) stated that systems safe with 1-10 spores/g failed when challenged with inoculum sizes 100 to 1000 times greater. Christiansen et al. (40) working with comminuted canned cured meat, found that the nitrite concentration necessary for complete botulinal inhibition was dependent on the spore inoculum level. With 90 spores/g, toxic samples were detected with 150 but not with 200 μg of nitrite. With 5,000 spores/g, toxin was detected with 400 but not with 500 μg of nitrite/g at 27 C.

Vacuum-packaged bacon with 100 spores/g showed no toxic samples during 32-day incubation at 20 C. With the same nitrite concentrations (100 and 200 μg/g) and 10,000 spores/g, three toxic samples were detected. Bacon samples inoculated via pickle and showing an average of 52 spores/g after processing were protected with 170 or 340 μg/g but not with 120 μg of nitrite/g or less (41). With increased spore concentration (4,300 spores/g) toxic samples were detected at all nitrite concentrations used and inoculation methods tested (via pickle or after slicing). However, there are instances that the spore level effect is limited or minor. Such instances could be due to other factors highly promoting growth. Roberts et al. (212) in a meat slurry system at pH 6.0 reported that the spore inoculum level (10^1, 10^3 or 10^5) affected only the extent of spoilage or toxicity at 15 C.

Heat processing and storage temperatures

The safety of all hermetically sealed, shelf-stable low-acid foods depends on a thermal process designed to destroy at least 10^{11} heat-resistant spores of *C. botulinum*. This is necessary to accomplish the classical 12D concept, (D value:time necessary to reduce the microbial population by 90%). To obtain this destruction in phosphate buffer at pH 7.0, a thermal treatment of 2.78 min at 250 F (121 C) is necessary ($F_0 = 2.78$ min). In food systems, this process is doubled due to economic spoilage of low acid foods from putrefactive anaerobes. Most cured meat products would be organoleptically unacceptable after such a thermal process ($F_0 = 2.78$ min) or even at a process of $F_0 = 1.0$ min. Processes of $F_0 = 0.05$-0.4 min are usually employed by most manufacturers. Such a process is 10-100 times lower than that necessary for low acid foods such as cured meats (150,190). Cured meat products receiving an adequate heat treatment are classified as commercially sterile and include deviled ham, corned beef spread, potted meat, Vienna sausage, canned corned beef, and corned beef hash (150).

The low thermal processes indicated above as being employed for most cured meat products have been proven to be adequate due to the supplementary effects of nitrite, salt and the very low incidence of anaerobic bacteria in raw meat (198,235). Thermal processes even less than $F_0 = 0.01$ min have been described to give shelf-stable cured meat products with nitrite concentrations much higher than those commonly employed or permitted in the United States and Canada (190). The current practices employed by the meat industry are based on excellent bacteriological studies related to the thermal processing of canned meat performed by Stumbo et al. (246,247,248), Gross et al. (105,106), Vinton et al. (285,286), Schack et al. (222) and Pivnick et al. (190,193). Summaries of the safe thermal processing of canned cured meats with regard to bacterial spores were given by Duncan (62) and Riemann (198). The above authors reaffirm the fact that the stability of cured meats is a complex interaction between the heat treatment and the curing agents. Pivnick et al. (190) reported on the interaction of heat treatment and spore inoculum size. In a ground pork system inoculated with *C. botulinum* type A and B spores, a thermal process of $F_0 = 0.15$ min did not prevent toxin production with 10^4 spores/g. An $F_0 = 0.30$ min prevented toxinogenesis with 10^4 spores/g but it did not with 10^6 spores/g inoculum size, which was inhibited by a thermal process of $F_0 = 0.60$ min. Viable spores were recovered after 18 months even though the meat was unspoiled and non-toxic.

It has been reported that the sensitivity of heat-damaged spores to inhibition by curing salts is higher than is the sensitivity of unheated spores (208,210). Ingram and Roberts (127) demonstrated that *C. botulinum* spores which were subjected to a sublethal heat process at 95 C were inhibited more by nitrite

heated in the recovery medium for 15 min at 115 C than by unheated nitrite. Ashworth et al. (9), working with a pasteurized meat system, reported that spores heated in the meat for up to 4 h at 80 C were inhibited by a nitrite level similar to that found to inhibit unheated spores inoculated into the meat after heating. The above observations were the reasons Jarvis et al. (132) suggested that, although spores heated (e.g. 90 C) are sensitized to the inhibitory action of salt and nitrite, heated spores at pasteurization temperatures (70-80 C) may not be sensitized to the inhibitory action of the curing salts. The same researchers (132) found lower sensitivity of spores to heated and unheated nitrite in a meat system, compared with sensitivity in culture media. This would suggest caution in drawing conclusions from data obtained in laboratory media in relation to potential effects in real meat systems.

Tompkin et al. (271), studying the effect of final internal processing temperatures within the range of 63 to 74 C, reported no alternations of the degree of botulinal inhibition in inoculated, perishable canned, comminuted, cured pork abused at 27 C. Erdman and Idziak (73) reported a botulism outbreak due to an "underprocessed" liver paste product.

The minimum temperature to prevent outgrowth of types A and B *C. botulinum* spores in cured meats would normally be less than 10 C (177). Christiansen et al. (40,41), working with canned comminuted cured meat and bacon, reported that no toxic samples were detected at 7 C, while 27 C permitted growth and toxin production. More toxic bacon samples were found at 30 than at 20 C with either 100 or 10,000 spores/g (44). Roberts et al. (212) studying a pH 6.0 meat slurry system over a 6-month storage, found more inhibition at 15 C than at 17.5 C, while little inhibition was observed at 20, 22.5 and 25 C even with 300 µg of nitrite/g. The salt-in-water concentration of the treatments was 1.8 or 3.5%.

The average optimum temperature for *C. botulinum* outgrowth is considered to be around 32 C, while the average room temperature is 22 C. Considering the above, the most common temperature chosen for *C. botulinum* studies in actual food or model systems has been 27 C. Temperatures around this value (27 C), while approximating real life product abuse temperatures, at the same time permit reasonably rapid outgrowth of thermally injured spores.

Salt and nitrate

As previously stated, salt was the first additive used for preservation of meat and that accidentally led to use of nitrate for the cured meat color development. Through the years it has been shown that nitrate serves only as a source of nitrite and that its direct effect on botulinal growth and toxin production is minor or negligible. Silliker et al. (236) reported that nitrate played no role in retarding putrid spoilage at all levels studied. On the contrary, it actively stimulated aerobic *Bacillus* spores. Duncan and Foster (64) reported that sodium nitrate had

no apparent effect on germination and outgrowth of PA 3679h spores at concentrations up to 2%. No practical antibotulinal activity by nitrate was found in the model system studied by Greenberg (98). In wieners ‚and comminuted canned cured meat, Hustad et al. (123) and Christiansen et al. (40) found the nitrate effect on botulism inhibition insufficient to be of practical importance. The USDA Expert Panel on Nitrites, Nitrates and Nitrosamines has recommended elimination of nitrate from all cured meat products except fermented sausage and dry-cured products. A 2-year period should be allowed for gathering of data to determine any need for nitrate in these two types of cured meat products (3). A need of 50-100 µg of nitrate/g in irradiation-sterilized (radappertized) ham was reported by Wierbicki and Heiligman (296). This nitrate was needed to assure color and flavor stability of product formulated with 25 µg of nitrite/g and preserved by irradiation. The same conclusions were reached by Wierbicki et al. (297), reporting that small amounts of nitrate had to be added to prevent fading of cured meat color and possibly to supplement the nitrite to scavenge electrons produced in meat by irradiation.

Salt (NaCl) has been shown to be one of the active ingredients in controlling *C. botulinum* growth in cured meats (30). The effect of salt is mostly associated and interrelated with other factors such as nitrite, pH, heat treatment, meat type, and spore level. More information on the interaction of the above factors will be given later, while a brief summary of the single effect of NaCl follows. Type A and B spores and vegetative inocula of *C. botulinum* can grow in media containing up to 10% NaCl, while type E grows only in NaCl concentrations less than 5-6% (172). Gould (93) reported that up to 8% NaCl had no effect on germination of six *Bacillus* species heat-shocked spores. However, concentrations ranging from 4 to 7% inhibited outgrowth, whereas concentrations of 10-15% progressively reduced and finally prevented germination. Gould (93) also noticed that the effect was similar at pH 6 and 7. Similar work was done with spores of PA 3679h strain by Duncan and Foster (64). It was found that salt concentrations above 6% prevented complete germination. In the presence of 3 to 6% NaCl most of the spores germinated and produced vegetative cells, but cell division was often blocked.

Pivnick et al. (190), working with canned, cured, shelf-stable luncheon meat inoculated with *C. botulinum*, concluded that higher salt concentrations were necessary to prevent botulinal outgrowth in the absence of nitrite than when nitrite was present. Brine levels of 5.8 to 6.1% were needed to prevent toxin formation with 0 or 75 µg of nitrite/g but less than 4.9% brine with 150 µg of nitrite/g. They concluded that, since the usual salt levels are close to 2.3%, salt alone is not a practical inhibitor of *C. botulinum* growth and toxin production, unless its concentration is in excess of 7%. Growther et al. (107) reported that "mildly salted" pork (containing about 4% salt in water) without nitrite or nitrate readily

supported *C. botulinum* growth, while "medium-salted" pork and bacon (containing about 5.5% salt in water) did not readily support growth of *C. botulinum*.

Effects on other bacteria and spoilage

Any inhibitory nitrite effect on other pathogenic microorganisms besides *C. botulinum* would be of great value, while inhibition of normal spoilage flora might be a disadvantage. Prevention of product spoilage for a longer time than botulinal toxin inhibition would extend the normal state of appearance of the products, and it might prevent the consumer from discarding toxic products.

Brooks et al. (*28*) reported reduced microbial growth on the surface of nitrite-treated bacon, while Tarr (*260,261,262*) listed several bacterial genera as being inhibited by 200 μg of nitrite/g at pH 6.0. The list of bacteria reported by Tarr included *Achromobacter*, *Aerobacter*, *Escherichia*, *Flavobacterium*, *Micrococcus* and *Pseudomonas*.

It is certain that the nitrite effect is not the same on all bacterial species. Research on the subject has shown that some bacteria are more resistant to nitrite than is *C. botulinum*. This has been reported for salmonellae (*34*), lactobacilli (*34,243*), *Clostridium perfringens* (*103*) and bacilli (*103*). However, when nitrite levels were increased, resistance was decreased or eliminated. Reduced survival of *C. perfringens* spores in meat containing nitrite was reported by Gough and Alford (*92*). The effect of nitrite on growth of salmonellae, *Staphylococcus* sp. and natural spoilage flora in frankfurters was evaluated by Bayne and Michener (*20*). Growth of the above organisms was at most only slightly faster in nitrite-free frankfurters at 20 C than in frankfurters with nitrite. Nitrite significantly reduced recovery of *C. perfringens* spores in cured ground pork (*221*). *C. perfringens* inhibition by nitrite has also been reported by Riha and Solberg (*200,201,202*). Nitrite concentration effect on total bacterial growth in vacuum-packaged bacon has shown that 15-60 μg of nitrite/g had very little effect on the lag phase while 120-170 μg of nitrite/g delayed bacterial growth for 4 to 5 weeks. In non-vacuum-packaged bacon, the nitrite level showed very little effect on bacterial growth (*114*).

Inhibitory effects of nitrite under anaerobic conditions were greater than under aerobic conditions on *Staphylococcus aureus* growth (*29,34,151*). The same greater nitrite effect under anaerobic conditions in retarding *S. aureus* growth was demonstrated by Barber and Deibel (*16*) in dry sausages. Labots (*417*) confirmed the above results by showing that growth of *S. aureus* in the surface layer of brine fermented sausages was lower and more affected by nitrite than growth in the surface layer of air-fermented sausages. That nitrite is more inhibitory under anaerobic than aerobic conditions has also been reported by Eddy and Ingram (*68*).

Product spoilage and gas production by putrefactive and gas producing organisms has often been used as an indication of *C. botulinum* growth (*204,268,269,270,271*).

A survey of the related literature indicates that botulinal toxin formation is not always associated with gas production and spoilage. Townsend et al. (*274*), working with several food products, reported toxin production at the next lower tested pH level below the lowest at which gas was formed, indicating that gas production was not a good index of botulinal growth and toxin formation in food products. Greenberg et al. (*102*) and Pivnick and Bird (*191*) have shown that cured meat products inoculated with *C. botulinum* became toxic before they were organoleptically spoiled. In canned comminuted cured meat, it was reported by Christiansen et al. (*40*) that not all toxic cans were swollen and not all swollen cans were toxic, but all toxic cans were putrid and proteolyzed. Collins-Thompson et al. (*44*) found one-third of all toxic bacon samples tested to have an acceptable odor and appearance. Roberts et al. (*212*), in their meat slurry system, detected toxin formation without overt spoilage, especially at 15 C.

Cured meat products-botulism safety

Commercially processed foods and cured meat products have shown a very good record of botulinal safety when compared to home canned foods and especially vegetables (Table 1). Greenberg (*98*) reported that in a 25-year period only nine botulism deaths occurred due to commercially processed foods compared to 700 from home-canned foods. In that period there was one death caused by underprocessed and canned liver paste, one by underprocessed canned vichyssoise, one by canned chicken vegetable soup, and two due to canned and vacuum-packaged unrefrigerated fish. Generally, commercially cured meat products in the United States have been proven botulism-safe. This is in contrast to the average of four outbreaks a year in France from home-cured ham (*224*). A variety of factors and conditions, and especially their interactions, have been reported as being responsible for such a safety record. Factors such as heat process, input and residual nitrite, NaCl, product pH, storage temperature, spore number and interrelated factors were reported by Spencer (*244*) as affecting and being responsible for the safety of canned cured meat products.

It has been stated several times that the microbial and botulinal stability of cured meat and fish products cannot be ascribed singly to any inhibitory parameter. It depends on the interacting effects of a number of factors, including salt concentration, water activity (a_W), heat treatment, injury of microorganisms, pH, Eh, product composition, nitrite concentration, microbial contamination, and packaging and storage conditions (*15,81,193,198,235,244,298*). Silliker et al. (*236*) surveyed the literature and concluded that no reasonable amount of any single curing ingredient or combination would completely prevent growth of *C. botulinum* and other spore-forming bacteria. From their results they (*236*) showed that the shelf-stability of canned comminuted cured meat resulted from the joint effect of nitrite, salt,

thermal injury of the spores and a low indigenous spore load. Ingram (125,126) summarized that in unheated systems the effect of nitrite in inhibiting botulinal growth depends on several significant factors including salt, pH, incubation temperature and number of microbial cells. Heated systems are a more complex situation, with the major inhibitory effect due to salt and nitrite, while the supplementary effect of heating to the order of $F_0 = 0.1-1.0$ min is of critical importance. The active ingredients of a curing mixture found by Bullman and Ayres (30) were salt and nitrite. Riemann (198) described an experiment where factors such as salt, nitrate, nitrite, pH, heat process (F_0) and spore level were tested. After 6 months at 30 C all factors, except nitrate were found to have a significant effect on spore development. Of the interactions, $F_0 \times NaCl$, $F_0 \times NaNO_2$, $NaCl \times NaNo_3 \times NaNO_2$, $NaCl \times pH$, and $NaCl \times NaNO_3$ showed significance, while nitrite and pH showed no interaction. Nitrate and pH alone showed no effect. Silliker et al. (236) indicated that some of the commonly employed thermal processes and curing salt concentrations would be inadequate if large numbers of anaerobic spores were present. The role of curing salts in preservation of canned cured meat products was studied by Duncan and Foster (63), and they found that their stability was a combined effect of heat treatment and curing salt mixtures. The significance of nitrite and pH was also discussed. Pivnick et al. (190) inoculated ground pork with C. botulinum A and B spores and thermally processed the product. Toxin production was affected by spore number, salt levels, nitrite concentration, and thermal processing. Meat inoculated with one spore per gram became toxic when nitrite and salt were omitted. Meat inoculated with 10^6 spores per gram remained non-toxic when protected with 6.1% salt in water and no nitrite or 4.6% salt in water and 300 μg of nitrite/g. The amount of heat processing necessary to prevent toxin production in meat containing 146 μg of nitrite/g and 5.5% brine depended on the concentration of spores. The inhibitory effects of pH, NaCl and $NaNO_2$ combinations at 35 C in a laboratory medium on C. botulinum type A, B, E and F vegetative cells were studied by Roberts and Ingram (211). The results indicated that growth inhibition was the result of the interaction of all three factors. The authors (211) also discussed the importance of such studies. Roberts (205) used the data of Roberts and Ingram (211) to measure the antimicrobial properties of cured meat systems to develop a model for predicting growth or inhibition in such systems. Roberts et al. (212) in their pasteurized pork slurry system showed that C. botulinum types A and B developed more slowly with higher nitrite concentrations and lower incubation temperatures. The relative effects of pH, salt and nitrite on the growth of PA 3679 spores inoculated into ground cured pork were studied by Nordin et al. (175). It was found that outgrowth increased with pH and decreased with nitrite and salt concentrations. The effect of 0-400 μg of nitrite/g was similar to that of 0-4% NaCl. The pH effect

in the range of 5 to 7 was greater than that of either salt or nitrite concentration. Certainly there exist combinations of factors such as salt and nitrite concentrations, pH, heat treatment, and incubation temperature that act synergistically to preclude the growth of the low number of spores found in meat products.

Nitrite mechanism

The microbiological role and effects of nitrite in the safety of cured meats have been extensively reviewed by Ingram (125,126) and Roberts (206). Although nitrite has been used in meat curing for many years and its effect in delaying C. botulinum toxin production is well established, the exact mechanism(s) through which nitrite performs that action is still unknown.

Several possible mechanisms have been proposed through the years but final conclusions have not been reached. Early reports (34,104,133,260,261,262) found nitrite to be more effective at lower pH (< 6.0) values and suggested that undissociated nitrous acid (HNO_2) might be the active form. Although admitting that the role of nitrite in maintaining stability was not fully understood, Johnston et al. (137) discussed four possible roles of nitrite which were later repeated and reviewed by Ingram (125,126). According to Johnston et al. (137) the role of nitrite might be: (a) to enhance destruction of spores by heat, (b) to increase spore germination during thermal processing with subsequent destruction of the germinated spores by heat, (c) to prevent germination of outgrowth of the spores and (d) to react with some type of meat component(s) to form a more inhibitory compound(s). The enhanced destruction of spores by heat was first mentioned by Jensen and Hess (135), suggesting that bacterial spores were more sensitive to heat in the presence of curing ingredients. Roberts and Ingram (210) examined the ability of aerobic (Bacillus) and anaerobic (Clostridium) spores to grow in media containing different concentrations of curing salts (NaCl, KNO_3, $NaNO_2$) after various degrees of heating. The data indicated that heating at realistic temperatures and in the presence of acceptable nitrite and salt concentrations had no effect on subsequent development of the spores. The nitrite effect was found to be pH-dependent, increasing ten-fold from pH 7.0 to pH 6.0. In another study, Duncan and Foster (63) studied the effects of salt, sodium nitrate and nitrite on the growth of PA 3679h strain. Heated spores were found less tolerant to all three curing agents than unheated spores. Spores heated in the presence of, but grown in media free of the curing salts, were found to be protected against heat injury by nitrate and sodium chloride, but not by nitrite. When the spores were both heated and grown in the presence of the curing agents, nitrate and salt increased their heat resistance at low concentrations (0.5 to 1.0%) and decreased it at concentrations of 2 to 4%. Sodium nitrite was found highly inhibitory, especially at pH 6.0. Ingram (125,126) stated that the nitrite effect was not

clear and the data were confusing. This increased sensitivity of spores reported by Duncan and Foster (63) when heated in the presence of nitrite was not confirmed by Ingram and Roberts (127) working with C. botulinum type A at pH 6.0. Ingram (125,126) stated that the possibility of enhanced spore destruction during heating in the presence of nitrite was now largely eliminated.

Although some of the spores are killed during thermal processing, some of them survive and can produce toxin. Silliker et al. (236), Riemann (198), and Christiansen et al. (40,41,43) found spores after thermal processing in the presence of curing salts, and Silliker et al. (236) calculated that 20% of the inoculated spores survive in cured meats. Pivnick and Chang (192) found C. botulinum spores surviving a heat treatment of $F_0 = 0.4$ min in a raw meat juice with 4.5% NaCl and 150 μg of sodium nitrite/g. It is generally well established in the literature that in either the presence or absence of nitrite, enough spores can survive thermal processing for future growth if conditions allow.

Germination and growth of spores of six Bacillus species in the presence of nitrite were studied by Gould (93). All species were reported to germinate in the presence of < 0.03% sodium nitrite (300 μg/g) at pH 6.0, and their development stopped immediately after germination and before lysis or rupture of the spore coats. Increased nitrite concentrations (0.075-0.25%) inhibited germination, and the effects were 3-5 times greater at pH 6.0 than at 7.0. Duncan and Foster (64) examined germination and outgrowth of PA 3679h spores in the presence of nitrite in microcultures. As much as 4% nitrite failed to prevent germination (complete loss of refractility) and swelling of the spores. Lower nitrite concentrations, 0.06% at pH 6.0 or 0.8 - 1.0% at pH 7.0, allowed emergence and elongation but prevented cell division, and the cells lysed. With nitrite levels above 0.06% (pH 6.0) or 1% (pH 7.0), the spores lost refractility and swelled, but vegetative cells did not emerge. In a subsequent report, Duncan and Foster (65) concluded that sodium nitrite stimulated germination of PA 3679h spores, indicating that the process was accelerated by using increased nitrite concentrations, a low pH and a high incubation temperature. A delay in germination (36-48 h) occurred with 0.01-0.02% nitrite at 37 C, while with 3.45% nitrite at 45 C and pH 6.0, most of the spores germinated within 1 h. With increasing pH the germination rate decreased, and at pH 8.0 it was negligible. A temperature of 60 C gave the highest germination rate, and hydroxylamine completely inhibited the nitrite-induced germination, while L-alanine-induced germination was inhibited by nitrite. Duncan (62) suggested that nitrite, instead of stimulating spore germination in cured meats, may actually inhibit germination when present at the low concentrations. In a more recent study with a canned, cured meat product, Christiansen et al. (43) examined the fate of C. botulinum spores in the presence of 50 or 156 μg of added sodium nitrite/g. Spore germination (loss of heat resistance)

occurred readily at both nitrite levels. Working with the same product, Tompkin et al. (270) also found spore germination at 27 C with 156 μg of nitrite/g. At lower temperatures in one trial, spore germination did not take place at 4.4 and 10 C with 156 μg of nitrite/g, whereas in a second trial with the same nitrite concentration, germination was recorded at 10 C. They suggested that this discrepancy might be due to variations between C. botulinum strains used to inoculate the meat. Baird-Parker and Baillie (15) reported that C. botulinum strains varied markedly in their resistance to sodium nitrite, and were differentiated into two groups: one including the most resistant strains growing in at least 150-200 μg/g at 25 C and a second including the heat sensitive, nonproteolytic, psychrotrophic types inhibited by 100-150 μg of nitrite/g. Generally, a close look at work related to C. botulinum spore germination and nitrite favors the conclusion that nitrite does not affect spore germination.

The possibility of an inhibitory nitrite effect on outgrowth of heat-damaged spores is more probable and logical. Roberts and Ingram (210), Roberts et al. (208), Duncan and Foster (63), Pivnick and Thacker (194), and Ingram and Roberts (127) have shown growth inhibition due to nitrite or salt. Interaction of nitrite, salt, pH, spore level and incubation temperature have also been implicated. Since it is almost certain that spore germination is not inhibited by nitrite in cured meat products and that botulinal toxicity delay due to nitrite is well established, the theory of outgrowth retardation by nitrite seems worthy.

Besides the possible inhibitory effect of nitrite in conjunction with other factors on the outgrowth of botulinal cells, another mechanism is the possible formation of a more inhibitory substance(s) from the reaction of nitrite with components of the system (125,126,137). It was observed by Castellani and Niven (34) and Gough and Alford (92) that sterilization of nitrite in the presence of glucose reduced the nitrite concentrations necessary to inhibit growth. Castellani and Niven (34) attributed the effect to production of more anaerobic conditions. In 1967, Perigo et al. (184) undertook extensive research to examine the inhibitory effect of sodium nitrite on growth of C. sporogenes (PA 3679 strain 8053) vegetative cells in a laboratory medium. When nitrite was added as a filter-sterilized solution to the medium, a ten-fold increase in its activity was calculated as the pH decreased from 6.8 to 5.8. Heating of nitrite in the basic medium for 20 min at temperatures above 90 C resulted in an increase in the inhibitory effect of the substrate, and much smaller nitrite concentrations were effective. The effect was observed in the temperature range 95-125 C, but a ten-fold greater concentration was found at 100-110 C at pH 6.0. In the summary, Perigo et al. (184) postulated that sufficient heating of nitrite in the medium produced some unknown inhibitory substance which differed from inorganic nitrite in three important respects. First, its

activity was less pH-dependent, compared to inorganic nitrite. Second, its response was less variable. Third, it was an extremely potent inhibitor, formed even with only 3.5 μg of nitrite/g heated for 20 min at 109 C. The final statement of the authors (184) was that "it would be naive to suppose that the mechanism described above could wholly account for the remarkable stability of sublethally processed cured meats, but it may well play an important complementary role to the spore sensitivity mechanism described by Roberts and Ingram (210)." In a subsequent study, Perigo and Roberts (185) tested thirty clostridial strains including fourteen strains of C. botulinum types A, B, E and F and eight strains of Clostridium welchii. The enhancement of the inhibitory effect of nitrite after heating in laboratory media was extended and confirmed. The inhibitory substance supposedly formed by autoclaving nitrite with laboratory media, was designated as "Perigo Factor" (PF) by Johnston et al. (137), and it is also known as "Perigo Inhibitor" (PI) or "Perigo Type Inhibitor" (PTI) for meat products (37). Roberts and Garcia (207) screened a range of Bacillus species and fecal streptococci and two strains of Salmonella typhimurium for their resistance to the Perigo Inhibitor. At pH 6.0 it inhibited nine of the 14 strains of Bacillus sp.; B. circulans, B. polymyxa, B. macerans, B. pantothenticus and Bacillus F were resistant. Streptococcus durans was inhibited, while Streptococcus faecalis, Streptococcus faecalis var. zymogenes and the salmonellae strains were not.

Roberts and Smart (213) examined whether nitrite heated in a laboratory medium was an equally effective inhibitor to clostridial spores as it had been shown for vegetative cells (184,185). Spores of C. botulinum types A and F and C. sporogenes were tested, and the effectiveness of heated nitrite was confirmed. It was also observed that the inhibitory activity of heated nitrite medium was not stable indefinitely. Growth sometimes took place on reinoculation with vegetative cells, and some spores remained viable over a 3-month period. A study was undertaken by Huhtanen (119) to develop a more rapid assay for Perigo-type Inhibitors, since the original procedure developed by Perigo et al. (184) required 10 days of incubation. A medium consisting of 0.5% yeast extract or tryptone, 0.2% glucose, 0.12% K_2HPO_4 and 0.1% cysteine-HCl or sodium thioglycollate was used and vegetative cells for C. botulinum type A were tested. It was found that yeast extract or tryptone, together with a reducing agent such as cysteine, sodium thioglycollate, or glucose autoclaved with nitrite at 15 psi for 15 min produced a Perigo Inhibitor. Tryptone was more active than yeast extract, and of the reducing agents tested, cysteine was more effective than thioglycollate which in turn was better than glucose. Enhancement of inhibition against C. perfringens by autoclaving nitrite in the medium has been shown by Riha and Solberg (200,201,202).

The possibility and evidence of the probable formation of a potent microbial inhibitor by heating nitrite in laboratory media gave rise to suggestions that a similar compound might be formed by heating nitrite in meat. Not long after the first report on the subject by Perigo et al. (184), a study was undertaken by Johnston et al. (137) to examine whether nitrite could interact with meat to form a compound which could prevent botulinal spore growth in meat products. Culture media as well as meat suspensions were heated with various concentrations of nitrite up to 200 μg/g. It was found that heating the medium at 110 C for 20 min with as little as 20 μg of nitrite/g produced C. botulinum inhibition. When meat was blended with medium and nitrite and heated, the inhibition at high nitrite inputs (> 150 μg/g) was attributed to the residual nitrite which was greater than 100 μg/g. The inhibitory substance in meat was found to be dialysable, while the Perigo Inhibitor in the medium was not. Addition of as little as 1% meat to culture medium interfered with development of the inhibitor, and 20% or more meat prevented it. Addition of non-fat meat solids to the culture medium with the inhibitor already present neutralized its activity, while fat and water-soluble meat extracts did not. Johnston et al. (137) concluded that the inhibitor produced in media was of little or no consequence in explaining the role of nitrite in the safety of commercially produced cured meats. The inhibitory activity of sodium nitrite on the C. botulinum outgrowth was also studied by Johnston and Loynes (136) in various bacteriological media and meat suspensions. Nitrite was heated at 110 C for 20 min in the media or meat suspension at pH 6.2. Perigo Factor was detected in the original Perigo medium (184) and reinforced clostridial medium when heated with 20 μg of nitrite/g, but not in liver veal medium and Wynne fluid medium even in the presence of 100 μg of nitrite/g. In 50% meat suspensions, 500, 150 and 50 μg of nitrite/g were required for inhibition when underprocessed, normal-processed and overprocessed, respectively. The inhibitory activity was dialysable in meat suspensions, liver veal medium and Wynne fluid medium but not in Perigo and reinforced clostridial medium. Addition of reducing agents to meat suspensions decreased the redox potential and increased inhibition without inducing the formation of the Perigo Factor. Jarvis et al. (132) reported that growth and toxin formation in a meat system were inhibited by 300-400 μg of unheated or by 200 μg of heated nitrite/g. In a culture medium only 20 μg of heated nitrite/g were necessary to inhibit a similar inoculum. Ashworth and Spencer (10) added nitrite directly to minced pork, heated it at 115 C for 20 min and subsequently challenged the system with a C. sporogenes inoculum. This system was more inhibitory than a similar one with the nitrite added after heating. The effect was observed at nitrite concentrations 150-300 μg/g and attempts to magnify it failed. Ashworth and Spencer (10) concluded that no evidence had been obtained which would implicate a Perigo effect in the safety of canned cured meats under practical conditions.

In another attempt to determine whether a Perigo-type factor was formed in meat, Chang et al. (38) autoclaved canned commercially formulated luncheon meat with various nitrite concentrations and stored them until all nitrite was depleted. The cans were then inoculated with a C. botulinum spore inoculum heated to $F_0 = 0.4$ min in a meat homogenate formulated with 4.0% NaCl and 150 μg of nitrite/g. An inhibitory effect was detected with increasing initial nitrite concentrations and lower spore inoculum. However, the effect was small since with only 10 spores/can and an initial nitrite concentration of up to 200 μg/g, one out of 16 cans showed growth. The model meat system of Ashworth and Spencer (10) was used by Ashworth et al. (9) to study the production of any antimicrobial effects with sodium nitrite heated under simulated commercial pasteurization conditions. Lower residual nitrite levels were found inhibitory to C. sporogenes when nitrite was heated with the meat at 115 C for 20 min, or 80 C for 4 h, or from 20 to 70 C in 4 h, compared to nitrite added unheated to the meat. Average residual nitrite levels of 64 to 149 μg/g in nitrite-heated systems and 91 to 234 μg/g in nitrite-unheated systems were necessary to inhibit growth. Chang and Akhtar (37) homogenized and adjusted to the same nitrite levels canned luncheon meat which had been formulated with various nitrite concentrations, heat-processed and stored for different periods. The homogenate was then inoculated with C. botulinum strain 13983B spores. They concluded that inhibition increased with increasing nitrite concentrations before processing and could not be accounted for by residual nitrite. Attempts by van Roon (282) to extract a Perigo Factor from luncheon meat failed. Grever (103) has suggested that the Perigo Inhibitor is destroyed by fat and adsorbed by muscle fibers. It has been stated by Ingram (125) that "the outcome of a substantial amount of work on the Perigo effect seems disappointing" and by the same author in 1976 (126) that "it seems that observations made in culture media may have little relevance to meat; for which reason it is better, for the time being, not to use the term Perigo Factor or Inhibitor in connection with meat." The necessity of heating to above 90 C and that the number of spores used makes little difference to inhibitory concentrations, are reasons for doubting the applicability of Perigo-type Inhibitors to meat systems.

Points of general agreement in Perigo-type work listed by Ingram (125) are: that a reducing agent (thioglycollate, ascorbic acid or cysteine) is necessary; that a protein hydrolysate, preferably casein, is necessary and that iron might be involved. Wasserman and Huhtanen (290) investigated the possibility that the Perigo Inhibitor might be certain volatile nitrosocompounds. After confirming the findings of Perigo et al. (184) they (290) tested against C. botulinum the inhibitory activity of various nitrosamines found in the medium or implicated in meat products. None of the nitrosocompounds examined inhibited C. botulinum.

Moran et al. (170) undertook a study to characterize chemically the nitrite inhibitor found by Riha and Solberg (200,201,202). It was found that cysteine and ferrous ions were reacting in some manner with nitrite under autoclaving to cause inhibition. The inhibition was lost when the inhibitor was added to cooked meat medium and it was not formed when the necessary components were autoclaved with the same medium. It was speculated that the inhibitor observed might be different than that in cured meats. Continuing their efforts, Moran et al. (170) investigated several compounds (S-nitrosocysteine, Roussin black and red salts, cysteine-Fe-NO complex) that could conceivably be produced by interaction of cysteine, ferrous ions and nitrite. S-nitrosocysteine inhibited the test organisms, but the amount formed in the test system was inadequate to explain the total inhibition observed. It should be mentioned that Incze et al. (124) found S-nitrosocysteine more inhibitory than nitrite in a beef bouillon-based medium. Roussin red salt was unstable in the test system, whereas Roussin black salt was inhibitory and could form in amounts sufficient to explain the inhibition recorded. Cysteine-Fe-NO complex was detected but the levels found were non-inhibitory. Moran et al. (170) concluded that the inhibition found may have been due to the combined effects of sublethal concentrations of each compound tested. Van Roon (282) suggested the possibility that iron-nitrosyl coordination complexes could be formed in canned cured meat products during heating. Roussin black salt and nitrosyl-cysteyl-ferrate were studied and found to inhibit growth of clostridial spores. Attempts to detect Roussin black salt in luncheon meat failed. Inhibition of a variety of microorganisms by Roussin black salt was also reported by Ashworth et al. (8), while Dobry-Duclaux (57,58) found low concentrations of the compound to inhibit the enzyme alcohol dehydrogenase.

Huhtanen and Wasserman (121) reported that inhibition of C. botulinum by nitrite was potentiated by the addition of Fe(II) or Fe(III) to the culture medium, and that the effect was more pronounced when the nitrite was added after autoclaving. It was suggested (121) that a potent anticlostridial inhibitor could be produced without autoclaving nitrite in the medium, that iron was the limiting factor and that sulfhydryl groups were probably necessary for its formation. Black iron nitrosyl-sulfide (INS) was tested by Huhtanen et al. (120) and showed a minimum inhibitory concentration of 0.16 mg/l compared to 80 mg/l for nitrite against C. botulinum in a tryptone-yeast extract medium. Addition of meat to the medium prevented inhibition, while autoclaving INS with the medium inactivated it, indicating that INS was different from the Perigo Factor. Van Roon and Olsman (283) found S-nitrosylcysteine and nitrite inhibitory to clostridia in canned cured pasteurized beef and ham products, while a cysteyl-nitric oxide ferrate complex was ineffective. Inhibition of [14C]uracil incorporation into ribonucleic acid of Bacillus cereus

during outgrowth was caused by a Perigo-type Inhibitor, nitrosothiols of thioglycollate and beta-mercaptoethanol (10). Inhibition occurred before and after germination and during outgrowth.

In spite of all the research conducted around Perigo-type inhibitors, Fe, and related compounds, no definite conclusions have been reached, and the value of such research in relation to real cured meat systems is debatable. Discussing the mechanism of action of nitrite, Ingram (126) stated the assumption that nitrite blocks energy metabolism by reacting with the amino groups of dehydrogenases. Ingram (126) also stated that work by Dainty and Meredith (51), while confirming that nitrite can inhibit various respiratory enzymes, suggested that it is "likely that the growth inhibitory effect of nitrite is because it generally inhibits the uptake of energy sources."

The inhibitory activity of reaction products of nitrite with degradation compounds of carbohydrates was investigated by Mirna and Coretti (165). Growth of *Micrococcus* sp. and *S. aureus* was inhibited by S-hydroxymethylfurfural at a concentration of 500 mg/l; *Enterobacter liquefaciens* showed inhibition at 2,000 mg/l only; and *Escherichia coli* was not inhibited by the above mass fractions of S-hydroxymethylfurfural. Of the other compounds examined, only 3,4-dihydroxyphenylalanine was inhibitory against three of the bacterial strains. Van Roon and Olsman (283) related clostridial inhibition to an increase in protein-bound nitrite during storage, and Lechowich et al. (150) stated that protein-bound nitrite contributes to the stability of cured meats.

A major issue in explaining the inhibitory activity of nitrite is the relative importance of the initial nitrite input versus that of the residual nitrite found in the product after processing and during storage. Greenberg (98) concluded that the level of nitrite at the time of product manufacture, rather than the residual nitrite concentration, was the key to botulinal protection. Greenberg (98) also stated that "it would appear that the nitrite either reacts with the spores, or that in the reaction with the meat component a substance(s) is formed which inhibits germination and/or outgrowth of the spores." Bowen and Deibel (26) and Christiansen et al. (40) concluded also that the initial nitrite level rather than the residual was the important factor in determining inhibition of botulinal toxin production, and Christiansen et al. (40) made a statement similar to that by Greenberg (98). From papers presented at the Second International Symposium on Nitrite in Meat Products (264) it was also concluded that the safety of cured meat products during storage was mostly determined by the concentration of nitrite added to the products before processing. Ingram (126) stated that "we do not know whether the best index of the inhibitory effect of nitrite is the initial or residual concentration, or some fraction of the amount of nitrite which disappeared." Ashworth and Spencer (10) and Chang and

Akhtar (37) reported that the inhibition found in the systems tested (minced pork and luncheon meat) could not be accounted for by residual nitrite levels alone. On the other hand Johnston et al. (137) suggested a high residual nitrite level as being responsible for the inhibition observed in meat suspensions.

Recently, a series of reports has appeared implicating residual nitrite or an intermediate, isoascorbate, and iron in the mechanism of botulinal safety of cured meats. Christiansen et al. (43) followed spore germination, cell survival and residual nitrite depletion during 27-C storage of a perishable canned cured pork product prepared with 50 or 156 μg of nitrite/g and 10^2 or 10^4 botulinal spores/g. The data indicated a race between death of the germinated cells and nitrite disappearance, and the time at which outgrowth occurred was dependent upon residual nitrite levels and surviving botulinal cells. It was stated (43) that "the data suggest that safety of these products is dependent upon sufficient residual nitrite until the viable cell level has decreased to a point at which growth can no longer be initiated." In another study with the same product, Tompkin et al. (270) examined the effect of prior refrigeration on botulinal outgrowth at abuse temperatures. It was demonstrated that botulinal protection decreased with storage at 10 C before incubation at 27 C. The decrease in inhibition was related to the decline in residual nitrite. However, the data that were presented (270) were inadequate to prove that residual nitrite, per se, was the key to inhibition, and it was stated, (270), that residual nitrite could be indirectly related to botulinal inhibition serving as a reservoir for a reactive intermediate such as nitrous acid. The possibility also exists that some inhibitory substance could be formed during processing involving nitrite and could be dissipated during storage simultaneously with residual nitrite. In a subsequent report, Tompkin et al. (271) examined the effect of adding hemoglobin or using different meat sources, on the inhibitory activity of nitrite against *C. botulinum* outgrowth. Addition of 1% hemoglobin to product formulated with 50 or 156 μg of nitrite/g, and replacing pork ham, beef round, veal or turkey meat with beef or pork hearts decreased or eliminated the inhibitory effect of nitrite. It was postulated (271) that the responses obtained were due to two different phenomena. Hemoglobin addition decreased residual nitrite which in turn decreased botulinal inhibition. A high level of available iron in heart meats also brought about a loss in botulinal inhibition. Tompkin et al. (271) offered the hypothesis that nitric oxide reacted with iron in the vegetative cells and blocked some essential metabolic step such as ferredoxin or some other iron-dependent enzyme. They (271) explained that the conclusion reached by Christiansen et al. (40), i.e., the nitrite level added at the time of formulation was the important factor in controlling botulinal outgrowth, was due to the pattern of botulinal swells observed which appeared to be unrelated to the levels of residual nitrite.

Continuing their efforts, Tompkin et al. (269) reported that addition of 0.02% isoascorbate markedly enhanced the antibotulinal effect of nitrite. A combination of 0.02% isoascorbate - 50 μg of nitrite/g gave protection similar to that obtained with 156 μg of nitrite/g, while isoascorbate alone was ineffective. Bowen et al. (25) found that sodium ascorbate in wieners did not decrease or potentiate the effectiveness of sodium nitrite to inhibit botulinal growth. Bowen and Deibel (26) found that ascorbate levels in the range of 0.1 to 0.2% in bacon decreased the ability of nitrite to inhibit botulinal outgrowth while lower ascorbate levels correlated with higher nitrite effectiveness. On the other hand, Growther et al. (107) reported that 0.2% sodium ascorbate did not reduce nitrite protection against *C. botulinum*. Increased inhibition due to nitrite by addition of reducing agents such as ascorbate was also reported by Johnston and Loynes (136) and Ashworth and Spencer (10). Expanding their efforts on isoascorbate Tompkin et al. (272) reported that isoascorbate enhanced nitrite inhibition due to its sequestering and not its antioxidant or reducing properties, and the same effect was produced with ascorbate, cysteine and EDTA. It was postulated (272) that moderate (0.02%) isoascorbate levels enhance nitrite inhibition of *C. botulinum* by sequestering a metal ion in the cured meat. Tompkin et al. (273) in a final report presented data showing that iron was critical to the inhibition of *C. botulinum* in perishable canned cured meat. The data indicated that nitric oxide reacted with some essential iron-containing compound within the germinated cell and prevented outgrowth, which would probably be related to injury of the electron transport system. It was concluded (273) that the degree of botulinal inhibition depends on the following factors: the residual nitrite level and viable botulinal cell counts at the time of temperature abuse, the iron available for repair of nitric oxide-damaged cells and/or the level of iron available outside the cell to react with nitric oxide and thus prevent injury. A summary of all this work was presented by Tompkin (265) in the 1978 Reciprocal Meat Conference.

Of all the possible mechanisms presented through the years and the vast amount of work reported, no single mechanism seems to explain entirely the nitrite effect on the safety of cured meat products and to apply in culture media and all the types of meat products. It is not safe to always conclude that what applies to culture media can be assumed as taking place in real meat systems, or that all the cured meat products are the same. On the other hand, results from work with meat systems such as inoculated packs are a problem to interpret since it is difficult, if not impossible, to know what changes are occurring inside the pack. A reasonable conclusion would be that most of the results reported are true and important for the conditions and systems tested in each particular case. The effectiveness of nitrite in delaying botulinal toxicity is probably due to one or more mechanisms, or one or more factors involved in each particular product or system considered. The remarkable safety of cured meat products experienced through the years can be accounted for by action of one or more nitrite-inhibition mechanisms involved, low incidence of botulinal spores in raw meats, heat process involved during product manufacture, effect of salt and other ingredients and proper handling and storage of the products.

Nitrite depletion

Recently residual nitrite has been directly or indirectly linked with the safety of cured meat products from botulinal toxicity (43,270,271). Also, nitrite is believed to react with secondary and tertiary amines and to form nitrosamines. In light of the above, an understanding of the fate of nitrite in cured meat products, and the factors influencing the rate of nitrite depletion, is essential.

As soon as nitrite is added to meat, its depletion starts. The depletion is continuous and its rate depends on product formulation, pH, time and temperature relationships during processing and subsequent storage (85,174). Nordin (174), working with canned ham, determined a relationship between the initial and final nitrite concentration, pH, temperature and length of storage. At 30 C the half-life of nitrite at pH 6.0 was 5 days. The rate of nitrite disappearance was followed in bacon by Herring (114) and in canned pork by Sebranek et al. (226). No simple relationship between nitrite level and time was found. This was probably the result of uncontrolled variables such as pH.

A large amount of the added nitrite is lost during processing. Fiddler et al. (80) · found 10-33% nitrite retention in commercially manufactured wieners after processing, while in another study (123) an average of 33% of the added nitrite remained in the wieners after manufacture. A survey of the literature showed a large variation in nitrite disappearance during and after processing (Table 3). Differences can be expected from one meat lot to the next, among laboratories, due to formulation differences, products examined, and storage temperatures. Nitrite depletion was shown to be directly proportional to the meat concentration (180). Higher nitrite losses were observed during processing when 1% hemoglobin was added to canned comminuted pork (271). In canned comminuted pork a large nitrite depletion was observed during formulation while cooking had little effect (40). However, nitrite disappearance in wieners during cooking was higher than during processing (123).

The temperature at which the product is stored influences the rate of nitrite depletion. The higher the storage temperature, the faster the rate of nitrite disappearance. This has been shown in products such as wieners (123), thuringer sausage (52), comminuted pork (40) and bacon (41).

Several reports have looked at nitrite depletion and found it to be rapid, but all have failed to account for most of the nitrite depleted (9,114,174,190). It is well

known that nitrosomyoglobin is formed from nitrite and myoglobin during curing. Formation of several other compounds when nitrite is added to meat has been proposed: nitrosothiol compounds (146); nitrosated amino acids, such as nitrosoproline (148); nitrotyrosine (145,300); and some gaseous derivatives such as nitric oxide (NO), nitrous oxide (N_2O) and nitrogen (N_2) (299). Fate of nitrite in whole meat and meat fractions was studied by Emi-Miwa et al. (72). Of the nitrite added, 66 to about 90% was traced as nitrite, nitrate, nitrosothiol, nitrosomyoglobin and gaseous nitrogen compounds. The remaining nitrite was unidentified and depended on both the curing period and the amount of ascorbate added. Ascorbate can act as a reducing compound and enhance the rate of nitrite depletion, and high concentrations have been recommended to prevent nitrosamine formation (3).

The specific interactions between nitrite and the sulfhydryl groups of meat protein have been studied in a model system consisting of myosin and nitrite (146). The rate of the reaction between nitrite and sulfhydryls was slow under pH and concentration conditions similar to those expected in meat. The findings led to the assumption that the direct reaction between nitrite and sulfhydryls in the myosin fraction can account for only a small proportion of the total nitrite lost during curing. A decreased loss of nitrite was recorded when the sulfhydryl groups in a chopped beef product were blocked (180).

Olsman and Krol (180) reported that in the pH range prevailing in meat products, only a small fraction of the nitrite was present as nitrous acid (HNO_2), which was considered as a leak through which the reservoir of nitrite was emptied. At pH values 6.0 or higher the nitrite loss followed first order kinetics, whereas at lower pH values the reaction was between first and second order.

Low molecular weight muscle cell components have been reported as active in nitrite reduction (263), while the ability of unsaturated fatty acids to combine with nitric oxide has been demonstrated (86) with no products being identified.

The effects of back and belly fat on nitrite disappearance were studied by Goutefongea et al. (94) with fat cured with ^{15}N-labelled nitrite. Ninety percent of the added nitrite was recovered as free nitrite. Connective tissue and lipid fraction extracted from the cured fat contained 6-9% and 2-5% nitrite, respectively. When fatty acids and glycerides were treated with ^{15}N-labelled nitrite, the amount of label incorporated was considerably higher in more unsaturated compounds. Woolford et al. (300) showed results suggesting that one of the major pathways for nitrite depletion in cured meat products might be through reaction with nonheme proteins, while Olsman and Krol (180) indicated that the concentration of protein-bound nitrite increases with decreasing pH.

Olsman (178), working with model meat emulsions of pH 5.35 to 5.80, reported that more than half of the nitrite lost during the first days of storage was recovered as protein-bound nitrite. This bound nitrite increased to a maximum and then gradually declined with kinetics similar to those for the depletion of free nitrite. Addition of Fe^{2+} at 0.1 or 1 mmol/kg resulted in more protein-bound nitrite, whereas EDTA showed the opposite effect. This effect was more pronounced at pH 6.2 than at pH 5.65, and it was completely lost at pH 5.1 (179). In 1974 Ando (4) reported that Fe^{2+} significantly decomposed nitrite in the absence of ascorbate, whereas Mg^{2+}, Ca^{2+} and Zn^{2+} enhanced nitrite decomposition to some extent only in the presence of ascorbate. The effects of Fe^{2+} and Fe^{3+} in the presence of ascorbate were considerable. Also glutamate, succinate, nicotinic

TABLE 3. *Variation in nitrite depletion.*[1]

Product	Added nitrite (μg /g)	Final cooking temperature (C)	Storage temperature (C)	Days after cooking	Replications		Additive	Nitrite depleted (% of added)	Reference
	50	68.5	—	0	A[2]	a[3]	—	30	(268)
Canned, communited pork			—	0	A	b	—	56	
			—	0	A	a	—	29	(212)
Pasteurized pork slurry	175	70.0	—	0	A	b	—	57	
			—	0	A	a	—	29	
			—	0	B	a	—	51	
			—	0	A	a	0%hemoglobin	40	(271)
Canned, comminuted pork	50	68.5	—	0	A	a	1%hemoglobin	88	
			—	0	A	a	—	36	(40)
Canned, comminuted pork	150	68.5	7.0	0				44	
				3				46	
				7				77	
				28				36	
			27.0	0	A	a	—	56	
				3				67	
				7				96	
				28				66	(123)
Wieners	150	71.0	27.0	0	B	b	—	89	
				3				96	
				7				97	
				28					

[1]Selected and calculated from the references listed.
[2]Laboratory.
[3]Meat lot.

acid, and nicotinamide enhanced nitrite decomposition in the presence of ascorbate. Monosodium and monopotassium orthophosphates, sodium hexametaphosphate, disodium pyrophosphate and sorbic acid also enhanced cured meat color formation (4).

Another route of nitrite depletion is the formation of nitrate. Herring (114) showed 30% of the ingoing nitrite in bacon to be converted to nitrate in less than 1 week. This nitrate formation increased until about the tenth week of storage when around 40% of the initial nitrite was converted to nitrate. In another study with the same product (bacon), nitrate was detected after processing and during storage at 7 and 27 C even though it was not in the product formulation (41). The quantity of nitrate found was related to the ingoing nitrite concentrations (41). In a study (87) on the fate of nitrite in a model system composed of nitrite, myoglobin and ascorbate, most of the nitrite nitrogen was recovered as nitrate. The remaining was found as nitrosomyoglobin or gases. These findings were confirmed by Lee et al. (152) in model and cured meat systems. Lee et al. (152) suggested that ascorbate and endogeneous meat reductants reduced metmyoglobin to myoglobin which was then oxidized simultaneously with nitrite to form nitrate.

The fate of nitrite in meat during curing was reviewed by Cassens et al. (33). It was stated that it is unacceptable to say that a large proportion of the nitrite "disappears." About 10-20% of the ingoing nitrite combines with porphyrin-containing compounds such as myoglobin. Other compounds shown to be formed are nitrate, gaseous products (N_2, NO, N_2O), nitrosothiols (protein-bound nitrite), and in the pH range of 2-3, S-nitrosocysteine can be formed through nitrite reaction with cysteine.

Generally the fate and rate of nitrite depletion differs from one system to another since different variables and several depletion pathways can be involved.

Nitrite toxicity-Nitrosamines

Concern over the nitrate and nitrite intake and its effect on human health goes back to the beginning of the century. In 1907 Richardson (197) asserted that most of the nitrate ingested was from vegetables. Kerr et al. (143) in their studies on the use of nitrite in meat curing gave special consideration to public health aspects. Nitrite is considered toxic at high concentrations and it is also implicated in carcinogenic nitrosamine formation. A recent report implicated nitrite itself to be a carcinogen (278). Used under the existing regulations, nitrite is not considered as a health hazard. Some rare toxic episodes have occurred, mostly due to accidental overuse. The lethal nitrite dose is 300 mg/kg of body weight (252). Nitrite can react as vasodilator and hypotensive agent (219); it can reduce the storage of vitamin A in the liver and it can also disturb thyroid function (71). DiFate (56) found nitrite to be mutagenic. It is well known that nitrite can oxidize hemoglobin to methemoglobin, thereby lowering the ability of the bloodstream to carry oxygen. This disturbance is called methemoglobinemia;

it can be fatal and most often occurs in infants. Two methemoglobinemia outbreaks involving 19 cases occurred in Los Angeles (36). They were traced to nitrite which had been repackaged and accidentally sold as monosodium glutamate (MSG).

On February 5, 1972 the FDA-USDA announced that nitrosopyrrolidine, a nitrosamine, was formed in retail-purchased and fried bacon. The levels recorded in the fried product ranged from 30 to 106 parts per billion (ppb), while the raw bacon was found free of nitrosopyrrolidine (114). Over 65 different nitrosamines have been found to be carcinogenic (158). Nitrosamines can be produced by a combination reaction of nitrite or nitrous acid and secondary or tertiary amines (225). Since cured meats contain both nitrite and amines, there is a potential for nitrosamine formation under the appropriate reaction conditions. Excellent reviews on the subject of nitrosamines are given by Sebranek and Cassens (225), and Crosby and Sawyer (49).

In recent years the concern over the nitrosamine question in cured meat products has greatly increased, and nitrite usage in meat curing has come under attack. Of all the cured meat products, fried bacon is the one product in which nitrosamines (mostly nitrosopyrrolidine) have been mostly found (232). Bacon is high in the amino acids proline and hydroxyproline, and other nitrosopyrrolidine precursors. The most likely pathway for nitrosopyrrolidine formation is that proline is first nitrosated and then decarboxylated (77). An excellent review on the subject is given by Gray (95). Nitrosopyrrolidine concentrations in bacon as high as 108 ppb have been reported (75), but the levels usually found fall in the range of 10 to 20 ppb (148). Nitrosopyrrolidine levels up to 139 ppb were confirmed in fried bacon by Havery et al. (111). It was also isolated from raw bacon (95,148). Wasserman et al. (292) reported finding nitrosopyrrolidine and nitrosodimethylamine, another nitrosamine, in 25 home-cooked bacon samples. Ten of the 25 samples tested contained nitrosamine levels above 10 and up to 39 ppb. Besides fried bacon, nitrosopyrrolidine was also found in some samples of severely fried (170 C, 12 min) country-style ham (100).

In other cured meat products nitrosamines have not been found or are only sporadically encountered. Fazio et al. (75) and Fiddler et al. (77) found nitrosopyrrolidine present in bacon but not in other cured meat products. Wiener samples tested for 14 nitrosamines at the 10 ppb sensitivity level were found negative (123). Canned comminuted pork (40), fermented sausage (147) and thuringer sausage (52) were also found nitrosamine-free. Wasserman et al. (289) reported occasional samples of commercial frankfurters at the retail level to show dimethylnitrosamine formation. Under controlled laboratory and normal heating conditions no nitrosamines were formed in frankfurters formulated with nitrite levels up to 750 μg/g. A nitrite concentration of 1500 μg/g was necessary for dimethylnitrosamine formation at levels of

10-11 ppb. A doubling of the smoking and heating process was necessary for the dimethylnitrosamine levels to reach 22 ppb (80). The researchers (80) concluded that frankfurters conforming to the United States Federal Regulations contained insufficient nitrite for nitrosamine formation.

Cured meat products can be divided into two groups on the basis of nitrosamine occurrence (96,220). One group includes bacon, where nitrosamines have mostly been found, and the other group includes ham, wieners, bologna and similar products which are less involved with nitrosamines. In bacon the nitrosopyrrolidine concentration has been found to be higher in the fat cookout than in the cooked edible portion (75,77,114,183,230). Removal of the fat before frying inhibited or at least diminished nitrosamine formation in the lean portion of bacon (77). The extent of nitrosamine formation in bacon is affected by ingoing nitrite concentration (232), cooking conditions (183) and post-processing age of bacon (111). Under certain conditions, several substances (ascorbate, ascorbyl palmitate, cysteine, glutathione, hydroquinone, alpha-tocopherol and tertiary butyhydroquinone) have been shown to decrease nitrosamine formation in cured meat products (3,78,79,97,99,114,167,231). Addition of nitrite to spice premixes resulted in formation of nitrosopiperidine from pepper, and nitrosopyrrolidine from paprika (229). Recently nitrite incorporation into spice premixes was disallowed in the U.S.

The nitrosopyrrolidine levels in bacon recently have been found to be lower in average than the original surveys (Table 4). Greenberg (100) reported that the average nitrosopyrrolidine in fried commercial U.S. bacon declined from 1972 to 1976 an average of 17 to 65 ppb. The same author (100) reported no correlation between nitrosopyrrolidine after frying with residual nitrite at the time of frying. Residual nitrite levels have also been declining through the years (Table 5). Cured meats constitute only a small proportion of the human nitrate and nitrite intake. In 1977 Rubin (220) reported

only 25-33% of the human nitrite intake comes from cured meats, while the rest comes from the saliva where it is formed from nitrate by the microflora of the mouth (255). Some common leafy vegetables such as spinach, lettuce and some root vegetables such as beets and radish are major nitrate sources. Also some drinking waters are high in nitrate (220,295). Another estimation (295) has indicated that only 10% of the ingested nitrate and 21% of the nitrite comes from cured meats, whereas vegetables contribute 86% of the nitrate and saliva 77% of the nitrite. A recent report (253) indicated that only 2% of the nitrite humans are exposed to comes from cured meats, another contributor being in vivo nitrite formation by microorganisms from degraded proteins. Tannenbaum et al. (255) found that under certain conditions salivary nitrite may reach concentrations of hundreds of $\mu g/g$. The importance of salivary nitrite to the in situ formation of nitrosamines has been discussed by Tannenbaum et al. (254). The kinetics of the reaction in model systems were studied by Mirvish (166) who showed that secondary amines and nitrite react only slowly at ambient temperatures and neutral pH values, but the reaction is rapid at pH values close to those of the human stomach. Some important considerations when dealing with toxic compounds such as nitrosamines are the significance of a 10-ppb level, the no-effect level, and possible advances in techniques to detect ppt (trillion) levels.

SORBATE

History, chemistry, safety

Sorbic acid was first discovered in 1859 in London by a German chemist named A. W. Hofmann. It was formed by the reaction of rowan berry oil with strong alkali. It was called sorbic acid after the scientific name of the moutain ash, *Sorbus aucuparia Linné* which is the parent plant of the rowan berry. The structure of the compound was clarified in the period 1870-1890 and it was first synthesized by O. Doebner in 1900. The antimicrobial properties of sorbic acid were first discovered in Germany in 1939 and in the U.S. in 1940 by E. Müller and C. M. Gooding, respectively. Industrial production of the compound started in the 1950's, and its use as a food preservative increased gradually after being permitted in most countries (cited from 156).

Sorbic acid is the trans-trans form of hexa-2,4-dienoic acid and has the structure $CH_3CH = CH \ CH = CH \ COOH$. It is a di-unsaturated, aliphatic, straight chain

TABLE 4. *Nitrosopyrrolidine (ppb) in bacon[1].*

Year	Average	Range	No. samples	No. determinations[2]
1973	31.8	10-67	6	18
1974	21.3	7-47	12	24
1976	4.3	0-9	16	42

[1]Calculated from data submitted to USDA (3).
[2]Multiple determinations from different laboratories.

TABLE 5. *Residual nitrate and nitrite in frankfurters and bacon.[1]*

	Nitrate ($\mu g/g$)				Nitrite ($\mu g/g$)			
	Frankfurters		Bacon		Frankfurters		Bacon	
Year	Average	Range	Average	Range	Average	Range	Average	Range
1936	710	570-880	2650	1550-3450	84	55-146	16	4-40
1937	714	340-1117	1200	300-1900	75	25-135	16	11-29
1970	—	—	—	—	50	0-105	33	3-199
1972	—	—	—	—	38	15-80	96	24-170
1977	—	—	—	—	4.2	1-11	5.5	1-12

[1]Calculated from data given (3,22).

monocarboxylic acid (7,156).

The solubility of sorbic acid in water at room temperature is relatively low (0.16%). It dissolves more easily in hot water or ethyl alcohol, which is an excellent solvent for the compound. Of the acids found in foods, acetic is by far the best solvent for sorbic acid. The pH of a sorbic acid-distilled water solution is 3.1, and in buffered solutions with pH above 3.1, more sorbic acid can be dissolved due to its partial conversion to its highly soluble salts. Sorbic acid is about three times more soluble in edible oils and fats than in water, and salt noticeably lowers its solubility. The major advantage of the alkali salts of sorbic acid is their good water solubility. Potassium sorbate, the salt most widely used in food applications, can be made into solutions of more than 50% in cold water without difficulty (91,156).

Sorbic acid is utilized in the body in a way similar to other fatty acids. Only the first step of β-oxidation is omitted, a, β-dehydrogenation, since sorbic acid already has an a, β double bond. The half-life of sorbic acid in the body is 40-110 min, depending on the dosage. No sorbic acid residues were found in the muscular tissues of domestic animals that were given feed containing sorbic acid. Under normal conditions of alimentation, it is completely oxidized to CO_2 and H_2O, yielding its potential energy as calories (53,54,156).

Deuel et al. (53) reported that sorbic acid was harmless to rats and dogs when incorporated in their diets to the extent of 5%. Its toxicity was lower than that of sodium benzoate which must be detoxified in the liver (53,109). The acute toxicity dose of sorbic acid (LD_{50}) is about 10 g/kg of body weight. The safety of sorbic acid use can be understood by comparing the above value with the corresponding LD_{50} of cooking salt, which is about 5 g/kg of body weight, and taking into consideration the respective quantities of salt and sorbic acid added to foods (156). Sorbic acid and its potassium salt have been cleared for use and listed as products "generally recognized as safe" (GRAS) by the FDA. On March 10, 1978 the FDA published a proposal to reaffirm the GRAS status of sorbic acid and potassium sorbate (276). The Select Committee concluded that these two substances demonstrated very low acute or chronic toxicity for experimental animals; and they were metabolized in the animal by the normal fatty acid pathway (276). The World Health Organization has confirmed the harmlessness of sorbic acid by stipulating for it the highest acceptable daily intake (25 mg/kg of body weight) among food preservatives (156).

Preservative action

Sorbic acid and its salts inhibit growth of fungi, yeasts and many bacteria, even though their action against bacteria is not as comprehensive as that against fungi and yeasts. The antimicrobial activity of sorbates depends on the pH of the substrate. At low pH values the amount of free undissociated acid, the effective form, increases (7,9,156,196). The inhibitory effect of sorbic acid against catalase-positive cultures was influenced by pH and it was found most effective in the pH range 5.0 to 5.5 (70). Gooding et al. (91) reported that the activity of sorbic acid in protecting foods against mold and yeast spoilage was higher in an acid environment. Raevuori (196) stated that the free acid enters the bacterial cell and inhibits several enzyme systems. In practice, effects against microorganisms can be obtained if the pH of the substrate is lower than 6.5. At that pH, the potassium salt is also hydrolyzed to free acid. In weakly acid environments, sorbic acid contains a considerable proportion of the undissociated form and thus sorbic acid is a better preservative at these hydrogen-ion concentrations compared to other preservative acids (156). The upper pH limit for sorbate efficacy has been reported to be 6.5; for propionates, 5.5; and for benzoates, 4.5. Parabens (esters of para-hydroxybenzoic acid) are generally used for foods with a pH of 7.0 or higher, but their astringent medicinal taste may be objectionable. The increased upper pH limit of sorbate efficacy (6.5), compared to other preservatives, extends its use in a wider variety of mildly acid foods. Sorbate can also frequently be used in more acidic foods to avoid the off-taste from benzoate, and/or in combination with benzoate to cover a broader range of microorganisms. The optimum effectiveness of sorbate, which is greater than that of benzoate and propionate is at pH values below 6.0. It also has some effectiveness at pH around 6.5, but at pH 7.0 and above it is ineffective. Sorbate is more effective than benzoate and propionate even at lower pH values (2.5-4.0) where they show their greatest effectiveness (7).

Lück (156) stated that "if the literature and practice of various countries are studied it will be found that sorbic acid has been tested for nearly all groups of food and also that it is used for all of these groups separately or collectively somewhere in the world." Sorbate has been used to preserve foodstuffs such as edible fat emulsions, cheese, meat and fish, fermented vegetables, pickled vegetables, tomato products, dried fruit, fruit juices and fruit syrups, drinks, fruit preserves, bakery goods, sugar and confectionery. Also it has found application on packaging materials as a fungistatic agent, in pharmaceuticals and cosmetic products and in animal feeds (156). The inhibitory properties of sorbic acid were first noted by Gooding (90). A concentration of 0.1-0.2% sorbic acid was used by Tanaka et al. (250) to preserve sausages. Boyd and Tarr (27) found better keeping quality in smoked fish with sorbic acid addition, and Amano et al. (2) found a tylosin-sorbic acid combination (40 μg/g-0.1%, respectively) most effective in preserving fish sausage at elevated temperatures (30 and 37 C). Flaked ice containing glycol diformate and sorbic acid was found somewhat effective in preserving poultry (141). The shelf-life of eviscerated, cut-up "dry-pack" poultry was extended by the synergistic effect of a two-step process in work reported by Perry et al. (186). The process consisted of acidic hydration and sorbic acid application. Control samples spoiled after 5 days at

refrigeration temperatures, whereas treated samples did not spoil until about 18 days. It was also noted that putrid odor development was suppressed markedly even during the latter days of storage and despite the eventual increase in microbial growth. The efficacy of potassium sorbate dip in extending shelf-life of broiler-parts and salmonellae growth was examined by Robach and Ivey (203). The total number of viable bacteria was significantly reduced with dipping in potassium sorbate solution. A 10% dip significantly reduced the total plate count compared to control parts after 5 days at 22 C. The 10% dip also resulted in salmonellae counts significantly lower than the untreated parts after 7 days at 10 C. A 5% or greater dip reduced the growth rate of salmonellae at 10 and 22 C. A 2.5% sorbate dip gave 0.05% sorbic acid concentration on poultry parts, while a 5.0 and a 10.0% dip gave a 0.13 and 0.31% sorbic acid concentration, respectively.

Gooding et al. (91) indicated salt and sugar to have a marked synergistic effect on sorbic acid fungistasis. In high sugar systems the increased sorbic acid effect occurred even at pH values above 6.5. The same authors (91) reported sorbic acid to be about four times as effective as propionates in protecting cheese, bread and cake products. Smith and Rollin (238) found sorbic acid superior to sodium benzoate as a fungistatic agent in protecting cheese and cheese products, which are generally very susceptible to mold spoilage. A level of 0.05% was shown to give full protection of the products.

Karelian pastry is a traditional foodstuff from Eastern Finland. Raevuori (196) examined the effect of sorbic acid and potassium sorbate in inhibiting growth of *Bacillus cereus* and *Bacillus subtilis* spores in the rice filling of Karelian pastry at 23 C. Addition of 0.2% sorbic acid or 0.4% potassium sorbate totally inhibited *B. cereus* growth, whereas *B. subtilis* growth was prevented by 0.1% sorbic acid or 0.26% potassium sorbate. Emard and Vaughn (70) found sorbic acid to inhibit *Salmonella* sp. in culture media. *Salmonella typhimurium* inactivation in media, milk and cheese was also reported by Park and Marth (181) and Park et al. (182).

The antimicrobial activity of sorbic acid has been shown to be selective. The compound has been found to effectively inhibit yeasts in cucumber fermentations and concurrently permit the normal growth of lactic acid-producing bacteria. This is not true in cases of a high sorbic acid concentration combined with a high initial brine level (46). It was first reported by Phillips and Mundt (188) and Jones and Harper (138) that 0.1% sorbic acid prevented growth of surface yeasts on cucumber fermentations without interfering with acid production. However, Borg et al. (23) reported that 0.1% sorbic acid inhibited growth of fermentative yeasts in cucumber fermentations as well as acid fermentation. On the other hand, Costilow et al. (47) showed that yeasts most prevalent in cucumber fermentations were completely inhibited by 0.01% sorbic acid in an 8% salt medium at pH 4.6. With increasing pH and/or decreasing salt concentration, higher sorbic acid concentrations were required for complete inhibition of some of the yeasts tested. In another study, Costilow et al. (45) demonstrated that cultures of *Pediococcus cerevisiae*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolated from cucumber fermentations were not greatly affected by sorbic acid concentrations up to 0.1%. It was concluded (46) that a sorbic acid concentration much lower than 0.1% would be effective against both subsurface and film-forming types of yeasts in cucumber fermentations.

In laboratory media, Emard and Vaughn (70) found that besides salmonellae, some *Streptococcus faecalis* strains were also inhibited by 0.12% sorbic acid, while *S. aureus* was inhibited by 0.07% sorbic acid in glucose yeast extract media and 0.12% in liver infusion media. Sorbic acid was also reported being effective in suppressing the growth of aerobic sporeforming bacilli (70,284).

Sorbic acid has been recommended as a selective agent in culture media for isolation of catalase-negative lactobacilli and clostridia (70,279,280,284). It was reported by Vaughn and Emard (284) and Emard and Vaughn (70) that sorbic acid-containing media could be used for selective enrichment and isolation of the catalase-negative lactic acid-producing bacteria. The authors suggested that sorbic acid media might be useful for enrichment and isolation of the catalase-negative clostridia. A concentration of 0.1% sorbic acid in liver infusion agar exerted a marked inhibitory effect on most catalase-positive cultures tested (70). The authors also reported some indication that the pH of the medium might affect the inhibitory activity of sorbic acid. No data showing possible usefulness of sorbic acid as a selective agent for clostridia were presented and the pH effect was not tested for clostridia. It was concluded (70) that the effectiveness of sorbic acid was dependent upon the concentration used, the basal medium, and the pH of the medium. A total of 47 cultures representing 20 species and types of *Clostridium*, including vegetative cells and spore suspensions, were tested for their resistance to sorbic acid in beef liver infusion medium by York and Vaughn (279). Vegetative cells and spores of *C. botulinum* types C, D and E were inhibited by 0.5% sorbic acid, while 3.0% sorbic acid did not inhibit *C. parabotulinum* types A and B. Nothing was mentioned by these workers (279) about the solubility of sorbic acid in the medium; the pH of the medium was 6.7-6.8; and considerable variation in resistance to sorbic acid was observed among the *Clostridium* species tested. In another study, Hansen and Appleman (109) tried to determine if sorbic acid was a growth stimulant for clostridia. It was reported that 0.12% sorbic, propionic, and caproic acids were neither inhibitory nor stimulatory to *C. sporogenes* and *C. botulinum* types A and B. Continuing their efforts, York and Vaughn (280) investigated the sorbic acid resistance of *C. parabotulinum* types A and B, and *C. botulinum* types C, D and E.

They concluded that sorbic acid was not a particularly effective inhibitor of food-poisoning bacteria in a suitable medium. These investigators (279,280) failed to report whether or not the growth of clostridia in the presence of sorbic acid was better or poorer compared to the growth in sorbic acid-free media.

Besides sorbic acid solubility, the pH of the medium is another factor that might have affected the conclusions reported from work related to sorbic acid effectiveness against clostridia. York and Vaughn (279) found that the ability of different clostridial spore cultures to germinate in 7 days was markedly decreased as the pH of the medium was reduced from 5.8 to 5.0. The authors stated that "the pH range must be increased to at least 6.0 or above if the sorbic acid medium is to be used for enrichment and isolation of the more fastidious species of *Clostridium*." However, in the higher pH range, 0.12% sorbic acid in liver infusion medium was not as effective in preventing growth of the catalase-positive microorganisms as in the recommended range of pH 5.0 to 5.5 (279). The same authors, York and Vaughn (280), reported that sorbic acid was more efficient at acid conditions (pH below 4.5), especially when the maximum concentration used was only 0.1% for *C. parabotulinum* types A and B and 1.0% for *C. botulinum* types C, D and E. In liver infusion, at pH 6.7-6.8, a sorbic acid concentration of 2.5% was necessary for inhibition to start, while 4.0% inhibited all strains tested. Generally, the studies recommending sorbic acid as a selective agent for the isolation of clostridia were made at high pH values, using sorbic acid to more strongly inhibit competing microorganisms and to eventually allow growth of clostridia.

York and Vaughn (279) reported that sorbic acid was used as a carbon source by some of the species tested, and the same authors (280) found that sorbic acid concentrations of 0.12 to 1.0% in the medium were used by all *C. parabotulinum* cultures examined. Melnick et al. (161) reported that a,β-unsaturated fatty acids such as sorbic acid were normal transitory metabolites in the oxidation of saturated fatty acids by molds, and that molds could also metabolize the compounds even during the period of growth inhibition. They suggested that since molds could metabolize sorbic acid, a "high" initial concentration was capable of inhibiting the dehydrogenase enzyme system in molds. They concluded that inhibition of that important enzyme system (dehydrogenase) was responsible for sorbic acid exhibiting fungistatic and under certain conditions even fungicidal activity.

Since sorbic acid is a fatty acid, a brief review of the effects of other fatty acids on bacteria, and especially clostridia, seems necessary. Humfeld, (122) in 1947, summarizing the pertinent literature, indicated that unsaturated C_{18} fatty acids, and particularly oleic, were known to inhibit a great variety of bacteria. Inhibition of germination of *C. botulinum* spores by as little as 0.1-1 μg of oleic, linoleic and linolenic acids/ml was reported by Foster and Wynne (83). Foster and Wynne

(82) also found that 10 μg of oleic acid/ml completely inhibited *C. botulinum* spore germination. Some variation in susceptibility among strains was reported, but all were affected, and linoleic and linolenic acids gave results comparable to oleic. Vegetative cells of *C. botulinum* were not inhibited by the acids, and starch neutralized their inhibitory effect. An oleic acid concentration of 100 μg/ml prevented germination over a 4.5-month period, and it was not sporicidal in distilled water but was in brain-heart medium. Spores of *C. perfringens* and PA 3679 were less inhibited; spores of *Clostridium histolyticum* and *Clostridium chauvei* were only slightly affected; and spores of aerobic species were unaffected (82). In 1952, Roth and Halvorson, (218) demonstrated that oxidative rancidity in lard and corn oil was a definite inhibitory factor for PA 3679, *C. botulinum* and *Clostridium pasteurianum* spore germination. The effect of methyl esters of oleic, linoleic and linolenic acids on spores of PA 3679 was also studied by the same scientists (218). Fresh unoxidized methyl esters at 0.01% concentration were not inhibitory, whereas the oxidized forms showed significant inhibition at the same concentration. Methyl-linoleate and methyl-linolenate caused 95% inhibition, and methyl-oleate 50% inhibition. All three acids were inhibitory in the rancid state and inhibition increased with unsaturation. A spore inhibition of the same magnitude as that of the fatty acids was observed with benzoyl peroxide. Addition of 0.01% catalase was able to partially overcome the inhibition of spore germination, indicating that the fatty acid action was actually due to peroxide. Sorbic acid however, is resistant to autoxidation (160). The relationship of the above to the antimicrobial activity of sorbic acid is not known. However, sorbic acid is also an unsaturated fatty acid (two double bonds) as is oleic, linoleic and linolenic, differing in that sorbic has only 6 carbons while the others have 18.

The antimicrobial action of sorbate against six *Bacillus* species was examined by Gould (93). At sodium sorbate concentrations of 0.015 to 0.05% and pH 6.0, germination occurred and spore walls were ruptured or were lysed and vegetative cells emerged and elongated but failed to multiply. A depressed rate of germination was observed at sodium sorbate concentrations above 0.04% and pH 6.0. The inhibitory effect of sorbate was five times larger at pH 6.0 than at pH 7.0. D-a-hydroxyisocaproic, L-a-hydroxyisocaproic and DL-a-hydroxyisocaproic acids (caproic acid is the saturated form of sorbic acid) in combination with L-alanine were shown to cause 74-87% inhibition of germination of *C. botulinum* type A and E spores (4).

The exact effects of sorbic acid on the growth of bacteria, and especially clostridia, and the mechanism of such effects, especially in food systems, are not fully understood. The findings reviewed here can give some indications or directions in the search for the answers.

Sorbate in meat products

The selectivity of bacterial inhibition by sorbic acid

and the early reports implicating it in enhancing, or at least not restricting, growth of clostridia might have been the reasons that the compound was neither tested nor used as an antimicrobial agent in meat products until recently. The only approved application of sorbic acid in meat products is that of dipping the dry sausage casings in a 2.5% potassium sorbate solution to inhibit mold growth on the surface of the product during the drying period.

Tompkin et al. (267) examined the possibility of increasing the public health hazard by using potassium sorbate to retard mold spoilage in cooked pork sausage. If sorbate enhanced clostridial growth at elevated temperatures, the product could become toxic. To the contrary, the data demonstrated that the addition of potassium sorbate reduced the public health hazard. Skinless, uncured sausage links with or without 0.1% (wt/wt) potassium sorbate were inoculated with salmonellae, *S. aureus*, *C. perfringens* and *C. botulinum* and stored at 27 C. The normal spoilage flora was delayed one day by sorbate, and salmonellae growth was markedly retarded. Growth of *S. aureus* was delayed 1 day with sorbate, after which growth occurred to the same extent as in product without sorbate. *C. perfringens* declined to undetectable levels within the first day in products with or without sorbate. The most important and probably unexpected finding was that sorbate retarded growth and toxin production by *C. botulinum*. Toxin was detected after 4 days in product without sorbate but not until after 10 days in product with sorbate.

One of the recommendations of the Expert Panel on Nitrites, Nitrates and Nitrosamines was that alternate preservatives with the potential to replace nitrite in cured meats should be evaluated. As a result of the general antimicrobial properties attributed to sorbate, the recent findings of Tompkin et al. (267) and the Expert Panel recommendation, research on the efficacy of sorbate to control botulinal toxicity in cured meat products has been initiated during the last 3 years.

Based on results of such research, on May 15, 1978 the USDA in accepting an April 27, 1978 petition, proposed that in the future, bacon may be produced with 40 μg nitrite/g and 0.26% potassium sorbate. The proposal will become effective in 1 year, unless data are presented to show that its adoption will result in bacon with inadequate botulinal protection or that the bacon will contain confirmable levels of nitrosamines after frying (277). A summary of the research that led to the above proposal follows.

Ivey et al. (131), in an effort to reduce the initial nitrite levels in the curing of bacon and still assure botulinal safety, tested low nitrite (0 and 40 μg/g) and potassium sorbate (0.13 and 0.26%) concentrations in product inoculated with 1100 spores per gram and incubated at 27 C for 110 days. Commercial bacon and bacon samples formulated with 80 and 120 μg of nitrite/g were included in the study. The results indicated that potassium sorbate significantly reduced the number of toxic-swollen packages and lengthened the time before toxicity was

observed. The presence or absence of 40 μg of nitrite/g had no significant effect on the sorbate inhibition. Nitrosopyrrolidine occurrence was reduced with decreased nitrite levels, and microbial growth on uninoculated samples was retarded by sorbate.

Tanaka et al. (251) tested nitrite, sorbate, sodium acid pyrophosphate (SAPP), pH and salt in a pork-macerate system. Sorbate did not show significant botulinal inhibition at pH 6.3, but it showed a rather strong effect at pH 5.5. At pH 6.0 some sorbate inhibition could be seen. Addition of low nitrite levels (25 or 50 μg/g) in the presence of sorbate and/or SAPP greatly increased the inhibitory effect. Sorbate and SAPP combined were much more inhibitory than sorbate or SAPP alone.

AF-2(2-(furyl)-3-(5-nitro-2-furyl)acrylamide) was used as a fish sausage preservative in Japan until it was prohibited in July, 1974. Since the above compound was replaced by sorbic acid, Wada et al. (287) examined the preservative effects of sorbic acid for fish sausage in connection with pH and storage temperature. The results indicated that addition of 0.1 or 0.2% sorbic acid prolonged the shelf-life of fish sausage by two or three times, respectively, at 30 C and pH 5.9. The authors concluded that a 0.2% sorbic acid level could preserve fish sausage for at least 2 months at about 10 C, if the pH was adjusted to the range of sorbic acid effectiveness.

As stated before and reported by Tanaka et al. (251), the inhibitory effect of sorbate is greater at lower pH values and inhibition diminishes at pH values above 6.0-6.5. Addition of sorbate and especially sorbic acid to foods causes a pH decrease in the product. Tompkin et al. (267) reported that uncured sausage links with 0.1% potassium sorbate had a pH of 6.4 while samples without sorbate had a pH of 7.1. Raevuori (196), working with the rice filling of Karelian pastry, presented data showing that addition of 0.4% potassium sorbate to the samples did not give a different pH than the control, either before or after baking, and after 6 days at 23 C. In contrast, addition of 0.3% sorbic acid decreased the pH by one unit and it did not change during baking and 6 days at 23 C. A question to be answered is whether the antimicrobial activity of sorbic acid is not only pH dependent, but that part of the effect is due to a restrictive pH for bacterial growth. Tompkin et al. (267) stated that the pH value of 6.4 in their sausage links with potassium sorbate was high enough to exclude the possibility that pH was a factor in inhibition of botulinal growth and toxin production. Therefore, the effectiveness recorded was attributed solely to sorbate.

The effectiveness of sorbic acid and low nitrite concentrations to inhibit botulinal growth in a canned comminuted pork product was studied by Ivey and Robach (130). Sorbic acid at the 0.2% level delayed growth and toxin production, while 0.1% sorbic acid was ineffective. Low nitrite concentrations (50 μg/g) and 0.1 or 0.2% sorbic acid greatly retarded growth and toxicity. Inclusion of either SAPP or sodium hexametaphosphate had a synergistic effect with sorbic acid. It was

stated that 0.2% sorbic acid and either phosphate with or without 50 μg of nitrite/g might be a potential alternative preservative to high nitrite levels in canned comminuted pork products.

The antibotulinal activity of sorbic acid has also been tested in a comminuted chicken product. Robach et al. (204) found that addition of 0.1 and 0.2% sorbic acid extended the time for swelling. Combinations of 20 μg of nitrite/g and 0.1 or 0.2% sorbic acid delayed botulinal growth even further. In another bacon study, Pierson (189) confirmed the effectiveness of potassium sorbate in delaying botulinal toxicity. A concentration of 0.13% potassium sorbate (equivalent to 0.10% sorbic acid) showed a minor effect, while 0.13% potassium sorbate in combination with 40 μg of nitrite/g was very effective. An even larger effect was found with 0.26% potassium sorbate (0.20% sorbic acid) and 40 μg of nitrite/g. In all cases the effectiveness increased in samples inoculated with sand-dried spores, compared to samples inoculated with aqueous spore suspensions.

Work in our laboratory (239,240,241,242) has indicated that sorbic acid (0.2%) inhibited botulinal spore germination (loss of heat resistance) in mechanically-deboned chicken meat, beef, and pork frankfurter emulsions abused at 27 C. Nitrite (20-156 μg/g), as indicated earlier in the text, did not affect spore germination, but at increased concentrations (156 μg/g) delayed toxin production. The above sorbic acid inhibitory effect on botulinal spore germination, outgrowth, and toxin production was pH-dependent and it started appearing at pH values below 6.0. When nitrite (40, 80, 156 μg/g) was added to the formulation, the sorbic acid effectiveness was greatly increased in magnitude and it started at pH values (6.20) higher than those for sorbic acid alone (< 6.0). Also, when sorbic acid (0.2%) was included in the formulation, nitrite depletion from the product was slower during temperature (27 C) abuse. This is important, since our studies indicated that even when sorbic acid (0.2%) was added in combination with nitrite (40-156 μg/g), botulinal toxicity developed when the residual nitrite in the product, and under our conditions, decreased to 5-15 μg/g. The above results, besides demonstrating the effectiveness of sorbic acid (0.2%)-nitrite (40-156 μg/g) combinations to extend the botulinal safety of emulsified cured meat products, can also facilitate the search for nitrite and/or sorbic acid mechanisms for such action.

An aspect of concern with incorporation of sorbate in food products is that of a potential undesirable flavor. Lück (156) reported that its flavor is neutral. In the study of Raevuori (196) organoleptic analysis of the sorbic acid-protected Karelian pastry was performed. A sorbic acid concentration of 0.08% in the rice filling could not be distinguished from the control, whereas a 0.15% concentration was easily detectable and judged as an off-flavor. It was recommended that a sorbic acid concentration of 0.1% could be introduced without altering the organoleptic quality of the product. In their

bacon study, Ivey et al. (131) using experienced judges to evaluate flavor, found that 0.26% potassium sorbate decreased product preference only slightly. Perry et al. (186) reported that sorbic acid did not impart off-flavors to poultry when used at recommended levels. To assure flavor acceptability of sorbic acid-treated products, more work is necessary with different products to establish no-effect levels and to possibly mask potential undesirable flavor.

Sorbate-nitrite combinations have been shown to greatly extend the botulinal safety of cured meat products, even more than nitrite or sorbate alone. One explanation of the effectiveness of such combinations, would be that nitrite and sorbate react and form a more potent inhibitor(s). Kada (139) reported that a mixture of nitrite and sorbic acid in an aqueous medium became positive upon heating in a sensitivity test named "rec-assay" (140) of Bacillus subtilis. According to the author, this indicated production of DNA-damaging compounds. Hayatsu et al. (112) and Namiki and Kada (173) further examined the possible reaction of nitrite and sorbic acid. Namiki and Kada (173) isolated and identified ethylnitrolic acid as a reaction product between a sodium nitrite solution and a sorbic acid partial suspension in distilled water in a 90 C water bath for 1 h. The pH of the mixture, from 4.3 at the beginning reached the value of 6.0 in the final state. It should be stressed that the reaction took place under special in vitro experimental conditions and in acid solution, as the authors stated (173). Testing ethylnitrolic acid against Escherichia coli, it was effective at 25-50 μg/ml, compared to 2000-4000 and 1500-3000 μg/ml for sorbic acid and nitrite, respectively. An extensive effort was undertaken by DiFate (56) to form ethylnitrolic acid by different methods and to test its mutagenicity. The results demonstrated that ethylnitrolic acid was not mutagenic, whereas nitrite was. DiFate (56) attributed the mutagenicity reported by Kada (139) and Namiki and Kada (173) to possibly free nitrite contamination in the ethylnitrolic acid solution. Mirna and Coretti (165) reported that the reaction of nitrite with carbonyl compounds containing an activated methylene group (like some ketones of fat degradation products) might lead to formation of nitrolic acids. Ethylnitrolic acid was tested against Micrococcus sp., Enterobacter liquefaciens, Escherichia coli and Staphylococcus aureus and inhibition was observed even at concentrations of 10 μg/g. Propylnitrolic acid inhibited the same microorganisms at about 100 μg/g (164). The relevance of the above to real cured meat products is unknown and debatable. However, the potential of such inhibitors being formed in cured meats by nitrite and sorbic acid exists and as the microbiological session of the Second International Symposium on Nitrite in Meat Products concluded and recommended, "attention should be directed especially to nitrolic acids" as possible botulinal inhibitors of the potent Perigo-type (264).

Sorbic acid can be considered as a unique food

additive since it is a metabolizable fatty acid and its use would represent one food protecting another. Usage of nitrite-sorbate mixtures to protect cured meats against botulinal toxicity is an attractive alternative due to the following factors (5): (a) with lower nitrite levels (e.g. 40 µg/g) the nitrosamine formation potential would be minimized, (b) C. botulinum would be inhibited at least as well or even better compared to present formulations, (c) the low nitrite level used would give the characteristic cured meat color and flavor, (d) the shelf-life of the products would increase, (e) sorbate would not cause health problems, as being a metabolizable GRAS substance and (f) the current processing procedures would not have to be changed.

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News and Events

Hearings Scheduled on Proposed Revision of Manufacturing Practices

Informal public hearings will be held at three locations around the country on the proposed revision of the Current Good Manufacturing Practices in Manufacturing, Processing, Packing of Holding Human Food Regulation ("Umbrella" GMP).

These hearings are scheduled for Chicago on Sept. 11, San Francisco on Oct. 3, and Atlanta on Oct. 24.

The proposed revisions in the Current Good Manufacturing Practices would update requirements for food production in such areas as sanitation, equipment, product coding, warehousing, distribution, ad recordkeeping.

Consumers and industry representatives are invited to attend the hearings. The FDA is especially inviting small businessmen to attend and testify as to the proposed changes may affect their business. Persons wishing to participate should submit the following information to the Office of the Hearing Clerk (HFA-305), Food and Drug Administration, Room 4-65, 5600 Fishers Lane, Rockville, MD 20857:

1) name, 2) street address, 3) city, state, zip code, 4) organization, 5) city in which you wish to participate, 6) number of minutes for presentation (maximum of 15 min.), 7) list of topics you wish to discuss.

Persons wishing to submit written comments only should submit four copies of their comments or, if having no organizational affiliation, one copy to the Office of the Hearing Clerk by Dec. 31, 1979.

Submitting news or calender items to the *Journal*?

Please make sure that you send them by the 10th of the month preceding desired publication. A number of items cannot be included in the *Journal* because they're too late for printer's deadlines.

Z. John Ordal Dies

Professor Z. John Ordal of the Department of Food Science at the University of Illinois, died of a heart attack on May 29, 1979. Professor Ordal received his B.A. at Luther College, Iowa, and his Ph.D. at the University of Minnesota, in Bacteriology and Chemical Engineering in 1940. Following post-doctoral work at Ohio State University and the Hormel Research Foundation, he spent two years as an associate in the Department of Bacteriology and Public Health at the University of Illinois College of Medicine. In 1946 he moved to Owens-Illinois Glass Company as supervisor of the Bacteriology group in the Process and Product Research Division, and in 1947 became chief of the Bacteriology Section of Economics Laboratory, Inc., of St. Paul, Minnesota. In 1949 he became associate professor of Food Microbiology at the University of Illinois and since 1957 has been full professor of Food Microbiology in the Department of Food Science. In 1970, he was also appointed full professor in the Department of Microbiology at the University of Illinois. His major research interests have been in physiology of the bacterial spore, injury and recovery of bacterial cells, psychrotrophic bacteria and production and utilization of microorganisms. Professor Ordal's research contributions have earned him an international recognition. His work on the bacterial endospore spans a quarter of a century. His work in explaining and characterizing the effect of physical stress on microbial cells and the conditions needed for repair or recovery has stimulated similar research in laboratories throughout the world. His earlier work on vacuum-packaging of fresh meats is now finding wide application in the wholesale distribution of fresh meats, particularly primal cuts of fresh beef. He was extremely effective in the training of graduate students, having been advisor to 31 Ph.D. candidates and 45 M.S. candidates. Most of these students have been extremely successful and are in responsible positions throughout the world.

Dr. Ordal received much recognition for contributions to food microbiology throughout his career. His most recent honor was to be selected as the 1979 Becton Dickinson Lecturer at the 79th American Society of Microbiologists Annual Meeting held in Los Angeles, in May 1979. Professor Ordal was a member of many professional societies and was elected a Fellow in both the American Academy for Microbiology and the Institute of Food Technologists. He edited several books in his field, published more than 15 book reviews and over 110 scientific papers in peer-reviewed journals. Some of these papers appeared in the *Journal of Food Protection*. He also served for 6 years on the Editorial Board of the *Journal*.

Perhaps his greatest professional impact was in his capacity to educate graduate students. He was a "professor's professor." His field of endeavor, university, colleagues, students and working associates were all enriched by his presence.

The Department of Food Science has established the Z. J. Ordal Food Microbiology Research Fund in his honor. This fund will be used to support graduate student research in the field to which he was dedicated. Donations may be sent to the University of Illinois Foundation, 224 Illini Union, Urbana, Illinois 61801, with appropriate notation as to the fund to which it is directed.

Coming Events

Sept. 13-14--INTERNATIONAL SYMPO-SIUM ON ANIMAL AND HUMAN INFLU-ENZA. Ecole Nationale Veterinaire D'Alfort, 7, avenue du General de Gaulle, 97 704 Maisons-Alfort cedex, France. Contact: Ch. Pilet, Secretariat of the Dept. of Microbiology, Ecole Nationale Veterinaire d'Alfort.

Sept. 13-14--MINNESOTA SANITAR-IANS CONFERENCE. Earle Brown Continuing Education Center, St. Paul Campus, University of Minnesota. Mastitis control, "STOP" program, quality control and energy conservation on the farm will be discussed. Awards banquet, 7 p.m., Sept. 13, Eagles Club No. 33, St. Paul. Contact: Edward A. Kaeder, Publicity Chairman, Minnesota Sanitarians Assoc., Mid-America Dairymen, Inc., 2424 Territorial Road, St. Paul, MN 55114, 612-646-2854.

Sept. 17-19--BAKING PRODUCTION TECHNOLOGY. Sponsored by American Institute of Baking. Dunfey Dallas Hotel, Dallas, TX. Fee: $300 per person. Contact: Registrar, AIB, 1213 Bakers Way, Manhattan, KS 66502, 913-537-4750.

Sept. 18-20--PROCESSING AND QUAL-ITY ASSURANCE UPDATE FOR THE CITRUS INDUSTRY. University of Florida, Gainesville. Sponsored by Cooperative Extension Service, Institute of Food and Agricultural Sciences, and IFT, Florida Section. Contact: R. F. Matthews, Food Technologist, Florida Cooperative Extension Service, 325 Food Science Bldg., University of Florida, Gainesville, FL 32611.

Sept. 18-20--WESTPACK '79. Convention Center, Anaheim, CA. Contact: Clapp & Poliak, Inc., 245 Park Ave., New York, NY 10017.

Sept. 19-20--NEW YORK STATE ASSO-CIATION OF MILK & FOOD SANI-TARIANS. Annual Meeting. Holiday Inn Arena, 2-8 Hawley Street, Binghamton, NY 13901. Sponsored by NYSAMFS, Cornell University Food Science Dept., New York State Dept. of Health, New York State Dept. of Agriculture and Markets. Contact: R. P. March, 124 Stocking Hall, Ithaca, NY 14853, 256-4550.

Sept. 19-20--WISCONSIN ASSOCIATION OF MILK AND FOOD SANITARIANS. Annual Meeting. Madison, WI. Sponsored by WAMFS. Wisconsin Dairy Plant Fieldmen's Association, Wisconsin Dairy Tech Society, Wisconsin Environmental Health Association, and Wisconsin Institute of Food Technologists. Contact: Neil Vassau, 4702 University Ave., Madison, WI 53705.

Sept. 23-29--XV INTERNATIONAL CON-GRESS OF REFRIGERATION. Venice, Italy. Contact: XV International Congress of Refrigeration, American Express Co. S.A.I., Conventions Service Italy, Piazza Mignanelli, 4, 00187-Rome, Italy.

Sept. 26-27--SOUTH DAKOTA STATE DAIRY CONVENTION. Downtown Holiday Inn, Sioux Falls, South Dakota 57100. Contact: Shirley W. Seas, Secretary, Dairy Science Department, South Dakota State University, Brookings, South Dakota 57007, 605-688-5420.

Sept. 27-28--NSF SEMINARS, Boston, Ma. See Feb. 11-12, 1980, NSF entry. Contact: Education Service, National Sanitation Foundation, P.O. Box 1468, Ann Arbor, MI 48106, 313-769-8010.

Sept. 28--SYMPOSIUM ON THE PRAC-TICAL APPLICATIONS OF MICROWAVE ENERGY. Kansas State University Union, KSU, Manhattan, KS 66506. Contact: D. Y. C. Fung, Chairman, or F. E. Cunningham, Co-Chairman, Call Hall, KSU, Manhattan, KS 66506, 913-532-5654.

Oct. 1-3--ADVANCED FOOD PLANT SANITATION COURSE. New Orleans, LA. Sponsored by American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502. Contact: Darrell Brensing, AIB, 913-537-4750.

Oct. 8-9--NSF SEMINARS, Nashville, TN. See Feb. 11-12, 1980, NSF entry. Contact: Education Service, National Sanitation Foundation, P.O Box 1468, Ann Arbor, MI 48106, 313-769-8010.

Oct. 9-11--FOOD SAFETY AND QUAL-ITY ASSURANCE IN FOOD SERVICES, Seminar sponsored by Capsule Laboratories. Radisson Hotel, St. Paul, MN. Contact: Darrell Bigalke, Capsule Laboratories, 840 Sibley Memorial Highway, St. Paul, MN 55118, 612-457-4926.

Oct. 14-17--24th ANNUAL ATLANTIC FISHERIES TECHNOLOGISTS CONFER-ENCE. Danvers, MA. Contact: Fred J. King, 1979 AFTC Secretary, Gloucester Laboratory, Northeast Fisheries Center, National Marine Fisheries Center, National Marine Fisheries Service, Emerson Ave., Gloucester, MA 01930, 617-281-3600, ext. 296.

Oct. 17--IOWA MILK AND FOOD SANI-TARIANS, Sheraton Inn, Cedar Rapids, IA. Contact: Hale Hansen, State Health Dept., Lucas Office Bldg., Des Moines, IA 50319.

Oct. 17-18--NEBRASKA DAIRY INDUS-TRIES ASSOCIATION, 25th Annual Convention. Holiday Inn, 72nd and Grover Streets, Omaha, NB. Contact: T. A. Evans, Exec. Secretary, 116 Filley Hall, East Campus, UN-L, Lincoln, NB, 68583.

Oct. 20-25--COMBINED CONFER-ENCES, EXPOSITIONS: National Environmental Sanitation & Maintenance Educational Conferences, Expositions of Environmental Management Association, The Green Industry Division, Food Sanitation Institute, Health Care Facilities Subsidiary, and Building Service Manager's Institute. Caribbean Gulf Resort Hotel, Clearwater Beach, FL. Contact, for any of the five conferences: EMA, 1701 Drew St., Clearwater Beach, FL, 33515, 813-446-1674.

Oct. 22-23--CALIFORNIA ASSOCIATION OF DAIRY AND MILK SANITARIANS, Annual Meeting. Hyatt House, San Jose, CA. Contact: Pat Dolan, Dairy Foods Consultants, 4009 Cayente Way, Sacramento, CA 95825.

Nov. 3-6--1979 AMERICAN MEAT IN-STITUTE CONVENTION. McCormick Place and The Conrad Hilton, Chicago. Contact: Judi Winslow, American Meat Institute, P.O. Box 3556, Washington, D.C. 20007, 703-841-2431.

Nov. 5-6--NSF SEMINARS, Reno NV. See Feb. 11-12, 1980, NSF entry. Contact: Education Service, National Sanitation Foundation, P.O. Box 1468, Ann Arbor, MI 48106, 313-769-8010.

Nov. 11-15--FOOD AND DAIRY EXPO '79. McCormick Place, Chicago, IL. Contact: Wes Dibbern, Dairy and Food Industries Supply Assoc., 5530 Wisconsin Ave., Suite 1050, Washington, D.C. 20015, 301-652-4420.

Nov. 27-29--INTERNATIONAL CONFER-ENCE ON UHT PROCESSING AND ASEP-TIC PACKAGING OF MILK AND MILK PRODUCTS. North Carolina State University, Raleigh, NC 27650. Contact: W. M. Roberts, Dept. of Food Science, NCSU, Raleigh, NC, 27650.

Dec. 3-4--NSF SEMINARS, Orlando, FL. See Feb. 11-12, 1980, NSF entry. Contact: Education Service, National Sanitation Foundation, P.O. Box 1468, Ann Arbor, MI 48106, 313-769-8010.

SOMEONE YOU SHOULD KNOW IN THE DAIRY INDUSTRY

John E. McCormack, D.V.M., Extension Veterinarian

Dr. McCormack operated a veterinary practice for 10 years in southwest Alabama. He has served on the teaching staff of veterinary colleges at Auburn University, Louisiana State University and the University of Georgia. He has been Extension veterinarian at the University of Georgia since 1976, and has written numerous cattle health articles for dairy publications and conducted many meetings on animal health matters for dairymen.

"The Extension specialist is an educator, a communicator, and a motivator to the dairy industry. Our job is supporting the basic unit of the Cooperative Extension Service; the county agents and their programs. In addition, we help local veterinarians by consulting, reinforcing their educational efforts, and providing updated information on research findings that help them provide better animal health care. Thus, the ultimate goal is to help dairymen, and other livestock owners, reap greater financial rewards from improved health care practices.

"Our educational efforts emphasize positively approached animal health programs.

How We Get Our Message Across

"Our basic educational tool is the dairy association meeting. We attend and present educational material at many of these each year, on a pre-planned basis with the county agent or dairy association official. Sometimes, however, it may be a quickly organized effort to present up-to-date material on an emerging health problem or disease.

"Since we feel that a regular scheduled program of reproductive tract examinations is important for maximum, long-range milk production, we emphasize this facet of dairy herd management. Usually these reproduction seminars or short courses for dairymen are held in the evening, sometimes in conjunction with a planned meal. We make certain the local veterinarians are involved in these programs. Specimens of reproductive organs, both normal and abnormal, are collected from a local slaughterhouse and are layed out on a table for the participants to look over and examine. We point out the normal anatomical structures of the reproductive tract, such as the ovary, and cervix and its rings, and other pertinent structures. Usually, we have several uteri in various stages of pregnancy.

"We encourage the dairymen to palpate these, then we incise the pregnant horn and examine the fetus after everyone has given the estimate of its age. This practical demonstration is supplemented with a slide presentation showing the basics of a reproduction program.

"This type seminar almost always creates a desire among some dairymen to enter into a regular program of reproductive exams. This is why the local veterinarian is an important part of the seminar. We motivate, the practitioner participates and together we instigate the program.

"Other programs in which we are routinely involved are vaccination schedules, parasite control programs and mastitis control. We cooperate with other dairy specialists and quality milk control officials in putting on milker schools and mastitis prevention programs.

"For instance, if high leucocyte counts and mastitis are a problem in an area, we may put on a program emphasizing milking hygiene, basic milking machine function, teat dipping, dry cow therapy and so forth. We ask the dairymen to bring milk to the meeting from a 'clean cow' and a known infected cow and we show them how to run a CMT on that milk.

"Educational meetings are a valuable teaching tool. However, one on one individual farm visits are even more beneficial, but are obviously done on a smaller scale. An example might be a situation where a dairyman has excessive numbers of cows off feed after calving, displaced abomasums, and breeding difficulties. We see this quite often in the form of the fat cow syndrome due to excessive feeding of grain and/or corn silage during the dry period. We cooperate with the local veterinarian on these visits and usually go with him on these trips.

"Telephone consultations are carried on daily with dairymen regarding some health problem or important decision. Examples include interpretation of laboratory information, the wisdom of instituting a BVD vaccination program or how to eliminate a foot disease problem.

"The message of the Extension veterinarian is: by utilizing the knowledge available to him, the dairyman can have healthier, more productive cows. This message is delivered in meetings, written articles, farm visits, and response to telephone calls. We feel a knowledgeable dairyman will be a better dairyman and continue to learn even more about his profession."