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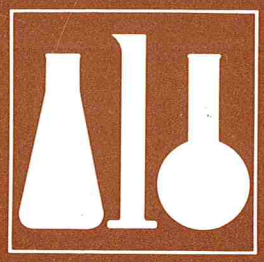

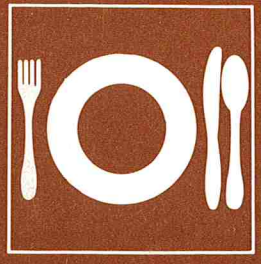

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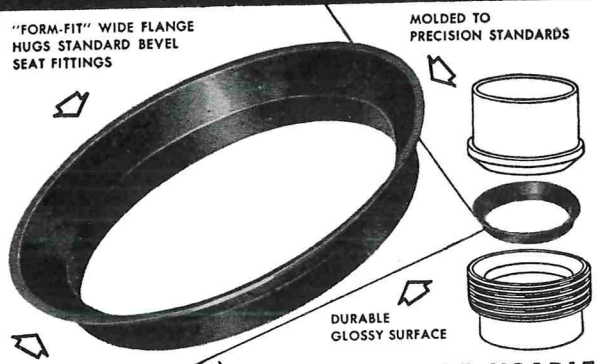


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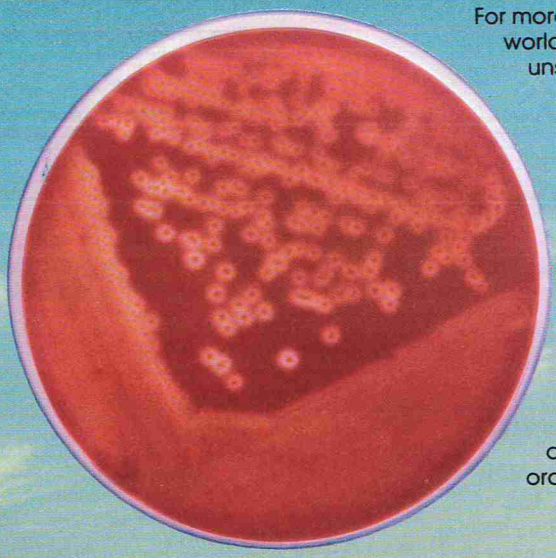

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*Journal of Food Protection Vol. 42, No. 10, Pages 778-779 (October, 1979)*


# Activity of NAD-Dependent Dehydrogenase Enzyme of Lactic Streptococci on Acetaldehyde and n-Hexanal[1]

**R. H. SCHMIDT, L. K. FARRON, T. R. BATEH and P. E. ARAUJO**

*Food Science and Human Nutrition Department, IFAS, University of Florida, Gainesville, Florida 32611*

(Received for publication December 15, 1978)

**ABSTRACT**

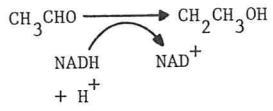
Alcohol dehydrogenase (alcohol:NAD oxidoreductase, E.C. 1.1.1.1) activity was observed on acetaldehyde and n-hexanal in homogenized cell extracts of *Streptococcus lactis* C2. Substrate inhibition was apparent at levels of n-hexanal above 4.0 mM. Increased centrifugal force from 12,000 × *g* for 20 min to 350,000 × *g* for 1 h resulted in increased specific activity in the cell-extract supernatant fluids. Aldehyde dehydrogenase (aldehyde:NAD oxidoreductose; E.C. 1.2.1.3) was not detected in any of the cell extracts. A possible involvement of the enzyme system with flavor modification in lactic-fermented oilseed milk is suggested.

Oilseed milk or beverage products generally are not widely accepted in many parts of the world because of well-documented grassy and beany off-flavors associated with these products (*8*). Lactic acid fermentation has been shown to improve or modify sensory acceptance of soy and peanut beverages (*1,8,10,13*). The mechanism of flavor improvement has not been investigated. It may involve a masking effect by lactic acid and/or other metabolites. It could also involve enzymatic modifications of volatile flavor compounds by the lactic bacteria.

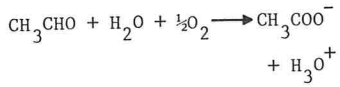
Volatile compounds normally associated with soybean or peanut preparations are generally aliphatic aldehydes (*9,11*). Modification of similar aldehydes by indigeneous enzymes of fruit and vegetables has been shown to affect the flavor by altering the aldehyde:alcohol equilibrium (*4*). A similar alteration in the acetaldehyde:ethanol equilibrium occurs in cultured dairy products (5). Since high levels of acetaldehyde are considered undesirable in most cultured products with the exception of yogurt (5), the ability of starter organisms to degrade acetaldehyde is extremely important. The possible mechanisms for acetaldehyde modification by lactic acid bacteria have been summarized in Fig. 1. Nicotinamide adenine dinucleotide (NAD)-dependent alcohol dehydrogenase (E.C. 1.1.1.1) and aldehyde oxidase (E.C. 1.2.3.1) activity on acetaldehyde have been reported for a variety of lactic acid bacteria (*6,12*). NAD-dependent acetaldehyde dehydrogenase (E.C. 1.2.1.3), not as extensively investigated, has been observed in *S. lactis* (*12*).

Since a relatively broad substrate specificity may be expected for the enzymes given in Fig. 1, it is conceivable that they could be involved in flavor modification in fermented oilseed products. Reactivity of these enzymes,

1. Alcohol Dehydrogenase (Alcohol:NAD Oxidoreductase)




2. Aldehyde Oxidase (Aldehyde:Oxygen Oxidoreductase)




3. Aldehyde Dehydrogenase (Aldehyde:NAD Oxidoreductase)

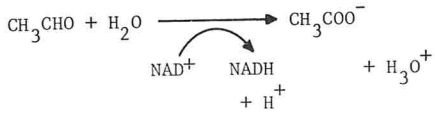



Figure 1. *Possible enzymatic mechanisms for acetaldehyde modification by lactic acid bacteria.*

however, varies with enzyme source and with substrate (*3,9*). This variability prompted investigations using substrates other than acetaldehyde for lactic streptococcal extracts.

In the present investigation, the reactivity of NAD-dependent enzymes (alcohol and aldehyde dehydrogenase) of *Streptococcus lactis* C2 was evaluated. Acetaldehyde and n-hexanal, a lipid-derived aldehyde associated with oilseed products (*9,11*), were used as substrates. The cellular location of the enzyme system was partially characterized by ultracentrifugation.

## MATERIALS AND METHODS

*S. lactis* C2, a strain with known ability to degrade acetaldehyde, (*6*), was obtained from the culture collection of the Department of Food Science and Nutrition, University of Minnesota. The organism was maintained in sterile 11% reconstituted nonfat dry milk. After three successive subculturings in Elliker broth (Difco, Detroit, MI), a 1.0% inoculation was made into 2.0 liters of the broth. Incubation was at 22 C for 18 h.

Cells were harvested by centrifugation in a Sorvall RC-5 centrifuge at 9000 × *g* for 20 min at 4 C. Pellets were resuspended and recentrifuged twice in 20 mM sodium phosphate buffer (pH 7.0). The final pellet was diluted 1:5 with phosphate buffer. A 30-ml aliquot of cell suspension was mixed with 30 g of size 12 glass beads and 0.2 ml of tri-n-butyl phosphate in a 75-ml Duran flask. The cell walls were ruptured in a Braun homogenizer for 2.5 min at 4000 oscillations/min with sufficient

[1]*Florida Agricultural Experiment Stations Journal Series No. 1568.*

$CO_2$ delivered for cooling (2). Cell disintegration was estimated at 99% since gram stain revealed homogeneous gram-negative material. Glass beads were removed from homogenized cell extracts by filtering through a coarse sintered glass funnel. Cell debris was removed by centrifugation at 12,000 × *g* for 30 min at 4 C. A portion of the supernatant fluid was then centrifuged in a Sorvall OTD-2 ultracentrifuge at 350,000 × *g* for 1 h at 2 C to remove membranes, ribosomes and related materials. Protein concentration of the supernatant fluids was determined according to the method of Lowry et al. (7).

Activity of alcohol dehydrogenase and aldehyde dehydrogenase was measured by following the rate of $NADH_2$ oxidation and NAD reduction at 340 nm at 25 C with a Beckman Model 25 spectrophotometer (6). Reaction mixtures (total volume 3.0 ml) contained 33.0 μM of $NADH_2$ or NAD, 0.13 to 6.25 mM acetaldehyde or n-hexanal (Aldrich Chemical Co., Milwaukee, WI), 20 mM phosphate buffer (pH 7.0) and 50 μl extract. Specific activity is reported as the absorbance (340 nm) change $min^{-1}$ mg protein in $extract^{-1}$.

## RESULTS AND DISCUSSION

### *Alcohol dehydrogenase activity*

The effect of increased substrate on alcohol dehydrogenase activity of *S. lactis* C2 is shown in Fig. 2. Specific enzyme activity reached an apparent maximum at 2.0 to 4.0 mM of acetaldehyde or n-hexanal. Slightly higher specific activity was observed on n-hexanal than for acetaldehyde at similar concentration. However, substrate inhibition was readily apparent with dramatic activity reduction at higher hexanal levels. This suggests that the *S. lactis* enzyme could be expected to actively degrade this aldehyde only in very specific concentration ranges. Slightly higher specific activity of alcohol dehydrogenase was observed in the 350,000 than in 12,000 -*g* cell-extract supernatant fluids (Table 1), suggesting a partial purification by the centrifugation or that the cellular location of the enzyme is probably cytoplasmic.

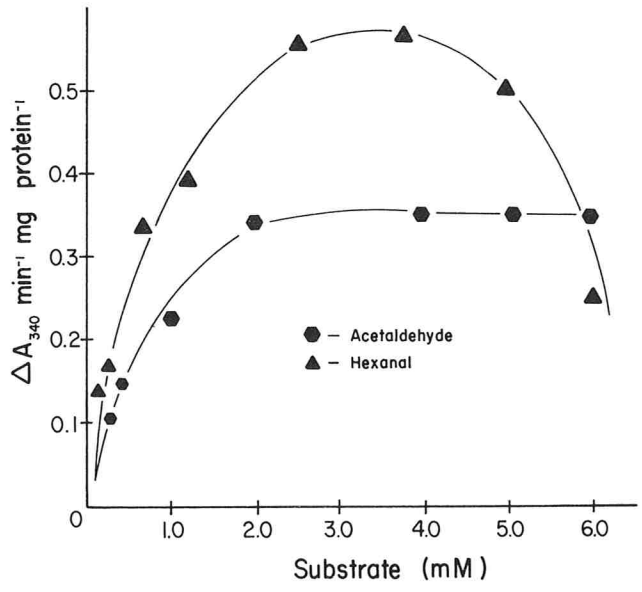



Figure 2. *Effect of increasing the substrate level (acetaldehyde or n-hexanal) on specific activity of NAD-dependent alcohol dehydrogenase activity of* S. lactis *C2.*

### *Aldehyde dehydrogenase activity*

Activity of NAD-dependent aldehyde dehydrogenase was not detectable in the *S. lactis*, C2 extracts under conditions of our experiments. Oxidative conversion of aldehydes to acid by lactic streptococci may follow the aldehyde oxidase pathway (Fig. 1) reported by Lees and Jago (6). This enzyme system should be investigated further.

While additional research is needed, it is apparent that NAD-dependent alcohol dehydrogenases could play a role in the flavor modification that occurs during lactic fermentation of oilseed milks. Their effectiveness would be dependent on the extent of off-flavor development, redox potential and the $NAD:NADH_2$ ratio in the system.

TABLE 1. *Effect of centrifugation on alcohol dehydrogenase activity of* S. lactis *C2 supernatant fluids.*

| Centrifugation force | Specific activity[a] |
|---|---|
| 12,000 g[b] | 0.35 |
| 350,000 g[c] | 0.81 |

[a] $\Delta A_{340}$ $min^{-1}$ mg protein in $extract^{-1}$.
[b] 7,500 rpm for 20 min (Sorvall RC-5).
[c] 60,000 rpm for 1 h (Sorvall OTD-2).

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# Potassium Sorbate as a Fungistatic Agent In Country Ham Processing

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(Received for publication January 5, 1979)

**ABSTRACT**

Sixty, seventy and ninety-day-old country cured hams were used to evaluate potassium sorbate as a fungistatic agent during aging and holding for market. A 1-min spray of 5% (w/v) potassium sorbate offered the lowest effective level for inhibition of fungal growth. Mold and yeast colony counts 30 days post-treatment were significantly lower than initial numbers but protection was lost by the 60th day under conditions conducive to fungal outgrowth (21 ± 5 C and 70 ± 5% relative humidity). Greater mold inhibition was noted when a 10% potassium sorbate spray was used under identical conditions. Less than 65% relative humidity inhibited mold growth on 120-day-old ham slices held at 7 C. Mold and yeast counts tended to be lower on hams treated after 60 days of processing than on hams treated after 90 days of processing. Residual concentrations of sorbic acid required to inhibit mold growth and yield an acceptable ham after 30 days storage were within the limit approved by the Food and Drug Administration for other food products.

Country cured ham has been a traditional meat product of the Southeastern United States for more than 300 years, and its commercial value has been realized for many generations. Currently, large scale commercial production is found in much of the Southeastern region. One of the recurring problems in production and distribution of country cured hams is the growth of molds and yeasts on ham surfaces during conditions of high relative humidity. Such conditions are fairly common in the Southeast.

Fungal growth on aged country ham was at one time thought to be indicative of proper aging and flavor development. However, recent studies have revealed that development of flavor in dry-cured ham is the result of enzymatic and chemical changes rather than the result of microbial growth (*9,11*).

For several years, larger processors have been expressing interest in expanding their sales to locations beyond local and regional markets, including overseas markets. Yet a consumer from outside the "country ham belt" may regard a moldy country ham as spoiled. More important is a possibility of the potential presence of mycotoxins. Several studies have revealed that a number of mold species isolated from country ham possess the capability of producing mycotoxins when grown in pure culture on laboratory media. These toxins include ochratoxin, sterigmatocrystin, citrinin and aflatoxin (*6,10,14,15,17*). A need, therefore, exists for an effective fungistatic agent for use on country cured hams and other dry-cured meats.

Sorbic acid and its potassium salt have long been used in foods as effective non-toxic inhibitors of fungal growth, including those genera that possess a mycotoxin-producing capability. Many investigations on use of sorbates in food products have resulted in their broad approval as antimicrobial agents in dairy, bakery and fruit preparations. The only meat products where use of sorbate is presently permitted are dry sausages (2.5% dip) and dog food patties (*3*). Studies conducted during the past 23 years have demonstrated the lack of toxicity of sorbates to rats, mice and dogs (*4,5,8,12*).

The purpose of this work was to determine the effectiveness of potassium sorbate as a fungistatic agent on country hams under conditions conducive to fungal growth.

## MATERIALS AND METHODS

### *Treatment of ham slices*

Ham slices were obtained from four country hams (long shank variety) that had been cured 30 days, held 30 days at 55 F to allow even distribution of salt (equalized) and aged 205 days under ambient conditions. Each ham was cut in half and the butt half was discarded. Ten 1.3-cm slices from the shank half of each ham were randomly paired within hams. One-liter sorbate solutions of 0, 2.5, 5, and 10% concentration were prepared at ambient temperature (25 C). One pair of slices from each ham was successively placed into each solution for 1 min. Another pair was dipped into the 10% sorbate solution for 2 sec. One slice from each pair was placed in storage at 22 ± 5 C and 70 ± 10% relative humidity to be examined at 60 days for mold growth and sorbate residuals.

### *Treatment of whole hams*

Twenty-eight 70-day-old country cured (packer-style cut) hams were used for studying the inhibitory properties of potassium sorbate applied by a 1-min spray of ambient temperature aqueous solutions at 0, 2.5, 5 and 10% concentrations. Analyses were as follows: (*a*) one ham per treatment level was analyzed on the day of treatment for sorbate residual, (*b*) three hams at each treatment level were analyzed at 30 and 60 days for sorbate residual, mold and yeast colony counts and visible mold.

Before spray treatment, the black pepper coating applied by the commercial producer was removed by scrubbing and washing to prevent it from masking mold development. Hams were air-dried overnight, bagged in cotton stockinettes, and hung shank end down. The hams were then sprayed for 1 min with appropriate concentrations of potassium sorbate using a stainless steel hand sprayer operated by compressed air (garden type). The hams were stored in a room held at 21.1 ± 0.5 C and 70 ± 5% relative humidity until removed for testing.

[1]*Present address: Route 3, Box 107, Bowling Green, KY 42101*
[2]*P.O. Box 130, 2105 Independence Blvd., Kansas City, MO 64124*
[3]*ITT Gwaltney Inc., P.O. Box 489, Smithfield, VA 23430*

*Sorbate analysis*

The method of analysis for sorbate residuals was developed from the methods of Bokus (*1*) and Wilamowski (*16*). Ham slices were trimmed of fat and skin and then were ground in a Waring blender for 15 sec. Samples for the whole ham study were obtained by surface trimming the entire lean ham face at a depth of 2-5 mm to obtain approximately 15 g of sample. The 15 g from each ham were then dry blended in a Waring blender for 15 sec. Ten grams of the ground ham were transferred to a second blender jar containing 100 ml of an alcoholic solution of metaphosphoric acid (5 g of $HPO_3$ in 250 ml of $H_2O$ and diluted to 1 liter with alcohol). The mixture was blended for 1 min and allowed to stand for 10 min. Contents of the blender jar were then vacuum-filtered through Whatman No. 3 filter paper. Five ml of the filtrate were transferred to a 250-ml separatory funnel and 100 ml of 1:1 petroleum ether-ethyl ether were added. This final ether solution contained the sorbic acid extracted from 500 mg of ham.

The mixture was shaken for 1 min, after which the ether layer was recovered and dried with 5 g of anhydrous sodium sulfate. Absorbance of the ether layer was determined spectrophotometrically at 250 nm using the extracts from untreated ham samples as a blank.

*Preparation of the standard curve*

The standard curve was prepared from stock solutions containing 0.134 g of potassium sorbate (equivalent to 0.1 g of sorbic acid) in 100 ml of deionized water. Aliquots of 1 to 6 ml were made up to 100 ml with the alcoholic metaphosphoric acid solution. Five ml from each flask were then shaken for 1 min with 100 ml of 1:1 petroleum ether-ethyl ether. The ether layer was recovered and dried with 5 g of anhydrous sodium sulfate.

Absorbance was determined at 250 nm versus a blank prepared with 5 ml of metaphosphoric acid solution and was plotted against mg of sorbic acid/100 ml of ether. A linear curve was obtained.

*Calculations: spectrophotometry*

Concentrations of sorbic acid in ham were calculated by the following formula:

$$\frac{(\text{mg sorbic acid}/100\text{ ml ether})}{500\text{ mg}} \times 2000 = \text{ppm sorbic acid in ham}$$

*Visible mold evaluations*

Subjective evaluations of visible mold on the ham slices were made at 8, 16, 24, 36 and 60 days. Whole hams were similarly evaluated at 30 and 60 days. Intensity of mold growth was rated on a scale of 0 to 5 where: 0 = no growth, 1 = very slight growth, 2 = slight growth, 3 = moderate growth, 4 = marked growth and 5 = intense growth.

*Enumeration of mold and yeast colonies*

A 25.8-$cm^2$ area of lean meat was swabbed using a 5.08²-cm template. The swab was placed in 10 ml of 0.85% saline solution and mixed. Aliquots of the solution, 1 ml and 0.1 ml, were surface-plated on acidified Czapek agar. Plates were incubated at 22 C for 5 days (*7*). Mold and yeast colonies were counted using a Quebec colony counter.

## RESULTS AND DISCUSSION

*Mold growth evaluation (slices)*

Mold growth was subjectively evaluated on the 0 to 5 scale at specific times following treatment and at various treatment levels (Table 1). Using ham slices as a test system, growth values between 0 and 1 represent optimum control; levels between 1 and 2 were acceptable; values above 2 were considered unacceptable.

Greater fungal growth was observed with increasing time and with decreasing levels of potassium sorbate. Factorial analysis of variance indicated significant differences at the P = .01 level for all three parameters: potassium sorbate level, days following treatment and source of the ham slice (Table 1), with the largest F value for the source of the slice. Slices that exhibited the most prolific fungal growth were obtained from Ham #52. The arithmetic growth mean of 2.32 was almost twice that of any other ham and about five times the minimum level, suggesting slices from Ham #52 were initially heavily contaminated with mold, and that sorbate treatment would be considerably less effective under these circumstances.

TABLE 1. *Factorial analysis of the effect of potassium sorbate level (L), days following treatment (D), and source of ham slices (S) on visible mold evaluation of 60-day ham slice study.*

| Factor | Growth means[a] | |
|---|---|---|
| *Potassium sorbate level (L) (%)* | | |
| 0 | 2.55 | |
| 2.5 | 1.10 | |
| 5 | 1.00 | F = 53.23**[c] |
| 10[b] | 0.76 | |
| 10 | 0.36 | |
| *Days following treatment (D)* | | |
| 8 | 0.26 | |
| 16 | 0.46 | |
| 24 | 1.45 | F = 42.92** |
| 36 | 1.70 | |
| 60 | 1.90 | |
| *Source of ham slice (S)* | | |
| Ham 37 | 0.45 | |
| Ham 40 | 0.65 | F = 67.85** |
| Ham 46 | 1.20 | |
| Ham 52 | 2.32 | |

[a]Growth expressed by visual evaluation on scale of 0 to 5: 0 = no growth, 1 = very slight, 2 = slight, 3 = moderate, 4 = marked, 5 = intense. Mean of 10 observations.
[b]Two second dip; all others are 1-min dips.
[c]**Denotes significant at 1% level.

*Sorbate residuals (slices)*

Residual studies were conducted to help elucidate the relationship between sorbate concentration and fungistasis over the 60-day study. Table 2 summarizes data from 32 ham slices taken from four cured hams. Sorbic acid residuals were determined immediately after dipping and after 60 days of storage. In some instances, wide variations were observed between samples subjected to identical treatment levels. A significant difference between the sample means was noted at the $P \leq 0.5$ level (Table 2). Variation within a treatment was attributed to the inherent irregularities of the ham slices and uneven absorption of sorbate.

Length of storage definitely influenced residual concentrations ($P < .01$). After 60 days, sorbic acid concentrations decreased by about 50% (583.5 to 250.0 ppm). These results differ from these of Boyd and Tarr (*2*) who found that sorbic acid residuals in smoked fish did not decrease appreciably over a period of 60 days at 25 C and 75% relative humidity.

*Mold and yeast evaluation (whole hams)*

The application of potassium sorbate to whole hams gave pronounced effects on levels of mold and yeast. In ham sprayed for 1 min with potassium sorbate (2.5, 5 or 10%), the initial yeast population was reduced to less than

1 colony forming unit/cm² (CFU/cm²), while the control hams sprayed only with plain water maintained a viable population of 1,100 yeasts CFU/cm² (Table 3). In contrast, mold counts on zero day were not significantly different at all treatment levels (0, 2.5, 5, and 10%) (Table 3); yet a slight reduction in numbers was noted. Reduction in yeast population appeared related to the washing of spores from the ham surface.

In a preliminary study, the spray (until visibly wet) applied to a lot of hams, identical to those in above study, did not significantly reduce the numbers of yeast or mold at all treatment levels (0, 2.5, 5, and 10%). Thus, the 1-min spray exposure appears to be the best method of application and one that is feasible in most commercial curing operations.

TABLE 3. *Statistical summary of mold and yeast colony counts on 70-day-old country hams at 0 days*[a].

| Potassium sorbate (%) | Mold | | Yeasts | |
|---|---|---|---|---|
| | Colony count (Mean) | Range | Colony count (Mean) | Range |
| 0 | 1.3[b] | 0-3.9 | 1,100 | 496-1,400 |
| 2.5 | 0.7 | 0-1.6 | 0.3 | 0-0.8 |
| 5 | 0.6 | 0-1.6 | 0.7 | 0.2-1.2 |
| 10 | 0 | 0 | 0.1 | 0-0.4 |
| | F —[c] | | F = 29.3**[d] | |

[a]All values expressed as colony forming units/cm².
[b]Mean of 4 observations from one ham.
[c]F — Denotes non-significance between treatment.
[d]**Denotes significance at 1% level.

After 30 days of storage, the mold and yeast colony counts increased. Mold on control hams (0% potassium sorbate) were too numerous to count while yeast counts ranged from 2,500 to 39,000/cm² (averaging 11,700 yeasts/cm²). The 10% potassium sorbate treatment showed mold counts ranging from 16 to 240 CFU/cm² with an average of 81 CFU/cm². Yeast counts at the 10% level ranged from 150 to 490 CFU/cm² with an average of 290 CFU/cm².

Table 4 gives growth means obtained by visual observation. At the end of the 30 days, the effect of sorbate treatment was significantly different at each level. The 5% sorbate concentration showing a 1.3 growth value (mean) would be acceptable but marginal for production of marketable hams. The 10% level produced a 0.7 growth value which is below the "very slight" category and would represent a very acceptable level in the market place. The fungistatic effect of this level is very desirable.

TABLE 4. *Summary of visible mold evaluation of 70-day-old country hams.*[a]

| Potassium sorbate (%) | 30 Days | | 60 Days | |
|---|---|---|---|---|
| | Growth (Mean) | Range | Growth (Mean) | Range |
| 0 | 4.3 | (4-5) | 4.7 | (4-5) |
| 2.5 | 3.3 | (3-4) | 4.0 | (4) |
| 5 | 1.3 | (1-2) | 4.0 | (4) |
| 10 | 0.7 | (0-1) | 4.0 | (3-5) |
| | F = 35.07**[b] | | F —[c] | |

[a]Growth expressed by visual evaluation on scale of 0 to 5: 0 = no growth, 1 = very slight, 2 = slight, 3 = moderate, 4 = marked, 5 = intense.
[b]**Denotes significance between treatment at 1% level.
[c]F — Denotes non-significance between treatment (60 days).

At the 60 days evaluation, fungal growth was approaching the intense level (Table 4), and there was no significant difference in the effects of treatment (0-10%). Thus, the inhibitory property of potassium sorbate was lost sometime between 30 and 60 days of storage in an ambient humid environment.

### *Sorbic acid residuals (whole hams)*

The sorbate residual data obtained from analysis of exterior lean meat (2-5 mm in depth) trimmed from the face of the whole 70-day-old country hams are summarized in Table 5. The effectiveness of the 1-min spray exposure was substantiated by the residuals shown on zero day. Although concentrations seemed fairly high, these values were from the exterior surface of the ham and would not reflect the concentration of the overall whole ham. Presumably these high residuals resulted in part from the stockinette cover aiding in retention of sorbate.

Thirty days after treatment a reduction in the concentrations of sorbate was evident (Table 5). The difference between treatments became less after 30 days of storage, but still statistically significant. On hams treated with 2.5 and 5% sorbate, there was mold growth on 27 of 28 hams studied. Molds in sufficiently high populations can metabolize the sorbate, thereby reducing its fungistatic effect (*13*). This may serve to explain why we found no significant difference between 2.5 and 5% residual levels at the end of 30 days. Since the 5% level only gives marginal fungal inhibition, it would appear that 142-228 ppm sorbic acid is about the lowest level for fungal inhibition. The 10% treatment with

TABLE 2. *Sorbic acid residuals from 60-day ham slice study.*[a]

| Potassium sorbate (%) | 0-Day | | 60-Day | | Average[b] |
|---|---|---|---|---|---|
| | Residual (Mean) | Range | Residual (Mean) | Range | |
| 2.5 | 247[c] | 224-270 | 135 | 130-140 | 191 |
| 5 | 422 | 400-444 | 276 | 178-374 | 349 |
| 10[d] | 625 | 590-660 | 268 | 200-336 | 337 |
| 10 | 1040 | 680-1400 | 323 | 270-376 | 682 |
| Average[e] | 584 | | 250 | | |

[a]All values expressed as ppm sorbic acid.
[b]For percent potassium sorbate, $F = 4.53$; $P \leq .05$.
[c]Mean of two observations.
[d]Two second dip; all others 1-min dip exposure.
[e]For storage time (Days), $F = 11.91$; $P \leq .01$.

TABLE 5. *Summary of sorbic acid residuals on 70-day country hams.*[a]

| Potassium sorbate (%) | 0 Days | 30 Days | | | 60 Days | | |
|---|---|---|---|---|---|---|---|
| | Mean | Mean | Range | S.D.[b] | Mean | Range | S.D. |
| 2.5 | 700[c] | 83 | 30-148 | 60.0 | 32 | 0-96 | 55.4 |
| 5 | 2100 | 173 | 142-288 | 48.5 | 50 | 0-100 | 50 |
| 10 | 2400 | 432 | 348-568 | 118.9 | 85 | 0-194 | 99 |
| | | | F = 13.82*[d] | | | F —[e] | |

[a]All values expressed as ppm sorbic acid.
[b]S.D. denotes standard deviation.
[c]One observation only.
[d]*Denotes significance at the 5% level for treatments (30 days).
[e]F — Denotes non-significant (60 days).

residuals of 348 to 568 ppm approached the optimum concentration.

After 60 days of storage, the residual levels of sorbate became quite low, explaining why we had fungal growth on every ham. At the highest treatment level (10%), our range of residuals was from 0 to 194 ppm. As shown in Table 5, there was no significant difference between treatment levels. These results, along with the 60-day visual mold evaluation, seem to reinforce the conclusion that the ability of sorbate to inhibit fungal growth on country hams is lost sometime between 30 and 60 days of storage in an ambient humid environment.

Based on 20-day residual data, it would appear that a minimum sorbate concentration of 400-500 ppm on the surface of the hams is required to limit fungal growth to an acceptable level. Our data indicate that an initial concentration of about 2400 is needed to assure an adequate concentration at 30 days. This initial level is within the range of 0.1-0.3% (by weight) already permitted by FDA in certain foods. Furthermore, we have analyzed only the exterior surface (2-5-mm depth) and the concentration in the interior of the ham would undoubtedly be much lower. Therefore, the total amount of sorbate consumed in a slice of treated ham with surface level of 2400 ppm would be well below that consumed with the same weight of processed product such as margarine where the sorbate concentration is 1000 ppm and uniformly distributed. Secondly, the level falls sharply during storage, being no more than 18% of the initial amount added after 30 days of storage on whole hams treated with 10% sorbate.

## SUMMARY

Potassium sorbate applied as a 10% solution was effective as a fungistat on country ham slices and on whole hams. The inhibitory effect lasted at least 30 days under conditions conducive to fungal development. However, by 60 days fungistatic property was lost primarily because the residual level of sorbate dropped, rapidly during storage. A 5% solution gave only marginal protection during the first 30 days. The 1-min spray exposure offered advantages for commercial use over the quick dip or spray because of its washing action to remove spores and the higher initial concentration of sorbate on the hams. The cotton stockinette used to bag the hams may have assisted in sorbate retention. The residual concentration of sorbate required to inhibit mold growth and yield an acceptable ham after 30 days storage was well within the limit approved by FDA for other food products.

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# Toxin Production by *Clostridium botulinum* in Shelf-Stable Pasteurized Process Cheese Spreads

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**ABSTRACT**

Five non-refrigerated, pasteurized process cheese spreads, considered shelf-stable, were studied for their ability to support growth and toxin production by spores of *Clostridium botulinum* types A and B. Based on pH and water activity ($a_w$) Cheese with Bacon, Limburger, Cheez Whiz, Old English, and Roka Blue cheese spreads were selected for the study. The pH ranged from 5.05 to 6.32 and the $a_w$ from 0.930 to 0.953. Fifty jars of each cheese spread were inoculated with 24,000 spores each; an additional 50 jars of the Cheese with Bacon spread received 460 spores each. The inoculum consisted of five type A and five type B strains in 0.1 ml of 0.85% NaCl. At 35 C, 46 jars of Limburger and 48 jars of Cheese with Bacon spread, which received the greater inoculum, became toxic starting at 83 and 50 days, respectively. One jar of Cheese with Bacon spread which received 460 spores became toxic. The average toxicity of the Limburger was 3000 MLD/ml of extract as compared with 54 MLD/ml for the Cheese with Bacon spread. Results of this study will be considered in determining whether these cheese spread products should be treated as low-acid canned foods under the Good Manufacturing Practice Regulations of the Food and Drug Administration.

Pasteurized process cheese spread has been marketed for many years as a shelf-stable product. It is usually sold in hermetically sealed glass jars. However, the question arises whether pasteurization alone is sufficient to ensure the safety of these foods or whether an additional procedure is required. Some of the processed cheese spreads have a pH above 4.6 and water activity ($a_w$) greater than 0.85. Therefore, under the Good Manufacturing Practices (GMP) for canned foods (*1*), these foods would be classified as low-acid and, as such, would require a sufficient heat process to destroy spores of *Clostridium botulinum* unless it can be shown that the spreads will not support spore growth.

Process cheese products have had a remarkably good safety record for many years; however, there is one case on record in which botulinum toxin was found in a commercially prepared cheese spread known as Liederkranz brand (*9*). Because of this one incidence of toxic cheese, a number of studies already have been made to determine the circumstances under which botulinum toxin might be produced in cheese.

In one series of such studies, the ability of the spores to grow and produce toxin was shown to be related to the pH, moisture and salt content of three types of surface-ripened cheeses (*12,13,14*). In another study, a canned Cheddar cheese spread with a favorable pH, salt and moisture content was experimentally inoculated with spores of *C. botulinum*. Although all cans contained viable spores at the end of the 6-month incubation period (*11*), none of the cans became toxic. In still another study, added toxin remained stable in processed, soft surface-ripened cheese for 2-4 years, and the added spores were detectable for 4-5 years (*7*). In a study of the factors which inhibit growth of *C. botulinum* spores, the extent of inhibition was related to the level of free fatty acids in type I, aged, surface-ripened cheese, but fatty acids alone were not completely inhibitory (*3*). Some cheeses have been reported to be inhibitory by virtue of antibiotic substances produced in the cheese by the bacteria responsible for ripening (*4,5,6*).

Thus, many factors influence the ability of *C. botulinum* to grow and produce toxin in cheese. Some mitigate against growth; others permit toxin production. The evidence shows, however, that *C. botulinum* toxin can be produced in cheese and may remain stable for long periods; spores may remain viable even longer. The purpose of this study, therefore, was to determine whether currently marketed, shelf-stable process cheese spreads in glass jars are capable of supporting growth and toxin production of *C. botulinum* spores. Results of this study will be considered in determining whether these spreads should be treated as low-acid canned foods under the GMP regulations.

## MATERIALS AND METHODS

### *pH determinations*

The pH of each type of cheese spread was determined by inserting the single probe electrode of a Fisher Accumet digital model 420 pH meter into the cheese. Multiple determinations were made on four jars of each type of cheese spread.

### *$a_w$ determinations*

The $a_w$ of cheese spreads was determined by the Fett-Vos method using Avicel (microcrystalline cellulose) as the moisture adsorbent, and comparing the results to standard curves of slurries of known relative humidity in equilibrium (*2,10*). Determinations were made on three jars of each type of cheese spread.

### *Spore suspensions*

A spore suspension consisting of five type A and five type B strains of *C. botulinum* was used as inoculum for the cheese spreads. Spore stocks of each of the 10 strains were separately grown in 350 ml of trypticase-peptone-glucose yeast extract broth in 500-ml Wheaton bottles at 35 C. When cultures were fully sporulated, the spores were harvested, washed three times in distilled water and concentrated by centrifugation. The spores were finally resuspended in a small volume of distilled water and stored at 4 C until needed, at which time each

strain was serially diluted to give a final concentration of $10^5$ spores/ml in 0.85% NaCl. A pool consisting of 5 ml of each of the 10 suspensions was heat-shocked at 80 C for 15 min and counted by the 3-tube MPN method to verify the count. The five type A strain designations were 62, 69, 78, 426 and 429; type B strain designations were 169, 383, 642, 999 and 8688.

*Inoculation of cheese spreads*

An area near the center on the lid of each jar to be inoculated was abraded with emory cloth and disinfected. A small hole was punched through the prepared area with a sterile punch and the inoculum was injected as far into the cheese as possible with a 1 ½-inch 18-gauge needle attached to a 1-ml syringe. Each jar was inoculated with 0.1 ml of the spore suspension, giving a total of 24,000 spores/jar. Later, additional jars were inoculated in the same manner with approximately 460 spores/jar. The needle reached to about the center of the jar and was rotated in a horizontal spiral in the cheese spread at the same time the plunger of the syringe was depressed to ensure some distribution of the spores. Vertical distribution resulted from withdrawal of the needle. The hole was immediately sealed with solder and the jars were incubated at 35 C.

*Toxicity tests*

In most instances, the cheese spread was tested when a change was observed in its appearance, e.g., gas production or separation into solid and liquid phases. Toxicity tests were made on extracts prepared from the entire jar of cheese. The contents of each jar were extracted with an equal volume of gel-phosphate buffer, pH 6.2. Serial dilutions of each extract were injected intraperitoneally (I.P.) into white Swiss Webster mice, 2 mice/dilution and 0.5 ml/mouse. Mice were observed up to 72 h for symptoms of botulism and death. The toxin was confirmed and the type determined by protecting separate groups of mice with 1 international unit of antitoxin/mouse contained in 0.5 ml of 0.85% NaCl, administered I.P., 30 min to 1 h before challenge with the cheese spread extract. Type A alone, type B alone, and type A and type B antitoxins combined were used. The antitoxin type or mixture which protected mice when the others died from botulism corresponded to the type or types of toxin in the extract (*8*).

## RESULTS AND DISCUSSION

The nine shelf-stable cheese spreads listed in Table 1 were found to be available on grocery store shelves in the Washington, D.C. area. All were in hermetically sealed glass jars. Four of the cheeses had a pH below 4.6 and were, therefore, unsuitable for study. The remaining five each had a pH above 5 and an $a_W$ above 0.85. Therefore, on the basis of both pH and $a_W$ these five cheese spreads were regarded as the most likely to be suitable for the growth of *C. botulinum*. Cheese with Bacon, Limburger, Cheez Whiz, Old English, and Roka Blue spreads were inoculated with 24,000 spores/jar.

Only the Cheese with Bacon and Limburger spreads supported growth and toxin production of *C. botulinum*. At the end of the 6-month incubation period, all 50 jars of the three remaining spreads, which still appeared normal, were tested for toxicity. None were toxic. Thirty jars of each were also cultured for viable spores of *C. botulinum* and all were positive.

TABLE 1. *pH and $a_W$ of representative shelf-stable, pasteurized process cheese spreads.*

| Type of cheese spread | pH | $a_W$ |
|---|---|---|
| Cheese with Bacon | 5.70-5.73 | 0.936-0.941 |
| Limburger | 6.26-6.32 | 0.952-0.953 |
| Cheez Whiz | 5.81-5.86 | 0.932 |
| Old English | 5.52-5.57 | 0.930-0.936 |
| Roka Blue | 5.05-5.09 | 0.944-0.945 |
| Cheese with Jalapeno Pepper | 4.34-4.36 | |
| Cheese with Pimiento | 4.33-4.43 | |
| Cheese with Pineapple | 4.15-4.17 | |
| Cheese with Olive and Pimiento | 4.22-4.23 | |

Toxicity results obtained with Cheese with Bacon spread and Limburger are shown in Table 2. Of the 50 jars of Cheese with Bacon spread, only two remained non-toxic; of the 50 Limburger, only four remained non-toxic at the end of the 6-month incubation. The first toxicity appeared much earlier in Cheese with Bacon spread than in Limburger, the former on day 50 and the latter on day 83, but the average toxicity per jar of Limburger was much greater than that of Cheese with Bacon spread. Average toxicity of Limburger was over 300 MLD/ml of extract, whereas that of Cheese with Bacon spread was only 54 MLD/ml. Both spreads supported growth and production of toxins A and B.

Table 2 also shows that more toxic jars contained type A toxin than type B. Although Cheese with Bacon spread had more jars positive for type B toxin than type A, in Limburger it was overwhelmingly the reverse with only one toxic jar not containing type A toxin. While this may not be of any great consequence, it appears that Cheese with Bacon spread favors type B toxin and Limburger, type A. Inoculation of additional 50 jars of Cheese with Bacon spread with 460 spores/jar showed that large numbers of spores are not required for growth and toxin production by *C. botulinum* in cheese spread. Although only one jar became toxic (and not until day 149), it had 10 MLD of toxin/ml of extract. This showed that, under suitable conditions, few spores are needed for toxin production. Moreover, at the end of the 6-month incubation, viable spores were detected in 6 of 18 jars examined.

All inoculated jars of cheese which appeared abnormal were compared organoleptically to uninoculated controls. After incubation, all jars of Cheese with Bacon spread shown in Table 2, including the two that remained non-toxic, contained a considerable amount of gas but the odor remained normal. Approximately half the jars of Limburger cheese spread showed a watery separation after incubation but retained their normal odor. The results of this study demonstrated that the spores of *C. botulinum* types A and B can grow and produce toxin in two shelf-stable cheese spreads currently on the market.

TABLE 2. *Toxicity of pasteurized process cheese spread samples inoculated with 24,000 spores/jar.*

| Type of cheese spread | Toxic jars | Days to first positive | Type of Toxin | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | A | | B | | A&B | |
| | | | No. | % | No. | % | No. | % |
| Cheese with Bacon | 48 | 50 | 6 | 12.5 | 16 | 33.3 | 26 | 54.2 |
| Limburger | 46 | 83 | 30 | 65.2 | 1 | 2.2 | 15 | 32.6 |

It is also significant that in one of these, as few as 460 spores were capable of growing and producing toxin. This serves to underscore the need for greater certainty of the safety of these products.

Possible modifications in the formulation of cheese spreads are currently being studied by the cheese industry with the aim of preventing the growth of *C. botulinum*, since a heat process adequate to destroy the spores would render the cheese product unacceptable. Under controlled conditions of pH, moisture, and salt concentration, formulation of cheese spreads with different emulsifiers and stabilizers may affect the ability of *C. botulinum* to grow and produce toxin. We have worked with finished cheese spreads and inoculated spores of *C. botulinum* into them, as have virtually all other workers cited in this paper. An additional question, therefore, is whether spores of *C. botulinum*, which may be present in raw materials or which may gain entrance into the spread at some stage of production, could survive the procedures used in making cheese spreads. Whatever the source of contamination, however, it has been shown that certain shelf-stable, pasteurized process cheese spreads could be a potential cause of *C. botulinum* food poisoning.

**ACKNOWLEDGMENT**

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## Outbreak of Gastroenteritis Associated with Lake Swimming

At least 239 cases of gastrointestinal illness occurred among persons who visited a recreational park in Macomb County, MI during July. The illness, determined to be viral in nature, was associated with swimming in the park's lake.

Following a report to the Macomb County Health Department on July 17 that several persons who had visited the park had become sick, news media requested information regarding other park visitors who had become ill. The health department received calls from 300 persons. Predominant symptoms they noted in relation to their illness were vomiting and/or diarrhea, nausea, abdominal cramps, headache, low-grade fever, and sore throat. Most persons recovered within one to two days. Following study of incubation periods of from 6 hours to 8 days, a park-associated case was defined as one in which the gastroenteritis occurred within three days after the park visit. There were 191 such cases.

Forty-eight additional park visitors developed the disease within four days of visiting the park, but each of these cases was associated with an earlier case in the same household.

Illness was not associated with consumption of water from the park's drinking facilities, nor with consumption of food or iced beverages purchases at either of the park's two concession stands. Of the 191 persons who became ill within three days of their park visit, 187 had visited one of the park's two beaches. These beaches are on opposite sides of the lake, separated by 3500 feet of water, suggesting widespread contamination of the water from July 14-16.

Routine sampling of the lake water on July 13 and 17 failed to reveal abnormal coliform counts. A sanitary investigation by the MCHD and the Michigan State Department of Natural Resources did not implicate faulty sewer lines or overflowing septic tanks as potential sources of fecal contamination. The lake was closed for swimming July 18 and reopened Aug. 9. No further cases of illness have been reported.

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# A Challenge of Pasteurized Process Cheese Spread with *Clostridium botulinum* spores

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**ABSTRACT**

Pasteurized process cheese spread with pimientos, packaged in glass jars, inoculated during processing with 1000 spores of *Clostridium botulinum* per gram, developed neither gas nor toxin at 52 or 54% moisture when sodium phosphate was used as the emulsifier. When sodium citrate was the emulsifier, the product developed gas at 52% moisture and became toxic at 54%. At 58% moisture the product became gassy and toxic with either emulsifier. Since the product is commercially manufactured at 52% moisture with phosphate emulsifier, a substantial margin of safety exists.

Outgrowth and toxin production of *Clostridium botulinum* in cheese systems has been reported to be dependent upon the variety of cheese, moisture, salt, pH and presence of added sodium phosphate or sodium citrate (*6-11*).

A study was undertaken to learn if *C. botulinum* (types A and B) is able to produce toxin in Cheddar-based pasteurized process cheese spreads as commercially produced. Because of the long history of freedom from *C. botulinum* toxin development in these products, it was anticipated that normally formulated product would not support toxin production. Additional batches were prepared with emulsifier variations and elevated moisture to learn the effect of such modifications.

## MATERIALS AND METHODS

*Bacterial cultures*

Four strains each of *C. botulinum* (types A and B) were used (strain numbers 56A, 62A, 69A, 90A, 113B, 169B, 213B, 13983B). Each strain was grown in a manner conducive to sporulation (*2*). Spores were harvested by centrifugation, thoroughly washed with sterile water and enumerated. Suitable dilutions with sterile water of each spore preparation were combined to produce a mixed inoculum consisting of approximately equal numbers of each strain.

*Antitoxin*

*C. botulinum* anti A/B toxin was obtained from Connaught Laboratories, Toronto, Canada.

*Enumeration of* C. botulinum

*C. botulinum* was enumerated by the three-tube Most Probable Number technique using trypticase peptone glucose yeast extract broth as the growth medium (*4*). Tubes showing bacterial growth were tested for the presence of toxin by the mouse protection assay (*4*). Only toxin-positive tubes were counted in determining the Most Probable Number of *C. botulinum*.

*Extraction and assay of toxin*

Twenty grams of each test sample were mixed with an equal volume of gelatin-phosphate buffer at pH 6.4. After thorough mixing with a magnetic stirrer, the mixture was centrifuged at $5000 \times g$ for 10 min and the aqueous supernatant fraction was tested for the presence of toxin. Previous work in this laboratory has shown that complete recovery of *added* toxin from such homogenates is affected by this procedure.

For the mouse protection assay, each of two mice was inoculated intraperitoneally with 0.5 ml of the extract from the test sample. Mice were held up to 4 days and examined for symptoms and death characteristic of *C. botulinum* intoxication. When death occurred, two additional mice were challenged with a sample-antitoxin mixture (types A and B) which was pre-incubated at 37 C for 30 min. Unprotected controls were used alongside the protected mice. Previous work in this laboratory has shown that non-specific deaths due to the cheese extract prepared as described are rare.

*Product*

A product conforming to the Federal Standard of Identity for pasteurized process cheese spread with fruits, vegetables or meats (*3*) was prepared. The commercial counterpart which served as the model for the experimental lots contains pimientos, a target moisture of 52.0%, about 2.0% sodium chloride and 2.5% disodium phosphate solids as the emulsifier. In this work all experimental batches were made to the same formula except that the moisture and emulsifier were varied.

The cheese blend consisted of barrel Cheddar cheese for manufacturing. Fifty percent of the blend was approximately 6 months old and 50% was approximately 10 months old.

The cheese was ground through an extruder mill using a 1/8-inch screen, blended without heat in a ribbon blender with addition of fluid sweet cream and oleoresin paprika and annatto color. The cheese mixture was drawn out of the blender into plastic lined containers and stored at 7 C until used the following week.

*Formulation*

Nine batches were prepared (Table 1). Batches 1 and 2 contained 36.9% solids from cheese and cream, 4.2% whey solids, 3.2% nonfat dry milk solids, 2.5% emulsifier solids, 1% added salt, 0.2% lactic acid solids and 0.5% pimiento solids. In Batch 3 the cheese and cream solids were reduced to 36.4% to offset the increased emulsifier solids of 3.0%.

Batches 4 and 5 contained 34.79% solids from cheese and cream, 4.0% whey solids, 3.0% nonfat dry milk solids, 2.5% emulsifier solids, 1% added salt, 0.2% lactic acid solids and 0.5% pimiento solids. In Batch 6 the cheese and cream solids were reduced to 34.3% to offset the increased emulsifier solids of 3.0%.

Batches 7 and 8 contained 31.45% solids from cheese and cream, 3.6% whey solids, 2.7% nonfat dry milk solids, 2.5% emulsifier solids, 1% added salt, 0.2% lactic acid solids and 0.5% pimiento solids. In Batch 9 the cheese and cream solids were reduced to 30.9% to offset the increased emulsifier solids of 3.0%.

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The pimientos were a standardized commercial preparation (concentrated to double solids). The pimientos were ground through a 1/8-inch plate, brined in 16% salt and heated to 88 C to aid in the incorporation of the salt. The lactic acid at 0.2% solids in all batches was added to the pimientos after brining and cooking.

*Processing procedure*

The cheese was cooked in a 20-gal steam jacketed stainless steel agitated kettle equipped with direct steam injection. The cooking sequence consisted of adding water, turning the agitator, adding half of the cheese blend, adding the dry emulsifier and the salt not contained in the brined pimientos, turning on the jacket steam to a pressure of 35 psi, adding the remaining cheese blend, bringing the temperature to 71-77 C with direct steam, adding additional water. Nonfat dry milk and whey were then added and the temperature increased to 88 C. The product was cycled through the valve on the bottom of the cooker, added back to the cooker and held for 2 min before filling uninoculated samples. The uninoculated samples were used for the chemical analysis.

*Inoculation*

Approximately 1000 spores were added per gram of product in the cooker, in total volume of 5 ml. After inoculation, the product was agitated for another 2 min, with 4 liters being drawn off every 30 sec through the bottom filling valve and added back to the cooker.

*Filling procedure*

The product was poured hot into 8-ounce glass containers using a stainless pitcher. The product was immediately capped with a lug closure with manual torque. Covers and glassware were previously sanitized with 200 ppm of chlorine and drained before filling. The filled product was air-cooled in a 7-C refrigerator overnight.

*Incubation and sampling schedule*

Samples were incubated at 30 C. Each experimental batch was examined for overt spoilage (gas, uneven color change, phase separation) at 0, 4, 8, 12, 16, 20, 24, 36 and 48 weeks. Samples were routinely tested for toxin at 0, 4, 24, 36 and 48 weeks. They were also tested for toxin in the intervening period when spoilage signs appeared. For toxin testing a minimum of five samples from each batch was used through the 20th week. Starting at the 24th week, a minimum of 10 samples per batch was used.

The initial number of viable *C. botulinum* organisms was determined by the three-tube Most Probable Number (MPN) method, on each of five jars from each of the nine variables.

All chemical analyses were performed in triplicate and averaged (Table 2). Moisture, fat and sodium chloride were determined by AOAC (5) methodology. Two moisture procedures are given in AOAC Methods. We used the 100-C vacuum oven procedure (AOAC 16.217). Water activity was measured on a Beckman Hygroline instrument, and pH by the procedure in *Standard Methods for the Examination of Dairy Products* (1).

## RESULTS AND DISCUSSION

The type of product under consideration is manufactured to contain a target level of 52% moisture, about 2% sodium chloride and 2.5% disodium phosphate as emulsifier. Pimientos may or may not be added, but they were used in all experimental batches in this study.

Table 3 summarizes observations for spoilage and results of the mouse assay for toxicity. It was apparent as inspections progressed that sodium citrate did not have the preservative effectiveness of either disodium phosphate or a mixture of disodium and trisodium phosphate.

In the lowest moisture group (Batches 1, 2 and 3), sodium citrate samples showed spoilage by 12 weeks, though toxicity did not develop through 48 weeks. None of the sodium phosphate samples showed either spoilage or toxicity through 48 weeks.

In the intermediate moisture group (Batches 4, 5 and 6), sodium citrate samples were toxic by 8 weeks, while no sodium phosphate samples showed either spoilage or toxicity through 48 weeks.

In the highest moisture group (Batches 7, 8 and 9), sodium citrate samples were toxic by 4 weeks, sodium phosphate by 8 weeks. All series darkened as incubation progressed, this being the normal Maillard reaction in systems containing reducing sugar (lactose here) and amino groups.

It is evident from these data that a substantial margin of safety against *C. botulinum* outgrowth and toxicity

TABLE 1. *Target levels of moisture, emulsifiers and lactic acid.*

| Batch | Target moisture level | 2.5% Disodium phosphate | 1.88% Disodium phosphate .63% Trisodium phosphate | 3.0% Sodium citrate | 0.2% Lactic acid |
|---|---|---|---|---|---|
| 1 | 51.5 | | X | | X |
| 2 | 51.5 | X | | | X |
| 3 | 51.5 | | | X | X |
| 4 | 54.0 | | X | | X |
| 5 | 54.0 | X | | | X |
| 6 | 54.0 | | | X | X |
| 7 | 58.0 | | X | | X |
| 8 | 58.0 | X | | | X |
| 9 | 58.0 | | | X | X |

TABLE 2. *Chemical analyses of the uninoculated product.*

| Batch | Emulsifier | pH | Moisture (%) | Water activity | Fat (%) | Salt (%) |
|---|---|---|---|---|---|---|
| 1 | di- & tri-sodium phosphates | 6.10 | 52.35 | .95 | 21.77 | 2.04 |
| 2 | disodium phosphate | 5.81 | 52.03 | .95 | 21.37 | 2.09 |
| 3 | sodium citrate | 5.80 | 49.67 | .95 | 21.88 | 2.19 |
| 4 | di- & tri-sodium phosphates | 6.00 | 53.57 | .95 | 21.04 | 2.14 |
| 5 | disodium phosphate | 5.85 | 53.42 | .95 | 20.77 | 2.04 |
| 6 | sodium citrate | 5.84 | 54.14 | .96 | 19.96 | 2.00 |
| 7 | di- & tri-sodium phosphates | 6.28 | 58.70 | .96 | 18.79 | 1.89 |
| 8 | disodium phosphate | 5.97 | 58.28 | .96 | 18.28 | 1.95 |
| 9 | sodium citrate | 5.90 | 59.16 | .96 | 17.64 | 1.91 |

TABLE 3. *Gas, toxin production and spoilage in the stored products.*

| Batch | % Moisture | Number of toxic samples/number of samples analyzed Time of inspection in weeks 0 | 4 | 8 | 12 | 16 | 20 | 24 | 36 | 48 | Initial MPN/G | Observation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 52.35 | 0/5 | 0/5 | —[a] | — | — | — | 0/10 | 0/10 | 0/20 | $0.66 \times 10^3$ | Darkening |
| 2 | 52.03 | 0/5 | 0/5 | — | — | — | — | 0/10 | 0/10 | 0/20 | $1.2 \times 10^3$ | Darkening |
| 3 | 49.67 | 0/5 | 0/5 | — | 0/5 | 0/5 | 0/5 | 0/10 | 0/10 | 0/20 | $1.0 \times 10^3$ | Gassy spoilage by week 12 |
| 4 | 53.57 | 0/5 | 0/5 | — | — | — | — | 0/10 | 0/10 | 0/20 | $0.64 \times 10^3$ | Darkening |
| 5 | 53.42 | 0/5 | 0/5 | — | — | — | — | 0/10 | 0/10 | 0/20 | $0.94 \times 10^3$ | Darkening |
| 6 | 54.14 | 0/5 | 0/5 | 5/5 | 10/10 | — | — | — | — | 9/10 | $1.7 \times 10^3$ | Gassy spoilage & phase separation by week 8 |
| 7 | 58.70 | 0/5 | 0/5 | 4/5 | 10/10 | — | — | — | — | 11/20 | $1.6 \times 10^3$ | Gassy spoilage & phase separation by week 8 |
| 8 | 58.28 | 0/5 | 0/5 | 2/5 | 5/10 | 6/10 | — | 10/10 | 6/10 | 3/12[b] | $1.3 \times 10^3$ | Gassy spoilage & phase separation by week 8 |
| 9 | 59.16 | 0/5 | 15/15 | Terminated | | | | | | | $1.3 \times 10^3$ | Gassy spoilage & phase separation by week 4 |

[a]Indicates not tested.
[b]All the remaining samples were analyzed.

exists in pasteurized process cheese spreads manufactured to a target moisture of 52%, with 2.5% of disodium phosphate emulsifier and 2% of sodium chloride. While pimiento was used in this work, commercial experience over several years employing other added flavor materials such as bacon, garlic, mustard and Worchestershire sauce shows equally good stability.

Elevation of pH is usually considered a factor in increasing possibility of *C. botulinum* spoilage in foods. In three batches in this series, pH was raised in a range of 0.15 to 0.31 unit by adding trisodium phosphate, without resulting in increased spoilage. One could conclude this system is not in a critical pH range.

While we were unable to induce spoilage in any phosphate-emulsified samples at moisture levels approaching 54%, spoilage and toxicity developed within 8 weeks of incubation at moistures slightly over 58%. It is evident there is a moisture limit beyond which a non-sterile system such as this cannot be expected to resist spoilage, and good manufacturing practice must take this into account.

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# Behavior of *Enterobacter aerogenes* and *Hafnia* Species During the Manufacture and Ripening of Camembert Cheese

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**ABSTRACT**

Camembert cheese was made from pasteurized milk contaminated with about $10^2$-$10^3$ cells of *Enterobacter aerogenes* or *Hafnia* sp. The coliform bacteria were enumerated with a Most Probable Number procedure and with violet red bile agar. Numbers of viable *E. aerogenes* decreased rapidly during ripening at 15.5 or 10 C when cheese was made with the commercial lactic starter cultures OD or C-5. No viable *E. aerogenes* was detected in cheese ripened at 10 C for 3 weeks. Ripening of cheese, made with starter culture OD or C-5, for 1 week at 15.5 C was accompanied by a decrease in numbers of viable *Hafnia* sp. to 10/g. The number of *Hafnia* sp. increased markedly during 7 weeks of further ripening at 10 C to yield cheese which contained numbers in excess of $10^7$ /g when ripening was almost completed. Growth of *Hafnia* sp. during the storage period coincided with an increase in pH of the cheese.

The possible presence of coliform bacteria in cheese has been recognized since 1895 when Russell (*15*) studied the defects caused by these organisms. These defects include off-odors and -flavors and excessively open texture resulting from gas produced by the bacteria. Coliform bacteria commonly are in raw milk, and, although killed by pasteurization, sometimes appear as contaminants in pasteurized milk. This, together with such conditions as improperly cleaned equipment, inadequate personal hygiene of workers in the cheese factory or a contaminated lactic starter culture, can lead to the presence of coliform bacteria in cheese. The last source should be extremely rare.

Survival of coliforms and other bacteria in cheese is related to pH (*1,13,15,16*). Coliforms prefer mildly acid to mildly alkaline conditions for maximum growth and survival in cheese. Cheese manufacturers rely on the ability of the lactic starter culture to successfully compete with coliforms, if present in milk, and produce sufficient acid to inhibit or inactivate them. However, a low pH, within limits, does not insure that all coliforms will be eliminated since some are more resistant to acidic conditions than are others. As cheese ripens, various areas of cheese may have different pH values. Mold-ripened cheese has a tendency to develop regions that are neutral or alkaline in pH and thus suitable conditions for growth and/or survival of coliforms may exist (*9*).

Enteropathogenic strains of *Escherichia coli* and other coliforms have been found in market milk and market cheese, especially in Camembert cheese from France, Sweden and Denmark (*2,18*) and also in cheese from the U.S. (*3*). Late in 1971, several outbreaks of acute gastroenteritis were reported in the U.S. These were associated with consumption of soft, ripened cheese of the Camembert-type that was contaminated with enteropathogenic *E. coli* (EEC). This was the first well-documented cheese-related outbreak of EEC food-borne illness in the U.S. (*11*). This incident prompted a renewed interest in the problem of coliforms and lead to studies by Frank et al. (*4*) and Park et al. (*14*) on the behavior of EEC in Camembert cheese and by Frank et al. (*5*) on the behavior of EEC in brick cheese.

Information is lacking on the fate of *Enterobacter aerogenes* and *Hafnia* sp. during the manufacture and ripening of Camembert cheese. This study was undertaken to determine growth or inactivation of these coliforms in Camembert cheese made with different commercial lactic starter cultures.

## MATERIALS AND METHODS

### *Cultures*

Cultures of *E. aerogenes* (MF-1) and *Hafnia* sp. (14-1) were obtained from the Food and Drug Administration. Each culture was transferred to nutrient broth daily for 3 days before it was used for an experiment. Incubation was at 35 C for 24 h. A sufficient amount of a 24-h-old nutrient broth culture (35-C incubation) was added to pasteurized milk to provide approximately 100 cells/ml of milk. Numbers of bacteria in broth were determined using a standard curve derived from absorbance measurements of nutrient broth containing *E. aerogenes* or *Hafnia* sp. and from plate counts of organisms. A spectrophotometer (Model 20, Bausch and Lomb, Rochester, New York) was used to make absorbance measurements. All necessary dilutions were made using sterile nutrient broth.

Lactic starter cultures used to make cheese included two commercial mixed strain lactic cultures obtained from the Marschall Division of Miles Laboratories, Inc., Madison, Wisconsin. These cultures were grown in sterile skimmilk at 21 C for 24 h, frozen at -57 C in 10-ml quantities and stored frozen until they were to be used. Each 10-ml quantity was subcultured once in 353 ml of sterile skimmilk and incubated at 21 C for 24 h before use in cheesemaking. The cultured skimmilk served as inoculum for 18 kg of pasteurized milk.

*Penicillium camembertii*, obtained from K. B. Raper, Department of Bacteriology, University of Wisconsin, Madison, was grown on slants of Czapek agar at 21 C for 1 week. Mold growth on one agar slant was blended (Waring blender) with 50 ml of sterile citrate-buffered distilled water and the mixture was added to 18 kg of milk at the start of cheesemaking.

### *Manufacture and sampling of cheese*

Camembert cheese was made according to procedures described by Park et al. (*14*). Cheese was made from 18-kg lots of pasteurized whole milk adjusted to 33 C; this temperature was maintained from the time

of inoculation until the cut curd was dipped into hoops. Before addition of 4.8 ml of commercial rennet extract (calf) to the milk, 363 ml of starter culture, blended mold from one agar slant and the coliform culture were added. The mixture was stirred, covered and allowed to stand undisturbed. Fifty minutes later the curd was cut and 20 min after cutting, the curd was dipped into eight hoops which when filled were held at room temperature. Six hours after addition of rennet extract to milk, the hoops of cheese were turned and holding at room temperature was continued. After 24 h, 69 g of salt were distributed over the surface of the cheese. The cheese was ripened for 1 week at 15.5 C with high humidity (80-90%) and turned daily to allow for mold development. Each cheese (approx. 275 g) was then wrapped in aluminum foil and ripened at 10 C for up to 8 additional weeks.

Each test coliform was used to inoculate two vats of milk which was made into cheese with one of the lactic starter cultures. This was repeated with the other lactic culture.

The schedule for sampling the cheese and for microbiological testing is indicated in Fig. 1 and 2. The following samples were taken: (*a*) milk after addition of rennet extract, (*b*) curd during dipping, (*c*) cheese

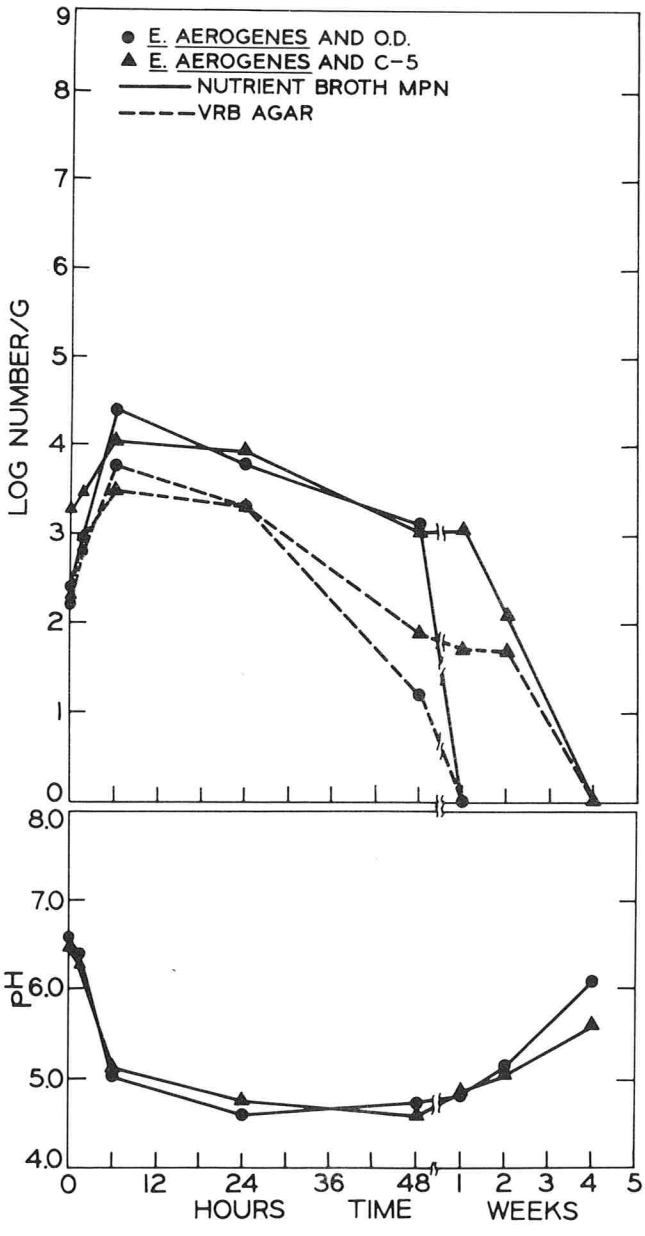


Figure 1. *Changes in pH and numbers of* Enterobacter aerogenes *(MF-1) during the manufacture and ripening of Camembert cheese made with lactic starter cultures OD or C-5.*

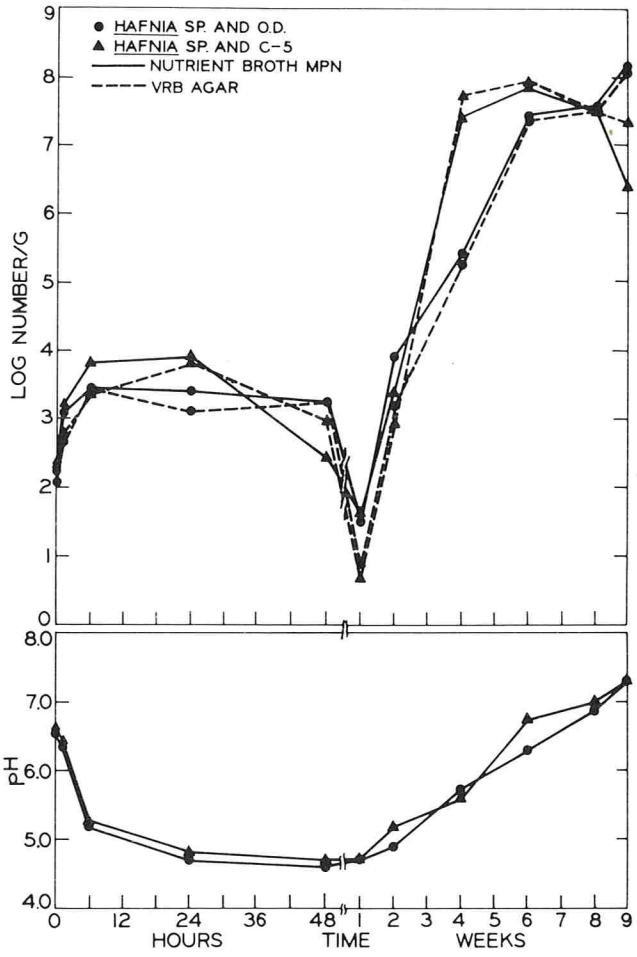


Figure 2. *Changes in pH and numbers of* Hafnia *sp. (14-1) during the manufacture and ripening of Camembert cheese made with lactic starter cultures OD or C-5.*

before salting, (*d*) cheese 1 day after salting, (*e*) cheese after 7 days at 15.5 C and (*f*) cheese after 1, 3, 5, 7 and 8 weeks at 10 C.

*Enumeration of coliform bacteria*

A Most Probable Number (MPN) technique and plating on a selective medium were used to enumerate *E. aerogenes* or *Hafnia* sp. in samples of milk, curd or cheese. Each value reported in Fig. 1 and 2 represents the average count obtained with the MPN technique or violet red bile agar (VRBA) in two trials. The MPN method involved (*a*) blending 20 g of cheese curd (Waring blender) for 3 min with 180 ml of sterile 2% sodium citrate solution, (*b*) making 1:10 dilutions in the sodium citrate solution, (*c*) adding 1-ml quantities of the appropriate dilutions to tubes containing 9 ml of sterile nutrient broth, (*d*) incubating tubes at 35 C for 24 h, (*e*) streaking material from each tube onto eosin-methylene blue (EMB) agar (Difco), and (*f*) incubating the plates at 35 C for 24 h.

Use of VRBA (Difco) and incubation of plates at 35 C for 24 h also served to enumerate *E. aerogenes* and *Hafnia* sp. VRBA in plates was allowed to solidify before being overlaid with an additional 5 ml of medium. Values in Fig. 1 and 2 represent the averages of duplicate platings and duplicate trials of cheesemaking.

*Measurement of moisture, NaCl and fat*

Approximately 3 g of cheese placed in a previously dried moisture dish made of aluminum foil were dried for 16 h in a forced-draft air oven at 110 C. Samples were then removed and placed in a desiccator to cool. The amount of weight lost was considered to be the amount of moisture in the cheese. Values reported are averages of duplicate trials. The Volhard method (*8*) was used to determine the amount of NaCl in cheese. Samples were done in triplicate and results reported are average

values. The amount of fat in the cheese was determined by the Mojonnier method (*12*). Experiments were done in duplicate and results reported are average values.

*Measurement of pH*

The pH values of the milk and curd were determined with a pH meter (Model 10, Corning, Corning, New York) equipped with a miniature combination glass electrode. The pH values of cheese were determined with a saturated calomel half-cell, gold electrode and a portable potentiometer (Leeds and Northrup, North Wales, Pennsylvania).

## RESULTS

Eight vats of Camembert cheese were made; two for each combination of lactic starter culture and coliform being studied. *E. aerogenes* (MF-1) was selected for these experiments because of its inability to survive in skimmilk during fermentation by the above mentioned lactic starter cultures (unpublished data). *Hafnia* sp. (14-1) was chosen because it was generally the hardiest of the coliform organisms tested in skimmilk during fermentations by lactic starter cultures (unpublished data).

*Behavior of E. aerogenes during cheese manufacture*

Relatively little growth of *E. aerogenes* occurred during the first 45-50 min of the cheesemaking operation (Fig. 1). Numbers of *E. aerogenes* increased during the interval between cutting the curd and turning the hooped cheese. Slightly larger numbers were attained when cheese was made with the lactic starter culture OD rather than with C-5. The increase during this time can be attributed both to bacterial growth and concentration of cells through entrapment in the curd (*14*). A 10- to 100-fold increase in numbers was detected when either lactic starter culture was used.

Numbers of *E. aerogenes* in cheese decreased slowly during the next 18 h until salting took place. The pH of the cheese dropped to 5.0 during the first 6 h and decreased further to 4.6-4.8 in the next 24-48 h. The resultant increase in acidity probably served to inactivate a portion of the coliform population. Other investigators also have noted that coliform bacteria are either inhibited or inactivated by pH values of 5.0 or below when they occur during production of Cheddar or cottage cheese or in other cultured products (*6,7,14,16,17*).

*Behavior of E. aerogenes during ripening of cheese*

After storage for 24 h, hoops were removed, salt (approximately 2%, wt/wt) was rubbed on the outside of the cheese, and cheese was ripened at 15.5 C and 80-90% relative humidity. During the first day of such ripening there was a further decrease in number of *E. aerogenes* in cheese made with either of the two starters (Fig. 1). Cheese made with culture OD showed a decrease of two logs in number of *E. aerogenes* during this 24-h period. A decrease of 1.5 logs in number was noted when C-5 was used as the starter culture. The pH of the cheese did not change appreciably during this period, but remained at 4.6-4.8.

The number of viable *E. aerogenes* continued to decrease at 15.5 C; however, it was more rapid than during the initial storage (Fig. 1). Cheese made with lactic culture OD was free of viable *E. aerogenes* at the end of 1 week at 15.5 C. In contrast, the number of *E. aerogenes* in cheese made with lactic culture C-5 decreased only slightly during the same time. Continued ripening at 10 C was marked by a further decrease in number of organisms until the cheese was free of viable *E. aerogenes* after 3 weeks. The pH of the cheese increased to 5.6-6.1 after 3 weeks at 10 C.

*Behavior of Hafnia sp. during cheese manufacture*

Behavior of *Hafnia* sp. was very similar to that of *E. aerogenes* during the first 6 h of cheese manufacture (Fig. 2). After turning of hooped cheese, numbers of *Hafnia* sp. in the cheese made with lactic culture OD decreased slightly for the next 18 h as did numbers of *E. aerogenes*. However, during this period there was a distinct increase in number of *Hafnia* sp. (.5 log/g) in cheese made with lactic culture C-5. The pH of the cheese decreased to 5.25 during the first 6 h and decreased further to 4.6-4.8 during the next 24-48 h, as did the pH of cheese containing *E. aerogenes*. Lactic culture OD appeared to inhibit *Hafnia* sp. more than did culture C-5 during the 24 h of cheese manufacture.

*Behavior of Hafnia sp. during ripening of cheese*

The number of *Hafnia* sp. in cheese made with lactic culture C-5 decreased by one log during the first day of ripening at 15.5 C (Fig. 2). This decrease in numbers was not as evident in cheese made with lactic culture OD. Further ripening at 15.5 C for 1 week resulted in a 100-fold decrease in the population of *Hafnia* sp. in cheese made with either lactic starter culture. The number of viable *Hafnia* sp. was approximately 10/g at this time.

Ripening at 10 C failed to eliminate all viable *Hafnia* sp. from cheese made with either lactic culture OD or C-5. Rapid growth of *Hafnia* sp. was clearly evident during the first 5 weeks of ripening at 10 C (Fig. 2). Increased growth coincided with a marked increase in the pH of cheese. The population of *Hafnia* sp. in cheese made with lactic culture OD increased from $10^1$ to $10^7$ /g during the 5-week period. Further holding at 10 C resulted in another increase in number of *Hafnia* sp. until more than $10^8$ /g were detected at the end of cheese ripening.

Cheese made with lactic culture C-5 contained numbers of *Hafnia* sp. in excess of $10^7$ /g after 5 weeks of ripening at 10 C. This was followed by a slight decrease in numbers during the final 3 weeks of ripening; however, the population of *Hafnia* sp. at the conclusion of ripening still exceeded $10^7$ /g. Ripening of cheese at 10 C for 8 weeks was accompanied by a steady increase in pH of cheese from 4.6 to 7.2

## DISCUSSION

Numbers of coliforms detected in samples of milk, curd and cheese generally were larger when the MPN procedure rather than VRBA plates was used. VRBA

may not have enumerated all coliforms present which were injured by acidic conditions. Acid-injured cells subjected to the MPN procedure were probably able to repair the injury during the 24-h incubation in nonselective nutrient broth. Use of the MPN procedure, even though it included plating on a selective medium (EMB), resulted in larger numbers of coliforms than when only a selective medium (VRBA) was used. Similar observations were reported by Frank et al. (*4,5*) when they studied the fate of EEC in Camembert and brick cheese.

Information on the ripening process required for production of soft cheese and on the physical properties of the final product is useful to explain how large numbers of coliforms can occur in the cheese. Growth of certain coliforms during ripening and subsequent extended survival of these organisms may, in part, be attributable to the high moisture and high pH of the cheese. Soft-ripened cheese has a moisture content generally greater than 50% (Table 1). After formation of the curd, ripening of the cheese entails degradation of protein through activity of the mold. Knoop and Peters (*10*) studied microscopic structures that exist in Camembert cheese during its ripening. They reported that hyphae of *P. camembertii* were seen only in the ripened areas of the cheese. Inward progression of ripening results from diffusion of extracellular enzymes originating at the surface of cheese where they are generated by the mold mycelium. This activity reduces the acidity of the curd and the pH of cheese increases from about 4.9 to 6.0 or more, depending on the lactic culture that was used. Thus, the acid produced originally by the lactic bacteria is neutralized and an improved environment is created for growth of coliforms. Those cheeses found to contain large numbers of coliform bacteria had elevated pH values in the range of 7.0 to 7.3 (Fig. 2) at the end of the ripening process.

TABLE 1. *Percent of moisture, NaCl, and fat in Camembert cheese made with the commercial lactic starter OD or C-5.*

| Coliform | Commercial lactic starter | Moisture (%) | NaCl (%) | Fat (%) |
|---|---|---|---|---|
| *E. aerogenes* | OD | 60.0 | 1.10 | 24.5 |
| *E. aerogenes* | C-5 | 56.5 | 1.59 | 23.8 |
| *Hafnia* sp. | OD | 52.5 | 1.30 | 27.6 |
| *Hafnia* sp. | C-5 | 60.5 | 1.27 | 23.2 |

Behavior of *E. aerogenes* in the manufacture of Camembert cheese, as described in this paper, generally was similar to that of EEC in Camembert cheese, as reported by Park et al. (*14*) and Frank et al. (*4*). However, of all the coliforms studied in this laboratory (*4,5,14*), the *Hafnia* spp. used in this study proved to be the only ones that were able to initiate growth in cheese and then attain rather substantial populations.

These data again emphasize the importance of active lactic cultures and of attention to sanitary practices in the manufacture of Camembert cheese.

**ACKNOWLEDGMENT**

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# Rapid Colorimetric Test for Alkaline Phosphatase in Dairy Products

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**ABSTRACT**

The Scharer rapid test for measuring alkaline phosphatase activity in milk and milk products was modified to include a photoelectric colorimeter in place of visual observation of color. Dairy products containing various amounts of added enzyme (3-15 μg phenol/ml or g) were prepared for analysis as per standard methods and analyzed by the rapid colorimetric test. A linear relationship was found between the percent of raw milk added and the enzyme activity with a correlation coefficient (r) range of 0.963 to 1.000. Hydrolysis of the substrate (disodium phenyl phosphate) by the surviving enzyme in different dairy products was similar. Recovery of the added enzyme varied, depending on the nature of the product. The method is quantitative, reproducible, and can be used as a rapid confirmatory procedure. Similarly, this method was applied to differentiating residual and reactivated enzyme in milk, cream and buttermilk. Compared to the Scharer rapid and the Rutgers visual methods, this test was more reliable in borderline cases because it eliminated the bias encountered in visual examination. A method was developed to analyze alkaline phosphatase in casein.

Alkaline phosphatase is a naturally occurring enzyme (E) in milk. Because thermal resistance of the enzyme is greater than that of pathogenic organisms commonly found in milk, a negative test for it indicates adequate pasteurization. Of the several visual and colorimetric methods for phosphatase (*1,5*), the Scharer rapid test (*2*), originally designed as a field test, now is used by many state and local health departments. The Pasteurized Milk Ordinance (*12*) has prescribed the Scharer rapid or its equivalent as the method for determining the enzyme activity in various dairy products. Although the method has greater appeal, it has the following drawbacks. (*a*) Commercial standards are not stable and require changing every 6 months. (*b*) The blue color of the standards is different from the color obtained with dairy products containing appreciable amounts of fat. (*c*) The standards are dispensed in 13- × 110-mm Pyrex test tubes, whereas the samples are prepared in 10- × 110-mm borosilicate glass tubes that provide different depths of color comparison. (*d*) The test is unreliable in borderline cases requiring the use of a confirmatory test (colorimetric) to obtain reliable data. (*e*) Similarly, the visual test has been found to be unreliable for detecting small differences in phenol concentration in the differential test for residual and reactivated phosphatases (*13*).

[1]*Mention of commercial products does not imply endorsement by the Food and Drug Administration.*

*Standard Methods for the Examination of Dairy Products* (*1*) has undergone many revisions, but only recently has any attempt been made to delete the use of commercial standards and recommend the use of phenol standards prepared at the time of analysis (*3*). To some extent, use of phenol standards may appear satisfactory but the bias encountered in visual examination of color still exists. Similarly, use of magnesium chloride ($MgCl_2$) has been deleted in favor of magnesium acetate, $Mg(C_2H_3O_2)_2$, in the differential test for phosphatase (*3,10*).

The industry trend has been toward ultra-high temperature, short-time processes, and therefore reactivation of the enzyme will be a prevailing problem.

The present investigation was undertaken to evaluate the Scharer rapid test and make it a rapid quantitative test by substituting an inexpensive colorimetric for the visual observation of colors.

## MATERIALS AND METHODS

### *Materials*

When needed, 4 to 10 liters of raw milk and 35% raw cream were collected from a local milk plant in sterile glass-stoppered bottles and stored at 4 C until use. Cream was standardized to 12% fat with milk.

Pasteurized milk, half and half cream, and other dairy products such as nonfat dry milk powder, cultured buttermilk, ice cream, creamed cottage cheese, Cheddar cheese, yogurt, chocolate milk, and butter were purchased from retail stores. Buttermilk was prepared from pasteurized cream by churning it in a Waring blender[1].

Casein was prepared from raw skim milk by isoelectric precipitation at pH 4.7 using 1 N HCl. The precipitated casein was divided into four portions. One served as the control, and the others were washed one, two, and three times, respectively, by dispersing them in acidified water (pH 4.7) equivalent to the original volume of milk. The casein was filtered through a Buchner funnel, pressed in between folds of a paper towel, and finally dried at 40 C overnight. The dried casein was pulverized in a blender and stored in a desiccator until use.

### *Equipment*

In general, the apparatus is the same as described for the modified spectrophotometric method (*1*) except as indicated under Method.

### *Photoelectric colorimetric*

Sargent-Welch, Model 4864 (or equivalent) was modified to accommodate 12 × 75-mm cuvettes (Coleman type) by mounting metal or rubber rings at the top and botton of the sample chamber. The bulb mount and holder were stabilized with rubber cement to reduce the meter shift.

### *Reagents*

All reagents are the same as described in the *Standard Methods* (*1*).

*Phenol standards*

The standard procedure (*1*) was modified to eliminate the necessity for diluting the butyl alcohol extract 1:1 before measuring absorbance. The modifications were as follows. (*a*) The stock solution of phenol was standardized as per *Standard Methods* (*4*). (*b*) The final working solution was prepared by diluting 10 ml of phenol solution (10 μg phenol/ml) to 100 ml with distilled water (this working standard was prepared within 2 h of analysis). (*c*) To a series of test tubes similar to those which will be used in the test procedure 0.0, 0.5, 1.0, 2.5, and 5.0 ml of phenol solution, respectively, were added. The volume of samples was made to 5.0 with distilled water. The phenol concentrations in these samples are equal to 0, 1, 2, 5, and 10 μg, respectively. (*d*) After adding 0.5 ml of buffer and 0.1 ml of Indophax solution and during incubation at 40 C, the samples were mixed at least once during the first minute. (*e*) The blue color was extracted with 3 ml of butyl alcohol by inversion of tubes six full turns making sure that each time the alcohol and the aqueous layers were mixed well. (*f*) After centrifugation, the alcohol layer was transferred to cuvettes using Pasteur pipettes, and the optical densities of samples were measured in the colorimeter and plotted against phenol equivalents (Fig. 1).

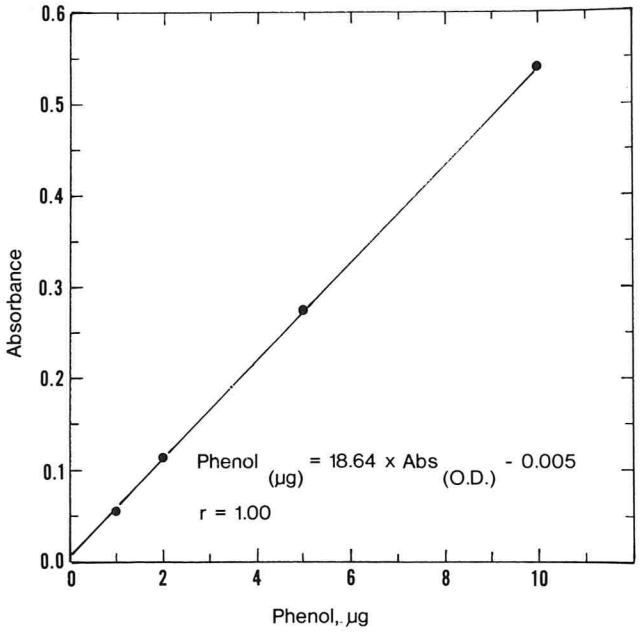


Figure 1. *Standard curve for phenol.*

*Heat treatment of milk or cream*

To produce a reactivatable product, milk or cream was heated to 104 to 110 C for < 1 sec in a continuous-flow heat-exchanger as described by Donnelly et al. (*7*) except that silicone oil (Dow Corning 200 fluid, industrial grade 100 cp) was used in the constant-temperature bath. The temperature of the product was continuously monitored using a thermocouple (copper-constantan) connected to a Bristol model 64-A-12PG-591-21 potentiometer. The heated product was stored overnight at 4 C before reactivation was studied.

*Reactivation*

The method of reactivating samples with magnesium acetate is the same as that described elsewhere (*3,10*).

*Sample preparation*

All products were prepared for analysis as per Standard Methods (*1*), except as follows. (*a*) A 3% casein solution was prepared by dispersing 3 g of casein in double strength carbonate-bicarbonate buffer. Dissolution of the casein was aided by placing the sample in an ultra-sonic bath. Alternatively, casein was dissolved in the buffer by grinding in an all-glass mortar and pestle. (*b*) The solution was quantitatively transferred to a volumetric flask and made to 100 ml with the buffer. (*c*) Ice cream was melted and allowed to stand in the refrigerator overnight for the air bubbles to subside; the liquid portion was used for analysis. (*d*) Melted butter was constantly stirred gently with a magnetic bar to keep the serum and the fat as homogeneous as possible before pipetting.

*Phosphatase determination*

In general, the method is the same as described in Standard Methods (*1*) except as follows. (*a*) Pipette 0.5 ml of the sample into 12 × 120-mm test tubes capable of withstanding centrifugation, using a 1.0-ml graduated (0.01- or 0.1-ml divisions) Mohr type pipette. (*b*) Add 0.1 ml of $Mg(C_2H_3O_2)_2$ (10 mg Mg/ml) to casein samples *only*. (*c*) Add purified, buffered substrate, mix well, and place the samples in a water bath equipped with a mechanical agitator or stirrer. (*d*) Allow a 1-min warm-up time, mixing the samples once during this period and then allowing them to stand in the water bath for 15 min. (*e*) Add 0.1 ml of Indophax solution or 0.1 ml CQC and 2 drops of the catalyst to the samples. (*f*) Mix the samples and place them immediately in the water bath for 5 min. (*g*) Add 3 ml of neutralized n-butyl alcohol, and mix by inversion six full turns. (*h*) Allow the samples to stand in the ice-water bath for another 5 min for the alcohol layer to separate. (*i*) At this point, the samples may be examined visually using the phenol standards prepared as described earlier. (*j*) Centrifuge the samples for 5 min and remove the clear alcohol layer to cuvettes, using Pasteur pipettes. (*k*) Measure the optical density in the colorimeter, using the red filter.

## RESULTS AND DISCUSSION

*Preliminary studies*

Early in the study, it became apparent that the commercial standards were variable, and therefore, their use was abandoned. Phenol standards (1, 2, and 5 μg) were prepared at the time of the milk sample analysis and used to compare the sample extracts. But these standards were not satisfactory because fat in milk samples imparted a greenish tint to butyl alcohol extracts, making it difficult to compare the blue color obtained with the phenol standards. In the visual comparison, the fact that the samples are turbid and the standards are clear causes some bias. The usefulness of the visual test even with the phenol standards for screening samples was limited to highly positive samples. An inexpensive photoelectric colorimeter was deemed essential to eliminate the bias encountered in the visual test for alkaline phosphatase activity in milk and milk products. This rapid test could be considered a confirmatory test for alkaline phosphatase.

*Phosphatase test rationale*

In the Scharer rapid test, samples with the added substrate are incubated for 15 min, whereas in the spectrophotometric methods (*1,2,5*), the incubation time is 60 min. The criterion for adequate pasteurization is that, when tested by the Scharer rapid test or its equivalent, the pasteurized products must contain less than 1 μg phenol per ml or g, whereas the equivalent phenol concentration for the modified spectrophotometric method is 2.3 μg.

Since the main objective of this investigation was to modify the Scharer rapid test to be reliable and quantitative in relation to the modified spectrophotometric method, the following were considered: (*a*) the proportion of the substrate (disodium phenylphosphate) hydrolyzed when different dairy products containing known amounts of added enzyme were incubated for 15

to 60 min, (*b*) the recovery of added enzyme and (*c*) reproducibility of analysis.

Fresh raw milk was obtained on different days and analyzed for alkaline phosphatase by the modified spectrophotometric method (*9*). A portion of the raw milk was heated to 95 C for 1 min and used for preparing samples. With other pasteurized dairy products, no heat treatment was applied, since the interfering substance control test was always negative. All products except chocolate milk were mixed with raw milk to obtain levels of 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5% raw milk. Based on the original analysis of raw milk, the computed average [E] and the standard deviation for all products at the respective levels were 0.00, 3.13 ± 0.27, 5.96 ± 0.57, 8.89 ± 0.93, 11.80 ± 1.36 and 14.78 ± 1.38 μg phenol/ml. Raw milk was added to portions of chocolate milk to levels of 0.5, 1.0, 1.5, 2.0 and 2.5%, and the computed [E] at these levels was 13.0, 26.0, 39.0, 52 and 65.0 μg phenol/ml, respectively. All samples were processed as described under Methods, and aliquots were incubated for periods of 15, 30, 45 and 60 min, respectively. Samples were analyzed for the [E] by the colorimetric method. The observed phenol concentrations after different incubation periods with respect to the percent of raw milk added were linearly correlated (r ranged from 0.963 to 1.000), indicating that the test yields quantitative results. Assuming that the amount of phenol liberated during 60 min of incubation was 100%, the relative concentrations of phenol liberated at other incubation periods were computed. Statistical analysis by analysis of variance indicated that the percent of phenol liberated at any given incubation period with respect to the percent of raw milk added was similar and not significantly different from the mean of that group. Therefore all data for each product at each incubation time were pooled, and the averages were calculated (Table 1) for further evaluation. The averages for the twelve products studied were ranked from 1 to 36, and Kruskal-Wallis' H was computed to be 2.25 (*6*), which was not significant at $\alpha = 0.05$. This indicates that the null hypothesis that the groups are equal cannot be rejected. The enzyme from raw milk added to dairy products behaves identically, regardless of the environment.

Table 2 shows the percent recovery of the added enzyme from each product. The average recovery of the added [E] from heated raw milk, pasteurized milk, nonfat dry milk, and buttermilk were quantitative at 60 min ranging from 98.8 to 110.7%. For these four products, the average recovery ranged from 34.7 to 41.3% at 15 min, which is in agreement with the data in Table 1. Cream gave a slightly low recovery of 89.8% at 60 min, whereas at 15 min the recovery was 35.7%, which was considered satisfactory. Cultured buttermilk gave satisfactory recoveries when analyzed within 2 h after addition of raw milk, but analysis of samples after storage at 4 C for periods of 24, 48, 72 or 144 h showed losses of enzyme activity of 24, 43, 55 and 77%,

TABLE 1. *Effect of incubation time on hydrolysis of disodium phenylphosphate in different dairy products.*[a]

| Product | Percent phenol liberated | | | |
|---|---|---|---|---|
| | 15 min | 30 min | 45 min | 60 min |
| Heated milk | 34.5 ± 2.5 (4)[b] | 63.8 ± 1.6 (23) | 83.9 ± 1.6 (35) | 100 |
| Pasteurized milk | 36.5 ± 3.3 (6) | 52.8 ± 5.4 (13) | 78.8 ± 4.5 (28) | 100 |
| Cream (half & half) | 40.1 ± 1.7 (10) | 58.3 ± 3.7 (17) | 79.7 ± 4.6 (29) | 100 |
| Nonfat dry milk | 41.4 ± 2.3 (11) | 58.9 ± 3.2 (18) | 80.6 ± 1.6 (31) | 100 |
| Creamed cottage cheese | 36.7 ± 4.4 (7) | 58.1 ± 3.6 (16) | 69.7 ± 3.9 (25) | 100 |
| Cultured buttermilk | 36.1 ± 2.9 (5) | 55.5 ± 1.6 (14½) | 76.7 ± 2.3 (27) | 100 |
| Ice cream | 32.4 ± 3.4 (2) | 55.5 ± 2.8 (14½) | 76.0 ± 2.7 (26) | 100 |
| Chocolate milk | 33.3 ± 1.5 (3) | 59.7 ± 2.8 (20) | 81.0 ± 2.0 (32) | 100 |
| Buttermilk | 41.8 ± 1.8 (12) | 60.8 ± 1.4 (22) | 80.0 ± 3.6 (30) | 100 |
| Yogurt | 31.4 ± 3.1 (1) | 59.3 ± 5.9 (19) | 81.1 ± 7.4 (33) | 100 |
| Butter | 39.1 ± 1.5 (8) | 66.5 ± 4.2 (24) | 88.2 ± 4.2 (36) | 100 |
| Cheddar cheese | 39.7 ± 2.3 (9) | 60.0 ± 4.9 (21) | 81.2 ± 2.3 (34) | 100 |

[a]Average for 5 levels of [E] ± std. dev.
[b](rank).

TABLE 2. *Recovery of added alkaline phosphatase from different dairy products.*[a]

| Product | Percent recovery | | | |
|---|---|---|---|---|
| | 15 min | 30 min | 45 min | 60 min |
| Heated milk | 36.0 ± 1.8 | 66.4 ± 3.8 | 87.3 ± 4.8 | 104.2 ± 5.2 |
| Pasteurized milk | 36.0 ± 1.4 | 55.8 ± 2.9 | 84.2 ± 4.4 | 110.7 ± 5.4 |
| Nonfat dry milk | 34.7 ± 2.4 | 55.8 ± 2.8 | 79.8 ± 3.7 | 101.0 ± 2.9 |
| Buttermilk | 41.3 ± 0.9 | 61.0 ± 2.0 | 80.0 ± 2.6 | 98.8 ± 2.1 |
| Cream (half & half) | 35.7 ± 0.6 | 52.4 ± 4.3 | 71.8 ± 6.2 | 89.8 ± 4.1 |
| Cultured buttermilk | 32.9 ± 2.0 | 51.3 ± 2.3 | 70.8 ± 1.1 | 92.4 ± 1.8 |
| Ice cream | 27.3 ± 2.8 | 46.7 ± 2.8 | 63.9 ± 2.2 | 84.1 ± 1.5 |
| Creamed cottage cheese | 21.8 ± 0.9 | 37.1 ± 2.1 | 45.8 ± 1.2 | 63.9 ± 1.7 |
| Cheddar cheese | 25.3 ± 0.9 | 38.4 ± 2.7 | 51.6 ± 2.8 | 63.7 ± 2.0 |
| Yogurt | 20.1 ± 1.5 | 37.4 ± 2.8 | 51.1 ± 3.1 | 63.2 ± 2.4 |
| Chocolate milk | 20.8 ± 0.4 | 34.8 ± 2.2 | 47.9 ± 1.7 | 59.5 ± 1.8 |
| Butter | 23.1 ± 0.6 | 39.2 ± 1.4 | 52.0 ± 2.2 | 59.2 ± 1.9 |

[a]Averages of five levels of raw milk added and standard deviation from the mean. All products were analyzed 24 h after mixing with raw milk and storage at 4 C, except cultured buttermilk, which was analyzed within 2 h after mixing with raw milk.

respectively, indicating that the E is inactivated by the acidity in the product. This was also evidenced in the poor recoveries obtained with acid products such as cheese and yogurt. The observed E activity in acid dairy products would be related to the age of the product and the extent of contamination with raw milk. Poor recoveries of the added E were also obtained with other acid dairy products tested. Viscosity of the products (cultured buttermilk, ice cream, yogurt and butter) and air bubbles incorporated into ice cream during manufacture affect quantitative transfer of the sample by pipetting. Sampling of butter was especially difficult because of the possible separation of the serum (the enzyme is associated with the serum phase of butter) from the fat during the melting of butter at 40 C. Transfer of a homogeneous sample was thus difficult. In addition, the pipette used for butter transfer had to be maintained in a warm condition to avoid solidification of butter inside the pipette. In spite of a negative test for the interference control sample, chocolate milk gave poor recoveries of the added E, a result that may be related to the synergistic effect of the constituents of chocolate on the enzyme. Satisfactory analysis of chocolate milk has been a perennial problem.

Although the recoveries of raw milk E from different dairy products varied, depending on the nature of the product and the experimental errors, analysis of the residual enzyme by this method is satisfactory.

*Reproducibility of analysis*

Pasteurized dairy products were mixed with raw milk to obtain alkaline phosphatase activity levels of 1 to 2 and 2 to 5 μg phenol/ml and prepared for analysis. Ten 0.5-ml aliquots of the same sample were analyzed for enzyme activity. Data (Table 3) show that for various dairy products the percent coefficient of variation among replicate analyses varied from 5.9 to 10.3% at level 1 and from 3.6 to 9.2% at level 2, respectively. These results were assumed to be satisfactory, considering the levels at which the [E] was analyzed.

*Sargent-Welch Colorimeter vs. Perkin-Elmer (Coleman 44) Spectrophotometer*

The absorbance readings of many of the samples were measured on both instruments for comparison using the red filter in the colorimeter and at a wavelength of 650 nm in the spectrophotometer. Statistical analysis of the data by student "t" test showed no significant differences, and the data were highly correlated (r = 0.998), indicating that the colorimeter was reliable and satisfactory.

TABLE 3. *Reproducibility (ag phenol/ml) of analysis.*[a]

| Product | Level 1 | Level 2 |
|---|---|---|
| Heated raw milk | 1.21 ± 0.09 ( 7.4%)[b] | 2.65 ± 0.10 (3.8%) |
| Pasteurized milk | 1.18 ± 0.07 ( 5.9%) | 2.20 ± 0.08 (3.6%) |
| Nonfat dry milk | 1.34 ± 0.13 ( 9.7%) | 2.36 ± 0.10 (4.2% |
| Half and half cream | 1.25 ± 0.08 ( 6.4%) | 2.32 ± 0.12 (5.2%) |
| Chocolate milk | 1.39 ± 0.13 ( 9.4%) | 2.63 ± 0.18 (6.8%) |
| Buttermilk | 1.56 ± 0.14 ( 9.0%) | 3.12 ± 0.15 (4.9%) |
| Cultured buttermilk | 1.44 ± 0.12 ( 8.3%) | 2.22 ± 0.16 (7.2%) |
| Creamed cottage cheese | 1.09 ± 0.09 ( 7.3%) | 2.25 ± 0.18 (8.0%) |
| Cheddar cheese | 1.36 ± 0.14 (10.3%) | 2.94 ± 0.27 (9.2%) |
| Yogurt | 1.33 ± 0.11 ( 8.3%) | 2.54 ± 0.10 (3.9%) |
| Ice cream | 1.96 ± 0.14 ( 7.4%) | 3.05 ± 0.19 (6.4%) |

[a]Average of 10 replicates ± std. dev.
[b](% coefficient variation).

*Casein*

Alkaline phosphatase in commercial casein intended for food use is not a potential problem in the United States. However, compliance regulations stipulate that the imported dry dairy products, including casein intended for food use, must show a negative test for this enzyme. Methods currently used (*1*) are not satisfactory for the analysis of casein.

To determine the conditions for satisfactory analysis, isoelectric casein was prepared as described under Methods. Atomic absorption analysis (*11*) of portions of 0 ×-, 1 ×-, 2 ×-, and 3 ×- washed caseins showed [Mg] of 240, 130, 15 and 4 mg/kg, respectively. A 3% solution of each preparation was prepared and analyzed for E activity by the modified spectrophotometric method (*1*) in the presence of 0.003 to 1.00 mg of Mg per test. Data in Fig. 2 show that in the absence of added Mg, the [E] in the samples were 2.1, 3.4, 3.1, and 2.3 μg phenol/ml for the 0 ×, 1 ×, 2 ×, and 3 × washed samples, respectively. Maximum [E] with unwashed casein was observed at a [Mg] of 0.134 mg. No increase in [E] was observed with further additions of Mg. The other three casein preparations showed a maximum [E] of about 30 μg phenol/ml at Mg of 0.973 to 1.094 mg, with an average of 1.03 mg. The number of washings of casein did not appear to affect the extent of rejuvenation of the E by Mg. The difference in [E] between unwashed and washed caseins may be related to the high nonprotein solids in the unwashed casein, which reduces the casein per unit weight and high [Mg], which may be inhibitory to enzyme activity.

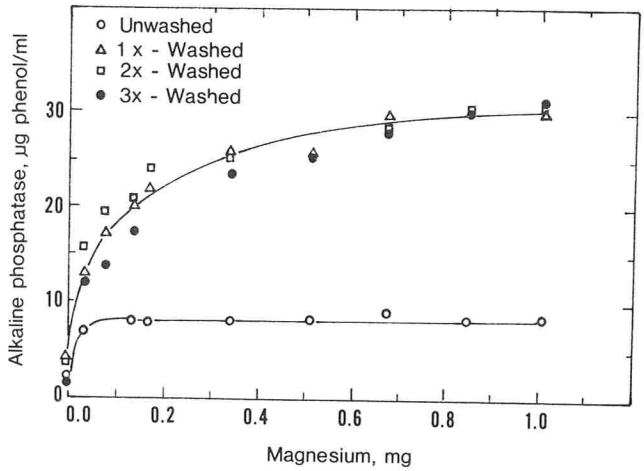


Figure 2. *Effect of magnesium acetate on alkaline phosphatase of casein.*

Another batch of unwashed and 2 × washed caseins was prepared and analyzed by the rapid colorimetric method. Results were:

| *Casein* | *μg phenol/ml* |
|---|---|
| Unwashed | 2.82 |
| Unwashed + Mg (1 mg) | 5.01 |
| Washed | 1.82 |
| Washed + Mg (1 mg) | 8.79 |

The coefficient of variation among ten replicates was less than 5%, indicating adequate control of experimental error.

*Differential test for residual and reactivated phosphatases*

The method is based on comparing results in testing a diluted (1 + 5) sample containing added Mg with the test results of an undiluted sample without the added Mg after storing at 34 C for 1 h. If the diluted sample shows less E activity than the undiluted sample, the test is considered positive for residual E. If the diluted sample shows greater E activity than the undiluted sample, the test is considered positive for reactivated E (*1*).

A recent study (*10*) showed that the AOAC method using $MgCl_2$ for reactivation was improved by the use of $Mg(C_2H_3O_2)_2$. Standard Methods (*1,3*) recommend spectrophotometric methods for differentiating residual and reactivated phosphatases. In practice, however, most state and local health laboratories use the Scharer rapid test (*13*). As indicated earlier, an improvement in the Scharer rapid test was deemed essential. The modified Scharer rapid test with colorimetric measurement of blue color could be used for this purpose.

Milk, cream, and buttermilk samples were heated to 104.4 ± 1.0 C, as described under Methods, and portions were mixed with the raw product to obtain levels of 0.0 to 0.5%. Additions of raw product were necessary to obtain residual E in the samples. Samples were reactivated with and without addition of $Mg(C_2H_3O_2)_2$ for 60 min at 34 C and analyzed for residual and reactivated E by (*a*) the Scharer rapid test, (*b*) the rapid colorimetric test, and (*c*) the Rutgers method (*8*).

In a preliminary study, a sample of 12% cream was reactivated and analyzed by the rapid colorimetric and modified spectrophotometric methods. The results were, respectively, 6.8 and 17.8 μg phenol/ml for the control, and 11.96 and 30.5 μg phenol/ml for the sample with Mg. The rapid test was satisfactory for the analysis of reactivated E.

Table 4 shows comparison of methods for differentiating reactivated from residual phosphatase in liquid dairy products. The ratio of correctly diagnosed to total samples for each method was: Scharer rapid test, 10/12; colorimetric method, 11/12; and Rutgers methods, 8/12. With the visual methods, most of the difficulty appeared to be related to borderline cases where the phosphatase activity of the control sample and the corresponding sample with Mg were very close. Also, in samples containing highly reactivatable phosphatase, Rutgers method yielded an intense pink color which made it difficult to recognize small changes in the color intensity of opaque solution. But in the Scharer rapid test, the blue color is clear and the sample is examined over a light that makes color comparison somewhat easier.

TABLE 4. *Comparison of methods for differentiating residual and reactivated alkaline phosphatase in liquid dairy products.*

| Sample | Raw milk (%) | Initial[a] E (μg phenol/ml) | Scharer rapid tests[b] | Rapid colorimetric test | Rutgers method |
|---|---|---|---|---|---|
| | | | Milk (3,020 μg phenol/ml)[c] | | |
| 1 | 0.0 | 0 | S > C (+) | C 2.7 (+) S 4.2 | S > C (+) |
| 2 | 0.2 | 2.1 | S = C (+) | C 4.8 (+) S 4.4 | S = C (+) |
| 3 | 0.3 | 3.6 | S < C (−) | C 6.0 (−) S 4.4 | S < C (−) |
| 4 | 0.5 | 5.4 | S < C (−) | C 8.1 (−) S 4.4 | S < C (−) |
| | | | 12% Cream (3,800 μg phenol/ml)[c] | | |
| 5 | 0.0 | 0.0 | S > C (+) | C 4.1 (+) S 6.9 | S > C (+) |
| 6 | 0.2 | 3.4 | S > C (+) | C 7.0 (+) S 7.3 | S > C (+) |
| 7 | 0.3 | 5.3 | S < C (−) | C 8.6 (−) S 7.6 | S ≤ C (±) |
| 8 | 0.5 | 8.8 | S < C (−) | C11.9 (−) S 8.0 | S < C (−) |
| | | | Buttermilk (7,800 μg phenol/ml)[c] | | |
| 9 | 0.0 | 0.0 | S > C (+) | C 2.7 (+) S 6.3 | S > C (+) |
| 10 | 0.2 | 5.9 | S < C (−) | C 8.4 (−) S 7.3 | S ≤ C (±) |
| 11 | 0.3 | 8.8 | S < C (−) | C10.2 (−) S 7.8 | S < C (−) |
| 12 | 0.5 | 13.4 | S < C (−) | C15.5 (−) S 9.0 | S < C (−) |

[a]Rapid colorimetric test.
[b]C = control, S = sample with Mg and diluted 1 + 5 with product heated to 95 C for 1 min. S > C = (+), reactivated; S < C = (−), residual.
[c]Modified spectrophotometric method.

Various factors, including initial [E] and processing temperature, affect reactivation of E (*10*). It has been reported that the reactivation of E in commercially processed heavy cream > half and half cream > milk (*8*). Therefore, the presence of small amounts of residual E in a highly reactivatable product may be difficult to predict, whether the samples are analyzed by the visual or the spectrophotometric methods. In addition, as indicated

elsewhere (*10*), the dilution ratio of 1 + 5 used for samples with added Mg is not a constant factor, and thus occasional failure of the method to predict the differential test may be expected. Further study is needed in this direction.

These investigations have provided information concerning the usefulness of the rapid colorimetric test for residual and reactivated E in dairy products. Collaborative studies are now underway to determine the reliability of the method, and the results will be reported in the near future.

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# Collaborative Study of Rapid Methods for Alkaline Phosphatase in Milk and Cream

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**ABSTRACT**

Determination of alkaline phosphatase activity in milk and cream by the modified Scharer rapid test with use of photoelectric colorimeter for measuring absorbance was collaboratively studied. Milk samples (skim milk, milk and cream) with and without added raw milk were sent to 12 collaborators to be tested by (*a*) the modified Scharer rapid test using commercial standards and phenol standards for comparing colors, (*b*) the rapid colorimetric test and (*c*) the Rutgers method. The latter method was used for comparison only. In the modified Scharer rapid test, based on the category of standards, 73.3% of the samples using the commercial standards and 71.6% of the samples using phenol standards were correctly diagnosed. When the scoring was based on positive or negative, 98.4 and 92.6% of the samples were correctly diagnosed. Results with the phenol standards were significantly lower than those observed with the commercial standards. There were no false-positive results, as all incorrect readings were below limit of detection. Most of the errors occurred when the expected value was 1.0 μg phenol/ml. Results were 100% correct for the Rutgers method, but there are only two choices for this method, and they correspond to positive or negative. Compared to the theoretical values, data obtained by the colorimetric method ranged from 1.5 to 18.1% high, with a coefficient of variation of 4.4 to 13.4%. These variations were assumed satisfactory considering the levels at which phosphatase was tested.

The pasteurized milk ordinance (PMO) (*4*) prescribes the use of the Scharer rapid test or its equivalent as described in *Standard Methods for the Examination of Dairy Products* (SMEDP) (*1*) for the analysis of phosphatase to detect pasteurization. In the 14th edition of SMEDP (*2*), the Scharer rapid test calls for use of phenol standards for color comparison instead of the commercial standards. The test used for differentiating residual and reactivated phosphatases requires that a spectrophotometric procedure be used. The fact that the rapid test is more reliable for the analysis of residual phosphatase and not for reactivated phosphatase has caused some concern. Most state central laboratories use the Scharer rapid test both for residual and reactivated phosphatases, but they have experienced difficulties. Recently, Murthy et al. (*6*) have published a rapid colorimetric test that could be used as a confirmatory test both for residual and reactivated phosphatases.

The purpose of this study was to test collaboratively (*a*) the usefulness of phenol standards for visual comparison of colors in the Scharer rapid test and (*b*) the adaptation of a photoelectric colorimeter in the rapid test for quantitative assay of phosphatase.

## MATERIALS AND METHODS

### *Samples*

Fresh samples of raw skim milk, milk, and cream were obtained, and portions were heated to 95 C for 1 min and cooled. Raw milk was analyzed for alkaline phosphatase activity (*6*), and portions were added to heated samples to produce five levels of alkaline phosphatase activity. Each sample was divided into two portions for a total of 10 samples and randomly numbered. Samples 1 and 5 were heated skim milk, samples 2 and 10 were heated cream with raw milk, samples 3 and 7 were heated milk with raw milk, samples 4 and 8 were heated milk with raw milk, and samples 6 and 9 were heated cream with raw milk. Samples were dispensed in 125-ml sterile polystyrene bottles and shipped to laboratories for analysis (*8*).

### *Reagents*

Phenol solution (1 mg/ml), sodium carbonate and sodium bicarbonate buffer salt mixture, disodium phenylphosphate, Indophax tablets, and Rutgers phosphatase test kits were distributed to laboratories. Other reagents were the same as described in SMEDP.

### *Equipment*

Equipment was the same as described in SMEDP except as follows: A Sargent-Welch model 4864 photoelectric colorimeter (modified to accommodate 12- × 75-mm cuvettes), and sufficient 12- × 75-mm cuvettes, which were sent to each laboratory for the duration of this study.

### *Procedure*

Pipette 0.5 ml of sample to test tubes using 1-ml graduated (0.01-0.1-ml divisions) pipette. Add 5 ml of purified, buffered substrate (disodium phenylphosphate), mix well and incubate in a water bath at 40 ± 1 C. Allow 1 min for warm-up time and during this period mix the sample at least once. Let samples stand in the water bath for an additional 15 min. Remove samples from the water bath, add 0.1 ml Indophax solution to each solution, mix well, and reincubate samples for exactly 5 min. Remove samples from water bath and cool in an ice-water bath for 5 min. Add 3 ml of neutralized and cold n-butyl alcohol. Extract indophenol blue color by inversion of tubes six full turns. Let samples stand in the ice-water bath for an additional 5 min for the alcohol layer to separate. Compare the blue color of the extract with (*a*) commercial standards, and (*b*) phenol standards (1, 2, and 5 μg) prepared at the time of analysis (*6*). Report results as < 1 μg, 1-2 μg, 2-5 μg, and/or > 5 μg.

After visual comparison of colors, transfer the samples to test tubes and centrifuge for 5 min (Note: if standard test tubes capable of withstanding centrifugation are used in place of the more fragile Scharer test tubes, there is no need for the transfer). Transfer the alcohol layer to the cuvettes using Pasteur pipettes and measure absorbance in a photoelectric colorimeter using the red filter and as per instructions in the instrument brochure.

Analyze the samples by the Rutgers method (*3*), and report results as < 0.1% or > 0.1% raw milk.

### *Statistical methods*

The colorimetric data for alkaline phosphatase were analyzed by the methods of Ostle (*7*) and Kendall (*5*). Presentation of components of variance is according to Youden and Steiner (*9*). All tests of significance were done at the $a = 0.05$ level.

## RESULTS AND DISCUSSION

Pretests of the method using two sets of four samples with known phosphatase activity were conducted to identify problem areas in the procedure. Five samples in duplicate (designated as unknown) were sent for analysis in the collaborative phase of the study. Tables 1 and 2 present data obtained with the modified Scharer rapid test using commercial and phenol standards, respectively. Assuming a phosphatase content of 2 μg phenol/ml for samples showing 1-2 or 2-5 μg phenol/ml, visual readings were compared to control readings and the percent correct results were computed. Similarly, the percent correct results when the samples are scored as positive or negative were computed (Tables 1 and 2). Based on the category of standards, 73.3% of the samples using commercial standards and 71.6% of the samples using phenol standards were correctly diagnosed. Based on positive or negative scoring, the corresponding results were 98.4 and 92.6%. Results with phenol standards were significantly lower than those obtained with commercial standards at $a = 0.05$ level. Most of the errors occurred when the phosphatase activity was near 1.0 μg phenol/ml. There were no false-positive results since all incorrect readings were false-negative.

Table 3 presents data obtained with the Rutgers method. Results were 100% correct, but there are only two choices for this method, and they correspond to positive or negative.

Table 4 presents absorbance readings of phenol standards prepared at the time of analysis of unknown milk samples. Statistical evaluation by analysis of variance (Table 5) indicated that variance between collaborators was significant at the $a = 0.05$ level.

Table 6 presents data obtained with the photoelectric

TABLE 1. *Alkaline phosphatase activity of milk samples determined by the modified Scharer rapid test using commercial standards.*

| | (μg phenol/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sample | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Item Control | 0.0 | 2.70 | 2.05 | 1.10 | 0.0 | 1.57 | 2.05 | 1.10 | 1.57 | 2.70 |
| Collaborator | | | | | | | | | | |
| 1 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | 1 - 2 | 2 - 5 | 2 | 2 - 5 | 2 - 5 |
| 2 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | 2 - 5 | 2 - 5 | 1 -2 | 1 - 2 | 2 - 5 |
| 3 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | 2 - 5 | 2 - 5 |
| 4 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | 2 - 5 | 2 - 5 | ∼ 2 | 2 - 5 | 2 - 5 |
| 5 | < 1 | 1 - 2 | 1 - 2 | ∼ 1 | < 1 | 1 - 2 | 1 - 2 | 1 - 2 | 1 | 1 - 2 |
| 6 | < 1 | > 5 | 5 | 2 - 5 | < 1 | 5 | 5 | 2 - 5 | 5 | > 5 |
| 7 | < 1 | 2 - 5 | 2 | 1 - 2 | < 1 | 1 - 2 | 2 - 5 | ∼ 1 | 1 - 2 | 2 - 5 |
| 8 | < 1 | 2 | 1 - 2 | 1 | < 1 | ∼ 1 | 1 - 2 | ∼ 1 | 1 | ∼ 2 |
| 9 | < 1 | 2 - 5 | 1 | 1 | < 1 | 1 | 2 - 5 | 1 | 2 | 2 - 5 |
| 10 | < 1 | 5 | 2 - 5 | 2 | < 1 | 2 - 5 | 2 - 5 | 2 | 2 - 5 | 5 |
| 11 | < 1 | 2 - 5 | 1 - 2 | < 1 | < 1 | 1 - 2 | 1 - 2 | < 1 | 1 - 2 | 2 - 5 |
| 12 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | ∼ 2 | 2 - 5 | 1 - 2 | 2 - 5 | 2 - 5 |
| Percent correct[a] results | 100 | 75 | 83 | 67 | 100 | 50 | 92 | 58 | 33 | 75 |
| Percent correct[b] results | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 92 | 92 | 100 |

[a]Assuming a value of ∼ 2 μg phenol for samples showing 1-2 or 2-3 μg phenol/ml. Average is 73.3.
[b]Based on positive or negative for alkaline phosphates. Average is 98.4.

TABLE 2. *Alkaline phosphatase activity in milk samples determined by the modified Scharer rapid test using phenol standards.*

| | (μg phenol/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sample | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Item Control | 0.0 | 2.70 | 2.05 | 1.10 | 0.0 | 1.57 | 2.05 | 1.10 | 1.57 | 2.70 |
| Collaborator | | | | | | | | | | |
| 1 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | ∼ 2 | 2 - 5 | ∼ 2 | 2 - 5 | 2 - 5 |
| 2 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | 1 - 2 | 2 - 5 | 1 - 2 | 1 - 2 | 2 - 5 |
| 3 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | 2 - 5 | 2 - 5 |
| 4 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | 2 - 5 | 2 - 5 | 2 | 2 - 5 | 2 - 5 |
| 5 | < 1 | 1 - 2 | 1 - 2 | 1 | < 1 | 1 - 2 | 1 - 2 | 1 - 2 | 1 | 1 - 2 |
| 6 | < 1 | 5 | 2 - 5 | 2 | < 1 | 2 - 5 | 2 - 5 | ∼ 2 | 2 - 5 | 5 |
| 7 | < 1 | 2 - 5 | 2 - 5 | 1 - 2 | < 1 | ∼ 2 | 2 - 5 | 1 - 2 | 1 - 2 | 2 - 5 |
| 8 | < 1 | ∼ 2 | 1 - 2 | < 1 | < 1 | 1 - 2 | 1 - 2 | < 1 | ∼ 1 | 1 - 2 |
| 9 | < 1 | 1 - 2 | < 1 | < 1 | < 1 | < 1 | 1 - 2 | < 1 | 1 | 1 |
| 10 | < 1 | 5 | 2 - 5 | 2 | < 1 | 2 - 5 | 2 - 5 | 2 | 2 - 5 | 5 |
| 11 | < 1 | 2 - 5 | 1 - 2 | < 1 | < 1 | 1 - 2 | 1 - 2 | < 1 | 1 - 2 | 2 - 5 |
| 12 | < 1 | ∼ 2 | 2 - 5 | ∼ 1 | < 1 | 1 - 2 | 2 - 5 | ∼ 1 | 2 - 5 | 2 - 5 |
| Percent correct[a] results | 100 | 67 | 92 | 50 | 100 | 58 | 100 | 58 | 33 | 58 |
| Percent correct[b] results | 100 | 100 | 92 | 75 | 100 | 92 | 100 | 75 | 92 | 100 |

[a]Assuming a value of ∼ 2 μg phenol for samples showing 1-2 or 2-5 μg phenol/ml. Average is 71.6.
[b]Based on positive or negative for alkaline phosphatase. Average is 92.6.

TABLE 4. *Absorbance readings of phenol standards.*

| Collaborator | Standards 1.0 | 2.0 | 5.0 |
|---|---|---|---|
| 1 | 0.052 | 0.118 | 0.295 |
| 2 | 0.070 | 0.120 | 0.290 |
| 3 | 0.060 | 0.115 | 0.280 |
| 4 | 0.055 | 0.115 | 0.270 |
| 5 | 0.041 | 0.120 | 0.290 |
| 6 | 0.050 | 0.102 | 0.245 |
| 7 | 0.056 | 0.114 | 0.276 |
| 8 | 0.051 | 0.102 | 0.264 |
| 9 | 0.061 | 0.102 | 0.260 |
| 10 | 0.055 | 0.100 | 0.260 |
| 11 | 0.055 | 0.100 | 0.235 |
| 12 | 0.061 | 0.128 | 0.310 |
| Average | 0.056 | 0.111 | 0.273 |
| Std. dev. | 0.007 | 0.010 | 0.022 |

colorimeter. A test for results that were significantly high or low was performed. The chi square ($\chi^2$) from rank correlation (5, 9) was 53.9 and significant. Collaborators 5 and 10 were determined to be significantly low and high, respectively, therefore their results were deleted from further evaluation.

TABLE 5. *Analysis of variance of data in Table 4.*

| Source | Degrees of freedom | Sum of squares | Mean square | Variance ratio |
|---|---|---|---|---|
| Collaborator | 11 | 0.00396 | 0.00036 | 2.8[a] |
| Phenol conc. | 2 | 0.30580 | 0.15290 | 1212.0[a] |
| Error | 22 | 0.00277 | 0.00013 | — |
| Total | 35 | 0.31254 | — | — |

[a]Significant at $a = 0.05$.

Analysis of variance of the colorimetric data (7) showed that the interaction effect resulting from collaborators and samples was not significant, whereas the variance resulting from collaborators was significant at the $a = 0.05$ level, indicating that the means are not equal.

Table 7 presents a summary of the colorimetric data. Compared with the theoretical values for phosphatase, the observed values ranged from 1.5 to 18.5% high. A 95% confidence limit based on the range of 10 observations was determined. The percent coefficient of variation ranged from 4.4 to 13.4%, which was

TABLE 3. *Alkaline phosphatase activity in milk samples determined by the Rutgers method.*

| | (Percentage) Sample[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Item Control[b] | 0.0 | 2.70 | 2.05 | 1.0 | 0.0 | 1.57 | 2.05 | 1.10 | 1.57 | 2.70 |
| Collaborator | | | | | | | | | | |
| 1 | < .1 | > .1 | > .1 | ∿.1 | < .1 | ∿.1 | > .1 | ∿.1 | > .1 | > .1 |
| 2 | < .1 | > .1 | > .1 | ∿.1 | < .1 | > .1 | > .1 | ∿.1 | ∿.1 | > .1 |
| 3 | < .1 | > .1 | > .1 | ∿.1 | < .1 | ∿.1 | > .1 | ∿.1 | ∿.1 | > .1 |
| 4 | < .1 | > .1 | > .1 | ∿.1 | < .1 | > .1 | > .1 | ∿.1 | > .1 | > .1 |
| 5 | < .1 | > .1 | > .1 | ∿.1 | < .1 | > .1 | > .1 | > .1 | ∿.1 | > .1 |
| 6 | < .1 | > .1 | > .1 | ∿.1 | < .1 | > .1 | > .1 | ∿.1 | ∿.1 | > .1 |
| 7 | < .1 | > .1 | > .1 | ∿.1 | < .1 | > .1 | > .1 | > .1 | > .1 | > .1 |
| 8 | < .1 | > .1 | > .1 | ∿.1 | < .1 | > .1 | > .1 | ∿.1 | > .1 | > .1 |
| 9 | < .1 | > .1 | > .1 | > .1 | < .1 | > .1 | > .1 | > .1 | > .1 | > .1 |
| 10 | < .1 | > .1 | > .1 | > .1 | < .1 | > .1 | > .1 | > .1 | > .1 | > .1 |
| 11 | < .1 | > .1 | > .1 | > .1 | < .1 | > .1 | > .1 | > .1 | > .1 | > .1 |
| 12 | < .1 | > .1 | > .1 | ⩾ .1 | < .1 | ⩾ .1 | > .1 | ⩾ .1 | > .1 | > .1 |

[a]If sample solution (with substrate) is less pink than standard solution, milk has been pasteurized to an extent equivalent to < 0.1% raw milk.
[b]Alkaline phosphatase activity, μg phenol/ml.

TABLE 6. *Alkaline phosphatase content of five samples in duplicate determined by the rapid colorimetric test.*

| | (μg phenol/ml) Sample | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Item Control | 0.0 | 2.70 | 2.05 | 1.10 | 0.0 | 1.57 | 2.05 | 1.10 | 1.57 | 2.70 |
| Collaborator | | | | | | | | | | |
| 1 | 0.0 | 3.30 | 2.10 | 1.2 | 0.0 | 1.6 | 2.2 | 1.4 | 2.1 | 3.2 |
| 2 | 0.0 | 3.5 | 2.4 | 1.2 | 0.0 | 1.9 | 2.0 | 1.2 | 1.6 | 3.5 |
| 3 | 0.0 | 3.7 | 2.2 | 1.15 | 0.0 | 2.1 | 2.25 | 1.3 | 1.9 | 3.55 |
| 4 | 0.0 | 4.0 | 2.5 | 1.2 | 0.0 | 2.2 | 2.6 | 1.2 | 2.7 | 3.9 |
| 5[a] | 0.2 | 1.4 | 0.8 | 0.9 | 0.0 | 1.0 | 1.5 | 1.2 | 0.8 | 1.9 |
| 6 | 0.0 | 3.45 | 2.0 | 1.4 | 0.0 | 2.2 | 2.1 | 1.4 | 1.9 | 3.65 |
| 7 | 0.0 | 3.25 | 2.0 | 1.5 | 0.0 | 1.81 | 2.35 | 1.6 | 2.0 | 3.65 |
| 8 | 0.0 | 2.83 | 2.13 | 1.13 | 0.0 | 1.73 | 2.08 | 1.15 | 1.32 | 2.60 |
| 9 | 0.0 | 2.38 | 1.05 | 0.5 | 0.0 | 1.05 | 2.05 | 1.05 | 1.05 | 2.60 |
| 10[a] | 0.0 | 5.8 | 4.05 | 2.4 | 0.0 | 3.96 | 3.47 | 2.0 | 2.8 | 4.84 |
| 11 | 0.0 | 2.8 | 1.8 | 0.6 | 0.0 | 1.4 | 1.9 | 0.6 | 1.55 | 2.8 |
| 12 | 0.0 | 2.53 | 1.95 | 1.39 | 0.0 | 1.29 | 2.01 | 1.40 | 1.65 | 2.77 |
| Average | 0.0 | 3.17 | 2.01 | 1.13 | 0.0 | 1.73 | 2.15 | 1.20 | 1.78 | 3.22 |
| Std. dev. | | 0.52 | 0.40 | 0.33 | | 0.39 | 0.20 | 0.27 | 0.46 | 0.49 |
| Percent correct[b] results | 100 | 92 | 75 | 100 | 100 | 100 | 100 | 92 | 92 | 100 |

[a]Data from analysts 5 and 10 were deleted as extreme values for all computations.
[b]Average is 95.0%.

TABLE 7. *Summary of colorimetric results.*

| Sample | Mean (μg phenol/ml) | 95% Confidence limits (μg phenol/ml) | % CV | Expected value (μg phenol/ml) | % Expected value | $S^2_{rep.}$ |
|---|---|---|---|---|---|---|
| 2, 10 | 3.20 | 2.65-3.63 | 4.4 | 2.70 | + 18.5 | 0.0201 |
| 3, 7 | 2.08 | 1.85-2.23 | 12.4 | 2.05 | + 1.5 | 0.0666 |
| 4, 8 | 1.18 | 0.78-1.40 | 11.6 | 1.10 | + 7.3 | 0.0188 |
| 6, 9 | 1.73 | 1.47-2.05 | 13.4 | 1.57 | + 10.2 | 0.0538 |

considered slightly high. As expected, there were some false-negative results (sample 4, collaborators 9 and 11; sample 8, collaborator 11) when the phosphatase activity was near 1.0 μg phenol/ml.

Results of this collaborative study demonstrate that the modified Scharer rapid test, using either the commercial or the phenol standards, was unsatisfactory for scoring the samples in different standard categories. However, when the samples were scored as positive or negative, the results with phenol standards were significantly lower (93 versus 98%) than those obtained with commercial standards. Measurement of absorbance of extracted color in a photoelectric colorimeter provided quantitative data that were slightly higher than the theoretical values. These variations were assumed satisfactory considering the levels at which the phosphatase test was carried out. The rapid colorimetric method should be useful as a confirmatory test in the analysis of milk and milk products.

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# Mycotoxins - Their Biosynthesis in Fungi: General Introduction

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Mycotoxins are toxic metabolites produced by fungi in foodstuffs that cause health problems in exposed humans and animals. Due to their wide-spread occurrence in food commodities and their distinct biological activities, mycotoxins have recently received recognition as a significant class of natural toxicants in foods.

The commodities that have been found susceptible to mycotoxin contamination include grains, oilseeds, treenuts, dehydrated fruits, and preserved animal products. Under practical conditions, contamination of certain mycotoxins can reach levels lethal to man and animals. Acute mycotoxicosis episodes involving large numbers of human and animal deaths have been well documented. At lower levels, mycotoxins are known to produce carcinogenic, mutagenic, teratogenic and hallucinogenic effects in animals.

Despite their significant animal toxicity, mycotoxins are secondary metabolites of their appropriate producing fungi, and the significance or benefit of their biosynthesis to the physiology and reproduction of these toxigenic fungi remains obscure. Questions arise then: (*a*) Why and how do fungi produce these secondary metabolites that happen to be so toxic to animals? (*b*) Is there any way that fungal secondary biosynthesis of mycotoxins can be altered and controlled? These have been intriguing and challenging questions faced by numerous natural product biochemists and microbiologists.

In a seminar organized for the 78th annual meeting of the American Society for Microbiology, addressing these questions, six prominent researchers in the field of microbiology of fungal toxigenicity were invited to speak on the mechanism of biosynthesis of six representative classes of mycotoxins — aflatoxins, patulin and penicillic acid, zearalenone, ochratoxins, trichothecenes, and ergot alkaloids. These six classes are currently of the greatest concern to food safety regulatory agencies as mycotoxins associated with human and animal health problems.

The presentations are now graciously elaborated by these experts in writing and published as a series to present an overall and comparative view of fungal secondary biosynthesis of mycotoxins. The information contained in this series will certainly shed light on the answer to the aforementioned challenging questions.

For general toxicology of mycotoxins, the reader is referred to the following monographs:

Toxicology - Biochemistry and Pathology of Mycotoxins. K. Uraguchi and M. Yamazaki (eds.). Halsted Press, Kodansha Ltd., Tokyo. 1978.

Mycotoxic Fungi, Mycotoxins, Mycotoxicosis. Vol. 1, 2, 3. T. D. Wyllie and L. G. Morehouse (eds.). Marcel Dekker, New York. 1977-1978.

Mycotoxins in Human and Animal Health. J. V. Rodricks, C. W. Hesseltine, and M. A. Mehlman (eds.). Pathotox Publishers, Park Forest South, Illinois. 1977.

Mycotoxins and Other Related Food Problems. J. V. Rodricks (ed.). Adv. Chem. Ser. 149. American Chemical Society, Washington, D.C. 1976.

Mycotoxins. I. F. H. Purchase (ed.). Elsevier Scientific, New York. 1974.

Microbial Toxins. A. Ciegler, S. Kadis, S. J. Ajl (eds.). Vol. 6. Academic Press, New York. 1971.

Aflatoxin - Scientific Background, Control, and Implications. L. A. Goldblatt (ed.). Academic Press, New York. 1969.

Biochemistry of Some Foodborne Microbial Toxins. R. I. Mateles and G. N. Wogan. M.I.T. Press, Cambridge, Massachusetts. 1969.

Mycotoxins in Foodstuffs. G. N. Wogan. M.I.T. Press, Cambridge, Massachusetts. 1965.

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# Mycotoxins - Their Biosynthesis in Fungi: Aflatoxins and other Bisfuranoids

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**ABSTRACT**

Aflatoxins are a family of highly toxic and carcinogenic secondary metabolites produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Biosynthetically, the aflatoxins are produced by a polyketide pathway. Most of the experimental data on aflatoxin biosynthesis are derived from $^{14}C$- and $^{13}C$-labeling experiments and use of blocked mutants. These data indicate that the general steps in aflatoxin biosynthesis are acetate → anthraquinones → sterigmatocystin → aflatoxin $B_1$. Many details of the pathway remain unresolved; it is hoped that further research, particularly with cell-free systems, will improve our understanding of the mechanism of formation of these important fungal metabolites.

What is now called the "aflatoxin problem" began in 1960 as a poultry disease of unknown etiology, dubbed "Turkey X disease". The subsequent association of Turkey X disease with a fungal toxin produced by the common molds *Aspergillus flavus* and *Aspergillus parasiticus*, and the resultant surge of interest in aflatoxins and other mycotoxins has been described many times; both Goldblatt (*30,31*) and Austwick (*4*) have given particularly detailed and eloquent accounts. Some experimental landmarks in the first decade of aflatoxin research are summarized in Table 1.

For the generalist it is readily apparent that aflatoxins are simply another group in a growing list of fungal secondary metabolites of economic importance. Secondary metabolites are an enigmatic family of organic compounds which are not easily defined in a few words. Among the properties of secondary metabolites are: a restricted taxonomic distribution, no discernable function in the metabolism of the producing organism and usually a limited time of synthesis at certain stages of the cell cycle after active growth has ceased (*17,18*). Secondary metabolites constitute a chemically diverse group, but they are all produced by a few intermediates of primary metabolism. Those secondary metabolites which are formed by condensation of an acetyl unit with three or more malonyl units, with concomittant decarboxylation, are called "polyketides". The polyketide hypothesis was first promulgated by Birch and Donovan (*14*) and later reviewed by Birch (*13*). Historical precedent for a theory of plant metabolites derived from two-carbon units goes to the British chemist, J. N. Collie, who first called these 2-carbon units "ketens" (*19*), and then in a revision of his hypothesis termed them "ketides" (*20*).

TABLE 1. *Experimental landmarks during the first decade of aflatoxin research.*

| Date | Landmark | References |
|---|---|---|
| 1960 | Turkey X disease | Blout (*15*) |
| 1961 | Isolation and identification of *A. flavus* from contaminated peanut meal; introduction of term "aflatoxin" | Sargeant et al. (*66*) |
| 1963 | Chemical structures of major aflatoxins elucidated | Asao et al. (*2*) |
| 1966 | Total synthesis of racemic aflatoxin $B_1$ | Buchi et al. (*16*) |
| 1970 | Derivation of entire carbon skeleton of aflatoxin $B_1$ from acetate | Biollaz et al. (*12*) |

In nature, the ability to produce polyketides is found mostly in the fungi, and among fungi it is largely restricted to the Ascomycetes and the Fungi Imperfecti (*77*). Polyketides are classified as triketides, tetraketides, pentaketides, etc., according to the number of repeating two-carbon units which have contributed to the biosynthesis of the particular compound. Aflatoxins are nonaketides. Credit for elucidation of the polyketide origin of aflatoxins goes to workers at Massachusetts Institute of Technology, whose early $^{14}C$-incorporation and degradation studies revealed that the entire carbon skeleton of aflatoxin $B_1$ was derived from acetate with methionine contributing the methoxy methyl group (*10,11,12,43*).

## INTERMEDIATES OF AFLATOXIN $B_1$ BIOSYNTHESIS

Early proposals concerning aflatoxin biosynthesis are summarized in Table 2. For the remainder of this discussion we shall concentrate on specific, identifiable

TABLE 2. *Early proposals concerning aflatoxin biosynthesis.*

| Year | Proposed precursor(s) | Experimental evidence | Reference |
|---|---|---|---|
| 1964 | Sterigmatocystin | Negative | Holker and Underwood (*39*) |
| 1964 | Aromatic amino acids | Equivocal | Adye and Mateles (*1*) |
| 1964 | Mevalonate | None | Moody (*56*) |
| 1965 | Kojic acid | None | Heathcote et al. (*36*) |
| 1965 | Averufin, 6-desoxyversicolorin A, sterigmatocystin | None | Thomas (*76*) |

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metabolites which are known as aflatoxin precursors. Portions of several previous papers have reviewed aflatoxin biosynthesis (*24,51,55,57,63*) and a number of other publications have been exclusively devoted to this topic (*37,38,54,61,80,81*). For a description of the relationship between primary metabolism and aflatoxin production, the review by Maggon et al. (*54*) is particularly helpful.

The same methods are used to study both primary and secondary metabolic pathways. These include the use of isotopically labeled precursors, blocked mutants, metabolic inhibitors, and cell-free systems. As is generally true of secondary metabolic pathways, most of the data available on aflatoxin $B_1$ biosynthesis are derived from experiments using labeled precursors and blocked mutants. However, and rather surprisingly, the organophosphate insecticide Dichlorvos has proven to be a specific and useful aflatoxin inhibitor (*40,82*). Moreover, a preliminary report by Uriah et al. (*78*) indicates that benzoic acid may also block a specific step in aflatoxin production. Application of cell-free systems to aflatoxin biosynthesis is still in its infancy, but refinement and expansion of the pioneering work of Singh and Hsieh (*71*) should soon remedy this situation. Finally, the *A. flavus* protoplast system being developed by Dutton and Anderson (*26*) may result in an efficient cell-free aflatoxin conversion.

To date there is experimental evidence for six intermediates in aflatoxin $B_1$ biosynthesis. The six intermediates, and the methods which have been used to verify their precursor status are presented in Table 3.

Five of these intermediates are polyhydroxyanthraquinones (averufin, versiconal hemiacetal acetate, versicolorin A, norsolorinic acid and a new uncharacterized yellow pigment), and four of them are accumulated by mutants of *A. parasiticus* with impaired aflatoxin biosynthesis (averufin, versicolorin A, norsolorinic acid and the uncharacterized yellow pigment). The uncharacterized yellow pigment is described in more detail below. Versiconal hemiacetal acetate, formerly misidentified as "versiconal acetate" in the earlier literature, accumulates when aflatoxigenic strains are treated with Dichlorvos (*21,29,68,82*). The sixth intermediate is the xanthone, sterigmatocystin, a natural metabolite of *A. versicolor*. Curiously, although sterigmatocystin shows the highest incorporation of $^{14}C$-label into aflatoxin $B_1$ of the known precursors, there is only one published report of sterigmatocystin from an aflatoxigenic species (*69*).

Retrospectively, the biosynthetic model proposed by Thomas in 1965 (*76*) can be praised for its perspicacity. In this scheme Thomas suggested that anthraquinones of the averufin or versicolorin type could be oxidized to the xanthone sterigmatocystin. Sterigmatocystin in turn is transformed into aflatoxin $B_1$ via oxidative cleavage, an aldol condensation, decarboxylation and dehydration. An almost identical scheme, supported by extensive experimental data, was presented 12 years later by Singh and Hsieh (*72*) who proposed the pathway: acetate → averufin → versiconal hemiacetal acetate → versicolorin A → sterigmatocystin → aflatoxin $B_1$.

An important question about the pathway has concerned formation of the $C_4$ bisfuran ring system of versicolorin A, sterigmatocystin and aflatoxin. Researchers applying carbon-13 nuclear magnetic resonance analysis to $^{13}C$-labeled averufin (*9,28,32,46*) versiconal hemiacetal acetate (*21,29*), sterigmatocystin (*22,58,59,70*) and aflatoxin $B_1$ (*22,59,75*) recognized the potential of this method for resolving the mechanism of formation.

Gorst-Allman et al. (*33,34*) have reviewed some of the differing proposals that explain the unique head-to-head linkage for the coupling of the dihydrofuran ring and the aromatic system in aflatoxin $B_1$ and propose the involvement of an epoxide intermediate. Continued application of judicious labeling experiments and cell-free systems should resolve the specific mechanisms of aflatoxin $B_1$ formation.

## NEW AFLATOXIN PRECURSOR

A new aflatoxin precursor has been isolated from a double mutant of *A. parasiticus*. This mutant was derived from the versicolorin A accumulating strain (*49*) by nitrosoguanidine treatment (*7*). The new mutant accumulates several pigmented metabolites; one of these is a yellow compound which has been purified, and $^{14}C$-labeling experiments show 10.2-28.5% incorporation of the yellow pigment into aflatoxin $B_1$ (See Table 3). The new aflatoxin intermediate has been partially characterized.

Evidence that the new yellow precursor is different from any known hydroxyanthraquinone intermediates is shown by comparison of chromatographic and physical

TABLE 3. *Known intermediates of aflatoxin biosynthesis. (References in parentheses).*

| Intermediate | Mutant block | Range of $^{14}C$ incorporation | Dichlorvos inhibition of label from intermediate into aflatoxin $B_1$ | Cell-free system available |
|---|---|---|---|---|
| Averufin | yes (*25*) | 7.4-49.4% (*52,53,72,82*) | yes (*82*) | no |
| Sterigmatocystin | no | 17.0-65% (*41,72,82*) | no (*82*) | yes (*71*) |
| Versiconal hemiacetal acetate | no | 8.0-13.7% (*72,82*) | yes (*82*) | no |
| Versicolorin A | yes (*49*) | 34.5-50.5% (*48,72*) | no (*8*) | no |
| Norsolorinic acid | partial (*23,50*) | 2.2% (*42*) | ? | no |
| Uncharacterized yellow pigment | yes (*7*) | 10.2-28.5%[a] | yes[a] | no |

[a]Bennett, J. W. and L. S. Lee. 1978. Previously unpublished data.

properties. Mobilities of the five hydroxyanthraquinone aflatoxin intermediates are reported in Table 4.

The new precursor melts at 228-230 C; it exhibits ultraviolet absorption maxima at 453, 315, 298, 262, 247, and 221 nm in ethanol. Infrared absorption (by KBr disc) are 3400, 2950, 2840, 1600, 1580, 1560, 1450, and 1390 $cm^{-1}$. The methyl ether derivative of the new precursor melts at 149-150 C. Nuclear magnetic resonance of the derivative indicate a 4-hydroxyanthraquinone with a substituted side chain. Comparison of the UV spectrum of the methyl ether derivative with the spectrum of tetra-O-methyldihydroaverythrin indicates a close similarity between the two compounds (*64*). Both exhibit maximum absorbance at 222 and 283 nm. However, biological activity and chromatographic behavior that indicate polar properties for the new precursor preclude assigning the structure of tetra-O-methyldihydroaverythrin to this new precursor. Extended investigations are underway in our laboratory to establish its structure and place this new precursor in the biosynthetic pathway for aflatoxin $B_1$ formation.

TABLE 4. *Thin layer chromatographic mobilities of hydroxyanthraquinone aflatoxin precursors.*

| Compound | Rf in benzene: acetic acid (9:1) | Rf in toluene: ethyl acetate: acetic acid (80:10:10) |
|---|---|---|
| Norsolorinic Acid | 0.52 | 0.82 |
| Averufin | 0.41 | 0.79 |
| Versicolorin A | 0.38 | 0.70 |
| New Precursor | 0.22 | 0.61 |
| Versiconal hemiacetal acetate | 0.08 | 0.42 |

## BIOSYNTHESIS OF AFLATOXINS $B_2$ AND $G_1$

Most research on aflatoxin biosynthesis has concerned production of aflatoxin $B_1$ which is the most plentiful and the most toxic of the aflatoxins. Labeling experiments from Heathcote et al. (*38*) indicate an interconversion of aflatoxin $B_1$ and aflatoxin $B_2$. However, reports from three laboratories (*60,67,79*) have noted strains of *A. flavus* which accumulate aflatoxin $B_2$ to the exclusion of aflatoxin $B_1$, providing genetic evidence that the pathway to these two compounds may diverge. An early study by Elsworthy et al. (*27*) showed the incorporation of $^{14}C$-label in the O-methyl group of dihydrosterigmatocystin into aflatoxin $B_2$ but not $B_1$ in *A. parasiticus*. To explain these data, Maggon et al. (*54*) have proposed a pathway to aflatoxin $B_2$ via versiconal, versicolorin C and 5-hydroxydihydrosterigmatocystin.

Strains capable of producing only G aflatoxins have not been reported in the literature, and were not noted in a large study of blocked aflatoxin mutants (*6*). Therefore, most workers concur with Biollaz et al. (*12*) that aflatoxin $G_1$ is derived oxidatively from aflatoxin $B_1$.

## OTHER BISFURANOIDS

Much less is known about bisfuranoids of the sterigmatocystin, austocystin, and polyhydroxyanthraquinone type than of the aflatoxins. Chemical and toxicity data for bisfuranoids were reviewed by Rodricks (*65*) through 1969, and more recently by several others (*35,47,62,73,74*). The $C^{13}$-nuclear magnetic resonance work on averufin and sterigmatocystin discussed above, and $^{14}C$-labeling studies by Hsieh and co-workers (*44,45*) indicate that a polyketide pathway terminating at sterigmatocystin in *A. versicolor*, is in other respects similar to the aflatoxin pathway found in *A. flavus* and *A. parasiticus*.

Known bisfuran metabolites are described largely from *Aspergillus* species, but two reports (*3,5*) suggest that these compounds may be more widespread in nature than is currently supposed. Should this be true, we await increased cognizance by toxicologists, biologists, biochemists, and other researchers of the molecular architecture and biotransformations of these interesting and economically important metabolites.

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## Increased Funds will Speed Research of Cheese Problems

The Cheese Research Institute at the University of Wisconsin-Madison is gearing up for full-time operation, thanks to a $76,000 increase in operating funds in the new state budget, according to institute director Norman Olson.

The additional funding will result in the hiring of two full-time research specialists and three part-time graduate students to assist the food scientists, economists and chemical engineers on the staff of the three-year-old institute, the only such facility in the national.

Olson says that the new funds will speed research for Wisconsin's large cheese industry and "will allow us to attack research problems more rapidly and efficiently than before.

"Although Wisconsin produces 38 percent of all cheese in the United States, few manufacturers are large enough to maintain their own research departments. Yet the industry is faced with challenges to keep down production costs, keep up quality and compete with improved versions of imitation cheese.

The institute aims to widen use of cheese by-products such as whey, which contains potentially usable protein, sugar and salts. Some whey is now used in foods and livestock feeds, but much becomes a troublesome and pollution-causing waste.

To improve the economic efficiency of cheese manufacturing, the institute will look for ways to reduce the amount of whey produced, speed up cheese ripening, and automate cheese manufacturing systems.

One short-term problem solved by the institute concerned a favorite pizza topping, mozzarella cheese. Producers, frozen food warehousers and the Refrigeration Research Foundation asked the institute to improve methods of freezing the cheese. The solution actually was in the thawing: mozzarella should be held several days after thawing to regain its high moisture content.

The institute staff includes Clyde H. Amundson, researcher in milk products and whey use; Myron P. Dean, UW-Extension food scientist; Elmer H. Marth, food microbiologist; Daryl Lund, food engineer; Thomas Richardson, food chemist; Truman Graf; dairy economist; Charles G. Hill, Jr., chemical engineer; and Olson, food scientist.

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# Mycotoxins - Their Biosynthesis in Fungi: Patulin and Related Carcinogenic Lactones

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**ABSTRACT**

A brief introduction to the mycotoxin, patulin is followed by a summary of the early contributions which demonstrated that patulin and its precursor, 6-methylsalicylic acid were derived from acetyl-CoA and hence are polyketides. The polyketide lactones, triacetic acid lactone, mycophenolic acid, patulin, penicillic acid and multicolic acid are compared with respect to their biosynthetic origins and the obscured sequence of acetate units in the latter three lactones is discussed. The current status of the patulin biosynthetic pathway is described with special emphasis on recent findings which have altered the late or post-gentisaldehyde portion of the pathway. This new appreciation of the patulin pathway is then extrapolated to the closely related γ-lactones, penicillic acid and multicolic acid. Pathways analogous to that of patulin are proposed for these two lactones and are discussed. These biosynthetic relationships are then related to the co-occurrence of some of these lactones in cultures of the same fungus.

## PATULIN

Patulin has a relatively long history. It was discovered in the midst of the excitement over penicillin, that is, during the years 1938-1943. Before its isolation as a product of *Penicillium patulum* (synonym: *Penicillium urticae*) by Raistrick's group in 1943 (*5*), it had been detected in the culture media of *Penicillium expansum* (*39*), *Aspergillus clavatus* (*40*) and *Penicillium claviforme* (*7*) and thus had been known variously as expansin, clavatin or claviformin. Patulin's potential as an antibiotic was significant since it is active against a wide variety of bacteria and fungi (*9,17,31,36*). Thus patulin inhibits *Bacillus* sp. about 10 times more effectively than does penicillin G; plant pathogenic fungi are particularly susceptible (*17,31*) and patulin is an effective insecticide (*11*), and antiviral (*12*) and anti-tumor agent (*9,31*). Unfortunately, patulin is generally very toxic to animals — a fact that was recognized soon after its discovery (*17,31*). Patulin also possesses phytotoxic activity (*9,15*). Since patulin has been shown to be mutagenic (*24,38*), teratogenic (*8*), and carcinogenic (*9,13*), it has been extensively studied in recent years as a relatively common mycotoxin. For example, patulin has been produced in the soil of wheat fields by *P. urticae* (*25*), in fruit and fruit products by *P. expansum* (*21*) and by *Byssochlamys* spp. (*4,28*) and in wild rice by *A. clavatus* (*22*).

In spite of its general toxicity, patulin continued to be of interest. Its relatively simple structure remained a challenge until 1949, when Woodward and Singh showed it to be a compact bicyclic molecule composed of a six-membered hemi-acetal ring and a five-membered lactone ring (Fig. 1). Patulin's toxicity is believed to be due to the alkylating ability of this lactone moiety with its *a*, *β γ*, *δ*-dienone structure (*8,9*).

Patulin's unusual structure led to an interest in its biosynthesis and Fig. 2 summarizes some of the early contributions which led to our present understanding of the patulin biosynthetic pathway. Birkinshaw in 1953 (*4*) was the first to suggest that the aromatic ring of the then hypothetical gentisaldehyde could be cleaved to yield an acyclic form which then cyclized to produce patulin. Two years later Arthur Birch et al. (*3*) used radiolabeled acetate to prove that four acetate molecules were

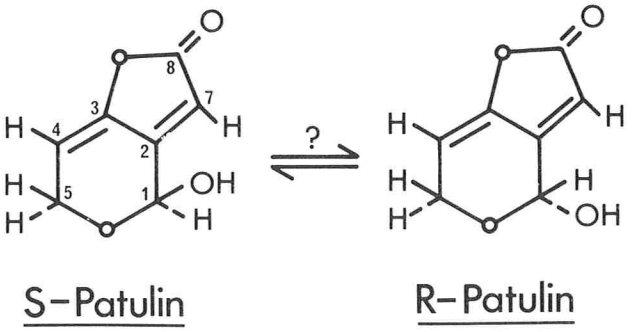


Figure 1. *Structure of patulin. Note that patulin is always isolated as a mixture of R and S stereoisomers.*

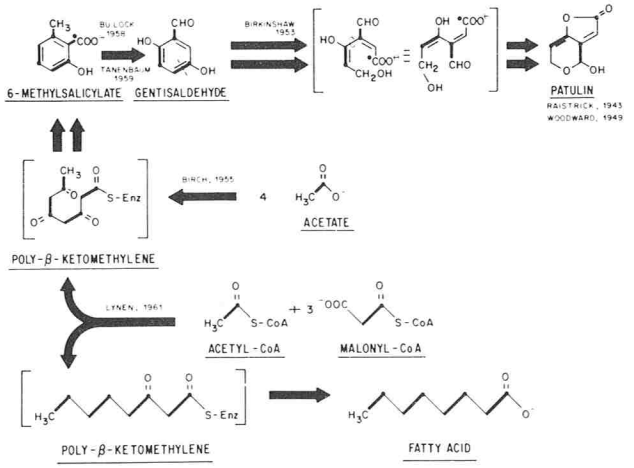


Figure 2. *Early contributions to our understanding of patulin biosynthesis. Each two carbon acetate unit is designated by a heavy line with the original carboxyl group designated by a solid dot. The postulated structures in square brackets have not been isolated.*

condensed in a head-to-tail fashion to yield 6-methylsalicylic acid, a co-metabolite of patulin. This discovery was important because it was the first proof that some cells could synthesize the aromatic ring from acetate rather than from carbohydrate via the more common shikimic acid route. Soon after this Bu'Lock (*6*), and Tanenbaum (*37*) separately showed that specifically labelled ( ● ) 6-methyl salicylic acid (Fig. 1) was converted to patulin in accord with Birkinshaw's earlier prediction. In 1961, Lynen and Tada (*23*) proposed that one molecule of acetyl-CoA and three of malonyl-CoA were condensed to yeild an enzyme-bound poly-β-ketomethylene chain which could be sequentially reduced to a fatty acid, or cyclized to 6-methylsalicylic acid, depending upon the multi-enzyme complex which was used. Subsequent evidence has supported this proposal (*14*). Since a large group of microbial and plant metabolites are also derived from a poly-ketomethylene chain, these metabolites have been collectively called "polyketides".

## SOME POLYKETIDE LACTONES

The biosynthetic origin of five polyketide lactones is shown in Fig. 3. The head-to-tail sequence of the original acetate units ( —● ) is indicated as is each acetate's carboxyl group ( ● ). The polyacetate origin of the first two lactones is relatively obvious, since the course of the polyacetate chain is apparent in even, mycophenolic acid in which two methylations, an oxidation, and an isoprenylation have occurred. If, however, the bottom three lactones (Fig. 3) are examined, their polyacetate origin is not obvious! This is because the head-to-tail distribution of acetates has been obscured by ring cleavage of an aromatic precursor in each case. Although much less is known about the biosynthetic pathways leading to penicillic acid and multicolic acid, recent additions to our knowledge of the patulin biosynthetic pathway suggest that all three of these lactones may be synthesized via analogous pathways.

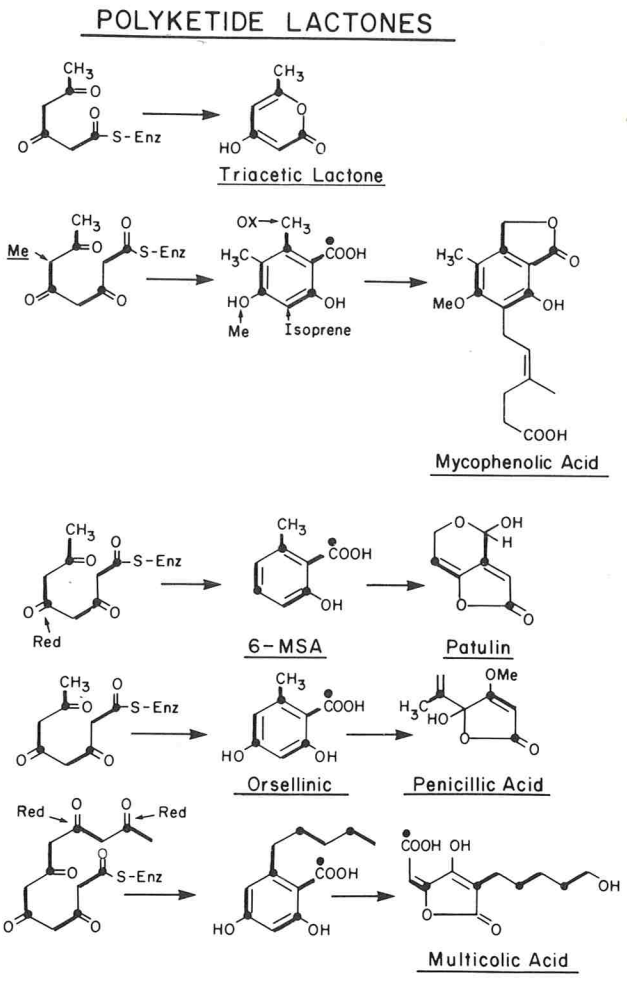


Figure 3. *Biosynthetically related lactones. The postulated enzyme bound poly-β-ketomethylene precursors, the first aromatic intermediate, if any, and the end products are illustrated in each case. The positions of the original acetate units are indicated as in Fig. 2, and the designations Me, Isoprene, OX, and Red refer to a methylation, an isoprenylation, an oxidation, or a reduction, respectively. 6-MSA is 6-methylsalicylic acid.*

## PATULIN BIOSYNTHESIS - AN UPDATE

Until recently (*33*) the literature version of the patulin pathway (*18*) was that shown in Fig. 4. The first committed metabolite of the pathway, 6-methylsalicylic acid, is decarboxylated, its methyl group is oxidized to an aldehyde group and the aromatic ring is hydroxylated to yield gentisaldehyde. Cleavage of the aromatic ring was then postulated to occur in analogy to the dioxygenase-mediated ring openings established in bacteria. Although never isolated, the resultant acyclic form of patulin ["pre-patulin"] could easily yield patulin.

In recent studies concerned with the patulin pathway's function (*32*) our attention was drawn to one of a number of patulin-minus mutants (J1) because of a marked coloration surrounding its colonies. When metabolites from shake cultures of this mutant were examined by TLC it was clear that unlike the parent, this mutant did not produce patulin but did produce two new metabolites

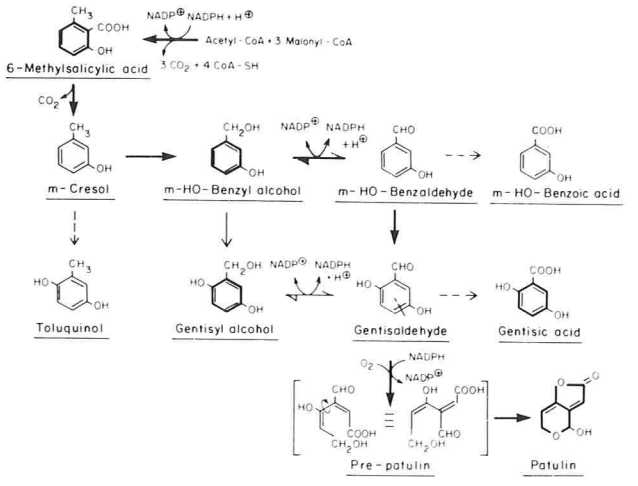


Figure 4. *The pre-1978 patulin biosynthetic pathway. The major route is designated by heavy arrows, an alternate route by light arrows and side reactions by dashed arrows. Metabolites which often accumulate in the culture medium are indicated by heavy structures while brackets indicate a hypothetical intermediate.*

which we called UII and UIII. Subsequent experiments (*19,33,34*) showed that radiolabeled acetate and gentisaldehyde were readily incorporated into these compounds and that both were, in turn, readily converted to patulin by the parent. The time course of these incorporations was determined by an invaluable technique which produced high-resolution thin layer radiochromatograms (*33*). This technique was augmented by use of washed whole-cell suspension feeding experiments in which 1 mM concentrations of the unknowns were used. Using this latter technique, another patulin-minus mutant, J2, which is blocked immediately after gentisaldehyde and does *not* produce UII or UIII, was found to convert UII and UIII into significant amounts of patulin by 30 min and by 3-5 h patulin yields of about 90% and 60%, respectively, were realized (*33,34*). Structure elucidation studies (*33,34*) have shown these unknowns (UII, UIII) to be an epoxyquinone and its related reduced derivative, respectively (Fig. 5). The first is a known phytotoxin called phyllostine (*29*), while the other is a new metabolite we have called isoepoxydon. A specific alcohol dehydrogenase interconverts these two metabolites in *P. urticae*. To accommodate these two metabolites as pathway intermediates we have postulated a mono-oxygenase-mediated conversion of gentisaldehyde to isoepoxydon or phyllostine followed by a ring-opening rearrangement which yields a postulated 7-membered lactone (Fig. 5). This lactone could then yield the acyclic and cyclic forms of patulin. A further clarification of this post-phyllostine part of the pathway is anticipated from studies of another mutant (S11) which appears to be blocked even later in the pathway (Fig. 5).

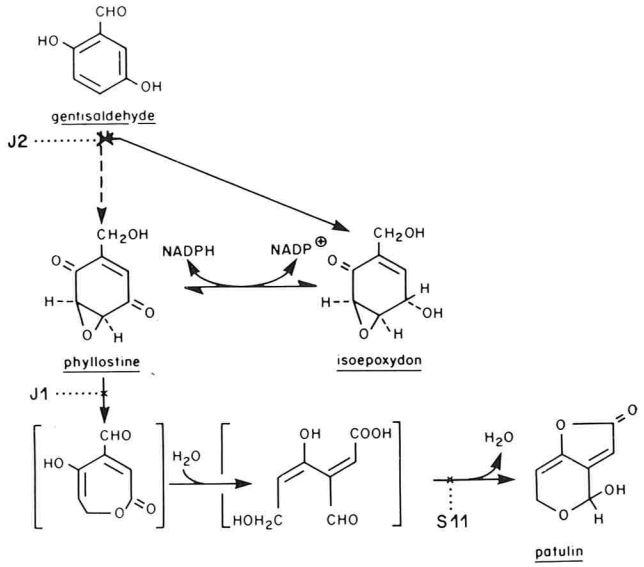


Figure 5. *Recent modifications to the post-gentisaldehyde portion of the patulin pathway. The solid arrows represent the most probable route, while the dashed arrow indicates an alternative possibility. The metabolites in square brackets are strictly hypothetical. The blocks in* P. uricae *mutants J2, J1, and S11 are indicated by dotted lines.*

## BIOSYNTHESIS OF PENICILLIC ACID AND MULTICOLIC ACID IN ANALOGY TO PATULIN BIOSYNTHESIS

Our altered understanding of the late part of the patulin pathway has provoked an interesting extrapolation of these findings to the biosynthesis of the related lactones penicillic acid and multicolic acid (Fig. 6,7). Beginning on the left in Fig. 6, an abbreviated route from 6-methylsalicylic acid to phyllostine is given. As previously noted, four steps are involved in the modification of ring substituents to yield gentisaldehyde. An internal redox reaction could then yield gentisquinone which is *postulated* here as the substrate for the epoxidase which produces phyllostine. Note that the other epoxide, isoepoxydon (Fig. 5) is omitted from this scheme since its position as an obligate intermediate is still obscure. The conversation of gentisquinone to isoepoxydon and then to phyllostine is a plausible alternative.

Penicillic acid biosynthesis is known to begin with the polyketide orsellinic acid (Fig. 3) and to also involve a number of ring substituent modifications (*1,2*). Thus the central pathway in Fig. 6 shows a methylation and an oxidative decarboxylation followed by an oxidation to a quinone, which is then postulated to yield an epoxyquinone. Multicolic acid is believed from [$^{13}$C]-acetate incorporation studies (*20*) to arise from a similar orsellinic acid-like precursor, 6-pentyresorcylic acid. Again by analogy, the hypothetical sequence at the right in Fig. 6 shows an oxidative decarboxylation, an oxidation to the quinone and finally an epoxidation to an epoxyquinone. Note that the original sequence of acetate

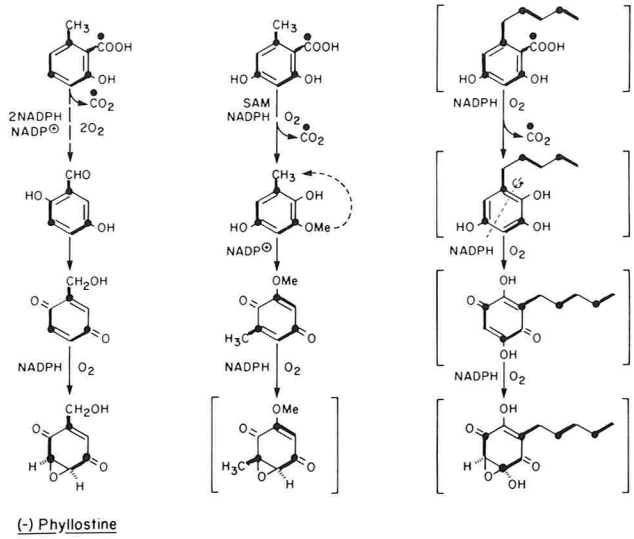


Figure. 6. *An extrapolation of the patulin pathway to the biosynthesis of other γ-lactones. Analogous pathways from the first aromatic precursor to an epoxyquinone intermediate are proposed for, from left to right, patulin, penicillic acid and multicolic acid. Large dashed arrows indicate two or more steps, while the small dash arrow indicates that the structure is rotated in the plane of the paper. The dashed line indicates the axis about which that structure is rotated out of the plane of the paper. The metabolites in square brackets have not been detected and are hypothetical. The positions of the original acetate units are again indicated as in Fig. 2.*

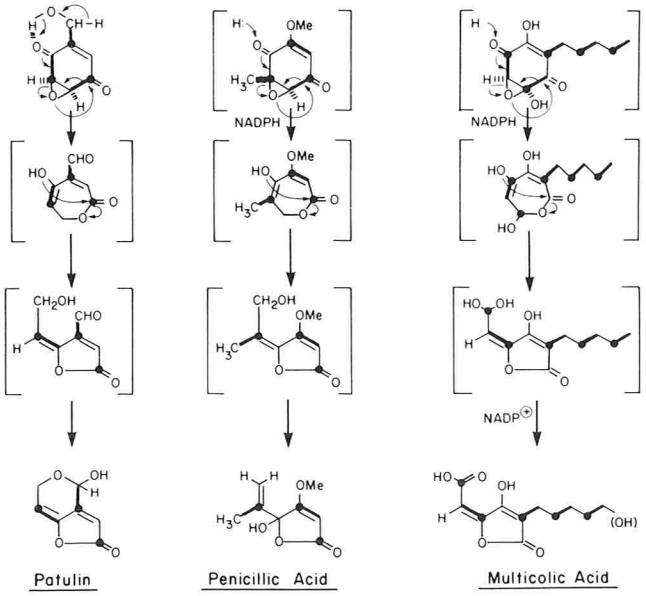


Figure 7. *A continuation of the proposed pathways for patulin, penicillic acid and multicolic acid. Analogous pathways for the conversion of epoxyquinones (Fig. 6) to known end-products are postulated. Proposed intermediates are indicated by square brackets and concerted reaction mechanisms are proposed although step-wise mechanisms are also possible. Acetate units are indicated as in Fig. 2.*

units ( — ● ) is still readily apparent in each of the epoxyquinones at the bottom of Fig. 6.

Since each of the above pathways involves the oxidative cleavage of an aromatic ring to yield similar lactones, it is not unreasonable to expect a similar ring opening mechanism to play a critical role in each pathway. Thus as postulated in Fig. 6, each pathway utilizes an epoxidation as a means of activating the aromatic ring before its cleavage. This cleavage can then occur in a concerted manner to yield a 7-membered lactone as indicated at the top of Fig. 7. Note that in each case this cleavage begins the process of obscuring the original sequence of acetate units. Identical rearrangements which yield a five-membered or γ-lactone follow, and one or two simple reactions then yield the known end products. Note that although the concerted mechanisms postulated in Fig. 7 can be subtly modified or rewritten as non-concerted mechanisms, the overall biosynthetic design would remain. Of most importance, however, is the fact that the pathways outlined in Fig. 6 and 7 correctly predict the position of the original acetate units in each of the end products in agreement with the extensive tracer experiments which have been carried out (*6,16,20,37*) on each of these lactones.

The obvious biosynthetic relationship between these lactones (Fig. 3) is supported by an examination of the fungi which produce them. It is well established that many mycotoxins such as patulin and penicillic acid (*9*) are produced by a relatively broad spectrum of fungi and that some fungal strains are capable of simultaneously producing structurally different but biosynthetically related mycotoxins. Thus *P. urticae* (syn. *P. patulum*) is known to produce patulin plus triacetic acid lactone (*30*), or griseofulvin (*35*), while *P. expansum* produces patulin and citrinin (*10*). All are polyketides of quite different structure. Given the close biosynthetic relationship between patulin and penicillic acid, it is noteworthy that until recently there have been no reports of the co-occurrence of these two mycotoxins. Olivigni and Bullerman (*26,27*) have, however, recently reported exactly such an occurrence in an atypical strain of *Penicillium roqueforti*.

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## ASAE Meeting Highlighted

"International Dimensions in Engineering," was the theme of the American Society of Agricultural Engineers' 1979 Summer Meeting. It was held at the University of Manitoba, Winnipeg, Manitoba, Canada, in late June.

Among the papers presented at that meeting were the following, which are just highlighted here. For more information about these or other papers, contact Roger Castenson, ASAE public relations manager, ASAE, Box 410, St. Joseph, MI 49085.

*Automation for Rotary Milking Parlors*

An electronic management system, developed by the National Institute of Agricultural Engineering, has been integrated with two automated rotary milking parlors at the National Institute for Research in Dairying. The systems take care of manual operations such as cow handling, platform control, feed and milking unit removal, as well as automatic cow identification and individual milk yield and body weight records. The microprocessor controlled system also tells the operator about antibiotic treatment, dry cows, and equipment failures.

*Energy Aspects of the New DASI Process for Milk Sterilization*

A new process for milk sterilization helps prevent spoiling without refrigeration. The result is savings of energy in refrigerated transport, storage, display, and home refrigerators. The ultra high temperature process is unique in its ability to overcome the off-flavor associated with conventional UHT processing. Energy usage for both conventional and new milk processing systems are evaluated and compared.

*Controlling Dairy Farm Electrical Loads for Off-Peak Metering with a Microprocessor*

An electrically complex farm enterprise often has difficulty taking advantage of time-of-day electrical metering. A microprocessor control system developed for a Michigan dairy automatically controls electrical equipment to take advantage of off-peak rates with minimum disruption to milking, feeding, and other operations. The cost of installing and maintaining the system is compared to anticipated energy cost savings.

*Hot Water Use in Milking Parlors*

Water heating provides a relatively simple opportunity for energy savings. Different types and sizes of dairies throughout the U.S. were monitored to determine their hot water usage and water and energy usage are summarized by various dairy categories.

*Agricultural Residues: Renewable Energy for Utility Power Companies*

A study performed for a major California utility power company on the utilization of agricultural and forest residues as a fuel source. The investigation concentrated on three areas: to determine which residues have the highest potential for use; to propose realistic systems to collect, transport, and convert residues; and to estimate the cost of producing energy from residues.

*Refeeding Processed Manure*

The use of animal manure as a feed ingredient and nutrient can help ease pollution problems, decrease feed costs, and increase supplies of available nitrogen and essential minerals. Collecting, processing, and incorporation of processed manure into livestock diets is reviewed.

*Selection Criteria for a Dairy Waste Management System*

In selecting a dairy waste management system or in modifying an existing system, a number of factors must be considered. These include the economic factors of labor and energy requirements as well as the social and legal implications of possible air or water pollution. In light of these considerations, a comprehensive network of dairy waste management alternatives for tie stall and free stall barns has been developed by three Canadian researchers.

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# Mycotoxins — Their Biosynthesis in Fungi: Ochratoxins — Metabolites of Combined Pathways

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**ABSTRACT**

Little work has been done on the biosynthesis of ochratoxins. Nevertheless, a scheme based on known chemical and enzymatic reactions and incorporating known facts about ochratoxin biosynthesis is proposed as a working model. The scheme calls for formation of an unbranched pentaketide from acetyl and malonyl CoA, its cyclization and aromatization to an isocoumarin, methylation and oxidation to the carboxy derivative which is chlorinated by chloroperoxidase before acyl activation for reaction with an ester of phenylalanine which arises via the shikimic acid pathway. The amide, ochratoxin A, is considered the final product of this biosynthetic pathway.

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It is customary to start a discussion such as this with some kind of justification for the effort. Because evaluation of the topic under consideration relies heavily on precedent, it seems inappropriate to be iconoclastic at the beginning. Therefore, let us begin by stating that ochratoxins are a family of toxins produced by several fungi, and until recently the ochratoxins were thought to be mainly laboratory curiosities, but now there is evidence for their causing nephropathy of humans, swine, and poultry (*6,7,12,15*). Ochratoxin does indeed appear menacing since it is twice as toxic in chickens as the rightly feared aflatoxin based on $LD_{50}$ dose and minimal-growth-inhibitory-dose (*12*). Thus, in studying ochratoxin we can indulge our intellectual curiosity without harming our social conscience.

The ability to produce ochratoxins is widespread (Table 1) among the aspergilli (*2,10*) and penicilli (*3*). The name ochratoxin is derived from the specific name of the fungus from which it was first isolated - *Aspergillus ochraceus*. From the viewpoint of unraveling the biosynthetic pathway, it is nice to have many producers with their inherent variations which can be exploited for a fuller and easier understanding of ochratoxigenesis. The advantages of such a situation have been demonstrated amply by the workers on aflatoxin.

TABLE 1. *Organisms which produce ochratoxin.*

| | |
|---|---|
| *Aspergillus ochraceus* | *Penicillium commune* |
| *Aspergillus melleus* | *Penicillium cyclopium* |
| *Aspergillus sulphureus* | *Penicillium purpurescens* |
| *Aspergillus osteanus* | *Penicillium palitans* |
| *Aspergillus petrakii* | *Penicillium viridicatum* |
| *Aspergillus sclerotiorum* | *Penicillium variable* |
| *Aspergillus alliaceus* | |

The commonly recognized, name-bearing members of the ochratoxin family are shown in Fig. 1. In addition, there are several other naturally occurring derivatives that must be considered in any proposed biosynthetic scheme. For the moment, notice that ochratoxin A, which occurs more abundantly and is more toxic than the others, is a chlorinated derivative of ochratoxin B; that ochratoxin C is the ethyl ester of ochratoxin A; that A, B, and C are amides of phenylalanine and substituted isocoumaric acids; that ochratoxin *a* is the non-amino acid portion of A and C, and that ochratoxin *β* is the non-amino acid portion of ochratoxin B. Now, the title of this paper can be more fully appreciated. In the biosynthesis of ochratoxins we must account for the synthesis of an amino acid - phenylalanine, for a substituted isocoumarin derivative, for a halogenation process, for an amide bond, for an ethyl ester of phenylalanine, plus any quirks discovered along the way, and not necessarily in that order. It should be noted now that the literature based on ochratoxin biosynthesis is very small and incomplete. Much, if not most, of what is presented is speculative and presumptive, but hopefully, these uncertainties will be identified.

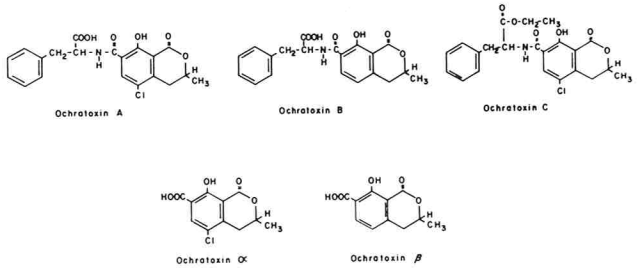
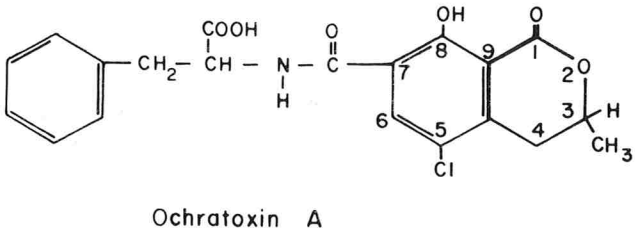



Figure 1. *Commonly recognized members of the ochratoxin family.*

Figure 2 shows the structure of ochratoxin A with the isocoumarin ring numbered according to IUPAC nomenclature. Searcy et al. (*21*) in their pioneering work used another numbering system that has been perpetuated to facilitate comparisons but we believe the time has come to use standard terminology where reasonable and possible. The isocoumarin portion of ochratoxin A has the synonyms of ochratoxin *a* and 5-chloro-7-carboxy-mellein as well as the official name of 5-chloro-7-carboxy-8-hydroxy-3,4-dihydro-3R-methyl isocoumarin. The synonyms will be used in a fashion that, hopefully, is consistent with both understanding and the literature.




Ochratoxin A

Figure 2. *IUPAC numbering system for the isocoumarin portion of ochratoxin A.*

The well-known shikimic acid pathway for the biosynthesis of phenylalanine (*16*) is shown in Fig. 3. It provides for the cyclization of a 7-carbon aldonic acid to shikimic acid which has a pyruvic acid side chain added during aromatization prefactory to a final transamination. As Cole (*4*) pointed out, this is a presumed pathway which has not actually been demonstrated during ochratoxin biosynthesis. However, its wide-spread occurrence in other species and forms of life makes it reasonable to assume its functioning. What is known is that phenylalanine, once formed, is incorporated in toto into ochratoxin. This was established by the appropriate experiments using labeled material (*17,21,24*).

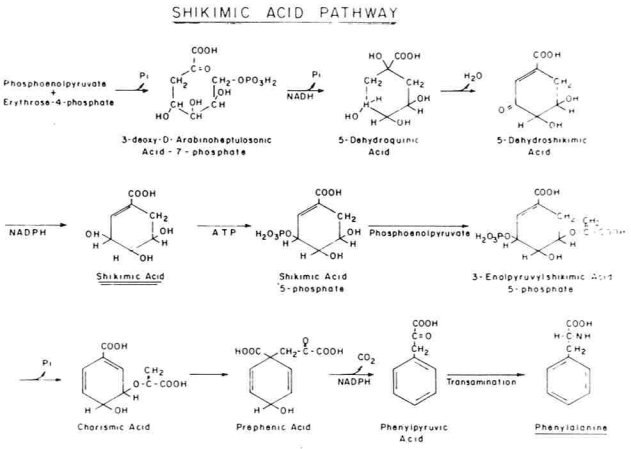



Figure 3. *Biosynthesis of phenylalanine by the shikimic acid pathway.*

Now let us consider the isocoumarin portion of ochratoxins and its biosynthesis. Table 2 lists some mycotoxins that have been reported to be synthesized via a polyketide or polyacetate pathway (*25*). Aside from the listing of ochratoxins, note penicillic acid, citrinin and mellein are produced by fungi which also produce ochratoxin. Further, remember that ochratoxins have the same ring structure as mellein. The biosynthesis of

TABLE 2. *Some mycotoxins synthesized by polyketide pathways.*

| | |
|---|---|
| Ochratoxin A, B, and C | Fumigatin |
| Aflatoxins | Kojic acid |
| Penicillic acid | Mycophenolic acid |
| Citrinin | Pyrenophorin |
| Mellein | Spinulosin |
| Curvularin | Zinniol |
| Zearalenone | Maltoryzine |
| Alternariol | Citreoviridin |
| Patulin | Versicolorin |
| Terreic acid | |

mellein via the polyketide pathway (*25*) is shown in Fig. 4. It starts with condensation of acetyl CoA and malonyl CoA and proceeds on the enzyme surface by stepwise, head-to-tail addition of acetyl units from malonyl CoA until a pentaketide is formed. This enzyme-bound polyacetate cyclizes via unknown steps to mellein, the product. All reactions are presumed to occur on the enzyme because free intermediates have not been detected.

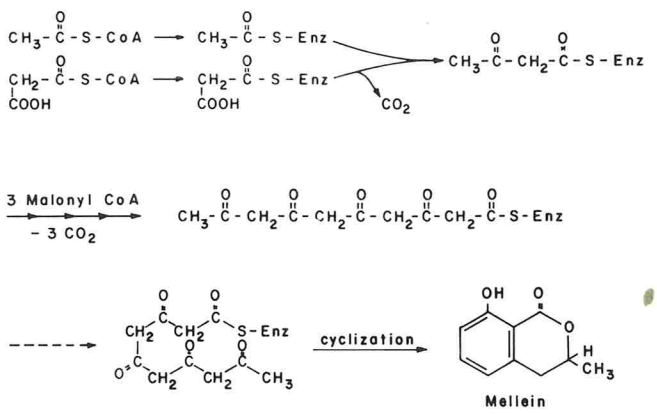



Figure 4. *Biosynthesis of mellein via a polyketide pathway.*

The structure of 7-carboxymellein is given in the center of Fig. 5. This compound has been isolated from ochratoxin fermentations. It is also called ochratoxin $\beta$, the dechloro-isocoumaric acid portion of ochratoxin B. Notice that it can be considered a pentaketide plus one carbon. Two biosynthetic pathways for 7-carboxymellein have been proposed (*21,24*). The one on the left (Fig. 5) has the C-1 from methionine adding to a single polyacetate chain and becoming the carboxy carbon. The one on the right has the C-1 addition becoming a ring carbon. Further, this scheme on the right has two independent acetate chains joining to become a branched pentaketide.

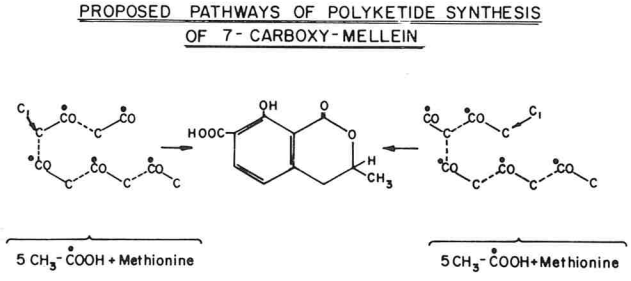



Figure 5. *Proposed pathways for polyketide synthesis of 7-carboxymellein. The dark circles by the alternate carbon atoms are for purposes of easy identification.*

Needless to say there was good and ample evidence for both views. Without giving a historical development, the current view favors a single unbranched polyacetate precursor for the ring structure, that is, the scheme on the left. The pertinent evidence for this view can be summarized by saying (*a*) that carbons 5, 7, 9 which are alternating carbons in the benzene ring, are derived from

the methyl group of acetate (*21*), (*b*) the 7-carboxyl carbon is derived from the methyl group of methionine not the C-1 of acetate as required in the branched chain scheme (*24*), (*c*) further, the carboxy carbon can be derived from formate (*17*); neither methionine nor formate are incorporated into C-1 or any other carbons of the mellein ring structure and (*d*) finally, C-3, the alcoholic carbon of the lactone ring, comes from C-1 of acetate (*24*).

This evidence is translated in Fig. 6 which gives the derivation of each carbon in 7-carboxymellein or ochratoxin $\beta$. The carboxy group is shown as coming from formic acid which is probably the precursor of methionine which can also give rise to the carboxy group. The other carbons are shown as a single unbranched chain made by head-to-tail combinations of acetic acid. It should be mentioned that the labeling experiments upon which this depiction is based have a common weakness of rather low percentage incorporation of precursor into product but this is counterbalanced by very low if any randomization of the label.

DERIVATION OF CARBON IN 7-CARBOXY-MELLEIN

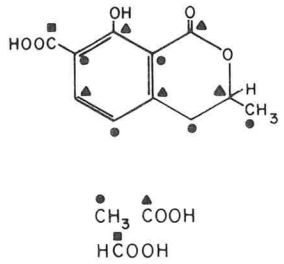



Figure 6. *Derivation of carbon atoms in 7-carboxymellein.*

Let us review briefly the manner in which the carboxy group could arise from formic acid. There is a well-known series of enzymatic reactions in which tetrahydrofolate is formylated and reduced to methyl tetrahydrofolate before transmethylation with homocysteine to give rise to methionine (*16,20,25*). Figure 7 shows formation of S-adenosyl-methionine, which is the actual alkylating agent by which methyl groups are introduced biosynthetically. These presumed reactions are based on their widespread occurrence in other species and life forms, not on their demonstration during ochratoxin formation. The bottom reaction (Fig. 7) shows the methylation of mellein ortho to the phenolic group. The product, 7-methylmellein, has not yet been isolated from an ochratoxin fermentation. Its oxidation product, 7-carboxymellein or ochratoxin $\beta$, has been isolated from ochratoxigenic fungi (*13*). This proposed methylation of the aromatic intermediate, mellein, rather than of a polyketide chain intermediate is supported by several bits of evidence. Actually, one can reasonably envision the alkylation occurring on the polyketide chain before cyclization and aromatization. However, alkylation of chains usually gives rise to multiple methyl groups as in the biosynthesis of citrinin (*1*). The proposed

ALKYLATION AND OXIDATION OF MELLEIN

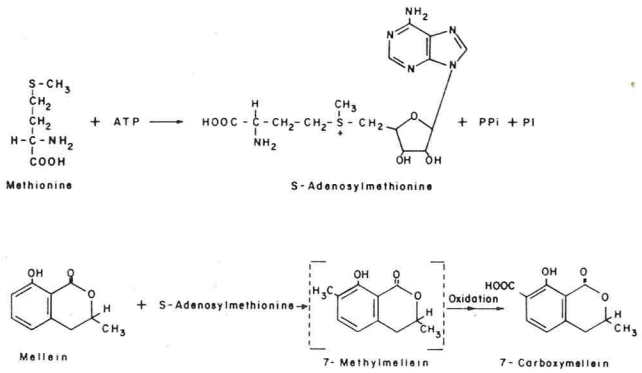
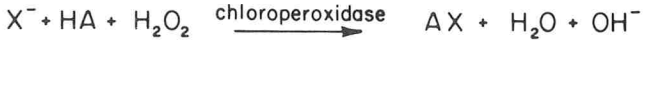



Figure 7. *Formation of the carboxy group in 7-carboxymellein. Incomplete brackets around 7-methylmellein indicate it has not been isolated from an ochratoxin fermentation.*

scheme also agrees with the fact that a phenolic group such as occurs on mellein is ortho-directing and this is the position where the carboxy group occurs. Finally, the presumed precursor, mellein, is formed by ochratoxigenic strains (*18*) and the chain alkylation scheme does not account easily for the production of mellein. It appears best to make a virtue of necessity.

The structures of ochratoxin A, C and *a* (Fig. 1) show they are chlorinated compounds. The chlorination step can be accounted for by assuming the intervention of an enzyme called chloroperoxidase. Figure 8 shows the type reaction catalyzed by this enzyme (*9*). Chloroperoxidase has low-substrate specificity and while ochratoxigenic fungi have not been examined for it, chloroperoxidase occurs widely in plants, animals and microorganisms and represents the agent invoked in the biosynthesis of over 200 naturally occurring halogenated compounds (*22*). The donor for this enzyme in ochratoxin synthesis would be the chloride ion. Figure 9 shows the proposed reaction whereby 7-carboxymellein becomes 5-chloro-7-carboxymellein or ochratoxin *a*. It is characteristic of organisms with chloroperoxidase that for every chlorinated compound they also produce the dechloro derivative. This relationship holds for the ochratoxins. The reaction drawn here has not been demonstrated; indeed, whether chlorination occurs early or late in biosynthesis of ochratoxin has not even been a subject of speculation.




X⁻ = Halogen Donor
HA = Halogen Acceptor

Figure 8. *Type of reaction of chloroperoxidase.*

CHLORINATION OF 7-CARBOXY-MELLEIN

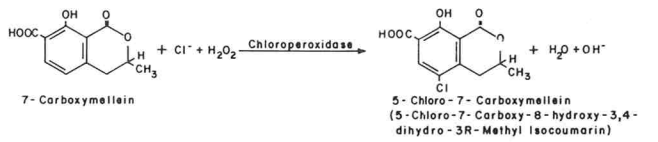



Figure 9. *Proposed chlorination of 7-carboxymellein.*

However, there are several reasons for thinking it occurs as the last substitution on the already formed aromatic nucleus. In other and better investigated natural systems, chlorination occurs after cyclization (*25*). The chlorine in ochratoxin is para to the phenolic group as would be expected in the presence of an ortho blocking group such as the 7-carboxy (*14*). Because chlorination results in ring deactivation, it usually terminates a series of substitutions. Finally, chlorinated isocoumarins other than those in Fig. 1 have not been reported in ochratoxigenic fungi.

Thus, the biosynthesis of the two main parts of ochratoxins has been accounted for now. What remains is the joining of the parts. This joining reaction has been demonstrated in a crude enzyme preparation by an enzyme complex called ochratoxin synthetase (*8*). The details are unknown except that ATP and $Mg^{++}$ are required. The overall reaction is the formation of an amide bond between the carboxy group of the mellein derivative and the amino group of phenylalanine. The mechanism by which this occurs is unknown but there are several hints to be found in comparative biochemistry. The most common amide group in nature occurs in proteins as a peptide bond and with the mechanism of protein synthesis being so well known it would be simple to propose an analogous reaction. However, the cofactor requirements of ochratoxin biosynthesis differ from protein synthesis which requires GTP (*16*). Further, strict analogy with protein synthesis would require transfer RNA of ochratoxin-*a* as well as the amino acyl t-RNA form of phenylalanine and there does not seem to be precedent for compounds such as ochratoxin *a* t-RNA. From a chemical viewpoint, the simplest and most obvious route of synthesis would be conversion of the carboxy group of ochratoxin *a* to the acyl form with subsequent displacement by the amino group of phenylalanine. Reasonable precedent for such acyl transfer in amide formation exists in the mycotoxin viridicatin (*25*). Before such a scheme can function, we must overcome one characteristic of amino acids at physiological pH values. They exist in the charged *zwitter ion* form which cannot participate in an acyl displacement reaction. Both nature and chemist accomplish the suppression of the zwitter ion form by converting the acid group of the amino acid to an acyl derivative such as an ester. The fact that ochratoxin C has an ethyl ester of phenylalanine now becomes very suggestive.

Esters are formed by several routes in microorganisms. Reversal of the customary esterase reaction,

$$RCOOH + R'OH \rightarrow RCOOR',$$

seems unlikely at first but it depends on the equilibrium constant. This route of biosynthesis has been invoked for esters of volatile fatty acids (*11*). The methylase reaction,

$$RCOOH + \text{S-adenosylmethionine} \rightarrow RCOOCH_3,$$

in which the alcohol portion of the ester is activated has precedent in protein biosynthesis (*20*). Acyl activation,

$$RCOOH \xrightarrow{ATP} [RCOP] + R'OH \rightarrow RCOOR',$$

leading to ester formation accomplishes the same end and appears commonly in nature (*16*). It is not essential with the present lack of information to select one of these routes just as long as we have an ester of phenylalanine with a free amine group to participate in a nucleophilic acyl displacement.

A generalized nucleophilic acyl displacement reaction (*19*) is shown in Fig. 10. The carbonyl group of the reactant holds the substituents in a planar triangle which is approached by the entering nucleophilic group. The attraction results in the formation of a tetrahedral intermediate from which the least basic group is displaced. Note that alcohols and amines would readily displace anhydrides.

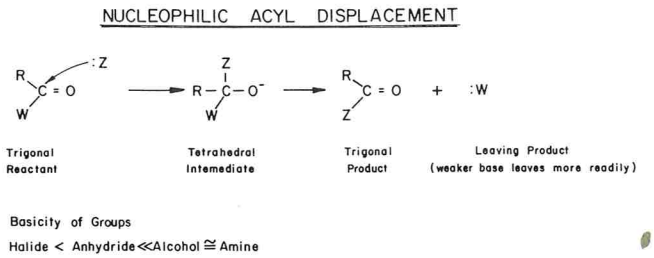



Figure 10. *Generalized reaction for nucleophilic acyl displacement.*

That carboxylic acid activation for participation in displacement reactions is a widely used strategem in biosynthesis is illustrated in Fig. 11. The fatty acid adenylate reacts with a sulphydryl group of coenzyme A to form the fatty acyl Co-A intermediates (*16*). γ-Glutamyl phosphate and amino acyl adenylate react with amines to form amides. In each case the reactant is

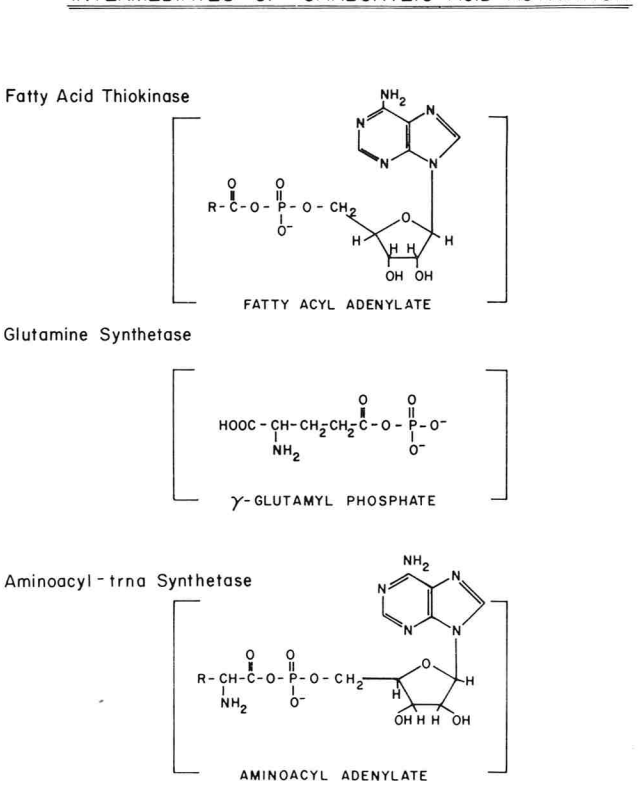



Figure 11. *Intermediates with activated acid groups used in biosynthetic displacement reactions. Brackets indicate the compounds and their reactions occur on the enzyme surface.*

a mixed anhydride and phosphate is the leaving group. These principles have been applied to a proposed biosynthetic pathway (Fig. 12) for the ochratoxin synthetase reactions which result in ochratoxin A production. Phenylalanine becomes an ethyl ester via a phosphorylated intermediate. Ochratoxin *a* undergoes acyl activation to a mixed anhydride from which the leaving phosphate group is displaced by the amine group of the ethyl ester of phenylalanine. The product is ochratoxin C which by esterase or transesterification results in the desired ochratoxin A. It should be noted that the methyl esters of ochratoxins A and B have been reported to occur naturally (*23*) and methylation and transmethylation reactions in general are much better studied and understood than those of the ethyl group. The methyl esters would also suppress zwitter ion formation as required in this proposed pathway. Hence it would be easy and tempting to include them as key intermediates in a comprehensive biosynthetic scheme, but somehow ochratoxin C which is an ethyl ester must be accounted for. Perhaps there is a methyl ester pathway independent of the ethyl ester pathway or perhaps there is a common pathway with a ramification at the end such as transesterification, but crystal balls seldom offer perfect vision.

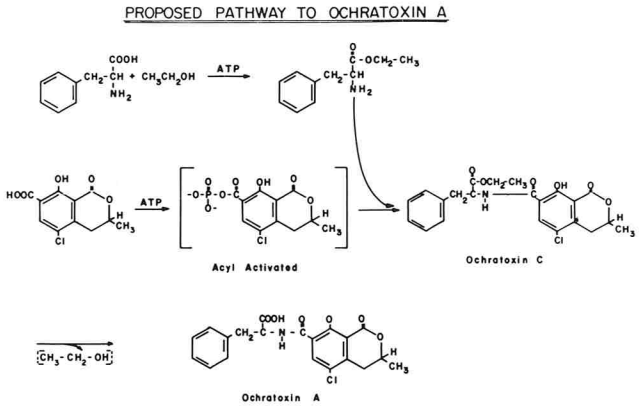



Figure 12. *Proposed ochratoxin synthetase reactions.*

A proposed scheme for the biosynthesis of ochratoxins is summarized in Fig. 13. Briefly, the scheme envisions the formation of an unbranched pentaketide from acetyl and malonyl CoA, its cyclization and aromatization to an isocoumarin, methylation, and oxidation to the carboxy derivative which is chlorinated before acyl activation for reaction with an ester of phenylalanine which arises via the shikimic acid pathway. Ochratoxin A is considered the terminal biosynthetic product. Practically none of the steps have been demonstrated satisfactorily despite the assurance with which they are depicted. The compounds in dashed brackets have not been reported to occur naturally and the compounds in solid brackets would be expected to occur on the enzyme surface and not be liberated as free intermediates. It should be noted that the scheme as presented does not include 4-hydroxymellein or 4-hydroxyochratoxin A which have been isolated from ochratoxigenic fungi (*5,13,18*). However, the

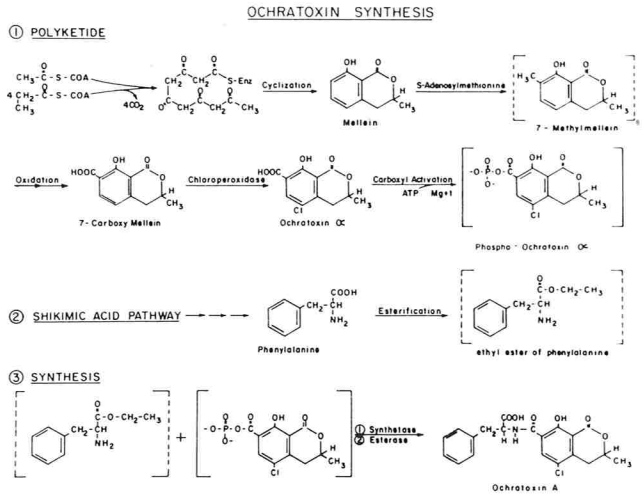



Figure 13. *A proposed general scheme for the biosynthesis of ochratoxins.*

scheme is sufficiently flexible to incorporate formation of an additional hydroxy group during or after cyclization which would remain intact during the ochratoxin synthetase reactions. Despite its weaknesses, the scheme appears to explain and accommodate known facts and compounds, and it relies heavily on analogy to well-known chemical and enzymatic reactions. Hopefully, it will suggest some enlightening experiments. If so, it will have served its purpose.

**ACKNOWLEDGMENTS**

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## Drug-Resistant Disease Organisms Are a Growing Health Problem

Disease organisms that show increasing resistance to many drugs are of growing concern to health officials, doctors, and veterinarians.

The widespread use of antibiotics for treating or preventing disease in both humans and animals has contributed to the buildup of drug-resistant strains of bacteria and viruses.

In addition, the low-level use of antibiotics in feeds to promote growth of livestock and poultry has been found to cause some bacteria to develop a resistance that persists long after the antibiotic pressure is discontinued.

A variety of antibiotics have been used in animal feeds during the past 25 years. And they have had a tremendous economic impact on the animal industries by enabling them to produce increasing amounts of high-quality protein on less feed.

But all the gains made in disease control and increased food production have been at the expense of having a growing number of disease-causing organisms resistant to a number of drugs.

"Resistance that is transferred by R-plasmids (cell fragments) is of great concern because there is convincing evidence that multiple drug resistance can be transferred from resistant to susceptible bacteria," according to Dr. L. C. Grumbles, with the College of Veterinary Medicine at Texas A&M University.

"Antibacterials, primarily antibiotics and sulfonamides, have been used at relatively low levels for 25 years in livestock and poultry feed," says Grumbles who has conducted extensive veterinary research for the Texas Agricultural Experiment Station.

"They have been used to increase the rate of growth and reduce both disease and the total feed required per pound of meat produced.

"It has been estimated that the use of antibiotics in livestock and poultry production has an annual value of $3,200 million. In the U.S. alone the value to the U.S. consumer was estimated at $2,100 million.

"If antibiotics could not be used in feed, it is further estimated that 103 million bushels of corn equivalent or an additional 2 million acres would be required to maintain beef and pork production, based on 1975 average yields."

So, it's easy to see that such use has resulted in larger supplies of meat at lower prices than would have been possible without antibiotics.

And, thus far, there have been very few incidents in which danger to human health has resulted from bacterial resistance in animals.

Following the ban in the United Kingdom on the use of antibiotics in feed, scientists found there was little change in numbers of resistant bacteria because they had established an ability to compete with the sensitive ones.

One scientist who studied the matter concluded that the responsibility for the spread of resistance in human clinical medicine rests primarily with those who are using antibiotics in clinical practice, not the feed industry.

Nevertheless, in the U.S. there is widespread concern about resistance in organisms such as *Staphylococcus aureus*. Whatever the origin, this organism is present on the skin of many animals and may cause diseases such as mastitis in dairy cattle and arthritis in poultry.

In humans, infections with drug resistant staphylococci are invariably serious and frequently fatal. There is mounting evidence that people who work in contact with animals are often carriers of this drug-resistant organism.

And there is also evidence that certain types of hospital staphylococci originated in animals that had received penicillin or tetracycline for treatment of some infectious process.

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# Mycotoxins - Their Biosynthesis in Fungi: Zearalenone Biosynthesis[1]

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**ABSTRACT**

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone] is biosynthesized by *Fusarium roseum* by head-to-tail condensation of acetate units via the acetate-malonyl-CoA (polyketide) pathway. Incorporation and distribution of 1-[$^{14}$C]acetate into zearalenone was demonstrated by degradation of the molecule into the aromatic ring, $CO_2$, oxalic, succinic and glutaric acids. Incorporation and distribution of 1-[$^{13}$C]acetate and 1,2-[$^{13}$C]acetate was demonstrated by $^{13}$C-NMR and confirmed the conclusion reached by $^{14}$C-incorporation studies. $CO_2$ is rapidly fixed by cultures of *F. roseum*, presumably by phosphoenolpyruvate carboxykinase and pyruvate carboxylase and incorporated into zearalenone. The wild-type isolates of *F. roseum* normally produce optimum amounts of zearalenone on a solid medium such as rice and at lower temperatures (10-14 C), although mutants are available that produce copious amounts in liquid medium. Some wild-type isolates also produce zearalenone optimally at 20-25 C.

Zearalenone is a sex hormone produced by various species of *Fusarium*, the most notable of which is *Fusarium roseum* (Link emend) Sny. & Hans. 'Graminearum'. This secondary metabolite became an object of intensive study when Stob et al. (*9*) and Christensen et al. (*1*) reported finding this mycotoxin responsible for causing the hyperestrogenic syndrome in swine. Since then Urry et al. (*10*) have chemically identified the molecule as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β resorcylic acid lactone (Fig. 1).

Zearalenone fluoresces a blue-green at about 450 nm when irradiated with ultraviolet light, which allows easy detection of this substance by thin-layer chromatography. It is also easily detected by gas-liquid chromatography, high-pressure liquid chromatography and mass spectroscopy. Further, there are two isomers of zearalenone, i.e. *cis* and *trans*. The *trans* isomer is naturally occurring and can be easily identified by its infrared spectrum because of absorbance at 980 cm$^{-1}$, representing the trans double bond. Although the *cis* isomer does not occur naturally, it is more active biologically than the *trans* in inciting a uterotropic response in the rat (*6*).

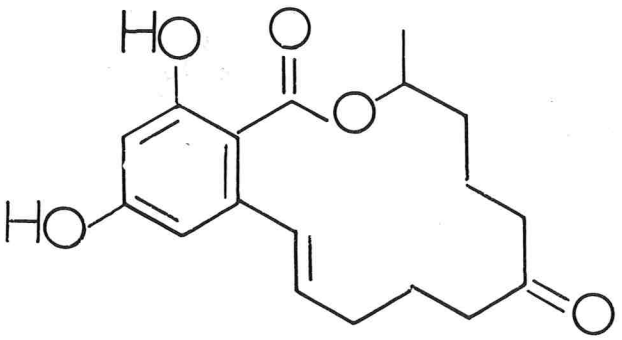


Figure 1. *Chemical structure of zearalenone: 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid μ-lactone.*

Mycotoxicologists are normally impressed with the fact that zearalenone is an estrogen and causes infertility in animals, particularly swine. Microbiologists are equally impressed, but in addition emphasize the fact that zearalenone regulates production of perithecia in *Fusarium*. Unlike the other mycotoxins mentioned in this symposium, zearalenone does play a known role in the fungus that produces it and that is the regulation of the sexual stage. Wolf and Mirocha (*11*) were the first to demonstrate the perithecia-regulating role of this hormone in *F. roseum*.

Close inspection of the structure of zearalenone suggests a head-to-tail condensation of acetate units as all even-numbered carbons are so arranged as to have a hydroxyl or ketone group corresponding to the $C_1$ carbon of acetate. This molecule is not a steroid although its large undecenyl side chain can be folded or manipulated to mimic conformation of a steroid molecule.

A few comments about the biosynthetic production of zearalenone. The isolates normally obtained from nature (wild-type) usually produce maximum amounts of zearalenone when grown at lower temperatures, i.e. 10-14 C. However, there are some isolates that produce copious amounts of zearalenone at room temperature. Acquisition of such isolates is desirable because of the ease with which the molecule can be synthesized with only a room-temperature requirement. However, we have a real problem in production because most isolates only produce maximum amounts of zearalenone when grown on a solid medium such as rice or corn but not in a defined liquid medium (*2*). However, to study biosynthesis of zearalenone, a solid substance is perhaps the better choice because in nature the organism normally produces zearalenone on solids. To our knowledge, the wild type does not produce any appreciable amount of zearalenone in liquid culture.

Results of biosynthetic studies reported here were derived from experiments conducted on a solid rice

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substrate. The moisture content was maintained between 40-50% on a fresh-weight basis. The isolate used was *F. roseum* 'Graminearum' (Isolate Mapletone No. 10). This isolate was sensitive to excess moisture for production of zearalenone in that a final water content in the rice of 60% after autoclaving reduced the yield of zearalenone. The fungus was grown in Boston Brown bottles (1 liter) at 14 C until the mycelium completely overgrew the rice kernels, causing formation of a solid mycelial mass cylindrical in form. The mass of mycelium and rice grains was then sliced into 1 cm × 10-cm discs, placed into sterile petri plates and incubated with various radioactive substrates.

Lieberman (*5*) first initiated the study of zearalenone biosynthesis by empirically selecting various radioactive precursors and studying their incorporation. He selected [1-$^{14}$C]seneciotic acid ($\beta$-methyl-crotonic acid), DL-[2-$^{14}$C]mevalonic acid, [G-$^{14}$C]shikimic acid, [2-$^{14}$C]malonic acid, and [1-$^{14}$C]acetic acid and found that only the latter two (as expected) were rapidly incorporated. In a detailed study of 1-[$^{14}$C]acetate incorporation, Lieberman (*5*) found a significant incorporation into zearalenone within 1 min and an almost linear incorporation up to 60 min (Fig. 2). Malonic acid competed with acetate (as it should) for incorporation into zearalenone. Malonic acid was introduced into the medium as diethylmalonate and was incubated simultaneously with 1-[$^{14}$C]acetate. This incorporation of radio-active precursor-product relationship confirmed the original hypothesis that zearalenone

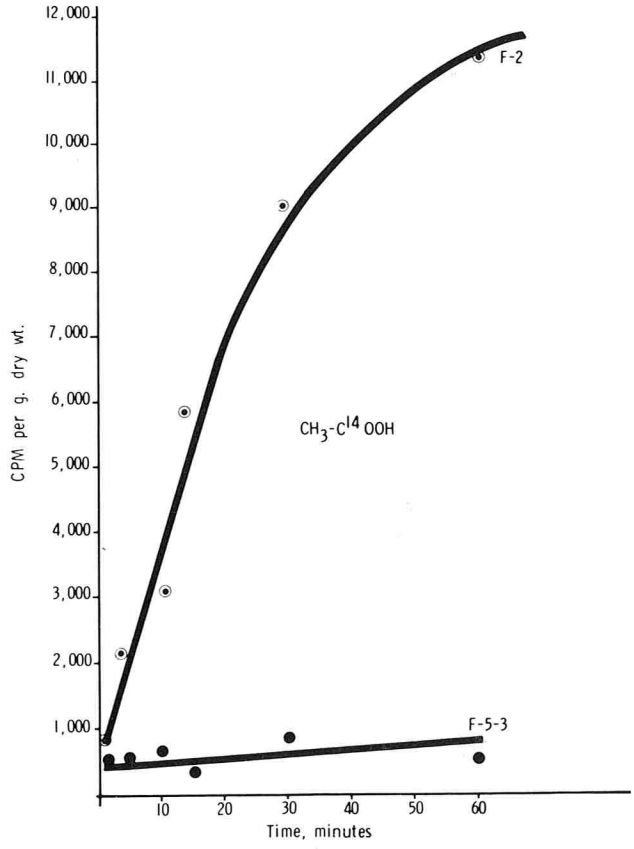


Figure 2. *Incorporation of 1-[$^{14}$C]acetate into zearalenone when measured for periods of 1, 5, 10, 15, 30 and 60 min.*

is biosynthesized via a head-to-tail condensation of acetate units or through the acetate-malonyl-Coenzyme A (polyketide) pathway of metabolism.

Steele et al. (*8*) added additional proof of acetate incorporation into zearalenone by degrading the radioactive molecule formed from 1-[$^{14}$C]acetate and studying the pattern of $^{14}$C incorporation. The molecule was degraded with NaOH and $HNO_3$ and the fragments which resulted were studied (Fig. 3).

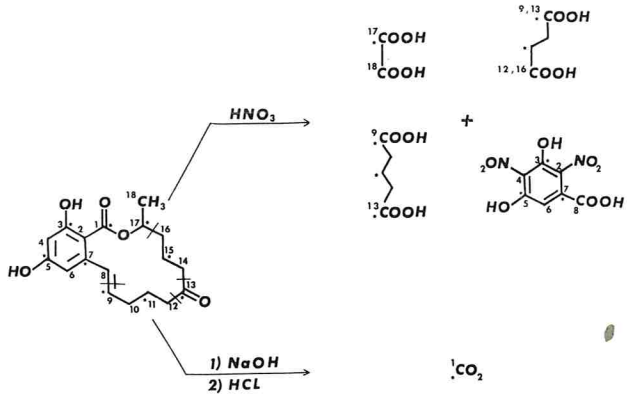


Figure 3. *Theoretical scheme of chemical degradation of zearalenone after treatment with base and $HNO_3$. The asterisk marks the location of $^{14}$C-labeled carbon derived from 1-[$^{14}$C]acetate provided that biosynthesis took place via the acetate-malonate pathway. (After Steele et al., 8).*

Theoretically, carbon dioxide derived from the lactone carbonyl should contain one-ninth of the total activity and have a relative molar activity (RMA) of one; 0.97 was recovered. Various combinations of cleavages yielded oxalic, succinic and glutaric acids. Glutaric acid arises from carbons 9-13 and 13-17 and should have three-ninths of the activity (RMS = 3); 3.11 was actually obtained. Succinic acid arises from carbons 9-13 or 12-16 and should have two-ninths (RMS = 2) of the total activity; 1.85 was obtained. Oxalic acid can arise from many sources including the aromatic ring, but it must arise from one even-numbered and one odd-numbered carbon and thus have a RMA of one; 0.74 was obtained. The methyl ester of oxalic acid (derivative isolated and measured) is volatile and some was lost during isolation, explaining the low molar activity value.

Regardless, any glutaric acid isolated from the degradation should have three of the five carbons labeled; succinic acid should have two of the four labeled; oxalic acid should be labeled. Zearalenone has 18 carbon atoms and if derived from 1-[$^{14}$C]acetate, it should have an RMA of nine. The aromatic ring did not lend itself towards isotope analysis and thus its value was obtained mathematically.

Final proof of isotope distribution in zearalenone was obtained from the $^{13}$C-NMR spectral study involving incorporation of [1-$^{13}$C] and [1,2-$^{13}$C]acetates (*7*).

The comparison was made with the $^{13}$C-NMR spectra of zearalenone with natural $^{13}$C abundance and that of enriched material. Table 1 shows the enrichment ratio of zearalenone derived from [1-$^{13}$C]acetate. It is seen that all

TABLE 1. *Percent $^{13}C$-enrichment of the carbons of zearalenone derived from [1-$^{13}C$]acetate.*

| Position | δ C (ppm from TMS) | Normalized intensities[1] (mm) Natural abundance a | [1-$^{13}C$]acetate derived (b) | % Enrichment 1.1 (b-a)/a |
|---|---|---|---|---|
| C-1 | 103.8 | — | — | |
| C-2 | 166.7 | 12.9 | 29.2 | 1.4 |
| C-3 | 102.9 | 104.8 | 123.6 | |
| C-4 | 163.6 | 16.9 | 46.1 | 1.9 |
| C-5 | 109.5 | 125.0 | 115.0 | |
| C-6 | 144.8 | 8.0 | 13.0 | 0.8 |
| C-1′ | 134.2 | 154.8 | 143.8 | |
| C-2′ | 133.2 | 116.9 | 382.1 | 1.5 |
| C-3′ | 31.8 | 169.4 | 173.0 | |
| C-4′ | 22.9 | 102.4 | 247.2 | 1.5 |
| C-5′ | 43.5 | 166.9 | 178.7 | |
| C-6′ | 210.6 | 23.4 | 38.2 | 0.7 |
| C-7′ | 36.9 | 162.9 | 176.4 | |
| C-8′ | 21.7 | 102.4 | 212.4 | 1.2 |
| C-9′ | 35.5 | 133.9 | 123.6 | |
| C-10′ | 74.4 | 138.7 | 284.3 | 1.1 |
| C-11′ | 21.0 | 100.0 | 100.0 | |
| C-12′ | 172.6 | 16.9 | 23.6 | 0.4 |

[1]Intensities are normalized to C-11′.

the even-numbered carbons (those derived from the C-1 carbon of acetate) have a high enrichment ratio whereas those derived from the C-2 of acetate (odd numbers) do not. Nine alternate (even numbered) carbon atoms are enriched almost uniformly and thus confirm the results obtained from $^{14}C$-degradation studies. Moreover, the $^{13}C$-NMR study also confirmed the presence of enrichment of carbons 2, 4 and 6 of the aromatic ring, which was not possible in the $^{14}C$ study.

Two alternative methods of biosynthesis of zearalenone are possible, one being derivation through head-to-tail condensation of nine acetate units (Fig. 4, pathway A) and the other through the octaketide intermediate (derived from 8 acetate units) with $CO_2$ being incorporated by direct carboxylation and the C-11 methyl group obtained from a methyl donor such as methionine (Fig. 4, pathway B). If the latter was true, [1,2-$^{13}C$]acetate experiments should show that C-10 is labeled but not C-11. Conversely, if C-10 and C-11 were obtained from an intact acetate group, they should come from the same molecule of acetate and both contain a labeled carbon from 1,2-[$^{13}C$]acetate. A comparison of the $^{13}C$-NMR spectra was made between single and double-labeled $^{13}C$-acetate-derived zearalenone which indicated that both C-10 and C-11 have doublets or side bands arising from adjacent $^{13}C$-$^{13}C$ coupling (Fig. 5A and B). Similarly, carbons 4' and 8' as well as the lactone carbonyl (C-12') were associated with side bands. Thus it appears to be clear that the entire zearalenone molecule is derived from intact acetate units by the conventional head-to-tail method of condensation.

Wolf et al. (*11*) demonstrated that the biosynthesis of zearalenone by *Fusarium* is inhibited by Dichlorvos (dichlorovinyl dimethylphosphate) without appreciably affecting growth of the mycelium. Dichlorvos inhibits the synthesis of aflatoxin by *Aspergillus flavus* in a similar manner as both metabolites originate from a polyketide pathway of biosynthesis. However, whether or not any

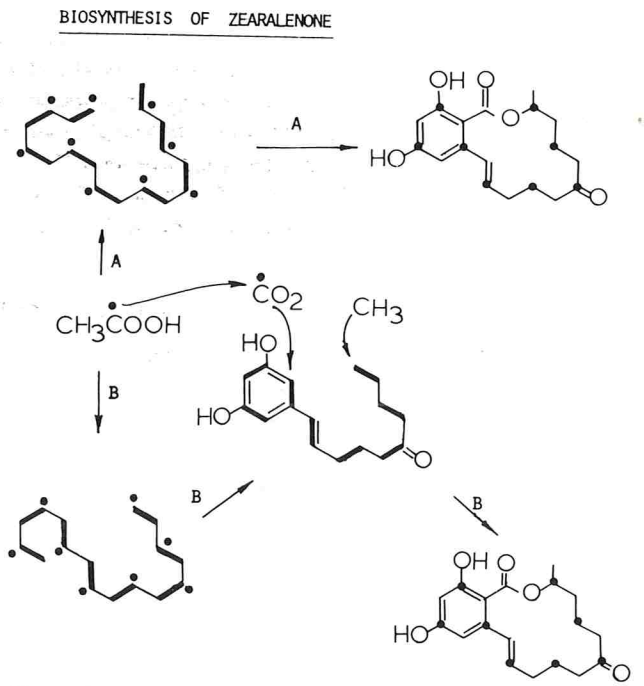


Figure 4. *(A) - Biosynthesis of zearalenone through the condensation of 9 acetate units. (B) - Biosynthesis through the octaketide intermediate derived from 8 acetate units and direct carboxylation at C-12′ and methylation of C-11′.*

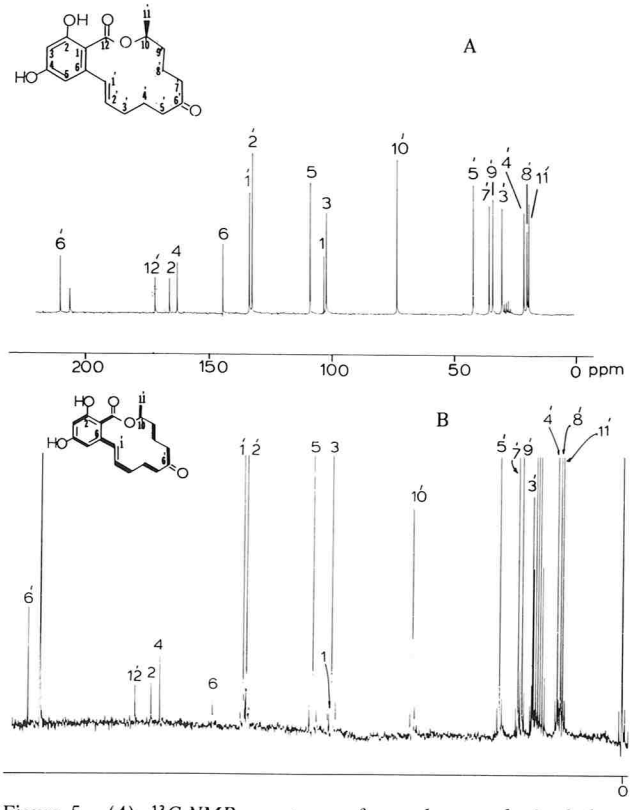


Figure 5. *(A) - $^{13}C$-NMR spectrum of zearalenone obtained from 1[$^{13}C$]acetate. (B) - $^{13}C$-NMR spectrum of zearalenone obtained from 1,2-[$^{13}C$]acetate. Note doublets at carbons 10, 11, 8 and 4, not present in a single labeled zearalenone shown in A.*

particular step in zearalenone biosynthesis is inhibited as in aflatoxin is not known.

Wolf et al. (*11*) reasoned that if zearalenone controls the formation of perithecia, and dichlorvos inhibits

zearalenone synthesis, then dichlorvos should also inhibit perithecia production. When dichlorvos was added to young cultures of *F. roseum*, as predicted, perithecia formation was inhibited. However, when dichlorvos and zearalenone were added simultaneously to cultures of *Fusarium*, the inhibition caused by dichlorvos in the absence of zearalenone was prevented.

## INCORPORATION OF $CO_2$ INTO ZEARALENONE

Lieberman (*5*) also found that $^{14}CO_2$ was rapidly fixed and incorporated (within 3 min) into zearalenone. In other experiments where $^{13}CO_2$ was used, one of the products of fixation was malic acid. Analysis by mass spectroscopy showed a 17% incorporation of $^{13}CO_2$ into malate via carbon 4 of malic acid (Fig. 6).

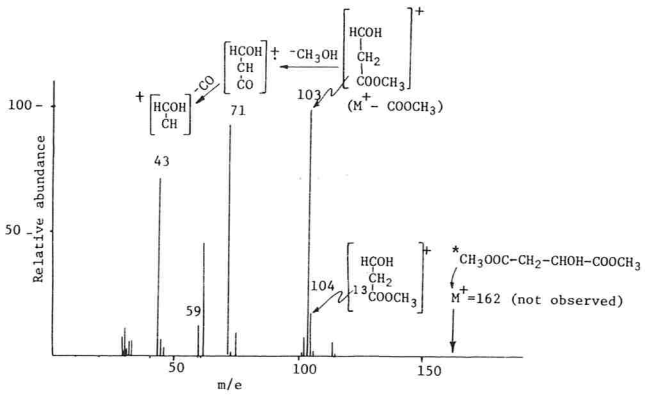


Figure 6. *Mass spectrum of $^{13}C$-enriched dimethylmalate obtained from a zearalenone-producing culture of* Fusarium roseum. *Carbon 1 of dimethylmalate is lost immediately, leaving m/e + 103 which contains the fixed $^{13}CO_2$ on carbon 4. The analysis was made on a combination gas chromatograph-mass spectrometer (LKB-9000). The dimethyl derivative of malic acid was used for analysis.*

*Fusarium culmorum* is capable of fixing $CO_2$ via the enzymes phosphoenolpyruvate carboxykinase and pyruvate carboxylase as demonstrated by Larmour and Marchant (*4*). Carbon dioxide fixation by fungi is a common phenomenon and the amount of $CO_2$ fixed is appreciable. *F. roseum*, like *F. culmorum*, also fixes $CO_2$ presumably through the same enzyme systems. Both of these enzymes fix $CO_2$ directly into oxalacetic acid. After fixation into oxalacetic acid, we speculate that, based on other plant systems, the molecule is rapidly converted into malic acid in a reversible mode by the enzyme malic dehydrogenase. Malic acid can be decarboxylated into $CO_2$ and acetate via the malic enzyme and then find its way into zearalenone via the polyketide pathway.

## PREPARATION OF $^{14}C$-LABELED ZEARALENONE WITH HIGH SPECIFIC ACTIVITY

One of the tests of any biosynthetic scheme is the preparation of radiolabeled product with sufficient specific activity to conduct metabolic studies. Hagler and Mirocha (*3*), using cultures of *F. roseum* 'Gibbosum' growing on rice amended with 4 mCi of 1-[$^{14}C$]acetate, produced 1.5 g of zearalenone with a specific activity of 46.5 $\mu$Ci/mmole. One limiting factor in this effort was the fact that a solid substrate had to be used which precluded adequate distribution of acetate throughout the culture as one would obtain in a liquid medium.

### ACKNOWLEDGMENT

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# Mycotoxins - Their Biosynthesis in Fungi: Biosynthesis of the Trichothecenes

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**ABSTRACT**

The biosynthetic pathway as currently postulated for trichothecin, a model 12,13-epoxy-$\Delta^9$-trichothecene, involves the following sequence: 3 mevalonic acids → farnesyl pyrophosphate → trichodiene → trichodiol → 12,13-epoxytrichothec-9-ene → trichodermol → 12,13-epoxy,4$\beta$, 8$\alpha$-dihydroxy-trichothec-9-ene → trichothecolone → trichothecin.

The trichothecenes are a group of closely related sesquiterpenoid mycotoxins with cytotoxic, phytotoxic, antifungal, and insecticidal activity. Currently, there are almost 40 of these compounds known that occur naturally. All possess a 12,13-epoxy-$\Delta^9$-trichothecene nucleus named after the first member of the group to be isolated--trichothecin. Trichothecenes are produced by a number of genera, but of most practical importance are those synthesized by the various *Fusarium* spp. and *Stachybotrys atra*. The structure, stereochemistry, and numbering system of trichothecin, an ester of isocrotonic acid, are shown in Fig. 1.

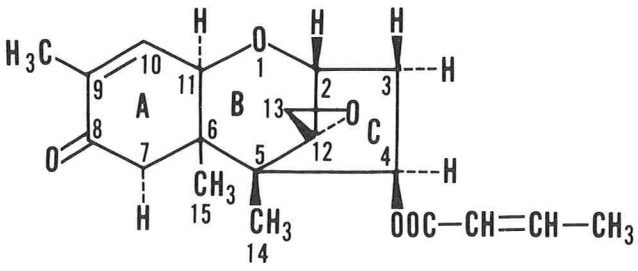

Figure 1. *Structure of trichothecin.*

It has been well established that the trichothecene skeleton is formed from three molecules of mevalonate via the usual pathway of lipid biosynthesis involving isopentenyl, geranyl, and farnesyl pyrophosphate (*2,3,6, 10,11,16,17,21-23,26*). The open chain farnesyl skeleton cyclizes to form the parent hydrocarbon of the trichothecene series, trichodiene (Fig. 2). In farnesyl pyrophosphate the configuration about the double bond at C6,7 may be cis or trans, which could result in two possible configurations on the initial folding of the molecule (Fig. 3). Achillidelis et al. (*1,2*) used two approaches to resolve this important problem. In feeding experiments with *Trichoderma sporulosum* and *Trichothecium roseum*, doubly labeled mevalonates of known $^3H$:$^{14}C$ ratio were employed, and the ratio of the two isotopes were determined in the metabolites and in their degradation products. From these data they could determine the number and location of the mevalonoid hydrogen atoms incorporated, thereby deducing information on the post-farnesyl pyrophosphate step. In the second approach, specifically labeled geranyl and farnesyl pyrophosphate were employed, thereby determining if a specific hydrogen or carbon atom originated in the distal, central, or terminal prenyl unit. This is illustrated in Fig. 3 where C-1,2 of farnesyl pyrophosphate were $^{14}C$ labeled. If the folding were cis, labels at C-1,2 would wind up at C-7,8 in the trichothecene nucleus; whereas if trans, labels would be found at C10,11. Achilladelis et al. showed experimentally that labeling occurred at C10,11, supporting the trans configuration of farnesyl pyrophosphate (Fig. 3).

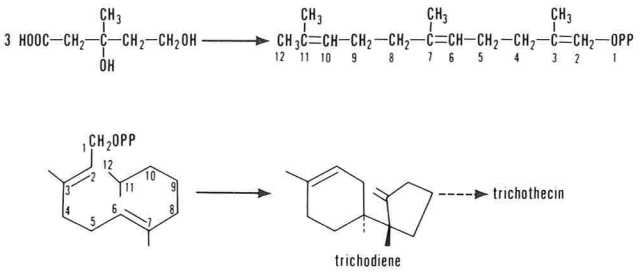


Figure 2. *General scheme for the biosynthesis of trichothecin.*

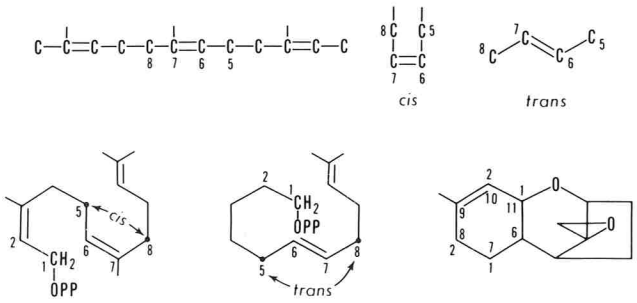


Figure 3. *The initial folding of farnesyl pyrophosphate.*

The structure of trichodiene (Fig. 4) was established by Nozoe and Machida (*24*) who postulated that it represented the biosynthetic precursor of the trichothecenes. Evans et al. (*13*) and Evans and Hanson (*12*) showed that to form the six-membered ring of

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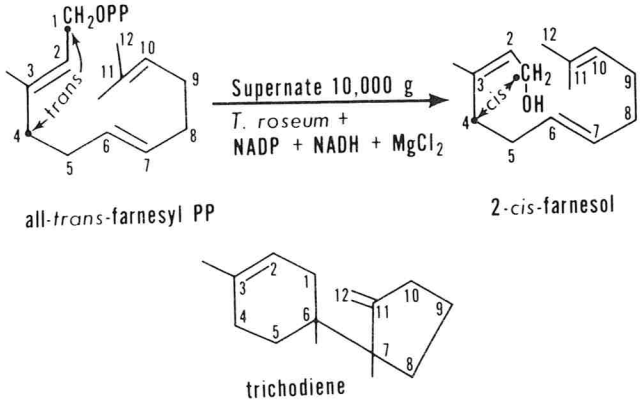



Figure 4. *Cyclization of farnesyl pyrophosphate to trichodiene.*

trichodiene, all-trans-farnesyl pyrophosphate must first be converted into a 2-cis-farnesyl unit. They used a cell-free system from 3-day-old shake cultures of *T. roseum* supplemented with reduced pyridine nucleotides to carry out the conversion (Fig. 4).

The sequence between farnesyl and trichothecin was confused until recently because what appeared to be an attractive intermediate, bis-aboline, had been proposed. However, labeling experiments of Achilladelis et al. (*2*) and the work of Nozoe and Machida (*24,25*), Machida and Nozoe (*18*), and Forrester and Money (*15*) eliminated this possibility. Instead, it was suggested that the cyclization of 2-cis-trans-farnesyl pyrophosphate may be concerted, with attack of an enzyme at C-10 initiating cyclization (Fig. 5). In the concerted cyclization sequence, a hydrogen transfer occurs from $C_6$ to $C_{10}$ causing removal of the enzyme and also possibily initiating a methyl group rearrangement to trichodiene.

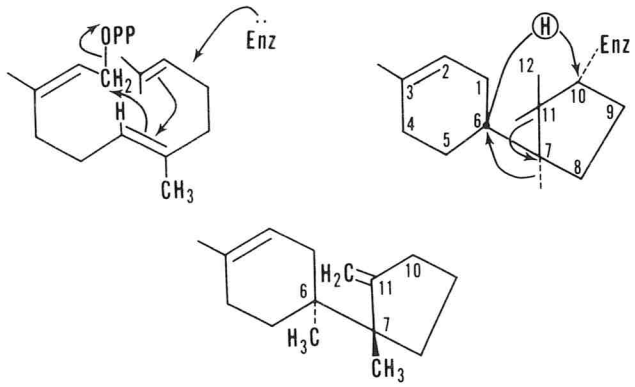


Figure 5. *Mechanism of cyclization of farnesyl pyrophosphate to trichodiene.*

Achilladelis and his colleagues (*2*) could explain the final structure of trichodiene by postulating two 1,2-methyl shifts. A double methyl group shift had previously been postulated to occur by Jones and Lowe (*17*) in their studies on the biosynthesis of trichothecin. In an earlier study, two 1,2-methyl shifts were demonstrated in the cyclization of squalene to lanosterol (*9,20*).

In 1970, Nozoe's group in Japan isolated from *T. roseum* a number of new trichothecene-type compounds that were of obvious biogenetic significance. Among these were trichodiene and trichodiol. The mechanism of formation of trichodiene to trichodiol has not been established, although it would be tempting to suggest hydroperoxide formation at C-11 as a fleeting intermediate (Fig. 6). The relationship between trichodiol and the more elaborate derivatives is clear since trichodiol needs only to cyclize at C-11 to give the trichothecene nucleus. Again, the mechanism of formation of the pyran nucleus has not been determined, and these experiments need to be done.

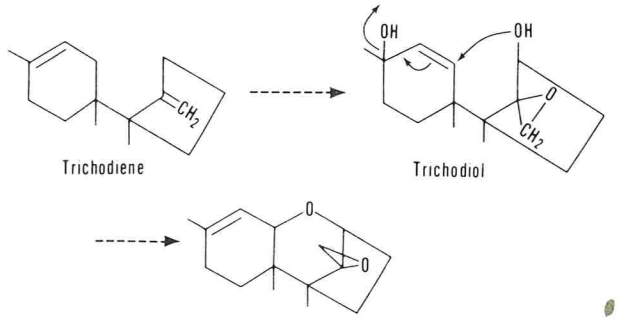



Figure 6. *Possible scheme for the biosynthesis of the trichothecene ring system.*

The carbonyl moiety at C-8 in trichothecin can be postulated to arise by a number of schemes (*14*). The most complex was that the C-8 and C-11 oxygen functions were related and that they arose by rearrangement of a $\Delta^9$-8,11-epidioxide, itself formed from a ring A diene. There are precedents for this in a related series of compounds, the cuprenenes. A second hypothesis involved a 7,8 epoxide, such as found in crotocin, a trichothecene which co-occurs with trichothecin in *T. roseum*. A third proposal encompasses simple hydroxylation at C-8 followed by oxidation. These three proposals are summarized in Fig. 7.

Scheme 1 (Fig. 7) involving the epidioxide is ruled out because trichodermol, which lacks a C-8 carbonyl group but does have a -OH at C-4, is readily transformed to trichothecin by *T. roseum* (Fig. 8). This probably excludes oxygenation at C-8 before formation of the trichothecene skeleton and also shows that once the skeleton is formed, oxidation at C-4 precedes that at C-8.

In formation of a 7,8-epoxide as in crotocin (Fig. 7), hydrogen atoms would be removed from C-7 and C-8.

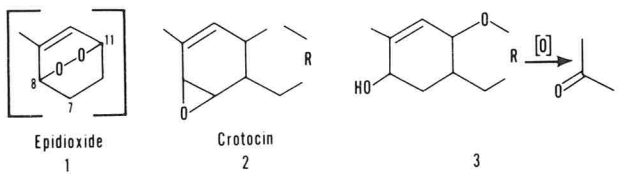



Figure 7. *Formation of a keto-moiety at carbon-8 in the trichothecene ring system.*

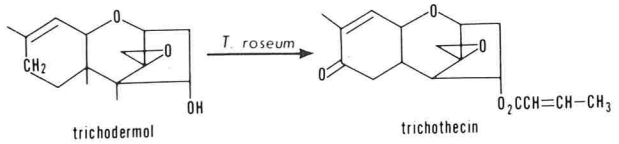



Figure 8. *Bioconversion of trichodermol to trichothecin.*

During the subsequent isomerization of the epoxide to form the carbonyl group, a C-8 hydrogen atom originating from C-2 of mevalonate would migrate to C-7 (Fig. 9). However, labeling data do not support this hypothesis.

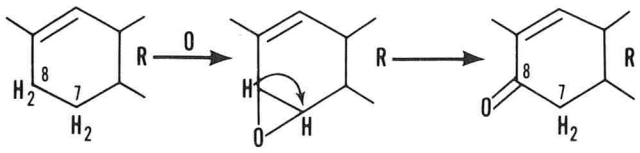


Figure 9. *Mechanism of epoxidation at carbon-8.*

The third route to trichothecin involves hydroxylation of trichodermol at C-8 followed by oxidation. Incubation of 12,13-epoxy-4$\beta$,8$a$-dihydroxytrichothec-9-ene with *T. roseum* results in formation of trichothecolone with a 6.6% incorporation (*14*). Trichothecolone, which co-occurs with trichothecin in *T. roseum*, was converted into trichothecin by the fungus in 27% yield. Therefore, esterification is probably one of the last stages of biosynthesis.

In summary, the biosynthetic pathway as currently known for trichothecin involves the following sequence:

3 mevalonic acids → farnesyl pyrophosphate → trichodiene → trichodiol → 12,13-epoxytrichothec-9-ene → trichodermol → 12,13-epoxy-4$\beta$,8$a$-dihydroxytrichothec-9-ene → trichothecolone → trichothecin (Fig. 10).

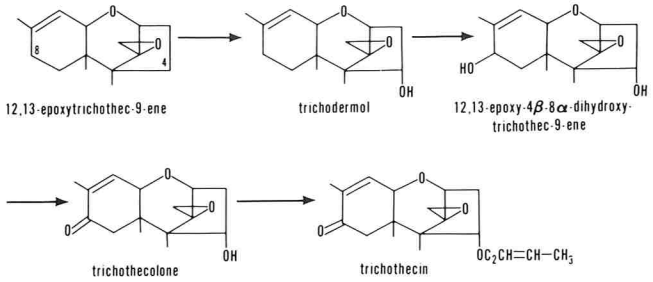



Figure 10. *Biosynthesis of trichothecin.*

The biosynthesis of the verrucarins and roridins have been investigated extensively at Tamm's laboratory in Basel and his publications should be consulted for detail (*4,5,8,21-23*). The di- and triester macrocyclic moieties of the more complex verrucarins and roridins have been shown by these investigators to be derived from mevalonic acid. Verrucarol appears to be the sesquiterpene common to both the verrucarins and roridins and is obtained on base hydrolysis of the parent compounds (*21,22*) (Fig. 11).

What has been outlined up to this point presents a relatively satisfying explanation for the general biosynthesis of the 12,13-epoxytrichothecenes. However, a paper published recently by Breitenstein and Tamm (*7*) has raised a possible question. These investigators isolated a new metabolite in low yield, verrucarin K, from *Myrothecium verrucaria* (Fig. 12). This compound represents the first natural trichothecene derivative lacking the 12,13-epoxy group, this moiety being replaced by an exocyclic double bond.

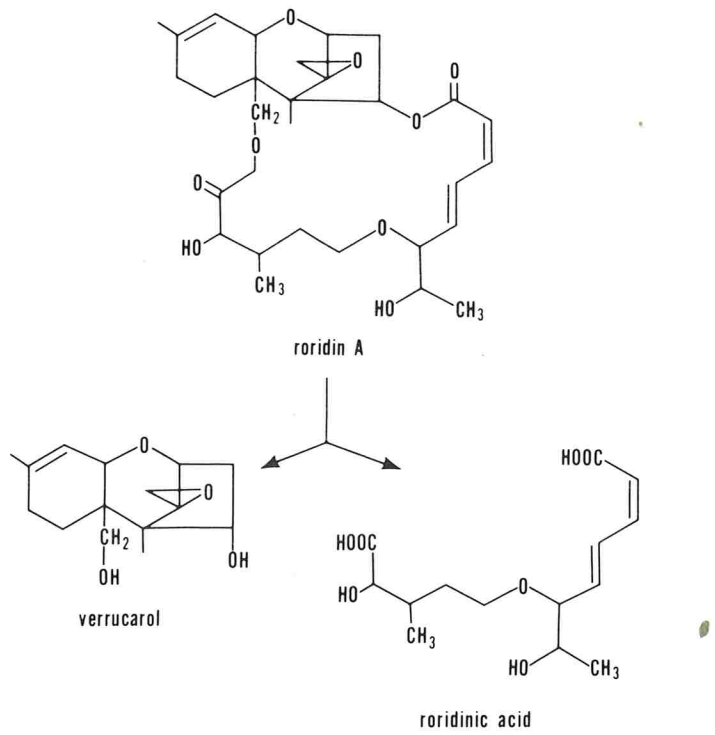



Figure 11. *Structure of roridin A.*

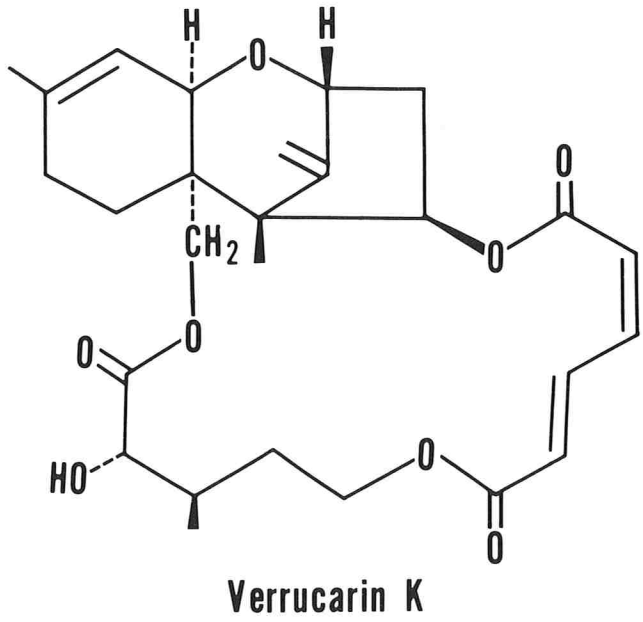



Figure 12. *Structure of verrucarin K.*

If the biogenesis of verrucarin K involves trichodermol as an intermediate as is postulated for all known 12,13-epoxytrichothecenes, then reductive removal of the preformed epoxy function would be required at a later stage. Tamm presented no experimental evidence to preclude this possibility. However, an alternative to the reaction sequence previously shown can be postulated by the direct cyclization of an intermediate, trichodiol, shown in a previous figure to the trichotheca-9,12-diene system (Fig. 13).

A compound very similar to trichodiol (hydroxydienone) was an actual key intermediate for the cyclization

reaction in a biomimetic total synthesis by Masuoka and Kamikawa (*19*) (Fig. 14).

One can also postulate, under the circumstances, that there is possibly more than one road to Rome.

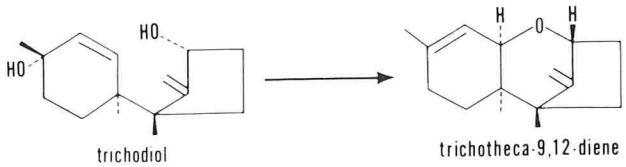
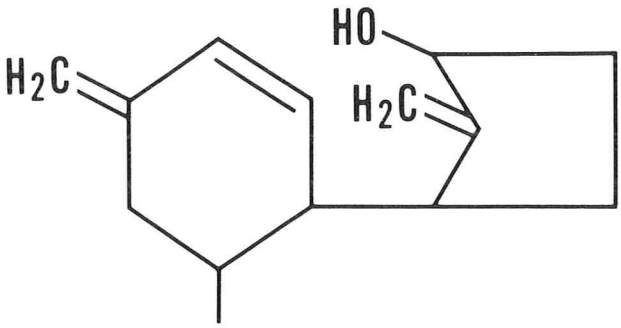



Figure 13. *Direct cyclization of trichodiol.*




hydroxydienone

Figure 14. *Structure of hydroxydienone.*

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# Mycotoxins — Their Biosynthesis in Fungi: Ergot Alkaloids

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**ABSTRACT**

Biosynthesis of ergot alkaloids is discussed from the standpoint of biosynthetic precursors and intermediates as well as known biosynthetic mechanisms. Emphasis is given to work concerning regulation of alkaloid production, including the role of tryptophan as an inducer of alkaloid synthesis. A postulation is proposed to explain the significance of induction in terms of evolution and survival of the organism which also takes into account the finding that the biosynthesis of tryptophan is under regulatory control in the fungal strain which was investigated. Recent studies using protoplasts of ergot have also shown that endproduct regulation of alkaloid synthesis may be a significant phenomenon.

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Mycotoxicoses caused by ingestion of ergotized rye have been known for centuries. The toxic effects in man have usually resulted from eating bread made from rye flour which was contaminated with sclerotia of *Claviceps purpurea* (Fries) Tulasne. From the accounts of ergotism reviewed by Barger (*2*) and Bové (*6*), it is apparent that during the Middle Ages one form of this mycotoxicosis, referred to as St. Anthony's fire or *ignis sacer*, produced vasoconstriction of the peripheral blood vessels, resulting in intense burning and pain in the extremities with eventual dry gangrene followed by the falling away of the affected portions of the body. Another form of the toxicity resulted in more prominent CNS effects which included hallucinations and convulsions. In connection with this, an interesting recent speculation is that the young women accused of being witches in the Salem witchcraft trails of 1692 were actually suffering from the convulsive type of ergotism (*9*).

## MYCOLOGY

Ergot infects a wide range of wild and cultivated grasses; however, rye, *Secale cereale*, is a common host plant. On the plant the fungus forms a sclerotium which may have the same general configuration as the seed but is larger, dark colored, and hard. The sclerotia are the resting or overwintering stage of the fungus and in the spring they germinate, sending up flesh-colored fruiting bodies which rarely exceed 25 mm in length. These fruiting bodies produce the sexual spores (ascospores) which are carried by the wind to florets of grass inflorescences where the germinating ascospore enters the developing ovary of the flower via the stigma. After an incubation period of 2 to 7 days a sweet, sticky exudate called honey-dew is produced from each of the infected flowers. The honey-dew contains conidia and since it attracts insects, the fungus is dispersed to other flowers with resulting fresh infections. At the end of the growing season the production of conidia and the secretion of honey-dew ceases and the sclerotia are formed (*27*).

## THERAPEUTIC USE

The mycotoxins produced by the ergot fungus are alkaloids, some of which have valuable therapeutic applications. The effects which have given ergot alkaloids importance in medicine are related to their ability to stimulate both vascular and non-vascular smooth muscle. Stimulation of the vascular smooth muscle causes constriction of the blood vessels in the vascular bed, and the resulting constriction of intracranial arteries is useful in the treatment of migraine and other types of cluster headache. Ergotamine (Fig. 1) is an agent of choice in the treatment of acute attacks of migraine.

The sensitivity of the smooth muscle of the uterus to stimulation by ergot increases along with the stage of gestation, so the resulting contractions at the end of the third trimester of pregnancy may induce labor. Midwives and physicians have used ergot for this purpose for centuries. At the present time, however, ergot compounds such as ergonovine (Fig. 1) are used only postpartum to produce firm uterine contractions and decrease uterine bleeding. They are also used to control hemorrhage associated with abortion.

An extremely potent hallucinogenic activity is exhibited by lysergic acid diethylamide (LSD). This compound was synthesized by Hofmann and Stoll (*40*) and Hofmann accidently discovered its hallucinogenic activity by inhaling the crystal dust while crystallizing LSD in the laboratory (*26*). LSD can be considered a semi-synthetic compound, since lysergic acid (Fig. 1) is prepared from the naturally occurring alkaloids and then used as starting material for the chemical synthesis. Another drug material which is also semi-synthetic and which has important therapeutic potential is Lergotrile® (Fig. 1). This compound is synthesized using elymoclavine (Fig. 2) as a starting material. Lergotrile® is representative of a group of ergot alkaloids and related compounds that inhibit prolactin release from the anterior pituitary (*17*). The clinical significance of these

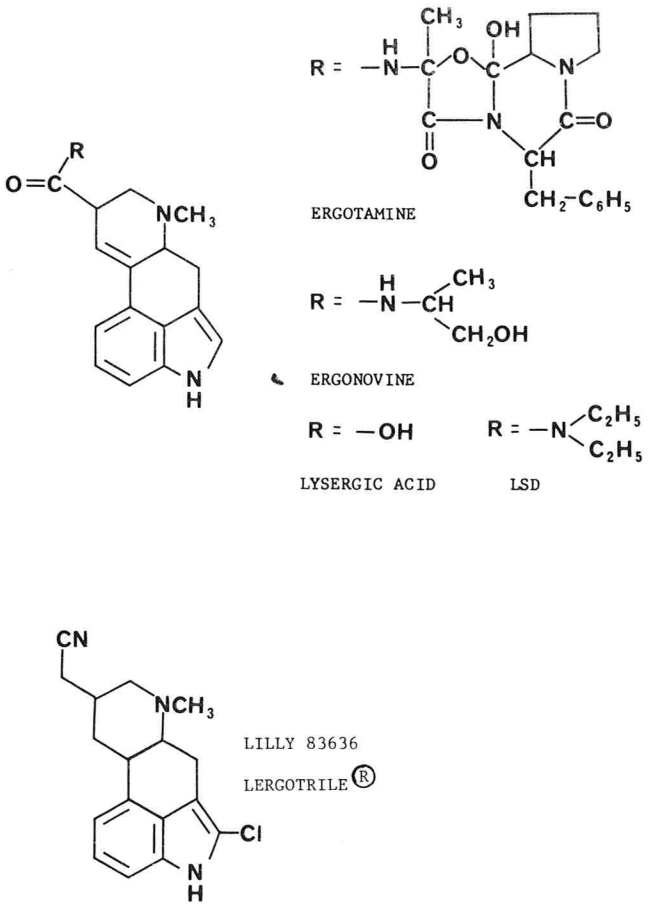


Figure 1. *Examples of ergot compounds of therapeutic importance.*

drugs is still being evaluated but they have potential in the treatment of breast cancer, galactorrhea, amenorrhea, acromegaly and Parkinsonism (*10*).

The commercially important ergot alkaloids are obtained either by isolation from ergot sclerotia which have been produced through field cultivation on rye or by fermentation of the ergot fungus with subsequent chemical alteration of the fermentation products. For example, the more chemically complicated peptide alkaloids such as ergotamine are obtained from sclerotia whereas partial synthesis from lysergic acid is the usual procedure for the production of alkaloids with less complicated side chains such as ergonovine. Most of the lysergic acid used for this purpose is produced by fermentation (*22*).

## BIOSYNTHESIS

The importance of fermentation in the production of ergot alkaloids has been a stimulus for studies to elucidate the biosynthetic pathway leading to these compounds as well as to determine the regulatory mechanisms involved in their formation. The current knowledge on the biosynthetic pathway leading to the tetracyclic ergoline ring system of the ergot alkaloids is summarized in Fig. 2. Floss (*16*) has written a comprehensive review that presents an in-depth analysis of the experimental evidence that supports this

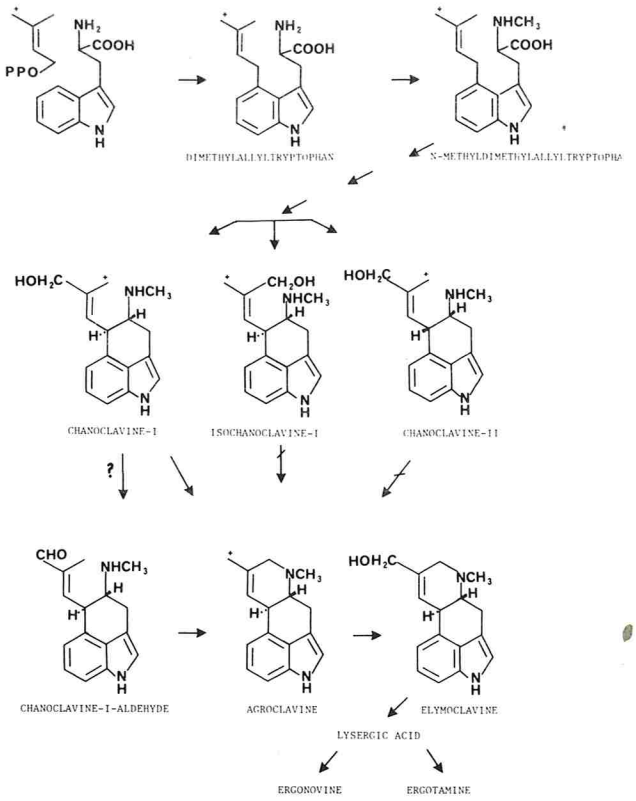


Figure 2. *Ergot alkaloid biosynthetic pathway.*

biosynthetic pathway. Essentially the biosynthetic precursors are tryptophan which was first found to be incorporated into the alkaloids when Mothes et al. (*32*) injected DL-tryptophan-$\beta$-$^{14}$C into the internodes of rye plants which had been inoculated with ergot. Subsequent isolation and hydrolysis of the radioactive alkaloids formed in the mature sclerotia revealed that radioactivity resided in the lysergic acid. Three different groups (*12,24,41*) almost simultaneously demonstrated that mevalonic acid is efficiently incorporated into the ergoline ring system, showing the biosynthetic involvement of an isoprene unit in the form of dimethylallylpyrophosphate. Finally Baxter et al. (*4*) established that methionine supplies the methyl on the N-methyl group.

The first pathway-specific step in the biosynthesis is the isoprenylation of the 4-position of tryptophan to form dimethylallyltryptophan. Plieninger et al. (*35*) showed that radioactively labeled dimethylallyltryptophan prepared synthetically was incorporated into the alkaloids. Other workers were able to isolate dimethylallyltryptophan from ergot cultures in which alkaloid synthesis had been inhibited by exclusion of oxygen (*36*) or by addition of the methionine antagonist ethionine (*1*). Finally, the enzyme, dimethylallyltryptophan (DMAT) synthetase, which catalyzes the isoprenylation reaction, has been isolated from *Claviceps* (*25*).

Very recent evidence indicates that the next intermediate in the pathway arises from the N-methylation of dimethylallyltryptophan to give $N_a$-methyl-4-dimethylallyltryptophan. Barrow and Quigley (*3*) have isolated this compound from *Claviceps*. Also, Otsuka et al. (*34*)

have fed $N_a$-methyl-4-dimethylallyl tryptophan (amino-$^{15}N$, N-$CD_3$) to a culture of *Claviceps* and the mass spectral analysis of the isolated elymoclavine showed the presence of 29% of the molecular ion species enriched with $^{15}N$ and three deuterium atoms. This result shows unequivocally that $N_a$-methyl-4-dimethylallyltryptophan can be converted intact into tetracyclic ergolines. These findings also support earlier work by Fehr (*14*) in which he found that N-demethylchanoclavine-I was not incorporated into agroclavine or elymoclavine, suggesting that N-methylation may occur before C-ring closure on the way to form chanoclavine.

The next anticipated step in the biosynthesis would be the decarboxylation of $N_a$-methyl-4-dimethylallyltryptophan with a subsequent formation of ring C of the chanoclavines. The number of intermediates and enzymatic conversions involved in these events is still unknown; however, the biosynthetic role of the chanoclavines, tricyclic compounds found in the fungus, is known. The stereoisomers of chanoclavine are chanoclavine-I, isochanoclavine-I and chanoclavine-II (Fig. 2). Chanoclavine-I was shown by Gröger et al. (*23*) to be efficiently incorporated into agroclavine, elymoclavine and lysergic acid methylcarbinolamide. In addition these workers demonstrated that chanoclavine-I was incorporated into elymoclavine with higher dilution than into agroclavine, supporting a pathway of chanoclavine-I → agroclavine → elymoclavine. As indicated in Fig. 2 neither chanoclavine-II nor isochanoclavine-I are incorporated into agroclavine or elymoclavine (*15,18*). A possible intermediate between chanoclavine-I and agroclavine is chanoclavine-I-aldehyde. Floss et al. (*21*) have demonstrated a 40% incorporation of the aldehyde into elymoclavine when the aldehyde group was labeled with tritium; however, it has not been possible to demonstrate the occurrence of chanoclavine-I-aldehyde in the fungus.

Concerning the biosynthetic mechanism of alkaloid formation, there is evidence that two isomerizations in the isoprenoid moiety takes place during formation of the tetracyclic ergolines (*19*). Mevalonic-2-$^{14}C$ acid labels the *trans*-carbon atom of dimethylallylpyrophosphate and, as can be seen in Fig. 2, the *trans*-carbon atom of the isoprenoid moiety of agroclavine is also labeled. Rather than this labeling pattern being a result of a biosynthetic mechanism without isomerization it is, instead, a result of a *cis-trans* isomerization going from chanoclavine-I to agroclavine, and experiments with mevalonic acids stereospecifically tritiated at C-4 indicate that another *cis-trans* isomerization occurs earlier in the pathway.

Using both sclerotia and cultures of the ergot fungus, elymoclavine has been shown to be the precursor of lysergic acid derivatives (*33*). Several investigators (*29,31*) have demonstrated that lysergic acid serves as a precursor to the peptide alkaloids and the various peptide side chains of these compounds arise from the respective amino acids.

## INDUCTION

In 1964 it was suggested that tryptophan not only serves as a precursor to the ergot alkaloids but may also act as an inducer of the alkaloid-synthesizing enzymes (*20*). This proposal resulted from observations that tryptophan stimulated alkaloid production if added early during the fermentation period before the onset of alkaloid synthesis, but not when it was added after the start of alkaloid synthesis. It was also demonstrated that methyl analogs of tryptophan which were not alkaloid precursors had a stimulatory effect on alkaloid production. In addition it was found that mycelium grown in the presence of tryptophan or tryptophan analogs retained an ability to produce more alkaloid than the controls even after it had been transferred into fresh culture medium not containing tryptophan or its effectors.

Other evidence supporting the possibility of induction was provided by Bu'Lock and Barr (*8*) when they found that protein synthesis was necessary to maintain alkaloid production. In addition, using tryptophan-supplemented cultures, they found that the second differential of the alkaloid production curve, which would indicate the rate of the appearance and disappearance of an enzyme(s) limiting the rate of alkaloid synthesis, closely paralleled the experimental curve for internal tryptophan concentration, suggesting a direct relationship between the rate of synthesis of this enzyme and the amount of tryptophan within the mycelium. In this regard, we observed an increase in the endogenous free-tryptophan pool before the onset of alkaloid production (*39*).

Other laboratories and our own have also provided evidence supporting an induction of ergot alkaloid synthesis by tryptophan. The stimulation of alkaloid synthesis by addition of tryptophan at the beginning of the growth phase has been observed by Vining (*42*). He found that addition of radioactively labeled L-tryptophan during the period of rapid alkaloid synthesis resulted in an efficient incorporation of radioactivity into alkaloids; however, there was only a slight increase in yield of alkaloid, leading Vining to suggest that tryptophan stimulates alkaloid production through the activity of the alkaloid-synthesizing enzyme system.

Employing a culture medium which did not contain yeast extract and carrying out parallel fermentations with tryptophan and 5-methyltryptophan added at the beginning of the culture period, we (*37*) were able to obtain a greater stimulation of alkaloid production over the control than had previously been reported (*20*). At day 11 in the culture period the tryptophan-containing cultures showed a six-fold increase in alkaloid and the 5-methyl-tryptophan-containing cultures a four-fold increase over the control cultures.

Whereas the methyl-substituted tryptophans used in these earlier works were never as active as tryptophan itself in increasing alkaloid production, we obtained an analog, thiotryptophan [β-(1-benzothien-3-yl)-alanine] which, as seen in Fig. 3, consistently equaled or exceeded

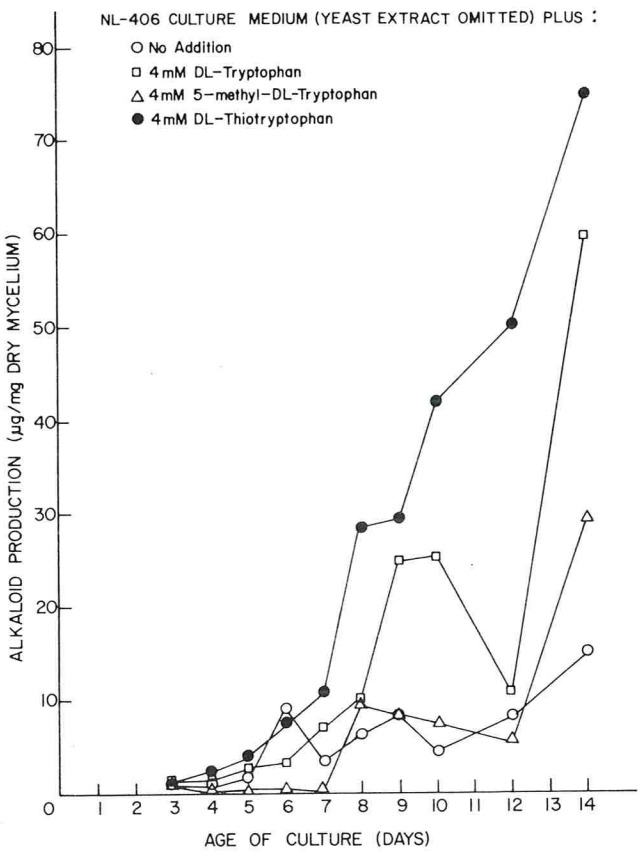


Figure 3. *Induction of alkaloid production in* Claviceps *species, strain SD 58, by tryptophan and its analogs.*

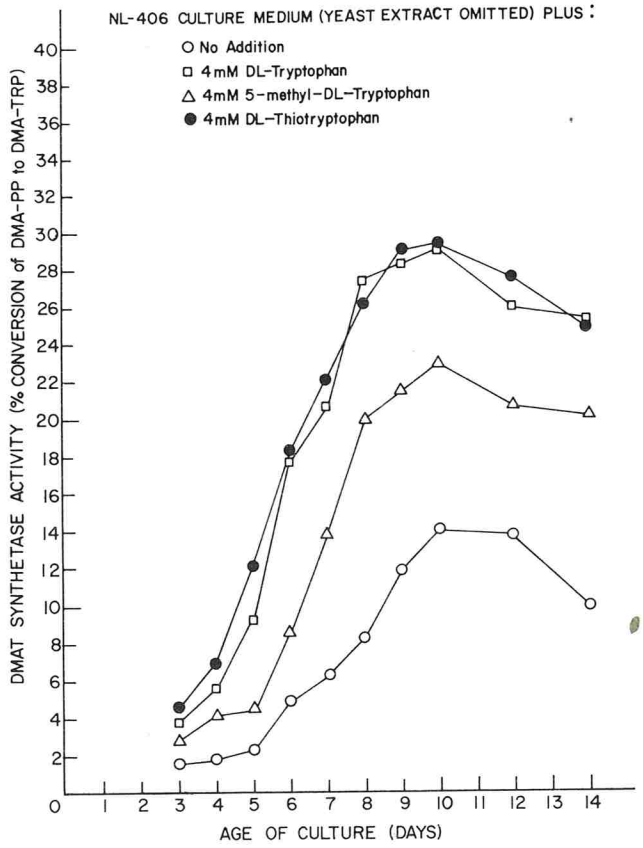


Figure 4. *Time course showing DMAT synthetase activity, based on milligrams of protein, in induced cultures of* Claviceps *species, strain SD 58.*

tryptophan in its ability to increase alkaloid production. Thiotryptophan contains a sulfur atom which substitutes for the indolic nitrogen atom of tryptophan, and experimental evidence indicates that it does not serve as a substrate for the alkaloid-synthesizing enzymes (*28*). Cultures which were induced with tryptophan, 5-methyltryptophan, and thiotryptophan were sampled daily over the course of the fermentation. The alkaloid titer of the cultures was determined and cell-free extracts were prepared from mycelium and assayed for activity levels of the first pathway-specific enzyme for alkaloid synthesis, DMAT synthetase. The results from these assays are illustrated in Fig. 3 and 4 and show a clear parallel between DMAT synthetase levels and alkaloid production leading us to believe that the induction effect involves de novo synthesis of this enzyme (*28*).

To obtain the full induction effect, time-course studies show that tryptophan must be added to cultures during the first 24 h of the fermentation, and that a 12-h exposure period is sufficient for the maximum effect (*38*). The effects, higher enzyme levels and greater alkaloid synthesis, are observed after the end of the active growth phase, which is 4-6 days later. Apparently the gene expression for induction is programmed by tryptophan early in the growth phase but suppressed in some way until later in the culture period. The suppression may be linked to phosphate inhibition, since it is known that in the presence of increased levels of phosphate alkaloid synthesis is almost completely repressed (*12*). Addition of ten times the normal level of inorganic phosphate to normal or thiotryptophan-induced cultures at any time up to day 11 of the fermentation will block any further alkaloid synthesis (*38*); however, high levels of tryptophan can partially overcome the phosphate inhibition of alkaloid synthesis (*28*).

## REGULATION OF TRYPTOPHAN BIOSYNTHESIS

The central role of tryptophan in ergot alkaloid biosynthesis has led us to investigate the degree to which tryptophan biosynthesis is regulated in the fungus. Since effectors of tryptophan such as 5-methyltryptophan usually inhibit tryptophan biosynthesis either through feedback inhibition or repression, the fact that in ergot these compounds stimulate the formation of alkaloids indicates a lack of regulation in tryptophan biosynthesis. As indicated in Table 1, we (*28*) found when we assayed for activity of certain key enzymes of tryptophan biosynthesis in induced cultures that the biosynthesis of tryptophan is under regulatory control in *Claviceps* species, strain SD 58. It appears that thiotryptophan is rather ineffective as a substitute for tryptophan in end-product regulation. As might be expected, tryptophan is the most effective in inhibiting the enzymes, whereas 5-methyltryptophan approaches the ability of the parent compound in controlling tryptophan biosynthesis. These results could also explain why 5-methyltryptophan is consistently much less effective in

stimulating ergot alkaloid synthesis than tryptophan, whereas thiotryptophan is equally as effective or, in some experiments, more effective than the parent compound (Fig. 3). Apparently, 5-methyltryptophan, while triggering formation of alkaloid synthesizing enzymes, limits tryptophan synthesis by false feedback inhibition and/or repression resulting in a limitation of precursor tryptophan for alkaloid synthesis.

## EVOLUTIONARY SIGNIFICANCE OF INDUCTION

It is reasonable to speculate that tryptophan induction of alkaloid biosynthesis in ergot has offered a selection advantage for the organisms in evolution. One of the more attractive hypotheses as to what this advantage might be is that proposed by Bu'Lock (*7*), who states that it is not the secondary metabolites (alkaloids) in themselves that are important but rather the actual activity of secondary biosynthesis. The argument is that during the growth phase of the organism there is an absence of secondary biosynthesis, not because of a lack of appropriate precursors, but because of the absence of the required enzymes. When growth is halted by some circumstance such as the using-up of a nutrient essential to growth, an imbalance results in primary biosynthesis and this may initiate formation of enzymes of secondary metabolism. If primary metabolites accumulate to a certain level, metabolic regulation phenomena such as feedback inhibition or repression would shut down the biosynthetic machinery of primary metabolism. Repression would result in a cessation of the synthesis of enzymes; however, if some of the primary metabolites are siphoned off into secondary metabolism, this may allow for the continued de novo synthesis of a limited amount of the enzymes of primary metabolism which would be a distinct advantage in maintaining the integrity of the cell and thus increase the survival opportunities for the organisms.

In the case of ergot, as illustrated in Fig. 5, during the growth phase tryptophan is utilized in the formation of protein. When growth ceases, tryptophan would no longer be incorporated into protein and would be expected to accumulate to levels that would shut down its biosynthesis. Indeed, we (*28*) have obtained evidence that exogenously fed tryptophan and 5-methyltryptophan do inhibit the tryptophan biosynthetic pathway (Table 1). However, since tryptophan also induces alkaloid synthesis, it is possible that enough tryptophan is removed from the endogenous tryptophan pool through alkaloid biosynthesis, so that the pool does not

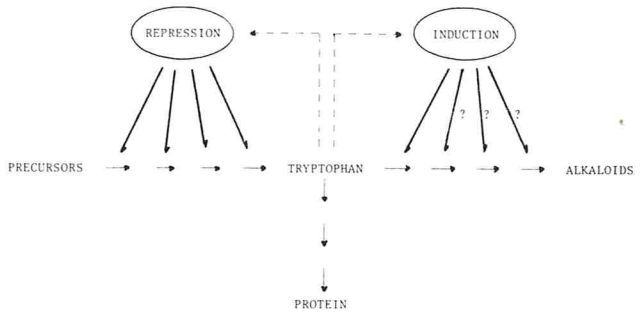


Figure 5. *Postulated mechanism for the evolutionary significance of tryptophan induction of alkaloid biosynthesis in the ergot fungus.*

reach levels that would cause repression of tryptophan biosynthesis. This would be a stabilizing influence since a consequence of this would be that primary metabolism would continue to operate and the substrates for protein synthesis would continue to be formed.

## END-PRODUCT INHIBITION

It has been shown by Heinstein et al. (*25*) that DMAT synthetase isolated from *Claviceps* species SD 58 is inhibited by physiological concentrations of agroclavine and elymoclavine, the end products of the alkaloid biosynthetic pathway in this strain of the organism. A later enzyme in the pathway, chanoclavine-I-cyclase, was also inhibited slightly by elymoclavine (*13*). Anthranilate synthetase was also sensitive to inhibition by elymoclavine (*30*). Since millimolar concentrations of elymoclavine accumulate in the culture medium towards the end of the fermentation, it is possible that end-product inhibition could stop the production of alkaloids. We (*11*) conducted experiments to determine whether end-product regulation was a significant phenomenon in vivo. Our results using intact cells of the fungus were variable; however, utilizing protoplasts of the fungus we found a significant difference of incorporation of L-(methylene-$^{14}$C)-tryptophan into alkaloids in the presence of physiological levels of elymoclavine as compared to controls without elymoclavine (Fig. 6). In addition DMAT synthetase activity was measured in cell-free extracts of protoplasts which had been exposed to elymoclavine and which had been carefully dialyzed to remove residual alkaloid. As shown in Fig. 7, there is little difference in the enzyme activity level between the controls and the treated protoplasts; consequently, it appears that elymoclavine acts through an inhibition of DMAT synthetase rather than a repression of enzyme synthesis.

TABLE 1. *Effect of tryptophan and its analogs on enzyme activities in* Claviceps *species, SD 58.*

| Additives | Enzymes[a] | | | |
|---|---|---|---|---|
| | DAHP synthetase | Anthranilate synthetase | Tryptophan synthetase | DMAT synthetase |
| 4mM DL-Tryptophan | 49[b] | 24 | 23 | 400 |
| 4mM 5-Methyl-DL-tryptophan | 60 | 34 | 24 | 209 |
| 4mM DL-Thiotryptophan | 92 | 94 | 50 | 522 |

[a]Obtained from five-day-old mycelium.
[b]Expressed as percent of enzyme activity as compared to enzymes from control cultures which contained no additives.

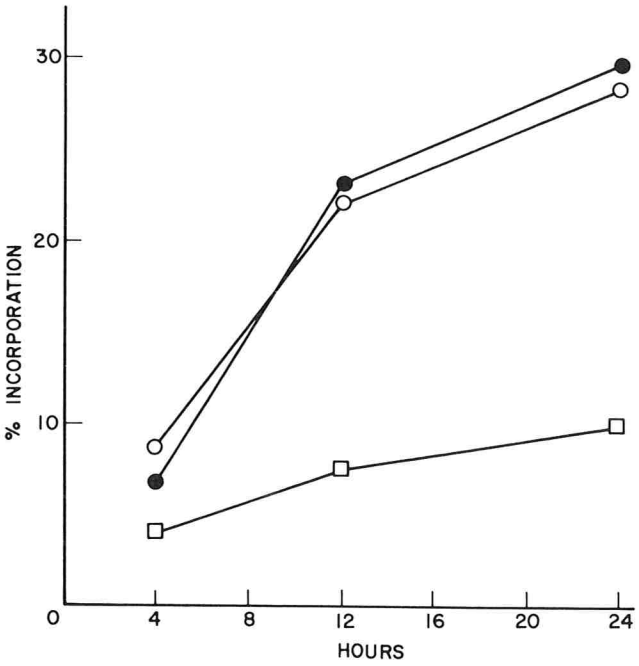


Figure 6. *Incorporation of L-tryptophan into ergot alkaloids by protoplasts of* Claviceps *prepared from 4-day-old-mycelium. The incubation consisted of flasks with 0.7 M KCl* ( ● ) *and with 4 mM potassium succinate* ( ○ ) *respectively as controls; and with 4 mM elymoclavine succinate* ( □ ). *Each flask was sampled at 4, 12, and 24 h after the addition of 2 μ Ci L-(methylene-$^{14}C$)-tryptophan.*

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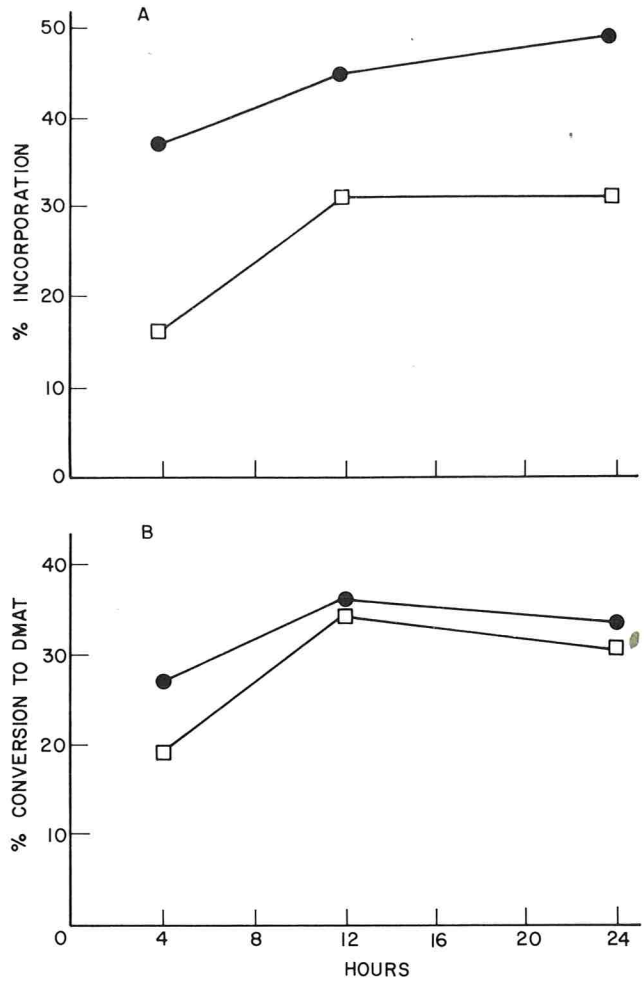


Figure 7. *The effect of removal of residual alkaloid from enzyme preparations obtained from protoplasts. (A) Incorporation of L-tryptophan into ergot alkaloids by protoplasts of* Claviceps *using the same conditions as described in Fig. 6. Incubation consisted of control with 4 mM potassium succinate* ( ◉ ) *and with 4 mM elymoclavine succinate* ( □ ). *(B) DMAT synthetase activity in enzyme extracts dialyzed for 20 h to remove residual alkaloid. Incubations were the same as in (A).*

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## Drug-Resistant Organisms, *con't. from p. 820*

Workers in a penicillin factory had 100 percent of nasal staphylococci carriers with resistant organisms while in the general public 88 percent of carriers had penicillin *susceptible* organisms.

In another study, the sensitivity pattern of *Staph. aurens* isolated from animals was checked. Of 621 samples about two-thirds were resistant to at least one of the 11 drugs tested.

Resistance was more frequent to penicillin (60%) followed by streptomycin (34.7%) and tetracycline (32.8%) in treated animals.

Two especially significant discoveries were 1) the high prevalence of resistance to penicillin, streptomycin, and tetracycline regardless of the host of origin, and 2) the low to negligible prevalence of resistance to the other antimicrobial agents among isolates from dogs and horses.

Other organisms of equal concern are *E. coli*, which are normal inhabitants of most animal intestines. It, under certain conditions, may attack its host with serious or fatal results. Resistance in this organism complicates treatment.

Its widespread presence and the ability to transfer resistance to other organisms are additional complications.

Grumbles stresses that he is not advocating discontinuing use of antibiotics where needed and effective. He is cautioning that in view of present knowledge added care should be exercised in their use.

One possibility is the development of parallel antibiotic usage — those used in animal feeds would not be used in treatment of disease.

Two such antibiotics are presently coming into use for swine and poultry feed.

*Editor's Note — Any questions regarding this column should be addressed to Science Writer, Dept. of Agricultural Communications, Texas A&M University, College Station, Texas 77843.*

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# Abstracts of Papers Presented at the Sixty-Sixth Annual Meeting of IAMFES

**Orlando, Florida, August 12-16, 1979**

Abstract of nearly all papers given at the 66th Annual Meeting appear on this and the following pages. The complete text of many of these papers will appear in future issues of the *Journal of Food Protection.*

## CONTRIBUTED PAPERS

**Nutritional and microbial changes during production of tostones [fried plantains].** R. J. Alvarez, J. A. Koburger, and H. Appledorf. *Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL 32611.*

Tostones (fried plantains) are prepared and consumed by a large proportion of the Latin population in Florida and popularity of this food is increasing in numerous ethnic restaurants. However, little is known about the microbial and nutritional quality of this product, particularly as affected by processing. Proximate and mineral composition, microbial flora, pH and water activity were followed during the various steps involved in their preparation to elucidate the answers. Tostones are prepared by peeling and cutting the plantains into slices, soaking in a brine, deep-fat frying, drying and flattening, and refrying until golden brown. Fat, protein, ash, crude fiber and carbohydrate increased during preparation. P, Na, Hg, Se and Zn also increased, whereas, K and Fe decreased. Changes in water activity and pH were not significantly pronounced. *Bacillus* and *Penicillium* were the only organisms isolated from the finished product. The final product contained approximately 50 bacteria and 15 fungi per gram. The final product was 26.5% moisture, 21.5% fat, 2.5% protein, 1.0% ash, 0.5% crude fiber, 48% carbohydrate and 395 Kcal/100 g.

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**Correcting coliform problems of pasteurized milk.** Sidney E. Barnard. *The Pennsylvania State University, 9 Borland Laboratory, University Park, PA 16802.*

Heat treatment of milk by either vat or high temperature - short time methods destroys coliforms. Therefore, they should not be present in pasteurized milk. Presence of coliforms indicates contamination after pasteurization. When coliforms are present, they usually are accompanied by psychrotrophs which will cause spoilage. Some plants seldom have problems of coliforms in their milk even after storage of samples for 10 days at 45 F. Many of these plants use hot water sterilization of all processing, pasteurized storage and filling equipment. All milk contact surfaces must be flooded with hot water at a temperature of above 170 F and circulated for more than 5 min. Experience has shown the common sources of problems.

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**Getting good preliminary-incubation counts.** Sidney E. Barnard. *The Pennsylvania State University, 9 Borland Laboratory, University Park, PA 16802.*

A 40-year-old test to determine raw milk quality is finally being widely used. A Preliminary Incubation (P.I.) count provides an indication of milk production methods and the potential shelf life of raw milk. The procedure involves incubating samples of raw milk from individual farms at 55 F for 18 h. Results can be expected to be as low as those for the Standard Plate Count. Experience from many milk supplies indicates that P.I. bacterial counts below 100,000 per ml are acceptable. However, initially counts of milk from some farms may exceed 1,000,000 per ml. Causes of high P.I. counts include dirty cows, poor udder washing practices, slow cooling or storage temperatures above 40 F, failure to thoroughly clean equipment twice each day, neglecting to sanitize equipment before use and a contaminated water supply.

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**Some characteristics of acid injury and recovery of *Salmonella barielly* in a model system.** L. C. Blankenship. *U.S. Dept. of Agriculture, Science and Education Administration Federal Research-Southern Region, Richard B. Russell Agr. Research Center, Athens, GA 30604.*

The occurrence of acid-injured microorganisms in foods and the need for attention to recovery procedures for their detection has been recognized. Studies of specific cellular injury characteristics caused by acid treatment and specific recovery requirements have been limited. We studied some of the characteristics of acid injury and recovery of *Salmonella barielly* in a model system. Cells grown in tryptic-soy broth were washed three times in sterile saline-sucrose medium before acid treatment in saline-sucrose + acetic acid. Injury was determined by comparison of counts on nutrient agar to those on violet red bile agar + 1% NaCl. Injury and death rates were temperature and acid concentration dependent. Recovery rates were greater in peptones and casamino acids than in complete media, such as, nutrient broth, brain-heart infusion broth, tryptic-soy broth or lactose broth. The effect of a variety of inhibitors and antimetabolites on acid-injured cells recovering in casamino acids was determined. Inhibitors of protein and RNA synthesis, as well as of electron transport adversely affected recovery. Thus, several cellular metabolic systems appear to be damaged by acid treatment and energy generating systems seem to be required for recovery.

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**Sensory, shelf-life, microbial, and chemical evaluations of creamed cottage cheese treated with sorbates.** F. W. Bodyfelt. *Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331.*

Several experiments with sorbate-treated creamed cottage cheese indicated a significant extension of product shelf life through inhibition of psychrotrophic bacteria. At treatment levels of 0.06 and 0.075% sorbic acid and 0.075% potassium sorbate, one lot of cottage cheese had a shelf-life of at least 4 weeks; untreated controls spoiled within 2 weeks. Though three experienced judges occasionally critiqued treated samples for a slight "aftertaste" or slight bitter taste, the preference panel (24 judgments) did not register any such objections to flavor. This would seem to indicate that a concentration of 0.075% sorbic acid or potassium sorbate in creamed cottage cheese would not be objectionable from a flavor acceptance standpoint. The predominant species of microorganisms isolated from pychrotroph plate counts were identified as *Pseudomonas fluorescens* and other *Pseudomonas*

sp., with limited species of *Alcaligenes, Chromobacterium* and *Pasteurella.*

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**Implementation of a plan to achieve reciprocity between foodservice manager certification programs.** C. Dee Clingman. *National Institute for the Foodservice Industry, 20 N. Wacker Drive, Suite 2620, Chicago, IL 60606.*

The National Institute for the Foodservice Industry (NIFI), under contract with the U.S. Food and Drug Administration, developed and implemented a plan to achieve reciprocal recognition of certificates attesting to foodservice sanitation training on an interstate basis. NIFI established an Advisory Committee representing the foodservice industry, education and regulatory agencies to provide technical guidance on the project. In field evaluations of sanitation training, courses were completed to determine the status of such training in a variety of statewide programs. Analysis of research evidence enabled NIFI, with the advice of its Advisory Committee, to develop a plan to uniformly recognize certification of foodservice management personnel by various jurisdictions. This plan was tested and refined using an experimental group with recommendations as to its continuance provided to FDA.

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**Methods for the effective recovery of *Salmonella* of fish and shellfish.** J. Y. D'Aoust and R. Gélinas. *Health Protection Branch, Health and Welfare Canada, New Research Center, Tunney's Pasture, Ottawa, Ontario, Canada, K1A 0L2.*

The efficacy of two methods for detection of *Salmonella* in 29 fish and 231 shellfish samples was evaluated using replicate samples (100 g) of food homogenate. In method A, samples were preenriched 3 h in lactose broth, selectively enriched overnight in selenite cystine (35 C) and plated on brilliant green sulfa, xylose lysine desoxycholate and Hektoen enteric agar media. In method B, overnight nutrient broth cultures were enriched in tetrathionate brilliant green (43 C) and selenite cystine (35 C) broths and plated on brilliant green sulfa and bismuth sulfite agar media. *Salmonella* was recovered from 6 (4%) shrimp samples of which five were detected by method B alone; the single positive sample detected by method A was negative by method B. Infected shrimp samples did not carry coagulase-positive staphylococci and plate counts ranged from $10^5$ to $10^7$ cells/g; two of the six positive samples contained no detectable *Escherichia coli.* Our results suggest that short preenrichment incubation periods are not reliable and that tetrathionate brilliant green is superior to selenite cystine for the effective recovery of *Salmonella* in shellfish.

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**Proteolytic and lipolytic activities of some toxigenic and nontoxigenic aspergilli and penicillia.** S. M. El-Gendy and E. H. Marth. *Department of Food Science, University of Wisconsin-Madison, Madison, WI 53706.*

Molds are of special importance in the food industry because of their usefulness in producing some foods, the spoilage they sometimes cause and the capability of many to produce toxins. Eighteen strains of *Aspergillus flavus* or *Aspergillus parasiticus*, one of *Aspergillus ochraceus* and 12 strains or species of *Penicillium,* many of them isolated from cheese, were evaluated for their proteolytic and lipolytic activities. Strains of *A. flavus* exhibited considerable proteolytic and little lipolytic activity, whereas the reverse was true for *A. parasiticus.* Of the *Penicillium* cultures tested, 10 exhibited considerable lipolytic activity, but only five had considerable proteolytic activity. Two cultures, *Penicillium patulum* M59 and *Penicillium* sp. 8, were markedly lipolytic and proteolytic. Of the other cultures, greatest lipolytic activity was associated with *Penicillium roqueforti* 849, *Penicillium* sp. 33, *A. parasiticus* NRRL 3145, and NRRL 465 and *A. ochraceus* NRRL 3174, whereas greatest proteolytic activity was associated with *P. patulum* M59, *Penicillium* sp. 25 and *A. flavus* WB500, 4018, 4098 and NRRL 5565.

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**Survival and growth of *Clostridium* species in the presence of hydrogen peroxide.** S. M. El-Gendy, T. Nassib, H. Abed-El-Gellel, and N. El-Hoda Hanafy. *Dairy Department, Faculty of Agriculture, Assiut University, Assiut, Egypt.*

Some bacteria in the genus *Clostridium* can occur as contaminants in milk. If cheese is made from milk with such contaminants, the bacteria can cause the "late gas" or "late blowing" defect in the cheese. Since hydrogen peroxide can be used to treat milk to be used for cheesemaking, this investigation was initiated to determine effects of the peroxide on viability and growth of *Clostridium tyrobutyricum* NIZO, *C. tyrobutyricum* 114, *Clostridium perfringens* 1115 and *Clostridium sporogenes* Tg. Presence of 0.01% hydrogen peroxide in litmus milk retarded but did not prevent growth of and gas production by the clostridia. Presence of 0.02% peroxide inhibited growth and gas production when litmus milk contained, per milliliter, 50 or 100 spores of any of the clostridia being studied. These numbers of spores are greater than those normally found in raw milk produced under ordinary conditions. Further work is warranted on use of hydrogen peroxide to prevent the "late-gas" defect in cheese.

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**Development of a production spray machine for the application of potassium sorbate on baked goods.** C. S. Hickey. *Monsanto Company, 800 N. Lindbergh Blvd. St. Louis, MO 63166.*

Laboratory-scale sorbate spray equipment was developed to study surface application of potassium sorbate on baked goods. The equipment is operated at 2.5 times conveyor speed of an actual production line to provide a separation of the production for complete spray coverage. The stainless steel unit is 40 inches long (24 inches the actual spraying section and the rest feed conveyor), 20 inches wide, and 15 inches deep. The pump will deliver from 30 to 100 psi pressure. The pressure used was determined by conveyor speed. Liquid nozzles are used, providing a particle size of 150 $\mu$. The number of nozzles and their position were changed based upon the shape of the product and their arrangement on a production conveyor. The spray unit was found to deposit a uniform reproducible concentration on each baked good.

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**Preservation of baked goods by sorbic acid and/or potassium sorbate.** C. S. Hickey. *Monsanto Co., 800 N. Lindbergh Blvd. St. Louis, MO 63166.*

The effect of sorbic acid (SA) and/or potassium sorbate (KS) on surface growth of *Aspergillus* sp. and *Penicillium* sp. at 27 C and 95% RH was compared to calcium propionate (CP) in multigrain loaf bread, English muffins and pizza crust. Each baked good was inoculated with approximately $5.0 \times 10^2$ spores/item. Loaves of bread (pH 5.6) containing no preservative molded in 3.3 days, and with 0.156% (flour basis) C.P. 4.5 days. Spraying 0.05 g of KS/100 in$^2$ surface area (S. ar.) on loaves containing 0.048% (flour basis) S.A. yielded 12.0 days with no mold. When 0.1 g/100 in$^2$ S. ar. was sprayed on loaves lasted 22.7 days. English muffins (pH 5.5) with no preservatives molded in 3 days, and with 1.0% (flour basis) C.P., in 6 days. Addition of 0.1 g of KS/100 in$^2$ S. ar. to English muffins containing 0.1% (flour basis) KS molded in 8 days, with 0.4 g of KS/100 in$^2$ S. ar. they lasted 26 days. Pizza crusts (pH 6.8) with no preservative molded in 2 days, and with 0.3% (flour basis), C.P., in 2.5 days. Addition of 0.05 g of KS/100 in$^2$ S. ar. pizza crusts containing 0.06% (flour basis) KS molded 5.7 days.

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**Stack pouring of plates: A potential source of error.** John A. Koburger. *Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL 32611.*

To maximize available countertop space, pouring of petri dishes in stacks of two or more is not common. Our laboratory routinely plated all dilutions in triplicate and then poured the plates in stacks of three. Following incubation, it was not uncommon to find one of the plates yielding a markedly higher count than the other two plates in the stack. Various explanations for this phenomenon were explored. Contaminated glassware and media, oxygen availability and incubation conditions were examined without success. Rate of cooling of the medium within the stacked plates was finally investigated and found to be the cause of the erratic results. From an initial temperature of 46 C, the medium in the plate against the countertop reached 36 C within 30 sec, whereas the other plates in the stack took as long as 4.6 min to reach this temperature. Results indicate that the prolonged exposure of the microorganisms in the stacked plates to the warm agar during the extended cooling was responsible for this phenomenon.

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***Escherichia coli* enterotoxin production in beef broth at 15 to 50 C.** J. Lovett, J. M. Bisha, and P. L. Spaulding. *Food and Drug Administration, 1090 Tusculum Ave., Cincinnati, OH 45226.*

Elsewhere we reported that enterotoxigenic *Escherichia coli* strains grown in beef broth and in milk do not produce enterotoxins at 5 to 15 C. In this series of experiments we examined the ability of an enterotoxigenic ($LT^+/ST^+$) *E. coli* to produce enterotoxin in the temperature range of 15 to 50 C. Cultures were incubated as stationary cultures for 9 days at 15 to 35 C and for 48 h at temperatures above 35 C. Heat-labile (LT) enterotoxin was detected, using the tissue culture (Y-1) assay, at 25 to 40 C. Heat-stable (ST) enterotoxin was detected, using the infant mouse assay, at 30 C and 35 C only. We conclude from our studies that preformed *E. coli* enterotoxins in protein foods is an unlikely health hazard. Exposure of foods at the optimum toxin producing temperature (35 C) for at least 24 h is required for toxin production. Departure from this optimum in either direction further delays the appearance of enterotoxin.

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**Simple technique for determination of D values for *Bacillus stearothermophilus* in liquid systems.** E. M. Mikolajcik and Kathleen T. Rajkowski. *The Ohio State University, 2121 Fyffe Road, Columbus, OH 43210.*

A simple, rapid, highly reproducible procedure for determination of heat resistance of *Bacillus stearothermophilus* spores at temperatures > 100 C in milk and soy protein base formulas was developed. Formula (5 ml) is added to a serum bottle with a rubber stopper and crimped aluminum cap. The formula is agitated during heating in a thermostatically-controlled oil bath, using a wrist action shaker. At the desired temperature, a spore suspension is injected through the rubber stopper using a high pressure GLC syringe. At selected time intervals, a sample is withdrawn from the bottle with a similar sterile syringe. The number of survivors is determined by plating on TSA which yields higher counts than SPC agar or DTA. D values for milk base formula were 14, 3.2, and 1.2 min at 115, 121, and 125 C. Values for soy base were 28.3, 3.9, and 1.5 min, respectively. The serum bottle technique yielded a higher D value than the capillary tube method. The difference was significant at $p = 0.05$ but not at $p = 0.01$. Maximum heat activation of the spores was at 95 C - 10 min in the milk base and at 100 C - 5 min in the soy base.

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**Short-chain fatty acids as sanitizers for beef.** E. A. Quartey-Papafio, R. T. Marshall and M. E. Anderson. *Department of Food Science and Nutrition, 203 Eckles Hall, University of Missouri, Columbia, MO 65211.*

Formic, acetic and propionic acids in combinations and alone were tested in vitro for antimicrobial efficacy against two pseudomonads, two coliforms, a *Streptococcus* sp., a *Micrococcus* sp., a spore-forming bottom yeast and three film yeasts. All were isolated from beef steak after spraying with 3% acetic acid. Also used was a species of *Enterobacter*. $2 \times 10^5$ cells were transferred to 1 ml of sanitizer and held therein for 60 sec (also 10 min for yeasts) before transferring 350 to 500 cells to recovery broth. Variables were pH, concentration and time. Formic (2%) and acetic (3%) acids gave highest decreases in viability with no effect of pH between 2.0 and the normal pH. The most effective sanitizers were sprayed onto beef which was tested for bacterial count before and after storage at 7 C for 7 days. Adding ascorbic acid to formic acid to reduce oxidation of meat pigments further lowered counts. Color was not improved by 1% added ascorbic acid, but with 5%, discoloration was limited.

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**Inhibition of *Staphylococcus aureus* by potassium sorbate in combination with sodium chloride, TBHQ, BHA and EDTA.** M. C. Robach. *Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63166.*

The effect of potassium sorbate alone and combined with sodium chloride, tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) and ethylenediamine tetra-acetic acid (EDTA) on growth of two strains of *Staphylococcus aureus* (S-6 and 12600) was studied. The growth studies were performed in trypticase soy broth (pH 6.0) at 37 C. Growth of the test organisms was monitored by plate counts using Standard Plate Count agar incubated at 37 C. Certain combinations of sorbate with NaCl resulted in synergistic inhibition of growth of both strains. The combination of sorbate and TBHQ also resulted in synergistic inhibition of the growth of strain 12600, but 25 ppm of TBHQ alone inhibited growth of strain S-6. Certain combinations of sorbate and BHA were synergistic against growth of both strains. Addition of EDTA did not potentiate sorbate's activity against growth of strain S-6 but was synergistic with sorbate against the growth of strain 12600.

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**Toxigenic *Yersinia enterocolitica* in retail pork products.** D. A. Schiemann and M. Latvala. *Ontario Ministry of Health, Environmental Bacteriology Laboratory, Box 9000 Terminal A, Toronto, Ontario M5W 1R5.*

The occurrence of *Yersinia enterocolitica* in retail pork products was investigated by the examination of 69 processed and 129 raw meat samples using two enrichment methods for isolation. Modified Rappaport broth incubated at 22 C for 7 days was far superior for isolation compared to cold enrichment (4 C) in phosphate-buffered saline for 21 days followed by modified Rappaport broth at 22 C for 2 days. Five processed (7%) and 63 raw (49%) pork products contained *Y. enterocolitica*. Sixty-five percent of fresh pork tongues were positive, and 46% of these contained serotype 0:3 which is frequently associated with human yersiniosis in Canada. The only other product containing serotype 0:3 was a sample of fresh pork ears. Only one isolation of serotype 0:8 was obtained, originating from ground pork. A selected number of isolates were examined for toxigenicity by the suckling mouse assay. Raw pork tongues represent the only identified vehicle for transmission of human types of *Y. enterocolitica* from the swine reservoir.

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**Effect of interrupted incubation on the total plate count of raw milk.** K. L. Smith and R. L. Richter, Cleo Cook and Doris Merchetti. *University of Florida, Gainesville, Florida Department of Agriculture and Consumer Services, Tallahassee, FL.*

To determine the effect of interrupted incubation on raw milk bacterial counts, plates were poured Thursday, incubated until Friday afternoon, refrigerated over the weekend and the 48-h 32-C incubation period was completed commencing Monday afternoon. Plates were poured Friday, refrigerated until Monday morning when the 48-h 32-C incubation period was begun. Control counts were conducted according to Standard Methods. Although the geometric mean of 100 Thursday samples was about 11% less than that of the controls, the difference was not statistically significant. The Friday samples were significantly less than the control counts after 48 h incubation, but were significantly higher than the controls after 72 h. Weight loss in the agar due to evaporation was not affected by the amount of agar poured in the plates; however, it was higher on the Thursday and Friday plates than on the controls. Moisture evaporation did not appear to have any effect on the corresponding plate counts.

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**Fermentation of soy and winged bean milks by lactic acid bacteria.** L. A. Smoot, V. V. Garcia, and M. D. Pierson. *Department of Food Science & Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060.*

Growth of *Lactobacillus acidophilus, L. bulgaricus, L. plantarum,* and *Streptococcus thermophilus* was compared in soymilk and winged bean milk. The two sterile milk products were prepared by a hot-grind process from either whole soybeans (Essex variety) or whole winged beans (Chimbu variety). Both types of milk were inoculated and incubated for 48 h at 30 or 37 C. Growth of lactic acid bacteria and changes in pH and titratable acidity were monitored. In general, the four species of lactic acid bacteria grew well in the two test milks. Acid production by the four cultures was adequate (pH $< 5.2$) to produce a firm gelatinous curd within 24 h of incubation in both the soymilk and winged bean milk while maximum cell numbers of $10^8$ or greater were attained within 24 to 36 h of incubation. Upon sensory evaluation, an American taste panel and an Oriental taste panel preferred ($P < .01$) the soymilk to the winged bean milk with only the soymilk product being rated acceptable (Oriental panel). When the two milks were fermented by *L. acidophilus* to a yogurt-like product, both the American and Oriental taste panels preferred the soybean yogurt ($P < .01$); neither panel rated these products acceptable.

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**Growth of *Staphylococcus*, enterotoxin and thermostable nuclease production in anaerobically inoculated canned salmon and sardines.** A. K. Stersky, H. Robern, T. Gleeson, C. Park, R. Szabo and C. Thacker. *Bureau of Microbial Hazards, Food Directorate, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, K1A 0L2.*

Cans of salmon and sardines were inoculated anaerobically with $10^1$-$10^3$ cells of *Staphylococcus aureus* and incubated at room temperature for 4 months. *S. aureus* counts reached their maxima after 1 week with $10^8$/g in salmon and $10^9$/g in sardines; after 4 months the count declined by three log cycles. Toxin levels in canned salmon rose to 2 μg/100 g in 1 week and increased to 2.5 μg/100 g after 4 months. The maximum for toxin in sardines, however, was 3.2 μg/100 g after 4 days. In sardines, TNase reached 12 μg/100 g after 2 days and disappeared after 2 months; in salmon it reach 100 μg/100 g in 1 week and declined to 2-4 μg/100 g after 2 months. When the cans were opened after 4-7 days and 4 months of storage, spoilage was not obvious to all panelists and, therefore, considerable numbers of the population may be at risk when contaminated cans of fish are served in meals.

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**Variation in toxin levels and counts in Emmentaler cheese contaminated with *Staphylococcus aureus*.** E. Todd, R. Szabo, H. Robern, T. Gleeson, N. Dickie, M. Akhtar, C. Park and D. Clark. *Bureau of Microbial Hazards, Food Directorate, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario.*

In 1976 Canadian-made Emmentaler cheese caused at least four incidents of illness. Subsequent investigation indicated that many lots were contaminated with *Staphylococcus aureus*, but samples gave various counts and toxin levels. As a result of this, one or more wheels of 59 lots were analyzed for staphylococcal counts, TNase and enterotoxin production. *S. aureus* in counts between $10^1$ and $10^8$/g was found in 51 of these lots. All the cultures isolated were TNase positive, but only 31 of the lots showed detectable amounts of TNase in them. Enterotoxin type B, an unusual type causing illness, was found in 20 of 21 lots analyzed for toxin. Wide variations were observed both in counts (0-3 log differences) and toxin levels (0 to 8.0 μg/100 g cheese) between wheels of the same lot for 16 lots, and also within wheels when 4 to 6 samples per wheel for 9 lots were examined. If the presence of *S. aureus* is due to starter contamination then a more uniform distribution of both counts and toxin levels throughout wheels would be expected. Sampling plans for Emmentaler and other Swiss-type cheese may have to be altered to allow for such variations.

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**Analysis of animal feed ingredients and soil amendment products produced from cattle manure for selected trace metals using atomic absorption spectrophotometry.** W. V. Willis, Amer El-Ahraf, V. V. Dutt, and Khairy Aref. *Department of Health Science and Human Ecology, California State College, San Bernardino, CA 92407.*

Procedures for wet and dry ashing of silage, protein, and soil amendment products from processed manure and determination of Cu, Cr, Fe, Mn, Ni and Zn by AAS are described. Results are given for these products and for NBS orchard leaves. Mineralization efficiency studies show the wet ashing method to be 95% effective; metal recovery studies show both methods give generally high ($> 90\%$) recoveries. The dry method is preferred as being simpler. Only Ni showed matrix effects, which were removed by addition of $H_3PO_4$. Both standard addition and direct interpolation techniques gave similar results. Metal concentrations increased in the order silage, protein, and soil amendment fractions. The silage fraction had metal concentrations comparable to those found in feedlot rations, except for high iron; the soil amendment fraction had concentrations roughly comparable to reported manure values. The study is significant in that it examines important elements in food protection of byproducts produced from animals consuming recycled agricultural waste.

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**Relationships of microbial quality of retail meat samples and sanitary conditions.** C. Jane Wyatt and V. Guy. *Oregon State University, Department of Food Science and Technology, Corvallis, OR 97331.*

A sanitation profile scoring form for evaluating sanitation in retail food stores was designed. The profile was tested in ten Oregon retail markets to evaluate its ability to reflect sanitary conditions. At the time of inspection, samples of meat processed in-store were purchased for microbiological analysis to explore the feasibility of bacterial quality as a measurement of sanitary conditions. Microbiological tests performed included APC, coliform, *E. coli, Staphylococcus aureus, Clostridium perfringens,* and *Salmonella.* Certain deficiencies were noted in the profile designed. The findings indicate many of the factors traditionally looked at by regulatory inspectors have little to do with total store sanitation and food protection. Recommendations are made for improvements in regulatory inspections. Data show no correlation

exists between microbiological quality of products processed in the store and total store profile sanitary conditions. Seventy percent of the products sampled exceeded bacterial load guidelines currently enforced in Oregon (OAR 603-26-400). These "high" counts appear to be more directly related to poor temperature control. The cost of sampling meat products as a "tool for sanitation" is questioned.

## INVITED PAPERS

**Status of the model retail food store sanitation ordinance.** K. J. Baker. *U.S. Food and Drug Administration, Bureau of Foods, Division of Retail Food Protection, 200 'C' Street, S.W., Washington, D.C. 20204.*

This is a status report on the Model Retail Food Store Sanitation Ordinance developed by the U.S. Food and Drug Administration for adoption by State and local regulatory agencies. The Ordinance is currently under final review by the federal agency. Approval is expected by the end of the calendar year. It is the first time the federal government has developed specific recommendations for the sanitary operation of retail food stores. The Ordinance has been under development since 1973. It contains information on some General Provisions, Food Care, Personnel, Equipment and Utensils, Cleaning, Sanitization and Storage of Equipment and Utensils, Sanitary Facilities and Controls, Construction and Maintenance of Physical Facilities, and Compliance Procedures. Several drafts preceded the original proposal which was available for public comment in October, 1977 with a subsequent revised edition available for comment in October, 1978. All comments received were given due consideration and the final version prepared for agency clearance. FDA believes the Ordinance is practical and enforceable. If the recommendations are implemented by the retail food store industry and equitably enforced by the regulatory agencies, the marketplace should be a safer place in which to retail food which meets the expectations of the buyer.

**Rancid milk — Is it here to stay?** David K. Bandler. *Department of Food Science, Cornell University, 11 Stocking Hall, Ithaca, NY 14853.*

The average acid degree value for New York farm milk is above .90. It has shown a steady increase since data was first collected in 1975. The principal causes are: (*a*) pipeline air leaks, (*b*) pipelines risers, (*c*) excessive foam and (*d*) freeze-on in bulk tanks. High numbers of psychrotrophic microorganisms also contribute to elevated ADVs in pasteurized milk. Samples from 60 milk processing plants were examined for total bacteria, coliforms and psychrotrophs, as well as flavor and acid degree value within 36 h of pasteurization, and again at the "sell-by" date. Over 90% of the samples scored good-to-excellent (8-10) when fresh. When rechecked at the end of the shelf-life period, over 50% of the samples were rated poor (5.5-0). Fresh pasteurized milk had an average acid degree value of 1.1. This increased to 1.6 when held at a constant 44 F (6.7 C) until the product "sell-by" date. During the same holding period, bacteria counts went from less than 1,000 to over 12,000,000/ml.

**Investigation of waterborne illness.** Frank L. Bryan. *Center for Disease Control, Atlanta, GA 30333.*

A waterborne disease investigation starts at the time complaints, illness reports, water test results, or epidemiologic logs indicate a possible association with drinking or contacting water. Diagnosis must be verified by getting a detailed case history and collecting specimens from ill persons and others at risk from possible exposure. From the initial information on the first few reported cases, epidemiologic associations are made to determine whether or not an outbreak occurred and to form bases for hypotheses about the cause, source, and mode of transmission. The hypotheses must be confirmed or refuted by subsequent investigation. This includes finding and interviewing additional persons who were at risk from exposure to the suspect water and conducting a field investigation to identify sources and avenues of contamination. The field investigation consists of (*a*) interviewing individuals responsible for quality of suspect water about operations and events that could have led to problems, (*b*) reviewing available records of layout, operations, and quality control for indications of possible problems, (*c*) collecting water samples, (*d*) conducting a sanitary survey of ground water sources, surface waters, treatment operations, and storage and distribution facilities as appropriate and (*e*) identifying factors that contributed to the outbreak.

**Food safety through hazard control.** Joe M. Byrnes. *Kraft Inc., 500 Peshtigo Court, Chicago, IL 60690.*

A review of the basic concepts used in developing a Hazard Analysis and Critical Control Point (HACCP) program and how a program such as this can document and organize your quality assurance program. In reviewing these concepts, the pending food legislation is discussed along with the impact it can have on the food industry and quality assurance programs.

**Fieldmen's and sanitarian's challenges.** Myron P. Dean. *Food Science Department, University of Wisconsin, Madison, WI 53706.*

Objective of fieldmen and sanitarians is to establish procedures which will give assurance that milk and milk products are safe; satisfactory in composition, flavor and appearance; meet regulatory standards and will maintain these attributes until consumed. In addition, they must use the most effective and efficient methods available. Present economic conditions and the need for more efficient use of our dwindling natural resources is going to make it mandatory for us to build on the concept of effective and efficient methods in reaching this objective. This means the necessity of being allowed to deviate from traditional methods and be willing to accept changes without sacrificing program effectiveness and still fulfill the objective. The challenges given to accomplish this are: become proficient in your field, establish priorities, accept change and strive for less government intervention.

**Public participation: An introduction.** R. Alexander Grant. *Food and Drug Administration, (HF-7), 5600 Fishers Lane, Rockville, MD 20857.*

Public participation has become an increasingly important activity within the Food and Drug Administration (FDA). Translating a concept such as public participation into an operational reality, however, presents a constant challenge to federal as well as state and local governments. FDA has developed a model Public Participation Program that provides a variety of ways for consumers to become involved in the Agency's decisionmaking process. The FDA program is policy-oriented and strives to achieve five basic objectives. The ultimate goal of this program is to provide consumers with the opportunity to participate in and impact on FDA's decisions concerning major health issues. Realizing this goal will enable the Agency to ensure that its policies and regulations are responsive to expressed consumer concerns.

**Sanitizers in the food industry.** J. J. Jezeski. *Department of Dairy Science, University of Florida, Gainesville, FL 32611.*

A review of the factors involved in selection and application of appropriate sanitizing agents and procedures in the harvesting, processing, and distribution of foods. This includes a discussion of the following topics: (*a*) The several objectives in sanitizing from the standpoint of both the numbers and types of microorganisms making up the target populations. (*b*) The primary factors involved in action of sanitizing processes dealing with both those related to the target microorganisms and those related to the basic characteristics and mode of action of the sanitizing agents. (*c*) Operational factors which cover the methods of delivery of the microbiocidal agents, the characteristics of the sites of application and the consequences of improper choices either of sanitizer or application techniques. (*d*) The roles of the principal agencies (FDA, EPA and USDA) controlling the sanitizing procedures in food processing plants as well as the principal specific regulations of concern to the food processor will also be considered.

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**Aflatoxin in milk and milk products.** E. H. Marth. *Department of Food Science, University of Wisconsin, Madison, WI 53706.*

Aflatoxin (there are four major types designated as $B_1$, $B_2$, $G_1$ and $G_2$) is a highly toxic and hepatocarcinogenic secondary metabolite produced by the common molds *Aspergillus flavus* and *Aspergillus parasiticus* during growth on feeds or foods. The 7-day oral $LD_{50}$ value for $B_1$, the most toxic and most common form of aflatoxin, is approximately 18 $\mu$g/50 g of body weight of most experimental animals. When cows consume feed contaminated with aflatoxin, $B_1$ is hydroxylated in the liver and the resultant new compound is called $M_1$. Approximately 1 to 3% of the amount of $B_1$ consumed by the cow appears in milk as $M_1$. The 7-day oral $LD_{50}$ value for $M_1$ is 16 $\mu$g/50 g of body weight of experimental animals, however, $M_1$ is much less carcinogenic than $B_1$. According to a directive by the Food and Drug Administration, milk entering interstate commerce may not contain more than 0.5 ppb of aflatoxin $M_1$. Ordinary processing does not inactivate or remove aflatoxin in milk. Manufacture of products based on concentrating constituents of milk also will cause concentration of aflatoxin. Thus a gram of cheese will contain four to five times as much aflatoxin as a gram of milk from which the cheese was made. Growth of toxigenic aspergilli on finished dairy products, e.g. cheese, under certain environmental conditions can lead to presence of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$ and others in such foods.

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**Monitoring of Florida food supply.** Martha E. Rhodes. *Food Laboratory, Division of Chemistry, Florida Dept. of Agriculture & Consumer Services, Tallahassee, FL.*

Routine analytical surveillance of the safety and quality of foods moving in intrastate commerce is the responsibility of the Food Laboratory of the Florida Department of Agriculture and Consumer Services. Sampling varies from consumer complaints, directed sampling of problem processors, to random testing of selected commodities and individual processors. High risk, perishable foods, consumer complaints and foods from firms with poor sanitation are analyzed on a priority basis by the Food Chemistry, Meat and Automated Chemistry, and the Microbiology Sections. Violations are detected in 23-28% of all samples analyzed, and the individual adulterations and misbrandings of various commodities are discussed. Problems enumerated are rodent contamination of bakery samples; undeclared presence of preservatives and saccharin, increased incidence of *Salmonella* in poultry, yeast problems in the beverage industry, molds in corn products, aflatoxin and other mycotoxins in corn products and milk, honey adulteration, substitution of fish species and meat additives.

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## Menu Enforcement Program Effective, Study Shows

The Washington, D.C. Department of Environmental Services has released a study which shows that the city's accuracy-in-menu enforcement program has substantially reduced misrepresentations on menus in the city's restaurants.

The study, conducted about six months after the program began, showed that only 49 percent of restaurants surveyed had menu disparities, compared with 84.5 percent during an original study.

Further, the average number of disparities was reduced from five to two, and the greatest number of disparities was reduced from 26 to eight.

Under the accuracy-in-menu enforcement program, food sanitation inspectors take points off sanitation scores of restaurants which offer foods on their menus and cannot substantiate that they serve what they offer.

In conjunction with the program, the DES Environmental Health Administration also prepared a menu dictionary to educate consumers about terms used by restaurants. Several thousand copies have been provided to persons from the District and all over the nation who have requested them.

In the original study, the department found numerous menu disparities, ranging from identifying turkey salad on menus as chicken salad to identifying domestic products as imported. In many cases, inexpensive cuts of meat were misrepresented as more expensive cuts.

The accuracy-in-menus enforcement program began last July, and the re-survey was conducted in January. The second study included half of the 318 restaurants which were checked in the original survey.

The team also surveyed 24 snack bars, delicatessens, cafeterias and fast-food outlets in the vicinity of the restaurants re-surveyed and found disparities in 25 percent of these establishments, with an average of one.

The substitution of cured pork shoulder picnic for the more expensive cured ham continues to be a major source of misrepresentations, the study noted.

In addition to the enforcement program and the menu dictionary, the department has held seminars with the local hotel and restaurant associations to help them develop accurate menu language, and has provided individual consultations with restaurant managers.

Wholesale distributors of meat and seafood also have been notified of the program to prevent misrepresentation of foods on the wholesale level.

# News and Events

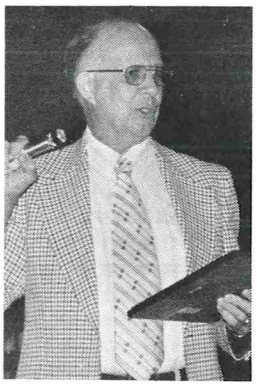
## Pennsylvania Fieldmen Meet, Discuss Three Mile Island

One of the more timely presentations at the 37th Annual Dairy Fieldmen's Conference, held in June at the Pennsylvania State University, was Margaret Riley's "Nuclear Contamination."

Riley, a member of the Pennsylvania Dept. of Environmental Resources, discussed the Three Mile Island nuclear accident and its effect on milk and dairying in the surrounding area. "Milk is a good collector and indicator of other products in the environment," she explained. Iodine-131, one of the most important radioactive isotopes from an agricultural point-of-view, is monitored in milk supplies as an indication of the amount of radiation in the environment. High levels of I-131 were not found in the milk the morning following the accident, Riley emphasized, although milk supplies will continue to be monitored for two to four more years. A point Riley stressed is that perception of radiation risk varies according to how a person receives the radiation. The risk is seen to be greater if radiation is involuntarily received, as in the case of the Three Mile Island accident, than if it is voluntarily taken, as in the case of medical x-rays even though the doses may be the same, she said.

Ray Shipp, with Agronomy Extension at Penn State, discussed "Applying Septic Tank Sludge on Land." Many persons get sludge from sewage treatment plants for their gardens or farms, Shipp said, but many sludges are too high in heavy metals concentration.

Among factors to be considered before spreading sludge are site evaluation, chemical analysis of the soil, application rates, and maximum metal loadings, Shipp said. Some municipalities' sludge is five times or more higher than it should be for certain heavy metal concentrations. "There are lots of benefits in using sludge," among them higher crop yields, "but there are lots of potential areas for problems," he cautioned.



The Pennsylvania Dairy Fieldmen honored George Watrous, above left, and Clarence Nagle, above right, at their 37th Annual Conference in June. Watrous received the Distinguished Service Award while Nagle was named Pennsylvania Dairy Sanitarian. Entertainment at the Annual Awards Banquet featured the Knight Shadows Barbershop Quartet, below.

Also on the program at the Pennsylvania Fieldmen's Conference were presentations on repair and maintenance of parlors and milkhouses, milk hauler surveillance, weight and fat accounting, "the ideal dairy sanitarian," cleaning and sanitizing farm milking equipment, the revised pasteurized milk ordinance, recycling energy in the milk center, transient voltage, and bulk tank applications and antibiotic testing.

Award winners at this year's conference included Dr. George H. Watrous, Jr., and Clarence Nagle. Dr. Watrous, retired professor of food science at Penn State, received the Pennsylvania Distinguished Service Award. He served as advisor to the state association, coordinated its annual conference for 20 years, provided materials and assistance to many persons in the dairy industry on problems of quality, flavor and processing. Dr. Watrous also served as a speaker at many state and regional association meetings. Nagle, dairy sanitarian for the Johnstown Sanitary Dairy in Johnstown, PA, received the Pennsylvania Dairy Sanitarians Award. With over 20 years' experience in plant, laboratory, and field work, he's responsible for 170 farms. He's also active in many of the committees of the association and has served as its president.

## Procedures to Investigate Waterborne Illness Now Available

"Procedures to Investigate Waterborne Illness," a manual prepared by IAMFES, provides techniques for complete investigation of an outbreak of waterborne illness. The manual describes procedures for the establishment of administrative protocol for surveillance of waterborne disease, delegation of responsibility, and the training and equipping of staff. Investigation procedures are presented in a format and sequence to be followed in routine investigations.

The manual is available for $1.75 per copy (reduced prices for larger quantities) at: IAMFES, P.O. Box 701, Ames, IA 50010. Investigative forms are also available in pads of 100 copies.

## Subscription Price, Manuscript Service Charge to Increase

Following increases in production and mailing costs of the *Journal of Food Protection*, the Executive Board of IAMFES voted at the Annual Meeting to increase subscription rates and manuscript service charges for the *Journal.*

Beginning with 1980, volume 43, a *Journal* subsciption will be $50 per year. The increase will not affect the cost of membership in the IAMFES with its subscription to the *Journal.* The new manuscript service charge for publication of papers in the *Journal* is $35 per page.

## Clingman Joins Red Lobster

Charles Dee Clingman has been named Director of Quality Control for Red Lobster restaurants, headquartered in Orlando, FL.

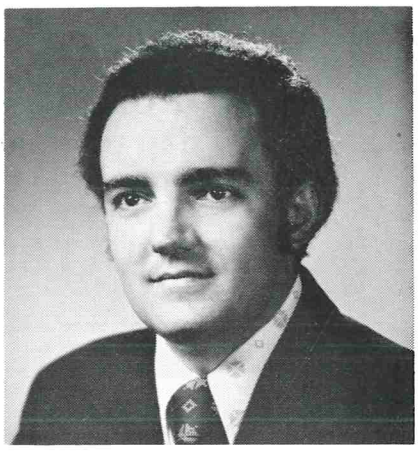
A native of Dayton, Ohio, Clingman formerly served as Director of Food Protection for the National Institute for the Foodservice Industry, (NIFI). Previously he had served in the Bureau of Environmental Health for the state of Ohio.

Clingman, who earned a masters degree in Environmental Health Engineering from the University of Cincinnati, developed and instituted the nation's first Foodservice Manager Sanitation Training and Certification program in 1973.

In 1974, he received an award from the Ohio Environmental Health Association for his contributions to Environmental Health Science.

At Red Lobster, Clingman's Quality Control Department is responsible for testing and evaluating all food products to make certain they meet company standards and specifications. The department also provides quality control training courses for restaurant managers and reviews individual restaurant quality control procedures on an ongoing basis.

Clingman, a member of IAMFES and chairman of the *Journal of Food Protection* Foodservice Committee, replaces Dave Hetterly who has been promoted to General Mills Restaurant Group Director of Quality Control.



Charles Dee Clingman

## U. of Wisconsin Offers Course in Cheese Technology

A new course covering the chemistry, microbiology and technology of cheese will be offered by the University of Wisconsin-Madison. The two-credit course, Food Science 390-375-4, will be offered each year for one week immediately preceding the second semester of the academic year. The first session will be held January 14-22, 1980.

The course has been designed to provide in-depth training in cheese technology. It is also structured for persons not presently enrolled as students who could benefit from a college level course which can be completed in a week. The schedule of the course allows students to be involved in the entire cheese manufacturing process.

Prerequisites for the course are one year of college chemistry, one course in bacteriology, and permission of the instructor. A course in organic chemistry and/or biochemistry and dairy or food microbiology would be helpful.

Persons interested in more information should contact Prof. Olson, 608-263-2001. To enroll in the course, contact in writing:

Prof. Norman F. Olson
Dept. of Food Science
Babcock Hall, 1605 Linden Drive
University of Wisconsin-Madison
Madison, WI 53706

## Fung to Chair KSU Food Science Grad Program

Dr. Daniel Y. C. Fung, Assistant Professor of Food Microbiology, Department of Animal Sciences and Industry has been elected to a four-year term as chairman of the Food Science Graduate Program at the Kansas State University, Manhattan, Kansas. The Food Science Graduate Program was established in 1966 through interdepartment efforts. Currently there are about 45 faculty from 10 departments participating in the program with about 35 graduate students working toward M.S. and Ph.D. degrees. Dr. Fung will provide leadership in administration and development of the program and will be assisted by Dr. J. Marshall, program secretary, as well as a 9-member coordinating committee.

## PMO Available from Printing Office

The *Grade A Pasteurized Milk Ordinance - 1978 Recommendations of the United States Public Health Service/Food and Drug Administration* and related documents are not available from the government printing office.

The documents may be purchased at the following address: U.S. Government Printing Office, Washington, DC 20402. Titles, stock numbers, and prices are as follows:

Grade A Pasteurized Milk Ordinance - 1978 Recommendations of the United States Public Health Service/Food and Drug Administration - SN 017-001-00419-7, Price $5.50.

Grade A Condensed and Dry Milk Products and Condensed and Dry Whey - 1978 Recommended Sanitation Ordinance for Condensed and Dry Milk Products and Condensed and Dry Whey used in Grade A Pasteurized Milk Products - SN 017-001-00420-1, Price $3.75.

Methods of Making Sanitation Ratings of Milk Supplies - 1978. Edition - SN 017-001-00421-9, Price $1.75.

Fabrication of Single-Service Containers and Closures for Milk and Milk Products - 1978 Edition - SN 017-001-00413-8, Price $1.25.

Evaluation of Milk Laboratories - 1978 Edition - SN 017-001-00412-0, Price $1.50.

Cover and Index Tabs for use with 3-ring binders - PMO - SN 017-001-00422-7, Price $2.75. DMO - SN 017-001-00423-5, Price $3.00.



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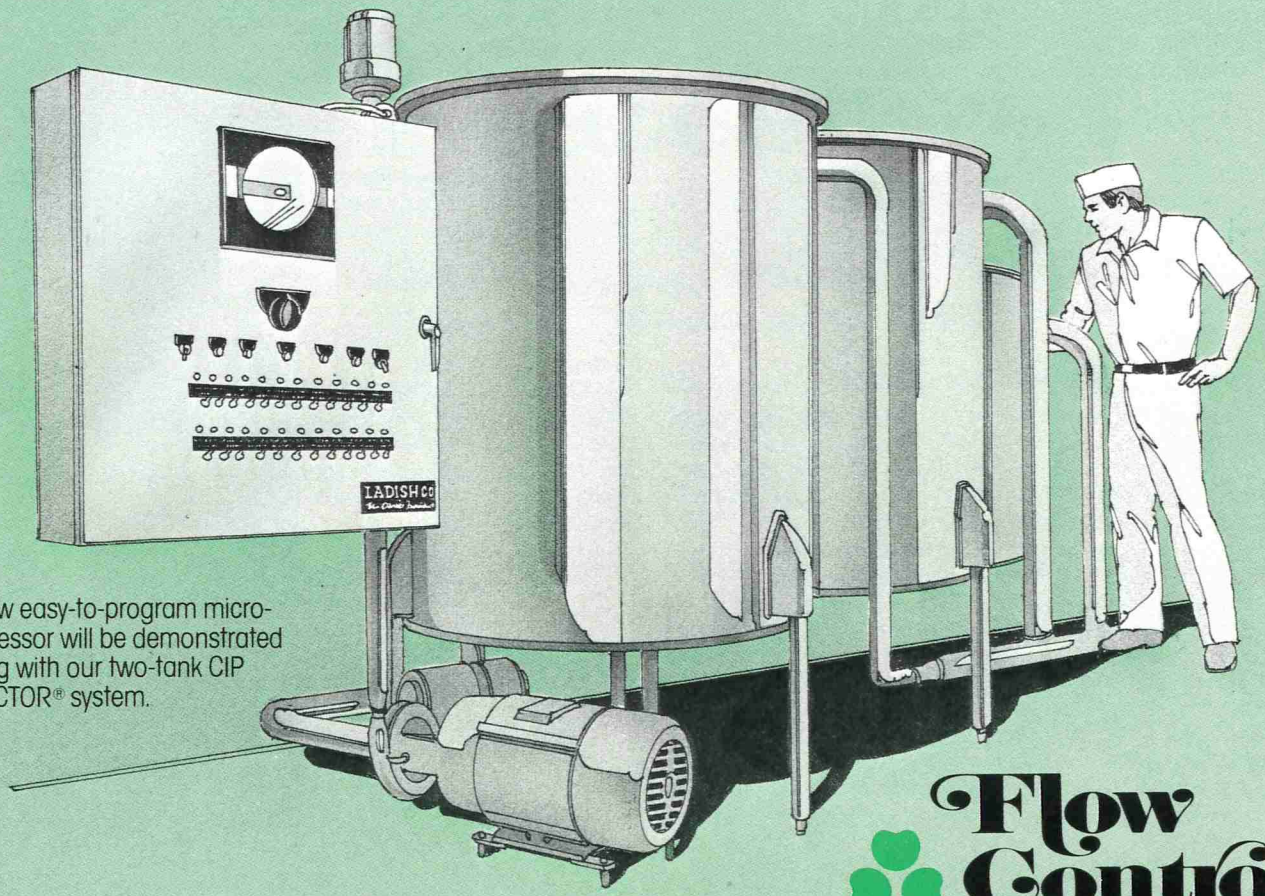


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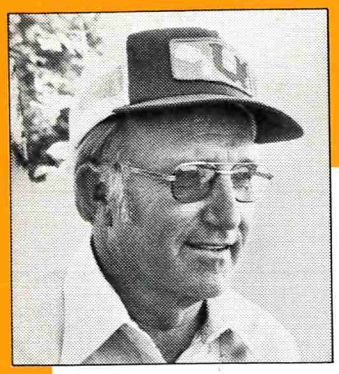

***Ed Kaeder, Milk Quality Fieldman***

*Mr. Kaeder is Field Supervisor, member services for Mid-America Dairymen Inc., Northern Division—a regional dairy cooperative. He was born and raised on a dairy farm and graduated from the University of Minnesota in dairy husbandry in 1944. He worked as an extension fieldman before becoming involved in milk quality control more than 30 years ago.*

"The anatomy of a good fieldman involves being familiar with milk products from the cow all the way through to the customer's table. Since fieldmen work with dairy farmers whose livelihood is production of milk, it is only right to note that milk is probably the most regulated agricultural product in America today. In many cases, government regulates the flow of products, sets minimum prices, and makes rules under which we operate. Basically the fieldman functions as the milk plant's personal contact with its members and is a goodwill ambassador.

"There are many reasons a fieldman will contact members, but the main reason is usually quality. Other calls may be about Grade A requirements, herd health, flavor control, farm building plans, milking equipment installations, arrangements for purchase of equipment, member relations dealing with problems and complaints.

## Knowledge And Testing Important

"There are many tools for the fieldman to use in assisting the producer of high quality milk. Among these are the numerous tests done by every dairy plant. The fieldman must be familiar with these tests: raw count, pasteurized count, cell counts and keeping quality.

"Care of milking equipment is important as well because no other piece of equipment on the farm will get an unsuspecting or careless operator into trouble faster than a faulty or poorly operated milking machine. Lack of proper sanitation will increase bacteria counts, and poor operation will contribute to poor udder health.

"The fieldman must be thoroughly familiar with cleaning and sanitizing compounds and their various uses. He must be in a position to help the dairy producer set up a cleaning program to be followed after each milking, and assure there are no shortcuts in the procedure.

"Requirements for the production of Grade A milk are spelled out in detail in the Grade A Pasteurized Milk Ordinance (PMO), a publication of the FDA and U.S. Public Health Service. We must be able to translate and interpret these requirements for members so they can and will maintain a Grade A status.

"We are involved in herd health with dairymen too, especially in areas relating to causes and prevention of mastitis. We must be able to make use of tools and testing devices because they can tell a great deal and be an aid in educating the dairyman.

"The problem of antibiotics in the milk supply seems to require more and more of our time and attention. Processors can't make cheese and cultured products from antibiotic milk because desirable bacteria will not grow, and any drug residues are unacceptable in milk.

"Working closely with milk haulers is another important aspect for the fieldman since most haulers have the closest and most frequent contact with members. A conscientious hauler is invaluable to us in performing effective fieldwork for the dairyman.

"Today dairy farmers account for only about 0.1% of the population. The distance between active dairy farms becomes longer and, in the interest of energy conservation, and making the most of the dairyman's time, a daily plan of farm calls in a given area is outlined so a minimum amount of time and miles will be expended in driving.

"In summary, I will say that the anatomy of a good fieldman is public relations. In this analogy public relations means selling yourself by being interested, optimistic and enthusiastic about the job at hand, and being informed on all aspects of milk, the industry and your organization."