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IAMFES
65th Annual Meeting
Hilton Airport Plaza
Kansas City, Missouri
August 13-17, 1978
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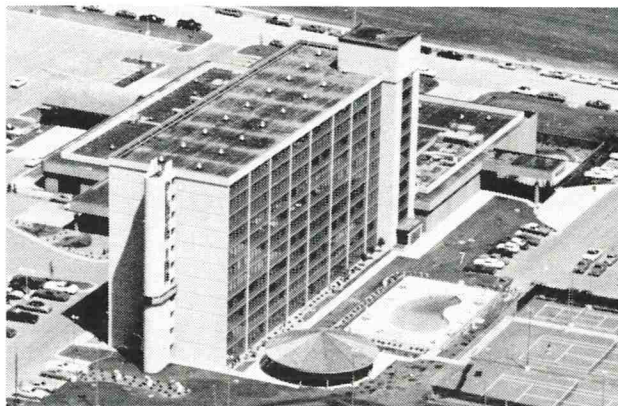
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Microbial Contamination of Vegetables Irrigated with Sewage Effluent by the Drip Method

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ABSTRACT

The effect of the drip irrigation method on microbial contamination of vegetables irrigated with wastewater was evaluated. The density of microbial contamination on surfaces of cucumbers and eggplants irrigated with sewage effluent was determined, using a controlled washing procedure. The fecal coliform count on vegetables irrigated with wastewater was 38-fold higher than on vegetables irrigated with fresh water. However, agrotechnical manipulations of the drip method, such as sub-irrigation or covering the soil and the drip lines with plastic sheets, reduced bacterial contamination considerably. Similarly, the bacterial contamination on vegetables which were irrigated with sewage effluent during the first stage of growth (up to flowering) and subsequently with fresh water was not different from the contamination on vegetables which were irrigated with fresh water. The viral contamination on 27 vegetable samples which were collected from sewage irrigated experimental plots was below the level of detection. It is proposed that the drip method may be considered for wastewater irrigation of crops in accordance with generally accepted public health criteria.

Land application of wastewater is prevalent in many parts of the world. The usefulness of this practice for agricultural production is especially significant in arid zones where the shortage in irrigation water obstructs expansion of food production. Wastewater irrigation is usually regulated by the Public Health authorities of the different countries (15). In Israel, the guiding principle for these regulations is to allow wastewater irrigation of crops consumed after peeling or processing or not intended for human consumption (12).

The most commonly used land application methods include the following modes of irrigation: spray, overland flow and ridge and furrow. All of these methods entail possible contamination of the crops and their environment either by direct contact or via an aerosol effect. This is especially true for spray irrigation (8,9,14). Since economical considerations make the complete renovation of wastewater to microbiological standards approaching

those of drinking water impractical for many countries, different approaches and new techniques for possible uses of wastewater including irrigation have to be developed.

The drip irrigation method is a fairly recent innovation in irrigation technology and its special merits were described by Goldberg et al. (6,7). It involves the lateral spread of water on the irrigated surface by conducting the water under pressure to a relatively closely-spaced grid of emitters or drippers and discharging the water through these drippers at virtually zero pressure. The drippers are mounted on 12-16-mm diameter polyethylene tubing which are stretched along the rows of the crop. Ozrad et al. (11) have concluded that the method is applicable for sewage effluent irrigation in spite of the presence of suspended solids which may clog the drip outlets.

The outstanding features of this method of irrigation include a highly controlled out-put rate of the water applied directly to the root zone areas, a lack of surface flow, and a uniform distribution of the water along the length of the crop rows. This and additional agrotechnical manipulations such as drip irrigation under plastic sheets which cover the soil or subsurface irrigation, may reduce crop contamination from wastewater irrigation. Since the level of risk to the public health from contaminated crops depends primarily on the infection dose (2,16), a reduction in contamination density opens new options for wastewater utilization in agriculture.

In this paper, the results of our studies on microbial contamination of eggplants (*Solanum melongena*) and cucumbers (*Cucumis sativus*) irrigated with sewage effluent using different drip treatments are reported. The field experiments were conducted in the Arava desert near the city of Eilat, Israel. The area receives very little or no rainfall and the underground water is relatively high in salt content (600-750 ppm Cl⁻). Fresh water for drinking purposes is supplied to the population from desalination plants. This made use of wastewater in this area essential for agricultural production.

¹Food Science Laboratory

²Environmental Health Laboratory

³Department of Soil and Water Science

MATERIALS AND METHODS

The agricultural work was directed by Mr. Ozrad of the Regional Council of Eilat, during the spring seasons of 1972-1974. Cultivation and other agricultural activities were done in accordance with the usual practices common in the area. The areas of experimental plots (at least 500 m²) were irrigated daily by a drip system which was equipped with internal spiral drippers of a minimal discharge rate of 4 or 8 liters/h and were spaced at 50 or 100 cm from each other, respectively. The amount of water applied was in accordance with evapotranspiration measurements (6). The soil was covered before planting with transparent polyethylene sheets 0.03 mm thick and 1.1 m wide. The layout of the different treatments in the field was designed to minimize cross-contamination by winds.

The microbial contamination on vegetables from five drip irrigation treatments was determined: (a) irrigation with sewage effluent on a non-covered soil surface; (b) irrigation with sewage effluent on the soil surface below a plastic sheet cover; (c) sub-surface irrigation with sewage effluent and soil covered with plastic sheets; (d) irrigation with sewage effluent on the soil surface until flowering of the plants and subsequent irrigation with fresh water; and (e) irrigation on the soil surface with fresh water (control).

Collection and handling of samples

Vegetable samples were collected with sterile disposable gloves, packed in sterile polyethylene bags, transported to the laboratory in ice chests and analyzed on the same day.

Determination of microbial contamination of vegetables

Some of the preliminary experiments for selecting the method were done with vegetables infected by immersing in sewage effluent followed by air drying (for 1-2 h) at room temperature (13). Five hundred to 1000 g of vegetables were placed in stainless steel jars measuring 12 × 18 cm with a 2000-ml capacity which contained 500 ml of a sterile refrigerated washing solution (0.85% NaCl in 0.02 M phosphate buffer, pH 8.0).

The jars were shaken on a horizontal shaker at a rate of approximately 160 strokes per minute for 7 min. At the end of this time, the vegetables were aseptically transferred to a second jar containing fresh solution for an additional shaking period. The above operation was repeated several times, and the number of bacteria and viruses eluted from the vegetables into the buffer solution, determined. Some samples of vegetables were peeled aseptically with sterile scalpels and tweezers and 100 g of the peel homogenized with 900 ml of the buffer washing solution.

Bacterial assays. Coliform and fecal coliform bacteria in the washing solutions, homogenized peel and in the sewage effluent were determined with the "three tubes" fermentation technique for the most probable number (MPN) determination (1).

Viral assays. (a) *Sewage effluent:* To a 9-ml volume of each sewage effluent sample, 1 ml of antibiotics, containing 200 mg of streptomycin, 2 × 10⁵ units of penicillin, 4 mg of neomycin and 5 mg of kanamycin were added. The treated sample was directly inoculated and assayed for plaque forming units (PFU) and the remainder (ca. 2 liters) concentrated

by the phase separation method (17). If the 9 ml samples led to negative results, the concentrated samples were then assayed (18). (b) *Vegetable eluent:* The eluent buffers from washed vegetables were concentrated by aluminum hydroxide precipitation as previously described by Fattal et al. (4) and assayed as above. All samples were maintained at -70 C until seeding on BGM cells (3).

RESULTS

Characterization of the effluent used for irrigation

The sewage effluent used for irrigation originated in the Eilat sewage treatment system which included an anaerobic settling pond and two consecutive oxidation ponds of 3 days detention time. The effluent was pumped to the agricultural experimental plots and passed through both, a screen and a gravel filter (6), before entering the dripping lines. The quality of the effluent was periodically determined and some of the physical, chemical and microbiological quality data are summarized in Table 1. The general quality of the effluent was poor and resembled the effect which may be obtained with a primary treatment only. This was most probably a result of an excessive load on the sewage treatment system. Several 100-ml samples of fresh water, were examined as well and no fecal coliforms or viruses were found.

Procedure for determining the density of microbial contamination on vegetables

Other investigators (5) had previously noted that the laboratory methods for determination of microbial density on vegetable surfaces are inadequate. The difficulty in developing such methods stems from large variability in shape and consistency of the vegetables and sometimes from the large volume of vegetable inner tissue which may be practically sterile. Our working assumption was that contamination of vegetables is limited to the surface area and a controlled washing procedure was developed accordingly.

To assess the efficiency of our method, recovery of bacteria and viruses by repeated shaking in buffer solutions was determined. The results (Table 2) show that most of the recoverable microorganisms were released in two washings and that subsequent washings resulted in but a small addition to the overall recovery. In a number

TABLE 1. *Some physical, chemical and microbiological quality data of the effluent which was used for drip irrigation of vegetables*¹.

Parameter	No. of samples	Mean values	Std. dev range or
Biochemical oxygen demand (mg/l)	5	85	± 26.6
Chemical oxygen demand "	5	235	± 130.0
Total solids "	5	2026	± 343.4
Suspended solids "	5	70	± 58.6
Soluble solids "	5	1955	± 307.8
Faecal coliforms (MPN/100 ml) ²	9	1.1 × 10 ⁶	(0.2 - 46 × 10 ⁵)
Enteroviruses (PFU/100 ml) ³	6	109	(17-439)

¹Samples were taken for analysis at the point of the dripping lines entry. The chemical data were supplied by Mr. Rami Halperin, Chief Sanitation Engineer, Ministry of Health, Jerusalem.

²MPN, most probable number.

³PFU, plaque forming unit.

of washing experiments it was ascertained that multiplication of bacteria during the shaking period did not occur and that the final recovery could not be improved by modifications such as a change in ratio of washing solution volume to sample size, or extension of the duration of each washing up to 15 min or addition of glass beads to the solution in which the vegetables were shaken (13).

The absolute recovery efficiency of this shaking procedure was evaluated by enumerating the bacteria which remained on the vegetables after washing. This was done by analyzing homogenized peel samples of previously washed vegetables (Table 3). In four of five experiments, a recovery of at least 70% was obtained from two washings. It is noteworthy that when the wash recovery was nil, the number of remaining bacteria was likewise zero. This ensured false negative results to be unlikely. Accordingly, enumerations of microbial contamination of the vegetables were done with a two-washings procedure.

Contamination of vegetables grown with sewage effluent irrigation

In spite of the differences between the creeping-like growth tendency of the cucumber and the more erect growth of the eggplant, the observed contamination densities values of the two vegetables were not significantly different. Accordingly the data were pooled and averaged for analysis of the effects of the different irrigation treatments.

In Tables 4 and 5 the contamination densities on vegetables which were irrigated with effluent, by different drip irrigation treatments and fresh water are compared. Coliform bacteria were abundant on the vegetables from all the different drip treatments, and the differences in the contamination densities were of little significance (Table 4). This fact could be attributed to the general abundance of this group in the environment and to large variations among replica samples.

On the other hand, the general density of the fecal coliforms on the vegetables was nearly 10-fold lower and the effect of the different irrigation treatments on the contamination density was significant (Table 5). Irrigation with sewage effluent on the soil surface resulted in a higher contamination of 108 mean MPN/100 g. This contamination was reduced to 13 mean MPN/100 g on vegetables sub-irrigated in soil which was covered with plastic sheets. The contamination density on vegetables which were irrigated with sewage effluent during the

TABLE 3. Efficiency of bacterial recovery from the surface of contaminated cucumber by successive shaking in buffer solution.

Experiment No.	Fecal coliform count (MPN/100 g)		% Residual contamination ³ ($\frac{B}{A+B} \times 100$)
	Recovery obtained by two washings ¹	Residual contamination ²	
	(A) Irrigation with sewage effluent	(B)	
1	0	0	0
2	0	0	0
3	2.8×10^2	1.1×10^2	28.2
4	3.8×10^2	1.6×10^1	4.0
5	2.6×10^3	5.0×10^3	65.8

¹The experiments were performed with cucumbers from sewage irrigated plots.

²The residual contamination on the washed cucumbers was determined by peeling the cucumbers and homogenizing peel samples in a phosphate buffer solution which was then analyzed as described in Materials and Methods.

³Calculated from the total recovery obtained (A + B).

TABLE 4. Coliform contamination on vegetables variously irrigated with sewage effluent and fresh water by the drip method.¹

	Irrigation with sewage effluent			
	Throughout the growing season		Up to flowering	
	On surface, soil exposed	Subsurface soil covered	On surface soil exposed	Irrigation with fresh water
Number of Samples	20	18	11	20
Contamination level (log mean MPN/100g)	1592	2620	166	172
Std. dev	± 1245	± 1.7	± 32.5	± 8.6
Variance analysis	Significant at $\alpha = 0.1$			

¹Cucumbers and eggplants were collected from the experimental plots and the density of bacterial contamination which was released to the buffer solution in two consecutive washings was determined as described in Materials and Methods.

TABLE 5. Fecal coliform contamination on vegetables variously irrigated with sewage effluent and fresh water by the drip method.¹

	Irrigation with sewage effluent			
	Throughout the growing season		Up to flowering	
	On surface, soiled exposed	Subsurface, soil covered	On surface soil exposed	Irrigation with fresh water
Number of Samples	21	19	11	20
Contamination level (log mean MPN/100g)	108	13.3	2.2	2.8
Std. Dev	± 6.7	± 1.8	± 2.5	± 0.54
Variance analysis	significant at $\alpha = 0.01$			
Multiple range test, significance level of 0.01	A	AB	B	B

¹See legend to Table 4.

TABLE 2. Mean accumulative recovery rate of bacteria and viruses from contaminated vegetables obtained by shaking in buffer solution in a successive manner¹.

	%Recovery at each of five washings (count/100 g)									
	1		2		3		4		5	
	Bacteria	PFU	Bacteria	PFU	Bacteria	PFU	Bacteria	PFU	Bacteria	PFU
Mean	74	84	90	95	93	98	97	99	100	100
Std. dev.	± 19.7	± 14.8	± 7.6	± 4.4	± 6.8	± 2.1	± 3.4	± 1.1	—	—

¹The vegetables used in the experiments were cucumbers and eggplants which had been infected by immersing in sewage effluent or samples which had been collected from sewage effluent irrigated fields. The bacterial results represent mean MPN values of fecal coliforms determined in 18 experiments. The viral results represent mean PFU of enteroviruses determined in 15 experiments.

early stage of growth (up to flowering), only, and subsequently with fresh water was similar to the density on the vegetables which were irrigated with fresh water: 2 and 3 mean MPN/100 g, respectively.

The effect of covering the soil with plastic sheets on contamination density of the vegetables irrigated with sewage effluent is summarized in Table 6. The results indicate a nearly 13-fold reduction in the fecal coliform contamination of the vegetables which were protected by the plastic sheets cover.

The enterovirus contamination density on the sewage effluent irrigated vegetables was below the level of recovery in all of the samples (Table 7). Although enteroviruses were present in all effluent samples (see also Table 1), not one of the 27 vegetable samples was positive for enterovirus contamination. Eleven vegetable samples irrigated with fresh water were also negative for enteroviruses.

TABLE 6. *The effect of soil cover on fecal coliform contamination on vegetables irrigated with sewage effluent¹.*

	Condition of the soil	
	Exposed	Covered with plastic sheets
Number of samples	37	24
Contamination level (log mean MPN/100 g)	389	30
Std. dev	±45.7	±29.5
Variance analysis;	significant at $\alpha = 0.01$	

¹Cucumbers and eggplants from both, the exposed and covered soil were irrigated with the drip method on the soil surface. The density of bacterial contamination which was released to the buffer solution in two consecutive washings was determined as described in Materials and Methods.

TABLE 7. *Enterovirus contamination in the sewage effluent used for irrigation and on the vegetables¹.*

Material	Number of samples	Positive samples
Effluent	17	17
Vegetables	27	0

¹Cucumbers and eggplants from both the exposed and covered soil irrigated plots and analyzed as described in Materials and Methods.

DISCUSSION

The general conclusion that may be drawn from the results of this investigation is that agrotechnical manipulations of the irrigation method may reduce the microbial contamination of vegetables irrigated with sewage effluent. The contamination density of fecal coliforms on vegetables irrigated with sewage effluent on uncovered soil surfaces was 38-fold higher than on the vegetables from the control plots irrigated with fresh water (Table 5). The vegetables which were grown with sewage effluent irrigation in covered soil, were contaminated only 10-fold higher than vegetables from the control plots (Tables 5 and 6) and those which were grown with sub-surface effluent irrigation in covered soil were contaminated only 5-fold higher than vegetables from the control plots (Table 5). No differences were found between the contamination densities of vegetables from the control plots and of vegetables which were

irrigated with effluent only during the first stage of growth (up to flowering).

The significance of these data to public health needs further clarification. However, it does provide support for our assumption that drip irrigation with certain manipulations may allow use of contaminated wastewater for irrigation purposes with a resultant low level of crop contamination.

It is clear, that reduction of contamination in itself, is not sufficient to ensure the safety of crops irrigated with contaminated wastewater for general public use. Also, the incomplete recoveries of the pathogenic microorganisms from the vegetables (Table 3 and 7) may not permit a full assessment of their microbiological quality. This is particularly relevant for evaluating the risks from enteroviruses which may be infectious in small numbers (19). In addition, evaluation of the significance to public health of the survival of pathogenic microorganisms in the environment is necessary (5,10,14). This has been done with simulated field epidemics which were created by inoculating the wastewater used for irrigation with a high titer of traceable marker organisms. The results of this study are to be published.

Considering our observations and the previously shown agrotechnical advantages of drip irrigation (6,7), it is suggested that the drip method be considered as an alternative for spray and ridge and furrow irrigation for those crops which are generally approved for sewage irrigation at the present time.

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Effectiveness of Sampling Methods for *Salmonella* Detection on Processed Broilers

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ABSTRACT

A total of 240 processed broiler carcasses (water-chilled and unfrozen) were each sampled by three methods (whole-carcass rinse, neck-skin rinse, and macerated neck skin) for detection of *Salmonella*. In addition to this, various procedures were compared: destructive (incubating the entire carcass with the rinse fluid) versus non-destructive (incubating the rinse water with concentrated lactose or selenite cystine broth added after removal of the carcass) sampling and pre-enrichment versus no pre-enrichment during *Salmonella* detection procedures. There was no significant difference ($p < 0.05$) between the percentage of *Salmonella*-positive carcasses obtained by destructive sampling and the percentage obtained by non-destructive samples of whole carcasses. There was also no significant difference ($p < 0.05$) in results obtained by rinsing and blending excised neck-skin samples. There was highly significant difference ($p = 0.001$), however, between whole carcass and neck-skin analyses. With whole-carcass sampling, 45% of the carcasses were positive for the presence of *Salmonella* while with rinsing or blending the neck skin of these same carcasses, only 11% and 12%, respectively, were positive for the organism. Pre-enrichment of the whole carcass, of the whole-carcass rinse, or of the neck-skin samples did not result in significantly greater percentages of positive results than did direct enrichment of these samples.

Several sampling methods and various laboratory procedures have been used to detect salmonellae on raw, water-chilled, unfrozen broiler carcasses. Sampling procedures that significantly alter the physical integrity of the carcass or result in its contact with an adulterating substance are considered to be destructive, e.g., maceration of the whole carcass (18) and incubation of the whole carcass with rinse fluid in lactose broth (6). Non-destructive techniques include surface swabbing of one or more areas of the skin, visceral cavity, or anatomical part (4, 20); carcass rinsing that does not adulterate the carcass (3); and rinsing or homogenization of pieces of tissue (1, 19, 24) that when excised do not affect the appearance or weight of the carcass.

The laboratory procedures used by some have included a pre-enrichment step (10, 11, 13, 21), while it has been

omitted in others (23). The high percentage of salmonellae isolated by Van Schothorst et al. (24) and clostridia isolated by Mead and Impey (16) from samples of neck skin suggests that this tissue may be a reliable sampling site. The primary objectives of this study were (a) to compare the effectiveness of different methods of sampling small sections of excised neck-skin tissue and whole-carcass rinsing procedures and (b) to obtain additional information concerning the need for pre-enrichment of samples and for incubation of the carcass for the detection of salmonellae-contaminated ready-to-cook broiler carcasses. The carcasses used in this study were water-chilled and unfrozen.

MATERIALS AND METHODS

Processed broiler carcasses were procured from four different locations (two processing plants and two retail outlets). Lots of 20 birds each were acquired from each site and these birds were randomly divided into four equal groups of five birds each. From each carcass, two pieces of neck skin (5 cm² each, weighing 1-4 g) were excised; one piece was blended in 50 ml of diluent for 10 sec and the other was rinsed in 50 ml of diluent by shaking 75 times in a 1-ft arc using 25 glass beads in a wide mouth 4-oz. screw-cap jar (7). The three samples (two neck-skin samples and the whole carcass from which they were removed) were then handled in the following manner for each of the four groups:

Group 1 (Pre-enrichment - Destructive)

The whole carcass was placed in a polyethylene bag with 300 ml Lactose Broth (Difco)¹ and vigorously shaken for 1 min. Carcass plus the broth was then incubated in the bag for 24 h at 37 C (6). Neck-skin samples were blended or shaken in 50 ml of Lactose Broth. For blended samples the entire blender jars were incubated for 24 h at 37 C and for rinsed neck-skin samples, the wide-mouthed 4-oz. screw-cap bottles plus the neck skin, glass beads, and broth were all incubated for 24 h at 37 C. After incubation, from each sample, 10 ml was transferred to 90 ml of Selenite Cystine Broth (Difco) as the enrichment medium and incubated for 24 h at 37 C. Then two 3-mm loopsfull were streaked onto duplicate plates of BG Sulfa Agar (Difco), and the plates were incubated for 24 h at 37 C. Five typical colonies were picked from each plate and inoculated into Lysine Iron Agar (Difco) slants which were incubated for 24 h at 37 C. Isolates exhibiting typical reactions were checked with *Salmonella* Poly O antiserum (Difco). Three ml of Brain Heart Infusion (BHI) Broth (Difco) and six Minitek (BioQuest)

¹Mention of specific brand names does not imply endorsement by the authors of institutions at which they are employed to the exclusion of others not mentioned.

biochemical discs [glucose, lactose, sucrose, mannitol, maltose, and dulcitol (Cox and Williams, 8)] were inoculated with Poly O-positive cultures. After 24 h at 37 C the BHI cultures were tested with *Salmonella* Poly H antiserum (Difco). Then cultures still considered to be *Salmonella* following Poly O, Poly H and biochemical testing were sent to the Diagnostic Bacteriology Laboratory, National Animal Disease Center (Ames, Iowa) for final confirmation and determination of the serotype.

Group 2 (Pre-enrichment - Non-destructive)

Whole carcasses were vigorously shaken in 270 ml of sterile water. The carcass was then drained into the rinse fluid and removed. Concentrated (10 ×) Lactose Broth was added to the rinse water to yield a single-strength pre-enrichment broth (3). The procedure for sampling the neck skin pieces from this group of carcasses was the same as for Group 1.

Group 3 (No Pre-enrichment - Destructive)

Whole-carcass samples were shaken in 300 ml of Selenite Cystine Broth. We used Selenite Cystine Broth (Difco) as our enrichment medium in this study because previous work (9) suggested that it was superior to TT Broth (Difco), Selenite Brilliant Green Broth (Difco), and Selenite Brilliant Green Sulfa Broth (Difco) for recovering very low levels of artificially inoculated *Salmonella typhimurium* from fresh, processed broiler carcasses. Neck-skin samples from this group of carcasses were blended or rinsed in 50 ml of Selenite Cystine Broth. Otherwise the procedure was identical to that of Group 1.

Group 4 (No Pre-enrichment - Non-destructive)

Samples in this group were treated exactly as those in Group 2 except Selenite Cystine Broth was used in place of Lactose Broth in the same quantity and concentration.

Procedures common to all samples were as follows: (a) incubation for 24 h at 37 C in Selenite Cystine Broth, (b) incubation on BG Sulfa Agar for 24-48 h at 37 C, (c) incubation on Lysine Iron Agar slants for 24 h at 37 C, (d) serological testing (O and H), (e) biochemical testing, and (f) confirmation and serotyping at a National Serotyping Laboratory.

The data were subjected to a Chi-Square Analysis to determine whether or not the sampling procedures tested significantly affect the detection of *Salmonella* on processed poultry carcasses.

RESULTS AND DISCUSSION

There was no significant difference ($p < 0.05$) between the number of *Salmonella*-positive carcasses detected when pre-enrichment was used during culturing and the number found when pre-enrichment was not used (Table 1). A pre-enrichment step in analyzing poultry carcasses for *Salmonella* has been used or recommended (5, 10, 12-15, 21). Others have used or recommended direct enrichment of fresh poultry carcasses (2, 23). Thomason and Dodd (22) compared lactose pre-enrichment and direct enrichment in the detection of salmonellae in raw meat and poultry and concluded that pre-enrichment offered no advantage. Our findings support that conclusion. In our tests, pre-enrichment, which requires extra time, apparently was unnecessary.

TABLE 1. Comparison of destructive to non-destructive sampling and pre-enrichment to no pre-enrichment with whole-carcass rinsing for determining incidence of *Salmonella*-positive fresh broiler carcasses.

Sampling procedure	Pre-enrichment	No Pre-enrichment	Total
Destructive	29/60 (48%)	24/60 (40%)	53/120 (44%)
Non-destructive	26/60 (43%)	28/60 (47%)	54/120 (45%)
Total	55/120 (46%)	52/120 (43%)	

The number of *Salmonella*-positive carcasses did not differ between procedures in which the whole carcass was (destructive sampling) or was not (non-destructive sampling) incubated in the medium (Table 1). Much less space in the incubator is required for samples without than for those with the carcass. Furthermore, carcasses rinsed for 1 min with sterile water are not adulterated or unfit for human consumption.

Rinsing of the whole carcass was compared to rinsing or blending of neck-skin pieces (Table 2). There was no significant difference ($p < 0.05$) between rinsing or blending (macercating) of the neck-skin samples. In 240 carcasses, *Salmonella* was detected on 26 (11%) rinsed and on 29 (12%) blended neck-skin samples. Using macerated neck-skin samples of frozen broilers, Van Schothorst et al. (24) isolated *Salmonella* from 50% (23/46). A total of 46 different carcasses of the 240 examined (19%) were classified as *Salmonella*-positive with the neck-skin sampling procedure in our study (rinse plus blend samples). Some carcasses were positive with both methods. Our study differed from that of Van Schothorst et al. (24) in many respects, for example weight of neck-skin samples (1-4 g and 10 g, respectively) and number of carcasses sampled (240 and 46, respectively). These and other differences might partly explain the difference between the levels of detection in the studies. We found no significant difference in numbers of *Salmonella*-positive carcasses detected with and without pre-enrichment of neck-skin samples.

TABLE 2. Comparison of neck-skin to whole-carcass sampling for determining incidence of *Salmonella*-positive fresh broiler carcasses

Pre-enrichment	Sampling Method		
	Whole carcass rinse	Rinse	Blended
Yes	55/120 (46%)	11/120 (9%)	15/120 (13%)
No	52/120 (43%)	15/120 (13%)	14/120 (12%)

The number of *Salmonella*-positive carcasses detected was significantly higher ($p = 0.001$) for any of the whole-carcass rinsing procedures than for neck-skin sampling. Whole-carcass rinsing detected *Salmonella* on 107 of 240 (45%) of the carcasses examined, whereas rinsing and blending of the neck-skin samples of these same carcasses detected only 11 and 12%, respectively. These results apparently disagree with those of Van Schothorst et al. (24) who detected more *Salmonella*-positive samples examining the neck skin of frozen broilers than by sampling the rinse water. However, Van Schothorst et al. (24) separately analyzed drip and rinse water; we used samples that were composites of these (drip was not drained before rinsing solution was added). If the number of *Salmonella* isolations from both their rinse and drip water samples were combined, Van Schothorst et al. (24) found a total of 34/46 (74%) carcasses were *Salmonella*-positive. Therefore, the findings of these two studies are somewhat similar. In fact, Van Schothorst et al. (24) recommended the examination of the rinse-drip water to detect *Salmonella*

on chickens. Based on the findings of our study, we feel that a nondestructive whole carcass rinse using direct enrichment with Selenite Cystine can be used for detecting salmonellae on fresh chickens.

In 77 of the 240 carcasses tested in this study the whole-carcass rinsing method was positive for *Salmonella*, while both neck-skin sampling methods were negative. Neck-skin samples were positive and carcass rinse negative in the same carcass only 16 times. In only four of the 240 carcasses did both of the neck-skin samples and the whole-carcass rinse all produce positive results.

The same serotype was found in the whole-carcass rinse and the neck skin for 25 of the birds sampled, while different serotypes were found on five carcasses. For one carcass, *Salmonella kentucky* was isolated from the whole-carcass rinse, *Salmonella californica* from the blended neck skin, and *Salmonella bredeney* from the neck-skin rinse.

The sampling of neck skin from processed broiler carcasses may be a practical, efficient, non-destructive method for determination of an aerobic plate count, *Enterobacteriaceae* count or for enumeration of *Escherichia coli*. Our data, however, strongly indicate that for detection of *Salmonella* on broiler carcasses, whole-carcass rinsing is superior to neck-skin sampling. The highest number of *Salmonella*-positive birds will be obtained when the part sampled is the whole carcass. Since *Salmonella* are usually present in small numbers on broiler carcasses (17, 21), any less sensitive sampling method will obviously reveal a lower incidence. Also, all of the rinse (including any drip) should be incubated, because when numbers of cells are low, incubation of a small aliquot (especially one as small as 10 ml) after rinsing is not adequate and could result in a gross underestimation of the incidence of *Salmonella*-contaminated carcasses among processed broilers. Our studies also indicate that direct enrichment of unfrozen broiler carcass samples into Selenite Cystine Broth is as effective as pre-enrichment with Lactose Broth. This suggests that processing operations such as scalding (ca. 55 C for 1-2 min) and slush ice continuous chilling do not result in sublethal injury to salmonellae on broiler carcasses to the extent that pre-enrichment is necessary. The effectiveness of this direct enrichment procedure for surface frozen as well as deep frozen carcasses, that may harbor more severely injured salmonellae, is currently being evaluated in our laboratory.

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Aflatoxin Production in Black Currant, Blueberry and Strawberry Jams

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ABSTRACT

Unsweetened and sweetened (20 and 44% sucrose) black currant, blueberry and strawberry jams with spores of *Aspergillus parasiticus* NRRL 2999 were incubated at different temperatures and atmospheres for 0.5, 1, 2, and 6 months. Hyphal dry weight, pH of medium and aflatoxin production were examined. Also, the aflatoxin distribution between mold and jam layers was examined in jam with uncontrolled and controlled pH (initial pH 3.1-3.6 and 5.6 respectively) and in 20% yeast extract sucrose broth (initial pH 5.6) after 2 weeks of incubation. Aflatoxin was observed in black currant and strawberry jams stored at 22 and 30 C, but not in blueberry jam. Addition of sugar prevented production of aflatoxin in detectable amounts, although it enhanced fungal growth. Storage at 4 C resulted in a marked reduction in fungal growth. The high CO₂ atmosphere prevented production of aflatoxin in detectable amounts in black currant and blueberry jams but not in strawberry jam. Raising the initial pH of the stored jam caused an increase in aflatoxin synthesis, although the amount of fungal mycelium, in contrast was reduced. Aflatoxin synthesis as a function of fungal growth was significantly weaker in the jams than in the yeast extract sucrose broth. The results imply that the jam raw materials, particularly blueberry, contain substances inhibiting production of aflatoxins. Alternatively, it is also possible that the jam materials contain only small amounts of nutrients necessary for synthesis of aflatoxin.

Research on the aflatoxin contents of foods has been concentrated on foods other than berries, although berries and berry products are known to be highly susceptible to molding, particularly home-prepared products. It has been shown that some *Aspergillus flavus* or *Aspergillus parasiticus* strains are capable of producing aflatoxin in significant concentrations at 7 to 10 C (16). Although most consumers nowadays return moldy food products bought from shops, they may nevertheless use moldy home-prepared jam after first removing the mold layer. Removal of the moldy layer from foods does not remove all of the aflatoxin, as the toxin has been shown to diffuse from the mold layer deep into the food itself (5,8).

In the food industry it may be necessary, because of the short season of berry harvesting, to store products in many cases for long periods before processing. Molding of berries during storage, apart from being a health risk, also represents a major financial loss.

The aim of this research was to investigate the susceptibility of black currant, blueberry and strawberry jams to molding in different storage conditions, and to monitor aflatoxin production during storage using *A. parasiticus* NRRL 2999 as a model strain with known good aflatoxin-producing qualities even at low temperatures.

MATERIALS AND METHODS

Organism

A. parasiticus NRRL 2999 (formerly known as: *A. flavus* V 3734/10), was received from Dr. Harwig of the Health Protection Branch, Department of National Health and Welfare, Ottawa, Canada.

Reagents

The fungus was grown on Potato Dextrose (PD) agar (Difco) for production of spore suspensions. The aflatoxin production properties of the organism were examined by cultivating in yeast extract sucrose (YES) broth, containing 2.0% yeast extract (Difco) and 20% sucrose in 1000 ml of distilled water at pH 5.6. Spores were suspended from the PD agar into a 0.05% aqueous solution of Tween 80 (G. T. Gurr) for inoculation of the berry jams. Diatomaceous earth (Kieselgur, E. Merck) was used during sintered glass filtration. Thin layer plates (precoated silica gel 60 TLC plates, E. Merck) were used in the assay method for aflatoxins. Pure crystalline aflatoxins B₁, B₂, G₁ and G₂ were obtained from Calbiochem. Standards were dissolved in benzene-2% acetone to a final concentration of 0.5 to 1.0 µg/ml.

Preparation of spore suspension for inoculation

Fungal spores were washed from heavily sporulating PD plates (30 C, 3 to 4 days) with 100 ml of Tween 80 solution and 1.0 ml of this suspension (10⁶ CFU/ml) was used as inoculum for berry jams and YES broth.

Preparation of berry jam samples

Frozen black currants (*Rubus nigrum* L.) blueberries (*Vaccinium myrtillus* L.) and strawberries (*Fragaria × ananassa* Duch.) were used for production of jams by boiling the berries for 20 min. Before boiling 10% (vol/vol) water was added to the black currants and blueberries, and in the case of the sweetened jams 20 or 44% (wt/vol) sucrose was also added before boiling. In the experiments to investigate the effect of pH on aflatoxin production, 1.0 N NaOH was added to some of the jams after boiling so that the pH rose to 5.6. Samples of 100 ml of jam were transferred into sterile 150-ml screw-cap jars (internal diameter 54 mm) and covers were loosely closed.

Experimental procedure

Effect of different variables on mold growth and aflatoxin production. Some of the unsweetened black currant, blueberry and

strawberry jams were incubated in a normal atmosphere at 4 C or 22 C, while some were stored in a 10% CO₂ atmosphere at 22 C, in a Uni-Trol CO₂-Incubator (model 329, Forma Scientific). Sweetened jam samples were incubated in a normal atmosphere at 22 C. Duplicate samples were taken of each jam type, in each set of storage conditions, after 0.5, 1, 2, and 6 months of storage. Some jars of unsweetened jams of each type received, instead of the spore suspension a known amount of aflatoxin standard (B₁48, B₂42, G₁56 and G₂43 µg/100 ml). Some of these jars were stored in a normal atmosphere at 4 or 22 C and some in a 10% CO₂ atmosphere at 22 C. These groups of control samples (2 jars per berry type per storage paradigm) were examined after 6 months of storage for residual aflatoxin. The maintenance of the ability of the organism to produce aflatoxin was examined after 6 months of incubation by cultivating spores, taken from the 6-month samples, in YES broth (30 C, 2 weeks) and assaying both hyphal growth and aflatoxin concentration.

Effect of pH on fungal growth and aflatoxin production. One lot of jams not treated with NaOH (initial pH of blackberry and blueberry jams 3.2 and of strawberry jams 3.6) and one lot treated with NaOH to an initial pH value of 5.6 were incubated at 22 C, and corresponding lots were incubated at 30 C. Samples from each group (2 jars per berry type) were examined after 2 weeks of incubation. The organism was also grown in two duplicate samples of YES broth at pH 5.6 (30 C, 2 weeks) to provide a control measure of optimal aflatoxin production. After incubation, samples from the different experimental and control groups were stored at -30 C before determination of dry weight, pH and aflatoxin concentration of jam or broth and of hyphal fractions.

Aflatoxin assay

From mycelium. Media and mycelium were separated and mycelial dry weight (60 C, 24 h) was measured. Aflatoxin was extracted from dry milled mycelium by the method of Shih and Marth (14). The chloroform extract of the berries was evaporated to dryness under a gentle stream of N₂ on a steam bath. The dry extract was redissolved in benzene-2%-acetonitrile. Thin layer chromatographic plates were developed in an unlined and unequilibrated tank (40 ml of H₂O in a separate vial) with chloroform-acetone (9:1 vol/vol). The concentration of aflatoxin was measured by visual estimation under long wave UV-light (366 nm, Uvis, Desaga).

From culture media. Samples of 10 g of culture media were extracted with 50 ml of chloroform on a wrist action shaker (Griffin and George) for 30 min. The mixture was filtered under reduced pressure through a sintered glass filter (G2) covered with 10 g of diatomaceous earth. The extract was first dried over 10 g of anhydrous Na₂SO₄ for 30 min and then evaporated to dryness under a gentle stream of N₂ on a steam bath. A thin layer chromatographic plate with black currant, strawberry, or YES - broth sample aliquots was developed once in an unlined tank with ether and then in an unlined and unequilibrated tank with chloroform-acetone (9:1 vol/vol). A plate with blueberry sample aliquots was developed in an unlined tank first with ether, then with benzene and finally in an unlined and unequilibrated tank with chloroform-acetone (9:1 vol/vol). The concentration of aflatoxin was measured by visual estimation under longwave UV-light (366 nm).

RESULTS

Effect of different variables on mold growth and aflatoxin production.

As can be seen from the results expressed in Fig. 1, the test organism *A. parasiticus* grew best in black currant and least well in blueberry jam under several different paradigms of storage incubation. In the case of the strawberry jam samples the organism grew better with increasing sugar concentration, whereas in the case of black currant and blueberry jams growth was poorer in the 44% sweetened than in the unsweetened samples (Fig. 1). The 10% CO₂ atmosphere did not inhibit hyphal growth in any type of jam (Fig. 1).

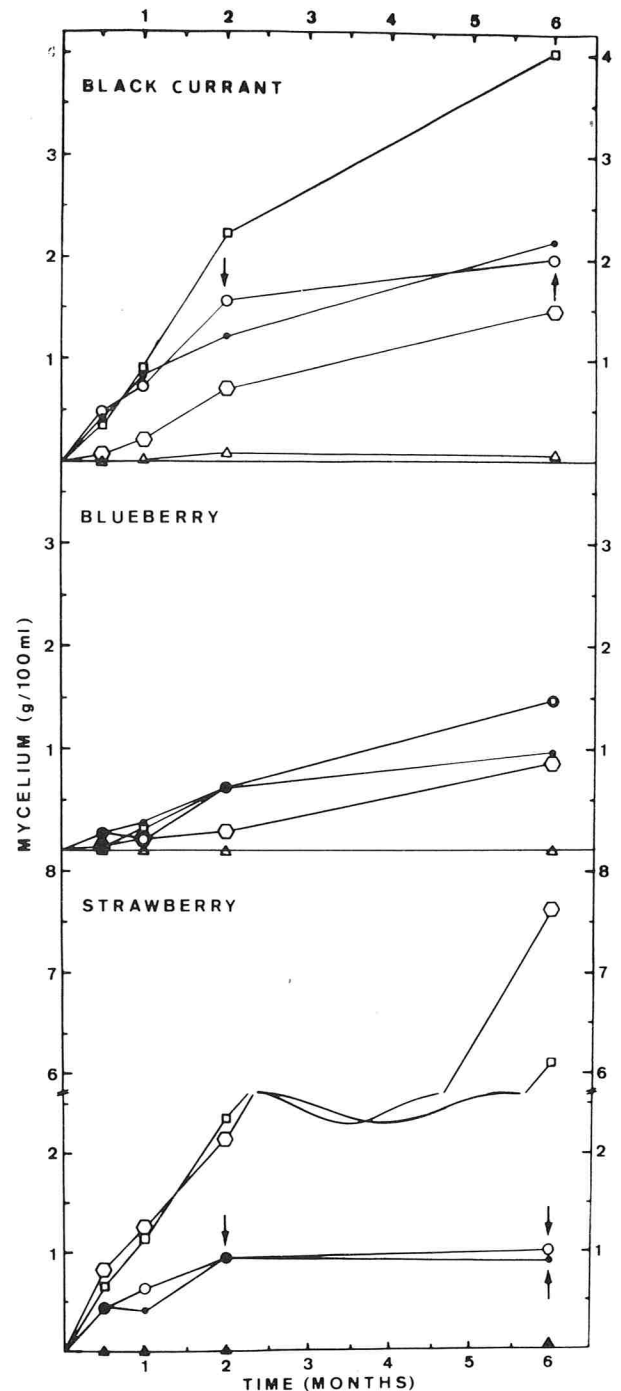


Figure 1. Mycelial growth and aflatoxin production (↓ = aflatoxin positive sample) by *A. parasiticus* in different berry jams at various storage conditions.

Symbols: Δ = 4 C; \circ = 22 C; \square = 20% sucrose at 22 C; \diamond = 44% sucrose at 22 C; \bullet = 10% CO₂ at 22 C.

Detectable amounts of aflatoxin were observed only in unsweetened black currant and strawberry jam samples stored for 2 and 6 months at 22 C. The aflatoxin concentration of strawberry jam samples increased with time of storage (11 µg/kg at 2 months and 50 µg/kg at 6 months), whereas the black currant jams contained less aflatoxin after 6 months (1 µg/kg) than after 2 months

(7 µg/kg). Of the jams stored in a 10% CO₂ atmosphere, traces of aflatoxin were detected only in some samples of strawberry jam.

Increase in the temperature of incubation caused a corresponding increase in both hyphal growth and toxin production in the jams with unaltered pH values (3.2 to 3.6), with the exception of the blueberry jam samples (Table 1). Raising of the initial pH to 5.6 caused an increase in aflatoxin production in the jams, despite the fact that hyphal growth was somewhat retarded by the raised pH. In the jams treated with NaOH the effect of increased temperature on toxin production by the test organism was more dependent on the type of berry than in the case of jams with unaltered initial pH (Table 1). On the other hand, the effect of raised temperature on growth was the same in the NaOH-treated and non-treated samples. In spite of poor production of aflatoxin in the berry jams, spores of *A. parasiticus* isolated from the jams after 6 months of storage grew well and produced aflatoxin as well in YES-broth (about 960 µg/g medium) as before inoculation of the fungus into the jam.

Distribution of aflatoxin content between medium and mycelium.

In jams incubated at 22 and 30 C the layer of fungal mycelium contained more aflatoxin than the jam itself. Similar results were recorded for samples grown in YES broth (Table 1). However, in both the NaOH-treated strawberry jam samples and also the 20% YES broth cultivations, the amount of aflatoxin in the mycelium decreased and the amount in the growth medium correspondingly increased, when the temperature of incubation was increased from 22 to 30 C.

Keeping qualities of aflatoxin in different berry jams.

Aflatoxin was found to be most stable in the strawberry jam samples at all temperatures (Table 2). Of the different aflatoxin standards added, B₂ and G₂ were found to keep better than B₁ and G₁. All four standard types of aflatoxin were found after 6 months of storage only in black currant and strawberry jams stored at 4 C and in blueberry jam samples stored in a 10% CO₂ atmosphere.

DISCUSSION

The results obtained indicate that, although hyphal growth in the berry jams was quite vigorous, the fungus was capable of producing only small amounts of aflatoxin in such media. Similar results have been obtained by other workers (6,17). In several other fruit varieties (1,8,17), as well as in many other agricultural products (4,10), fungi have been shown to produce high levels of aflatoxin.

Although the model *Aspergillus* strain chosen was known to be capable of producing significant concentrations of aflatoxin even at refrigerator temperatures (16), the organism grew only very poorly or not at all in the berry jams at 4 C. Aflatoxin was not produced at all at this temperature. The results of this research, as well as those of earlier work concerning the relationship between aflatoxin production and temperature (2,12), indicate that aflatoxin risk in the storage of berry jams can be significantly reduced by the use of a sufficiently low storage temperature.

Industrial-scale storage of berries, use of high CO₂ atmosphere to prevent growth and toxin production by aflatoxigenic fungi may have practical significance. In

TABLE 1. Aflatoxin content in medium and mycelium after growth of *Aspergillus parasiticus* in black currant, blueberry and strawberry without (A) or with (B) NaOH-treatment and in YES-broth at various temperatures for 14 days.

Medium	Initial pH	Dry weight of mycelium (g/100 ml)	22 C Aflatoxin		Final pH	Dry weight of mycelium (g/100 ml)	30 C Aflatoxin		Final pH
			Mycelium (µg/g)	Medium (µg/g)			Mycelium (µg/g)	Medium (µg/g)	
(A)									
Black currant	3.1	1.0	0.2	tr ^a	3.4	1.9	28	0.01	3.6
Blueberry	3.2	0.7	0	0	3.2	0.7	0	0	3.3
Strawberry	3.6	0.9	18	0.7	3.8	1.1	23	1.4	4.2
(B)									
Black currant	5.6	0.7	2	0.1	5.1	0.8	1	0.06	5.2
Blueberry	5.6	0.5	0.2	0	5.3	0.4	0.3	0	5.3
Strawberry	5.6	0.5	54	3.2	5.2	0.7	44	4.5	5.5
(Control)									
20% YES-broth	5.6	2.6	1800	67	4.5	3.2	1100	100	4.7

^atr = traces.

TABLE 2. Stability of aflatoxin in berry jams stored for 6 months.

Aflatoxin standard	Recovery of added aflatoxin standards (%)								
	4 C			22 C			22 C in 10% CO ₂		
	Black currant	Blue-berry	Straw-berry	Black currant	Blue-berry	Straw-berry	Black currant	Blue-berry	Straw-berry
B ₁	6	0	21	0	0	0	0	2	2
B ₂	35	50	110	38	31	36	17	38	48
G ₁	7	0	20	0	0	0	0	11	0
G ₂	49	44	44	37	26	35	16	28	42
B ₁ + B ₂ + G ₁ + G ₂	23	21	46	17	13	16	7	18	20

this experiment the CO₂ concentration used was too low. It was insufficient to prevent fungal growth, despite the fact that aflatoxin production was significantly reduced. In experiments carried out on the storage of peanuts, a combination of 60% CO₂ concentration and 86% relative humidity was enough to prevent both molding of the nuts and aflatoxin production (9). In combination with reduced storage temperatures, lower CO₂ concentrations have also been sufficient to prevent aflatoxin production and fungal growth.

Although the 20 and 44% sugar additions did not prevent growth of *A. parasiticus* in the berry jams, they did prevent production of detectable amounts of aflatoxin. Using artificial nutrient media, Shih and Marth (13) reported that a 30% addition of glucose resulted in maximal levels of lipid and aflatoxin, whereas in terms of fungal dry weight an addition of 10% glucose resulted in the best growth levels. Aflatoxin production has been reported in YES broth at 25 C even in the presence of 50% sucrose (3). According to the observations of Orth (11), the a_w values of the jams (0.91 to 0.98) seem not to be limiting factors for aflatoxin biosynthesis. Thus, on the basis of the results reported above it would appear that the berry jam materials contain some other factors inhibiting synthesis of aflatoxin. This conclusion is also supported by the observations on the effect of pH. Raising of the pH improved aflatoxin synthesis, but the synthesis was still only 1/25 of the amount produced when the strain was grown in YES broth, although hyphal growth was as much as 1/5 of that observed in YES broth. One possible limiting factor for aflatoxin production may be the low level of zinc present in the berry material (mostly 1-2 mg/kg). Zinc has been shown to play an important part in aflatoxin synthesis (7).

It is probable that *A. parasiticus* actually produced more aflatoxin than that observed in the samples as the organism has been shown to destroy some of its own aflatoxin (15). Also, it was found in this work that the aflatoxin concentration in the control samples decreased over the 6 months storage period by over 50% (Table 2). As these samples were free of fungal contamination, the decrease in aflatoxin was presumably a result of chemical activity taking place within the jam during storage.

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Failure of *Clostridium botulinum* to Grow in Fresh Mushrooms Packaged in Plastic Film Overwraps with Holes

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ABSTRACT

Fresh, commercially grown mushrooms were inoculated in the stem with *Clostridium botulinum* spores (2.5×10^4 of each of four type A with 1×10^5 of each of four type B strains). One lb (454 g) of mushrooms, including two spore-inoculated ones, was packaged in paperboard trays and overwrapped with a polyvinyl chloride film. The packages were incubated 6 days at 24-26 C after making one or two holes of 1/8 inch (3.175 mm) diameter in the overwrap of test packages. Botulinum toxin, either type A only or mixed with type B, was found in the spore-inoculated mushrooms of all 28 control packages (no hole), but was not detected in any from the 123 packages with one hole and the 47 with two holes. The $O_2\%$ inside the packages after incubation averaged 1.5 for the controls, 4.0 for packages with one hole and 6.2 for the 2-hole package group.

A potential botulism problem exists when a food contains viable *Clostridium botulinum*, has the nutrients, is in or develops the anaerobic state required for growth of the organism, and is held at a temperature at which the organism can multiply.

These conditions could exist concurrently in fresh mushrooms packaged by overwrapping with a semi-permeable plastic film. Fresh mushrooms are natural carriers of *C. botulinum* (2). Finding of botulinum toxin in canned mushrooms produced by several companies (3) indicates that mushrooms satisfy the nutritional requirements of the organism. Packaged fresh mushrooms are not normally refrigerated in stores and might be left at room temperature after being purchased. When held in this manner, the atmosphere inside the packages equilibrates within a few hours to 1 to 2% O_2 from the mushrooms using the gas for respiration and from the overwrap film restricting its entrance from the outside (6,7).

In inoculated pack studies, botulinum toxin could be demonstrated within 3 to 4 days when fresh mushrooms were inoculated with as few as 1,000 type A spores, repackaged and held at 20 C. At the time toxin was first detectable, the mushrooms appeared edible (7). The report included a preliminary suggestion that toxin formation might be prevented when one hole of 1/8 inch

(3.175 mm) diameter is made in the overwrap. This report amplifies that suggestion.

MATERIALS AND METHODS

Mushrooms

Agaricus bisporus was obtained directly from growers in different geographic areas of the United States. Supplier I was located in the state of Wisconsin, II in Illinois, III and IV in different parts of Pennsylvania, V. in Oregon, and VI in California.

Shipments were bulk lots in paperboard cartons. Mushrooms of suppliers I and II were received on day of harvest; all others were received the day after being cut. Those of I were brought directly to the laboratory and of III were sent by refrigerated truck. All others were airshipped without refrigeration.

At least two different shipments on different days were obtained from each grower. Test were started (inoculated with spores) on day of receipt except that one shipment from V was refrigerated overnight.

Spores

Stock spore suspensions of *C. botulinum* were prepared and enumerated as described previously (7). Spores of type A strains (56A, 62A, 69A, and 90A) and type B strains (113B, 169B, 213B, and 13983B) were combined so that 20 μ l of the mixture would contain 5×10^5 spores represented by 2.5×10^4 of each type A and 1×10^5 of each type B strain.

The spores were injected into the stem of mushrooms by distributing the 20 μ l inoculum in 10 well-separated sites. Injections were made with a 50 μ l capacity Hamilton syringe-pipette. The working spore suspension was heated 15 min at 80 C immediately before use to destroy residual toxin.

Packaging and incubation

Packaging of mushrooms followed commercial practice. Two spore-inoculated mushrooms were surrounded with uninoculated ones in paper pulp trays designed to hold 1 lb (454 g) of mushrooms. The filled box was then completely overwrapped by heat-sealing the packaging film on the bottom of the tray with a single role wrapping machine (loaned by Dr. D. G. James, Borden Chemical, North Andover, Mass.). The wrapping film was a plasticized polyvinyl chloride film with O_2 permeability of 1320 $cm^3/100$ inch² (645 cm^3)/atm/24 h and is used commercially.

A soldering iron with a tip of 1/8 inch (3.175 mm) diameter was used to make holes in the wrapping film. Holes not appreciably larger than the tip could be made consistently after trials determined the heating and cooling times required to get the tip to proper temperature. The perforation was placed near the geometric center of one of the long sides of the packages; for two-hole packages, one hole was made on each of the sides.

The packages were held at 24-26 C until spoilage made mushrooms unacceptable for human consumption. This deterioration stage was reached by the sixth day.

Oxygen analysis

Immediately before opening a package to collect the inoculated mushrooms, 12 ml of gas was sampled from inside with a hypodermic syringe (disposable, airtight) fitted with a 1½ in (3.81 cm) long needle. The tray was entered by pushing the needle through the film and side of the tray, the needle point inserted as far as possible, and the sample taken from among mushrooms. The samples were saved by immediately transferring 10.5 ml into a 10-ml serum bottle closed with an air-tight rubber diaphragm. These bottles were prepared by three cycles of evacuating with a vacuum pump and flushing with O₂-free N₂ before final evacuation.

Oxygen content was determined by a modification of published procedures (5,8). The gas chromatograph was a Packard model 419 fitted with a thermal conductivity detector (Packard Instrument Co., Downers Grove, Ill.) The stainless steel coiled columns of 9 ft × 1/8 inch outside diameter (274 cm × 3.175 mm) were packaged with Carbosieve B of 120/140 mesh (Supelco, Inc., Bellefonte, Pa.). Before the first trial of the day, columns were heated to 365 C at increments of 30 C/min and then cooled.

Carrier gas was He (laboratory grade, Chemetron Corp., Chicago, Ill.) whose trace O₂ was removed by passing through an Oxy-Trap (Regis Chemical Co., Morton Grove, Ill.) placed in the gas line ahead of a Packard gas filter (Cat. #7600088). Gas flow was 50 ml/min, inlet temperature 40 C, detector temperature 200 C, thermal conductivity 200 mA, attenuation 16, and sample volume 0.4 ml. The sequence of the oven programming was 25 C for 3 min following sample injection, from 25 to 165 C at the rate of 30 C/min, final 1 min hold, and cooling time of 8 min. Recordings were made with a Linear Instruments model 282 (Cole Parmer, Chicago, Ill.) running at 0.5 in (12.7 mm)/min; the area under the O₂ peak was simultaneously recorded with the integrator.

Oxygen in samples was calculated from a plot made of areas of O₂ peaks (abscissa) vs O₂ percentages obtained with standards of 0.5 to 20% O₂ in CO₂. This straight line plot had a slope of 0.0764 and passed through the origin. Percent oxygen in a sample is the area of its O₂ peak multiplied by the slope.

Toxicity tests

Stems of the two inoculated mushrooms of a package were extracted and 0.5 ml of extract was injected intraperitoneally into each of two mice (7). Extracts that killed were retested in three other mouse pairs that were protected with 1 international unit of type A antitoxin, type B antitoxin, or their mixture, as well as in a pair not given antitoxin. Antitoxins (Center for Disease Control, Atlanta, Ga.) were injected in 0.4 ml before the extract.

The mouse lethal agent was considered to be botulinum toxin if the

unprotected pair died and that protected with the antitoxin mixture survived. The toxin type is indicated by the responses of the pairs protected separately with A or B antitoxin. The sample has a mixture of types A and B toxin if both pairs die. If only one of the two pairs die, the toxin is of the type homologous to the protecting antitoxin.

RESULTS

Difficulty was encountered with extracts of mushrooms from one grower. These extracts had nonbotulinum, mouse killing agent(s); even 1/20 dilutions killed mice protected with a mixture of antitoxins to all known toxin types. Such extracts were frozen (-20 C) overnight in tubes, thawed without shaking, and the top fluid portion used for retests. This procedure has been used to detect relatively low levels of *C. botulinum* type E toxin in specimens containing interfering amounts of nonspecific lethal factors (1).

Of the packages made of each mushroom shipment, three were used as controls without a hole in the wrapping film. One was held at 4 C; the other two were incubated. The refrigerated controls were consistently negative for botulinum toxin and showed that the spore suspension did not contain toxin.

Table 1 gives the consolidated results. Presence of botulinum toxin in all 28 of the incubated controls (no hole) shows that the different result of the test samples is due to the packaging. Among these controls, 17 had type A toxin only, 11 had type B in addition, and none had B only.

About equal numbers of one- and two-hole packages were tested with the initial shipments of mushrooms, but study of the two-hole ones was gradually eliminated when the accumulating data showed that toxin was not produced even in packages with one hole. A total of 123 packages with one hole and 47 with two holes were examined. Botulinum toxin was not formed in mushrooms of any of these packages.

The O₂ concentration inside the packages after incubation averaged 1.5% (range of 0.9 to 2.5) in packages with intact overwrap, 4.0% (1.4-6.6) in those with one hole and 6.2% (4.9-7.3 range) in two-hole

TABLE 1. *Botulinum* toxin formation in mushrooms packaged with holes in overwrapping film. Mushrooms inoculated with mixture of spores of *C. botulinum* types A and B and held 6 days at 24-26 C. O₂ concentrations are those inside packages at end of incubation period.

Source	Mushrooms	# Shipments	Holes	Packages	# Tested	O ₂ % ¹	Results	Toxin ²
I		3	0		6	1.4 (0.9 - 2.0)	2 = A; 4 = AB	
			1		24	4.0 (1.9 - 6.3)	nil	
			2		15	6.0 (4.9 - 7.0)	"	
II		3	0		6	1.5 (1.0 - 2.0)	4 = A; 2 = AB	
			1		23	3.9 (2.3 - 6.6)	nil	
			2		22	6.3 (5.0 - 7.3)	"	
III		2	0		4	1.4 (1.1 - 1.7)	2 = A; 2 = AB	
			1		20	3.3 (2.3 - 4.6)	nil	
			2		8	6.2 (4.9 - 6.9)	"	
IV		2	0		4	1.6 (1.4 - 1.8)	2 = A; 2 = AB	
			1		20	3.3 (2.2 - 4.0)	nil	
			2		2	5.7 (5.6 - 5.8)	"	
V		2	0		4	1.6 (1.3 - 2.5)	3 = A; 1 = AB	
			1		20	5.0 (3.8 - 6.5)	nil	
VI		2	0		4	1.8 (1.4 - 2.0)	4 = A	
			1		16	4.3 (3.2 - 5.7)	nil	

¹Average with extremes in ().

²Number of packages with indicated toxin types.

packages.

DISCUSSION

The type A spore inoculum in the tests was 100 times more than is required for generally reproducible toxin production when mushrooms are packaged with intact overwrap (7). Nevertheless, toxin formation did not occur when one hole was present in the wrapping film.

Type A grew in spore-inoculated mushrooms of all incubated controls but type B grew in only 40% of these packages. This difference occurred despite the inoculum including 1×10^5 spores of the 169B strain which is able to grow when used alone at the 1×10^4 /mushroom level. The results agree with the previous finding (7) that type B strains do not grow as well as type A in fresh mushrooms. Of interest is that when *C. botulinum* and/or toxin were found in canned mushrooms (3), type B was always present and the less frequently found A was always associated with type B. The total 5×10^5 spores/mushroom of the present study compares with the 1×10^3 type B spores that would be in a 1-lb package if the natural *C. botulinum* contamination is 214/100 g of rinsed mushrooms (2).

The number of holes in the wrapping film correlates with the average O_2 % of the package groups. However, the values of individual one-hole packages overlapped those of the controls and two-hole packages. Because the age of mushrooms at harvest and transit conditions were not identical, the freshness of mushrooms in the different shipment lots varied somewhat. These variations could have influenced O_2 utilization during the tests; the overlapping was much less frequent among package groups made of the same mushroom shipment. In some samplings, air could have leaked in or the needle point could have been partially inside a mushroom. These considerations suggest that the more meaningful data are the averages of the different packaging methods.

A *C. botulinum* strain failed to grow in a broth medium that was equilibrated with pure O_2 of 1.14 cm pressure (4) or equivalent to air containing 1.5% O_2 . In

the present study, type A toxin was produced even when the atmosphere within the packages was higher than this O_2 concentration. The observations are not necessarily contradictory. Suitability for anaerobic growth is not determined by O_2 only; when O_2 % of itself is slightly higher than that permitting growth, sulfhydryls and other compounds of mushrooms probably participate in lowering the oxidation-reduction potential to a level at which *C. botulinum* can grow.

An important benefit of packaging mushrooms in film overwraps is the increased shelf life that results from the procedure. Our observations and those of commercial growers are that the shelf life is not significantly shortened when the packaging is done with one or two holes in the overwrapping film.

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Heat Resistance of *Clostridium botulinum* Type B Spores Grown from Isolates from Commercially Canned Mushrooms¹

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ABSTRACT

The heat resistance of ten *Clostridium botulinum* type B spore crops was determined in mushroom puree and 0.067M Sorenson phosphate buffer (pH 7). The spore crops were grown from *Clostridium botulinum* isolates obtained from commercially canned mushrooms. The D-values for all of the *C. botulinum* spore crops were overall slightly higher in the buffer than in mushroom puree. The mean D(110.0 C)-value for the ten spore crops in buffer was 1.17 min and for the spores in mushroom puree the mean D(110.0 C)-value was 0.78 min. The mean D(115.6 C)-value in buffer for the ten spore crops was 0.24 min compared to a mean D(115.6 C)-value of 0.19 min for spores in mushroom puree. The *C. botulinum* type B spores tested in this study had a heat resistance that was less than the classical heat resistance for *C. botulinum* spores.

During 1973 and 1974, the Food and Drug Administration (FDA) conducted a survey of all domestic and imported canned mushrooms held in warehouses in the United States. As a result of this survey canned mushrooms from nine commercial canners were found to contain *Clostridium botulinum* (I). The cans were from seven U.S. producers and two foreign producers.

The objective of this study was to determine the wet-heat resistance of the *Clostridium botulinum* type B spore crops produced from the isolates obtained from commercially canned mushrooms. Heat resistance tests were done at 110 C (230 F) and 115.6 C (240 F) with the spores suspended in two different substrates: mushroom puree and 0.067 M Sorenson phosphate buffer (pH 7.0).

The heat resistance of these *Clostridium botulinum* spore crops can be used as an aid in ascertaining if the recent detection of botulinum toxin in commercially canned mushrooms (I) was due to the failure to deliver a minimum botulinum cook or to post-processing contamination.

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MATERIALS AND METHODS

Spores

Ten cultures of *Clostridium botulinum* type B isolated from commercially canned mushrooms were used to produce 10 spore crops.

The cultures were grown in Stumbo's beef heart casein medium (6) in 100-ml bottles, incubated at 30 C for 15 to 20 days followed by refrigeration for 7 to 20 days. The spore crops were harvested by filtration through sterile cheese cloth layered with glass wool and centrifuged. All spore crops were washed five times with sterile distilled water and were not treated with lysozyme. The spores were suspended in sterile distilled water, stored at 4 C, and counted by the 3-tube MPN procedure using trypticase-peptone-glucose-yeast extract broth. The spore codes and the codes for the producers of the cans of mushrooms that were the source of the isolates are shown in Table 1.

TABLE 1. *Clostridium botulinum* type B isolates.

Univ. of Minn. spore code	FDA strain designation	Company
SKAF	002-642G-1A	A
SKBF	083-383G-2	B
SKCF	083-384G-2	B
SKDF	083-424G-7	B
SKEF	039-612G-10	C
SKFF	011-273G-E	D
SKGF	083-384G-4	B
SKHF	038-390G-6	B
SKIF	083-390G-12	B
SKJF	038-424G-4	B

Substrate preparation

Sorenson 0.067M phosphate buffer, pH 7 (2). Two-ml quantities of the buffer in 18 × 150 mm screw-capped glass tubes were autoclaved and stored at 4 C and used within 48 h of preparation. On the day of a test, tubes containing buffer were removed from the refrigerator and allowed to reach room temperature. Five min before heating .05 ml of the appropriate spore crop was added to the buffer in each of the glass test tubes using an Eppendorf pipette. Inoculation of the spores was done in a laminar flow hood. The buffer and spore inoculum were mixed and tubes were loaded into the assigned retort and were heated at the predetermined temperature for the designated time.

Mushroom puree. A single case of 4-oz. cans of commercially produced mushrooms stems and pieces, stored at 4 C was used throughout this study. Opening of the cans and other aseptic manipulating were carried out in a laminar flow hood. Before a heating test the content of one can was removed aseptically and blended (Waring Aseptic Dispersall, Model AS-1) with 1.0 ml of spore crop for

1 min at low speed. The puree was kept in the blender container at less than 20 C for the duration of the test period, which never exceeded 1 h. The pH of the puree ranged from 6.3 to 6.5.

To facilitate removal of specified amounts of inoculated puree from the blender container and the subsequent transfer to glass test tubes, a 10-ml syringe was fitted with a 130 mm × 4-mm (ID) stainless steel tube. The free end of the stainless steel tube was placed into a needle valve with an "O"-ring packing located at the base of the blender container and the syringe was filled with the puree. The puree was then delivered in 2.0 ± 0.1 -ml amounts to the bottom of each of five test tubes.

The inoculated mushroom puree was added to glass tubes 5 min before heating. The tubes were then loaded into the assigned retort and heated at the predetermined temperature for the designated time.

Heat resistance testing

Heat resistance testing was done using the multiple replicate fraction-negative method (3) with screw-cap test tubes as the test unit. To do each fraction-negative test, six 18 × 150-mm screw-cap test tubes containing approximately 10^7 spores in the appropriate substrate were heated for five or six heating times. The tubes were heated in miniature retorts at 110 C (230 F) and 115.6 C (240 F). At the end of the heating period tubes were quickly transferred to 80-C water bath and held in this water bath for 2 min.

Twenty ml of pork infusion agar (6) at 80 C was added to each tube followed by 5 ml of a 1:1 paraffin-mineral oil mixture. The tubes were incubated at 30 C for 60 days. Visually observed gas formation and/or colony formation were used as indication of survival.

The Spearman-Kärber (SK) method was used for D-value calculation (5). At the shortest heating time all the units must be positive and at the longest heating time all the units must be negative. At the intermediate times various fractions of the replicate tubes would be negative. To obtain data usable by the SK method a preliminary test with widely spaced heating times was first used. Subsequent tests were done until data usable by the SK method were obtained.

Determining the initial numbers of spores

The initial number of spores (N_0) for the fraction-negative tests was determined by serial dilutions and pour plate method using pork infusion agar (PIA). For tests with the buffer substrate, serial dilutions of 1-ml aliquots from three unheated tubes were made with Butterfield's phosphate buffer, pH 7.2. The N_0 in the mushroom puree was determined by serial dilutions of three 1-ml aliquots removed from the puree in the blender container.

Two aliquots (0.1 and 1.0 ml) from each dilution bottle were plated in duplicate and 20 ml of PIA was added to the plates. The plates were

inverted, placed in anaerobic jars under a hydrogen-carbon dioxide atmosphere (GasPak) and incubated at 30 C for 48 h.

RESULTS AND DISCUSSION

The D-value results for each of the 10 spore crops heated at 110 and 115.6 C in 0.067 M Sorensen phosphate buffer (pH 7.0) and in mushroom puree are presented in Table 2. The response of the 10 spore crops to the four test conditions was variable.

The D-value data in Table 2 were statistically analyzed; there was no significant correlation between the D-values for spores heated in buffer and in mushroom puree at either 110 or 115.6 C, or between the D-values for the spores heated in mushroom puree at 110 and 115.6 C. The most significant correlation was between the values for the spores heated in buffer at 110 and 115.6 C. The results of the statistical analysis were basically the same when the D-values were analyzed by either of several methods: (a) the product moment correlation on the D-value, or on the logarithm of the D-value, (b) Spearman's rank correlation, or (c) Kendall's Tau.

Considering the correlation between the D-values for the spores in buffer at 110 and 115.6 C, heating *C. botulinum* spores in mushroom puree produced results that were quite variable. The variation may have been due to an interaction between the spores and the mushroom puree during heating or during incubation.

When heated in buffer, the several spore crops showed a range of heat resistance levels at 110 and 115.6 C, which suggests that there were several *C. botulinum* type B variants in the group.

For the purpose of evaluating mushroom sterilization processes, the results of tests of all 10 of the spore crops were examined. The D(110 C)-values for the spores heated in mushroom puree ranged from 0.49 to 0.99 min and in buffer from 1.02 to 1.38 min. The D(115.6 C)-value for the spores heated in mushroom puree ranged

TABLE 2. D-value results for the ten *Clostridium botulinum* type B spore crops heated at 110.0 and 115.6 C in mushroom puree and 0.067M phosphate buffer.

Spore suspension code	110.0 C Tests				115.6 C Tests			
	Mushroom puree		0.067M phosphate buffer, pH 7		Mushroom puree		0.067M phosphate buffer (pH 7)	
	D-Value (min)	95% C.I. ^a (min)	D-Value (min)	95% C.I. (min)	D-Value (min)	95% C.I. ^a (min)	D-Value (min)	95% C.I. ^a (min)
SKAF	.99	0.84 - 1.14	1.13	1.01 - 1.25	.14	0.09 - 0.20	.22	0.19 - 0.26
SKBF	.87	0.69 - 1.04	1.08	0.89 - 1.26	.12	0.09 - 0.16	.22	0.19 - 0.26
			1.10	0.98 - 1.21	.18	0.15 - 0.22	.19	0.14 - 0.23
SKCF	.68	0.50 - 0.86	1.02	0.86 - 1.18	.19	0.15 - 0.22	.22	0.18 - 0.25
			1.06	0.91 - 1.21	.14	0.08 - 0.20	.24 ^b	—
SKDF	.68	0.50 - 0.86	1.26	1.13 - 1.40	.15	0.11 - 0.20	.22	0.16 - 0.28
			1.23	1.10 - 1.37	.18	0.15 - 0.22	.11	0.05 - 0.17
SKEF	.49	0.30 - 0.69	1.10	0.95 - 1.25	.30	0.24 - 0.36	.35	0.30 - 0.39
SKFF	.78	0.64 - 0.91			.14	0.10 - 0.18	.18	0.13 - 0.22
SKGF	.89	0.77 - 1.01	1.22	1.04 - 1.39	.39	0.33 - 0.44	.27	0.21 - 0.34
SKHF	.96	0.87 - 1.05			.12	0.09 - 0.15	.32	0.25 - 0.39
SKIF	.79	0.70 - 0.88	1.28	1.14 - 1.42	.12	0.09 - 0.15	.29	0.21 - 0.37
SKJF	.58	0.44 - 0.70	1.38	1.27 - 1.50	.19	0.16 - 0.23	.29	
Mean	.78		1.17		.19		.24	
Range	.49 - .99		1.02 - 1.38		.12 - .39		.11 - .35	

^a95% confidence for the D-value.

^bData not sufficient to calculate variance of the D-value.

from 0.12 to 0.39 min and in buffer from 0.11 to 0.35 min.

There were marked differences between D-values for spores heated in buffer compared to spores heated in mushroom puree. At 110 C the mean D-value for spores heated in mushroom puree was 0.78 min and in buffer 1.17 min. At 115.6 C the mean D-value for the spores heated in mushroom puree was 0.19 min and in buffer 0.24 min.

The mean width of the D-value confidence interval (CI) expressed as a percent of the D-value was 39% for mushroom puree at 110 C, 25% for buffer at 110 C, 49% for mushroom puree at 115.6 C, and 48% for buffer at 115.6 C. The D-values for the spores heated in buffer at 110 C showed the smallest mean width of the CI. For the spores heated in buffer the difference between the mean D-value CI at 110 and 115.6 C was quite large, 25 versus 48%. The effect of normal variation of time and temperature, that is the same regardless of test time and temperature, probably produced a correspondingly greater percent error in the results at 115.6 C because of shorter heating times. The wider mean CI for the spores heated in mushroom puree at 110 C compared to buffer at 110 C suggests that the mushroom puree contributes to variability.

By extrapolation equivalent D(121.1 C)-values were calculated from D(110.0 C)-values and D(115.6 C)-values for the 10 spore crops tested using a z of 10 C. These values are presented in Table 3. The mean D(121.1 C)-values for spores heated in the mushroom puree were .05 min and .06 min calculated from D(110.0 C) and

D(115.6 C)-values, respectively. The mean D(121.1 C)-values for spores heated in the 0.067 M phosphate buffer were .07 min and .09 min calculated from D(110.0 C) and D(115.6 C) values, respectively.

The spore crops tested in this study were not very heat resistant compared to the classical D(121.1 C)-value (calculated from the data of Esty and Meyer by Schmidt) of 0.2 min (4). The spores in this study heated in a mushroom puree or in buffer had D(121.1 C)-values that were 15 to 55% of the classical value. The mean D(121.1 C)-value for spores heated in mushroom puree was about 25% of the classical resistance value and for the buffer was about 40% of the classical value. The largest equivalent D(121.1 C)-value was 0.11 min for strain SKHF heated in mushroom puree and for strain SKJF heated in 0.067 M phosphate buffer.

The isolates for these spore crops were obtained from canned mushrooms that had been processed in canning plants with deficiencies in equipment (inaccurate or broken thermometers, faulty piping, etc.) and in operating procedures (poor fill control, inadequate venting, etc.) (1). If we assume that the initial number of *C. botulinum* spores per can was 100 and that cans containing toxin were found at the rate of one can per 100,000 can-lot; the F_0 -value that would produce these conditions if the spores had D(121.1 C)-values of 0.03 to 0.11 min would be 0.21 to 0.77 min. An acceptable F_0 for public health safety for low-acid canned is 3.0 min. Since all of the *C. botulinum* spore crops tested had significant heat resistance the possibility is very high that the spores survived the heat processes and that they did not leak into the container after processing.

TABLE 3. Extrapolated D(121.1 C)-Values for the ten Clostridium botulinum type B spore crops calculated from D(110.0 C)-values and D(115.6 C)-values using a z-value of 10 C.

Spore code	Calculated from D(110.0 C)-value		Calculated from D(115.6 C)-value	
	Mushroom puree	0.067M phosphate buffer, pH 7	Mushroom puree	0.067M phosphate buffer, pH 7
	D(121.1 C) min	D(121.1 C) min	D(121.1 C) min	D(121.1 C) min
SKAF	.08	.09	.04	.06
SKBF	.07	.08	.03	.06
SKCF		.09	.05	.06
SKDF	.05	.08	.04	
		.10	.05	.07
		.10	.03	.06
SKEF	.04	.09		.03
SKFF	.06		.08	.10
SKGF	.07	.09	.04	.05
SKHF	.07		.11	.08
SKIF	.06	.10	.03	.09
SKJF	.04	.11	.05	.08
Mean	.06	.09	.05	.07
Range	.04 - .08	.08 - .11	.03 - .11	.03 - .10

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Fate of *Clostridium botulinum* in Perishable Canned Cured Meat at Abuse Temperature

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ABSTRACT

Three experiments are described wherein perishable canned cured pork was prepared with 50 or 156 $\mu\text{g/g}$ added sodium nitrite and 10^2 or 10^4 botulinal spores/g. Spore germination (i.e. loss of heat resistance), cell survival, and nitrite depletion were followed during abusive storage at 27 C. Spore germination occurred readily at both levels of added nitrite. The data indicate a race between death of the germinated cells and nitrite depletion. The time at which botulinal outgrowth occurs depends upon the relative levels of residual nitrite and surviving botulinal cells.

There have been numerous studies on the effect of nitrite on botulinal outgrowth in cured meats. The criteria used for measuring this effect have included swelling of packages or cans, production of toxin, and development of a putrid aroma. However, the events which occur between the time of manufacture and the endpoint criteria have received little attention.

The purpose of this research was to follow the dynamics of residual nitrite depletion and botulinal spore germination, death, and outgrowth in a perishable cured meat product. This should lead to greater understanding of the means by which nitrite inhibits *Clostridium botulinum*. In addition, the relevance of residual nitrite to botulinal inhibition in perishable cured meat is elucidated.

METHODS

Inoculum

The *C. botulinum* spore inoculum consisted of a mixture of five type A (33A, 36A, 52A, 77A, and 12885A) and five type B (ATCC 7949, 41B, 53B, 213B, and Lamanna B) strains prepared as previously described (1). The mixed spore suspension was heat-shocked at 80 C for 15 min to destroy vegetative cells and toxin. The spore suspension was then added to the meat during formulation to levels of 10^2 or 10^4 spores per g of product.

Formulation and processing

Perishable canned comminuted cured pork was formulated with salt, water, and sugar; inoculated; processed; and chilled as previously described (1). Sodium isoascorbate (Pfizer, Inc., New York) and sodium nitrite were added at levels of .02% and 50 or 156 $\mu\text{g/g}$, respectively, on

the basis of the weight of meat in the formulation.

Three experiments were conducted which investigated the effect of inoculum level (10^2 and 10^4 spores/g) and the level of added sodium nitrite (50 and 156 $\mu\text{g/g}$).

Holding conditions

The product was placed at 27 C on the day of manufacture.

Microbiological and chemical analyses

Five normal-appearing cans were removed at each sampling time and tested for botulinal spore and vegetative (i.e., heat sensitive) cell levels. Botulinal spore levels were obtained by heating a 1:10 dilution of the product at 80 C for 15 min before doing MPN determinations. An unheated portion was analyzed to obtain total viable botulinal counts (spores plus vegetative cells). Modified peptone colloid (2) was used as the recovery medium. The MPN procedure consisted of three tubes per dilution. Blackening of the recovery medium accompanied with a putrid aroma was considered adequate evidence of botulinal growth in the medium.

Residual sodium nitrite was assayed as described earlier (1). Two cans were assayed per sampling time.

RESULTS AND DISCUSSION

Results of the first test (Table 1) clearly show the ability of *C. botulinum* spores to germinate rapidly in the presence of nitrite. The spore inoculum level in this test was $10^2/\text{g}$. The level of added sodium nitrite was 156 $\mu\text{g/g}$. The samples of canned, uncooked product show that the spore inoculum had rapidly changed to heat sensitive cells. After cooking, the spore count was about 1/g or less, and declined throughout the test period as germination continued. Counts of heat sensitive cells (germinated spores) also declined through 33 days of storage. Although the residual sodium nitrite level decreased to less than 6 $\mu\text{g/g}$ of meat within 28 days, evidence of botulinal outgrowth was not detected until after 43 days when two of five cans had counts greater than $10^3/\text{g}$; the other three cans had counts less than 1/g. At 50 days of storage, five of 65 cans remaining in incubation swelled.

The first test was repeated using a higher inoculum level of spores ($10^4/\text{g}$). Again, germination occurred rapidly. The spore levels decreased from 4,600/g after 1 day to 38/g after 10 days and 3.2/g at 28 days (Table 2).

The total botulinal count also decreased during the first 7 days. At 10 days botulinal outgrowth was detected in one of five cans. Two swelled cans occurred after 28 days. At 7 days the residual sodium nitrite level was 32 µg/g. When growth was detected, the level was 20 µg/g. Thus, growth occurred between 32 and 20 µg/g of residual nitrite compared to less than 6 µg/g in the first test.

TABLE 1. Fate of *C. botulinum* in perishable canned cured pork with 156 µg/g sodium nitrite and 100 spores per g.

Days at 27 C	Residual NaNO ₂	Geometric means of 5 cans/sampling time	
		Spores/g	Spores & vegetative cells/g
0 (Before cook)	NT ^a	13	96
0 (After cook)	94.5	.32	20
2	67.5	1.5	26
5	33.5	.38	6.9
7	21.5	.72	10
15	10	.36	3.1
22	7.5	< .3	.36
28	5.5	.32	1.1
33	NT	.037	1.0
43	NT	.047	14.3

^aNT = Not tested.

TABLE 2. Fate of *C. botulinum* in perishable canned cured pork formulated with 156 µg/g sodium nitrite and 100 spores per g.

Days at 27 C	Residual NaNO ₂	Geometric means of 5 cans/sampling time		
		Spores/g	Spores & vegetative cells/g	
0 (After cook)	NT ^a	NT	18,000	
1	95	4,600	30,000	
2	78	580	3,700	
4	49	65	1,300	
7	32	32	370	
			No. of cans out of 5	
			<1000/g	>100,000/g
10	20	38	4	1
15	11	36	3	2
21	7	7.3	4	1
28	5	3.2	4	1
36	5	< 30	3	2
43	5	<3(4),240(1)	3	1

^aNT = Not tested.

Rapid outgrowth occurred also in a third test wherein a low inoculum level (10²/g) was used in conjunction with a reduced level of sodium nitrite (Table 3). Growth was evident after 5 days at which time the residual sodium nitrite level was between 26 and 13 µg/g and the viable botulinal count had decreased to 30/g or less.

TABLE 3. Fate of *C. botulinum* in perishable canned cured pork formulated with 50 µg/g sodium nitrite and 100 spores per g.

Days at 27 C	Residual NaNO ₂	Geometric means of 5 cans/sampling time	
		Spores/g	Spores & vegetative cells/g
0 (Before cook)	NT ^a	NT	240
0 (After cook)	33	NT	23
1	26	4.0	30
5	13	1.1	94
7	11	.52	82
12	6	.37	1000
18	NT	2.3	48000

^aNT = Not tested.

These data show the fate of *C. botulinum* spores in a perishable canned cured meat subjected to abuse immediately after processing. The results show that the inhibition pattern reflects a race between nitrite depletion and the rate of death of germinated spores. In addition, 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.

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Evaluation of Raw Milk Quality Tests

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ABSTRACT

A study was done to evaluate raw milk quality tests in an attempt to answer some questions regarding values or standards by which milk acceptance is determined. Initially, of 315 individual producer samples, 226 (72%) had proteolytic psychrotrophs. More than 90% of these psychrotrophs were of the genus *Pseudomonas*. All samples were obtained from four dairy companies processing milk in Georgia. One dairy had individual producers whose milk samples contained a large percentage of thermoduric psychrotrophs. "Bitter" flavors were more prevalent in samples collected from this dairy than in samples from the other three dairies. The degree of measurable proteolysis (Hull value) was directly correlated with the incidence of the naturally occurring "bitter" flavor. The mean Hull value for milk from this dairy (30.62 µg/ml) was 12 - 14 µg higher than the other three means. The mean Hull value for the "bitter" samples was 46 µg/ml.

Much has been said and done concerning the quality of the raw milk supply in this country. Obviously, dairy products possessing good flavors and good shelf-life cannot consistently be produced when made from raw milk of less than ideal bacteriological quality. In addition, even though normal high-temperature-short-time pasteurization and certainly ultra-high temperature processing kill a large percentage of the microorganisms in milk, there is ample evidence to indicate that heat-stable enzymes liberated by some of these microorganisms while in the raw milk are present in the pasteurized product (1,10,14). These heat-stable enzymes can subsequently bring about off-flavors in products having a bacterial count meeting legal standards. In addition, the raw product may already be off-flavored due to prolonged storage during which time the enzymes are biochemically active (1,14).

While there is almost universal agreement concerning the desirability of having high quality raw milk, there appears to be a decided lack of agreement as to the best way of determining the quality of this milk. For many

years the Standard Plate Count (SPC) has been a factor in ascertaining the bacteriological acceptability of the raw product. Johns (7,8) notes that the psychrotrophic bacteria count (PBC), in contrast to SPC, better reflects whether or not milk has been produced under sanitary conditions. The PBC, which calls for 10 days of incubation at 7 C, is considered by most to be impractical from a time standpoint but still serves as the "standard" by which the newer, more rapid tests are compared.

Two of the more promising tests, which are basically designed to measure the number of psychrotrophs in milk, are: (a) Preliminary Incubation (PI) of samples at 13 C for 18 h followed by SPC, and (b) Microscopic Colony Count (MCC). This is a test perfected by Juffs and Babel (9) in which milk and media at 45 C are mixed and placed on a glass slide. After solidification the slides are incubated at either 7 or 21 C (48 or 72 h at 7 C and 13.5 or 16.5 h at 21 C). The slides are then dried, stained, and the colonies counted under a low power microscope.

Other methods are available such as the one recently described by Oliveria and Parmelee (11) which is based on incubation of plates at 21 C for 25 h. An extremely high correlation (.992) was reported when the method was compared to the standard PBC.

Thus, while there are admittedly other, perhaps equally promising tests, the above two serve to illustrate the trend. In addition, each dairy foods company has some type of test to predict how long each product will last before development of off-flavors, i.e., a predictor of shelf-life. Since termination of shelf-life is in many instances characterized by psychrotrophic-like off-flavors, tests are all remarkably similar in the factors which are being tested and/or enumerated.

Finally, there are continual questions between producer or producer organization and processors regarding values or standards by which milk is accepted. These standards, whether they be acidity, flavor, dye reduction, etc., vary tremendously and cause doubt either by the producer or the processor as to their absolute certainty.

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Thus, this study was initiated to evaluate these "raw milk quality" tests, both bacteriological tests and others designed to measure such factors as the protease activity (6), acid degree value (ADV) (13), titratable acidity (TA) (4), and flavor (presence or absence of off-flavors).

MATERIALS AND METHODS

A preliminary study was done to determine the number of raw milk samples which contained microorganisms capable of exhibiting proteolysis on Standard Methods Agar (SMA) supplemented with 1% nonfat dry milk (NFDM) (2). A total of 315 individual producer samples were evaluated by spreading 0.1 ml of milk on the surface of the medium. All plates were incubated at 4 C for 7 days rather than the 21 C - 72 h incubation described in *Standard Methods for the Evaluation of Dairy Products* (2). Proteolysis was detected by clear zones around the colonies with the zones being enhanced by adding several milliliters of 0.72 N trichloroacetic acid (TCA).

As a further screening tool, 57 of the 315 samples exhibiting proteolysis on SMA + 1% NFDM were selected at random to identify the predominant microorganism present in the sample after refrigerated incubation. Use was made of the gram reaction, catalase test, oxidase test, motility in Sulfide-Indole-Motility (SIM) medium (Difco Laboratories, Detroit, Michigan), and the IMVIC series of tests for identification of members of the families *Enterobacteriaceae* and *Pseudomonadaceae* (3).

Following these screening studies, a study was initiated which is outlined in Fig. 1. Laboratory pasteurization was accomplished by heating milk samples to 74 C for 17 sec followed by placing the tubed samples in an ice water bath for rapid cooling.

Milk samples

Grade A raw milk was obtained from four large dairies which collect and process milk in the state of Georgia. Individual producer samples were used in the study. The samples, consisting of 22-30 producer samples for each testing period, were brought to the dairies by the farm tank pickup driver. Samples were then collected from the dairy plant and transported under refrigerated conditions to the University laboratory for final testing. There were two batches collected from each of the four dairies.

Bacteriological evaluation

Coliform counts, SPC, and PBC were done in accordance with *Standard Methods* (2). The MCC procedure was done as described by Juffs and Babel (9). All counts were converted to log values for analysis.

Flavor

All samples were evaluated organoleptically and recorded as being either "bitter" or "nonbitter." Only the raw samples and not the heat-treated ones were tasted since psychrotrophs can produce this off-flavor if sufficient numbers are present regardless if the samples are

subsequently pasteurized (14).

Protease activity

The protease activity of each raw milk sample was measured by the Hull method (6). Hull values, which are a measure of the degree of proteolysis having taken place in a sample, were reported in micrograms of tyrosine released per milliliter of milk. In addition, the samples were spread on SMA + 1% NFDM. Following incubation (4 C - 7 days) proteolysis was indicated by zones of clearing of at least 2 mm around each isolated colony. Again, bacterial generic identification was made, this time on 33% of the 215 total samples.

Statistical analyses

Data were analyzed statistically to evaluate differences among dairies, test factors, and within tests. Means, standard deviations, and coefficients of variability were done on all data with correlations determined on pertinent factors. Use was made of the F test to determine significance and Duncan's new Multiple Range Test (DNMRT) for means separation (12).

RESULTS AND DISCUSSION

The data in Table 1 show results of the preliminary study. Of 315 individual producer samples, 226 (72%) had microorganisms capable of visible proteolysis on SMA + 1% NFDM after 7 days at 4 C. These organisms were thought to be proteolytic psychrotrophs. Of these 226, 57 (25%) samples were randomly selected to determine the types of microorganisms present. Of the 57, 55 samples (96%) had members of the genus *Pseudomonas* as the predominant bacteria in the microflora. One sample had a member of the *Bacillus* genus and one sample had *Escherichia* as the primary psychrotroph.

TABLE 1. Screening of raw milk samples for the presence of proteolytic psychrotrophs

I. Proteolysis	
No. samples	Positive samples ^a (%)
315	226(72)
II. Identification of Bacteria Responsible for Proteolysis	
No. samples examined	
57	
Genus	Frequency (%)
<i>Pseudomonas</i>	55 (96)
<i>Bacillus</i>	1 (2)
<i>Escherichia</i>	1 (2)

^aPositive = Exhibited zones of proteolysis on SMA + 1% NFDM after 7 days at 4 C.

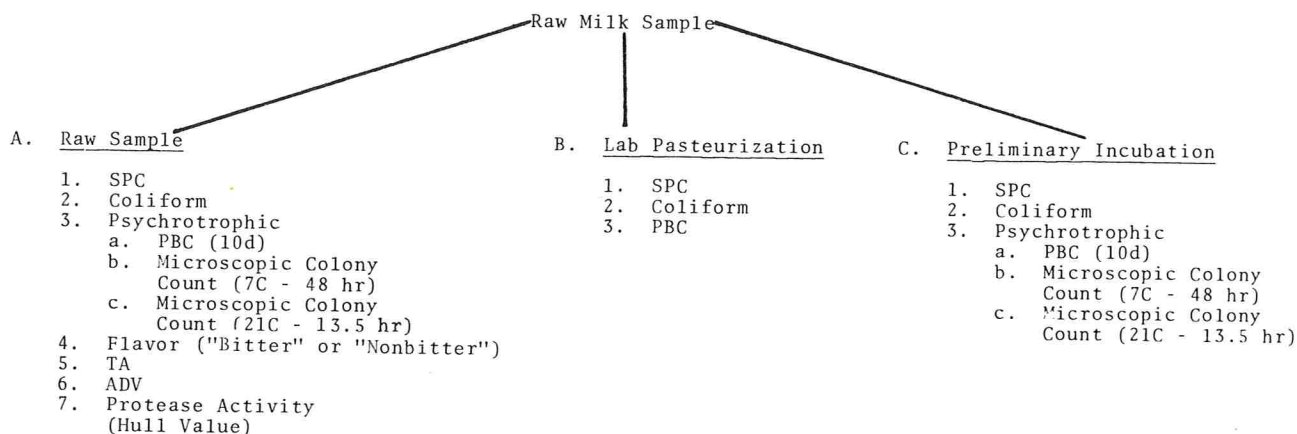


Figure 1. Evaluation of individual producer samples of raw milk.

In the second portion of the study, analyses were first made on each of the test parameters over dairy and sampling period. The primary reason for analyzing the data with regard to dairies was to illustrate the problem existing with one of the dairies that was not evident with the other three. At the time of the study, three of the four dairies admitted raw and finished products had a "bitter" flavor. This was a major reason for the study. When an analysis of variance was done on each parameter, a means separation test (DNMRT) was done on each significant ($P < 0.05$) F value. When considering SPC of raw milk samples, highly significant ($P < 0.01$) differences existed among dairies. Data in Table 2 reflect the specific differences among means. Producers from

TABLE 2. Separation of SPC and PBC means of raw milk by DNMRT.

Dairy	Log count (SPC Mean)	Dairy	Log count (PBC Mean)
2	3.8963 a*	2	3.1659 a
3	3.8352 a	4	2.8881 b
1	3.5483 b	3	2.7689 b
4	3.4478 b	1	2.4952 c

* Any means not followed by the same lower case value differ significantly ($P < 0.05$).

dairies #4 and 1 had SPC values significantly ($P < 0.05$) lower than those for dairies #3 and 2.

With regard to PBC values, another highly significant (< 0.01) difference was noted among dairies (Table 2). Thus dairy #1 had producers providing milk with significantly ($P < 0.05$) lower psychrotrophic counts than did the other three dairies. There were no significant differences noted among dairies for coliforms.

When considering laboratory pasteurization (74 C - 17 sec) of the samples, the SPC, coliform count, and PBC all differed significantly ($P < 0.01$) among dairies. A combined means separation using DNMRT is shown in Table 3. While dairy #1 had the lowest raw PBC and the next-to-lowest SPC, apparently a large percentage of the psychrotrophic microflora was thermotolerant — a great hindrance to prolonged shelf-life.

TABLE 3. Separation of log means of SPC, coliform count, and PBC of lab pasteurized milk samples by DNMRT.

Dairy	SPC	Dairy	Coliform	Dairy	PBC
1	2.2108 a*	1	0.2912 a	1	0.7181 a
2	1.9245 b	2	0.1137 b	2	0.5086 b
3	1.6338 c	4	0.0099 b	3	0.4231 b
4	1.4495 c	3	0.0055 b	4	0.0000 c

* All means not followed by the same lower case letter differ significantly ($P < 0.05$).

When considering samples which were incubated at 13 C for 18 h (Preliminary Incubation — PI), there were no significant differences noted among dairies for the SPC. There were significant differences in coliform counts for the samples following PI. Dairy #4 had significantly ($P < 0.05$) higher coliform log counts (3.0097) than did the other three dairies. When considering PBC for the PI samples, comparisons of means for the dairies and the different methods of

checking for psychrotrophs are shown in Table 4. Logarithmic counts were similar for the three methods with all three indicating milk from dairy #1 having significantly ($P < 0.05$) lower PBC. The MCC with incubation at 7 C - 48 h resulted in the same placement of dairies as did the standard PBC.

TABLE 4. Separation of log means of PBC and MCC by DNMRT of raw milk samples incubated at 13 C for 18 hours.

Dairy	PBC	Dairy	MCC-1 ¹	Dairy	MCC-2 ²
2	4.5100 a ³	2	4.2084 a	3	4.6372 a
4	4.2201 a	4	4.1191 a b	2	4.3840 b
3	4.1688 a	3	4.0493 b c	4	4.1631 b c
1	3.2690 b	1	3.9352 c	1	4.0255 c

¹ Microscopic Colony Count 7 C - 48 h.

² Microscopic Colony Count 21 C - 13.5 h.

³ All means not followed by the same lower case letter differ significantly ($P < 0.05$).

In observing the nonmicrobiological measurements (Fig. 1), the overall mean TA was .146% and did not appear to affect any other factors to any appreciable extent. The overall mean ADV was .602 with no significant differences noted among dairies. The mean separations are shown in Table 5. The data indicate that dairy #1 had a significantly ($P < 0.05$) greater number of samples exhibiting a "bitter" flavor than the other three dairies. This corresponded to the higher Hull mean (30.62 μg of tyrosine released per milliliter of milk) which was likewise significantly ($P < 0.05$) higher than the values for other dairies.

As would be expected, the PBCs of milk samples which had been subjected to PI were significantly ($P < 0.05$) higher than of those samples which had been plated immediately upon collection at the laboratory. The SPC likewise was increased in approximately the same ratio. Both sets of these counts are shown in Table 6. Thus, the criteria established by Johns (8) were certainly met in that the counts averaged at least four times higher after PI than before.

TABLE 5. Separation of flavor means and protease activity means (Hull values) of raw milk samples by use of DNMRT.

Dairy	Flavor ¹	Dairy	Hull Values ²
1	0.133 a ³	1	30.62 a
3	0.036 b	4	17.31 b
2	0.019 b	3	17.09 b
4	0.000 c	2	15.83 b

¹ Flavor 0 = Non bitter; 1 = Bitter.

² Hull Value = $\mu\text{g}/\text{ml}$; Higher value means greater proteolysis.

³ All means not followed by the same lower case letter differ significantly ($P < 0.05$).

Also in Table 6, a comparison was made of the three methods for detecting psychrotrophic bacteria. From these data one might conclude that the MCC technique may be used with the realization that the counts will be, in most instances, somewhat higher than counts obtained by the standard PBC method. Juffs and Babel (9) took this into account with establishment of appropriate correction factors. The saving in time helps to make selection of this technique justifiable for routine use.

TABLE 6. Comparison of log counts of SPC and psychrotrophic counts before and after PI.¹

Type of Count	Before PI	After PI
1. SPC	3.6835	4.4302
2. Psychrotrophic		
a. PBC (7 C - 10d)	2.8152 a ²	4.0117 a
b. MCC (7 C - 48 h)	3.8368 b	4.0715 a
c. MCC (21 C - 13.5 h)	3.9013 b	4.2994 b

¹ Preliminary Incubation at 13 C for 18 h.

² All means not followed by the same lower case letter differ significantly ($P < 0.05$).

In looking at all possible correlations, several observations may be noted, with the two factors showing the most promise being flavor and protease activity (Hull values reported). The correlations with level of significance following each are shown in Table 7. Only a few of the possible relationships are illustrated. For the most part, weak correlations appeared with high levels of significance. Examination of a larger number of samples and looking for specific factors could strengthen these

TABLE 7. Correlation coefficients for flavor and protease activity when compared to related factors.

Comparison	Correlation coefficient/ Probability
Flavor vs. ADV	.12/.07
Flavor vs. PBC (after heat treatment)	.16/.02
Flavor vs. Coliform Count (after heat treatment)	.11/.09
Flavor vs. Protease activity	.48/.0001
Protease activity vs. SPC (after heat treatment)	.15/.02
Protease activity vs. PBC (after heat treatment)	.14/.03
Protease activity vs. Coliform Count (after heat treatment)	.19/.005

questionable relationships. In looking at the relationships mentioned as being of extreme importance, i.e., protease activity vs. flavor ("bitter" or "nonbitter"), a correlation coefficient (r) of 0.48 was observed with a probability of 0.0001. This means that as the protease activity increased (elevated Hull values as $\mu\text{g/ml}$), there was a corresponding increase in the number of samples possessing a recognizable "bitter" flavor. In regions of the country having problems with a "bitter" flavor in the milk supply, measurement of the protease activity appears to be an effective screening tool in detecting samples with too much measurable released tyrosine. More work is needed to specifically define exact relationships such as suggested "cutoff" values to be used by the dairy processor. A determination as to the exact number of proteolytic psychrotrophs required to achieve this value is also needed. Work is presently being done in this laboratory in this regard.

From the data contained in this study, 11 samples (5% of total) were "bitter" in flavor. The mean Hull value for these 11 samples was 46 $\mu\text{g/ml}$. Eight of the 11 samples had values greater than 46 while three samples had values less than 46. Only four samples not having a distinguishable "bitter" flavor had values in excess of 46 $\mu\text{g/ml}$ although two of these samples were called "rancid." Whether the value 46 $\mu\text{g/ml}$ will prove to be a

true "standard" remains to be seen but under the conditions of this study, the value of 46 $\mu\text{g/ml}$ would appear to be too high for an internal standard since the product in almost every instance would already possess an off-flavor. Therefore, a lower value such as 30 $\mu\text{g/ml}$ would be more realistic since milk with values above this figure would be much more susceptible to development of a "bitter" flavor than would samples having lower values. Of course, this value will vary to some degree for every laboratory doing the test due to establishment of standard curves and different instrumentation.

CONCLUSIONS

In this study, a large dairy processor was receiving milk which contained a large percentage of thermophilic psychrotrophs. "Bitter" flavors were more prevalent in samples collected from this dairy than in samples from the other three dairies included in the study. The degree of measurable proteolysis (Hull test) was directly correlated with the incidence of the naturally occurring "bitter" flavors.

Preliminary incubation (PI) (13 C for 18 h) of raw milk samples increased the counts significantly ($P < 0.05$) when compared to counts on the milk plated immediately upon receipt. Use of this procedure in an overall control program has been recommended by Johns (8).

The Microscopic Colony Count (MCC) both at 7 C - 48 h and 21 C - 13.5 h, was compared to the psychrotrophic bacteria count (PBC) as a means of estimating the number of psychrotrophic bacteria present in raw milk samples. Before PI, the two MCC procedures correlated better with each other ($4 = .62/\text{probability} = .0001$) than either with the "standard" procedure (MCC - 7 C vs. PBC = .15/.02; MCC - 21 C vs. PBC = .29/.0001). After PI MCC - 21 C correlated slightly better with the "standard" procedure (.37/.0001) than did MCC - 7 C (.25/.005).

Based on the conditions of this study, the authors recommend use of the MCC technique at 21 C for 13.5 h or 16.5 h (depending on work schedule) for routine screening of raw milk. The advantage of the saving in time is too significant to ignore in addition to the adequacy in detecting high psychrotrophic numbers.

Use of PI has a definite place in the quality control of finished products and can be effectively used in pinpointing psychrotrophic problems at the producer level. According to these data (Table 4), however, PI is of no benefit when using the MCC technique.

Determination of the protease activity of incoming milk (raw or pasteurized shipments such as cream) can be a valuable screening tool to prevent acceptance of contaminated product. Use of the Hull test is suggested.

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Comparative Evaluation of the Difco Disc Assay Method for Detecting Penicillin in Milk and Milk Products

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ABSTRACT

The Difco disc assay method to detect antibiotic residues was evaluated and compared with the *Bacillus subtilis* disc assay and the *Sarcinia lutea* cylinder plate methods. The Difco method, using both color change and zone of inhibition as indicator, was able to detect penicillin to levels as low as 0.002 units per ml. Of 5200 raw milk samples analyzed for presence of penicillin in a commercial laboratory, the *B. subtilis* disc assay method identified and confirmed as positive 12 samples (0.239%). The Difco disc assay method identified 61 samples as positive for inhibitor, of which 48 (0.92%) were confirmed as positive for penicillin. Of 37 of these latter samples, analyzed also by the *S. lutea* cylinder plate method, 29 (78.4%) yielded positive results. The Difco procedure was found effective as a test method for detecting penicillin in finished fluid milk products.

In the proposed revision of the Grade A Pasteurized Milk Ordinance, the Food and Drug Administration would make the *Sarcinia lutea* cylinder plate method (2) an official procedure in the control of antibiotic residues in finished commingled raw milk and grade A milk products. The disc assay, using *Bacillus subtilis* as test organism, remains the method of choice for monitoring antibiotic residues in producer raw milk. In the latter case, speed and simplicity are the important factors. For finished product evaluation, sensitivity looms as the important criterion in selection of a specific method of analysis. Any method that combines all three attributes—simplicity, speed, and sensitivity—would appear to have value as a tool in the on-going effort to control antibiotic residues in milk and dairy products.

For a number of years researchers have been investigating culture plate, disc-assay techniques for penicillin detection using a variety of test organisms, among them strains of *Bacillus stearothermophilus*. Although these organisms have been found to be very sensitive to a number of antibiotics, they have also been found to be difficult to maintain and handle in routine laboratory work. Recently, however, better methods of

maintaining spore suspensions of *B. stearothermophilus* have been introduced. One method provides for agar-seeded spores to be stored in ampules and the test procedure carried out directly in the ampules (6). Kaufmann (4) has described an adaptation of the International Dairy Federation method (3). In a disc assay using *B. stearothermophilus* var. *calidolactis*, he was able to detect 0.004 units of penicillin/ml of milk after 7 days storage of seeded culture plates. On fresh plates sensitivity increased to 0.002 units per ml. Difco Laboratories (P.O. Box 1058 A, Detroit, Michigan 48232) has further modified this procedure and made available for routine laboratory work, a spore suspension which can be stored and used for preparing seeded plates for disc assay analyses. The work reported herein is an evaluation of this procedure under commercial laboratory operations and a comparison of it to both the disc assay using *B. subtilis* and the *S. lutea* cylinder plate method.

MATERIALS AND METHODS

Raw milk samples were procured in the on-going operation of Dairy Quality Control Institute, Inc. In total, 5200 such samples were analyzed over about 1 month's time. Both the rapid method disc assay using *B. subtilis* (1) and the Difco disc assay using *B. stearothermophilus* were applied to these samples for the detection of inhibitors. All positive samples by either test were subjected to confirmatory testing for penicillin as directed in *Standard Methods for the Examination of Dairy Products* (1). In addition, 37 of the samples that were positive by the Difco procedure were frozen and retained for analysis by the *S. lutea* cylinder plate method (2). In addition to raw milk samples, various finished products were also tested by the Difco method. These included whole milk, 2% fat milk, 1% fat milk, skimmilk, and half and half, whipping cream, and chocolate milk products.

As a part of the evaluation of the Difco method, the sensitivity of the test was measured using standardized dilutions of penicillin in reconstituted nonfat dry milk. Both this method and the *S. lutea* cylinder plate method were checked for reliability in rancid milks with Acid Degree Values of 2.7, 3.3, 4.1, and 6.1.

To determine the effect of storage of seeded plates on the sensitivity and reliability of the Difco method, a number of plates were prepared and stored at 5 C. At weekly intervals over a period of 3 weeks sublots

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of these culture plates were tested against standardized dilutions of penicillin.

With no essential changes, procedures outlined by Difco Laboratories were followed in handling of spore suspensions, seeding of plates, and analytical work. The method utilized a separate spore suspension from which seeded analytical plates are prepared. The analytical plates consist of indicator agar which allows readings to be made both in terms of zone of inhibition and color change (purple/blue against a yellow background).

Washed, standardized *B. stearothermophilus* spore suspensions, given the name Bacto — Thermospore Suspension PM, come from the manufacturer in use-size ampules. Held in this form, spores can be stored considerable lengths of time. Indicator agar (termed Bacto-PM Indicator agar) is made up of nutrient agar (23 g), tryptic soy broth (3 g), dextrose (5 g), Tween 80 (1 g), and brom cresol purple (0.06 g). The medium should be adjusted to and maintained at a pH of 7.6 ± 0.1 .

Indicator agar plates are seeded with spore suspension in the following way: Thirty-two grams of Bacto-PM Indicator Agar are mixed into 1000 ml of Purified Water USP, heated to boiling, cooled to 55-60 C and dispensed into containers in 100-ml portions. The medium need not be sterilized as other organisms do not interfere in the test at the high incubation temperature that is used.

Contents of one ampule of Bacto-Thermospore Suspension PM are added to 1000 ml of indicator agar at 55-60 C. The mixture is swirled to obtain a uniform suspension, and poured into sterile, flat-bottomed, 100-mm diameter petri dishes. These should be at room temperature during pouring. Six-ml portions of seeded agar are added to each plate, the plate tilted and rotated to achieve a uniform layer of agar. For plates of 150-mm size, 14-ml amounts of agar must be used. All plates are allowed to solidify on a flat surface. Covers may be left slightly ajar to facilitate drying and hardening, which takes 15-30 min.

For quantifying results, penicillin standards were prepared as follows: potassium penicillin G was added to 100 ml of distilled water to obtain a dilution of 100 units/ml. One ml of this solution in 100 ml of purified water yielded a concentration of 1.0 unit/ml. From this solution standards were prepared in reconstituted nonfat dry milk at 10% solids. The milk was pre-tested to assure that it was free of penicillin and other inhibitors. If used immediately, the reconstituted milk need not be sterilized. Using the stock solution of 0.1 unit penicillin per ml, standard penicillin/milk mixtures of 0.002, 0.003, 0.004, and 0.005 units/ml may be prepared by adding 0.5, 1.0, 1.0, and 1.0 ml of penicillin solution to 24.5, 32.0, 24.0 and 19.0 ml of milk, respectively. In this work dilutions of 0.005-0.001 were prepared for sensitivity evaluation.

One-half inch discs were handled in the following manner. Sterile forceps were used, discs were touched to milk samples and allowed to fill by capillary action. Excess milk was removed by touching the disc to the rim of the container. Discs were then placed on plates around the outer periphery about 1/2 inch from the edge and pressed firmly in place.

All plates were incubated in the inverted position at 65 ± 1 C for 2 h and 40 min before reading. In many cases plates appeared readable at 2 h and 30 min. However, they were not officially read until a lapsed time of 2 h and 40 min. The manufacturer suggests incubation between 2 h and 40 min and 2 h and 55 min.

Incubators should be equipped with air circulating devices and humidity control, and plates should not be read later than 2 h and 55 min after incubation starts because of the possibility of zone and color alterations that may occur. Plates are read in the inverted position. Difco suggests that a purple color may be taken as evidence of a positive reaction at the lowest levels of sensitivity. A zone of inhibition will not necessarily accompany this minimum-level detection point.

RESULTS AND DISCUSSION

In sensitivity tests the Difco disc assay method showed a tinge of purple color on the edge of the disc, though with a scarcely recognizable zone per se, at 0.002 unit of penicillin per ml. At 0.003 unit per ml the color

change was more distinct, and a more definitive zone could be detected. At no levels of rancidity within the range 2.7 to 6.1 Acid Degree Value was there any noticeable effect on test results. This was also true for the *S. lutea* cylinder plate method.

Table 1 shows the incidence of penicillin residues noted in 5200 commercial raw milk samples as detected by the disc assay method using *B. subtilis* and the Difco method using *B. stearothermophilus*. The former detected 12 positive samples (0.23%) of which all were confirmed for presence of penicillin. The Difco method yielded 61 samples identified as positive for inhibitor of which 48 (0.92%) were confirmed for penicillin. Thus, while the total percentage of positive identifications was not great, the Difco method did in fact detect four times as many samples of penicillin-contaminated milk as did the other method. Estimates of amounts of penicillin indicated that the samples detected as positive by the Difco and not by the *B. subtilis* method were in all cases at or below the minimum level of detectability usually assigned to the latter procedure; i.e., 0.05 unit/ml

TABLE 1. Incidence of penicillin residues in 5200 samples of commercial raw milk as detected by *B. subtilis* and *B. stearothermophilus* disc assay methods.

Method	No. of positive samples detected	No. of positive samples confirmed	Percent of samples confirmed
Disc Assay (<i>B. subtilis</i>)	12	12	0.23
Disc Assay (<i>B. stearothermophilus</i>)	61	48	0.92

When 37 samples identified and confirmed as positive for penicillin by the Difco method were also subjected to testing by the *S. lutea* cylinder plate method, the latter identified 29 samples as positive; the difference was presumed to be due to differences in test sensitivity. Since these samples were frozen and stored for future analysis by the cylinder plate method, they were also checked again at the time of testing by the Difco procedure. All but one showed positive results, i.e. presence of penicillin. The one, upon confirmatory testing, indicated presence of a naturally occurring inhibitor.

On 160 samples of finished products; i.e., whole milk, 2% fat milk, 1% fat milk, skimmilk, half and half, whipping cream, chocolate milk and chocolate drink, all obtained and tested in routine operations of the Dairy Quality Control Institute Laboratory, only one sample of milk was positive for presence of penicillin and this at 0.002 unit/ml, the lowest level of detectability. Sensitivity tests in pasteurized whole milk indicated that the Difco procedure was equally sensitive with this type of finished product as with raw milk.

Seeded plates were stored for 3 weeks. Lots were checked each week for sensitivity, using standardized concentrations of penicillin in reconstituted nonfat dry

milk. No decrease in sensitivity was noted after 1 week of storage. Upon two weeks of storage, the minimum level of detectability had risen to 0.004 unit of penicillin per ml. At 3 weeks of storage, a very small halo could be noted only at 0.005 unit of penicillin per ml.

Overall, the Difco procedure proved to be simple to do, and essentially as fast and somewhat more sensitive than any currently-used method for penicillin detection. It was also found to be practical for use in a commercial dairy testing laboratory.

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Bacteriological Survey of Raw "Soul Foods" Available in South Carolina

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ABSTRACT

Bacterial counts of "soul foods" obtained from 10 farm families and 10 supermarkets were determined. Total plate count means for farm family vegetables ranged from 1.5×10^6 /g to 2.9×10^6 /g, and 2.0×10^2 /g to 3.1×10^5 /g for vegetables purchased at the retail sales establishments. Pig offal samples with the highest total plate counts were pigtailed, 4.8×10^8 /g and maws, 4.5×10^8 /g from farm families; and pigtailed, 2.8×10^9 /g, neckbones, 2.4×10^9 /g from the retail sources. Fecal coliforms were not detected in any of the vegetable samples but were isolated from 24% of the pig offals. Sixty percent of all of the foods were positive for *Salmonella* and 96% were positive for coagulase-positive *Staphylococcus aureus*. *Clostridium perfringens* was only detected in one sample, chitterlings from a retail source.

The foundation of "soul foods" in the United States came from the black Africans. When kidnapped and enslaved, they brought with them techniques for preparing their foods. Various parts of slaughtered pig normally discarded by slave owners on southern plantations were salvaged by the slaves and used for food. These foods, when prepared by African cooking methods, along with procedures learned from the American Indians and caucasian colonists, continue to be used today primarily by black Americans and are known collectively as "soul foods" (2).

There appears to be little published information about the bacteriological quality of raw "soul foods." Therefore, this survey was designed to determine the bacterial content of "soul foods" available to consumers in a section of South Carolina. The foods were examined for numbers of total aerobic bacteria, fecal coliforms, salmonellae, *Staphylococcus aureus* and *Clostridium perfringens*. The bacteriological quality of the foods obtained from local supermarkets and from farm families was compared.

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MATERIALS AND METHODS

Collection of samples

For this investigation, "soul foods" surveyed included the following processed and semi-processed swine offals: chitterlings (washed intestines), fatback, hamknuckles, jaws, maws (stomach of swine), neckbones, pigears, pigfeet, pigtailed, liver puddings, sausage (loose), sausage (encased), and cracklings (skin residue after fat is rendered out by cooking). Also tested were *Brassica oleracea* (collard greens) and *Pisum sativum* var. *arvense* (field peas). The field peas were shelled and dried.

From 10 supermarkets and 10 farm families in Orangeburg and Calhoun Counties of South Carolina food samples were randomly selected. The foods, obtained from the farm families, were collected aseptically. Foods purchased at supermarkets were packaged in the stores in accordance with the normal procedures of the stores. Food samples were refrigerated and analyzed within 72 h after collection.

Bacteriological analysis

Three samples from each of 10 sources within the farm families and retail outlets were analyzed. A 10-g portion of food was blended with 90 ml of 0.1% peptone dilution fluid, pH 7.2, at high speed in a Waring Blendor for 2 min. Two subsamples from each sample were decimally diluted from 10^{-1} to 10^{-8} and cultivated in triplicate from each dilution on or in the following types of media for: (a) total counts on pour plates of Tryptone Glucose Yeast Extract agar (BBL); (b) fecal coliforms by MPN technique in EC medium (Difco); (c) *S. aureus* on spread plates of Baird-Parker medium (BBL); (d) *Salmonella* by MPN technique in Lactose broth (Difco) and Selenite Cysteine broth (Difco); (e) *C. perfringens* on pour plates of SFP medium (BBL). The incubation temperatures were 45 C for the fecal coliforms, 28 C for *Salmonella*, *S. aureus* and total bacterial populations, and 37 C anaerobically for *C. perfringens*. The specific methods used for cultivation and identification of all these groups of bacteria were those described in the *Bacteriological Analytical Manual for Foods* (1). Isolates from Selenite Cysteine broth showing reactions typical of *Salmonella* on Brilliant Green (Difco), Sallmonella-Shigella (Difco), Bismuth Sulfite (Difco), Triple Sugar Iron (Difco) and Lysine (Difco) agar media were further characterized by a series of biochemical tests and by both Spicer-Edwards H antisera (Difco) and *Salmonella* O antisera (Difco).

RESULTS AND DISCUSSION

All of the foods tested harbored some bacterial flora, as expected, but no fecal coliforms, salmonellae, *S. aureus* or *C. perfringens* were detected in field peas from

supermarkets (Table 1) or in maws and encased sausage from farm families (Table 2). One or more of these bacterial groups was found in all of the other food items examined.

The generally lower total counts on vegetables from supermarkets (Table 1) probably reflect a more extensive washing treatment which these foods received before analysis. Surprisingly, while no fecal coliforms were found on these foods, fairly high numbers of salmonellae, $8 \times 10^2/g$ to $8 \times 10^3/g$, were detected. The 10-fold higher coagulase-positive *S. aureus* count for the farm family collard greens probably reflects more human contact with this product.

Tables 2 and 3 summarize results of the bacteriological examinations of pig offal samples from farm

families and supermarkets. Farm family samples which had lower total counts than supermarkets samples were chitterlings, neckbones, pigears, pigfeet and pigtails. These are offals which are cleaned carefully before human consumption and the cleaning process could determine the quantity of bacterial flora detected.

The MPN counts of fecal coliforms and salmonellae from the raw pig offal sources are also summarized in Tables 2 and 3. There apparently was greater incidence of the latter bacterial group in the supermarket than the farm family samples. Coagulase-positive *S. aureus* counts were 2 to 5 logs higher for all samples except the fatback samples from the supermarket sources compared to farm family sources. *C. perfringens* was found in only one food, supermarket chitterlings, at a

TABLE 1. Mean bacterial counts^a per gram of raw vegetables obtained from farm families and supermarkets.

Source	Foods	Total counts	Fecal coliforms ^b	Salmonellae ^b	Coagulase-positive <i>S. aureus</i>	<i>C. perfringens</i>
Farm family	Collard greens ^c	2.9×10^6	*	8.0×10^3	2.0×10^3	*
Farm family	Field peas ^d	1.5×10^6	*	*	1.9×10^3	*
Supermarket	Collard greens ^c	3.1×10^5	*	8.0×10^2	9.7×10^2	*
Supermarket	Field peas ^d	2.0×10^2	*	*	*	*

^aMeans are geometric averages of 30 samples.

^bMost Probable Number/g of food.

^c*Brassica oleracea*.

^d*Pisum sativum* var. *arvense*.

* Not detected.

TABLE 2. Mean bacterial counts^a per gram of raw pig offals obtained from farm families.

Foods	Total counts	Fecal coliforms ^b	Salmonellae ^b	Coagulase-positive <i>S. aureus</i>	<i>C. perfringens</i>
Chitterlings	5.4×10^6	4.8×10^5	1.2×10^3	*	*
Fatback	1.4×10^8	*	*	1.4×10^3	*
Hamknuckles	1.4×10^7	*	*	1.7×10^2	*
Jaws	6.9×10^5	*	9.3×10^1	1.3×10^3	*
Maws	4.5×10^8	*	*	*	*
Neckbones	1.9×10^6	*	*	1.6×10^2	*
Pigears	2.7×10^6	*	*	1.1×10^3	*
Pigfeet	5.4×10^5	*	*	4.8×10^2	*
Pigtails	4.8×10^8	4.3×10^1	*	3.3×10^2	*
Puddings (liver)	2.7×10^8	*	1.7×10^2	1.2×10^4	*
Sausage (loose)	6.2×10^7	1.1×10^3	*	*	*
Sausage (encased)	2.2×10^7	*	*	*	*
Cracklings (cooked)	6.8×10^2	*	*	1×10^1	*

^aMeans are geometric averages of 30 samples.

^bMost Probable Number/g of food.

* Not detected.

TABLE 3. Mean bacterial counts^a per gram of raw pig offals purchased at supermarkets.

Foods	Total counts	Fecal coliforms ^b	Salmonellae ^b	Coagulase-positive <i>S. aureus</i>	<i>C. perfringens</i>
Chitterlings	8.3×10^7	1.6×10^4	3.3×10^5	1.5×10^5	2.0×10^5
Fatback	2.8×10^4	*	*	4.2×10^2	*
Hamknuckles	3.2×10^6	*	2.0×10^1	8.4×10^6	*
Jaws	4.9×10^5	*	2.2×10^1	4.3×10^3	*
Maws	3.2×10^7	7.6×10^4	2.8×10^4	5.0×10^5	*
Neckbones	2.4×10^9	*	4.2×10^2	1.4×10^5	*
Pigears	8.1×10^6	*	4.4×10^2	2.5×10^5	*
Pigfeet	7.9×10^7	2.6×10^2	3.2×10^2	2.6×10^5	*
Pigtails	2.8×10^9	*	1.0×10^2	1.3×10^6	*
Puddings (liver)	2.1×10^7	*	6.0×10^1	1.1×10^4	*
Sausage (loose)	1.0×10^7	*	2.4×10^4	2.8×10^4	*
Sausage (encased)	1.6×10^5	*	*	2.0×10^3	*
Cracklings (cooked)	1.0×10^1	*	*	1.0×10^1	*

^aMeans are geometric averages of 30 samples.

^bMost Probable Number/g of food.

* Not detected.

level of $2.0 \times 10^5/g$. Supermarket cracklings showed minimal bacterial contamination with total and *S. aureus* counts of just 10/g. The total bacterial content of farm family cracklings was slightly higher, 680/g, while the *S. aureus* content was the same as that of the supermarket cracklings.

The aforementioned data show that these raw foods harbor considerable numbers of bacteria of public health significance. The high *S. aureus* counts probably reflect the human handling these foods receive. It seems obvious that these foods, if exposed to time-temperature abuse during preparation before consumption, can be a serious source of foodborne illness. Also, persons preparing these items for consumption should be made more fully aware of the potential of these foods to serve as sources of cross-contamination of other foods.

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A Research Note

Single or Multiple Antibiotic-Amended Media to Enumerate Yeasts and Molds¹

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ABSTRACT

Gentamicin, chloramphenicol and chlortetracycline were evaluated singly and in combination for their ability to control bacteria in media to enumerate yeast and molds. No single antibiotic tested at 50 or 100 ppm was totally effective in controlling bacteria. Chlortetracycline in combination with either chloramphenicol or gentamicin inhibited the contaminating bacteria as long as the antibiotics were added after autoclaving. It is recommended that for routine use, reliance not be placed upon a single antibiotic to control bacteria when enumerating fungi.

The question continues to arise as to the need for single or multiple antibiotics in media to control bacterial growth during the routine evaluation of foods for their fungal populations. In the most recent edition of the *Bacteriological Analytical Manual* (8) it is recommended that 40 ppm chlortetracycline HCl be used and that 40 ppm chloramphenicol may also be added "if bacteria are present which are resistant to chlortetracycline HCl". The *Compendium of Methods for the Microbiological Examination of Foods* (7) discusses the use of antibiotic-supplemented media and recommends that a combination of 100 ppm each of chloramphenicol and chlortetracycline HCl be added to the medium.

There are sufficient data in the literature to indicate that a single antibiotic is inadequate to control bacteria during enumeration of fungi (3-6), yet procedures employing single antibiotics are still being recommended. The type of sample analyzed with its resident flora will play a major role in dictating the choice and concentration of inhibitor needed. However, any inhibitor system must be effective in routine use, without concern for growth of contaminating bacteria. Therefore, a study was undertaken to specifically evaluate the use of single and multiple combinations of chloramphenicol, chlortetracycline and gentamicin with both Plate Count and Rose Bengal Agar and to determine the effect of steam sterilization on their

inhibitory properties.

The basis for selecting gentamicin and chloramphenicol for this study was their resistance to heat, whereas, chlortetracycline was selected because of its known effectiveness in controlling bacteria.

MATERIALS AND METHODS

All samples were obtained in the Gainesville, Florida, area. Preparation and plating of samples followed methods outlined in the *Compendium* (7). Antibiotic solutions were prepared in distilled water so that the addition of 2 ml of solution per 100 ml of medium would give a concentration of 50 or 100 ppm. Both Plate Count and Rose Bengal Agar (Difco) were used with incubation at 25 C for 5 days. Antibiotics were added before and after autoclaving at 121 C for 15 min. If possible, at least 10 colonies which resembled bacterial colonies were picked from each set of countable plates from each test medium and gram-stained. The percentage of colonies examined which were bacteria was then calculated.

RESULTS AND DISCUSSION

Data for addition of 50 and 100 ppm of the antibiotic preparations after sterilization to Plate Count and Rose Bengal Agar are listed in Table 1. Within limits of the method used to determine the efficacy of the antibiotics, it was found that growth of bacteria occurred in both media with the rose bengal dye (35 ppm) appearing to have only a minimal effect on suppressing bacterial growth. However, as has been reported by other workers, there was a slight reduction in colony size of most of the molds due to the rose bengal. Bacterial contamination was generally greater on the 50- than on the 100-ppm supplemented medium as would be expected with the isolates being mainly gram-negative rods. Gentamicin and chloramphenicol were less effective in controlling bacteria than was chlortetracycline. However, bacterial growth was found on chlortetracycline plates from three of the 20 samples. These kinds of samples (ground beef and oysters) would not routinely be analyzed for fungal content and were included because of their usual high levels of gram-negative bacteria. Nevertheless, it is

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conceivable that organisms of this kind could be found in most any food due to the wide distribution of bacteria in the environment.

When combinations of the three antibiotics were added at 100 ppm after autoclaving (Table 2), a marked reduction in plates containing bacteria was observed. No bacteria were found in any of the combinations containing chlortetracycline; however, the chloramphenicol/gentamicin combination showed limited bacterial growth in two of the samples when cultured on Plate Count Agar but not on Rose Bengal Agar.

When the antibiotic combinations were added before autoclaving, bacteria developed on all media (Table 2),

although chloramphenicol and gentamicin have been reported as being heat-stable (1). Those combinations which included chlortetracycline proved to be the least effective following sterilization.

This study supports existing data pointing out the limited usefulness of a single antibiotic in media to control bacterial growth during enumeration of fungi. Each selective plating system has its limitations; however, there is adequate information available to give guidance in selection of a combination of inhibitors that will routinely yield bacteria-free plates during enumeration of fungi.

TABLE 1. Percentage of isolates identified as bacteria from media supplemented with either 50 or 100 ppm antibiotic after sterilization.

Sample	Gentamicin		Chloramphenicol		Chlortetracycline	
	50 ppm	100 ppm	50 ppm	100 ppm	50 ppm	100 ppm
<i>Rose Bengal Agar</i>						
Breaded shrimp	80	5	30	20	—	—
Soil	— ¹	—	30	20	—	—
Calamondin	—	—	—	—	—	—
Beef	70	90	100	60	—	—
Fish sticks	—	—	30	80	—	—
Stuffed flounder	30	10	—	—	—	—
Shrimp sticks	—	—	—	—	—	—
Chicken	40	30	30	10	—	—
Ground beef	10	—	40	20	60	40
Raw oysters	40	30	30	10	—	—
<i>Plate Count Agar</i>						
Chicken	50	30	80	60	—	—
Ground beef	20	10	60	70	10	—
Raw oysters	100	70	100	90	30	10
cooked shrimp	—	—	—	—	—	—
Soil	25	—	50	—	—	—
Beef	30	10	—	—	—	—
Shrimp sticks	100	—	—	—	—	—
Pork	70	70	60	60	—	—
Deviled crab	—	30	—	—	—	—
Pond water	80	80	70	15	—	—

¹No bacteria found

TABLE 2. Percentage of isolates identified as bacteria from media supplemented with combinations of antibiotics (100 ppm each) added before and after autoclaving.

Sample	Ct/G ¹		Antibiotics		Ca/G	
	PC ²	RBA	PC	RBA	PC	RBA
<i>Added before autoclaving</i>						
Chicken	10	—	100	50	—	—
Pork	— ³	—	80	40	—	—
Ground beef	20	40	90	30	10	—
Lamb	20	20	100	60	—	10
Raw oysters	90	60	80	100	10	40
<i>Added after autoclaving</i>						
Chicken	—	—	—	—	—	—
Ground beef	—	—	—	—	—	—
Pork	—	—	—	—	—	—
Lamb	—	—	—	—	—	—
Raw oysters	—	—	—	—	—	—
Soil	—	—	—	—	—	—
Pond water	—	—	—	—	—	—
Breaded shrimp	—	—	—	—	—	—
Beef	—	—	—	—	10	—
Stuffed flounder	—	—	—	—	20	—

¹CT = Chlortetracycline, G = gentamicin, Ca = chloramphenicol.

²PC = Plate Count Agar, RBA = Rose Bengal Agar.

³No bacteria found.

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Interaction of Mycotoxins with Copper-Folin Reagent

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ABSTRACT

To study the interference by mycotoxins in protein measurements, solutions of various concentrations of aflatoxin B₁, citrinin, cytochalasin B, ochratoxin A, patulin, penicillic acid, rubratoxins A and B, T-2 toxin and zearalenone were tested for a positive copper-Folin reaction. Except for T-2 toxin, all mycotoxins tested developed a blue color, characteristic of proteins, in the copper-Folin solution. The Lowry reaction for the mycotoxins was linear over the concentration range of 20-100 µg/ml for most of these toxins. Aflatoxin B₁ and citrinin at 2 µg/ml and zearalenone at 0.5 mg/ml developed a blue color in the copper-Folin solution. The linear relationship between color development and toxin concentration plus high sensitivity makes the copper-Folin reaction a potential analytical method for these toxins but only in the absence of protein or other copper-Folin positive materials.

Secondary fungal metabolites have gained significant recognition as public health hazards in the last 15 years because of widespread occurrence in foods and feeds (3,10,11). Several are potent carcinogenic agents and epidemiological evidence suggests that aflatoxin B₁ may be responsible for liver cancer in certain human populations in Africa and Asia (2,6,9). Other mycotoxins are carcinogenic, mutagenic and teratogenic, at least in laboratory animals (4,11). The chemistry and biology of mycotoxins has been reviewed recently (7,8).

A number of *in vivo* and *in vitro* studies have reported that mycotoxins inhibit protein synthesis, microsomal enzymes and a variety of biological systems that rely on protein quantitation as part of the analytical procedure (7,11). In 1951 Lowry et al. (5) described use of the Folin phenol reagent and copper for measuring protein. The main disadvantage of this test was that color development was not "strictly" proportional to protein concentration. Recently we noted interference by citrinin in our Lowry protein analysis. This paper presents data on citrinin and other mycotoxins that react with the copper-Folin complex, developing a blue color around 750 nm, the suggested wavelength for measuring optical density for protein determination. Data also are presented on potential complications arising in protein measurements by the Lowry method in the presence of mycotoxins.

MATERIALS AND METHODS

Citrinin, cytochalasin B, ochratoxin A, patulin, penicillic acid, rubratoxins A and B, T-2 toxin and zearalenone were dissolved in 95% ethanol while aflatoxin B₁ was dissolved in a 1:1 mixture of dimethyl sulfoxide (DMSO) and ethanol. The purity of the toxins was verified by melting point and mass spectra. Aqueous solutions of bovine serum albumin (BSA) were used as the standard for color development with the copper-Folin reagent.

The protein method of Lowry et al. (5) was used to develop color in solutions containing various concentrations of a single toxin or BSA and for solutions containing a mixture of BSA and a toxin. The intensity of the color developed was measured at least 30 min after addition of 1 N phenol solution (diluted Folin's reagent). Spectra over the range of 400 to 900 nm for the mycotoxins were obtained on Beckman Acta III spectrophotometer. Absorbance of individual mycotoxins at 750 nm was measured with both the Acta III and a Bausch and Lomb spectronic 20.

A study also was undertaken to determine if elevated protein content was observed in BSA solutions containing a mycotoxin. Zearalenone (5 and 10 µg/ml) was dissolved in 2% Na₂CO₃ in 0.1 N NaOH, citrinin (10 and 20 µg/ml), in 5% NaHCO₃, and patulin (10 and 20 µg/ml), in 0.9% NaCl. Toxins were added to solutions of BSA which ranged in concentration from 5 to 30 µg/ml. Corresponding BSA solutions containing no toxin were the standards.

RESULTS AND DISCUSSION

A blue color, characteristic of a positive Lowry reaction, was obtained for all the mycotoxins tested except T-2 toxin. Maximum color developed within 30 min and remained stable for 2 h. A broad absorption peak around 750 nm was observed for all positive-reacting toxins. Development of the blue color of the copper-Folin reagent in the presence of a toxin suggested formation of a copper-toxin complex. The presence of conjugation and the location of the functional groups in the structure determine the ability of a compound to complex with a metal. Recently citrinin has been reported to react with copper (II) to form chelates (1).

The relative order of reactivity of the mycotoxins to the copper-Folin solution is presented in Table 1. Zearalenone was the most reactive. As low as 0.5 µg/ml of this toxin can be measured spectrophotometrically. Citrinin and aflatoxin B₁ followed by patulin were the next most sensitive toxins. Two-three µg/ml of these toxins could be

measured. Ochratoxin A and penicillic acid could not be measured below 5 $\mu\text{g/ml}$ whereas 15 μg of cytochalasin B or rubratoxin A/ml were necessary for detection. Except

TABLE 1. Order of reactivity exhibited by mycotoxins, dissolved in ethanol, to the copper-Folin reagent.

Toxin	$E_{1\%}^{1\text{cm}}$	Lower detection limit ($\mu\text{g/ml}$)
Zearalenone	525	0.5
Citrinin	266	2
Aflatoxin B ₁ ^b	233	2
Patulin	137	3
Ochratoxin A	68	5
Penicillic acid	66	5
Rubratoxin A	20	15
Cytochalasin B	15	15
Rubratoxin B	8	20

^a $E_{1\%}^{1\text{cm}}$ is the mean of 3-4 $E_{1\%}^{1\text{cm}}$ values (750 nm) at concentrations of 19.0,

30.0, 38.0 and 77.0 $\mu\text{g/ml}$ of the toxin.

^cDissolved in a 1:1 mixture of DMSO/ethanol.

for T-2 toxin, which developed no color in the presence of the copper-Folin reagent, rubratoxin B was the least sensitive of the toxins tested.

Plotting of the spectral data after linear regression analysis showed that all the mycotoxins tested except aflatoxin B₁ exhibited linearity over approximately the same concentration range (Fig. 1). Zearalenone was linear over the range of 0.5-75 μg zearalenone/ml of ethanol whereas the range for citrinin, patulin and rubratoxin B was 5-100, 10-100 and 30-100 $\mu\text{g/ml}$, respectively. The remaining mycotoxins tested were linear over a 20-100 $\mu\text{g/ml}$ range. No attempt was made to measure absorbance of solutions containing more than 100 $\mu\text{g/ml}$ of a toxin. The absence of linearity for aflatoxin B₁ over this concentration range resulted from its low solubility in the DMSO:ethanol mixture (precipitation occurred above 50 $\mu\text{g/ml}$). Zearalenone, citrinin and patulin, when dissolved in water also showed a linear relationship in their reaction with the copper-Folin solution (Fig. 2).

Data demonstrating the interaction of zearalenone, citrinin and patulin with BSA are presented in Fig. 3. In each instance, absorbance was greater in solutions of protein plus a toxin versus protein solutions without toxin. The effect was accumulative and more pronounced when absorption of the contaminating toxin was within 0.3 absorption units of the pure protein solution. Thus with 5 and 10 $\mu\text{g/ml}$ of zearalenone (Fig. 3A) and with 10 and 20 $\mu\text{g/ml}$ of citrinin (Fig. 3B) or patulin (Fig. 3C), the measurement of protein at levels of 30 μg protein/ml and lower showed greater absorbance than the protein solution without a toxin. Absorbance of the toxin contaminated protein solution was linear and its slope calculated by linear regression was within experimental error to that calculated for the protein solution alone over the same concentration range. With greater protein concentrations, the absorbance of toxin-contaminated protein solutions was higher than that of pure protein solutions but not additive.

The copper-Folin reaction has been used for almost three decades as a standard method for protein

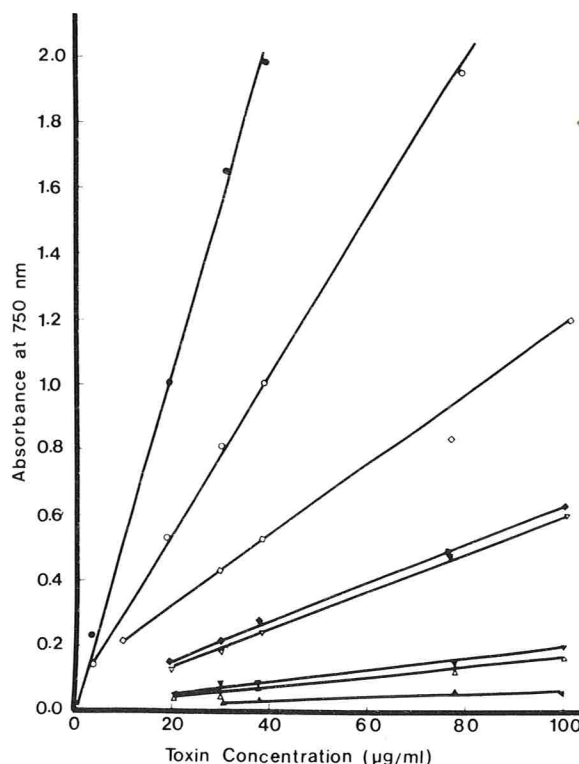


Figure 1. Linear regression plot of data from the copper-Folin reaction with different mycotoxins in ethanol. The correlation coefficient was at least 0.96 for all toxins.

Zearalenone (●—●), citrinin (○—○), patulin (◇—◇), penicillic acid (▽—▽), ochratoxin A (◆—◆), rubratoxin A (▼—▼), cytochalasin B (△—△) and rubratoxin B (▲—▲).

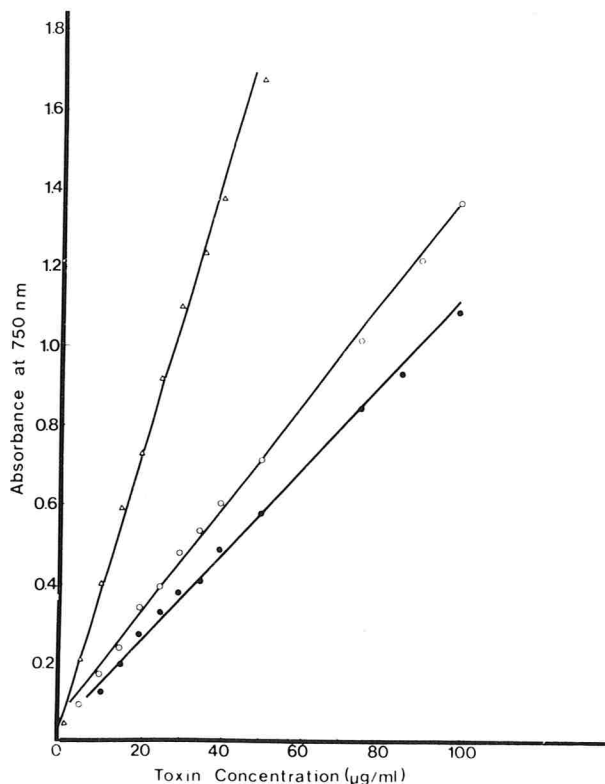


Figure 2. Copper-Folin reaction with aqueous solutions of citrinin (○—○), patulin (●—●) and zearalenone (△—△).

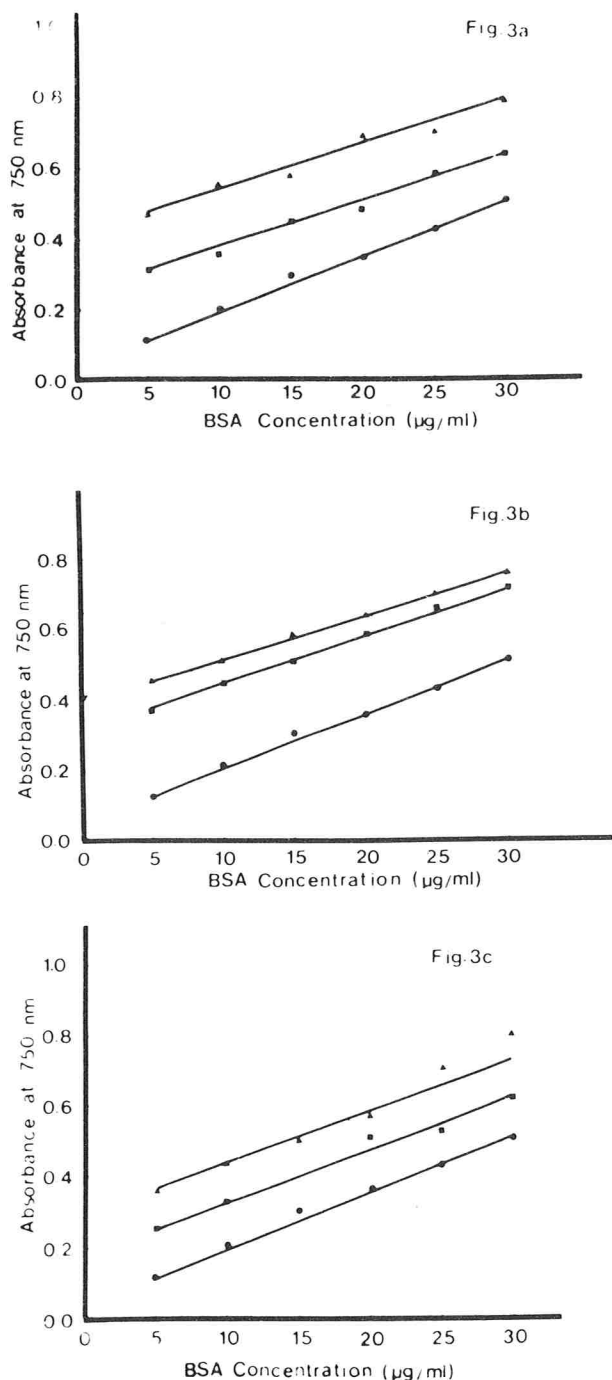


Figure 3. Effect of different concentrations of mycotoxins on color development by the copper-Folin reaction of BSA. BSA and citrinin were dissolved in water, patulin in .9% NaCl solution and zearalenone was dissolved in 2% Na_2CO_3 in 0.1 N NaOH. Figure 3A. BSA (● — ●) with 5 $\mu\text{g}/\text{ml}$ (■ — ■) and 10 $\mu\text{g}/\text{ml}$ (▲ — ▲) zearalenone. Figure 3B. BSA (● — ●) with 10 $\mu\text{g}/\text{ml}$ (■ — ■) and 20 $\mu\text{g}/\text{ml}$ (▲ — ▲) of citrinin. Figure 3C. BSA (● — ●) with 10 $\mu\text{g}/\text{ml}$ (■ — ■) and 20 $\mu\text{g}/\text{ml}$ (▲ — ▲) of patulin.

estimation in biological systems (5). Although contamination of protein solutions by mycotoxins is not a common problem, the possibility exists for complications

to arise in protein measurements of tissues used for the study of distribution of a given toxin in animals. Since small quantities of these toxins develop appreciable color, the measurement of low protein concentrations in toxin-contaminated protein samples can cause complications. In the event higher than normal protein values are obtained, specific checks for these toxins should be made. Also toxin-contaminated glassware presents a potential problem. The copper-Folin reagent reacted readily with all the mycotoxins tested except T-2 toxin. The intensity of the color developed was constant after 30 min and was linear over a wide concentration range of the toxins. Furthermore, the method is sufficiently sensitive to develop color in solutions containing low toxin concentrations. These advantages make this simple colorimetric test a potential, nonspecific method of assay for these toxins but only in absence of protein.

ACKNOWLEDGMENTS

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Mycotoxins — A Real or Potential Problem — Introduction

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It has been 17 years since the original report first appeared in England implicating the aflatoxins in "turkey X" disease. The 007 fashion leading to identification of the causal organism, *Aspergillus flavus*, isolation and characterization of the responsible compounds followed by the demonstration of aflatoxin B₁ as an extremely potent carcinogen and the significance of this compound in animal and human health rapidly focused worldwide involvement and attention upon the mycotoxins. It is interesting to note that, although knowledge of mold toxicoses developed only recently and is limited, one of the oldest foodborne diseases recognized was a mold infection. Ergotism known a thousand years ago as St. Anthony's Fire, was a disease which killed thousands of people in Europe (1). This disease is caused by the fungus, *Claviceps purpurea* which grows on rye. The rye grain becomes completely replaced by sclerotia and turns black. Ingestion of between 1 and 1.5 g diseased rye grain daily can result in symptoms that range from peripheral thrombosis to gangrene of the extremities to nervous system involvement, lysis and death. It was not for hundreds of years until the discolored grain was recognized by Kaspar Schwenckfeld in the 1600's as being the cause of the disease. This mold-induced foodborne disease continued to occur right into the nineteenth century in Europe and America and was last recorded on a large scale in Russia in the 1920's and 30's.

Considerable evidence, mostly epidemiological, is developing which would suggest that other mycotoxins indeed are of concern to humans. Data suggesting that the aflatoxins were ingested by population groups of several countries and that this ingestion can be related to hepatic diseases, particularly cancer, has come from three types of studies. The first of these consists of surveys of various food commodities for toxigenic fungi and for aflatoxin levels. A second general source of information has come from unusual outbreaks of disease among groups of people in which the cause was apparently related to consumption of a common type of

food that was mold-contaminated and contained measurable levels of aflatoxin. Third, there have been reports of somewhat isolated cases where one or a few individuals, often children, became ill after consuming aflatoxin-laden food. Such reports which encompass one or more of these types of studies, have emerged from Uganda, Taiwan, Kenya, Swaziland, Mozambique, the Philippines, Southeast Asia, Senegal and India. With the exception of the Indian studies, all of these relate to epidemiological evidence supporting the relationship between liver cancer and aflatoxin. The Indian report describes outbreaks of acute toxic hepatitis with high fatality rates in adult humans; death was usually sudden and in most instances was preceded by massive gastrointestinal bleeding. Males were affected twice as commonly as females.

Reye's syndrome, first described in 1963 as encephalopathy and fatty degeneration of the viscera, has since been recognized as a major cause of morbidity and mortality among infants and children. This disease syndrome and possible linkage to aflatoxin has been suggested.

Balkan nephropathy, another case in which epidemiological evidence is developing to relate mycotoxins to a human disease, is an endemic disease of people living in close proximity to the Danube River in Yugoslavia, Rumania and Bulgaria. Krogh (3) has indicated that 8 to 20% of the foodstuffs in one endemic village in Yugoslavia contained ochratoxin A, a well known nephrotoxin of swine and poultry; however, the late J. M. Barnes and his co-workers (2) from the U.K. recently published a report showing that *Penicillium verrucosum* var. *cyclopium* was the isolate obtained most frequently from food samples and other materials collected from five endemic areas of the disease. Culture extracts which did not contain ochratoxin A force-fed to young rats caused subtle histological changes in renal tissue.

This seminar was conceived as a means to communicate the status of the mycotoxin problem in man and in his animals and to convey information about some of the

current ongoing research in the mycotoxin field. As the title, "Mycotoxins — A Real or Potential Problem?", would indicate some hesitation exists on the part of workers in this field as to the importance of these compounds in agriculture, in animal health and particularly in human health. Hopefully, this hesitation can now be dispelled

Dr. Hamilton, who has long been concerned with mycotoxicoses in real life, particularly in poultry, will deal with some of the fallacies in our understanding of mycotoxins. Dr. Hamilton will address the need to apply Koch's postulates as they relate to mycotoxins and will respond to the important question "What is a safe level of a mycotoxin?" The concept of a mycotoxin residue or "zero tolerance," and food safety also will be addressed by Dr. Hamilton. This will be followed by a discussion by Dr. Peter Scott, who will describe the occurrence of mycotoxins in feeds and ingredients and who will discuss their biogenic origin. The recent discovery that the mycotoxicoses, alimentary toxic aleukia and stachy-bachtrytoxicosis, reported in the Soviet literature in the early 40's, may have been due to tricothecene poisoning suggests an emerging problem because of the widespread occurrence of these mycotoxins. The tricothecenes, derivatives of the 12, 13 epoxy Δ^9 9-tricothecene ring system, may be involved in toxicoses throughout the world, although there is distinct evidence implicating these compounds in only a few outbreaks. Dr. Ciegler will describe these outbreaks and will discuss this important group of compounds.

The best evidence to date suggests that mycotoxins are a clear and present hazard to man's animals, be they domesticated or companions. A major problem in veterinary medicine includes diagnosis of mycotoxicoses of livestock. In such case livestock production, and thus profit, is reduced. Dr. Cysewski will describe some of the very interesting studies that he and his colleagues at the National Animal Disease Laboratory have undertaken.

Finally the suggestion that mycotoxins could be responsible for human liver cancer and other human diseases opened up a whole new and important field of investigation. The concept that mold-produced toxins could cause chronic diseases in man was a major advance in thought, and it has proved to be the trigger which

unleashed an avalanche of research in this field. In the context of human disease, particularly in developing nations, the idea that fungi might be responsible for chronic disease, particularly liver cancer, has received enthusiastic support. There are a number of factors which make study of such a hypothesis uniquely difficult. Not least among these is the inability to use the human organisms as an experimental model. Dr. Benjamin Wilson of Vanderbilt University School of Medicine will discuss field surveys and epidemiological and experimental data attempting to examine the possible relationship of mycotoxin contamination of foodstuffs in the incidence of human diseases. The role of native food customs and regional climatic factors appear important and these also will be discussed by Dr. Wilson.

This seminar has the promise of being an occasion which will be remembered because we have all the ingredients necessary for success. We have five dynamic researchers assembled to discuss a subject which is not only scientifically interesting but which is of great concern in many countries throughout the world. Finally, we have an audience with an interest in this subject, thus giving us the opportunity of exchanging ideas and learning.

ACKNOWLEDGMENTS

The seminar on "Mycotoxins — A Real or Potential Problem" was held at the Annual Meeting of the American Society for Microbiology, New Orleans, Louisiana, May 11, 1977. Papers given at the seminar and described in this Introduction will appear in the *Journal of Food Protection*.

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Hazards of Mycotoxins to Public Health

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ABSTRACT

Since the discovery of aflatoxins, the ultimate question concerning mycotoxins in general is whether they relate to human as well as animal health. Sources of information on this question include results of several food and health surveys done in various countries. These studies have examined the possible relationship of mycotoxin contamination of foodstuffs and the incidence of endemic human diseases in geographically defined regions. In addition, reports linking toxin contamination of dietary items and isolated cases of disease have also come from certain countries. Most of these reports have been concerned with aflatoxins which are among the more easily detected mycotoxins in foods. Suitable analytical methods for the more obscure mycotoxins are sorely needed. Certain recognized diseases which might be attributable to mycotoxins require further investigation. Factors favoring mycotoxin contamination of human foods are usually quite obvious, involving improper harvesting and methods of food storage that favor fungus contamination and growth. Use of fermented foods and beverages by native populations may offer certain risks to health; the role of regional food customs and climatic factors favoring fungus contamination of foods are also important. Proper inspection and control of food by governmental agencies as protective measures should be emphasized.

Before discovery of the aflatoxins, the question was frequently asked whether toxins played a significant role in diseases of livestock and other animals. The pioneering work of Forgacs and his associates (20) in the United States and Mortimer, Taylor, Hodges, and others (54) in New Zealand helped to answer this question at least for certain animal diseases. After discovery of aflatoxins and demonstration of their carcinogenicity in laboratory species, the next important question frequently asked was, "Are mycotoxins involved in human health?" The answer, we know now, is a definite, "Yes". However, the proper response to a logical follow-up question is not so clear; namely, "To what extent are mycotoxins, particularly aflatoxins, affecting human health?" The importance of these queries is obvious when one considers that environmental agents may be causing many of the neoplasms of man (48). Of course, our concern with mycotoxins is not confined to their carcinogenic potential since many different tissues and organs of susceptible animals may be targets of the wide

variety of fungus toxins now recognized, and several disease manifestations may ensue from their toxic action.

It is obvious that an assessment of human susceptibility to mycotoxins is difficult to make since we dare not purposely employ humans in toxicological experiments. Thus, investigators have attempted to extrapolate animal data to man, realizing that species variations often make such data of limited value. The use of sub-human primates is generally thought to allow for more meaningful extrapolations. Perhaps most useful estimates of man's susceptibility and involvement are gained from analyzing the mycotoxin levels of human foods and observing the type and severity of diseases associated with their consumption (12).

Although ingestion of moldy cereals, vegetables, or nuts is probably the most important mode of contact with mycotoxins for both man and animals, other means of acquiring intoxications must also be considered. These include skin contact with certain dermally toxic compounds, consumption of contaminated meat or milk, and, on occasion, aerosol inhalation of dust containing fungus elements and toxins. Laboratory personnel working with toxigenic fungus cultures or their extracted toxins should be aware of the health hazards inherent in many experimental manipulations done in the laboratory.

As with animals, the nutritional status of man might be expected to play a role in his susceptibility to mycotoxins (42). The effect of this factor, however, is not always predictable on the basis of present knowledge derived from animal studies.

In highly civilized societies, food that is obviously moldy or that is organoleptically objectionable, is likely to be rejected. However, toxin may be present in certain foods or beverages that do not present these warning signs to the senses of sight, smell, or taste. As with animals, the element of hunger can be expected to favor ingestion of contaminated food by man where no alternative is available. In primitive societies food shortages, along with lack of knowledge as to how foods

should be cultivated, harvested, selected for use, properly stored, or held after cooking can augment the intake of undesirable foods. Moreover, use of primitive fermentation procedures for both food and beverage could conceivably favor formation of microbial toxins. Conditions that lead to mycotoxin contamination of foods have been reviewed by Hesseltine (25).

Surveys designed to reveal the possible relationship of mycotoxins in food and human diseases are sometimes made less meaningful by the presence of other deleterious environmental agents. Specifically, one must consider practices such as use of toxigenic native medicinal plants which can produce pathological lesions somewhat similar to those caused by certain mycotoxins (16). Similarly, it is often difficult to differentiate among lesions caused by infectious agents, such as endemic infectious hepatitis virus, and hepatotoxic fungus metabolites.

HISTORICAL ASPECTS

By definition, classical ergot poisoning in man was also a mycotoxicosis. This being the case human involvement in fungus intoxications has a history dating back several centuries. Ergotism from food, which apparently can manifest itself by both a hallucinogenic state and by gangrene development in the limbs, has an interesting history. Somewhat belated recognition of cereal grain infected with *Claviceps purpurea* as the cause of this disease led to measures that have largely eliminated ergotism in man in most parts of the world.

The pictorially documented mass outbreak of hallucinogenic disease in Pont St. Esprit, France, in 1951 by *Life* magazine (4) was considered by some experts to be due to contamination of flour by a mercury compound rather than ergot as first commonly assumed. The unusual mass affliction and the prolonged trauma among those residents who consumed the bread of madness were the subjects of a book entitled, *The Day of St. Anthony's Fire* (21). An extensive review of the problem of ergot and ergotism was provided by Barger (7). A condensed historical account, along with extensive coverage of the chemical compounds extracted from cultures of *C. purpurea*, have been written by Van Rensburg and Altenkirk (58).

The early interest of Japanese investigators in fungus toxins was based, in part, on the knowledge that mold-contaminated food had apparently caused illness and death, several decades ago, among groups of people in Japan and other rice-eating countries. For example, it is strongly suspected by several Japanese scientists that acute cardiac beriberi was related to use of moldy rice, and that a subsequent decrease in cases coincided with institution of rice inspection by the governments involved (56). Cardiac beriberi was frequently encountered in Japan in the latter part of the 17th century and beginning of the 18th century, but is now rarely seen in that country.

Uraguchi and coworkers (57) have documented outbreaks of disease in the Orient which may have been

caused by moldy food: (a) In 1971 a large number of Korean immigrants in Tunghwang, Manchuria ate microbially damaged corn over a period of a few months, and many died as a result. Findings at necropsy included degenerative changes in the liver and heart. (b) In the autumn of 1952, within the suburbs of Tokyo, some 25 persons were made ill from eating rice contaminated with *Fusarium* sp. The illness which included nausea, vomiting, and drowsiness, was similar to outbreaks of poisoning occurring in other districts. This author speculated further that the high incidence of primary hepatic carcinoma in tropical countries of Asia was probably related to eating damaged rice.

Saito and Ohtsubo (43) described an outbreak of human disease occurring in Tochigi and Kochi prefectures of Tokyo in 1955 attributed to deteriorated rice containing toxigenic *Fusarium* species. More than 40 persons were affected, suffering nausea, vomiting, and diarrhea. Similar poisonings occurred several times in Hokkaido in the post-war years and in 1946 in Tokyo. Imported wheat flour containing *Fusarium graminearum* was isolated but not proven to be the toxigenic agent.

Most persons familiar with mycotoxins are probably well informed on the human mycotoxicoses that occurred in the Soviet Union during the second World War. Overwintered grain infected with *Fusarium* sp. was the cause of a syndrome known as alimentary toxic aleukia (ATA) (22). ATA developed in increasingly severe stages with the continued intake of toxic food. More recent studies have shown that the fungus isolates responsible for ATA are capable of producing trichothecenes (53), a fact that correlates well with the pathological picture in animals injected or fed the purified compounds. Stachybotryotoxicosis, a disease of horses and man that occurs in Russia and other countries, is caused by *Stachybotrys atra*, which also produces trichothecenes.

AFLATOXINS AS AGENTS OF HUMAN DISEASE

Undoubtedly the initial discovery of aflatoxins in the 1960's (13) and preliminary assessments of their toxic potential, especially knowledge of their carcinogenicity in rats (46), were major events leading to worldwide recognition of the importance of fungus toxins to both animal and human health. Soon thereafter the words "mycotoxin" and "mycotoxicosis" were coined, and their meanings gained considerable significance. A factor aiding all phases of research in aflatoxins is their remarkable fluorescence under long wave ultraviolet light. This property has enabled their detection as naturally occurring contaminants of several foods used by man or animals in many parts of the world (62). This property has also led to methods for quantitation of aflatoxin in food samples. The analytical data in turn have been related to the incidence or extent of liver disease in various human populations.

Public health implications for these compounds were realized early. Dr. Max Milner of UNICEF, of the United

Nations Organization, convened a meeting of interested scientists in New York City in October, 1962, to help assess the hazards of aflatoxin to human health. Under consideration was suspension of the ARLAC infant food program in Nigeria since the peanut meal component was likely at that time to be contaminated with these metabolites of *Aspergillus flavus*.

Since their discovery, aflatoxins, more than any other mycotoxins, have been considered as prime threats to health. This is especially true in tropical countries of the world where the humidity is high and living conditions are often primitive. In several of these areas post-necrotic cirrhosis of the liver and primary hepatoma show a relatively high incidence and appear to have an environmental etiology (33).

Data suggesting that aflatoxin¹ was undoubtedly ingested by population groups of several countries and was related to hepatic disease have come from three types of studies. The first of these consisted of surveys of various food commodities for toxigenic fungi and for aflatoxin levels. The frequency and levels of toxin in foods were then related to previously recorded incidence of human disease, mostly primary hepatoma, in the selected region. More specific information has been provided where individual prepared food samples were analyzed for toxin and compared to the hepatoma case rate. Where an individual's intake of aflatoxin could be determined, secretion of toxin in breast milk, or excretion in urine, were also sometimes measured. A second general source of information has come from unusual outbreaks of disease among groups of people in which the cause was apparently related to consumption of a common type of food that was mold-contaminated and contained measurable levels of aflatoxin. Thirdly, there have been reports of somewhat isolated cases where one or a few persons, usually children, have become ill after consuming aflatoxin-laden food. From these cases the aflatoxin levels of the food were determined which, along with knowledge of the period of ingestion, gave an estimate of total dosage.

Possible sources of erroneous information in each of these situations are obvious, but in spite of imprecise data collection methods, information has been gained indicating correlation between aflatoxin levels of foods and incidence of human liver diseases.

The following are brief resumés of some of the surveys, epidemics, and individual cases that have been reported over the last decade from different geographical regions of the world

Uganda

Alpert and coworkers (2) surveyed the incidence of primary hepatoma in various tribal regions of Uganda in 1966-1967. The incidence of liver disease, particularly liver cancer, was considered to be high in that country (1). Aflatoxin levels were determined in 480 food samples

stored for consumption between harvests. Approximately 30% of these contained detectable levels of aflatoxins and 3.7% had more than 1 mcg/kg. The primary hepatoma rate was fairly uniform over most of the country, ranging from 1.4 to 3.0 cases per 100,000 population per year except for Karamoja region of the Northern Province which had a rate of 15.0. Of 105 food samples analyzed in this area, 43.8% were aflatoxin-positive which was significantly higher than for most other regions sampled. Assuming a daily food consumption of 500 g of staple grain, per capita ingestion in Karamoja could be on the order of 0.02 to 2.0 mg daily, a level known to be hepatotoxic to monkeys. Karamoja has a dry semi-desert climate whose annual rainfall is concentrated into a short rainy season once or twice a year, turning the semi-desert region into mud fields for several days or weeks. Thus, the microenvironment of the food storage area was considered to be a major factor determining mold growth.

The Toro region, containing the Bwamba tribe, had considerably higher frequency of aflatoxin as well as higher average amounts of aflatoxin in food samples. However, no data on cancer incidence were available for this region.

The authors believed that both poverty and food scarcity among tribes living in Karamoja also contributed to chronic ingestion of foods that were moldy and contained relatively high levels of aflatoxin. There was no appreciable difference in the aflatoxin content of food stored in the raw form or as milled or cooked preparations. Neither the type of storage nor its duration seemed to affect the aflatoxin content. Three of five foods fermented and stored for use in beer production were heavily contaminated.

Taiwan

The incidence of primary liver cancer has been high in Taiwan compared to that of the United States and Europe. The climate of the island is generally warm and humid which tends to favor mold growth on foods. Nearly all agricultural products are sun-dried in the field and stored in damp storehouses. A survey in 1966 of market rice, sweet potatoes, and peanuts revealed occasional contamination of sweet potatoes and peanuts by aflatoxin, but none was found in market rice samples. However, moldy rice samples found to contain aflatoxin-producing fungi were believed to have caused illness of 25 persons, among 39 members of three farm families from Shung-chi township of Taipei county. Three of the 39 died of undiagnosed illness. This report by Tung and Ling (55) offers only very speculative information on the role of aflatoxin in the illnesses of these persons. However, other Taiwanese investigators have supplied information to the present author strongly suggesting that moldy food is a factor in the relatively high incidence of liver cancer on Taiwan. Some cases that were diagnosed early were amenable to partial hepatectomy.

¹The singular "aflatoxin" is often used generically to include all of the closely related compounds implied in the plural of this word.

Kenya

Peers and Linsell (35) divided Murang'a district of Kenya into three geographical areas based on altitudes (high, middle, and low) and analyzed for aflatoxin levels in foods prepared for human ingestion. The sampling continued over a period of 21 months to afford some measure of seasonal and annual replication. The data obtained were compared with national cancer registration data for this district provided by the Kenya Cancer Registry for the years 1967-1970. Diagnosis of hepatocellular cancer was based on histological findings, a positive alpha-fetoprotein test, or clinical diagnosis within 6 months of death when the first two criteria were not obtainable. A statistically significant association between ingested levels of aflatoxin and liver cancer cases in the designated altitude areas was obtained which required certain qualifications, because of possibly incomplete data, before the etiological significance was considered. There was an increasing frequency of both aflatoxin-contaminated diets and mean levels of food contamination as the area altitudes decreased which also corresponded with increased incidence of liver cancer. These workers cautioned that the Murang'a district findings covered only a small portion of possible associations of dietary aflatoxin and liver cancer cases and that additional studies should be done in areas with higher rates of liver disease or higher food levels of aflatoxin to test the strength and consistency of this association.

Swaziland

Primary liver cancer, particularly in males, has long been a serious problem in this small African country. Cancer Registry figures for 1964 to 1968 represent a crude rate of 8.6 per 100,000 for males and 1.6 for females. The incidence among the small number of Shangaan immigrants living in the same environment was considerably higher than the Swazis in the age group of 25 to 64 years. An attempt was made by Keen and Martin (26) to correlate the presence of aflatoxin in peanuts with the prevalence of primary hepatoma in four geographical divisions. The incidence of liver cancer decreased with increasing altitude; the risk of acquiring liver cancer in the lowveld was at least four times greater than in the highveld. Forty percent of all peanut samples collected contained aflatoxin, and the geographical sources of positive samples correlated well with the incidence of primary liver cancer in the different areas. The increased prevalence of hepatoma in the Shangaans was attributed to their peculiar habit of eating larger quantities of peanuts. Better harvesting and storage methods in the southern region of Swaziland apparently resulted in a smaller percentage of aflatoxin-positive peanut samples along with a lower number of primary liver cancers, as recorded by the Registry.

Peers et al. (36) extended their previous survey work in Kenya to Swaziland where three major and one minor altitude areas were selected for study. As before, "food

from the plate" samples were obtained. The three major areas consisted of the highveld, middleveld, and lowveld regions mentioned above, whereas the Lebambe range in the extreme eastern part of the country made up the fourth area. This latter region approximates the middleveld in altitude but resembles the lowveld in respect to vegetation in many places. The primary liver cancer crude rates for all ages in Swaziland for 1964-1968 was 4.9/100,000 compared with 3.3/100,000 for all ages in the Murang'a district of Kenya for 1967-1970. Here, as in the Murang'a survey, the principal significant factor relating to aflatoxin contamination frequency and mean toxin levels was the altitude of respective areas in which food samples were collected. These workers found, as had Keen and Martin (26), that the northern parts of each region showed greater contamination incidence, but there was no significant difference in mean contamination levels between the north-south regions. Surprisingly, there was a lack of seasonal effect on the aflatoxin content of the diets. Peanuts, beans, and cultivated vegetables were included more frequently in the diets containing aflatoxins. Maize could not be specifically incriminated as a source of aflatoxin since it was included in almost all of the plate samples.

It was not possible from this study to show an increased risk for liver cancer among the Shangaans. Moreover, although there was an increased frequency of liver cancer in the northern portions of the high and middleveld areas, this was not true of the country as a whole. This study clearly demonstrated, however, that, at least in males, a logarithmic relationship existed between liver cancer incidence and aflatoxin ingestion.

Mozambique

According to Purchase and Goncalves (39), liver cancer is common among the relatively stable population of the Inhambane district of Mozambique. Based on 101 cases encountered in 1968 at the Chicucque Hospital, the liver cancer rate was 16 per 100,000 per annum with the male to female ratio 2.1 to 1. Maize, peanuts, rice, beans, manioc and cashew nuts were the staples drawn from nine families of liver cancer patients and from other family sources. Although the total number of food samples was small, there was a somewhat higher incidence of positive samples obtained from the families of cancer victims.

Van Rensburg and coworkers (59), in a 1974 report, gave additional data suggesting the Inhambane district had the highest known primary hepatoma rate in the world based on hospital registrations and data on occurrence of the disease from health records of gold miners in the study area. Their figures for the 1964-1968 period were 35.5 cases/100,000/year; and for 1968-1971, 25.4/100,000/year. The male to female ratio was 2 to 1. Aflatoxin assays on 880 meals, collected at random, revealed that 9.3% contained aflatoxin. The mean level of all prepared food was 7.8 mcg/kg wet weight, and the

mean daily per capita consumption was 222.5 ng/kg of body weight or 15.6 mcg/adult/day. These figures were compared with similar data from Kenya and Thailand. The study areas in Kenya had a hepatoma incidence of 3.2/100,000/year with an estimated intake of 7.8 ng/kg/day; and in Thailand, 7.3/100,000/year and 74 ng/kg/day. These pooled data indicated that over a wide range, cancer incidence appeared to be linearly related to the logarithm of the level of aflatoxin intake.

The Philippines

Campbell and Salamat (11) reported a survey carried out in 1967-1969 on aflatoxin contamination of a variety of foods including peanuts and peanut butter in the Philippines. They found that generally a better quality of peanut was selected for the sale of whole shelled kernels than was true for those used in peanut butter. The latter samples contained aflatoxin at a median value of 155 mcg/kg and a very high mean of 500 mcg/kg, whereas whole peanuts had a median of 17 mcg/kg. Candy containing peanuts was at an intermediate level of contamination. Highest concentrations of peanut aflatoxin were noted in the health region that included Manila (Region No. 3) where most of the locally produced peanut butter was consumed. Health region No. 6 included the island of Cebu, where maize was eaten by a large percentage of the people. This commodity was one of the more highly contaminated foods of that region. Both regions, No. 3 and No. 6, also had the highest rates of liver cancer.

Twenty-four hour urine samples collected from individuals eating peanut butter with known concentrations of aflatoxin contained only the M_1 toxin, presumably derived from B_1 in the food. It was calculated that not more than 1 to 4% of ingested B_1 appeared in the urine as M_1 , and that the minimum daily consumption of B_1 required to produce detectable levels of M_1 was 15 mcg/day. Human fecal and milk samples did not give positive results even when urine samples were positive for M_1 .

Southeast Asia

Shank and Wogan of MIT, along with various collaborators in Hong Kong and Thailand, carried out an extensive survey of fungus and aflatoxin contamination of prepared and market foods in Southeast Asia and compared the data with recorded incidence of liver carcinoma in various regions of that part of the world. In the initial phase (50) they screened more than 3000 food samples, showing that *Aspergillus* was the most common genus and that *A. flavus* was the predominant species occurring on foods. *Penicillium*, *Fusarium*, and *Rhizopus* were also frequently isolated. One hundred sixty-two isolates were obtained, and 49 of these produced toxins other than aflatoxins when grown on food materials. The extracts were fed orally to rats to determine toxicity.

Analysis of more than 2000 market foods and foodstuffs from Thailand (51), representing at least 170 different human foods, demonstrated that peanut

products were the food items most frequently and highly contaminated with aflatoxins. Somewhat less often involved were dried corn, millet, wheat, barley, Job's tear seeds, and dried chilli peppers. The frequency and extent of contamination of foods followed geographical distributions and seasonal trends. In Hong Kong different varieties of beans constituted the chief sources of aflatoxins among the foodstuffs examined. The frequency and levels of aflatoxin in Hong Kong foods were lower than in Thailand. Rice in both places was seldom contaminated and only at low levels.

Three rural areas of Thailand were selected for sampling and analyzing prepared foods for aflatoxin (52). One hundred forty-four randomly selected households (families) in nine villages were the sources of cooked food collected by three 2-day surveys over a period of one year. Estimates of individual human intake based on the results of these analyses were highest in the Singburi and Ratburi areas with respective annual means of 73 to 81 ng and 45 to 77 ng total aflatoxin/kg/body weight/day. Intakes as high as 1072 ng/kg were noted for some individuals. In the Songkhla area the aflatoxin intake was 10 to 14 times less than in Singburi. People in two of these areas (high and low liver cancer areas) were surveyed for incidence of confirmed primary hepatoma. In line with data on aflatoxin ingestion obtained from Ratburi, the incidence of liver cancer was six new cases/100,000/year. In the town of Ratburi itself, the rate was 12.3 new cases/100,000/year. However, in the Songkhla area only two new cases/100,000/year were confirmed, which is comparable to the rates noted in parts of the United States. The male to female ratio overall in Thailand has been reported as 6 to 1 while in the U.S. the ratio was estimated at 1.5 to 1.

Senegal

Payet et al. (34) described the effects of inadvertent administration of aflatoxin-contaminated peanut meal in Kwashiorkor therapy to two African infants, just under 12 months of age, for nearly a year. Each received 70 to 140 g of peanut meal daily for an estimated intake of 35 to 140 mcg aflatoxin/day (5 to 20 mcg/kg of body weight). The meal contained 0.5 to 1.0 mg of aflatoxin/kg (type not specified). The children were located for study 4 and 6 years later. Biopsies revealed a fibrotic liver in one child which persisted through the sixth year; the other had minor histological changes in the liver which appeared to resolve within the next 2 years.

India

Robinson (41) in 1967 reviewed the epidemiology and possible causative agents of infantile cirrhosis and presented evidence strongly indicating that aflatoxin may be the prime factor. The syndrome was first described in 1887 and is now recognized all over India. Similar or identical conditions have been noted in Ceylon, Indonesia, West Africa, Costa Rica, Trinidad, Israel, Lebanon, Syria, Egypt, West Indies, Burma, and the

Soviet Republic of Tadzhekistan. The disease frequently strikes several children of the same family. Most cases in India have been in infants and children of 1 to 2½ years of age.

The initial histopathological change is seen as infiltration of hepatic cells with fat, followed by disintegration of centrilobular cells. Fibrosis soon ensues with enlargement of the liver, and yellow urine. In the following stage the spleen becomes enlarged, and the liver becomes shrunken. Jaundice becomes more pronounced as portal obstruction develops, producing ascites and edema of the limbs.

Analyses of the milk of Indian mothers of cirrhotic children were positive for aflatoxin B₁ in three cases out of 43 examined; 12 others exhibited violet fluorescent spots at other R_f levels on thin layer chromatography plates, and three gave fluorescent spots with colors other than violet. The remainder were negative. Eighteen of 50 urine samples from cirrhotic children were considered positive for B₁. Urines from normal subjects gave negative results.

Amla et al. (3) described the condition of 20 children (19 kwashiorkor, 1 nephritis), ranging in age from 1.5 to 5 years, who received daily food supplements of 30 to 60 g of peanut meal later found to contain 0.3 mg of aflatoxin B₁/kg. This would amount to 9 to 13 mcg toxin or an average of 1.1 mcg/kg of body weight. The meal was given for a period varying from 5 days to 1 month. Three children who received the supplement for 17 days developed signs of liver cirrhosis that began as a fatty liver and developed into cirrhosis over a one-year period. The characteristic clinical signs and histopathological lesions noted in biopsies were not seen before 6 months.

Screenivasamurthy [cited by Campbell and Stoloff (47)] reported negative biopsy data for cirrhosis and negative clinical symptoms in kwashiorkor children receiving peanut meal containing 15 mcg/kg.

Krishnamachari and coworkers (29) described a 1974 outbreak of acute toxic hepatitis with high fatality rate in adult humans and dogs which was attributed to consumption of maize contaminated with aflatoxin. Analysis of the food indicated the affected persons, who were poorly nourished, may have eaten 2 to 6 mg of toxin daily for one month.

A total of 397 patients were studied in the adjacent states of Gujarat and Rajasthan, 106 of whom died. The contamination of grain was attributed to heavy rains in October and poor storage conditions. Death was usually sudden and in most instances was preceded by massive gastrointestinal bleeding. Males were affected twice as commonly as females. Dogs sharing the family diets were also stricken. Liver analyses did not show aflatoxin B₁ but did show other fluorescent spots. Of seven serum samples collected, two had detectable B₁ spots on thin layer plates, but all urine samples were negative. The outbreak of disease lasted only until the supply of moldy maize was exhausted. In afflicted families, persons who did not partake of the diet remained healthy.

Bile duct proliferation and periductal fibrosis were noted histologically. Jaundice, rapidly developing ascites, and portal hypertension were cardinal clinical signs.

The high levels of aflatoxin on the grain (6.5 to 15.6 ppm) would account for the massive gastrointestinal hemorrhaging which is similar to that recorded for dogs afflicted with hepatitis x (5) and experimental animals receiving high doses of aflatoxin orally (60).

AFLATOXINS AND REYE'S SYNDROME

Reye's syndrome, first described in 1963 as "encephalopathy and fatty degeneration of the viscera" (40) has since been recognized as a major cause of morbidity and mortality among infants and children (17). It is an acute disease that may affect those of a few months of age to adolescence. The disease often progresses from a mild prodromal viral illness with vomiting and abdominal pain due to influenza B or chickenpox, to cerebral involvement with coma. Blood ammonia levels are often high early in the course of the illness, whereas glucose levels are low. The mortality rate has been about 40%, but survivors may recover rapidly.

The Viral Diseases Division, Bureau of Epidemiology, at the Communicable Disease Center in Atlanta reported that 220 cases of Reye's syndrome were documented from 33 states and the District of Columbia for the period January 1 through March 31, 1977. In 30 of the cases, in which the outcome was known, 19 died, two survived with residual neurologic damage, and nine recovered completely.

One hundred thirty-nine cases of what appeared to be Reye's syndrome among children in Thailand were reported by Bourgeois et al (10) in 1969. Eighty percent of the hospitalized cases ended fatally. It has been estimated that several hundred children in Thailand, between the ages of 1 and 13 years, may die each year of this condition. The seasonal and geographic incidence of aflatoxins in Thai food markets seems to parallel frequency of the disease.

Aflatoxin analyses were reported by Shank and a group of Thai coworkers (49) on post-mortem specimens from 23 Thai children who died from acute encephalopathy and fatty degenerative changes in the visceral organs (EFDV), and from 15 children and adolescents who died of unrelated causes. Aflatoxin B₁ was found in one or more specimens from 22 of the 23 cases of EFDV. In two of the cases, very high concentrations of aflatoxin were found in liver specimens. A 2-year-old boy had 93 mcg of B₁/kg of liver, 123 mcg/kg in stomach and intestinal contents, and 8 mcg/ml of bile. Trace amounts of B₁ were demonstrated in post mortem specimens from 11 of the 15 control subjects.

Dvorackova et al. (18) found aflatoxin in the livers of two infants of three fatal cases presenting features of Reye's syndrome in Czechoslovakia. It was considered likely that the subjects were first exposed to aflatoxin during intrauterine life or soon after birth. Another group of investigators (14) detected what appeared to be

an aflatoxin metabolite in the liver of a 15-year-old girl who died of Reye's syndrome. Seven other patients with the same diagnosis did not show chromatographic evidence of aflatoxins in their liver samples.

Hayes (24) detected B₁, using high speed liquid chromatography, in the plasmas of three patients with Reye's syndrome recently at the University of Mississippi Medical Center. Concentrations of toxin ranged from 3.4 to about 12 ppb. Urine, liver, kidney, and brain samples were negative.

Harwig et al. (23) have reviewed various reports on Reye's syndrome and outlined the evidence supporting aflatoxin as an etiological factor. They called for submission of properly collected and transported liver specimens from suspected cases in Canada to the Health Protection Branch of the Canadian Health and Welfare Department.

It seems apparent that what is commonly diagnosed as Reye's syndrome could have more than one causative agent; i.e., a virus or a toxin, or possibly both agents acting in concert.

If the foregoing accounts are accurate in their implications, the evidence suggests that many thousands, if not millions, of people in different parts of the world may consume aflatoxins in several naturally contaminated foodstuffs. Children appear to be particularly susceptible and have, in several cases, been the victims of lethal quantities of toxin. The liver of man, as with primates and other animals, apparently is the principal target organ. Depending on the individual's age, levels of toxin intake, and the duration of dosing, the human response apparently may consist of acute to chronic hepatitis with clinical jaundice with fatty metamorphosis and necrosis of hepatocytes leading to death from internal hemorrhages. As in the case of chronic alcoholism, subacute to chronic intoxication from aflatoxin may cause liver cirrhosis. Also, as with other species, the human liver may show ductular cell proliferation and malignant neoplasia that ends in early death from the time hepatoma is first detected.

The importance of carefully planned human health surveys is evident as one studies data and conclusions from the foregoing reports. The necessity for compiling complete and accurate cancer statistics is matched by the need for valid analytical methods for aflatoxins in foods. Certain anomalous results, perhaps due to aflatoxin metabolites in human livers or urine specimens, need to be investigated in view of recently identified metabolic derivatives of these compounds. The possibility of false-positive data caused by unrelated substances from certain food sources should also be considered.

Finally, one must be aware of other means of acquiring poisoning from aflatoxins than through ingestion of contaminated foods. Those who work with these substances in the laboratory are prime subjects for accidental or careless exposure which can lead to skin contact, ingestion, and inhalation of toxins. Aerosols may be generated from mold dust, spills and pipetting of

toxin solutions, scraping thin layer plates, and several other potentially hazardous procedures. Dvorackova (19) reported the observation of alveolar cell carcinoma in a 68-year-old chemical engineer who had worked for 3 months on methods of sterilizing Brazilian peanut meal contaminated with *A. flavus*. Analysis of the excised lung demonstrated a blue fluorescent spot on thin layer chromatography whose R_f matched that from a commercially available sample of B₁.

In areas where dairy products are a major food commodity for man the possible periodic contamination of milk with aflatoxins B₁ or M₁ should be considered since livestock are often fed aflatoxin-containing corn or other moldy grains. Such contamination of commercial milk with M₁ was reported in South Africa by Purchase and coworkers (38) and in Germany (milk and cheese) by Kiermeir and coworkers (27,28). Processing of milk was shown to reduce the M₁ content.

In the United States extensive regulation of food production and marketing by various governmental agencies tends to decrease chances of aflatoxin entering our food supply to a hazardous extent. Phillips and Yourtee (37), however, have recently reported finding B₁ toxin in a liver biopsy specimen taken from a 56-year-old male Caucasian rural resident of Missouri who had cancer of the rectum and liver. It was estimated that 520 ng of B₁ aflatoxin was present per gram of wet liver. This incident as well and the finding of B₁ in Reye's syndrome patients suggests that more extensive surveys should be carried out to assess, if possible, the role of aflatoxins in human diseases in this country.

DERMOTOXIC FUNGUS METABOLITES

Mycotoxins known to produce dermal reactions in humans by direct contact are mainly of two sources. The first of these is pink-rotted celery infected with *Sclerotinia sclerotiorum*. Two compounds, 8-methoxy-psoralen and 4,5',8-trimethoxy-psoralen, produced by the fungus are capable of sensitizing the integument of celery harvesters with light skin so that subsequent exposure to light of about 320 to 400 nm wave length for a short period will give rise to bullous lesions (44). Photosensitization associated with food consumption by man has been reported but not well characterized (45). However, this syndrome is well known for animals that consume grass infected with *Pithomyces chartarum* and other naturally toxic plants. The photosensitization is thought to be caused by liver damage that results in accumulation of sensitizing serum and tissue levels of phyloerythrin, a metabolite of chlorophyll.

The second group of direct skin irritants belongs to the class of fungus metabolites known as 12,13-epoxytrichothecenes. These compounds are produced by several food-contaminating fungi including species of *Fusarium* (6). Many also exert toxic effects on bone marrow and other tissues as mentioned earlier for the disease

alimentary toxic aleukia. The author has known of two laboratory workers who suffered severe facial, upper limb, and trunk skin eruptions due to inadvertent direct skin contact with pure T-2 toxin or culture extracts of *F. graminearum*. It is quite clear why inhalation of trichothecene-containing fungus dust from grain, hay, or other contaminated plant material can produce upper respiratory tract irritation in humans as reported by Russian investigators for hay infected with *Stachybotrys atra* (*alternans*) (9).

HUMAN DISEASES WHICH MAY BE ATTRIBUTABLE TO FUNGUS TOXINS

Neurological diseases

There are now several tremorgenic mycotoxins recognized which are produced by food-contaminating fungi. The complex chemical structures of certain members have also been elucidated (15). It seems reasonable to suppose that these substances may be finding their way into human foods on occasion. Although they are potent neurotoxins, producing acute reactions in animals at dosages of a few milligrams/kg of body weight, it probably would require ingestion of a considerable amount of well-molded food at one sitting to produce noticeable effects in man.

In western Nigeria a seasonal affliction of natives, known as Ijesha Shakes, is a fairly common diagnosis upon hospital admission (63). The cause is unknown, although cyanogenic glycosides or tremorgen-containing foods are considered likely possibilities. Symptoms of the disease usually begin after eating, and nearly complete incapacitation is caused by intentional tremors, particularly of the legs, when the patient attempts to stand. The trembling may last for a few days followed by complete recovery. Certain outbreaks of "grass staggers" of sheep and cattle in New Zealand appear to be identical to the neurological disease signs induced by feeding pure toxin or mycelium of *Penicillium cyclopium* which contains the well-known tremorgenic toxin called penitrem A (61).

Balkan nephropathy

Balkan nephropathy is an endemic disease of people living in close proximity to the Danube River and its tributaries in Yugoslavia, Romania, and Bulgaria (32). It is estimated that there may be more than 20,000 cases within rural areas of these regions. The disease presents a clinical picture of slowly progressing renal failure, seldom causing sodium retention or systemic hypertension. The cause(s) of the disease remains unknown in spite of broad studies attempting to associate it with several possible causative agents. The possibility of a mycotoxin etiology has been attractive, and studies to ascertain the incidence of ochratoxin in foodstuffs used in the Balkan region have been made by at least two groups. Krogh (30) has indicated that 8 to 20% of the foodstuffs in one endemic village in Yugoslavia contained ochratoxin A, a well known nephrotoxic substance for swine and poultry in Scandinavia (31).

However, the late J. M. Barnes and five coworkers recently published a report (8) showing that *Penicillium verrucosum* var. *cyclopium* was the most frequent isolate from some 163 samples of foodstuff and other materials collected from five endemic areas of the disease. Liquid cultures of one of the isolates was force-fed to young rats and caused subtle histological changes in cells of the lower part of the proximal convoluted tubules. The location of the lesions and their morphology were considered to be almost identical to those in the disease of man. Speculation that the toxic principle(s) may be the cause of Balkan nephropathy will certainly lead to additional study.

This review has mentioned only a few of the many recognized mycotoxins that could be involved in human health. Undoubtedly, several other important metabolites remain undiscovered or unrecognized as toxins. A pressing need exists for suitable analytical methods permitting detection and quantitation of the more nondescript mycotoxins that may occur in human foods. Recommendations for further research on this and related problems were generated by a meeting of the United States-Japan Conference on Mycotoxins in Human and Animal Health held at College Park, Maryland in October, 1976. Continued research and vigilance on the part of governmental regulatory agencies concerning mycotoxins are required to afford protection to human and animal population.

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Mycotoxins in Feeds and Ingredients and their Origin

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ABSTRACT

Data from many parts of the world on incidence and levels of aflatoxins in feeds and feed ingredients indicate cause for concern, especially in tropical and sub-tropical regions and in those countries importing feedstuffs from these areas. The main products affected are peanut (groundnut) meal, cottonseed products, corn, copra products, and mixed feeds containing any of these components. Reports of the presence of the metabolite aflatoxin M₁ in dairy products provide further evidence of contaminated feeds. Although the main survey effort has been with the aflatoxins, surveys in several countries for other fungal toxins have revealed some contamination of certain grains or pasture grasses with ochratoxin A, citrinin, sterigmatocystin, patulin, penicillic acid, zearalenone, trichothecenes (T-2 toxin, deoxynivalenol, nivalenol, and diacetoxyscirpenol), alternariol and its methyl ether, PR toxin, kojic acid, ergot alkaloids, and sporidesmin. It is important to differentiate between biased and unbiased surveys in evaluation of these data. Mycotoxins usually result from improper storage of feedstuffs, although in certain cases they can also be formed before harvest. Tenuazonic acid has been detected in blast diseased rice plants. Dicoumarol may be produced in sweet clover as a result of fungal activity. Ipomeamarone and related compounds in the sweet potato are examples of toxins synthesized by plants in response to invasion by molds or to other stress conditions.

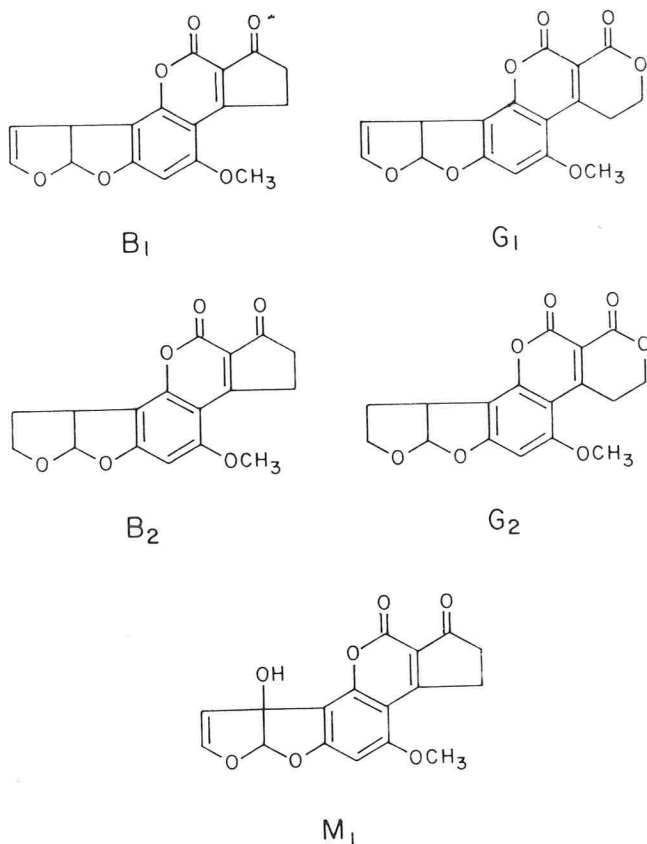
Investigations over the last 6 years have greatly expanded our knowledge of the natural occurrence of mycotoxins in grains and other feedstuffs. Surveys to date have concentrated on aflatoxins B₁, B₂, G₁, and G₂, ochratoxin A, and zearalenone (55,56,137,166). However, there is also evidence for contamination of grains or pasture grasses by aflatoxin M₁, ochratoxin B, citrinin, patulin, penicillic acid, sterigmatocystin, PR toxin, kojic acid, T-2 toxin, deoxynivalenol, diacetoxyscirpenol, sporidesmin, ergot alkaloids, alternariol and its mono-methyl ether, and tenuazonic acid. This review will be restricted almost entirely to the foregoing compounds, which have been isolated from or detected in the feedstuff by physicochemical procedures. For a discussion of other mycotoxins that have been isolated from fungal cultures and even in some instance associated with mycotoxicoses, but have not been proven present in the feedstuff itself, the reader is referred to recent books

on fungal toxins (118,126). In considering the data on natural occurrence of mycotoxins, it is important to distinguish between surveys that are unbiased, usually on grains in commercial channels and preferably over several years, and surveys or even single analyses biased because the samples were associated with mycotoxicoses in farm animals, showed mold growth, were collected near the floor of the storage container, or were improperly stored in general. Factors affecting mycotoxin formation both in storage and in the field have been reviewed by Hesseltine (57). Unbiased surveys give useful incidence data while biased analyses illustrate that contamination of a feedstuff by a particular mycotoxin is possible under natural conditions and, of course, gives information on the situation at hand. All quantitative analytical results are very dependent on good sampling of the feedstuff, major contamination may be present in only a few of the kernels of the sample of say, corn moving in commercial channels. Even sampling of a single storage container is a problem (65) and corn kernels highly contaminated with aflatoxin were often found adjacent to aflatoxin-free kernels with one "hot spot" that has been studied in detail (146).

Methods of analysis of grains and feeds for mycotoxins vary widely but usually depend on thin-layer chromatography for the final estimation (3,163). Sensitivity is generally highest for the aflatoxins, which can be detected in grains at concentrations of a few µg/kg.

AFLATOXINS [45]

Aflatoxins B₁, B₂, G₁, G₂ and M₁ (Fig. 1) are metabolites primarily of *Aspergillus flavus* and *Aspergillus parasiticus* (32). Of all known mycotoxins, they are of most concern to regulatory authorities on account of their carcinogenicity, particularly potent in the case of aflatoxin B₁ (20), and their implications regarding human health on a worldwide basis (22). Nevertheless, the aflatoxins are not necessarily the most important mycotoxins from the viewpoint of animal health (61).



AFLATOXINS

Figure 1. Chemical structures of aflatoxins B_1 , B_2 , G_1 , G_2 and M_1 .

Aflatoxins B_1 , B_2 , G_1 , and G_2 have been shown to occur naturally in many agricultural commodities that are used as feed ingredients (Table 1). The occurrence of

aflatoxins in human foods (19,22,74,83,86,143,166,167,190) will in general not be included in this review, except with reference to commodities such as commercial grains that are destined for both feed and food use. Usually aflatoxin B_1 is the predominant aflatoxin found in agricultural commodities. Aflatoxins G_1 and G_2 are rarely found in cottonseed. Aflatoxin M_1 has been detected in corn accompanying aflatoxins B_1 and B_2 in very minor amounts (148). It has been demonstrated that aflatoxins may be formed before harvest, at least in corn, cottonseed, and soybeans, and insect damage appears to be a critical factor in field infection. Peanuts (groundnuts) are highly susceptible to aflatoxin contamination. The outbreaks of "turkey X disease" in England in 1960 that provided the impetus for systematic mycotoxin research were traced to imported Brazilian groundnut meal (20). Copra is another important commodity liable to be contaminated with aflatoxins. Data on the other feed ingredients listed in Table 1 are limited.

Selected examples of the reported occurrence of aflatoxins in U.S. grains, other feed ingredients and feeds are given in Table 2. Incidences and levels of aflatoxins in wheat, oats, sorghum, soybeans and rice have not appeared to be a problem in national surveys, although higher incidences in the last three grains have been reported in localized surveys. It may be noted that a level as high as 180 μg of aflatoxins/kg was found in soybeans imported to Italy from the U.S.A. (104). Aflatoxin in corn grown in the Southern U.S.A. definitely is a problem as several surveys have shown (145). This is a good example of the phenomenon of geographic distribution of mycotoxins. There can also be a marked increase in incidence of feed contamination by the time the corn reaches the feed trough (157). A concentration

TABLE 1. Agricultural commodities [feed ingredients] with demonstrated natural contamination by aflatoxin

Commodity	Location	References
Corn (stored)	U.S.A., France, Australia, etc.	18, 66, 84, 145
Corn (field)	U.S.A.	145
Peanut meal	U.S.A., Africa, Indonesia, India, Taiwan, Brazil, etc.; imports to Europe, Japan	23, 66, 88
Cottonseed meal and other products	U.S.A., Central America, India, Sudan, etc.; imports to Europe	48, 66, 130, 167, 177
Cottonseed (before harvest)	U.S.A.	128
Copra	India, Ceylon (Sri Lanka), etc.; imports to U.S.A., Europe	2, 9, 66, 79
Rye	France	84
Oats, sorghum, wheat	France, U.S.A., Australia	18, 66, 84, 131, 150
Barley	France, Britain, Australia	18, 84, 155
Millet	Australia	66
Malt sprouts	Germany	82
Soybean (meal)	U.S.A., France, Germany	10, 72, 84, 136
Sunflower seed (meal)	Italy (imported from Yugoslavia), France, etc.	66, 84, 104
Safflower meal	India	66
Rapeseed	Italy (imported from France)	104
Rice	U.S.A., etc.	66, 131, 166
Linseed (meal)	U.S.A., Germany	1, 72
Alfalfa	Canada, Brazil	105, 115
Cocoa cake	France, Sweden (imports)	84, 110
Sugar scrap	Germany	72
Cassava chips	Indonesia	66
Sesame (cake)	Italy (imported from Sudan), India	66, 104

of 101 mg (ppm) of aflatoxin B₁/kg has been found in one sample of farm-stored corn, the highest level recorded (51). Significant levels of contamination of cottonseed with aflatoxin have been reported in Arizona and Southern California (167). Cottonseed meal was involved in incidents of trout hepatoma in the Western

states in the early 1960's (50). Peanut meal may of course contain aflatoxins, although data on meal from segregation-2 peanuts does not appear to have been published (31), contrasting with extensive data available on shelled peanuts destined for human consumption (167). A Japanese survey found up to 50 ppb of aflatoxin

TABLE 2. *Examples of incidence of aflatoxins in commercial grains, feed ingredients, and feeds in the U.S.A.*

Samples	Location	Incidence	Levels (µg/kg)	References
Wheat (1964 crop)	U.S.A.	0-2/531	9 B ₁ + G ₁	150
Wheat (1970-73 crops)	U.S.A.	0/848	—	149
Grain sorghum (1964 crop)	U.S.A.	0-6/533	≤38 B ₁ + G ₁	150
Sorghum (1969-71 crops)	Texas	11/261	3-20	131
Oats	U.S.A.	0-3/304	6 B ₁	150
Soybeans (1964-65 crops)	U.S.A.	2/866	10-11 B ₁ + G ₁	151
Soybeans (1971 crop)	Maryland	14/28	4-81	10
Rice	U.S.A.	1/157	5	166
Rice (1969-71 crops)	Texas	47/425	≤282	131
Corn (1964-65 crops)	U.S.A.	30-35/1311	≤37 B ₁ + G ₁	151
Corn (1967 crop)	U.S.A.	6/283	12-25 B ₁	153
Corn (1969-70 crops)	Southern U.S.A.	21/60	≤348 B ₁ + B ₂	152
Corn (farm, country elevators, 1972 crop)	Corn Belt	4/169	Tr-20 Total	169
Corn (farm, country elevators, 1972 crop)	Southeast U.S.A., Appalachia	51/115	Tr->100 Total	169
Cottonseed meal (1964-66 crop years)	U.S.A.	604/3218	151-1500 B ₁	166,167
Feeds (all types)	Florida	42/825	≤200 B ₁	23
Feeds (50-60% corn, 1972-74, associated with aflatoxicosis)	N. Carolina	94/278	60-15,000 B ₁ (in corn)	158
Feed (troughs)		91%		
Feed (mill)	N. Carolina	52%	100-10,000	157
Corn ingredient (all associated with aflatoxicosis)		30%		

TABLE 3. *Examples of incidence of aflatoxins in feeds and ingredients in countries other than the U.S.A.*

Location	Samples	Incidence	Levels (µg/kg)	References
Canada	Feeds and ingredients (moldy or suspected association with mycotoxicoses)	1/100	50 B ₁	115
France	Corn (moldy, 1974 crop)	2/75	≤10 B ₁	28
France	Various grains	47/157	≤225 B ₁	84
France	Mixed feeds	43/126	≤2,000 B ₁	63
France	Peanut cake	19/25	≤4,500 B ₁	63
Denmark	Imported peanut products	45/52	≤3,465 Total	77
Sweden	Peanut cake (stored)	10/10	12-28,440 Total	109
Norway	Peanut meal (imported) 1968-73)	86/86	10-3,300 Total	41
Finland	Peanut meal (imported)	18/24	22-4,056 Total	79
Finland	Imported copra	10/16	10-100 B ₁ + B ₂	79
Denmark	Imported cottonseed products	40/120	5-120 B ₁	48
Britain	Feeds (excluding peanut; suspect)	4/123	40-270 Total	155
Yugoslavia	Corn (1975 crop)	0/191	—	6
Poland	Feed grains	0/150	—	69
Poland	Various feeds and ingredients	39/306	≤2,000 B ₁	170
Germany	Feeds and ingredients	45/100	7-300 B ₁	72
Ireland	Oats	0/26	—	185
Czechoslovakia	Feedstuffs (1969-73)	263/575	?	66
<i>1970-1976 Tropical Products Institute data (selected)</i>				
Africa	Peanut cake and meal	1,594/2,965	?	5
Asia	Feeds	237/415	?	
Asia	Oilseeds	24,840/25,565	100-1,000	
Australia	Feeds	35/84	?	
Europe	Feeds	62/547	?	
Europe	Oilseeds	7,371/8,133	?	
South America	Oilseeds	(mainly groundnut)	?	
South America	Feeds	258/461	?	
South America	Feeds	1/23	?	

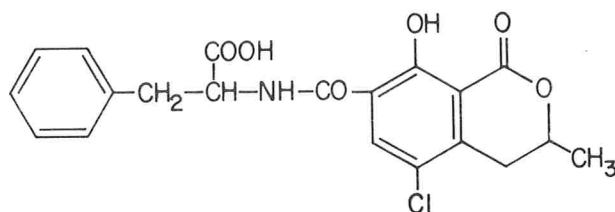
B₁ in peanut meal imported from the U.S.A. (93). There is always the possibility when considering analyses of imported commodities that contamination increased or came about during the ocean voyage.

In the rest of the world, peanut meal is an important feed ingredient in many countries. High levels and incidences of aflatoxins are evident. Table 3 gives examples of this contamination in feeds and feed ingredients, particularly in oilseeds and their products. Maximum levels of aflatoxin B₁ permitted in complete feeds by the European Economic Community now range from 10 to 50 µg/kg (66). Certain European countries, such as Norway and Denmark, have virtually ceased importation of groundnuts and groundnut meal because of even more stringent regulations (66,190). Such control measures involve economic hardship for some exporting countries. Oilseed meals can be decontaminated by ammoniation under pressure (11,44), a process that is in use to some extent on cottonseed meal in the southwest U.S.A. (167). Feed grains in Europe do not generally appear to be seriously contaminated with aflatoxins, apart from one report from France (84).

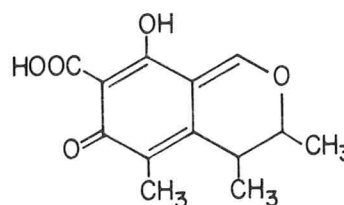
Presence of aflatoxin B₁ in feed for dairy cattle can result in appearance of its still carcinogenic metabolite aflatoxin M₁ in milk. In addition to the various experimental data on this conversion (117), a qualitative relationship was recently demonstrated in Germany with naturally contaminated feeds and milk (72). Reports of the incidence of M₁ in dairy products are summarized in Table 4. Such data provide impetus for aflatoxin control programs for feeds. In the U.S.A., localized problems of aflatoxin M₁ in milk have occurred in Arizona due to use of contaminated cottonseed meal in the feed (176). There appear to be no reports on the natural occurrence of residues of aflatoxin B₁ itself in meat, although these have been demonstrated after experimental feeding of the mycotoxin to farm animals (117). Parenthetically, aflatoxin B₁ has been reported in human liver (22,33,113,125).

OCHRATOXIN A AND CITRININ

These two mycotoxins may be considered together to emphasize that they can co-occur naturally in grains and that they are both nephrotoxic to experimental animals (54,75,107). There is also a structural similarity in their basic ring systems (Fig. 2). Ochratoxin A was first isolated from *Aspergillus ochraceus* (95,96) and subsequently found to be produced (together with the dechloro analog ochratoxin B) by other members of this group, *A. sulphureus*, *A. melleus*, *A. sclerotiorum*, *A. alliaceus*, *A. ostianus* and *A. petrakii* (25,58). *Penicillium viridicatum*, *P. palitans*, *P. cyclopium*, *P. commune*, *P. variabile* and *P. purpurescens* are also reported fungal



OCHRATOXIN A



CITRININ

Figure 2. Chemical structures of ochratoxin A and citrinin.

TABLE 4. Incidences of aflatoxins in dairy products

Aflatoxin	Product	Location	Incidence	Levels (µg/kg or l)	References
M	Retail milk	South Africa (groundnut area)	5/21	Tr-0.16	120
B ₁ , M ₁	Milk powder	South Africa	0/56	<8, <1	90
M	Milk	U.S.A. (Colorado)	5%	?	46
M	Milk	U.S.A.	0/approx. 400	<1	15
M ₁	Milk products (1973)	U.S.A. (Arizona S. California	16/16 (0/36)	0.05-0.4 (fluid milk basis)	176
M	Milk products	France	0/29	—	14
M ₁	Milk (1972)	Germany	28/61	0.04-0.25	70
M ₁	Dried milk products	Germany	8/166	0.67-2	108
M ₁	Milk powder (1972-73)	Germany	74/118	Tr-4	68
M ₁	Milk (1972-74)	Germany	117/260	0.05-0.33	114
M ₁	Milk powder (1972-74)	Germany	30/41	0.2-2.0	114
M ₁	Yogurt (")	Germany	44/54	0.05-0.47	114
M ₁	Various cheeses (")	Germany	172/356	0.1-1.30	114
M ₁	Bulk milk (1976)	Germany	79/419	0.05-0.54	72
M ₁	Various cheeses (1976)	Germany	136/197	0.02-0.23	71
M ₁	European cheese	U.S.A. (imports)	8/120	0.1-1.0	168
M	Dried milk (1973)	Norway	0/15	—	190

sources of ochratoxin A (26,140,181). *P. viridicatum* has been associated with natural production of ochratoxin A in wheat in Canada and the U.S.A. (140,149). Natural occurrence of ochratoxin A in grains and feeds appears so far to be limited to countries with a temperate or continental climate (Table 5). The highest levels of ochratoxin A have been found in biased surveys of Canadian and Danish grains, while the highest incidence so far reported was in grains from a village in Yugoslavia affected with human nephropathy (76). The presence of ochratoxin A in feed grains is also evident from residues found in animal products in Denmark and Sweden (Table 6). In addition to the grains for food use included in Table 5, ochratoxin A has also been detected in beans destined for human consumption, a sample of moldy peanuts, and in trace amounts in green coffee beans (87,140,180). Roasting of coffee beans would destroy most of the ochratoxin A (42,87).

Citrinin was originally obtained by Hetherington and Raistrick (59) from *Penicillium citrinum*. Other fungal species known to produce this mycotoxin are *P. lividum*, *P. implicatum*, *P. velutinum*, *P. fellutanum*, *P. citreoviride*, *P. jensenii*, *P. canescens*, *P. steckii*, *P. notatum*, *P. expansum*, *P. claviforme*, *P. viridicatum*, *P. palitans*, *Aspergillus terreus*, *A. candidus*, *A. niveus* (75), *A. carneus* (24), *Clavariopsis aquatica* and *Blennoria* sp. (16). Instances of reported natural occurrence (Table 7) of citrinin in feed grains are fewer than for ochratoxin A; in Denmark and Canada the responsible fungus appears to be *P. viridicatum* (40,140). Citrinin is also produced by an Australian tropical plant, *Crotalaria crispata* (38).

STERIGMATOCYSTIN

Interest in sterigmatocystin, which is structurally related to the aflatoxins (Fig. 3), has arisen because of its carcinogenic properties (182), but it has not been found

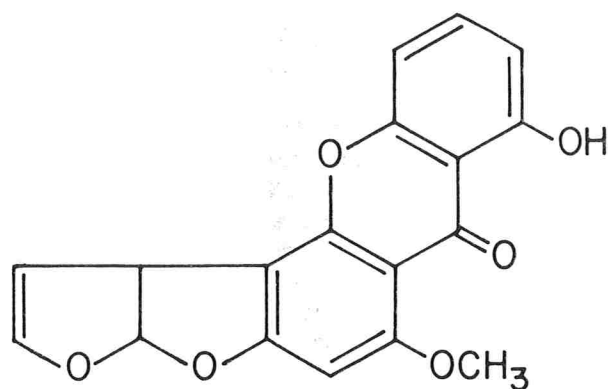
TABLE 5. Natural occurrence of ochratoxin A in grains and feeds.

Location	Sample(s)	Incidence	Levels ($\mu\text{g}/\text{kg}$)	References
U.S.A.	Commercial corn (1967 crop)	1/283	110-150	153
U.S.A.	Export corn	3 ^a /293	83-166	154
U.S.A.	Barley (1971 crop)	23/180	10-37	39
U.S.A.	Wheat (1970-73 crops)	11/848	Tr-115	149
U.S.A.	Corn (associated with mycotoxicosis in poultry)	7 cases	$\leq 16,000$	52
Canada	Grains (heated)	19/30	20-27,000	139, 140
Canada	Mixed feeds (suspect)	2/7	20; 530	140
Canada	Feedstuffs (moldy or suspect)	7/95 (wheat, hay)	30-6,000	115
Denmark	Barley, some oats (associated with nephrotoxicosis in pigs)	19/33	28-27,500	80
Denmark	Barley, some oats	3/50	9-189	80
Denmark	Horse beans (moldy)	1 sample	?	91
Sweden	Barley and oats (feed)	7/84	16-410	78
Sweden	Barley (food)	3/110	≤ 11	67
Sweden	Oats (food)	0/79	—	67
Britain	Feeds (suspect, excluding groundnut meal)	3/188	Tr-?	155
France	Corn (1973 crop)	12/463	15-200	43
France	Corn (moldy, 1974 crop)	2/75	?	28
Poland	Feed grains (1974 crop)	8/150	50-200	69
Yugoslavia	Corn (1975 crop)	50/191	45-5,100	6
Yugoslavia	Grains (food)	11-20%	?	76
Japan	Rice (food)	1/21	50	174

^aTraces of ochratoxin B in 2 samples

TABLE 6. Residues of ochratoxin A in animal products.

Origin	Animal	Incidence	Level ($\mu\text{g}/\text{kg}$)	Incidence	level ($\mu\text{g}/\text{kg}$)	References
Denmark (feed naturally contaminated)	Pig	18/19	<i>Kidney</i>		?	49
			<67	7/8		
Sweden (abnormal kidneys)	Pig	32/129	2-104			129
Denmark (abnormal kidneys)	Poultry	5/14	<i>Muscular tissue</i>		4-29	34
Yugoslavia	Pig	?	?			76



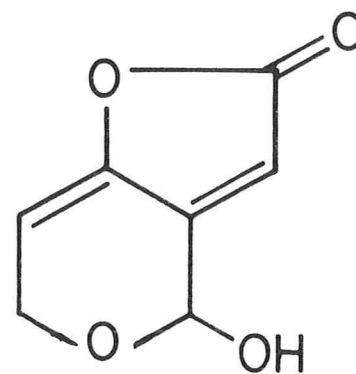
STERIGMATOCYSTIN

Figure 3. Chemical structure of sterigmatocystin.

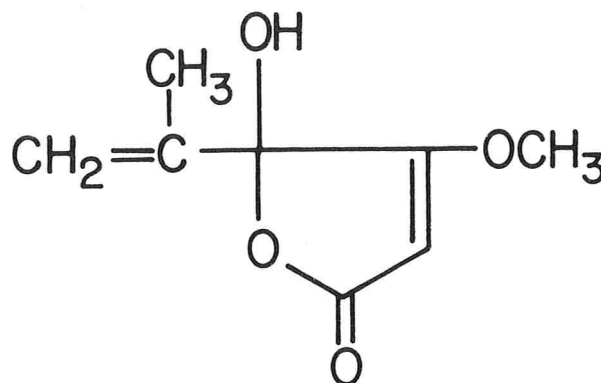
to occur naturally in grains to any great extent (Table 8). Occurrence of sterigmatocystin in moldy coffee beans (119) and in some food samples from Mozambique (182) has also been reported. The most important fungal source is *Aspergillus versicolor*, which contaminated the Canadian wheat sample that was the first reported case of natural occurrence of sterigmatocystin (140). Other species that produce this metabolite are *A. nidulans* (182), *A. unguis* (103), *A. flavus*, *A. parasiticus*, *A. chevalieri*, *A. ruber*, *A. amstelodami* (134), *Penicillium luteum* (30) and *Bipolaris sorokiniana* (121).

PATULIN AND PENICILLIC ACID

Patulin and penicillic acid are carcinogenic to rodents on repeated subcutaneous injection and both have conjugated carbonyl systems (Fig. 4) that react readily with sulfhydryl compounds (188). Hence they would not have been expected to occur naturally to any great extent in grains. This supposition appears to be wrong. Patulin



PATULIN



PENICILLIC ACID

Figure 4. Chemical structures of patulin and penicillic acid.

TABLE 7. Natural occurrence of citrinin.

Location	Sample(s)	Incidence	Levels ($\mu\text{g}/\text{kg}$)	References
Canada	Wheat and other grains (moldy)	13/18 samples with ochratoxin A	70-80,000	140
Denmark	Barley and oats (associated with swine nephrotoxicosis)	3/33	160-2,000	80
India	Groundnuts (damaged and undamaged pods)	16/160	Tr-1,400	171
Britain	Feed (suspect)	1 sample	?	53

TABLE 8. Natural occurrence and incidence of sterigmatocystin in grains and feeds.

Location	Sample(s)	Incidence	Levels ($\mu\text{g}/\text{kg}$) ¹	References
Canada	Wheat (moldy)	1 sample	300	140
Japan	Rice (long storage)	12/37	800-16,300	92
Britain	Feeds (suspect, excluding groundnut meal)	3/188 (barley)	$\leq 3,000$	155
Poland	Feed grains (1974 crop)	0/150	—	69
U.S.A.	Small grains	0/457	—	165

has been found by Escoula (36) in 17 of 34 samples of moldy silage at levels of 1.5-40 mg/kg (ppm), while penicillic acid (5-231 $\mu\text{g}/\text{kg}$) was detected in 7 of 20 stored U.S. corn samples from the 1972 crop (173) and in all of 48 selected samples of "blue eye" corn at levels of 5-184 $\mu\text{g}/\text{kg}$ (166). Non-feed materials that have been contaminated naturally by patulin are apple juice (138,164), moldy baked goods (122), and wheat straw residues (111). Thorpe and Johnson (173) found penicillic acid in five selected samples of dried beans (11-179 $\mu\text{g}/\text{kg}$) and it has also been detected in tobacco (159).

The following fungal species are reported to produce patulin: *Aspergillus clavatus*, *A. giganteus*, *A. terreus*, *Penicillium claviforme*, *P. patulum* (*P. urticae*), *P. griseo-fulvum*, *P. expansum* (*P. leucopus*), *P. divergens*, *P. melinii*, *P. equinum*, *P. novae-zeelandiae*, *P. cyclopium*, *P. lanosum*, *P. lapidosum* (138,188), *P. cyaneo-fulvum* (12), *P. rivolii* (13), *Byssoschlamys nivea*, *B. fulva* and *Paecilomyces varioti* (the conidial phase of *B. fulva*) (37). The list of species that form penicillic acid is even longer: *Aspergillus ochraceus*, *A. melleus*, (*A. quercinus*), *A. sulphureus*, *A. ostianus*, *A. alliaceus*, *A. sclerotiorum*, *Penicillium puberulum*, *P. cyclopium*, *P. thomii*, *P. suaveolens* (*P. roqueforti*), *P. martensii*, *P. baarnense*, *P. madriti*, *P. simplicissimum*, *P. janthinellum*, *P. aurantio-virens*, *P. palitans*, *P. viridicatum*, *P. olivino-viride*, *P. expansum*, *P. fennelliae*, *P. commune*, *P. lividum*, *P. griseum*, *P. lilacinum*, *P. canescens* and *Paecilomyces ehrlichii* (188).

FUSARIUM MYCOTOXINS

Zearalenone is an estrogenic and growth-promoting resorcylic acid lactone (Fig. 5) produced by *Fusarium roseum* (*F. graminearum*), *F. tricinctum* (*F. poae*, *F. sporotrichioides*), *F. oxysporum*, *F. moniliforme* (98),

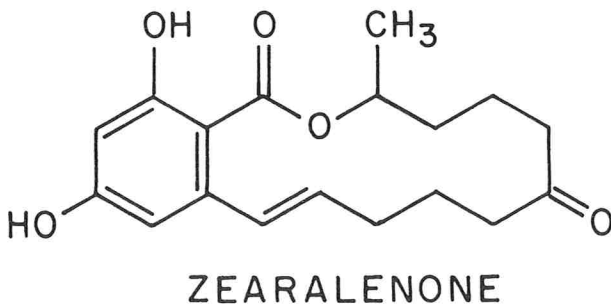


Figure 5. Chemical structure of zearalenone.

F. solani (85), *F. nivale*, *F. avenaceum*, *F. sambucinum* and *F. culmorum* (47). *F. roseum* appears to be the most important source of natural zearalenone in the U.S.A., while *F. culmorum* was the most frequently isolated producer of zearalenone in a study of English barley (47). Zearalenone occurs not infrequently in grains and feeds (Table 9). Incidence in U.S. corn varies with the crop year, a typical feature of mycotoxin occurrence. Extremely high concentrations (up to 2909 ppm) have been found in feeds associated with estrogenism and other disorders in swine and dairy cattle (98). Like the

afatoxins, zearalenone can occur in corn before harvest (21). Although in general, countries that have problems with zearalenone and associated mycotoxins also have a temperate climate, there are apparent exceptions notably Zambia, where up to 4.6 mg of zearalenone/liter was found in maize beer (89). Even in Zambia there is a cold period, which if combined with the rainy season as in 1973-74, affords favorable conditions for *Fusarium* growth and zearalenone production in the corn. Other human foodstuffs in which zearalenone has been detected are tomatoes (160) and pecans (135).

T-2 toxin, diacetoxyscirpenol, nivalenol and deoxynivalenol (vomitoxin, Rd-toxin) (Figure 6) are just four of over 30 known trichothecenes, but they are among the most toxic (8) and to date, are the only ones shown to occur naturally in grains and feeds, notably in samples associated with mycotoxicoses or feed refusal (Table 10). The Canadian outbreak of fusariotoxicosis included in

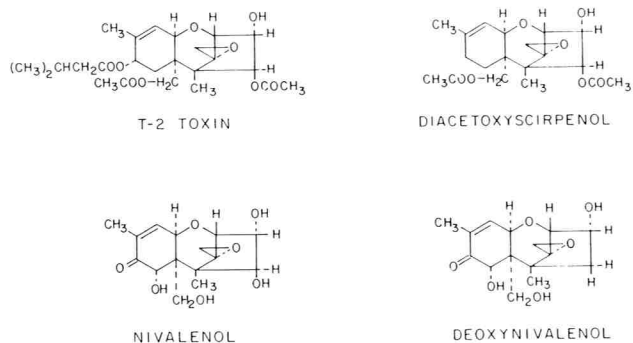


Figure 6. Chemical structures of 4 trichothecenes.

Table 10 was attributed to barley overwintered in the field in the Peace River District of Northern Alberta/British Columbia (116), an area with very low winter temperatures, and provides a good example of conditions that would favor T-2 toxin production (156). Identifications of other trichothecenes in feedstuffs will depend on improved methodology. The four mycotoxins are produced by one or more of the following species: *Fusarium roseum*, *F. tricinctum*, *F. nivale*, *F. solani*, *F. lateritium*, *F. culmorum*, *Trichoderma viride* and *T. lignorum* (7,8,101,156,191). Co-occurrence of zearalenone and trichothecenes in the same fungal strain has been reported (101).

MISCELLANEOUS MYCOTOXINS AND OTHER FUNGALLY ASSOCIATED TOXINS

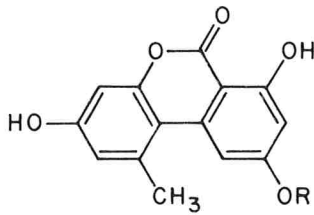
Other mycotoxins known to occur naturally in grains or pasture grasses are listed in Table 11, together with fungi that produce them. Structures are shown in Fig. 7. The ergot alkaloids are responsible for what is probably the best known mycotoxicosis, ergotism, which has had devastating effects on man as far back as the Middle Ages (123). Outbreaks of lameness and gangrene of the feet in cattle grazing on ergotized pastures have been associated with ergotamine in the ergots (94). Spori-

TABLE 9. *Natural occurrence of zearalenone in grains and feeds.*

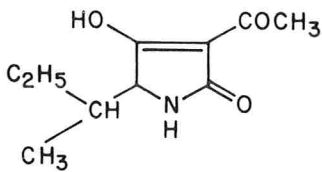
Location	Sample(s)	Incidence	Levels ($\mu\text{g}/\text{kg}$)	References
U.S.A.	Commercial corn (1967 crop)	2/283	800; >1,250	153
U.S.A.	Export corn	5/293	450-750	154
U.S.A.	Corn (1972 crop)	38/223	100-5,000	35
U.S.A.	Corn (1973 crop)			
	- marketable	19/315	38-204	169
	(Corn Belt.....)	17/169)		
	- damaged	4/57	97-10,400	169
U.S.A.	Field corn	2/10	100	21
(Indiana)	(infected, 1965)			
U.S.A.	Sorghum (associated with bovine abortion)	1 sample	12,000	102
(Oklahoma)				
U.S.A.	Sorghum (moldy, 1973)	2/2	?	133
U.S.A.	Wheat (1975 crop)	19/42	360-11,050	147
(Virginia)				
U.S.A.	Feeds (associated with mycotoxicoses in animals, 1968-70)	28/65	100-2,909,000	98
U.S.A., Canada	Feeds (associated with hyperestrogenisms in swine and turkeys, includes sesame meal.....)	14/14	65-5,600	101
U.S.A.	Feeds (containing trichothecenes)	7/9	1,500 Tr-3,600	101
Mexico	Corn (food)	6/139	?	99
Finland	Feed (associated with infertility in cows)	1 sample	25,000	73, 127
England	Hay (associated with infertility in cattle)	1 sample	14,000	100
Scotland	Barley and feed (associated with mycotoxicosis in pigs)	2/12	500; 750	97
Britain	Feeds (suspect)	1/188 (barley)	Tr	155
Britain	Barley	At least 3 samples	?	47
France	Corn (stored wet)	1 sample	2,350	64
France	Corn (moldy, 1974 crop)	62/75	\leq 170,000	28
Yugoslavia	Corn (1972 crop, associated with feed refusal)	6/6	1,300-20,000	Scott, P.M. (unpublished)
Yugoslavia	Corn (associated with mycotoxicoses in swine)	?	2,310-35,600	112
Yugoslavia	Corn (1975 crop)	5/191	43-10,000	6
Hungary	Feed grains (1970-)	200/?	?	85
Poland	Feed grains (1974 crop)	0/150	—	69
Japan (imports from U.S.A.)	Corn	2/10	18-230	172
Japan (imports from Thailand)	Corn	3/16	670-1,020	172
Japan (imports from South Africa)	Corn	1/3	50	172
Zambia	Corn (1974 crop)	10-12/20	100-800	89

TABLE 10. *Natural occurrence of trichothecenes.*

Location	Sample(s)	Toxin	Incidence	Levels ($\mu\text{g}/\text{kg}$)	References
U.S.A. (North)	Corn (1972 crop)	"Skin irritant"	93/173	\leq 1,000 (as T-2 toxin)	35
U.S.A. (Wisconsin)	Corn (associated with mycotoxicosis in cattle)	T-2 toxin	1 sample	2,000	60
U.S.A.	Corn (associated with vomiting, refusal in swine)	Deoxynivalenol (vomitoxin)	3 samples	>3,000 >18,000	62, 178, 179
U.S.A. (North)	Feeds (associated with animal mycotoxicoses or refusal)	Diacetoxyscirpenol	2 samples	380; 500	101
		T-2 toxin	1 sample	76	101
		Deoxynivalenol	6 samples	40-1,800	101
Canada	Barley (associated with mycotoxicosis in geese, etc.)	T-2 toxin	1 sample	25,000	116
Japan	Barley (moldy)	Nivalenol		?	106
		Deoxynivalenol		7,300	106
Yugoslavia	Corn (1975 crop)	"Skin irritant"	16/191	500-5,000?	6

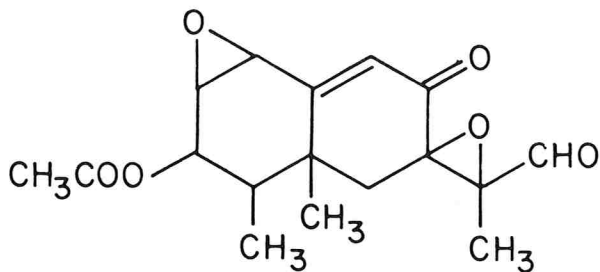


ALTERNARIOL (R=H)
ALTERNARIOL MONOMETHYL ETHER (R=CH₃)

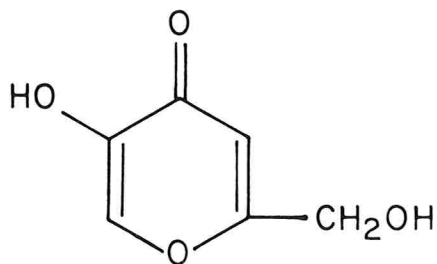


TENUAZONIC ACID

Figure 7a. Chemical structures of alternariol and its monomethyl ether and tenuazonic acid.

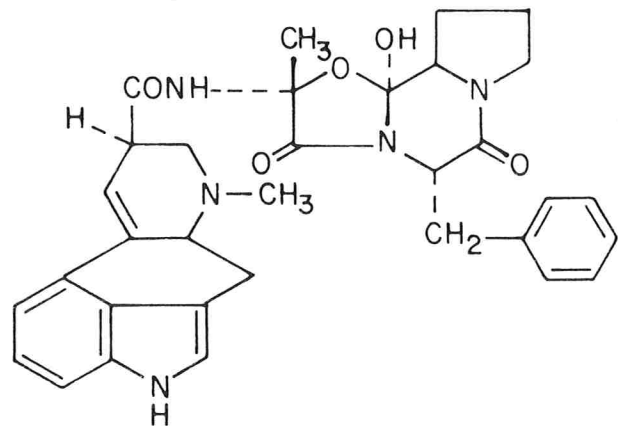


PR TOXIN

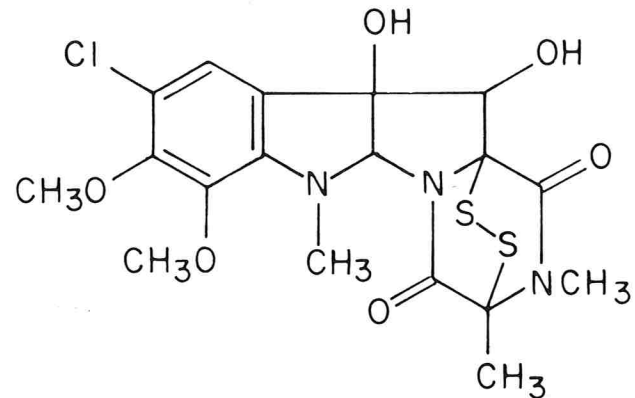


KOJIC ACID

Figure 7c. Chemical structure of PR toxin and kojic acid.

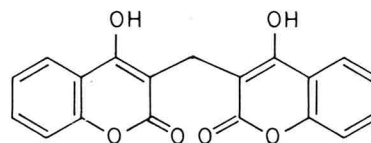


ERGOTAMINE



SPORIDESMIN

Figure 7b. Chemical structures of ergotamine and sporidesmin.



3,3'-METHYLENEBIS (4-HYDROXYCOUMARIN)

Figure 8. Chemical structure of dicoumarol.

desmin is responsible for facial eczema, a liver disease of sheep and cattle in New Zealand and Australia (17).

Dicoumarol or 3,3'-methylenebis (4-hydroxycoumarin) (Fig. 8) is a hemorrhagic agent that has been isolated from sweet clover infected with certain fungi (124). Such sweet clover has caused many fatalities in cattle in Canada and the U.S.A. Dicoumarol is formed

from 4-hydroxycoumarin produced by the fungus and exogenous formaldehyde, and it might be regarded as a mycotoxin if the definition were broadened to include toxins whose formation is dependent on fungal activity.

No review on fungally related toxins in feedstuffs would be complete without mentioning toxic plant metabolites, including phytoalexins, that can be induced by stress conditions, such as fungal infection (81). For example, ipomeamarone and related compounds (Fig. 9) have been detected in damaged sweet potatoes, including retail samples for human consumption (29,186). In particular, ipomeanine and 4-ipomeanol contribute to a "lung edema factor" postulated to be responsible for an interstitial pneumonia in cattle fed moldy sweet potatoes (186).

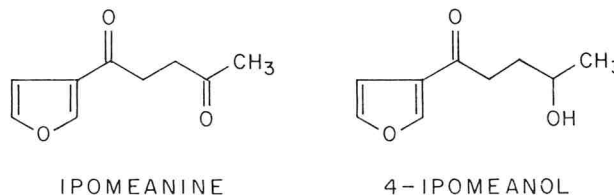
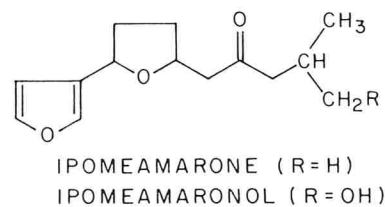


Figure 9. Chemical structures of ipomeamarone and related compounds.

TABLE 11. Natural occurrence of miscellaneous mycotoxins.

Toxin(s)	Producing fungi	Occurrence	References
Alternariol, alternariol monomethyl ether	<i>Alternaria tenuis</i> , <i>A. dauci</i>	Weathered grain sorghum (Kansas: 21/28, trace - 5 ppm); pecan pickouts	132, 141, 142
Tenuazonic acid	<i>Alternaria tenuis</i> , <i>Aspergillus tamarii</i> , <i>Sphaeropsidales</i> sp., <i>Pyricularia oryzae</i> <i>Phoma sorghina</i>	Diseased rice plants (Japan: 4/6, up to 379 ppm)	144, 161, 175
Ergot alkaloids (ergotamine, etc.)	<i>Claviceps</i> spp., <i>Aspergillus</i> spp., <i>Penicillium</i> spp.	Ergots, ergot-infected pasture grass	94, 123, 189
Sporidesmin	<i>Pithomyces chartarum</i>	0.1% in spores on dead pasture grass	4, 17
PR toxin	<i>Penicillium roqueforti</i>	Silage (Wisconsin: 1/7, 8 ppm)	162, 183, 184
Kojic acid	<i>Aspergillus flavus</i> , <i>A. oryzae</i>	Moldy corn (Trace, 1 sample)	27, 187

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Trichothecenes: Occurrence and Toxicoses

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ABSTRACT

The Δ^9 -12,13-epoxy-trichothecenes are a group of 37 naturally occurring sesquiterpenoids produced by species of *Fusarium*, *Stachybotrys*, *Myrothecium*, *Trichoderma*, and *Cephalosporium*. They are responsible for a variety of mycotoxicoses in man and animals that may be manifested by severe dermatitis, vomiting, bloody diarrhea, decreased weight gains, extensive hemorrhaging, and death. Outbreaks, which have occurred in Russia, Europe, the United States, Japan, and Korea, usually result after cereal grains in the field have been exposed to prolonged cool and wet weather. Hence, these mycotoxicoses do not appear to be a problem of faulty grain storage, since they involve field fungi primarily rather than storage molds.

The trichothecenes are a family of about 37 naturally occurring sesquiterpenoids produced by various species of fungi. They are derivatives of the trichothecane ring system (Fig. 1a) named after trichothecin, the first member of the group to be isolated. All of the naturally occurring toxins contain an olefinic bond at 9, 10 and an epoxy group at 12, 13 and, therefore, may be characterized as 12, 13-epoxy-trichothecenes (Fig. 1b). In addition, most of these toxins have at least one -OH or ester group at position 4 as in Fig. 1b. The compounds can be subdivided into groupings based on substitution at position 8 and the presence (or absence) of a macrocyclic ring (Fig. 2-4).

Additional subgroupings are possible, e. g., the presence or absence of an epoxide between positions 7 and 8 as in crotochin and crotochol.

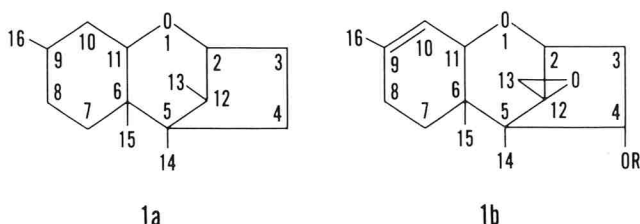


Figure 1. Basic ring structures of trichothecane and 12,13-epoxy-trichothecenes.

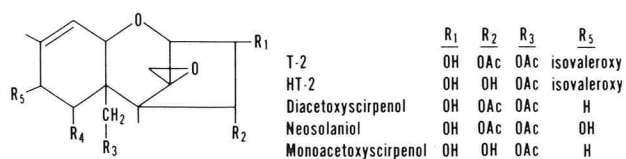


Figure 2. Structures of 8-keto trichothecenes.

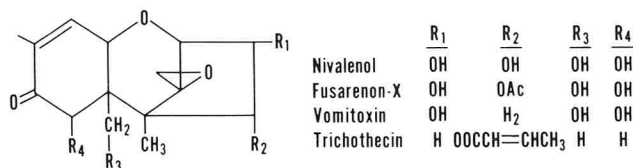


Figure 3. Structures of trichothecenes substituted at position 8.

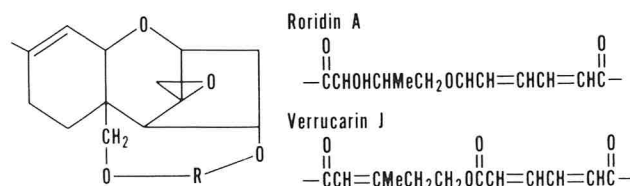


Figure 4. Structures of macrocyclic trichothecenes.

FUNGAL SOURCES

The 12, 13-epoxytrichothecenes are produced by members of a number of fungal genera including *Fusarium*, *Stachybotrys*, *Myrothecium*, *Trichoderma*, and *Cephalosporium*; but pragmatically, with respect to serious contamination of food or feedstuffs, we will be concerned primarily with members of the genera *Fusarium* and *Stachybotrys*. Considerable confusion exists concerning the taxonomy of the fusaria, and I am not qualified, nor is this the time and place, to discuss this aspect; I will use, in general, the simplified nomenclature of Snyder and Hansen who recognized only nine species of *Fusarium* (26).

TOXICITY

The 12, 13-epoxytrichothecenes are toxic to animals, plants, insects, fungi, protozoa, tumor cells, and cultured cells. However, as a class they are rather poor antibiotics. In humans and animals, many of these compounds can cause a severe dermatitis, vomiting, bloody diarrhea, decreased weight, extensive hemorrhaging, and death. Pathological lesions in animals include cellular damage and karyorrhexis to the proliferating tissues of intestinal mucosa, bone marrow, spleen, testis, and ovary. On a molecular level, they may cause inhibition of protein and DNA synthesis. The LD₅₀ of some select trichothecenes is shown in Table 1.

TABLE 1. LD₅₀ of select trichothecenes

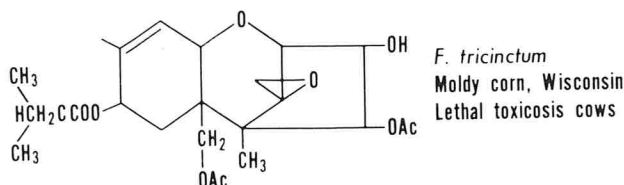
Toxin	Mice (mg/kg)
Fusarenon-X	3.3
Nivalenol	4.1
Vomitoxin	50.0
T-2	5.2
HT-2	9.0
Diacetoxyscirpenol	23.0
Trichothecin	< 250
Verrucaric A	0.5-0.75

INCIDENCE

What is the incidence of trichothecenes in agricultural commodities, and what role do these compounds play in mycotoxicoses? These questions should be separated from the incidence of fungi capable of producing trichothecenes that can be isolated from various commodities. Put differently, it is a question of direct vs. circumstantial evidence and the two are often confused in the literature. Direct evidence, particularly that also implicating a mycotoxicosis, is often extremely difficult to obtain.

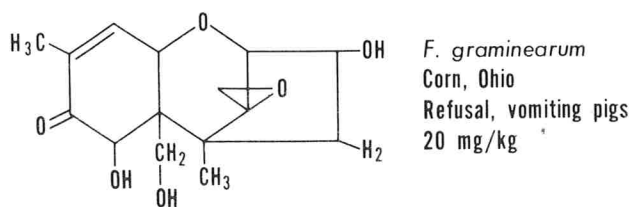
To the best of my knowledge, there are only a few documented reports of trichothecenes actually being detected in contaminated commodities. These are summarized in the following figures (Fig. 5-8).

Although only a few reports have appeared in the literature, considerable circumstantial evidence exists to link *Fusarium*-contaminated food and feeds to a variety of mycotoxicoses. *Fusarium* contamination of commodities is common throughout the world, particularly in geographical regions subject to cool wet periods. Intoxications of man and/or animals ascribed to consumption of *Fusarium*-contaminated commodities have been reported extensively over a long time span,



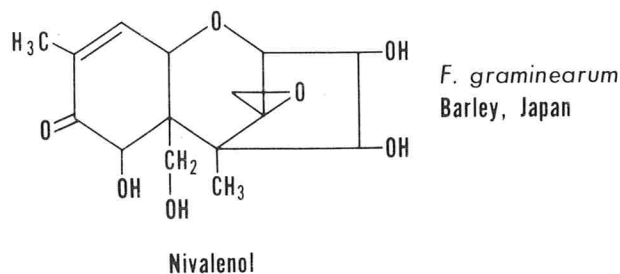
Hsu et al. [9]

Figure 5. Natural occurrence of T-2 toxin.



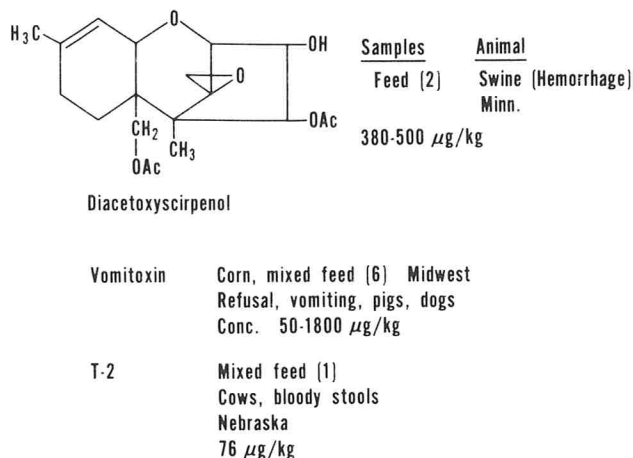
Vesonder et al. [32, 33]

Figure 6. Natural occurrence of vomitoxin (deoxynivalenol).



Morooka et al. [19]

Figure 7. Natural occurrence of vomitoxin and nivalenol.



Mirocha et al. [17]

Figure 8. Natural occurrence of trichothecenes in feedstuffs.

particularly in Japan, Korea, Russia, and the United States. The best known of these intoxications was the outbreak of alimentary toxic aleukia (ATA) in Russia during WWII involving thousands of people; however, cases had been recorded in Eastern Siberia as early as 1913. The disease was ascribed to toxic steroids in *Fusarium*-contaminated grains, but it was later shown that one of these toxic steroid preparations actually contained 12, 13-epoxytrichothecenes. Yagen (38) offered compelling evidence that T-2 toxin produced by *Fusarium poae* and *Fusarium sporotrichiodes* (*Fusarium tricinctum*) was the causative compound.

Another aspect of fusariotoxicoses prevalent in the United States involves the refusal-vomiting phenomena affecting pigs fed corn infected with *Fusarium graminearum*. Vomiting and nausea have also been reported among humans in Japan from consuming *Fusarium*-

contaminated rice or wheat products (23) and in Russia from eating bread baked with scabbed wheat (4,20). The literature is extremely confusing on the causative toxin; various investigators have shown that a number of trichothecenes can elicit vomiting in laboratory animals. I suspect that most trichothecenes can cause vomiting on ingestion. However, this is not the point; the question is rather which toxins actually occurring in the field are causing toxicoses; which are major, which are minor; do they interact? To date, definitive experiments to clarify all these questions have not been carried out.

The refusal-vomition phenomenon has been described as caused by *F. poae* which produced T-2 toxin (5,15) or by presence of fusaranone-x or nivalenol (29,30). Vesonder and his colleagues (32,33) at our laboratory and later Ishii et al. (10) have definitively shown that *F. graminearum* which produced vomitoxin (deoxynivalenon) in corn was the cause of both refusal and vomiting. Morooka et al. (19) earlier had isolated vomitoxin from contaminated barley but had not characterized it. The most recent outbreak of fusariotoxicosis on large scale was in 1972 and involved corn in a region stretching from Pennsylvania to Nebraska. FDA personnel analyzed 223 samples of this corn (7), and 93 of 173 samples contained a skin irritant by the rabbit skin test. We have started an analysis of 10 of these samples and have found vomitoxin and T-2 in some; these assays are continuing.

Previous outbreaks of fusariotoxicoses of cereal grains in the United States which caused intoxication in swine occurred in 1928, 1957, 1958, 1964, 1965; an outbreak in 1963 caused nausea, vomiting, abdominal pain, and diarrhea in humans (3).

T-2 toxin produced by *F. tricinatum* has received most attention among the trichothecenes for a variety of reasons; mycotoxicoses believed caused by a trichothecene are often ascribed to this compound, even without evidence. However, in the United States *F. graminearum*, a non T-2 producer, occurs more often than *F. tricinatum*. Nevertheless, a well-documented case where T-2 toxin caused deaths among dairy cows in Wisconsin was reported by Hsu et al. (9).

A disease syndrome in poultry and fancy pigeons (characterized by raised yellowish-white lesions in the oral cavity, on the feet and shanks, and around the eyes) observed in the Southeast could be mimicked by feeding chickens small concentrations of T-2 toxin (35-37). However, T-2 toxin was not isolated from the feed involved.

The most dramatic mycotoxicosis, probably involving T-2 toxin as well as other trichothecenes, was the previously mentioned ATA that occurred in Russia during WWII and earlier. Because of the inadequate state of the scientific art at that period, the toxic agents involved were reported to be steroids (11). More recent work has clarified the situation so that strong circumstantial evidence implicates T-2 toxin as the major toxin involved (12,16,39).

F. tricinatum capable of producing T-2 toxin and a toxic butenolide have been isolated from toxic fescue grass (8). However, the dry-type gangrene which occurred in cattle eating this contaminated forage has not been reproduced by administration of these substances.

Fusariotoxicoses have been reported throughout the world; Tables 2 and 3 partly illustrate the problem as occurring in Sweden, Korea, and Japan.

Stachybotryotoxicosis, a haemorrhagic disease of horses, cattle, and swine consuming hay or other cellulosic feeds molded with *Stachybotrys atra*, has been reported to occur extensively in Russia, in the Balkan countries, and possibly in Israel and Finland (14,18,25,27,28,31). Experimental stachybotryotoxicosis has been developed in poultry (21,24). In addition, peasants in these areas have suffered severe vesicant effects from sleeping on contaminated hay. Eppley and Bailey (6) at FDA have found five trichothecenes produced by *S. atra* which probably account for the symptoms observed. *Stachybotrys* and stachybotryotoxicosis have recently been reviewed in extenso (22).

In addition to the preceding mycotoxicoses apparently caused by various species of *Fusarium*, *Fusarium moniliforme* on moldy corn appears to be implicated in equine leucoencephalomalacia in South Africa, Egypt, and in Louisiana, where swine as well as horses are affected (1,13). We have isolated *F. moniliforme* from toxic Louisiana corn, but we have not as yet been able to detect the presence of trichothecenes. Herein lies the crux of the overall problem—practical analytical methods for detection of trichothecenes in contaminated agricultural commodities have not been developed. It is obvious from the widespread occurrence of the fusaria and their enormous capacity for toxin synthesis that a potentially serious fusariotoxicosis problem can exist throughout the world. However, defining the magnitude of the problem awaits development of assays to permit the necessary surveys.

Those outbreaks which can attributed to trichothecenes have been dramatic. Less obvious and perhaps

TABLE 2. *Fusariotoxicoses in Sweden*

Fungus	Source	Host	Symptoms
<i>Fusarium moniliforme</i>	Oats, feed	Horse, chicken	Colic, gastric, growth loss
<i>Fusarium tricinatum</i>	Hay, feed	Sheep, cow, pig, chicken,	Gastric, respiratory, anorexia, growth loss
<i>Fusarium sporotrichiodes</i>	Hay	Cow	Respiratory
<i>Fusarium poae</i>	Hay, oats, feeds	Cow, horse, pig, chicken	Colic, gastric, anorexia, growth loss

more important are toxicoses exerting insidious effects. Boonchuvit et al. (2) showed an increased mortality in chickens dosed with T-2 toxin and later subjected to infection with *Salmonella* species; hens exposed to T-2 exhibit lowered egg production, thinner shells, and concomitant reduced feed efficiency and weight gains (34). Similar loss of feed efficiency and weight gains have been noted on farms where pigs have been fed corn contaminated with *F. graminearum*.

Another neglected aspect deserving attention is potential synergistic activity among the trichothecenes. Many fusaria and *S. atra* can produce more than one toxin, e.g., *F. tricinctum*: T-2 toxin, HT-2 toxin,

neosolaniol, diacetoxyscirpenol; *F. roseum gibbosum*: monoacetoxyscirpenol, scirpentriol; *S. atra*: satratoxin G and H, plus others; *F. nivale*: nivalenol, fusarenol, nivalenol diacetate; *F. roseum*: nivalenol, vomitoxin, monoacetoxyldeoxynivalenol. Under the circumstances, it is not surprising that the following variety of diseases described under numerous terms all turn out to be primarily trichothecene toxicoses: ATA, refusal-vomition phenomenon, haemorrhagic syndrome, moldy bean hull toxicosis, akakabibo toxicosis, cereal scab or scabby grain toxicosis, drunk bread (intoxicating bread) toxicosis, stachybotryotoxicosis, dendrodochiotoxicosis (myrotheciotoxicosis), and fusariotoxicosis.

TABLE 3. *Fusariotoxicoses in Japan and Korea*

Date	Grain	Host	Symptoms
1932	Wheat	Horse, man	Vomiting, diarrhea
1946	Flour	Man	Vomiting
1950	Wheat	Man	Vomiting
1954	Oats	Horses	—
1955	Wheat	Man	Vomiting
1956	Wheat	Man	Nausea, vomiting, diarrhea, chills
1963	Wheat, oats corn, barley	Horses, sheep, man, pigs, dogs	Vomiting, diarrhea
1969	Fodder	Horses	—
1970	Wheat	Chicken	—

Fusaria isolated: *F. graminearum*, *F. nivale*, *F. poae*, *F. oxysporum*, and others.

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Fallacies in Our Understanding of Mycotoxins

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ABSTRACT

It is arguable that mycotoxins offer a new challenge to the logical framework on which microbiology is erected. The time honored concepts of cause and effect are not as clear and simple with mycotoxins as we would wish and frequently assume. We have nondescript diseases with multiple nondescript causes. It is a rare field outbreak in which everything equals what is seen in the laboratory. A microbiologist working with mycotoxins is like a microbiologist working with mixed cultures, he not only feels lost but to a certain extent he is lost with the customary road signs vague and obscured or even misleading. Nevertheless, mycotoxicology reflects reality with all its excitement and complexity as do few other areas of science. Mycotoxins are a real and present hazard as they have been for ages. It is up to us to question, acknowledge, and correct the fallacies hindering our goal of relieving mankind of the affliction of mycotoxins.

It is a fortunate scientist who can address this topic with its built-in opportunity to say that everybody else is out of step. Actually, our understanding of mycotoxins changes every year. Consequently, the fallacies with which we are burdened change with passing time. This is particularly true in a rapidly developing area such as mycotoxicology. Table 1 readily shows this point.

TABLE 1. *Statements about aflatoxin by respected scientist.*

Date	Statement
1971	<i>A. flavus</i> does not occur in USA corn
1973	<i>A. flavus</i> but not aflatoxin occurs in USA corn
1975	Aflatoxin occurs in USA but only in Southern corn
1977	Aflatoxin occurs in USA corn even in my laboratory

This respected scientist will be unidentified, at least here, not because he would mind but because I wish to divorce my comments from personalities. What is one man's fallacy is another man's truth, and I need all the friends I can keep. Let us all have the grace of this nameless scientist and change our views with new facts. I must confess it has been pointed out to me that my understanding of mycotoxins has been deficient on occasion. What hurt was that my graduate students did the pointing. Hence, I hope you are prepared for a discussion by the blind leading the blind.

KOCH'S POSTULATES

Many of the fallacies in mycotoxicology can be grouped around misapplication and misunderstanding of Koch's postulates as they apply to mycotoxins. Table 2 shows my interpretation of Koch's postulates as they apply to mycotoxins. In considering the first postulate,

TABLE 2. *Koch's postulates in mycotoxicology.*

1. Find the mycotoxin in suspect substrate from the toxicosis outbreak.
2. Find in the substrate a fungus that produces the toxin.
3. Induce the toxicosis in experimental animals by ingesting or contacting the toxin.

a rather clear set of symptoms not associated with known diseases must be recognized before a toxicosis is even suspected. To prove it is a new toxicosis, a toxic factor must be isolated, which implies an assay. To call it a mycotoxin, production by a fungus found in feed or feed ingredients must be established (second postulate). Finally, the suspect mycotoxin must be introduced into animals and symptoms matching those occurring in the field must be elicited. Once a mycotoxicosis is established as a disease entity, there are some strict constructionists who would have us repeat the process for each new diagnosis of that mycotoxicosis and further would introduce the refinement that the concentration of mycotoxin found in the feed must equal that required to produce the symptoms in the laboratory. For reasons to be elaborated later, such stringencies do not appear reasonable or necessary.

Let us consider the so called first case of aflatoxicosis or the turkey "X" disease outbreak in southern England in 1960. A close reading of the original reports (1,6) gives an inconsistent and not completely clear picture of what symptoms were actually occurring in the field outbreak. Nevertheless, an epidemiological analysis (2) traced the problem to imported peanut meal which microscopic examination proved to be laced through with hyphae. *Aspergillus parasiticus* (nee *flavus*) was isolated and found to produce a toxic principle. The toxic principle

was split into the now well-known four components called aflatoxins B₁, B₂, G₁, and G₂. The pure components were extremely toxic to ducklings and rats. Enterprising scientists found that aflatoxin caused hepatomas in rats and everybody was off and running after the pot of gold at the foot of the rainbow. What seems amazing is that, as far as I can determine, nobody put aflatoxin back into turkeys to see if the "X" disease was indeed aflatoxicosis. What about subsequent cases? Does the proper scientist need to repeat the laboratory animal experimentation on each occasion? Surely not, if the investigator is familiar with the disease he is investigating; otherwise, the accumulation of scientific information through the ages is invalid. Is the finding of toxin necessary if the symptoms are present? Not if the symptoms are unique such as the oral lesions caused in chickens by T-2 toxin (11). No other known disease causes such lesions. Remember that bile ductule proliferation in ducklings at one time was the definitive answer for the occurrence of aflatoxin (2). At that time chemical analyses for aflatoxin were suspect. Development and acceptance of reliable chemical assays for aflatoxin were based on ease and practicality and were not necessitated by logic. Certainly all early chemical assays were justified by their correlation with the symptoms observed in ducklings. Based on personal experience, I will say that under field conditions, the symptoms can be a more reliable guide than something is wrong than the results of a chemical assay. Thus, it appears that the rigorous logic of Koch is not always necessary and reasonable *after* a mycotoxicosis is established as a defined entity.

Even if these considerations were wrong, it could not be stipulated that the level of toxin found in feed must equal that required to produce the disease under laboratory conditions. Such a stipulation would require that mycotoxins occur uniformly in feed, that the sampling be representative, that the toxin be stable, that the sensitivity of the assay approximate the sensitivity of the animals, that the laboratory conditions for the animals are the same as the field conditions, that the toxin does not interact (in the statistical sense) with other substances and conditions, that the effects in animals are large enough to measure in the usually small numbers of experimental laboratory animals, and that the laboratory animal approximate the farm animal or human as the case may be. All of these assumptions are demonstrably if not self-evidently false.

SAFE LEVELS

This assignment to discuss fallacies was accepted after receiving in one week 17 phone calls in which the callers essentially wanted to know a safe or unsafe level of a mycotoxin. For example, "I have "X" amount of "Y" toxin in my corn, can I feed it or should I sell it to my neighbor?" This question of safe or unsafe levels is a simple, obvious, and practical question, but the answer hinges on all the assumptions just mentioned above. Let it be said that I think the question betrays a mind un-

aware of reality and that a person giving an answer is equally unaware of reality. Here is my answer to the question.

The nonuniformity of the occurrence of mycotoxins in feed is familiar to all who have looked into feed bins or sampled them, but Table 3 shows some data demonstrating it. The samples were taken at one time from a

TABLE 3. *Nonuniformity of occurrence of mycotoxins in feed.*

Sample	Source	Aflatoxin (ppb)
1	Tank periphery	120
2	Tank periphery	350
3	Tank periphery	35
4	Tank core	< 20
5	Tank core	< 20
6	Tank core	< 20

single on-farm storage tank containing broiler chicken starter mash, a material in which the particle size effect is minimal. The samples taken from the periphery of the tank, that is from within 6 inches of the sides of the tank, contained aflatoxin while samples taken from the core or center of the tank were free of aflatoxin. This spatial inequality is not proposed as a general phenomenon although it did explain large variation between samples from this tank.

Why did the positive samples from this tank vary 10-fold in aflatoxin concentration? Perhaps it was an error in sampling or analysis. Perhaps the sites represented sunny versus shady sides of the tank and consequently localized differences in temperature and moisture. Maybe the negative samples from the core reflected a laminar flow of the feed induced by peripheral friction and consequent peripheral stagnation.

Consider the results of analysis on the core samples which are indicated as < 20 ppb and not as negative or zero. This is not because of FDA preference for feed assaying below that magic level. It is because, in my experience, you cannot reliably detect below that amount in chicken feed. Many occasional samples of chicken feed occur in which it is impossible to detect 100 ppb using a standard method. The reason for the lack of claimed sensitivity in chicken feed is ingredients such as alfalfa meal, fish meal, meat scrap meal, fermentation residues, and moldy corn which contain high levels of numerous interfering substances.

In view of the points just made about the data in this table, it appears impossible to say what level of aflatoxin was consumed by the birds eating feed from this tank. Also, consider the birds are from 3 to 150 meters away from the tank and the feed must be conveyed to the birds thus offering additional opportunity for confusion. Surely these considerations confound any correlation expected between the laboratory and these field results.

Probe sampling is praised as the ultimate for mycotoxin analyses. Results in Table 4 show that while it may be superior, it is not perfect. Three independent samples from a single 5000-bushel corn storage bin gave results ranging from negative to 2 ppm. Once again, the negative result is recorded as less than a specific value

TABLE 4. *Inadequacy of probe sampling for detection of mycotoxins.*

Corn sample ^a	Aflatoxin (ppb)
1	250
2	< 10
3	2000

^aEach sample of 20 kg was obtained with a 1 meter probe from 6 sites in a storage bin.

which reflects the interfering substances that can occur in moldy corn. These are remarkable results in view of each sample being combined material from six independent sites. Assuming the assay was valid, aflatoxin clearly was localized and not evenly distributed in this storage tank. Clearly the probe sampling technique was inadequate to overcome the uneven distribution. The comparatively large sample size (20 kg) in this experiment should be noted. Whitaker et al. (10) demonstrated that to negate the particle size effect in material like peanuts and corn, it is necessary to take samples of near 20 kg or about a bushel yet rarely are field samples exceeding 1 kg sent to the laboratory for analysis. Once again, in view of these points, what level of aflatoxin will animals ingest in feed made of corn from this bin? There can be little doubt that aflatoxin will be consumed with this corn, but how safe or unsafe is this corn? Do you feed it or do you bury it and possibly go bankrupt either way?

It seems peculiar that we generally accept analytical results unquestioningly and immediately make comments such as "That level of toxin 'X' is below what Scientist 'Y' used in his study; therefore, the disease is not caused by toxin 'X'." Table 5 shows the results of the assay of spiked samples split between six commercial laboratories. These laboratories provide the assays used

TABLE 5. *Assays of spiked samples by commercial laboratories.*

Laboratory	1971	1976
A	60	478
B	20	527
C	neg.	680
D	neg.	310
E	neg.	192
F	neg.	—

^aSamples contained 470 ppb of added aflatoxin B₁.

by many in the poultry industry to make decisions about mycotoxin problems and control. Indeed, the samples in this survey were submitted by regular customers and the laboratories were unaware of the nature of the samples. As can be seen, 1971 was a bad year with only two of the six laboratories reporting any aflatoxin in feed that contained 470 ppb added B₁. Results of the 1976 survey were more satisfying and hopefully reflect a real improvement. Even so, there was a 350% difference between the low and high value. Laboratory F is no longer in business. Although not shown here, assay results with feed containing 47 or 4.7 ppb of added aflatoxin had even greater variation. The feed used as a carrier for the aflatoxin was a starter mash selected for its very low amount of interfering substances and which

permitted the detection of 2 ppb aflatoxin in our laboratory. Now, did failure to find aflatoxin in suspect feed in 1971 mean there was no aflatoxicosis in 1971? No more than the discovery of aflatoxin in 1961 means that aflatoxicosis did not exist before 1961. Surely the symptoms in the animals were a better guide to the existence of the disease in 1971 than were laboratory tests (8).

Now some undoubtedly will say, "What can you expect of commercial laboratories?" On the other hand, results of surveys of university and government laboratories are no better, as those who have participated in blind studies know. I truly regret the arrogance that all too many of us have towards the results of other people. How many times have we heard "His results are no good, he didn't have them confirmed by Joe Schnook"? Another example is a comment made once too often the first time it was made, "That Indian study is no good because the FDA wasn't asked to confirm it." Not only is the arrogance insufferable, but the idea that any laboratory is the sole repository of knowledge and technique is insupportable and contrary to scientific history, logic and spirit. If you don't like someone's data, point out the errors in them and even better get your own data, but don't belittle or knife the investigator.

Table 6 shows one reason why laboratories have troubles with mycotoxin assays. This one laboratory received 18 different materials for analysis in just 1 week. No single assay procedure is equally adaptable to such diverse substances. Of course, there are separate approved assays for certain products but these are limited

TABLE 6. *Materials received for aflatoxin assay during one week by one laboratory.*

1. chicken feed	10. corn
2. dog feed	11. oats
3. pig feed	12. wheat
4. cocoa beans	13. hay
5. peanut meal	14. corn gluten meal
6. chicken litter	15. "cookie" meal
7. chicken livers	16. fish meal
8. dried eggs	17. silage
9. rice hulls	18. alfalfa meal

in number and expensive. While there is the potential for developing modifications suitable for all materials encountered, what is to be done with material such as chicken feed whose composition varies weekly and which may contain up to 15 ingredients? Another weakness with assays is that the methods and their modifications are developed using normal feed or ingredients, and moldy materials have different characteristics and interfering substances which vary depending on the predominant mold and growth conditions. Thus the methods are most likely to fail with the materials most likely to contain mycotoxins.

Before leaving assay problems consider Table 7. This shows industrial experience with the BGY or black light test for aflatoxin in corn. Company A reported that 10% of the corn shipments received were BGY positive, and when this BGY-positive corn was analyzed for aflatoxin

TABLE 7. *Industrial experience with BGY test.*

Company	BGY positive (%)	False positives (% of BGY +)
A	10	10 (\bar{X} = 80 ppb)
B	37	85
C	0	—
D	< 2	100
E	21	—
F	30	\bar{X} = 250 ppb

content using accepted methodology, only 10% of the positives proved to be false. The truly positive samples contained a mean aflatoxin concentration of 80 ppb. Obviously, the BGY test was valuable to this company. On the other hand, companies B and D found it to be practically worthless. Companies C, E, and F did not report percentage false positives. Company C did not have any BGY-positive corn and company F had a high level of aflatoxin in the BGY-positive corn although the percentage of false positives was not reported by this company. Assuming that both the BGY and quantitative analyses were correct, and as we have seen this may not be a good assumption, the value of the BGY test depends on the company. Also, the data show that the prevalence of aflatoxin-containing corn is irregular as are the levels of aflatoxin.

Another assay procedure which is widely used in the feed animal industries as a measure of the mycotoxin hazard is mold spore counts. The inherent weaknesses of this assumed correlation should be obvious, but Table 8 shows data obtained with some field samples. Clearly, there was no significant correlation in these data. However, there is one poultry firm that has used this test for 5 years with successful results. If the mold count increased dramatically above the normal background level, the firm experienced poor productivity in a few weeks about one-third of the time. If the *Fusarium* counts exceeded 100/g, productivity problems appeared in 2-3 weeks with a correlation coefficient of 1.0. My first reaction was that the method is unsound and could not work, but this company with millions of dollars at risk was reluctant to discard the method and who can fault success. Certainly, study in this area is needed.

TABLE 8. *Invalidity of mold spore counts as measure of fungal activity.*

Corn sample	Aflatoxin (ppm)	Spore count
1	neg.	1×10^5
2	0.05	4×10^6
3	0.45	7×10^3
4	6.0	9×10^2
5	9.0	2×10^7
6	15.0	2×10^4

Let us consider again the proposal that the level of mycotoxin found in suspect feed must equal that necessary to produce the field symptoms in laboratory animals before the field condition can be called a mycotoxicosis. The invalidity of this requirement was indicated by the finding (7) that increasing the dietary

protein level of chickens from 20 to 30% gave essentially complete protection against the growth inhibitory effect of 5 ppm aflatoxin. Assume an investigator did not know about the presence of aflatoxin in the 30% protein diet, would he say the birds were normal and were not consuming aflatoxin? On the other hand, assume the investigator did not know about the 30% protein level which is above normal, he would probably say that 5 ppm aflatoxin has no effect on birds and that to have significant aflatoxicosis under field conditions, the level of dietary aflatoxin must exceed 5 ppm.

Consider also that over a dozen things beside protein have been reported to interact with aflatoxicosis (4,6,7,9) making it difficult to extrapolate from field to laboratory and vice versa. For example, a rarely considered but important aspect is the genetic background of the animal. A survey of six inbred lines of chickens showed that acute oral LD₅₀ varied from 6.5 to 16.5 $\mu\text{g}/\text{kg}$ (6). A single production company may use half-a-dozen different strains of chickens. I have found three different strains in the same chicken house, none of which were used in laboratory studies. Different strains can show entirely different symptoms; for example, one of the strains just mentioned did not display hypocarotenoidemia which is considered a characteristic of aflatoxicosis (9). What does this strain-dependent variation in symptoms do to a strict interpretation of Koch's postulates? Finally, a survey of farm animals for aflatoxicosis revealed not a single symptom occurred in all of the cases (8).

Additional facets of our misunderstandings about mycotoxins are shown in Table 9. In a mill considered normal, 2% of feed samples had detectable aflatoxin and the highest level was 6 ppb. This feed gave a nominal conversion of 2.00 with a final body weight of 2.04 kg. In a problem mill under the same management and diet formulation, 30% of the feed samples contained aflatoxin at levels ranging up to 30 ppb. This feed gave a 1% increase in feed conversion and 2% decrease in body weight in comparison to the feed from the normal mill.

TABLE 9. *Field evidence of safe level of aflatoxin.^a*

Parameter	Normal mill	Problem mill
Frequency of B ₁ (%)	2	30
Range (ppb)	0-6	0-30
Feed conversion ratio	2.00	2.02
Body weight (kg)	2.04	2.00

^aData for each mill involved more than 10,000,000 birds.

These effects are so small that enormous numbers of birds must be under study before significance can be attached to the effects; for example, 10 million but not 1 million were satisfactory here. While the effects were significant economically, no diagnostician could detect anything wrong in the affected flock because the effects were too slight and subtle. Now reconsider the question of safe or unsafe levels of aflatoxin. Could we say 6 but not 30 ppb are safe? Remember the assay problems discussed earlier, remember that even in the problem mill only 30%

of the samples had detectable aflatoxin. What about the strains of birds used? What about the symptoms? They are not specific which leads us to the most important thing. We are assuming that aflatoxin is causing the problem. Yet, we do not know that the feed is free of other toxicants. In an unrelated case I found corn which had been rejected on the basis of a BGY test. On analysis the corn contained 40 ppb which is not an alarming amount, but any question of feeding the material was quickly resolved by also finding 6000 ppb of ochratoxin. Finally, consider the mind-boggling aspects of designing experiments to study the situation presented in Table 9. Where do Koch's postulates fit? What is a safe level?

CONTROL OF MYCOTOXINS

Fallacies in mycotoxicology do, indeed, change. Until the landmark investigation of Lillehoj et al. (5) showed aflatoxin originating in corn still on the stalk, mycotoxicologists thought aflatoxin was strictly a storage problem and by storage we understood after harvest but before manufacture of feed. This pioneering study opened new vistas of investigation and control. Indeed one now hears that control will be achieved only in the corn fields. However, a survey (8) of an integrated broiler chicken operation afflicted with clinical aflatoxicosis found the frequency of aflatoxin in arriving corn to be 30%, in freshly manufactured feed to be 52%, and in feed being consumed by the chickens to be 91%. In this instance it was obvious that the problem was originating in arriving corn and was being magnified during manufacture and storage before consumption. Thus, aflatoxin formation occurs all along the feed chain; correspondingly, control efforts can be exerted at all links in the chain.

The effectiveness of simple control measures was demonstrated in a field study which showed the effect of cleaning and disinfecting all feed handling equipment from the mill to the bird (3). The clean-up procedures improved final body weight about 5%, serum carotenoids about 20%, and the carcass grades showed a similar improvement. However, this approach is not a cure-all since the labor required can be exorbitant in comparison with the economic benefit.

A time honored approach to the control of fungal problems is the use of antifungal agents. Table 10 shows the antifungal agents that have been available to the poultry industry. The efficacy of these is questioned by some, but not the people who use them with economic

benefits. The main problem is the regulatory agencies. FDA has removed gentian violet from use in chicken feed while you can buy it over the counter and give it to your child. This regulatory action was a major blow to efforts in control of the introduction of carcinogenic mycotoxins into the food chain because gentian violet was the cheapest, most widely used, and according to users, the most effective mold inhibitor for animal feeds. EPA appears on the verge of preventing the use of CuSO_4 in chicken feed because it is a heavy metal and might pollute the environment. FDA Commissioner Kennedy has started a drive to prevent the use of mycostatin and all other antibiotics in animal feeds under the guise of preventing an admittedly theoretical hazard to human health. This leaves calcium propionate which is not perfect as anyone who has seen a loaf of moldy bread knows, propylene glycol which is too expensive for widespread use, and propionic acid which is the current favorite because of low price and high effectiveness. I suppose these regulatory actions reflect an opinion that mycotoxins present little, if any, hazard to humans or animals. Somewhere in all of this, there must be some fallacies, but the time and energy for their exposure is limited.

ACKNOWLEDGMENT

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TABLE 10. *Antifungal agents available to treat animal feed.*

Agent	Treatment cost (\$/ton)
Gentian violet ^a	0.50
Propionic acid	0.85
Copper sulfate	1.25
Calcium propionate	1.60
Propylene glycol	3.00
Mycostatin	13.00

^aNot legally available at this time.

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SUNDAY, AUGUST 13, 1978

1:00- 5:00 p.m.	Registration—Gallery of Nations Foyer
1:30- 5:30 p.m.	Executive Board—Board Room
6:00- 7:00 p.m.	Early Bird Reception—Japanese and European Consulate Room
8:00-10:00 p.m.	Executive Board—Board Room

MONDAY, AUGUST 14, 1978

8:00 a.m.- 5:00 p.m.	Registration—Gallery of Na- tions Foyer
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Special Meetings

9:00 a.m.-12:00 Noon	Executive Board—Board Room
	1. Report on Local Arrange- ments
	2. Report of Executive Sec- retary
	3. Report of Sanitarians Joint Council
9:00 a.m.-4:00 p.m.	Journal Management Com- mittee, Parlor 108
9:00 a.m.- 4:00 p.m.	Committee on Communicable Diseases Affecting Man (Mon. through Thursday), Parlor 208
10:00 a.m.-12:00	Missouri Association of Milk, Food & Environmental Sani- tarians and Missouri Envi- ronmental Health Associa- tion, South Litton Ballroom
12:00- 1:00 p.m.	Luncheon—Kansas Associa- tion of Sanitarians and Mis- souri Milk, Food and Envi- ronmental Sanitarians and Missouri Environmental Health Association, North Litton Ballroom
12:00- 5:00 p.m.	Luncheon and Meeting—Milk- ing Machines Manufacturers' Association, Parlor 121

1:00- 1:30 p.m.	Kansas Association of Sanitarians Business Meeting, North Litton Ballroom
1:00- 4:00 p.m.	Methods for Making Sanitation Ratings Committee (NCIAS), Parlor 220
1:00- 5:30 p.m.	National Conference on Interstate Milk Shipments, Parlor 109
1:00- 3:00 p.m.	Council of State Sanitarian Registration Agencies, Parlor 121
1:00- 4:00 p.m.	Food Equipment/Sanitary Standards Committee, Parlor 221
1:00- 3:00 p.m.	Affiliate Council, Japanese Consulate Room
	1. Update of IAMFES affairs, including "jointure"—Earl O. Wright
	2. Discussion of "Jointure"—membership
	3. Future Affiliate Programming—David Fry
	4. Response of Affiliates to Journal
	5. Awards Committee Report—Harold Thompson
	6. Discussion of potential new awards
	A. Certificates of Merit
	B. Recognition of students
	7. Report from Affiliate with largest membership increase
	8. Election of Affiliate Council Officers
	9. Other pertinent topics
2:00- 5:00 p.m.	Foodservice Journal Committee, Parlor 308
3:00- 5:00 p.m.	Farm Methods Committee, European Consulate
3:00- 5:00 p.m.	Sanitarians Joint Council, Parlor 121
3:30- 5:30 p.m.	Executive Board, Board Room
	1. Regular Agenda
	2. Report of Journal Management Committee
	3. Committee Chairmen
	4. Report of Affiliate Council
	5. Meet with Past Presidents
6:00-10:00 p.m.	Fun on the Farm—Buses leave promptly at 6:00 p.m.

Individual Committee Meetings are open to all registrants.

TUESDAY, AUGUST 15, 1978

8:00 a.m.-5:00 p.m.	Registration—Gallery of Nations Foyer
8:00 a.m.- 9:00 a.m.	Executive Board—Board Room

Morning-General Session-Litton Ballroom Howard Hutchings—Presiding

9:30 a.m.	INVOCATION—C. Bronson Lane
9:35 a.m.	ADDRESS OF WELCOME—Charles B. Wheeler, Mayor, Kansas City, Mo. (Introduced by Dr. Richard Bierry, Director of Health, Kansas City, Mo. Health Department)
9:55 a.m.	PRESIDENTIAL ADDRESS—David D. Fry, T. G. Lee Foods, Orlando, FL
10:25 a.m.	KEYNOTE ADDRESS—A JUSTIFIABLE FOOD-ENERGY-LEGISLATIVE TRIANGLE—Nan Unklesbay, Univ. of Missouri, Columbia, MO
11:10 a.m.	COMMUNICATIONS—KEY TO PUBLIC RELATIONS—Arthur W. Nesbitt, Nasco International, Ft. Atkinson, WI
11:40 a.m.	ANNOUNCEMENTS Program Highlights Local Arrangements—Wendell Allen New Nominating Committee Other Announcements

TUESDAY, AUGUST 15, 1978

Afternoon-Milk Sanitation Session-North Litton Ballroom Richard P. March—Presiding

1:30 p.m.	DOOR PRIZE DRAWING
1:40 p.m.	STABILITY OF POLYVINYLCHLORIDE TUBING IN DAIRY PROCESSING OPERATIONS—Jane S. Mueller and R. L. Bradley, Jr*., Univ. of Wisconsin, Madison, WI
2:00 p.m.	GOAT MILK PRODUCTION AND PROBLEMS—Judy Kapture, Countryside Publications, Ltd., Waterloo, WI
2:30 p.m.	PBB—THE MICHIGAN PROGRAM—K. Van Patten, Michigan Dept. of Agriculture, Lansing, MI
3:00 p.m.	INHIBITION OF <i>CLOSTRIDIUM BOTULINUM</i> TYPE A AND B SPORES BY PHENOLIC ANTIOXIDANTS—M. C. Robach* and M. D. Pierson, Monsanto Co., St. Louis, MO and Virginia Polytechnic Institute and State Univ., Blacksburg, VA
3:15 p.m.	MILK BREAK
3:30 p.m.	ULTRA FILTRATION AND REVERSE OSMOSIS—Neil C. Beaton, Dorr-Oliver Co., Stamford, CN.
4:00 p.m.	KIDS, CARTONS AND QUALITY—C. Bronson Lane, Dairy and Food Nutrition Council of Florida, Orlando, FL

- 4:30 p.m. TOXICITY OF CHLORINATED COMPOUNDS FOUND IN DRINKING WATER ASSESSED BY A FERTILE EGG INJECTION TECHNIQUE—M. Hekmati, R. L. Bradley, Jr.* and M. L. Sunde, Univ. of Wisconsin, Madison, WI

TUESDAY, AUGUST 15, 1978

**Afternoon-Food Sanitation Section-South Litton Ballroom
Howard Hutchings, Presiding**

- 1:30 p.m. DOOR PRIZE DRAWING
1:40 p.m. THE ENVIRONMENTAL HEALTH PROFESSIONAL—CAN HE COMMUNICATE AND MOTIVATE?—C. Dee Clingman, National Institute for the Foodservice Industry, Chicago, IL
2:00 p.m. DESIGNING TOMORROW'S FOODS—Joe Edmondson, Univ. of Missouri, Columbia, MO
2:30 p.m. FOOD PROTECTION FOR THE 80's—F. F. Busta, Univ. of Minnesota, St. Paul, MN
3:00 p.m. FOODBORNE DISEASE—DATA FROM SIX COUNTRIES—E. Todd, Food-Borne Disease Reporting Centre, Ottawa, Ontario, Canada
3:15 p.m. MILK BREAK
3:30 p.m. DEVELOPMENT OF A RECIPROCITY SYSTEM FOR FOODSERVICE MANAGEMENT TRAINING AND CERTIFICATION PROGRAMS—C. Dee Clingman, National Institute for the Foodservice Industry, Chicago, IL
3:50 p.m. QUALITY STANDARDS FOR RETAIL MEATS, Gail Holland, Meat Packers Council of Canada, Islington, Ontario, Canada and Robert Winslow, Safeway Stores, Inc., Oakland, CA

TUESDAY EVENING, AUGUST 15, 1978

Evening Discussion Groups

- 7:00- 9:00 p.m. FOOD SANITATION—Joe Edmondson, Moderator—South Litton Ballroom
7:00- 9:00 p.m. MILK SANITATION—Bob Marshall, Moderator—North Litton Ballroom

WEDNESDAY, AUGUST 16, 1978

**General Session-Litton Ballroom
Dave Fry—Presiding**

- 8:30 a.m. DOOR PRIZE
8:40 a.m. KEEP AMERICA BEAUTIFUL—Charles F. Vogt, Public Works Dept., Kansas City, MO.
9:10 a.m. SANITARY PROCESSING OF EGG PRODUCTS—Dwight Bergquist, Hen-

- ningsen Foods, Inc., Omaha, NE
9:40 a.m. MILK BREAK
9:55 a.m. DOOR PRIZE
10:00 a.m. ANNUAL BUSINESS MEETING; Ivan Parkin—Parliamentarian
1. Report of Executive Secretary
2. Report of Secretary-Treasurer
3. Committee Reports
4. 3-A Symbol Council Report
5. Report of Resolutions Committee
6. Report of Affiliate Council
7. Old Business
8. New Business
9. Election of Officers

**WEDNESDAY AFTERNOON-MILK SANITATION
SECTION-AUGUST 16, 1978**

**Mexican-Scandinavian and Canadian Room
Charles Neighbors—Presiding**

- 1:30 p.m. DOOR PRIZE
1:40 p.m. COST OF QUALITY IN A FOOD PLANT—C. S. Gelda, The Bordon Company, Ltd., Tillsonburg, Ontario, Canada
2:00 p.m. 3-A SANITATION CRITERIA AND THEIR DEVELOPMENT—D. H. Williams, Dairy and Food Industries Assn., Washington, D.C.
2:30 p.m. INTERNATIONAL DAIRY FEDERATION—K. G. Savage, International Dairy Federation, Victoria, British Columbia, Canada
3:00 p.m. MILK BREAK
3:15 p.m. THE INHIBITION OF *SALMONELLA TYPHIMURIUM* AND *STAPHYLOCOCCUS AUREUS* BY BUTYLATED HYDROXYANISOLE AND THE PROPYL ESTER OF P-HYDROXY-BENZOIC ACID—K. R. Vantassell,* L. A. Smoot, and M. D. Pierson, Virginia Polytechnic Institute and State Univ., Blacksburg, VA
3:35 p.m. PSYCHROTROPIC BACTERIA IN RELATION TO KEEPING QUALITY OF MILK PRODUCTS—Maribeth Cousin, The Great Atlantic & Pacific Tea Co., Inc., Horseheads, N.Y.
4:05 p.m. BACTERIAL NUMBERS IN MILK: WHAT DO THEY TELL US?—Robert Marshall, Univ. of Missouri, Columbia, MO

**WEDNESDAY AFTERNOON-MILK PRODUCTION
SECTION-EUROPEAN CONSULATE ROOM**

Sidney Beal—Presiding

- 1:30 p.m. DOOR PRIZE
1:40 p.m. OPPORTUNITIES TO IMPROVE MILK QUALITY—Bill LaGrange, Iowa State Univ., Ames, IA

- 2:20 p.m. REFRIGERATION—ENERGY CONVERTER—Darl Evans, Babson Brothers Co., Oak Brook, IL
- 2:55 p.m. BRUCELLOSIS—Francis J. Drazek DVM, Cornell Univ., Ithaca, N.Y.
- 3:30 p.m. MILK BREAK
- 3:45 p.m. FUNCTIONS OF CLEANERS—James J. Jezeski, H. B. Fuller Co., Minneapolis, MN
- 4:20 p.m. BUSINESS MEETING—NATIONAL ASSOCIATION OF DAIRY FIELD-MAN

WEDNESDAY AFTERNOON, AUGUST 16, 1978

**Food Sanitation Section
Japanese Consulate Room
Michael Sanford—Presiding**

- 1:30 p.m. DOOR PRIZE
- 1:40 p.m. EFFECTS OF FOAM-REDUCING METHODS ON RECOVERY OF INDICATOR ORGANISMS FROM FOODS—A. K. Stersky* and C. Thacker, Food Directorate, Health Protection Branch, Canada, Tunney's Pasture, Ottawa
- 2:00 p.m. INNOVATIVE WATER SAVING IN CANNERIES—Allen Katsuyama, National Food Processors Assoc., Berkeley, CA
- 2:30 p.m. SANITATION PROBLEM OF SOFT DRINK MIXING MACHINES—Bob Gregory, Springfield-Green County Public Health Center, Springfield, MO
- 3:15 p.m. MILK BREAK
- 3:30 p.m. THE EXTRACT-RELEASE VOLUME METHOD AS A TEST OF MICROBIAL QUALITY OF CHICKEN—Y. W. Huang* and J. C. Ayres, Univ. of Georgia, Athens, GA
- 3:50 p.m. RETORTABLE FOOD POUCHES—Gerald Schulz and Herbert Hollender, U.S. Army Natick Research and Development Command, Natick, MA
- 4:25 p.m. INHIBITION OF *VIBRIO PARAHAE-MOLYTICUS* BY SORBIC ACID IN CRAB MEAT AND FLOUNDER HOMOGENATES—M. C. Robach* and C. S. Hickey, Monsanto Company, St. Louis, MO

- 3:00- 6:00 p.m. MASTITIS COUNCIL EXECUTIVE BOARD—Room to be announced

WEDNESDAY EVENING, AUGUST 16, 1978

- 7:00 p.m. ANNUAL AWARDS BANQUET—LITTON BALLROOM

THURSDAY, AUGUST 17, 1978

- 7:30 a.m. Executive Board, Breakfast Meeting—Mexican Consulate Room

**NATIONAL MASTITIS COUNCIL
1978 SUMMER MEETING PROGRAM**

South Litton Ballroom

- 8:00-12:00 a.m. Registration, National Mastitis Council—Litton Foyer
- 8:45 APPROACH TO MASTITIS CONTROL OF A MILK MARKETING COOPERATIVE—Dr. Don Rollins Technical Veterinary Advisor, Mid-America Dairymen, Inc., Springfield, Missouri
- 9:15 IMPLEMENTING A MASTITIS CONTROL PROGRAM IN COMMERCIAL DAIRY HERDS: EFFECTIVENESS OF EDUCATIONAL EFFORTS—Dr. William L. Crist, Dairy Science Extension Specialist, University of Kentucky, Lexington
- 10:00 Break
- 10:15 EXPERIENCES AND RESEARCH WITH SOMATIC CELL COUNTS IN DAIRY HERD IMPROVEMENT PROGRAMS—Dr. L. H. Schultz, Professor of Dairy Science, University of Wisconsin, Madison
- 11:00 VACCINATION FOR MASTITIS IN DAIRY CATTLE—Dr. J. Woodrow Panky, Assistant Professor of Microbiology, North Louisiana Hill Farm Experiment Station, Homer
- 11:45 Luncheon
- AFTERNOON PROGRAM**
- 1:15 TEAT ANATOMY AND UDDER DISEASE—Dr. John S. McDonald, Veterinary Medical Officer, National Animal Disease Center, Ames, Iowa
- 2:00 THE EFFECT OF A PHYSICAL BARRIER ON INTRAMAMMARY INFECTION RATES—Dr. Ralph J. Farnsworth, Associate Professor of Veterinary Medicine, University of Minnesota, St. Paul
- 2:45 Break
- 3:00 USING CMT TESTS TO PREDICT NUMBERS OF COWS TO REMOVE FROM PROBLEM HERDS—Dr. Robert T. Marshall, Professor, Food Science and Area of Microbiology, University of Missouri-Columbia

**ENTERTAINMENT
MEN AND WOMEN**

SUNDAY, AUGUST 13, 1978

- 6:00 p.m.- 7:00 p.m. Early Bird Reception—Japanese—European Consulate

MONDAY, AUGUST 14, 1978

- 6:00 p.m.-10:00 p.m. Fun on the Farm—Buses leave from Hotel promptly at 6:00 p.m.

TUESDAY, AUGUST 15, 1978

Open

Bus leaves promptly at 10:00
a.m.**WEDNESDAY, AUGUST 16, 1978**

6:00 p.m.- 7:00 p.m. Reception—Litton Foyer
7:00 p.m. Banquet and Entertainment

SPOUSE'S SPECIALITIES**MONDAY, AUGUST 14, 1978**

10:00 a.m. Tour—Harry S. Truman Li-
brary
12:30 p.m. Buffet Luncheon—K.C. Roy-
als Baseball Stadium with
tour of facilities

TUESDAY, AUGUST 15, 1978

10:00 a.m. Tour—Nelson Art Gallery
Bus Tour of Country Club
Plaza
12:30 p.m. Buffet Luncheon—American
Restaurant, Crown Center

WEDNESDAY, AUGUST 16, 1978

Pool Side Fashion Show—Hotel
Spouse's Hospitality: Parlor 120
(Monday through Thursday)

IAMFES Committee

Foundation Committee

Committee Members:

Orlowe M. Osten, Director, Dairy Industries Division, Minn.
Dept. of Agriculture State Office Bldg., St. Paul, Minn. 55155.
Parnell J. Skulborstad, Babson Bros. Co., 2100 South York
Rd., Oak Brook, Ill. 60521.
Fred E. Uetz, 395 Maitland Ave., Teaneck, N.J. 07666.

E. O. Wright, Ex-officio.

Objective:

To determine proper procedures for collecting and disbursing funds contributed to the IAMFES Foundation to further the educational and other goals of the Association.

Membership Committee

Committee Objectives:

To make every effort to increase the membership of the organization by bringing to the attention of all qualified persons the advantages of belonging to the International Association of Milk and Food Sanitarians, Inc., and to interest state milk and food sanitarians' organizations in the advantages of affiliation with the Association.

Committee Members:*Co-Chairmen:*

Ray A. Balknap, 79 Locust, Lakeside Park, Fort Mitchell,
KY 41017.

Harry Haverland, Director, Cincinnati Training Facility
EDRO-FDA, Room 8002, Federal Bldg., 550 Main St.,
Cincinnati, Ohio, 45202.

Harold Y. Heiskell, 3380 Sierra Oaks Drive, Sacramento, CA
95825.

Harold J. Barnum, 960 Leyden St., Denver, Colorado 80220.
Robert Bishop, 17812-147th Ave., S.E., Renton, Washington
98055.

Marion Causey, Jr., Director, Div. of Dairy Foods and Bottling
Plants, Bureau of Environmental Sanitation, J. Marion Sims
Bldg., Columbia, S.C. 29201.

M. R. Cooper, Virginia Dept. of Agriculture, Box 7,
Broadway, VA 22815.

William H. Gill (Alternate), Secretary-Treasurer, Virginia

Association of Sanitarians, 6702 Van Buren Ave., Richmond, VA.
23226.

Maurice Guerette, Div. of Food Control, N.Y. State Dept. of
Agriculture and Markets, Bldg. 8, State Campus, Albany, NY
12203.

Jim Harton, Dairy Div., Indiana State Board of Health, 1330
West Michigan Ave., Indianapolis, Ind. 46202.

William V. Hickey, 2737 Imperial St., Salt Lake City, Utah
84106.

Ralph Kirkland, P.O. Box 3384, Tampa, Fla. 33601.

R. P. March, 118 Stocking Hall, Cornell University, Ithaca, NY
14850.

A. N. Myhr, Associate Professor, University of Guelph,
Guelph, Ontario, Canada.

Sam Noles, P.O. 120, Jacksonville, Fla. 32201.

George Parker, Chief Deputy Dairy Commissioner, State
Arizona, 1601 West Jefferson, Phoenix, Ariz. 85007.

James E. Petit, Marketing Manager, Dairy, Food and Beverage
Products, Norton Company, P.O. Box 350, Akron, Ohio 44309

Alvin E. Tesdale, Secretary-Treasurer, Oregon Association of
Sanitarians, 5155-7th Ave., N.E., Salem, Oregon 97303.

George Van Wormer, Kraftco Corporation, Sealtest Foods
Div., P.O. Box 88, Hartford, Conn. 06102.

Eugene Viets, Chief, Food Sanitation, Dept. of Health,
Jefferson City, MO 65101.

Dick B. Whitehead, Mississippi State Board of Health, P.O.
Box 1700, Jackson, Miss. 39205.

Food Protection Committee

Committee Members:

John B. Gerberich, *Chairman*, Div. of Allied Health Professions, University of Wisconsin-Eau Claire, Wisc. 54701

K. J. Baker, Div. of Food Service Sanitation, PHS—Food & Drug Administration, 200 "C" St., S.W., Washington, D.C. 20204.

Robert Beck, Environmental Health Administration, 801 N. Capitol St., N.E., Suite 733, Washington, D.C. 20002.

Karl Erickson, Food Science Section, University of Wisconsin-Eau Claire, Eau Claire, Wisc. 54701.

William A. Grills, Assistant Chief, Div. of Food and Drugs, Illinois Dept. of Public Health, 535 West Jefferson St., Springfield, Ill. 62706.

Walter H. Jopke, Boynton Health Service, University of

Minnesota, Minneapolis, Minn. 55455.

Richard Jolley, Chief, Milk Inspection, Dept. of Agriculture, Mayo Bldg., Tallahassee, Fla. 32304.

Karl M. Jones, Environmental Health Officer, Purdue University, Student Hospital, Lafayette, Indiana 47907.

Michael R. Sanford, Director of Health Services, Municipal Bldg., Sixth and Broadway, Columbia, MO 65201.

Harold Wainess, Harold Wainess & Associates, 464 Central Ave., Northfield, IL 60093.

Robert L. Winslow, Manager, Safeway Stores, Inc., 4th & Jackson Sts., Oakland, CA 94660.

C. Dee Clingman, Director, Food Protection Programs, National Institute for the Food Service Industry, 120 South Riverside Plaza, Chicago, IL 60606.

IAMFES Representatives

IAMFES Representative to Conference of State Sanitary Engineers

Dick Whitehead

IAMFES Representative to International Dairy Federation

Harold Wainess, Harold Wainess and Associates, 464 Central Ave., Northfield, Illinois 60093.

IAMFES Representative to National Mastitis Council

Carl Webster, Mississippi Valley Products, 4117 Round St., Cedar Falls, IA 50613.

IAMFES Representative to Sanitarians Joint Council

Ray A. Belknap, HEW-FDA, 1090 Tusculum Ave., Cincinnati, Ohio 45226.

Harry Haverland FDA Training Institute, 1090 Tusculum Ave., Cincinnati, Ohio 45226.

Alternate-Vernal Packard, Dept. Food Science and Nutrition, University of Minnesota, St. Paul, MN 55101.

IAMFES Representatives to 3A Symbol

Henry V. Atherton, Dairy Building, University of Vermont, Burlington, Vermont 05401.

Pat J. Dolan, California Dept. of Agriculture, 1220 North St., Sacramento, Calif. 96814.

Orlowe M. Osten, Minnesota Dept. of Agriculture, 530 State Office Bldg., St. Paul, Minn. 55155.

Earl O. Wright, P.O. Box 701, Ames, Iowa 50010

IAMFES Affiliate Council

Chairman: Clair Gothard, Houston City Health Dept., 1115 N. Gregory, Houston, TX 77030.

Secretary: Vernal Packard, Dept. Food Science and Nutrition, University of Minnesota, St. Paul, MN 55101.

Cornell Food Science Professor Named Emeritus

Richard P. March has been named professor of food science emeritus after 29 years as a faculty member at the N.Y. State College of Agriculture and Life Sciences at Cornell University.

In 1974, March was honored with the "Education-Industry Award" of the International Association of Milk, Food and Environmental Sanitarians. He is currently serving as vice president of the organization and will become president in 1980.

March has been a specialist in milk quality, and in fluid milk handling and processing in the Department of Food Science at Cornell. He has served on numerous departmental committees and has

served the department as extension leader.

He has written a monthly column in the Cooperative Extension Service News since 1969 and recently completed a widely used training film for bulk milk haulers.

March has made a major contribution to the field of dairy sanitation through his efforts to establish uniform sanitary and quality control regulations in the northeastern states and to improve communication in this field.

In 1963, he was the recipient of the New York State Association of Milk and Food Sanitarian's Paul B. Brooks Memorial Award and in 1972 was honored with the Emmet R. Gauhn Award. He will continue to serve as executive secretary for this

state association and for the Northeast Dairy Practices Council.

During the 1975 sabbatical leave, March made a study of the food science extension programs at 12 land grant colleges throughout the United States.

March is also a member of the Finger Lakes Sanitarians Association, the American Dairy Science Association, the National Mastitis Council, the Institute of Food Technologists and Sigma Epsilon Phi.

March received degrees in dairy industry from the University of Massachusetts and Cornell University. He has been active in many church and community activities. He and his wife, Barbara, plan to remain in Ithaca.

Risk/Benefit Concept Applies to Food Supply Also, Say Food Technologists

Although there is a widespread belief that there should be no hazard whatsoever associated with the food supply, the idea of food safety includes a constant comparison between risks and benefits, according to the Institute of Food Technologists in Chicago.

The risk/benefit concept is causing much debate among research scientists and regulatory agencies, according to IFT, a scientific society representing some 18,000 members. In a newly released Scientific Status Summary, "Benefit" is defined as anything that contributes to an improvement in a condition, while "Risk" is divided into the categories of "vital" and "non-vital." In developed countries, risks from food are normally far from vital or life-threatening, but zero risk or absolute safety in any area, including food, is unattainable, according to the Summary.

DRF Establishes Memorial Award

The Executive Committee of the Dairy Remembrance Fund, by unanimous vote, has adopted the suggestion of the family of the late Everett Byers to make a second-place \$50 annual award in the National Dairy Products Evaluation Contest.

Everett Byers served as All-Products judge in the National Contest from 1974 through 1977, and for twelve years before as official ice cream judge.

Byers died enroute home from the 1977 contest in Denver.

Since retiring from Jewell Foods Co., Chicago, in 1976 Byers has resided in Girard, Kansas. His keen sense of quality dairy products was respected by all who knew and worked with him.

The family will fund the annual award by making a memorial donation to the Dairy Remembrance Fund.

"The goal of absolute safety is a worthy one," the IFT Summary went on, "and some industries have approached it. For example, more than 800 billion units of commercially canned food have been produced in North America since 1940, with only five deaths attributable to botulism from that food." Yet in attempting to prove absolute safety for a process or ingredient, "the best we can ever hope for is to show no harm in every situation attempted to date, or no harm in the situation in which a material is useful."

The Status Summary describes a number of situations in which attempts to insist on absolute safety have deprived consumers of demonstrable benefits without providing freedom of choice, and it urges that a broad-based judgement as to "acceptable risks" be part of any regulatory decision. Among these instances is the proposal to ban saccharin, and questions as to whether (and how) to increase the iron content of the diet. Iron-deficiency anemia is relatively common and could be alleviated by increased

One other annual award is made by DRF, the Robert Rosenbaum \$100 memorial award to the first place student in the All-Products category. This honors the founder of the fund. The Byers' award will be made to the second place student in All-Products.

The nationally organized non-profit DRF, which operates solely on voluntary contributions, was founded as a means of recognizing outstanding professional men and women. Scholastic standing and field of study (dairy, marketing, or food related majors) are prime considerations in granting loans. Loans are interest free until graduation, when interest is applied at 2 percent per year. As loans and interest are repaid to the Fund, new loans are made to deserving students, thus insuring perpetuity.

supplementation of the food supply. However, a very small proportion of the population suffers from a condition known as hemochromatosis, in which they absorb more iron than they need. They could thus be endangered by increased amounts of iron in bread, for example, while most consumers would benefit.

The Summary also points out that widespread attention is being paid to possible risks from various food additives, although far less is known about comparable or greater risks from natural components of the food supply. "Applying the Delaney clause to naturally occurring carcinogens in food would, in many cases, make specific foods unavailable," the IFT Communicator stated. "Since there is ample precedent for setting a tolerance level for a natural carcinogen in food . . . it would seem that similar reasoning could also be applied to foods containing added chemicals, if the risks were judged to be low enough."

The Summary describes the dilemma posed by compounds such as DES (diethylstilbestrol), used to fatten cattle at a faster rate than normal feeding practices would permit. DES is known to be carcinogenic, as are almost all estrogens. Yet estrogens are produced in the human body and are naturally present in many food stuffs in much larger quantities than ever found in livers of cattle fattened by this technique.

Decisions require comparison of the valuable protein produced at a saving of almost 8 billion pounds of grain feed per year vs. the predicted but undemonstrated slight increase in disease.

According to the Summary, produced by IFT's Expert Panel on Food Safety and Nutrition, "The ability of scientists to detect minute quantities of chemicals has outstripped their ability to interpret their findings. Chasing an ever-receding 'zero' level with improved analytical instruments could bring you to the ultimate questions: 'Does the presence of one molecule of a

(see Risk page 419)

TAES Takes a Look at Retort Pouches . . .

Consumers may soon be reaching for certain products on their grocer's shelves that are packaged in Flexible foil pouches, called "retort pouches."

The pouches will not require freezing or refrigeration but will still have the same shelf life as conventional containers such as cans and glass jars.

"The development of the retort pouch is definitely one of the most significant advances in food packaging since the advent of the rigid can more than 150 years ago," contends Al B. Wagner, Jr., food technologist with the Texas Agricultural Extension Service.

The retort pouch is composed of three layers of materials, including an inner layer of a polyolefin blend, a middle layer of foil, and an outer layer of polyester. The polyolefin is inert so it will not react with the food and also provides heat sealing capabilities. The foil is a good moisture and light barrier while the polyester adds durability. Initially, the pouch will be inserted into a paperboard carton for added protection and ease of merchandising.

The retort pouch has been approved by the Food and Drug Administration and the U.S. Department of Agriculture after being in the development and planning stages for more than 15 years.

"This type of packaging concept is not new as European food firms currently market and sell more than 150 million retort pouches annually," points out the food technologist. "In Japan, the sale of food in retortable pouches exceeds 600 million units annually. Canada is also marketing several food products in this type of pouch."

Wagner notes some of the advantages of the retort pouch packaging concept:

—Process times can be reduced

which yields products with improved texture, color and flavor and saves energy.

—Weight and bulk factors are reduced.

—Convenience in preparation favors the pouch since it can be heated in boiling water for three

to five minutes, thus eliminating messy pots and pans.

—Paperboard cartons containing the pouches have more total display area for labels and other information than traditional containers such as cans.

(see *Pouches* page 419)

. . . And More Energy from an Old Source

Photosynthetic bacteria, converting wastes into fuel, may be one answer to this Nation's growing dependence on increasingly expensive oil, according to a research report from the Texas Agricultural Experiment Station (TAES).

Solar power is one alternative that scientists are especially interested in because it's abundant, free, non-polluting, and inexhaustible.

Dozens of ways are being tested to utilize solar power and many of them are at present too expensive for use. But one way, photosynthesis, has been used to trap the sun's power for as long as there have been light and plants.

Photosynthesis is the process whereby plants grow using light from the sun and nutrients from the air and soil solution. Scientists are researching ways to encourage plants to reach maximum production and serve as a renewable fuel source.

One disadvantage, of course, is that "fuel" farms would be competing for resources with "food" farms.

A scientist with TAES is working to convert agricultural wastes into hydrogen by use of "anaerobic photosynthetic bacteria."

"These bacteria are not subject to the oxygen limitations of many photosynthetic systems," explains Dr. C. R. Benedict of the Department of Plant Sciences at Texas A&M University.

"Such bacteria, in the presence of sunlight, can efficiently convert organic wastes into hydrogen and carbon dioxide. In addition, the bacteria could be harvested as a protein source for livestock feed.

"Cotton gin trash, unusable as livestock feed, can serve as food for these bacteria while they produce hydrogen for fuel. And the residue, when exhausted for food for the bacteria, can then be burned to produce additional electricity.

"Our laboratory results show that hydrogen production on cotton-gin trash with these photosynthetic bacteria is equal or superior to other biological systems.

"However, before the converter prototype is adapted to a larger-scale plant model, we plan to do additional research in a number of areas. Current studies on the cost analysis and energy balance of the laboratory hydrogen converter will be used in the design of a large scale model for industrial use," Benedict concluded.

Gin trash from a crop as large as the United States' accumulates at gins in large quantities. In addition to quantity, gin trash has other favorable attributes as an energy source: it's cheap, present uses are economically marginal, and this new process would help our energy situation by converting a low-value resource into high-value energy.

ADA Urges Development of Nutrition Policy; Sees Need For Comprehensive Education Program

The current bewildering spate of printed and broadcast nutrition information—sound, unsound, and debatable—contributes to food fadism on the one hand and public apathy on the other.

Evidence shows that faulty nutrition and diet are risk factors that precipitate crippling and killing diseases, and many leaders in government and industry recognize nutrition education as a critical issue. To promote well-being through educated eating habits, The American Dietetic Association (ADA) recommends that nutrition professionals work with lawmakers to forge a program of nutrition education for the nation.

In a position paper on "The Scope and Thrust of Nutrition Education," published today in the *Journal of*

The American Dietetic Association, the ADA urges high priority for developing a National Nutrition Policy designed to include such a comprehensive education program. The program, integrated into educational, health, and food delivery systems, could be cost effective.

The ADA position paper calls for a kindergarten-through-high school plan that introduces nutrition concepts as part of basic education in order to influence lifelong attitudes and habits and to provide a tool for assessing new information. In the schools, ADA says, lunch facilities should double as laboratories for teaching nutrition, as they are now in a few school systems.

"With rapidly changing conditions influencing lifestyles and sour-

ces of nutrients," the ADA observes, "the consumer must be educated to accept nutritional well-being as a responsibility." The Association of more than 35,000 dietitians takes the position that qualified personnel should be assigned to plan, direct, coordinate, and evaluate the program. To protect the public, the ADA asserts that any legislation providing for nutrition education must specify the qualifications of the nutrition educator.

The objective of nutrition education is not only to transmit knowledge about nutritional needs and nutritive values, the position paper states, but also to motivate the public to change eating habits. The Registered Dietitian is the health professional who is uniquely prepared to do both, the ADA points out.

The ADA holds that basic course content and continuing education for health-related professionals should include current nutrition information, because "with the increasing level of sophistication of nutrition knowledge, health workers should be prepared to use the expertise of the Registered Dietitian as a consultant and educator for themselves and their patients or clients."

Quality health care requires nutrition education as an essential component of all programs—protective, clinical, and rehabilitative—according to the ADA, which recommends in part:

- Third party reimbursement plans should specify nutrition education and diet counseling as required reimbursible services.
- Nutrition education and diet counseling should be part of the array of services offered in health care settings.
- Public health agencies should be funded to strengthen leadership in developing the nutrition education component of protective health care services.
- Public, voluntary, and private health agencies' efforts should be coordinated to reach specified nutritionally vulnerable segments of the population.

University of Wisconsin Scientists Study Refrigerant's Effectiveness as Food Preservative

Gases of the general type used in refrigerating equipment and as propellants in aerosol cans may be useful food preservatives.

University of Wisconsin-Madison food scientists M. A. Cousin, E. H. Marth, and Owen Fennema have found that fluorocarbon-21 inactivates two yeasts which can cause food spoilage—*Saccharomyces bailii* and *Debaryomyces hansenii*. Earlier studies by Marth and Fennema with J. L. Middleton showed the material to be effective against another yeast, *Saccharomyces cerevisiae*, and several researchers reported that certain bacteria are killed by fluorocarbons.

Fluorocarbons are not very toxic, they're not expensive, they're easily removed from food and they can be recovered for further use. The researchers think this type of preservation could be useful with foods that are harmed by conventional means of preservation such as heating, freezing, drying, fermenting, or adding chemical preservations.

The fluorocarbon works best with acidic liquids such as fruit juices. Salt also enhances action of the fluorocarbon. Sugars don't seem to interfere with its activity, but protein and fats do.

With B. A. Prior, Fennema and Marth also studied use of certain clinical anesthetics for food preservation. Fluorocarbons also act as anesthetics but are not used with humans because of bad side effects.

The clinical anesthetics, such as halothane and cyclopropane, have the same advantages as fluorocarbons for food preservation—they are non-toxic, inert, moderately volatile, and recoverable for further use.

The researchers tested the anesthetics against *Escherichia coli*. Halothane was the most effective, and all worked best in a liquid state.

This research was supported by the U.S. Public Health Service and the Continental Can Co., and by UW-Madison.

Single Service Institute Opens Washington Office

The Single Service Institute, the national trade association of manufacturers of single-use paper and plastic food service and packaging products, has opened a branch office in Washington, D.C.

Charles W. Felix, Director of Environment, Health and Public Affairs, has been named to manage the Washington office. The headquarters of the Institute remain in New York City, where Mr. Felix was assigned before moving to Washington.

In opening the new office, Mr. Felix pointed to the growing import-

ance of on-the-spot representation in the nation's capital, adding that "we feel a Washington office will be helpful both to Single Service Institute members and to the various government agencies and officials whose work relates to our industry's products."

To mark the official start of business at the new office, the Institute held a reception for members and guests on March 16 at the National Association Executives Club in the Capital Hilton Hotel.

The new Washington branch office is located at 915 15th Street, N.W., Washington, D.C. 20005.

1978 Directory of Food Labs Available

A directory describing the capabilities and areas of expertise of nearly 400 food testing laboratories and consulting organizations has just been made available by the Institute of Food Technologists in Chicago.

The 1978 IFT "Regional Guide to Food Testing Laboratories and Consultants" is organized according to the six U.S. Census regions, to make it easier to locate needed services in

any given part of the country. It also contains a section listing non-U.S. organizations, for those having testing requirements abroad.

In addition to brief descriptions of the testing capabilities and consulting services for each organization, the directory carries the name and phone number of the director of each organization, and its full address.

The 40-page directory is available from IFT Regional Guide, Lockbox 94332, Chicago, IL 60690 for \$5.00 per copy, postpaid.

AMI Sets Stage for 1978

Reservations for exhibit space at the American Meat Institute 1978 Convention now are being accepted. The AMI Convention is America's largest meat trade and educational exposition.

The 1978 Convention will be held in Chicago, November 11-14. The exhibits and meetings will be held at McCormick Place. *The Palmer House* will be this year's headquarters hotel. The 1978 exhibit hours will be from 10 a.m. to 5 p.m. on Saturday and Sunday, November 11 and 12; from 12 noon to 4 p.m. on Monday, November 13; and from 11 a.m. to 3 p.m. on Tuesday, November 14.

Exhibit information on the 1978 Convention has been sent to AMI

associate members and prospective exhibitors. Registration information will be mailed in late June.

The AMI Convention has become known as the place to go to see what's new in meat processing equipment, supplies, and services. Last year's show drew crowds of more than 10,000 visitors from all segments of the meat industry. More than 92,000 net square feet of booth space was booked by over 270 firms for the 1977 Convention.

For additional information, contact Charles Yuska, American Meat Institute, P.O. Box 3556, Washington D.C. 20007, Telephone (703) 841-2444.

The American Meat Institute is the national trade association of the meat packing and processing industry.

(Pouches from page 417)

Of course, there are also some drawbacks to the retort pouch:

- High capital investment in proportion to speed of fill.
- Need for a program to educate consumers about the pouch, its capabilities, and use characteristics.
- Paperboard carton which is an added step in production and presently renders the total packaging cost higher than the can. However, in the future pouch materials are expected to increase at a slower rate than those for cans.

"The pouch will play a significant role in our food packaging system in the future," believes Wagner. "Of course, canned and frozen foods will continue to occupy a share of the market although frozen foods may decline due to high energy costs for frozen storage in retail stores."

(Risk from page 416)

carcinogen constitute grounds for removing a food from the market place?"

Although it doesn't propose a final solution as to how risk/benefit decisions should be made, the IFT Expert Panel points out that "Pressure on food production is increasing each year, and we will soon reach the point where every technological concept in food availability will be needed to keep up with the population growth. The need for a realistic approach to the risk/benefit problem will thus become even more important."

Correction:

Officers for the Ontario Milk and Food Sanitarians Association were incorrectly listed in the April 1978 Association Affairs section of the *Journal of Food Protection*. Following are the correct officers.

ONTARIO MILK AND FOOD SANITARIANS ASSOCIATION

Pres., Roger Wray Ontario
 Vice Pres., Gail (Evans) Holland Islington
 Past Pres., C. L. Duitschaever Guelph
 Secretary, Jeanne Bernard, 6 Norfolk N., Hamilton, Ontario
 Treas., Bruce Keown, Borden Co., 273 King St., Ingersoll, Ontario

Directors:

John Sterns Ron Osborne
 Ralph Abell Bruce Hamilton
 John Atkinson

June 1-2, 1978. NUTRITION AND THE AMERICAN FOOD SYSTEM. Shoreham Americana Hotel, Washington, D.C. Co-sponsored by Food Marketing Institute, Community Nutrition Institute, and Family Circle Magazine. Contact: Karen Brown, FMI, 1750 K St., NW, Washington, D.C. 20006.

June 4-7, 1978. 1978 ANNUAL MEETING OF THE INSTITUTE OF FOOD TECHNOLOGISTS, Dallas, Texas. Contact: Dan Weber, Director of Convention Services, IFT, 221 N. LaSalle St., Chicago, IL 60601.

June 7-10, 1978. NATIONAL FOOD BROKERS ASSOCIATION MANAGEMENT CONFERENCE. Miami, FL. Contact: NFBA, 1916 M St. NW, Washington, DC 20036.

June 14, 1978. WISCONSIN DAIRY PRODUCTS ASS'N. SUMMER CONFERENCE. Dell View Hotel, Lake Delton, WI. Contact: Norm R. Maier, WDPA, 324 Hamilton St., Madison, WI 53703.

June 18-21, 1978. RECIPROCAL MEAT CONFERENCE OF THE AMERICAN MEAT SCIENCE ASS'N. University of Connecticut, Storrs, CT. Contact: National Livestock and Meat Board, 444 N. Michigan Ave., Chicago, IL 60611.

June 20-23, 1978. FLORIDA DAIRY PRODUCTS ASS'N CONVENTION. Breakers Hotel, Palm Beach, FL. Contact: Joseph Antink, Suite 315, Bradshaw Bldg., Orlando, FL 32801.

June 26-30, 1978. 20th INTERNATIONAL DAIRY CONGRESS, Palais des Congres, Paris, France.

Contact: Herbert Forest, Director, Dairy Div., Agricultural Marketing Service, USDA, Washington, D.C. 20250.

July 9-13, 1978. AMERICAN DAIRY SCIENCE ASS'N/ AMERICAN SOCIETY OF ANIMAL HEALTH. Joint meeting, Michigan State University, East Lansing, MI. Contact: ADSA, 113 N. Neil St., Champaign, IL 61820.

July 17-21, 1978. ADVANCES IN FOOD AND APPLIED MICROBIOLOGY. Massachusetts Institute of Technology, Cambridge, MA. Contact: Director of Summer Session, Rm. E19-356, Massachusetts Institute of Technology, Cambridge, MA 02139.

July 24-28, 1978. FOOD PROCESSORS BASIC MICROBIOLOGY SHORT COURSE. Cruess Hall, University of California, Davis. Contact: Dr. Robert J. Price, Food Science and Tech. Dept., University of California, Davis, CA 95616.

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Faculty Position Announcement

The Department of Animal Sciences at the University of Kentucky has a vacancy in Dairy-Food Technology Extension. The person who fills this position should have a Ph.D. in Dairy Technology, Food Science or related area. A working knowledge of or experience in the dairy-food industry is desired. The position is 100% Extension; however, if the person filling the position so desired, a joint Extension-Research appointment could be arranged with up to 40% of the appointment Research.

Send complete resume, transcript of all college work and at least four references to: B. E. Langlois, Department of Animal Sciences, 204 Agriculture Science Center, South, University of Kentucky, Lexington, KY 40506. The position is now available and applications will be received until a suitable candidate is found. The University of Kentucky is an equal opportunity employer.

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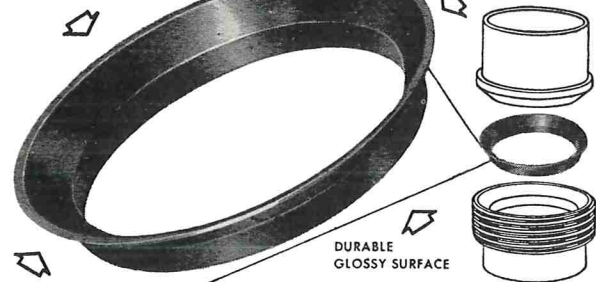
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*Dr. George C. Fisher, Head
Veterinary Services Laboratory,
Ontario Ministry of Agriculture & Food,
Kemptville, Ontario*



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Through Total Management, you coordinate planning to minimize emergencies, be they in health, equipment, or production. This is done with the help of available experts in six broad areas:

1. Soil Testing
2. Nutrition
3. Cow Performance
4. Equipment Maintenance
5. Herd Health Program
6. Record Keeping

Though some areas may appear more important than others, the exclusion or neglect of any one will result in less profit, or as a sudden problem in production or herd health—a problem easily avoided with proper management and teamwork.

The Team in Action

Total Management provides the farmer with

continually updated information, and assures that all areas are working to his benefit. Since the amount of information available is more than any one person can possibly handle, specialists are essential to apply the right facts to your needs. Broadly stated then, the TMC helps you make informed decisions and to put them into practice.

• HERD HEALTH

Use your local veterinarian in a systematic approach to a herd health program (specifically in respect to infertility and mastitis control).

• PRODUCTION GOALS

Set uniform production goals designed to meet the capabilities of the herd and farm unit with the assistance of your County Agent.

• RECORDS

Apply a unified approach to the keeping of records relating to herd health, nutrition, reproduction, and production—(with the help of a milk recording system such as D.H.I.A. or R.O.P.). University extension people are trained to provide guidance in this area.

• EQUIPMENT

Have your dairy equipment dealer perform periodic checks and adjustments of the milking system through a scheduled maintenance program. This will assure proper equipment operation for better production and improved herd health.

• FEEDING PROGRAM

Work with your nutrition specialist to develop an in-depth feeding program using nutrient analysis to determine year-round feeding according to production. Base this program on the production, storage, and utilization of high quality forages.

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