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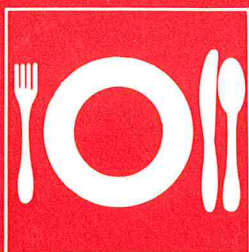
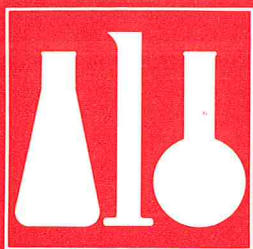


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Fate of Pathogens Inoculated onto Vacuum-Packaged Sliced Hams to Simulate Contamination During Packaging¹

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ABSTRACT

Ham and chopped ham from two manufacturers were contaminated with five enteropathogens: *Bacillus cereus*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*, at time of slicing and vacuum-packaging, to simulate contamination by manufacturer. Subsequent treatment of the samples, representing sound and undesirable retail handling and consumer use conditions, indicated marked differences in the fate of the pathogens between these products and within product type between the two manufacturers. Greatest differences were observed between the chopped ham products. All pathogens, except *C. perfringens*, grew actively in fresh ham and chopped ham with abusive holding at 30 and 21 C. After storage at 4 or 10 C for 30 days, *B. cereus* and *C. perfringens* were no longer detected, even after subsequent holding at 30 or 21 C for 24 h. *E. coli* survival and growth was variable, *S. typhimurium* survived well and grew under some conditions and *S. aureus* was generally inhibited at high levels of competition.

Luncheon meats such as ham and chopped ham are cooked or pasteurized during manufacture, markedly reducing their viable microbial load (25). However, *Bacillus* spp. are common in processed meats because of their wide distribution and resistance to thermal processing (14). Many of these products are sliced and vacuum-packaged for sale to the consumer. Slicing and packaging gives an opportunity for recontamination (23,25) and shelf-life usually ends as a result of microbial growth. Vacuum-packaging markedly influences the microbial flora that develops, selecting the lactic acid bacteria (1,12,14,31). Inhibition or selection may also be attributed to the pH, nitrite and salt concentrations, microbial competition, storage temperature and factors attributed to packaging of the product (7,18,20,21).

Proper storage and handling of these products after manufacture should inhibit growth of potentially pathogenic contaminants. In a study of the food poisoning potential of pathogens inoculated onto bologna sandwiches (29), severe temperature abuse was necessary to create potentially hazardous conditions. The object of this study was to simulate contamination of the product with pathogenic bacteria by the manufacturer, and to determine the food poisoning potential under abusive and ideal conditions of storage and handling at retail and consumer levels.

MATERIALS AND METHODS

Freshly sliced, but not packaged, ham and chopped ham were obtained from two manufacturers. The products were returned to the laboratory under refrigeration, and inoculated with low levels of enteropathogenic organisms (100 to 1,000 of each pathogen per slice), including: *Bacillus cereus* B4AC, *Clostridium perfringens* 8239-H, *Escherichia coli* 7A, *Staphylococcus aureus* S-6 (all obtained from Dr. A. Hauschild, Health Protection Branch, Health and Welfare Canada, Ottawa) and *Salmonella typhimurium* ATCC 13311. *C. perfringens* was grown in Cooked Meat medium (Difco) at 35 C for 24 h, all other pathogens were grown aerobically in Tryptic Soy broth (Difco) at 35 C. All cultures were diluted with 0.1% sterile peptone water to achieve required inoculum concentrations of approximately 100 to 1000/g, and then mixed to give the final inoculum. Batches of 10 slices were inoculated with 0.05 ml of inoculum mixture/slice. The test organisms in the mixture had previously been shown not to be inhibitory to one another. After inoculation, batches were vacuum-packaged in aluminum-nylon-polypropylene pouches (Cryovac Division, Grace Chemicals, Mississauga, Canada) under 26 lb/in² vacuum using a vacuum-packaging machine (CDL, Toronto, Canada; Model 100). Experiments were replicated three times for both products of both manufacturers.

Inoculated samples were stored at 4 C for 24 h, and at 4 and 10 C for 30 days. After storage, the packages were opened and an 11-g sample wedge was cut through all slices, and blended in a Waring Blendor with 99 ml of sterile, 0.1% peptone water for 2 min. Additional 11-g wedges were cut and placed in plastic sandwich bags (Zip Lock Seal, Dow Chemicals) and held at 30, 21 and 4 C for 24 h, to simulate possible sandwich handling. Following holding, samples were prepared for bacteriological analysis as described above. In addition, uninoculated controls were also prepared and handled as described for the inoculated samples.

Appropriate dilutions of inoculated and uninoculated (control) samples were prepared in sterile, 0.1% peptone water blanks and plated in duplicate onto growth media, to determine the following counts: (a) Total Aerobic Colony counts on Standard Plate Count agar (Difco), incubated at 35 C for 48 h (Standard Plate Count, SP35), 21 C for 72 h (Total Aerobic Plate Count, SP21) and 4 C for 10 days (Psychrotroph Count, PSY). (b) *B. cereus* on Mannitol Egg Yolk Phenol Red Polymyxin (MYP) agar (26) incubated at 30 C for 24 h, and heavily contaminated plates for 48 h. Colonies were confirmed using gram stain, catalase test and differential spore stain. (c) *C. perfringens* on Egg Yolk, Tryptose-Sulfite-Cycloserine (TSC) agar (15) incubated anaerobically (BBL "Gas-Paks" with H₂/CO₂ cartridges) at 35 C for 24 h. Isolates were confirmed using the same tests used for *B. cereus*. (d) *E. coli* and total *Enterobacteriaceae* and coliforms on Violet Red Bile (VRBA) agar (Difco) (27) and triplicate tubes of Lauryl Tryptose (LST) broth (Difco) incubated at 35 C for 24 h. For control samples with bile-precipitating colonies on VRBA, the gas-positive LST tubes were transferred to EC medium (Difco) and incubated at 45 ± 0.01 C for 24 and 48 h to obtain a differential presumptive *E. coli* count (16). (e) *S. aureus* on Baird-Parker (BP) medium, prepared according to Holbrook et al. (19), was incubated at 35 C for 48 h. Isolates were confirmed using Mannitol Salt agar (Difco) slants and coagulase tests

¹Supported by funds from Health and Welfare Canada, Research Programs Branch.

(17). (f) *S. typhimurium* on Brilliant Green (BGA) agar (Difco) and incubated at 35 C for 24 h. Samples were also inoculated into Nutrient broth for nonselective enrichment, and subsequently into Selenite Cystine broth (Difco) for selective enrichment. Isolates were confirmed with Salmonella O antiserum Poly A-I and Group B factors 1, 4, 5 and 12. (g) Presumptive group D streptococci on KF Streptococcus agar (Difco), incubated at 35 C for 48 h. (h) Lactic acid bacteria were enumerated on APT agar (Difco) incubated at 30 C for 48 h (9,11). Colonies were confirmed by gram stain and catalase test. (i) *Microbacterium thermosphactum* was determined on STAA agar (13), incubated at 21 C for 72 h, and flooded with 2-3 ml of oxidase reagent (N,N-Dimethyl-p-phenylene-diamine monohydrochloride, Sigma Chemicals), after counting, to eliminate any oxidase-positive colonies from the count.

pH was measured using a single probe electrode (Fisher Scientific Co., Cat. No. 13-639-90) placed between the meat slices. Readings were taken at five different places between the slices, and the mean pH value was recorded. Moisture was determined by an official method (4).

RESULTS

Mean pH values for the three samples of ham and chopped ham, for both manufacturers at different stages of the experimental process are shown in Table 1. Initial pH of all samples was > 6.0. After 30 days of storage the mean pH drop for ham samples was 0.52 and 0.38 for manufacturers A and B, respectively. For chopped ham, the mean pH drop was dramatically different, 0.74 and 0.03 for manufacturers A and B, respectively. During the

24-h holding of samples at 30, 21 and 4 C, most pH change occurred at 30 C; however, for stored (30 days at 4 and 10 C) chopped ham from manufacturer B, pH only dropped 0.37 at 30 C and 0.20 at 21 C. The pH on individual samples of ham and chopped ham did not fall below 5.71 and 5.39 during storage, or 5.45 and 5.39 during holding at 30 C.

Mean moisture content of ham from both manufacturers was 76%; while chopped ham had lower mean moisture contents, 70% for manufacturer A, 59% for manufacturer B.

The saprophytic flora of the control samples is shown in Tables 2 and 3. For ham samples (Table 2), differences between product from manufacturers A and B were principally in the standard plate count (SP35) and the psychrotroph count (PSY). Both manufacturers' products had total plate counts (SP21) of 10^4 /g, but in ham A the SP35 count predominated, while in ham B the PSY count predominated. Presumptive group D streptococci (KF) were < 100/g in ham A, and < 10/g for ham B. A ten-fold difference in lactic acid bacteria (APT) was observed between the products of the two manufacturers.

Assuming that a population of 10^8 to 10^9 /g represented maximum population, it can be seen (Table 2) from SP35, SP21, PSY and APT counts that the ham samples had reached maximum population after 30 days

TABLE 1. Changes in pH of ham and chopped ham under conditions simulating retail storage and consumer handling of the product.

Storage	Ham		Chopped ham	
	Manufacturer A	Manufacturer B	Manufacturer A	Manufacturer B
	(Mean pH)		(Mean pH)	
Fresh product	6.33	6.26	6.29	6.18
24 h holding at 30 C	6.07	5.97	5.80	6.13
21 C	6.23	6.40	6.27	6.32
4 C	6.23	6.28	6.27	6.20
30 day storage at 4 and 10 C	5.81	5.88	5.55	6.15
24 h holding at 30 C	5.58	5.82	5.47	5.78
21 C	5.63	5.85	5.51	5.95
4 C	5.69	5.88	5.53	6.12

TABLE 2. \log_{10} mean counts^a of saprophytic flora on control samples of vacuum-packaged, sliced ham from two manufacturers under conditions simulating retail storage and consumer handling of the product.

Storage	Manufacturer A						Manufacturer B					
	SP35	SP21	PSY	KF	APT	STAA (\log_{10} mean count/g)	SP35	SP21	PSY	KF	APT	STAA
Fresh product	4.67	4.58	2.33	2.82	4.42	3.00	3.38	4.24	3.49	< 1.0	3.47	3.12
24 h holding at 30C	9.84	9.75	5.33	6.52	9.73	< 2.0	9.51	9.51	7.15	5.14	9.47	3.67
21 C	6.67	6.43	4.46	4.52	6.73	4.82	6.52	7.18	4.25	1.89	6.64	5.75
4 C	5.02	4.70	2.22	3.15	4.50	< 2.0	3.51	4.11	3.74	1.69	3.69	3.23
30 days storage at 4 C	8.26	8.92	8.44	5.69	8.92	4.87	7.81	8.82	8.34	< 1.0	8.33	6.04
24 h holding at 30 C	8.68	8.71	8.12	7.96	8.58	2.84	7.86	8.72	8.13	1.12	8.13	3.51
21 C	8.35	8.69	8.39	7.52	8.61	4.90	7.84	8.77	8.42	1.00	8.45	5.83
4 C	8.31	8.48	8.40	7.52	8.50	5.14	7.80	8.68	8.10	< 1.0	8.31	5.43
30 days storage at 10 C	8.68	8.75	8.34	5.86	8.55	4.28	7.73	8.81	8.19	< 1.0	8.29	5.14
24 h holding at 30 C	8.61	8.64	7.65	8.60	8.60	3.36	9.68	8.56	7.81	2.13	8.34	4.95
21 C	8.91	8.85	7.85	7.52	8.80	4.22	9.26	8.78	8.55	2.80	8.47	4.60
4 C	8.53	8.59	8.07	7.52	8.49	4.83	8.73	8.73	7.49	3.20	8.47	4.47

^aKey: SP35 - Standard aerobic plate count, 35 C for 24 h; SP21 - Total aerobic plate count 21 C for 72 h; PSY - Psychrotroph count; KF - Presumptive group D *Streptococcus* count; APT - Lactic acid bacteria; STAA - *M. thermosphactum*.

of storage at 4 or 10 C, or holding fresh samples at 30 C for 24 h. KF counts in fresh ham from both manufacturers increased dramatically at 30 C in product from manufacturers A and B, and also at 21 C, and even at 4 C, for product from manufacturer A, but failed to predominate the population. After 30 days storage, KF count in ham A had increased to 10^5 /g, but little increase occurred in ham B. In ham A, with subsequent holding of stored product at 30, 21 or 4 C, KF counts increased to a predominant level among the bacterial flora. This did not occur in ham B. *M. thermosphactum* did not become a major component in the saprophytic flora of ham, and growth patterns in product from both manufacturers were similar, achieving maximum populations of 10^4 to 10^5 /g.

For chopped ham samples (Table 3), differences in saprophytic counts between products from the two manufacturers were less obvious. PSY counts were low in fresh chopped ham B. After storage for 30 days at 4 and 10 C, counts in chopped ham from manufacturer A had generally reached the maximum population, but

manufacturer B's product stored at 4 C had not reached maximum population. This was shown by subsequent increases in these counts when this product was held at 30, 21 and 4 C for 24 h. KF counts in fresh chopped ham were low, but with storage, these counts increased markedly in chopped ham A but not in B. *M. thermosphactum* were not detected in the fresh samples (< 100 /g), and increased in count in product stored at 4 C for 30 days, but not at 10 C for 30 days.

The numbers of pathogenic bacteria inoculated and surviving in ham and chopped ham are shown in Tables 4 and 5. Initial inocula were in the range of 10^2 to 10^3 of each pathogen per g. After 24 h of storage of the inoculated product at 4 C, detectable counts had decreased in many samples, but were generally still 10^2 /g. After storage for 30 days at 4 and 10 C, both *B. cereus* and *C. perfringens* in both products decreased below their minimum detectable level of 100/g. In contrast, *S. typhimurium* and *S. aureus* survived the 30 days of storage in both ham and chopped ham, with slight decreases in viable count in some instances. *E. coli*

TABLE 3. \log_{10} mean counts^a of saprophytic flora on control samples of vacuum-packaged, sliced chopped ham from two manufacturers under conditions simulating retail storage and consumer handling of the product.

Storage	Manufacturer A					Manufacturer B						
	SP35	SP21	PSY	KF	APT	STAA (\log_{10} mean count/g)	SP35	SP21	PSY	KF	APT	STAA
Fresh product	4.47	4.79	4.63	< 1.0	3.95	< 2.0	4.13	4.23	< 2.0	< 1.0	3.47	< 2.0
24 h holding at 30 C	9.62	9.78	6.62	6.51	9.61	< 2.0	9.62	9.41	3.83	< 1.0	9.68	< 2.0
21 C	7.12	6.28	5.81	5.56	7.01	5.46	5.15	5.12	2.60	< 1.0	5.07	< 2.0
4 C	3.69	4.46	3.89	1.30	3.58	< 2.0	4.72	4.88	4.20	< 1.0	3.81	< 2.0
30 days storage at 4 C	8.24	8.60	8.36	5.17	8.62	5.42	5.15	7.87	7.95	1.90	7.02	5.24
24 h holding at 30 C	8.25	8.54	8.90	5.00	8.35	2.66	9.06	9.35	8.23	< 1.0	9.28	5.08
21 C	8.03	8.82	8.55	4.59	8.54	4.52	7.66	8.22	8.68	< 1.0	8.56	6.86
4 C	8.05	8.56	8.38	4.71	7.27	5.52	6.12	8.15	6.76	< 1.0	7.43	4.66
30 days storage at 10 C	8.46	8.83	8.48	5.72	9.38	1.51	7.65	8.38	8.25	5.52	8.54	2.30
24 h holding at 30 C	8.98	8.49	8.01	7.42	7.39	< 2.0	9.41	9.50	8.08	3.50	9.68	< 2.0
21 C	8.28	8.44	8.21	6.52	8.40	< 2.0	8.51	8.72	8.23	< 1.0	8.74	6.05
4 C	8.32	8.65	8.25	6.52	8.46	< 2.0	7.30	7.87	7.29	3.15	7.63	3.45

^aKey: SP35 - Standard aerobic plate count, 35 C for 24 h; SP21 - Total aerobic plate count 21 C for 72 h; PSY - Psychrophilic count; KF - Presumptive group D *Streptococcus* count; APT - Lactic acid bacteria; STAA - *M. thermosphactum*.

TABLE 4. \log_{10} mean counts^a of pathogenic bacteria inoculated onto vacuum-packaged, sliced ham from two manufacturers under conditions simulating retail storage and consumer handling of the product.

Storage	Manufacturer A					Manufacturer B				
	MYP	TSC	VRBA	BGA	BP (\log_{10} mean count/g)	MYP	TSC	VRBA	BGA	BP
Initial inoculum/g	2.49	2.36	2.56	2.63	3.27	2.64	2.85	2.88	2.69	2.30
Fresh inoculated product	2.23	2.44	2.54	2.63	3.03	2.25	2.11	2.47	2.54	2.00
24 h holding at 30 C	8.21	5.93	8.32	8.47	9.48	8.39	2.87 ^b	8.57	8.56	8.84
21 C	5.22	1.47	5.42	3.14	5.77	5.41	1.91	5.46	2.85	4.23
4 C	4.00	1.69	2.34	2.36	1.23	2.51	2.17	2.43	2.43	2.07
30 days storage at 4 C	< 2.0	< 2.0	2.63	2.36	2.14	< 2.0	< 2.0	2.31	2.54	0.90
24 h holding at 30 C	< 2.0	< 2.0	8.24	3.98	5.22	< 2.0	2.30	7.70	3.97	4.22
21 C	< 2.0	< 2.0	3.06	1.82	3.14	< 2.0	< 2.0	3.26	2.39	< 2.0
4 C	< 2.0	< 2.0	1.22	2.11	2.03	< 2.0	< 2.0	2.59	2.25	1.39
30 days storage at 10 C	< 2.0	< 2.0	5.25	1.47	0.84	< 2.0	< 2.0	3.23	2.00	< 2.0
24 h holding at 30 C	< 2.0	< 2.0	5.23	5.00	4.88	< 2.0	< 2.0	7.93	6.52	4.03
21 C	< 2.0	< 2.0	6.26	1.23	2.09	< 2.0	< 2.0	4.42	2.00	1.77
4 C	< 2.0	< 2.0	5.09	< 2.0	2.39	< 2.0	< 2.0	2.62	1.84	1.43

^aKey: MYP - *B. cereus*; TSC - *C. perfringens*; VRBA - *E. coli*; BGA - *S. typhimurium*; BP - *S. aureus*.

^bOne batch had a high count of 10^6 - 10^7 .

also survived, with up to 1,000-fold increase in ham A stored at 10 C, and chopped ham A stored at 4 and 10 C.

Holding of fresh, inoculated ham (Table 4) for 24 h at 30, 21 and 4 C resulted in growth of all pathogens, except *C. perfringens*, generally to maximum population at 30 C, and to variable population levels at 21 C. The only growth observed for *C. perfringens* was in fresh ham and chopped ham from manufacturer A, stored at 30 C for 24 h. In ham A at 30 C, *C. perfringens* increased 1,000-fold, whereas in ham B this organism failed to grow in two trials, but grew to $10^6/g$ in the third trial. At 21 C most growth was recorded for *B. cereus*, *E. coli* and *S. aureus*, with up to 1,000-fold increase in population in the 24-h holding period.

Marked differences in pathogen growth were observed on fresh chopped ham from the two manufacturers (Table 5). In fresh chopped ham A held at 30 C for 24 h, all pathogens grew to high counts, except *C. perfringens*, which grew to $10^5/g$. At 21 C for 24 h, growth ranged from 100-fold increase for *S. typhimurium* and *S. aureus* to 10,000-fold increase for *B. cereus* and *E. coli*. At 21 C, *C. perfringens* failed to grow in these chopped hams. In fresh chopped ham B, only intermediate levels of pathogen growth occurred, even after holding at 30 C for 24 h. The only exception to this was *S. aureus*, which at 30 C for 24 h grew to maximum population. Growth of *E. coli* and *S. typhimurium* was almost entirely inhibited in chopped ham B.

Holding stored product at 30, 21 and 4 C for 24 h resulted in no detectable change of *B. cereus* and *C. perfringens*. Both remained below the lowest detectable count of 100/g. *E. coli* grew to significant populations at 30 C in ham originally stored at 4 C and in fresh chopped ham A. Samples stored at 10 C had already achieved high populations of *E. coli*. Chopped ham B, however, did not support *E. coli* growth even at 30 C. *S. typhimurium* and *S. aureus* increased in ham stored at 30 C, but only to populations ranging from 10^3 to $10^6/g$. In chopped ham, however, differences between manufac-

turer's product were again apparent. *S. aureus* in chopped ham B grew to $10^7/g$ at 30 C.

DISCUSSION AND CONCLUSIONS

Contamination of ham and chopped ham in this study was intended to simulate "manufacturer" contamination at time of slicing and vacuum-packaging. Storage of product at 4 C for 30 days represented ideal retail storage, while storage at 10 C for 30 days was intended to represent poor retail storage. Similarly, product held at 4 C for 24 h simulated ideal sandwich storage, while product held at 21 and 30 C simulated undesirable (abusive) storage of sandwiches. Meats in this study were not made into sandwiches because laboratory studies (unpublished data) indicated that pathogen growth was similar in sandwiches or under the simulated conditions of this study.

Product of two manufacturers' was selected for this study, based on apparent differences between manufacturer's products in the marketplace. Ham from these two manufacturers was similar from the standpoint of pH change and moisture content, but differences occurred in saprophytic flora. In contrast, chopped ham produced by these manufacturers differed markedly in pH and moisture content, making these essentially different products. Hence, it may be expected that differences in pathogen survival and/or growth might occur both between and within product types. Ham is described as an integral meat, in which small chunks of raw meat are formed into larger pieces by a natural exudate of soluble proteins (22). Chopped ham is either a finely or coarsely comminuted ham suspended in an emulsion base (3), with allowable carbohydrate filler up to 3%, and might therefore be expected to vary more than ham, between manufacturers.

The pH of these products seldom decreased below 5.5, even with storage for 30 days at 4 and 10 C, or with subsequent holding at 30, 21 and 4 C. Hence, pH alone can not be relied on as a protective factor against

TABLE 5. \log_{10} mean counts^a of pathogenic bacteria inoculated onto vacuum-packaged, sliced chopped ham from two manufacturers simulating retail storage and consumer handling of the product.

Storage	Manufacturer A					Manufacturer B				
	MYP	TSC	VRBA	BGA	BP (\log_{10} mean count/g)	MYP	TSC	VRBA	BGA	BP
Initial inoculum/g	2.79	2.72	2.52	2.59	2.64	2.81	2.72	2.68	2.93	2.49
Fresh inoculated product	2.48	2.23	2.53	2.81	3.32	2.54	2.18	2.68	2.83	2.78
24 h holding at 30 C	8.54	5.00	8.60	7.11	8.59	5.48	< 2.0	2.56	3.81	8.82
21 C	6.79	1.23	6.39	4.11	5.30	2.62	1.85	2.89	3.23	4.34
4 C	2.40	2.23	2.51	2.65	2.65	2.54	2.26	2.67	2.91	2.75
30 days storage at 4 C	< 2.0	< 2.0	5.38	2.54	2.57	< 2.0	< 2.0	1.93	2.34	2.67
24 h holding at 30 C	< 2.0	< 2.0	5.08	3.52	5.72	< 2.0	< 2.0	2.93	2.23	7.75
21 C	< 2.0	< 2.0	3.67	2.11	2.97	< 2.0	< 2.0	2.03	2.65	1.95
4 C	< 2.0	< 2.0	4.26	2.26	2.86	< 2.0	< 2.0	1.98	2.48	3.08
30 days storage at 10 C	< 2.0	< 2.0	5.26	1.23	1.40	< 2.0	< 2.0	2.08	2.65	2.94
24 h holding at 30 C	< 2.0	< 2.0	6.53	< 2.0	1.23	< 2.0	< 2.0	1.78	3.72	7.63
21 C	< 2.0	< 2.0	6.59	1.72	1.70	< 2.0	< 2.0	1.70	2.23	3.92
4 C	< 2.0	< 2.0	5.51	3.08	< 2.0	< 2.0	< 2.0	2.28	2.72	2.88

^aKey: MYP - *B. cereus*; TSC - *C. perfringens*; VRBA - *E. coli*; BGA - *S. typhimurium*; BP - *S. aureus*.

pathogen growth. Saprophytic microflora generally grew to large numbers in these products, providing competition for the pathogenic bacteria. However, this also could not be relied on as a protective factor because of low counts in fresh product and, in the case of chopped ham from manufacturer B stored for 30 days at 4 C, maximum population was not reached.

M. thermosphactum generally increased in count during storage, and in chopped ham this organism increased more at 4 than at 10 C. This conflicts with other reports for *M. thermosphactum* in which it was described as an aerobic organism, that did not increase under reduced oxygen conditions (24,30,32). However, during holding at 21 C, the optimum growth temperature of *M. thermosphactum* (5), counts increased but it failed to become a major component of the saprophytic flora of these products.

KF counts indicated that group D streptococci had the potential to grow in these products, and in certain instances, to predominate the population. Group D streptococci have been used to indicate fecal contamination (5,6) and *S. faecalis* has been implicated in outbreaks of food poisoning (33). However, the types of group D streptococci enumerated on KF in this study were not identified, hence their significance in these products cannot be interpreted fully. In a study of group D streptococci in bologna, the principal types were *Streptococcus faecium* var. *durans* and *Streptococcus faecalis* (28).

Fresh product contaminated with pathogens and subjected to abusive holding conditions allowed growth of pathogens, except *C. perfringens*, to potentially hazardous levels. However, with fresh ham, *C. perfringens* grew to 10^5 /g in one manufacturer's product and gave a variable growth response in the other manufacturer's product. Hence, the retarded growth or inhibition of *C. perfringens* was unpredictable. In general, for growth of enterotoxigenic pathogens to potentially pathogenic levels, holding for less than 24 h at 30 C or greater than 24 h at 21 C would be necessary.

In old product, i.e. stored 30 days at 4 or 10 C, *B. cereus* and *C. perfringens* decreased in count below detectable levels (< 100/g), and even with subsequent temperature abuse, failed to grow. *E. coli* increased in the products of manufacturer A, especially at 10 C, and in chopped ham, also at 4 C. In ham, the unexpected lack of growth of *E. coli* at 30 C, after storage at 10 C, might be due to competition. In manufacturer B's chopped ham some factor(s) appeared to inhibit *E. coli*. *Salmonella* was inhibited in chopped ham from both manufacturers, but contrary to expectation (2,8), *Salmonella* increased in ham, especially in product stored 30 days at 10 C. *S. aureus* growth in stored (old) ham was also inhibited, but in chopped ham, where growth of the saprophytic flora was incomplete, *S. aureus* growth occurred.

In general, contamination of ham and chopped ham with low levels of pathogens represents little or no hazard

from enterotoxigenic pathogens, which must grow to cause food poisoning, providing conditions of storage and holding are reasonable. In contrast, infective pathogenic bacteria survive, even the prolonged period of product storage during its shelf-life. Hence, contamination of product at manufacturer level represents a potential hazard, especially if the product is subsequently abused by poor consumer handling.

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Coming Events, *con't from p. 539*

Aug. 20-22--FREEZING OF BAKED AND UNBAKED PRODUCTS. American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502. Contact: Darrell Breising, AIB, 913-537-4750.

Aug. 29-31--FOURTH INTERNATIONAL IUPAC SYMPOSIUM ON MYCOTOXINS AND PHYCOTOXINS. Co-sponsored by World Health Organization and Swiss Society for Analytical and Applied Chemistry. Lausanne, Switzerland. For participation and poster presentation, contact: Prof. P. Krogh, Dept. of Veterinary Microbiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907 or Prof. D. Reymond, IUPAC, Case postale 88, 1814 La Tour de Peilz, Switzerland.

Sept. 10-13--2nd INTERNATIONAL CONFERENCE ON FOOD SERVICE SYSTEMS DESIGN. Harrogate, England. Contact: G. Glew, Catering Research Unit, Procter Dept. of Food Science, The University of Leeds, LS2 9JT, England.

Sept. 10-14--FOOD PROCESSORS ADVANCED MICROBIOLOGY SHORT COURSE. University of California, Davis. Fee \$200. Contact: John C. Bruhn, Dept. of Food Science and Technology, University of California, Davis, CA 95616, 916-752-2192.

Sept. 11-12--AMERICAN CULTURED DAIRY PRODUCTS INSTITUTE, Annual Meeting and Conference. Southeast Holiday Inn, Madison, WI. Contact: C. Bronson Lane, ACDPI, P.O. Box 7813, Orlando, FL 32854.

Sept. 11-14--MAINTENANCE MANAGEMENT. American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502. Contact: Darrell Breising, AIB, 913-537-4750.

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

Sept. 17-19--BAKING PRODUCTION TECHNOLOGY SEMINAR. Dallas, TX. Sponsored by American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502. Contact: Darrell Breising, AIB, 913-537-4750.

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

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

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

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

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

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

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

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

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

Influence of Carbon Source, Bile Salts and Incubation Temperature on Recovery of *Enterobacteriaceae* from Foods Using MacConkey-type Agars

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ABSTRACT

The plea made many years ago (27) to replace the ill-defined coli-aerogenes ('coliform') bacteria as indicator organisms in foods processed for safety with the *Enterobacteriaceae* which are taxonomically accurately defined and as a rule more abundant has of late been more generally accepted. This called for development of a rigorously standardized formula for violet red bile glucose agar and for assessment of the optimal incubation temperature. Four reference strains of *Enterobacteriaceae*, 120 samples of minced meat and 100 samples of frozen broiler chickens were used in these studies. Considerable differences in the performance of commercially available dried formulae, when used as poured plates were observed. These applied both to productivity and to the type of colony produced by a given pure culture. As expected, replacement of lactose plus glucose by an equimolar amount of glucose did not influence the performance of the medium. Intrinsic toxicity of some batches of medium to non-stressed *Enterobacteriaceae* appeared to be mainly responsible for substandard performance. It could be overcome by careful selection of the preparations of crystal violet and particularly the bile salts (39) used in the formulae. Incubation at 30 C led to higher confirmed colony counts in minced meat than at 37 C. However, incubation temperature did not greatly influence similar counts in broiler drip. This observation could be substantiated by identification of the types of *Enterobacteriaceae* isolated from the two commodities. Psychrotrophic species predominated in minced meats, which are often made from raw materials stored for some time under refrigeration, whereas mesophilic species were in the majority on frozen broilers, which are generally frozen shortly after slaughter.

In monitoring foods processed for safety (11,33), it is advantageous to replace the coli-aerogenes bacteria, indicator organisms used in the past, by the taxonomically better defined and more abundant *Enterobacteriaceae* (38). Subsequently it has been suggested to apply *Enterobacteriaceae* counts also to unprocessed foods (4,8,19,25,44). Extreme care should be exercised in doing so. Particularly, any conclusions about lack of safety or mishandling drawn from high counts should be based on studies on the mesophilic or psychrotrophic character of the *Enterobacteriaceae* isolated (28,38,40).

For enumeration of the entire *Enterobacteriaceae* group MacConkey's agar with glucose added was originally recommended (29). For various reasons it seemed better to replace lactose in MacConkey's medium with glucose. The effect of this substitution had to be assessed experimentally. In addition, we had repeatedly

*Brand names are presented for the purpose of identification of materials used in this investigation. Their mentioning does not imply recommendation.

observed, that the properties of the bile salts, and to a lesser extent of the triphenyl methane dyes, used in this type of medium could significantly influence its productivity (32,34,36,37,39). This investigation was designed to study this effect in more detail.

Furthermore, the temperature of incubation of MacConkey-type agar plates is worthy of consideration. Occurrence of psychrotrophic *Enterobacteriaceae* in foods has repeatedly been demonstrated (6,10,12,15,21,24,28,41,42,45,46,47,56). Incubation at 35-37 C leads to suppression of many of these strains (14) and hence to an underestimation of the degree of recontamination of products processed for safety. On the other hand, we observed that incubation at lower temperatures occasionally led to the growth of non-fermentative bacteria in MacConkey-type agars, even when these were overlaid with sterile agar (Harrewijn and Mossel, 1971, unpublished). In this investigation the influence of the incubation temperature in the range 30 to 37 C on gross and confirmed counts of typical colonies in MacConkey type agars was therefore also studied.

MATERIALS AND METHODS

Pure cultures

The pure cultures studied in this investigation originated from the collection of the Laboratory of Microbiology, Department of the Science of Food of Animal Origin. They consisted of *Escherichia coli*, *Klebsiella* sp., *Salmonella typhimurium* and *Shigella sonnei* cultures. Before use they were streaked on lactose lauryl sulfate agar (11) and incubated overnight at 37 C. After their mode of attack on lactose had been verified, their key properties were determined by deep stabbing into gram negative diagnostic tubes (36).

Minced meat samples

One hundred and twenty samples of minced meat were obtained from 10 different butcher's shops within 11 months. The minced meat was composed of approximately equal parts of pork and beef.

Samples of drip from frozen broilers

Ten samples from each of 10 different consignments of frozen broilers were defrosted for about 24 h at 4 ± 2 C. Thereupon the packs were hand-pressed (33) and about 5 ml of drip were withdrawn using a sterile syringe.

Culture media

In the pure culture studies two non-selective media, tryptone glucose yeast extract agar (Difco¹) and tryptone soya peptone agar (Oxoid¹) were used. The media were prepared following the manufacturers' instructions, and after sterilization, tempered at 47-51 C to prepare poured plates.

The violet red bile agars used in this investigation originated from four different manufacturers. They will be indicated as A, B, C and D. Media A and B contained 10 g of lactose/l (VRBL), and a further 10 g of glucose/l was added, as done previously (VRBLG). Medium C contained 10 g of glucose/l only (VRBG). From brand D two different formulae were tested, D₁ the lactose-containing formula with glucose added (VRBLG) and D₂ the medium containing glucose only (VRBG). For the remainder, the media were identical in composition, containing per 1 l of distilled water: peptone, 7 g; yeast extract, 3 g; NaCl, 5 g; bile salts, 1.5 g; neutral red, 30 mg; crystal violet, 2 mg; and agar 15 g.

All media were prepared by bringing to a boil to dissolve the ingredients, and were not further sterilized. One brand, chosen at random (A), was also tested after sterilization for 15 min at 121 C. This medium is indicated as A(S).

Plating procedure

Pure cultures were incubated overnight at 37 C on nutrient agar (Oxoid). From these a series of decimal dilutions in peptone-saline solution was prepared in such a way, that at least one could be expected to contain 150 ± 50 cfu/ml. As a rule, three successive decimal dilutions had to be examined to attain this objective.

Minced meat samples were homogenized in 25-g portions with 250 ml of peptone-saline solution in a "stomacher" and, after allowing resuscitation of stressed cells for 2 h at room temperature (20,30,49,50,51,52,59), further decimal dilutions up to 10^{-4} were prepared. A series of three dilutions was examined in every experiment, again with the purpose of obtaining at least one series of duplicate plates containing approximately 150 colonies.

Broiler drip was kept for 2 h at room temperature before dilutions were made, once more to allow resuscitation of cells injured by freezing.

One-ml portions of dilutions of pure cultures, minced meat and drip were transferred to 9-cm petri dishes, thoroughly mixed with 15 ml of tempered agar, and allowed to solidify. Subsequently the plates were overlaid with 10 ml of sterile medium, tempered at 47-51 C. Incubation was done at 30 or 37 C for 18-20 h.

RESULTS

Pure culture studies

The colony counts obtained with the various media have been summarized in Table 1. An assessment of the types of colonies is presented in Table 2. This criterion is of some relevance since at one time it was thought that the diameter of the colony and of the precipitation zone around it could help in recognition of particular *Enterobacteriaceae* types (19).

It is quite obvious that considerable differences in performance of the various media occurred. The differences in the numbers of colonies remained within reasonable limits, although the recovery was usually maximal in D₁ and D₂. Considerable differences in colony size, variation in colony size and in particular the character of the precipitation zones were observed. The media D₁ and D₂ were markedly better than the other brands. The type of carbon source, lactose + glucose or only glucose did not influence the counts or colony types.

TABLE 1. Recovery of pure cultures of *Enterobacteriaceae* in a variety of MacConkey-type selective agar media.

Strain	Number of replicates	Recovery, in vertical order min., max., mean, as fraction of colonies obtained from same inoculum in the non selective reference agar medium					
		Medium and type of sugar					
		A ^a L + G ^b	A (S) L + G	B L + G	C G	D ₁ L + G	D ₂ G
<i>Escherichia coli</i>	6			0.54	0.59	0.83	0.65
		NT	NT	0.83	0.90	1.02	0.92
				0.68	0.78	0.93	0.76
<i>Klebsiella</i>	4	0.42	—	0.30	0.24	0.49	0.43
		0.42	—	0.64	0.57	0.50	0.50
		0.42	0.31	0.47	0.41	0.50	0.46
<i>Salmonella typhimurium</i>	6	NT	NT	0.48	0.41	0.88	0.81
				0.84	0.90	1.15	0.91
				0.75	0.65	0.99	0.86
<i>Shigella sonnei</i>	4	0.85	—	1.09	0.83	1.02	0.87
		0.95	—	1.16	0.96	1.12	1.11
		0.89	0.98	1.13	0.90	1.07	0.99

^aA-D = different manufacturers (cf. text), (S) = medium sterilized instead of pasteurized, as is customary.

^bL = lactose, G = glucose, NT = not tested.

TABLE 2. Character of colonies obtained from pure cultures in a variety of MacConkey-type selective agar media.^a

Strain	Medium and type of sugar					
	A L + G	A (S) L + G	B L + G	C G	D ₁ L + G	D ₂ G
<i>Escherichia coli</i>			0.60	0.70	0.85	1.00
	NT	NT	0.50	0.50	1.0	0.87
			2	1	3	3
<i>Klebsiella</i>	0.50	0.50	0.75	0.50	1.0	0.75
	0	0	0.50	0	0.50	0.62
	0	0	2	0	3	2
<i>Salmonella typhimurium</i>			0.95	0.65	1.10	1.12
	NT	NT	1.2	0.50	1.0	1.50
			2	0	3	3
<i>Shigella sonnei</i>	0.75	0.75	1.0	1.0	1.3	1.12
	0	0.50	0.60	0.50	0.80	0.50
	0	0.50	2	1	3	2

^aParameter combination: colony size, average, mm;/ precipitation zone, do.:/quality of precipitation zone, expressed in 3 = excellent, 2 = good, 1 = fair, 0 = absent.

Consequently another medium component, most probably the bile preparation (5,7,37,39) influenced performance of the MacConkey-type media.

Lactose versus glucose at 30 and 37 C

To study the effect of lactose in comparison to that of glucose, agars purchased from manufacturer D were used in all experiments, because the pure culture studies reported in the previous section indicated that much more easily countable colonies were obtained from these agars. In Tables 3 and 4 the data obtained with VRBG and VRBL are recorded. Minced meat was used as the inoculum. Colony counts were confirmed by testing the square root of the total number of colonies obtained, chosen at random, for the criteria of *Enterobacteriaceae*: negative oxidase reaction and fermentative attack on glucose. Three-layer gram negative diagnostic tubes, as described elsewhere (36) were used for this purpose.

TABLE 3. Effect of glucose on the recovery of *Enterobacteriaceae* from minced meat using VRB agars^a incubated at 37 C.

Percentage of the samples	log ₁₀ cfu _{VRBG} - log ₁₀ cfu _{VRBL}
14	0.00
22	0.00-0.10
53	0.11-0.50
11	> 0.50

Av. log₁₀cfu_{VRBG net} = 3.61; av. log₁₀cfu_{VRBL net} = 3.53.

^aVRBG = violet red bile glucose agar; VRBL = violet red bile lactose agar; net = confirmed as *Enterobacteriaceae*.

TABLE 4. Effect of glucose on the recovery of *Enterobacteriaceae* from minced meat using VRB-agars incubated at 30 C.

Percentage of the samples	log ₁₀ cfu _{VRBG} - log ₁₀ cfu _{VRBL}
6	0.00
19	0.00-0.10
41	0.11-0.50
34	> 0.50

Av. log₁₀cfu_{VRBG net} = 4.26; av. log₁₀cfu_{VRBL net} = 3.92.
Difference 0.34.

In analyzing the data, the results of earlier studies on reproducibility of counts in MacConkey type agars (27,38) were used as the base line. These had indicated that the confidence interval of such counts was of the order of a factor 2; therefore differences below 0.3 log₁₀ were not considered as significant. Applying this approach to the data in Tables 3 and 4 it appears that net counts at 37 C did not depend on the carbon source, but those at 30 C did.

The figures in Tables 3 and 4 also indicate that net recoveries at the lower temperature are considerably higher than at 37 C. This is further analyzed in Tables 5 and 6. The differences between confirmed counts at 30 and 37 C were greater in VRBG than in VRBL.

As indicated earlier, we had observed in preliminary studies that the rate of confirmation as *Enterobacteriaceae* of typical colonies in MacConkey-type agars increased with increasing temperature of incubation. The data obtained in this investigation were analyzed for this effect in Tables 7-10. Such a difference was not

TABLE 5. Influence of the incubation temperature on the recovery of *Enterobacteriaceae* from minced meat using VRBG-agar.

Percentage of the samples	log ₁₀ cfu _{30 C} - log ₁₀ cfu _{37 C}
5	0.00
9	0.00-0.10
41	0.10-0.50
45	> 0.50

Av. log₁₀cfu_{30 C net} = 4.26; av. log₁₀cfu_{37 C net} = 3.61.
Difference 0.65.

TABLE 6. Influence of the incubation temperature on the recovery of *Enterobacteriaceae* from minced meat using VRBL-agar.

Percentage of the samples	log ₁₀ cfu _{30 C} - log ₁₀ cfu _{37 C}
15	0.00
23	0.00-0.10
36	0.10-0.50
26	> 0.50

Av. log₁₀cfu_{30 C net} = 3.92; av. log₁₀cfu_{37 C net} = 3.53.
Difference 0.39

TABLE 7. Confirmation rate of typical colonies obtained from minced meat using VRBG-agar incubated at 37 C.

Percentage of the samples	log ₁₀ cfu _{gross} - log ₁₀ cfu _{net}
71	0.00
5	0.01-0.05
5	0.06-0.10
19	0.10 max.

Av. log₁₀cfu_{net} = 3.61; av. log₁₀cfu_{gross} = 3.76.
Difference 0.15

TABLE 8. Confirmation rate of typical colonies obtained from minced meat using VRBG-agar incubated at 30 C.

Percentage of the samples	log ₁₀ cfu _{gross} - log ₁₀ cfu _{net}
75	0.00
6	0.01-0.05
6	0.06-0.10
13	0.10 max.

Av. log₁₀cfu_{net} = 4.26; av. log₁₀cfu_{gross} = 4.31.

TABLE 9. Confirmation rate of typical colonies obtained from minced meat using VRBL-agar incubated at 37 C.

Percentage of the samples	log ₁₀ cfu _{gross} - log ₁₀ cfu _{net}
86	0.00
2	0.01-0.05
7	0.06-0.10
5	> 0.10

Av. log₁₀cfu_{net} = 3.53; av. log₁₀cfu_{gross} = 3.55.

TABLE 10. Confirmation rate of typical colonies obtained from minced meat using VRBL-agar incubated at 30 C.

Percentage of the samples	log ₁₀ cfu _{gross} - log ₁₀ cfu _{net}
79	0.00
2	0.01-0.05
12	0.06-0.10
7	> 0.10

Av. log₁₀cfu_{net} = 3.91; av. log₁₀cfu_{gross} = 3.94.

observed in any of the data, the confirmation rates of typical colonies being very high in all instances. This disagreement with the results of previous studies may be caused by two factors. First, the earlier investigations had been carried out with quite different MacConkey-type agar formulae; as we have observed that some of such media lead to poorly discernible colonies, our previous results may have suffered from this shortcoming of the media used. In addition, minced meat might contain less interfering types of organisms than the commodities studied earlier, which were mainly dried foods.

Lactose plus glucose versus glucose at 30 and 37 C

The results of the experiments with pure cultures recorded in Tables 1 and 2, point to the absence of any influence of the use of glucose alone or in combination with lactose on the productivity of MacConkey-type agars as expected. This required yet further substantiation by the use of the two types of media for examination of foods containing considerable numbers of *Enterobacteriaceae*. Minced meat and chicken drip were chosen as such substrates.

The data obtained with minced meat are summarized in Table 11. Once more, considering differences below $\log_{10} = 0.30$ as non-significant because within the confidence interval of the mode of examination, the results for glucose and glucose plus lactose were identical. Again, the differences between net and gross counts were negligible. And, once more the counts at 30 C were higher than those at 37 C.

TABLE 11. The influence of carbon source and incubation temperature on the recovery of *Enterobacteriaceae* from minced meats.

Incubation temperature (C)	Gross (\log_{10} cfu/g)		Confirmed (\log_{10} cfu/g)	
	VRBLG	VRBG	VRBLG	VRBG
30	4.46	4.42	4.37	4.40
37	4.14	4.11	4.14	3.90

The results of the examination of the samples of broiler drip are summarized in Table 12. In agreement with the observations made on minced meat, gross and confirmed counts did not differ, and the carbon source did not exert any influence. However, with broiler drip the temperature of incubation did not influence the colony counts. We had observed earlier that the types of *Enterobacteriaceae* isolated from minced meat were quite different from those present in chicken drip. Among the latter *Escherichia coli* seemed to predominate, whereas from minced meat mostly psychrotrophic types were isolated. To investigate this matter in more detail, 72 colonies taken at random from plates of minced meat, and 68 picked similarly from plates of chicken drip were identified. The following procedure was used for this purpose: (a) the three layer tubes referred to earlier, allowing assessment of oxidase reaction, mode of attack on glucose, motility and formation of H_2S and indole; (b) slants of Simmons citrate agar, DNA agar, urea agar and gelatin agar. The

TABLE 12. The effects of carbon source and incubation temperature on the recovery of *Enterobacteriaceae* from broiler drip.

Incubation temperature (C)	Gross (\log_{10} cfu/g)		Confirmed (\log_{10} cfu/g)	
	VRBLG	VRBG	VRBLG	VRBG
30	1.97	2.03	1.97	2.03
37	1.96	1.94	1.96	1.94

results obtained are presented in Table 13. Over 75% of the isolates obtained from minced meat were typical psychrotrophic types. These are mostly considered to be primarily of aqueous or vegetable origin. Psychrotrophic types occurred only to a total of 30% in chicken drip, with *E. coli* predominating there. It is therefore not surprising that *Enterobacteriaceae* counts in chicken were almost equal at 37 C and 30 C, but higher at the lower temperature when minced meat was examined.

TABLE 13. Types of *Enterobacteriaceae* isolated from minced meat and drip from frozen broilers in violet red bile media at 30 C.

Species or genus ^a	Percentage of isolates from	
	Minced meat	Broiler drip
<i>Serratia liquefaciens</i> ^b	47	12
<i>Escherichia coli</i> ^b	9	35
<i>Enterobacter</i>	21	6
<i>Erwinia</i>	10	12
<i>Hafnia</i>	7	13
<i>Citrobacter</i>	4	13
<i>Klebsiella</i>	< 1	6
<i>Proteus</i>	< 1	3

^aThe order is that of decreasing summated frequency.

^bIncluding a minority of irregular types.

DISCUSSION

The investigations reported in this paper substantiate observations made repeatedly in the past.

First, we have demonstrated once more that indiscriminate use of dried formulae of MacConkey-type agars may lead to drastically reduced recoveries of *Enterobacteriaceae* and hence to serious underestimation of health or spoilage risks in foods. This confirms observations on similar and related media by numerous authors (3,13,22,23,26,34,43,53,54,55,57,58). These results call for constant monitoring of purchased dried culture media. Such testing should include (a) pure culture studies to assess productivity, and (b) practical challenge tests to estimate selectivity (31).

Furthermore, the possible influence of the incubation temperature on the results of testing for *Enterobacteriaceae* has been demonstrated once more. Incubation at ca. 30 C may lead to consistently higher results than at 37 C, as could be anticipated from physiological studies and ecological surveys carried out many years ago. The consequence of these observations for ecological studies are obvious. However, for practical monitoring the inference is less strict. Results of the bacteriological examination of foods depend strongly on the method by which they were obtained. Consequently Reference Values (35) carry little weight, unless accompanied by prescribed methods. Hence, any of the temperatures customarily used so far for enumeration of *Entero-*

bacteriaceae may be applied, provided Reference Values are adapted accordingly.

One new, although barely surprising, element emerged from our investigation. Using glucose or glucose plus lactose in MacConkey-type media does not in the least influence confirmed counts of *Enterobacteriaceae* at a given temperature. However, the composition of the media in other respects is of great significance. Particularly characteristics of the bile preparations may result in dramatic differences in recoveries (5,7,37,39). For practical purposes the conclusion is obvious: glucose-base media can be used in confidence, provided the bile preparation to be incorporated has been carefully selected by the manufacturer. Nevertheless it remains an obligation of the bacteriologist to check every new batch purchased from suppliers for productivity and selectivity, even when a manufacturer has previously been found to be offering satisfactory media.

With regard to the fundamental concept of replacing lactose by glucose, the data in Table 4 substantiate earlier findings (11) that this may lead to higher counts. However, this is not always the case, as the data in Table 3 show - for the simple reason that lactose-negative types of *Enterobacteriaceae* do not always occur in large numbers. Actually the types of *Enterobacteriaceae* colonizing proteinaceous staples may vary greatly, as the data in Table 13 and those of other research teams (9,47) clearly indicate. But in no instance will glucose media lead to lower results, as this study and other investigations (8,16) demonstrated beyond any doubt. Therefore, use of glucose-containing MacConkey-type media will result in data of considerably better consistency than those reported for similar media based on lactose (2,48).

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Penicillic Acid Production by Some Fungal Species in Relation to Water Activity and Temperature

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ABSTRACT

The combined effects of water activity (a_w) and temperature on growth of and penicillic acid (PA) production by strains of *Penicillium cyclopium*, *Penicillium martensii*, and *Aspergillus ochraceus* were determined. On malt agar media in which the a_w had been adjusted by addition of sucrose or glycerol, the minimum a_w for PA production by *P. cyclopium* and *A. ochraceus* was 0.97 and that of *P. martensii* was 0.99. The temperature range for PA production by *P. cyclopium* and *P. martensii* was 4-31 C, whereas that of *A. ochraceus* was 8-31 C. Optimum temperature for PA production by *P. cyclopium* and *A. ochraceus* varied with the strain tested and was 24-31 C. The only strain of *P. martensii* tested showed an optimum temperature of 16-24 C. On Gouda and Tilsiter cheese of 0.96-0.98 a_w , temperature ranges for growth of *P. cyclopium*, a common mold on cheese, were 0-24 and 4-16 C, respectively. When a strain of *P. cyclopium* known to be able to produce PA in culture media, was grown on Gouda cheese incubated at different temperatures, no PA was detectable in the moldy cheese at the time the average colony diameter was 30 mm. However, in a culture on Gouda cheese incubated for a prolonged time (42 days) at 16 C, PA was detectable. On poultry feed, *A. ochraceus* produced PA at a_w as low as 0.88, whereas the minimum a_w for PA production by *P. cyclopium* was 0.97.

Penicillic acid (PA) is a toxic fungal metabolite synthesized by a number of *penicillia* and *aspergilli*. The oral toxicity is low, but the toxin may be present in moldy foods in large quantities. Oral administration of lethal doses of PA induced fatty liver degeneration in quail and liver cell necrosis in mice (11). Rats injected subcutaneously with PA showed sarcomas (14).

PA has been detected in commercial corn and beans (29). Harvesting of high-moisture corn by a picker-sheller may cause considerable damage to the kernels, which favors growth of molds. The growth is apparent from a blue-green discoloration of the germ. Various *Penicillium* spp. are reported to cause this so-called blue-eye (8). Kurtzman and Ciegler (15) found that in blue-eye corn invaded by *Penicillium martensii*, large quantities of PA had been produced after it had been stored at high-water content and at low temperatures. *Penicillium cyclopium* is another PA producer associated with blue-eye disease of corn (8). Moreover, *P. cyclopium* was found to be the predominant mold in moist stored barley (19), dried beans (18), and cheese in warehouses or

on display in shops (23). Bullerman and Olivigni (4) isolated PA-producing *Penicillium* spp. from Cheddar cheese. In another study, Swiss cheese showing visible mold after incubation at low temperature contained small amounts of toxin (5). *Penicillium* cultures that were part of the fungal flora of mold-fermented dry sausage (salami), synthesized the toxin in liquid media. However, no PA was found in the sausage, presumably because of reaction of the toxin with certain amino acids (11).

Another PA producing mold frequently isolated from foodstuffs in Japan is *Aspergillus ochraceus* (25). This species invaded grain with a water content of more than 16% (6) and was found to be one of the predominant fungi of red and black peppers (7). *A. ochraceus* isolated from bread showed PA production when tested on laboratory media (3).

The most important factors determining fungal growth and therefore toxin production are temperature and water activity. Water activity (a_w) is defined as the equilibrium relative humidity of a substrate (27). The effect of temperature on production of PA has been studied by various investigators (12,15,17). However, quantitatively little is known about the influence of a_w on production of PA.

The purpose of this study was to determine the combined effects of temperature and a_w on growth of and PA production by some fungal species. Malt extract agar media with different a_w values obtained by adding sucrose or glycerol were used. Moreover, some natural substrates were inoculated to compare the results with those on malt extract agar media. *P. cyclopium* was cultivated on different types of hard cheese, whereas *A. ochraceus* was cultivated on poultry feed of different a_w values.

MATERIALS AND METHODS

Organisms and spore suspensions

Fungal strains were maintained as lyophilized cultures. *P. cyclopium* RIV 232, *A. ochraceus* RIV 86, two strains of *Penicillium notatum*, three strains of *Penicillium palitans* and three strains of *Penicillium roqueforti* originated from different kinds of moldy foods. Another 34 and 48 strains of *P. cyclopium* were isolated from moldy cheese from shops and warehouses, respectively. *P. cyclopium* RIV 127, *Penicillium viridicatum* RIV 80, and *A. ochraceus* RIV 45 had been received from Dr. J. Harwig, Health and Welfare Canada, Health Protection Branch, Ottawa and were originally labelled with the numbers 553, 183 and 132, respectively. *A. ochraceus* RIV 1215 had been received from Dr. C. W.

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Bacon, Richard B. Russell Agricultural Research Center, Athens, Georgia and was originally labeled with the number 107. A *P. cyclopium* isolate from a shelf in a cheese warehouse had been supplied by Mr. E. de Boer, Gist-Brocades, Delft, The Netherlands. Other strains of *Penicillium chrysogenum*, *Penicillium crustosum*, *P. cyclopium*, *P. martensii*, *P. notatum*, *Penicillium palitans*, *P. roqueforti*, and *Penicillium viridicatum* had been received from Dr. R. A. Samson, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. *P. cyclopium* isolates from cheese were determined according to Stolk and Samson (28) and Samson et al. (26). The identity of other *P. cyclopium* strains and other *Penicillium* species were checked according to Raper and Thom (24).

Cultures grown for 7-10 days at 24 C on malt extract agar (Oxoid) were washed with an aqueous solution of 6 g of sodium heptadecylsulphate (Tergitol-7, BDH)/l to prepare spore suspensions of ca. 10^6 spores per ml.

After the desired periods of incubation, cultures of these organisms on the substrate described below were extracted for PA analysis either immediately or after storage at -18 C for a maximum of 7 days.

Cultures on agar media

In a preliminary study, mold strains were tested for PA production at 24 C on two agar plates of each of the following media: malt extract agar medium (Oxoid) (ca. 1.00 a_w , pH 5.4, ME), malt extract agar medium (Oxoid) supplemented with 20 g of yeast extract/l (Oxoid) and 100 g of sucrose/l (0.99 a_w , pH 6.0, MEYS), 10 g of peptone/l (Oxoid) - 20 g of dextrose/l - agar medium (ca. 1.00 a_w , pH 6.2, PD), and minerals-tartrate-dextrose agar medium according to Bentley and Keil (2) (ca. 1.00 a_w , pH 3.9, MTD). Each plate was inoculated at three different spots with an inoculation needle dipped in a spore suspension. Incubation was terminated as soon as the colonies reached an average diameter of 30 mm.

High PA-producing strains of each fungal species, i.e. *P. cyclopium* CBS 434.73, *P. cyclopium* RIV 129, *P. martensii* RIV 159, *A. ochraceus* NRRL 5175, and *A. ochraceus* RIV 1215, were used to determine the environmental conditions for PA production. Various a_w conditions in malt extract agar were achieved by adding sucrose (MES-series) or glycerol (MEG-series). Preparations of agar plates, inoculation and a_w measurement have been described in a previous paper (20). The a_w measurement device consisted of a sample jar that was submerged in a temperature controlled water bath, thus facilitating equilibration of the vapor pressure around the sample, and was connected to a dew-point meter through a closed circuit. The accuracy of the device was 0.005 a_w . All measurements were done at 24 C. For each determination of rate of growth and PA production, two inoculated agar plates with three colonies each and one non-inoculated agar plate, which served as control for determination of the a_w after incubation, were used. They were enclosed in a 0.3-liter polyethylene bag (gauge, 0.04 mm). Cultures were grown at combinations of eight temperatures and six a_w values on MES or MEG. The temperature variation was 0.3 C. The growth rate of the mycelium was determined by daily measurement of two right-angled diameters of the colonies. The regression lines of colony diameter on days after inoculation were calculated for each a_w -temperature combination. The germination time was obtained by extrapolating the regression line to the X-axis (20). Where rates of growth were decreasing during incubation, initial rates of growth obtained by drawing the tangent are given. Incubation was terminated on the day the six colonies reached an average diameter of 30 mm or on the 35 days of incubation when they did not reach this size.

To assess whether or not the strains tested were representative of the mold species, the limiting a_w and temperature for growth and PA production were determined for some other strains of *P. cyclopium* and *A. ochraceus* which were grown on MES at the same time as the test strains.

To investigate whether a decrease of rate of growth might be due to lack of oxygen, two series of cultures of *P. cyclopium* RIV 129 were grown at combinations of different temperatures and water activities. In one series, cultures were enclosed in polyethylene bags until the colonies reached 30 mm in diameter according to the method described above, whereas in the other series the cultures were aerated daily by

opening the bag, replacing the air in the bag with fresh air, and closing it again.

Four replicate MES cultures of *P. cyclopium* CBS 434.73, grown at 0.99 a_w and 24 C, were analyzed for PA to determine the repeatability of the method.

To determine the effect of substrate on growth and PA production, MTD agar plates supplemented with dextrose yielding 0.99 a_w were inoculated with spores of *P. cyclopium* CBS 434.73. Incubation, storage, and extraction were as for MES or MEG cultures.

Screening *P. cyclopium* isolates from cheese for toxin production

Spore suspensions of *P. cyclopium* strains isolated from cheese and of a PA-producing strain of *P. cyclopium* isolated by de Boer from a warehouse shelf (13) were used to inoculate MTD agar plates. Two plates of agar medium were inoculated at three different spots with a needle covered with spores and then were incubated at 24 C for 14 days. The strains from warehouses were also screened for PA production by the method of de Boer (13). One hundred ml of a medium with 20 g of yeast extract (Oxoid)/l and 150 g of sucrose/l in 500-ml flasks were inoculated with 1 ml of a spore suspension and incubated as stationary cultures at 24 C for 9 days.

P. cyclopium cultures on cheese

In the first experiment, growth rate of and PA production by *P. cyclopium* CBS 434.73 was measured on Gouda cheese of 0.98 a_w and on Tilsiter cheese of 0.96 a_w . The 8-day-old Gouda cheese of 5.2 kg was obtained from a warehouse, whereas the Tilsiter cheese of 1.4 kg of unknown age was obtained from a shop. The cheeses were aseptically cut in slices of 40 × 40 × 4 mm. To obtain slices of similar a_w value, the central part and the outer layer of the cylindrical Gouda cheese and the outer layer of the brick-shaped Tilsiter cheese were discarded. The cultural method was similar to that of the agar cultures; for each determination of rate of growth and PA production, two inoculated slices of cheese with three colonies each and one non-inoculated control slice of cheese, were used. They were placed in three petri dishes, which were enclosed in a polyethylene bag. Cultures were incubated at six different temperatures until the six colonies reached an average diameter of 30 mm or until the 42nd day when they did not reach this size. Other incubated slices of Gouda cheese were incubated at 16 C for 20, 27, 34, and 42 days and of Tilsiter cheese for 30, 37, and 44 days; these colonies grew beyond 30 mm diameter.

A second experiment was carried out with potential PA-producing *P. cyclopium* strains RIV 212, CBS 161.42, NRRL 1888, as well as CBS 434.73. Seven-day-old Gouda cheese was obtained from a warehouse, stored at 4 C for 7 days, cut into slices and treated as described for the first cheese experiment. The a_w of the slices was 0.97. Inoculated slices were incubated at 16 C for 14, 28 and 42 days.

A. ochraceus cultures on poultry feed

Complete feed meal for laying hens was blended with water to a water content of 200, 260, 320, 380, 440, 500, and 620 g/kg (wet weight basis), giving after autoclaving water activities of 0.88, 0.93, 0.96, 0.97, 0.975, 0.98 and 0.99, respectively. The feed was autoclaved at 121 C for 1 h, thoroughly mixed, stored for 5 days and pressed into petri dishes to a thickness of 5 mm. Each plate was inoculated at three different spots with 1 μ l of a spore suspension of *A. ochraceus* RIV 1215. Feed plates of 0.99 a_w were incubated at eight different temperatures, whereas feed plates of 0.88, 0.93, 0.96, 0.97, 0.975, and 0.98 were incubated only at 24 C. In another experiment, feed plates of 0.95, 0.97, and 0.99 were inoculated with *P. cyclopium* CBS 434.73 and incubated at 24 C. For each a_w -temperature combination two inoculated plates and one non-inoculated control plate were enclosed in a polyethylene bag.

The method for determination of growth rate and germination time of mycelium was the same as that for cultures on agar. When the average diameter of the colonies reached 11, 17, 22, and 26 mm respectively, the bag was opened a short time to aerate the cultures. Incubation was terminated as soon as the colonies reached an average diameter of 30 mm. Total protein and amino acid content of the feed were determined by Mr. K. Terpstra, Spelderholt Institute for Poultry Research, Beekbergen, The Netherlands.

Extraction and determination of PA from agar and liquid media

After thawing, cultures were inactivated by adding 20 ml of chloroform to each pair of plates. The chloroform was allowed to stand in contact with the culture for at least 2 h at room temperature. For qualitative analyses, 20 μ l of the chloroform solution was spotted on a thin layer chromatography (TLC) plate next to a PA standard. The plate was developed in toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol) in an unsaturated tank, dried, exposed to ammonia vapor for 10 min and then held at 100 C for 2 min. The presence of PA was indicated by a blue fluorescent spot on the plate when exposed to long wave UV light at 365 nm (9).

For quantitative analysis, the chloroform was evaporated and the contents of the two inoculated agar plates were blended in 75 ml of H₂O by means of an homogenizer (Ultra Turrax). After centrifugation, 5 ml of 0.5 mol H₂SO₄/l was added to 5 ml of the supernatant fluid and the mixture was extracted twice with 50 ml of chloroform. The collected chloroform extracts were dried over Na₂SO₄, evaporated to dryness and the residue dissolved in 1 ml of chloroform. After semi-quantitative determination of the PA concentration by TLC, the sample solution was diluted to the desired concentration. Sample extract and PA standard were spotted next to each other on a silica gel TLC plate (Merck 60) with a thickness of 0.25 mm and developed in toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol) in an unsaturated tank. After development, the plate was exposed to ammonia vapor for 10 min and held at 100 C for 2 min.

The resulting blue fluorescent spot of PA was measured densitometrically by fluorescence measurement (excitation wavelength 365 nm, emission wave-length 430 nm). The limit of detection was ca. 0.1 mg of penicillic acid per two agar plates.

Extraction and determination of PA from cheese

Quantitative analysis was preceded by qualitative analysis of mycelium scrapings. Mycelium of six colonies together with the ca. 0.5-mm upper layer of cheese was suspended in 2 ml of chloroform. After at least 2 h at room temperature, 20 μ l of the chloroform extract were spotted on a TLC plate and the same procedure was carried out as for the qualitative check for PA on agar plates. If PA was detected in the mycelium scrapings, these were recombined with the scraped cheese slices and the extraction procedure was carried out as follows: 10 g of cheese were blended in 50 ml of acetonitrile and 1.5 ml of concentrated formic acid (5) by means of an Ultra-Turrax. After filtration through glass wool, the mixture was shaken twice with 50 ml of n-hexane in a separatory funnel. The n-hexane phase was discarded. Then 25 ml of water were added to the remaining solution and the water layer was extracted with 50 and 25 ml of chloroform, successively. The chloroform extracts were collected and extracted with 50 ml of a 30 g NaHCO₃/l solution in water. The NaHCO₃ solution was shaken with 10 ml of chloroform, which was discarded. The water phase was acidified to ca. pH 1 with 2 mol H₂SO₄/l and then extracted three times with 50 ml of chloroform. The collected chloroform extracts were dried over Na₂SO₄, evaporated to dryness and the residue was dissolved in 1 ml of chloroform. After preliminary visual determination of the PA concentration by one-dimensional TLC, the sample solution was diluted to the desired concentration and 5 μ l of the extract was spotted on a 66 x 66-mm Merck 60 TLC plate (thickness 0.25 mm), and developed two-dimensionally in diethyl ether (first direction) and toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol, second direction), both times in an unsaturated tank. Ammonia fumigation, heat treatment and densitometric measurement were carried out as for quantitative analysis of agar cultures. The limit of detection was ca. 2.5 mg/kg of cheese.

Extraction and determination of PA from poultry feed

The amounts of reagents used indicated below are for 50 g of dry weight of feed. They were proportionally increased or decreased when the amount of dry feed to be analyzed varied.

After thawing, 250 ml of acetonitrile and 25 ml of 0.1 mol H₃PO₄/l were added to the two inoculated feed plates. The mixture was blended by means of an Ultra-Turrax, and this was followed by filtration. Fifty ml of filtrate were shaken in a separatory funnel with 50 ml of

acetonitrile, together with 50 ml of a solution of 12.5 g of NaHCO₃/l and 30 g of NaCl/l in water and 50 ml of iso-octane. After mixing, three layers were formed of which the lower water phase was drained and shaken with 50 ml of chloroform. The chloroform phase was discarded and the water layer was acidified with 5 ml of 2 mol H₂SO₄/l and extracted three times with 50 ml of chloroform. The collected chloroform fractions were dried over Na₂SO₄, evaporated to dryness and the residue was dissolved into 0.5 ml of chloroform. TLC determination of PA was carried out as described for the analysis of agar cultures. The detection limit was 2.5 mg/kg of poultry feed.

To assess the effect of water content on the recovery of PA from feed, 3.8 mg of PA were added to four batches of 150 g moistened feed containing 380, 440, 500, and 620 g of water/kg which corresponded to 0.97, 0.975, 0.98, and 0.99 a_w. Extraction and analysis of each batch were done in duplicate.

RESULTS

Cultures on laboratory media

The results of the preliminary study on different agar media are shown in Table 1. PA-producing strains were found in the species of *P. cyclopium*, *P. martensii*, *P. viridicatum*, and *A. ochraceus*, but not in the species of *P. chrysogenum*, *P. crustosum*, *P. notatum*, *P. pallians* and *P. roqueforti*. On MTD agar medium, *P. cyclopium* produced more PA than on ME or PD. Enrichment of ME agar medium by addition of protein-rich yeast extract and sucrose (MEYS) did increase PA production by *P. martensii*, but did not influence PA production by *A. ochraceus*.

Results of the examination for PA production of *P. cyclopium* strains isolated from cheese are shown in Table 2. None of these strains produced PA on MTD agar medium or in YES liquid medium. However, the strain isolated from a cheese shelf produced PA on MTD agar medium but not in YES liquid medium.

Figure 1 shows examples of colony growth curves of cultures of *P. cyclopium*, *P. martensii*, and *A. ochraceus* on agar medium. Under certain conditions of a_w and temperature, the growth rate of *P. cyclopium* and *P. martensii* decreased after a period of time. This phenomenon could not be correlated with a_w or temperature and it occurred very rarely with cultures of *A. ochraceus*.

The average growth rate of colonies and PA production on MES and MEG agar medium at each combination of temperature and initial a_w are shown in Fig. 2-10. For cultures with decreasing growth rate the average initial growth rate is given in the figures. Optimum temperatures for PA production by *P. martensii* were 16-24 C, whereas *A. ochraceus* produced maximum quantities at 24-31 C. Optimum temperatures for PA production by *P. cyclopium* varied with the strain tested: strain CBS 434.73 had an optimum temperature of 24 C, whereas the optimum temperature of strain RIV 129 was 31 C (Fig. 11). Temperature ranges for PA production by *P. cyclopium*, *P. martensii*, and *A. ochraceus* were 4-31, 4-31, and 12-31 C, respectively.

Water activity had a profound effect on PA production. In the Fig. 2-11 all species showed PA production at 0.99 a_w only. However, additional cultures grown on MES at intermediate a_w values (0.96-0.98) showed that *A.*

ochraceus and *P. cyclopium* had a minimum a_w for PA production of 0.97. To demonstrate whether or not PA could have been produced on MES of 0.95 a_w , the possible disappearance of PA was checked by mixing 2 mg of toxin with a MES plate of 0.99 a_w and a MES plate of 0.95 a_w and incubating them for 60 h at 24 C. The recovery of toxin was 84 and 78%, respectively. *A. ochraceus* NRRL 5175, a low producer of PA compared to strain RIV 1215, showed no detectable PA production on MEG agar medium. Germination times of the three species were 0.5-2 days under favorable conditions for growth and they increased to 7-20 days under unfavorable conditions.

In Table 3 it is demonstrated that two other strains of *P. cyclopium* had limiting conditions for growth and PA production similar to strains of which the results are given in Fig. 2, 3, and 10. In contrast to *A. ochraceus* RIV 1215, other strains of *A. ochraceus* showed growth at 8 C and of which some produced PA. Therefore, the temperature range for PA production by *A. ochraceus* should be regarded as 8-31 C.

Fig. 10 and 11 show that there is no effect of aeration on growth; the time of decreased growth rate, noticed under some conditions of a_w and temperature, was quite comparable with those of the non-aerated cultures. However, an effect on PA production was demonstrated; aerated cultures at 4-31 C showed an increased PA

TABLE 2. *Penicillic acid production on different media by Penicillium cyclopium strains isolated from cheese in shops and from cheese and shelf in warehouses.*

Origin	Isolates Number	Medium ¹	
		MTD-agar	YES-liquid
Cheese in shops	34	0/34 ²	NT ³
Cheese in warehouses	48	0/48	0/8
Shelf in warehouse	1	1/1	0/1

¹Incubated at 24 C.

²Number of toxin producing isolates and isolates examined.

³Not tested.

production compared with that of non-aerated cultures.

The PA contents of the four replicate cultures of *P. cyclopium* CBS 434.73 differed by not more than 7% from the average when grown on MES of 0.99 a_w and at 24 C.

In Fig. 12, growth of and PA production by *P. cyclopium* CBS 434.73 on MTD, a mineral agar medium without protein, is shown. Temperature range and optimum temperature equalled those determined for cultures on MES agar medium; however, PA production on MTD was higher than that on MES.

Cultures on natural substrates

Figure 13 shows colony growth rate curves of *P. cyclopium* CBS 434.73 on two types of cheese. Initial rate of growth and PA production are summarized in Fig. 14. The temperature range for growth on Gouda cheese was 0-24 C, whereas on Tilsiter cheese, it was 4-16 C. Neither

TABLE 1. *Penicillic acid production (mg per six colonies) by various strains of different fungal species on various agar media incubated at 24 C.*

Species and strain no.	Agar medium ¹			
	ME	MEYS	PD	MTD
<i>P. cyclopium</i>				
RIV 127	4.6	— ²	0.4	8.0
RIV 156	< 0.1 ³	—	< 0.1	< 0.1
RIV 157	< 0.1	—	< 0.1	< 0.1
RIV 212	0.1	—	< 0.1	6.6
RIV 232	< 0.1	—	< 0.1	< 0.1
CBS 434.73	4.6	—	1.1	27
NRRL 1888	< 0.1	—	< 0.1	37
<i>P. martensii</i>				
RIV 158	0.5	1.3	0.1	—
RIV 159	1.7	11	< 0.1	—
RIV 160	< 0.1	3.4	0.8	—
RIV 161	< 0.1	< 0.1	0.2	—
<i>P. viridicatum</i>				
RIV 80	< 0.1	< 0.1	—	< 0.1
RIV 188	< 0.1	—	—	0.3
RIV 189	< 0.1	—	—	< 0.1
<i>A. ochraceus</i>				
RIV 45	< 0.1	< 0.1	—	—
RIV 86	8.9	9.0	—	—
CBS 263.67	5.8	5.3	—	—
CBS 588.69	< 0.1	< 0.1	—	—
CBS 589.68	19	15	—	—
NRRL 3174	23	29	—	—
NRRL 3519	< 0.1	< 0.1	—	—
NRRL 5175	16	13	—	—
ATCC 18641	32	15	—	—
<i>P. chrysogenum</i>	< 0.1 /2 ⁴	—	< 0.1 /2	—
<i>P. crustosum</i>	< 0.1 /4	—	—	< 0.1 /4
<i>P. notatum</i>	< 0.1 /2	—	< 0.1 /2	—
<i>P. palitans</i>	< 0.1 /8	—	< 0.1 /8	—
<i>P. roqueforti</i>	< 0.1 /4	—	< 0.1 /4	—

¹ME: malt extract agar, MEYS: malt extract yeast extract sucrose agar, PD: pepton dextrose agar, MTD minerals tartrate dextrose agar.

²Not tested.

³Detection level 0.1 mg per 6 colonies.

⁴Number of strains tested.

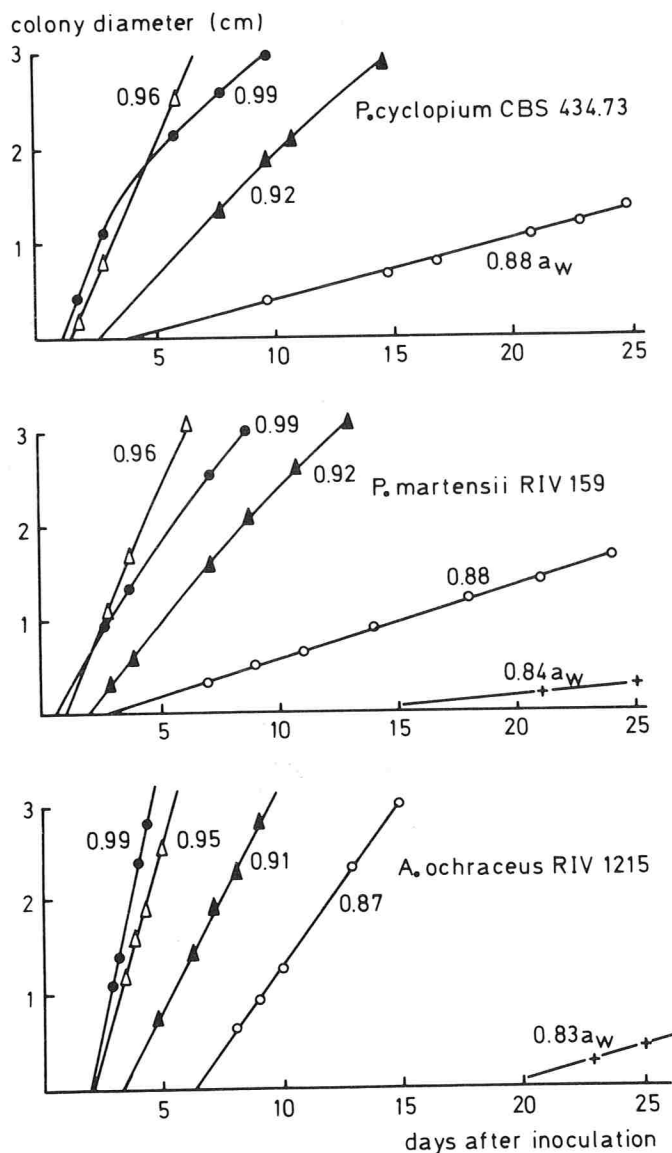


Figure 1. Growth of strains of three different fungal species on malt extract sucrose agar at various water activity levels and 24 C.

cultures on Gouda cheese nor cultures on Tilsiter cheese contained PA when extracted after reaching an average colony diameter of 30 mm. Cultures on Tilsiter cheese which were incubated at 16 C for longer periods up to 44 days, also did not contain PA. However, a culture of six colonies on Gouda cheese incubated for 42 days contained 4 μ g of PA. Results of cultures on Gouda cheese of 0.97 a_w of four potential PA-producing strains of *P. cyclopium* revealed that these strains did not produce PA on Gouda cheese at 16 C after 14, 28 and 42 days of incubation (Table 4).

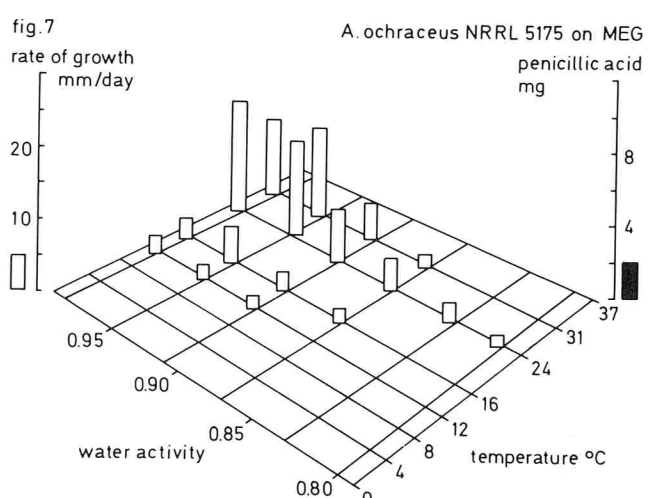
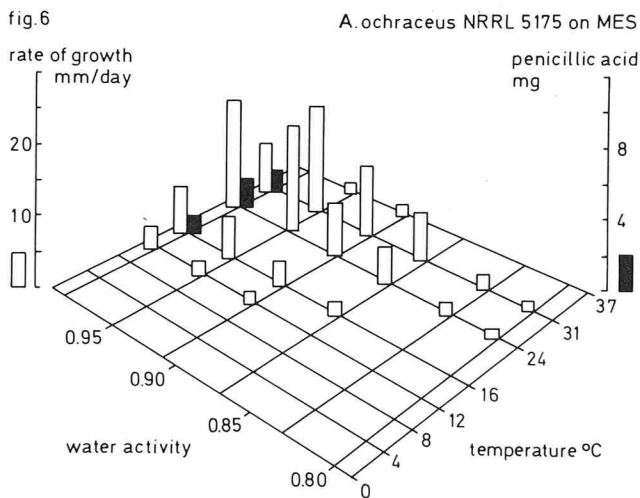
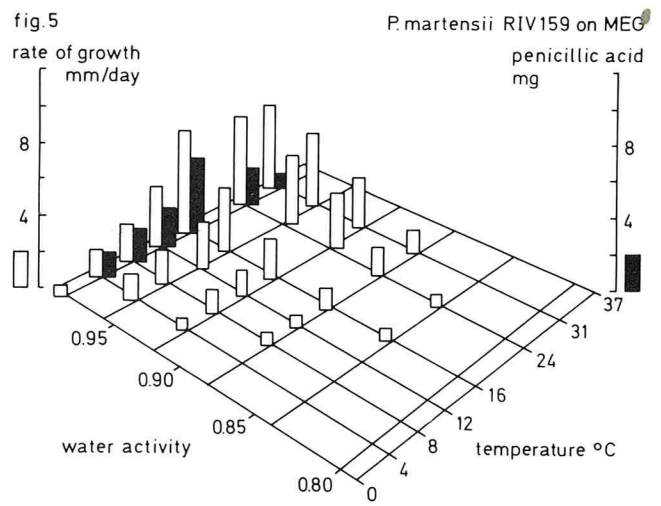
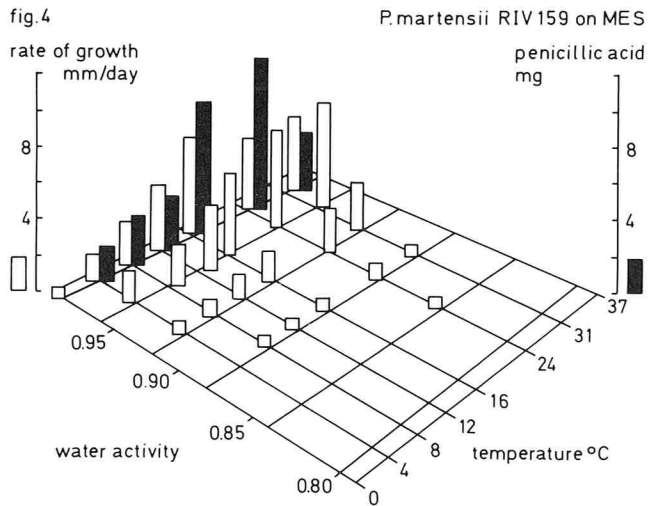
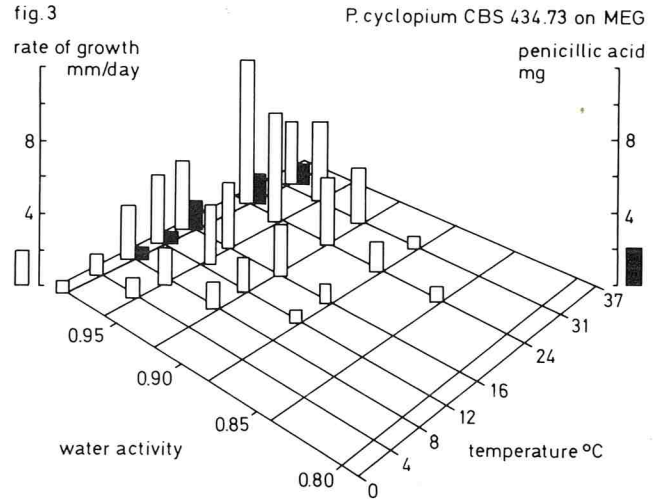
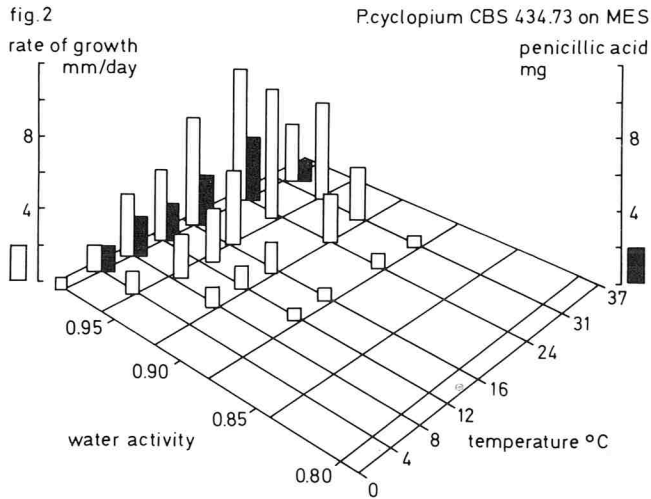
Growth rate of and PA production by *A. ochraceus* on poultry feed are shown in Fig. 15. At 0.99 a_w the temperature range for PA production was 12-24 C. When the water activity was adjusted to lower values, PA production was affected more than growth. PA could be detected at a water activity as low as 0.88. The PA production showed two a_w optima; one optimum at 0.99 a_w and one optimum at 0.975 a_w . This phenomenon was

repeatable and was not caused by the small variation in recovery of PA due to differences in moisture content of feed, because the average recovery of PA added to the batches of feed of 0.97, 0.975, 0.98 and 0.99 a_w was 82, 87, 89, and 92% respectively. The wide a_w range of toxin production could only be demonstrated in *A. ochraceus* RIV 1215 cultures on poultry feed; in another experiment, *P. cyclopium* CBS 434.73 produced toxin in poultry feed at 0.99 and 0.97, but not at 0.95 a_w . Because of the high protein content of poultry feed, reaction of toxin with several amino acids could be expected to result in only little detectable toxin. It was determined that the protein content of feed amounted to 200 g/kg of the dry weight (d.w.), and the total amount of reactive amino acids (11), i.e. cysteine, lysine, histidine, and arginine, was 28 g/kg of d.w. To explain the high amount of toxin found in poultry feed, leaching of toxin from mycelium and reactivity of toxin with poultry feed were examined. In one experiment, it was shown that mycelial pellets obtained from a liquid MES 0.99 a_w shaken culture contained only 0.5% of the initial total amount of toxin after draining the culture medium and washing the pellets with water. In another experiment, 2 mg of toxin was mixed with a plate of poultry feed of 0.99 a_w and a plate of 0.95 a_w and incubated for 60 h at 24 C. The recovery of toxin was 43 and 38% respectively.

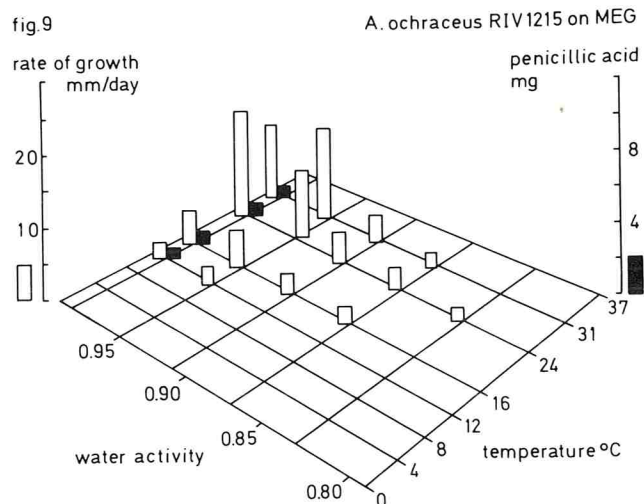
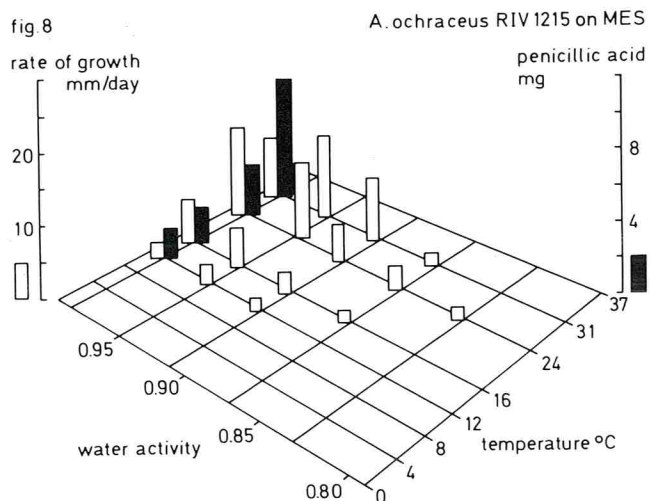
DISCUSSION

The temperature range for PA production almost equalled that for growth of the fungi tested. *P. cyclopium* and *P. martensii* produced PA over a wide temperature range starting at 4 C, whereas *A. ochraceus* produced PA starting at 8 C. The optimum temperature for PA production by *P. cyclopium* and *A. ochraceus* varied with the strain tested. This property was strain-related rather than species-related and had also been demonstrated with aflatoxin-producing strains of *Aspergillus flavus* and patulin-producing strains of *Penicillium patulum* in previous investigations (21,22). Aeration of *P. cyclopium* cultures stimulated PA production, but it did not influence the optimum temperature for PA production, nor the a_w range of PA production. The decrease in growth rate at the end of the incubation period was not abolished by extra supply of air, which indicated that there was no shortage of oxygen in the cultures.

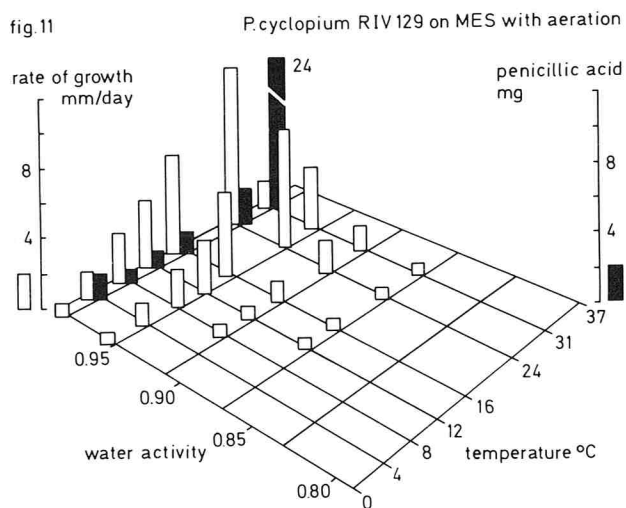
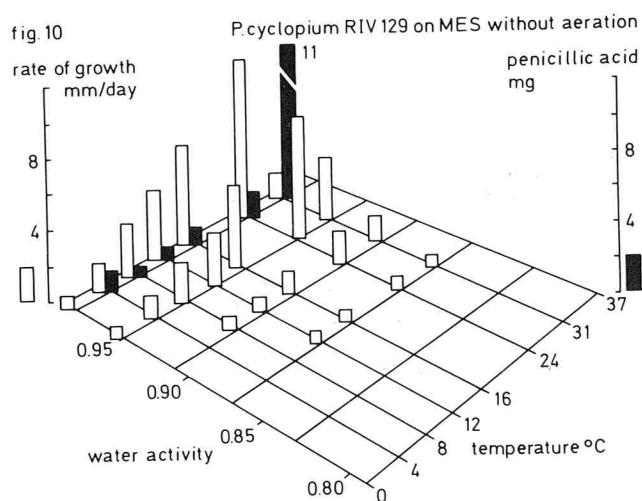
The substrate played an important role in the a_w range for PA production. On MES and MEG, *A. ochraceus* had a narrow a_w range for PA production, similar to those of *P. cyclopium* and *P. martensii*, but it had a wide a_w range on poultry feed, as was also found by Bacon et al. (1). The recovery studies with PA in MES and poultry feed demonstrate that the low amounts of PA found at low a_w could not be explained by an increase of instability of PA, but merely by a depression of the production of PA. The absence of PA in dry sausages ripened by penicillia capable of producing PA (10) should be explained by the impossibility of PA production by penicillia at 0.95 a_w ,



Figures 2-7. Growth of and penicillic acid production by three fungal species on malt extract sucrose agar (MES) and malt extract glycerol agar (MEG) under various conditions of water activity and temperature (the 1 mm black columns represent amounts of 0.1-0.4 mg of penicillic acid).



Figures 8 and 9. Growth of and penicillic acid production by *Aspergillus ochraceus* RIV 1215 on malt extract sucrose agar (MES) and malt extract glycerol agar (MEG) under various conditions of water activity and temperature (the 2 mm black columns represent amounts of 0.1-0.4 mg of penicillic acid).



Figures 10-11. Growth of and penicillic acid production by *Penicillium cyclopium* RIV 129 on malt extract sucrose agar (MES) and malt extract glycerol agar (MEG) under various conditions of water activity and temperature, with and without regular opening of culture bags (the 1 mm black columns represent amounts of 0.1-0.4 mg of penicillic acid).

TABLE 3. Growth of penicillic acid-producing molds and penicillic acid production on malt extract sucrose at unfavourable conditions of a_w and temperature.

Species and strain no.	0.99 a_w						0.95 a_w 24 (C)
	0	4	8	12	24	37(C)	
<i>P. cyclopium</i>							
RIV 127	G ¹	NT ²	NT	NT	G ³	NG ⁴	G-
RIV 129	G-	NT	NT	NT	G+	NG	G-
RIV 196	G-	NT	NT	NT	G+	NG	G-
CBS 434.73	G-	NT	NT	NT	G+	NG	G-
<i>A. ochraceus</i>							
RIV 86	NT	NG	G+	G+	G+	NG	G-
RIV 1215	NT	NG	NG	G+	G+	NG	G-
CBS 589.68	NT	NG	G+	G+	G+	NG	G-
NRRL 3174	NT	NG	G-	G+	G+	NG	G-
ATCC 18641	NT	NG	G-	G+	G+	NG	G-

¹Growth and no penicillic acid detected (< 0.1 mg per 6 colonies).

²Not tested.

³Growth and penicillic acid detected.

⁴No growth.

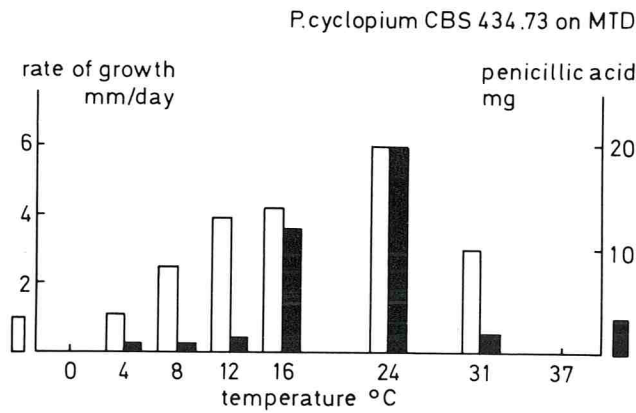


Figure 12. Growth of and penicillic acid production by *Penicillium cyclopium* CBS 434.73 on minerals tartrate dextrose agar (MTD) at different temperatures (the 1 mm black columns represent amounts of 0.1-0.7 mg of penicillic acid).

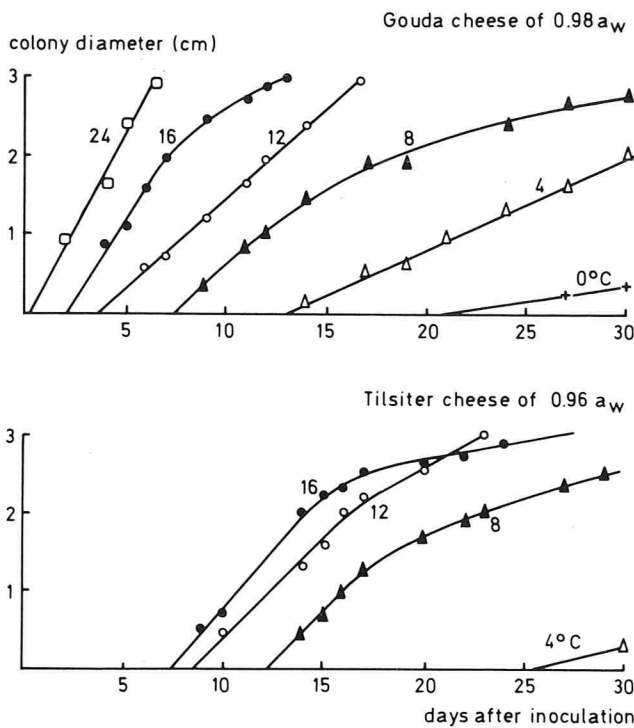


Figure 13. Growth of *Penicillium cyclopium* CBS 434.73 on slices of Gouda and Tilsiter cheese at different temperatures.

rather than by the instability of PA in the product. Although we used the same strain of *A. ochraceus* as Bacon (1) did, our results showed two a_w optima for PA production, e.g. 0.99 and 0.975 a_w, instead of an a_w optimum of 0.90, as observed by Bacon. This might be explained by a difference in the poultry feed used.

The PA production by *A. ochraceus* demonstrated at low a_w in poultry feed may be related to the composition of this substrate. Poultry feed is high in protein compared to MES and MEG, and this might suggest that protein stimulates PA production by this mold. However, this explanation is not supported by our results, where no increase of PA production was noticed in the yeast extract-enriched malt extract medium, MEYS, compared to malt extract medium, ME.

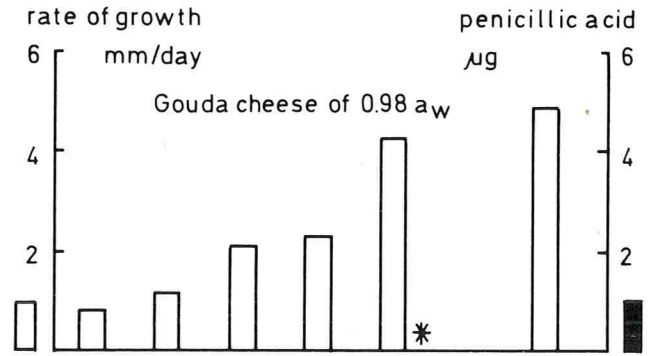


Figure 14. Growth of and penicillic acid production by *Penicillium cyclopium* CBS 434.73 on slices of Gouda and Tilsiter cheese at different temperatures (* after 6 weeks of incubation 4 µg of penicillic acid per 6 colonies were determined).

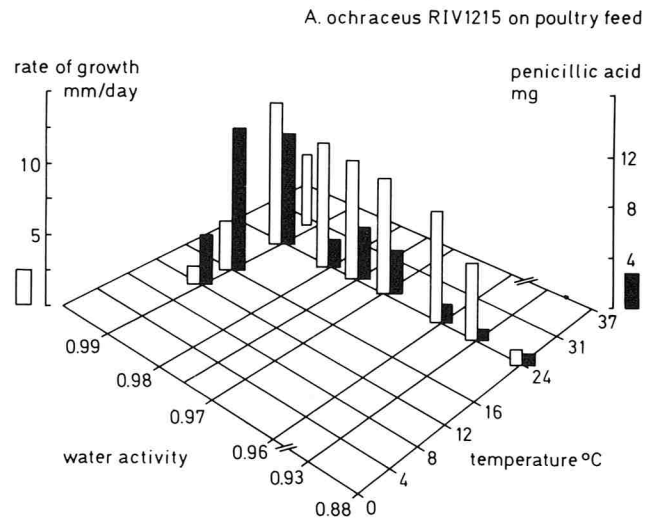


Figure 15. Growth of and penicillic acid production by *Aspergillus ochraceus* RIV 1215 on poultry feed under various conditions of water activity and temperature (the 1 mm black columns represent amounts of 0.1-0.6 mg of penicillic acid).

TABLE 4. Penicillic acid production by penicillic acid-producing strains of *Penicillium cyclopium* on 1 week old Gouda cheese of 0.97 a_w at 16 C.

Strain of <i>P. cyclopium</i>	Incubation period (days)		
	14	28	42
RIV 212	— ¹	—	—
CBS 161.42	—	—	—
CBS 434.73	—	—	—
NRRL 1888	—	—	—

¹No penicillic acid detected.

Therefore, an explanation for the substrate effect should be sought in other unknown factors in poultry feed.

In the survey of cheese, *P. cyclopium* was isolated most frequently from naturally moldy Gouda cheese ripening in warehouses or on display in shops (23). All strains except the one that was isolated from a cheese shelf failed to produce PA in media favorable for PA production. In several experiments with cheese inoculated with *P. cyclopium* strains of known ability to produce PA, PA could be detected in only one sample of cheese after a long incubation period. The absence of PA-producing *P. cyclopium* strains on cheese and the low production of PA in inoculated cheese suggest that there is little chance to detect PA in Gouda cheese molded by *P. cyclopium*. The small amount of PA in cheese can be explained by a depression of PA production in substrates low in carbohydrates (16) and the sub-optimal a_w of cheese for PA production. Besides, PA is unstable in cheese, possibly reacting with sulfhydryl compounds and becoming chemically undetectable (16). However, a large amount of toxin was found in cultures on protein- and carbohydrate-rich poultry feed containing reactive amino acids. It was demonstrated that the mycelium readily released the toxin, and yet the toxin was rather stable in poultry feed, as shown by a 43% recovery rate of PA after 60 h at 24 C. This indicates that the main reason for the small amount of PA in cheese is low production rather than instability of PA in the cheese.

It is concluded that the temperature and water activity as well as the nature of the substrate play an important role in production of PA. With regard to reactive mycotoxins such as penicillic acid one should keep in mind that the final amount of detectable toxin depends on how much is produced and how much is lost as a result of the toxin with substrate components.

ACKNOWLEDGMENTS

The authors thank Mr. K. Terpstra for the analysis of poultry feed and Dr. C. W. Bacon, Mr. E. de Boer, Dr. J. Harwig, and Dr. R. A. Samson for supplying fungal strains. Moreover, they are grateful to Dr. J. Harwig for correcting the manuscript.

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Ochratoxin A Production by Some Fungal Species in Relation to Water Activity and Temperature

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ABSTRACT

The effects of water activity (a_w) and temperature on growth of and ochratoxin A (OA) production by strains of *Aspergillus ochraceus*, *Penicillium cyclopium*, and *Penicillium viridicatum* were investigated. On agar media in which the a_w had been adjusted by addition of sucrose or glycerol, the minimum a_w values for OA production by *A. ochraceus*, *P. cyclopium* and *P. viridicatum* lay between 0.83-0.87, 0.87-0.90, and 0.83-0.86, respectively. At 24 C, optimum a_w values for OA production by *A. ochraceus* and *P. cyclopium* were 0.99 and 0.95-0.99, respectively, whereas that of *P. viridicatum* varied and was 0.95 and 0.99 for the two strains tested. At optimum a_w , the temperature range for OA production by *A. ochraceus* was 12-37 C, whereas that of *P. cyclopium* and *P. viridicatum* was 4-31 C. Optimum temperature for OA production by *A. ochraceus* was 31 C, whereas that of *P. cyclopium* and *P. viridicatum* was 24 C. On Edam cheese of 0.95 a_w , the minimum temperature for OA production by *P. cyclopium* was 20 C. On barley meal, *P. viridicatum* produced maximal quantities of OA at 0.97 a_w and could produce OA at temperatures as low as 12 C.

Ochratoxins are a group of structurally related secondary metabolites produced by molds belonging to several species of the genera *Aspergillus* and *Penicillium* (33). The most extensively studied compound of this group, ochratoxin A (OA), is toxic to many test animals, as has been reviewed by Chu (5). The main pathological changes after fatal dosing of animals, such as rats and chicks, with OA were confined primarily to damage to renal tubules and necrosis of kidneys and periportal liver cells. OA was also found to be teratogenic (3). According to Krogh et al. (15), OA may be a disease determinant of Balkan endemic nephropathy, a chronic kidney disease affecting rural populations in some areas of the Balkans.

The presence of OA has been demonstrated in a number of agricultural products. In the United States, corn (31), barley (9), and wheat (32) have been found to be contaminated with OA. In Sweden, OA-contaminated barley and oat products have been obtained from shops (13). In some districts of Denmark with a high incidence of swine nephropathy, OA was demonstrated in cereal grains, especially barley, used as feed (14). Residues of OA were found at slaughter in kidneys of pigs (16) and poultry (7) showing nephropathy changes.

OA-producing fungi have been characterized as storage fungi. *Penicillium viridicatum* has been frequently isolated from stored corn (4,20), dried beans (25),

fermented sausages (19), cured hams (17) and hard cheese (23). The evidence points to *P. viridicatum* as the main cause of OA contamination of grains (29), but *Aspergillus ochraceus* has also been associated with the presence of OA in corn (10) and green coffee beans (18). The fungus was one of the predominant species isolated from dried beans (21) and black pepper (4). Other OA-producing fungi are *Penicillium cyclopium* and *Penicillium palitans*. *P. cyclopium* was the chief species isolated from stored corn (20), hard cheese (23), and fermented and cured meat products (11). *P. palitans* has been isolated from fermented and cured meat products (11) and mixed feed containing OA (29).

Although much work has been done on the toxicity of OA and the examination of foodstuffs for OA, little is known about the environmental conditions for growth of and OA production by molds. The most important factors in safeguarding stored foodstuffs are water activity (a_w) and temperature. The first factor, defined as the equilibrium relative water vapor pressure of a substrate, is of great value as a measure for the amount of water available to microorganisms (30).

In the present study, cultures of various fungal species were grown on semi-synthetic media at different combinations of a_w and temperature to determine the limits and optimum conditions for mycelial growth and production of OA. Moreover, natural substrates were inoculated with OA-producing molds to estimate the possible influence of substrate.

MATERIALS AND METHODS

Organisms and spore suspensions

Fungal strains were maintained as lyophilized cultures. Strains of *A. ochraceus* and *P. cyclopium* had been received from the late D. I. Fennell, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois. Strains of *A. ochraceus* and *P. viridicatum* had been supplied by J. Harwig, Health and Welfare Canada, Health Protection Branch, Ottawa, of which strains RIV 28, 29, 80, 126 and 712 were originally labeled with the numbers 129, 136, 183, 182 and 583, respectively. Strains of *A. ochraceus*, *P. cyclopium*, *P. palitans*, and *P. viridicatum* had been received from R. A. Samson, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, of which the *P. cyclopium*, *P. palitans* and *P. viridicatum* strains were isolated from meat products by R. Hadlok, Giessen. G. F. R. Moreover, the laboratory's own strains, belonging to the four species and isolated from different kinds of foods, were used. Aspergilli were classified according to Raper and Fennell (27), penicillia according to Raper and Thom (26). It should be noted that in several European countries *P.*

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cyclopium and *P. viridicatum* are now classified as *P. verrucosum* var. *cyclopium* and *P. verrucosum* var. *verrucosum*, respectively, whereas *P. palitans* is classified as either *P. verrucosum* var. *cyclopium* or *P. verrucosum* var. *melanochlorum*, depending on the color of the colonies (28).

Cultures grown for 7-10 days at 24 C on malt extract agar (Oxoid) were washed with an aqueous solution of 6 g of sodium heptadecyl-sulfate (Tergitol-7, BDH)/l to prepare spore suspensions of ca. 10^6 spores per ml.

After the desired periods of incubation, cultures were stored at -18 C for 10-30 days until extracted for OA.

Cultures on agar media

In a preliminary study, mold strains were tested for OA production at 24 C on two agar plates of malt extract agar medium (Oxoid) (ca. 1.00 a_w , ME) and Czapek maize extract agar medium (ca. 1.00 a_w , CM). CM agar medium was prepared by mixing a sterilized solution of 70 g of Czapek Dox broth powder (Difco)/l and 30 g of agar no. 1 (Oxoid)/l with an equal volume of sterilized maize extract. The components were separately sterilized at 121 C for 20 min. The maize extract was prepared by boiling 200 g of crushed maize in 1 liter of water for 30 min, followed by centrifugation. Each plate was inoculated at three different spots with an inoculation needle dipped in a spore suspension. Incubation was terminated as soon as the colonies reached an average diameter of 30 mm.

A. ochraceus NRRL 3519, *P. cyclopium* NRRL 6065, *P. viridicatum* RIV 28 and *P. viridicatum* RIV 712 were used to determine the environmental conditions for mold growth and OA production. Agar media favoring high production of OA were chosen: *A. ochraceus* NRRL 3519 was cultured on malt extract agar supplemented with sucrose (MES-series) or glycerol (MEG-series) to obtain the desired a_w conditions, whereas the *Penicillium* species were cultured on Czapek maize extract agar supplemented with sucrose (CMS-series) or glycerol (CMG-series) to obtain the desired a_w conditions. The two agar series were used to demonstrate the effect of a_w on toxin production rather than the effect of concentration of the supplemented compound. To determine the effect of substrate on mold growth and OA production, *A. ochraceus* NRRL 3519 was also cultured on CMS at different conditions of a_w and temperature. Preparation of agar plates, inoculation, and a_w measurement have been described in a previous paper (22). The a_w measuring device consisted of a sample jar that was submerged in a temperature-controlled water bath, thus facilitating the equilibration of the vapor pressure around the sample, and was connected to a dewpoint hygrometer through a closed circuit. The accuracy of the device was 0.004 a_w . All measurements were done at 24 C. For each determination of rate of growth and OA production, two inoculated agar plates with three colonies each and one non-inoculated agar plate, which served as control for the a_w after incubation, were used. They were enclosed in a 0.3-liter polyethylene bag (gauge 0.04 mm). Cultures were grown at combinations of eight temperatures and six a_w values on MES and MEG or CMS and CMG. The variation of temperature was 0.3 C. The growth rate of the mycelium was determined by daily measurement of two right-angled diameters of the colonies. The regression lines of colony diameter on days after inoculation were calculated for each a_w -temperature combination. The germination time was obtained by extrapolating the regression line to the X-axis (22). Where rates of growth decreased during incubation, initial rates of growth obtained by drawing the tangent are given. Incubation of strains *A. ochraceus* and *P. viridicatum* was terminated on the day the six colonies reached an average diameter of 30 mm or on the 35th day of incubation when they did not reach this size. The incubation of *P. cyclopium* NRRL 6065 lasted until the average colony diameter reached a size of 20 mm, due to the slow growth rate of the strain.

To assess whether or not the strains tested were representative of the mold species, the limiting a_w and temperature for mold growth and OA production were determined for some other strains of *P. viridicatum*, which were grown on CMS at the same time as the test strain, *P. viridicatum* RIV 28.

Four replicate CMS cultures of *P. viridicatum* RIV 28, grown at 0.95 a_w and 24 C, were analyzed for OA to determine the repeatability of the method.

P. cyclopium cultures on cheese

Growth rate of and OA production by *P. cyclopium* NRRL 6065 was measured on Edam cheese of 0.95 a_w . The ca. 5-week-old brick-shaped cheese of 2.6 kg was obtained from a shop and was aseptically cut in slices of 40 × 40 × 4 mm. To obtain slices of similar a_w value, the outer layer of the cheese was discarded. The culture method was similar to that used for the agar cultures; for each determination of rate of growth and OA production, two inoculated slices of cheese with three colonies each and one non-inoculated control slice of cheese were used. They were placed in three petri dishes, which were enclosed in a polyethylene bag. Cultures were incubated at seven different temperatures until the six colonies reached an average diameter of 20 mm or until the 35th day when they did not reach this size.

P. viridicatum cultures on barley

Barley meal was blended with water to a moisture content of 144, 179, 236, 289, 341, 495 and 649 g/kg (wet weight basis), giving water activities of 0.80, 0.86, 0.91, 0.93, 0.95, 0.97 and 0.98, respectively, after autoclaving. The barley was autoclaved at 121 C for 1 h, thoroughly mixed, pressed into petri dishes to thickness of 5 mm and stored for 5 days. Each plate was inoculated at three different spots with 1 μ l of spore suspension of *P. viridicatum* RIV 28. Barley plates of 0.95 a_w were incubated at eight different temperatures, whereas barley plates of 0.80, 0.86, 0.91, 0.93, 0.97 and 0.98 were incubated only at 24 C. For each a_w -temperature combination two inoculated plates and one non-inoculated control plate were enclosed in a polyethylene bag. The method for determination of growth rate and germination time of mycelium was the same as that for cultures on agar. Incubation was terminated as soon as the colonies reached an average diameter of 30 mm or after 30 days when they did not reach this size.

Extraction and determination of OA from agar media

After thawing, cultures were inactivated by adding 20 ml of chloroform to each pair of plates. After shaking, the chloroform was allowed to stand in contact with the culture for at least 2 h at room temperature. For qualitative analysis, 20 μ l of the chloroform solution was spotted on a thin layer chromatography (TLC) plate next to an OA standard. The plate was developed in toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol) in an unsaturated tank, dried and exposed to ammonia vapor for 10 min. The presence of OA was indicated by a blue fluorescent spot on the plate when exposed to longwave UV light at 365 nm.

For quantitative analysis, the chloroform was decanted, the agar plates were washed twice with 10 ml of chloroform and the chloroform fractions were combined. The agar plates were blended with 50 ml of H₂O, 50 ml of chloroform and 5 ml of H₂SO₄ (0.5 mol/l) by means of a homogenizer (Ultra Turrax). After centrifugation, the mixture was transferred into a separatory funnel, and the chloroform layer was combined with the chloroform fractions collected before. The chloroform extracts were dried over anhydrous Na₂SO₄, evaporated to dryness and the residue was dissolved in 1 ml of chloroform. After preliminary visual determination of OA concentration by TLC, the sample solution was diluted to a measureable concentration. Twenty μ l of sample solution and OA standard were spotted next to each other on a silica gel TLC plate, Merck 60, with a thickness of 0.25 mm and developed in toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol) in an unsaturated tank. After development, the plate was exposed to ammonia vapor for 10 min and covered with a glass plate. The resulting blue fluorescent spot of OA was measured densitometrically by fluorescence measurement (excitation wavelength 365 nm, emission wavelength 430 nm). The limit of detection was ca. 0.1 μ g of OA per two agar plates.

Extraction and determination of ochratoxin A from cheese

Quantities of chemicals for extraction of OA indicated below are related to the weight of the cheese; they were proportionally increased or decreased when the amount of cheese to be analyzed varied.

Twenty-five g of cheese was blended in 125 ml of chloroform and 12.5 ml of H_3PO_4 (0.1 mol/l) for 90 sec by means of an Ultra Turrax (2). Ten g of diatomaceous earth (Celite) was added and the moisture was filtered. Two g of diatomaceous earth was mixed with 1 ml of $NaHCO_3$ (0.15 mol/l) in water and brought into a 700×17 -mm chromatographic column, fitted with a plug of glass wool (24). After tamping the column, a mixture of 50 ml of cheese extract and 40 ml of hexane was brought onto the column. The column was eluted with 75 ml of chloroform and the eluate was discarded. Ochratoxin A was eluted with 75 ml of chloroform-formic acid (99:1 vol/vol). Immediately thereafter the eluate was evaporated almost to dryness and the residue was dissolved in 1 ml of chloroform. Concentration of OA was determined as described for the quantitative analysis of agar plates. However, instead of one-dimensional TLC, two-dimensional TLC was applied with chloroform-methanol (8:2 vol/vol) and toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol) as first and second developing solvent, respectively. The limit of detection was ca. $0.05 \mu\text{g}$ per culture.

Extraction and analysis of ochratoxin A from barley

The quantities of chemicals indicated below are related to the dry weight of barley; they were proportionally increased or decreased when the amount of barley to be analyzed varied.

After thawing, barley from the pair of plates were blended with 250 ml of acetonitrile and 25 ml of H_3PO_4 (0.1 mol/l) by means of an Ultra Turrax, followed by filtration. Fifty ml of filtrate was shaken in a separatory funnel with 50 ml of acetonitrile, 50 ml of iso-octane and 50 ml of $NaHCO_3$ (0.15 mol/l) and $NaCl$ (0.5 mol/l) solution in water. After shaking, three layers were formed of which the lower phase was the water phase. The water phase was drained off, acidified with 5 ml of H_2SO_4 (2 mol/l), and extracted three times with 50 ml of chloroform. The combined chloroform fractions were dried with anhydrous Na_2SO_4 , evaporated to dryness and the residue was dissolved in 0.5 ml of chloroform. TLC determination of OA was carried out as described for the analysis of agar cultures. The detection limit was ca. $1 \mu\text{g}$ per culture.

RESULTS

Cultures on agar media

Results of the preliminary study of ME and CM agar medium are given in Table 1. One strain each of *A. ochraceus* and *P. cyclopium* and several strains of *P. viridicatum* produced OA, but none of the *P. palitans* strains tested produced OA. On ME, *A. ochraceus* produced more OA than on CM, whereas *P. cyclopium* and *P. viridicatum* produced more OA on CM than on ME.

Figure 1 shows examples of colony growth curves of cultures of *P. viridicatum* RIV 28 on CMS agar medium. Under certain conditions of a_w and temperature, growth rate decreased after a period of time. This phenomenon could not be correlated with a_w or temperature and it occurred rarely.

Average growth rate of colonies and OA production on agar media at each combination of temperature and initial a_w are shown in Fig. 2-9. For cultures with decreasing growth rate, the average initial growth rate of colonies is given in the figures. Optimum a_w value for OA production by *A. ochraceus*, *P. cyclopium* and *P. viridicatum* RIV 712 was 0.99. However, that of *P. viridicatum* RIV 28 was 0.95. The minimum a_w value for OA production by *A. ochraceus* and *P. viridicatum* RIV 28 lay between 0.83 and 0.87, that of *P. viridicatum* RIV 712 between 0.83 and 0.86 and that of *P. cyclopium* between 0.87 and 0.90. The minimum a_w value for mold

TABLE 1. Production of ochratoxin A (in μg per 6 colonies) by various strains of different fungal species on malt extract agar (ME) and Czapek maize extract agar (CM) incubated at 24 C.

Species and strain no.	Agar medium	
	ME	CM
<i>A. ochraceus</i>		
NRRL 3519	410	8
13 other strains	< 0.1	— ^a
<i>P. cyclopium</i>		
NRRL 6065	24	47
5 other strains	< 0.1	—
<i>P. palitans</i>		
13 strains	< 0.1	—
<i>P. viridicatum</i>		
RIV 28	3.8	230
RIV 29	0.7	—
RIV 80	0.1	—
RIV 126	1.0	—
RIV 188	2.6	—
RIV 712	0.8	220
4 other strains	< 0.1	—

^aNot tested.

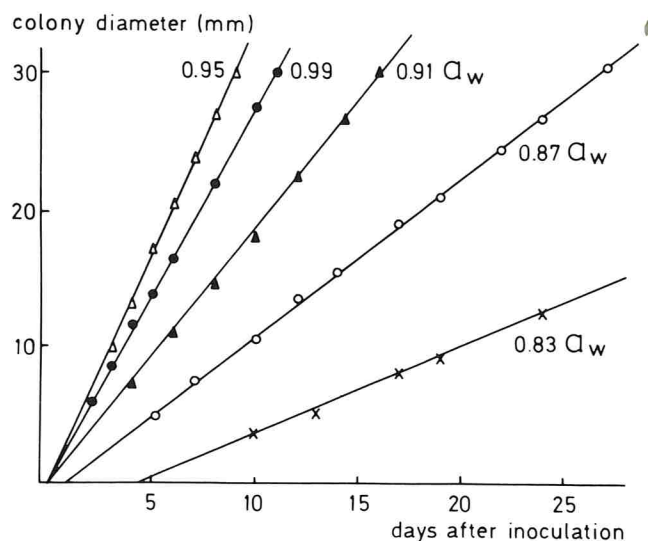
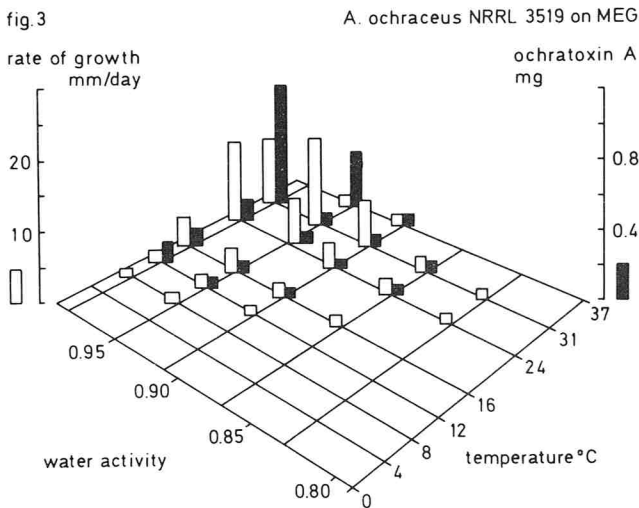
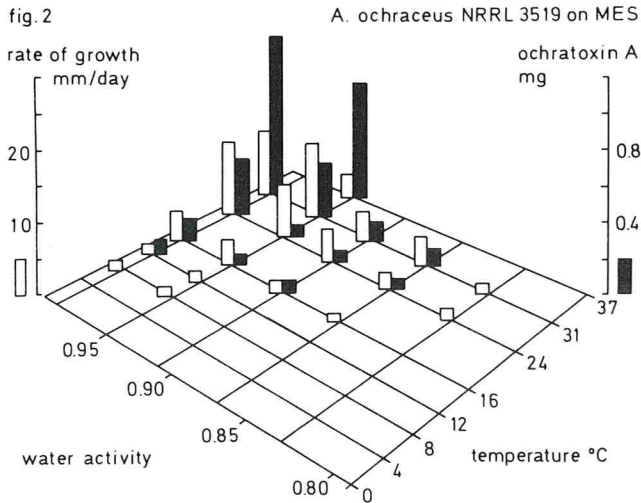


Figure 1. Growth of *Penicillium viridicatum* RIV 28 on Czapek maize extract sucrose agar at various water activity levels and 24 C.

growth lay between 0.79 and 0.83 and did not differ significantly for the different fungal species. Optimum temperatures for OA production by *A. ochraceus* were 31 or 37 C, depending on a_w , whereas *P. cyclopium* produced maximum quantities at 24 C. *P. viridicatum* RIV 28 produced more OA on CMS at 24 C than at 16 C, but no difference was noticed on CMG. Temperature ranges for OA production by *A. ochraceus*, *P. cyclopium* and *P. viridicatum* were 12-37, 4-31 and 4-31 C, respectively. The temperature range for mold growth by *A. ochraceus*, 8-37 C, was higher than that of *P. cyclopium* and *P. viridicatum*, which was 0-31 C.

Germination times of *A. ochraceus* and *P. viridicatum* were 0.5-2 days under optimum conditions for mold growth and they increased to 12-14 days under unfavorable conditions. Those for *P. cyclopium* were 1-2 days and 21 days, respectively.

Data in Table 2 demonstrated that two other strains of *P. viridicatum* had limiting conditions for mold growth and OA production similar to those for strain RIV 28.



Figures 2-3. Growth of and ochratoxin A production by *Aspergillus ochraceus* NRRL 3519 on malt extract sucrose agar (MES) and malt extract glycerol agar (MEG) under various conditions of water activity and temperature (the 1 mm black columns represent amounts of 0.1-40 μg of ochratoxin A).

However, two strains failed to produce toxin at 4 C and did not grow at 31 C.

The OA contents of the four replicate cultures of *P. viridicatum* RIV 28 differed by not more than 15% from the average when grown on CMS of 0.95 a_w at 24 C. The difference between initial and final a_w of substrates could amount to 0.006.

Cultures on natural substrates

Figure 10 shows average growth rate of and OA production by *P. cyclopium* NRRL 6065 on Edam cheese of 0.95 a_w . The temperature range for OA production was 20-24 C, whereas that for mold growth was 0-24 C.

Growth rate of and OA production by *P. viridicatum* on moistened barley meal are shown in Fig. 11. At 0.95 a_w the temperature range for OA production was 12-24, whereas that for mold growth was 0-31. Optimum a_w for OA production as well as for mold growth lay at 0.97. OA could be detected at 0.91 a_w , but not at 0.86.

DISCUSSION

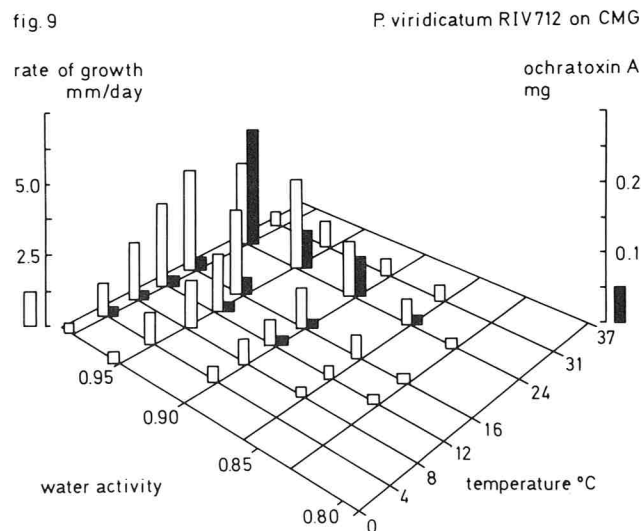
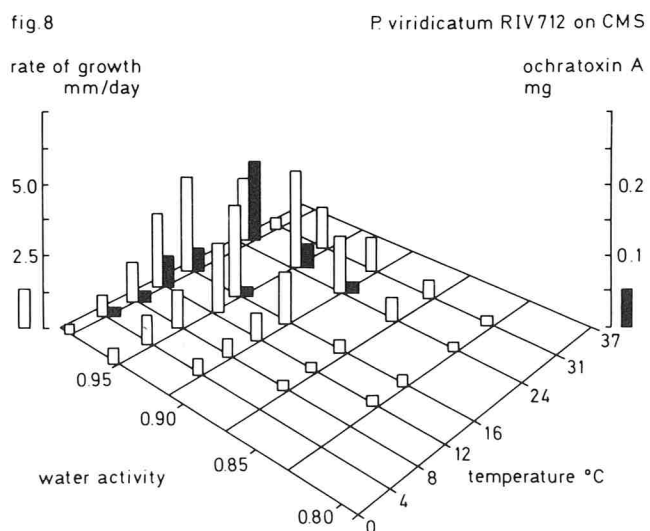
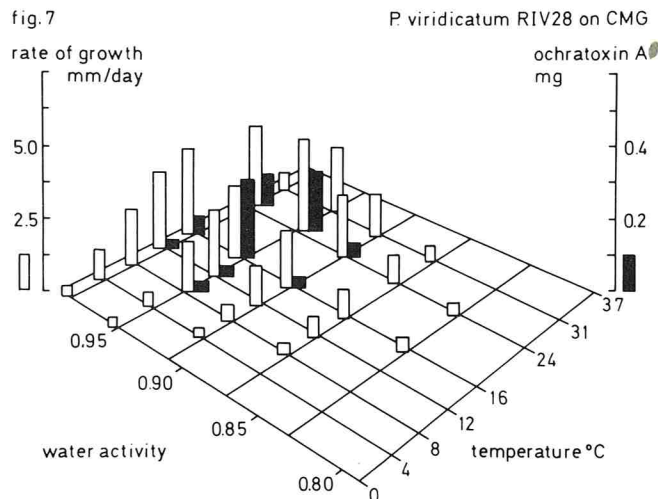
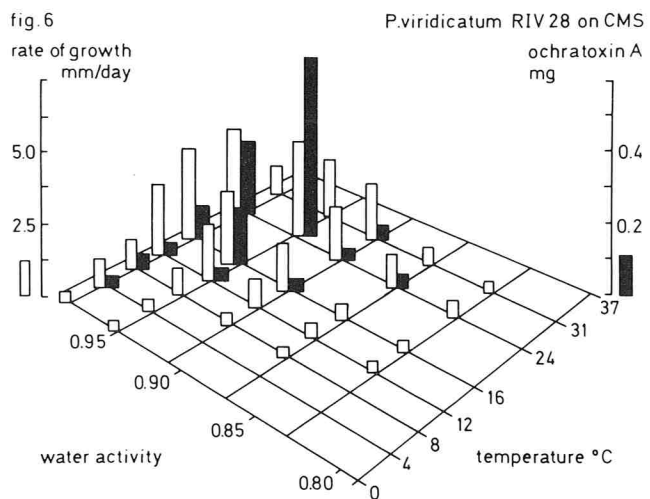
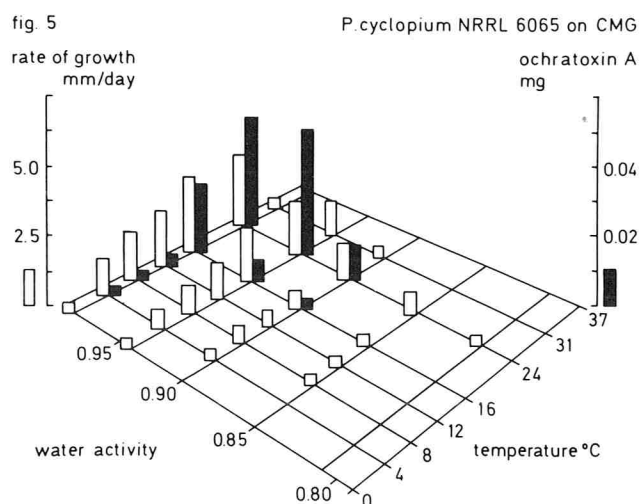
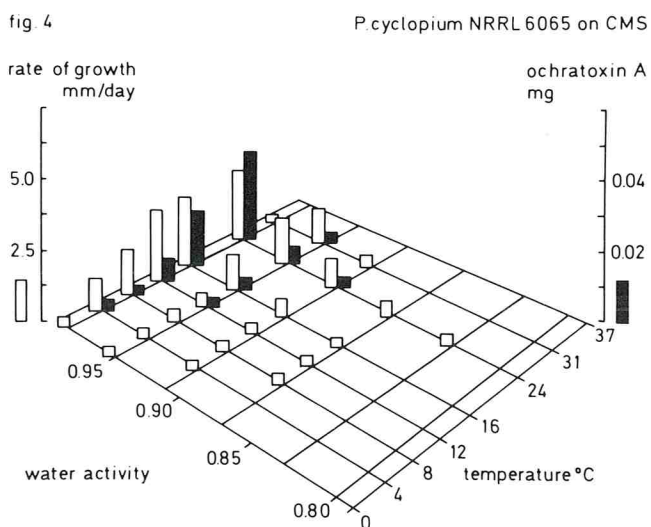
The a_w range for OA production was similar for the three fungal species tested. In general, 0.99 a_w was optimal for OA production. The difference between the optimum a_w for OA production by *P. viridicatum* RIV 28 and *P. viridicatum* RIV 712 indicate that this feature may be strain-related. The effect of a_w on OA production was independent of substrate, since results obtained with cultures of *P. viridicatum* RIV 28 on Czapek maize extract agar and on barley meal were similar. This was also true for results obtained with cultures of *P. cyclopium* NRRL 6065 on Czapek maize extract agar and cheese, and those of *A. ochraceus* NRRL 3519 on MES, MEG and CMS.

Data in figures show that the penicillia tested can grow and produce OA at lower temperatures than *A. ochraceus*. Therefore penicillia may thrive in moderate climates, whereas *A. ochraceus* may be found especially in tropical areas. This has been indicated by the association of *P. viridicatum* with the presence of OA in Canadian (29) and U.S. (34) wheat and Danish barley (34), and the predominance of *A. ochraceus* in black pepper (4) and green coffee beans containing OA (18).

Our results demonstrate a minimum a_w of 0.86 - 0.90 for OA production and confirm the data of other investigators using wheat and barley (12) or poultry feed (1) as substrate. However, they contrast with results reported by Escher et al. (8), who inoculated *A. ochraceus* on cured ham and found that even more toxin was produced in ham of a moisture in equilibrium with 70-75% ERH than in ham with more moisture. Although unlikely, this might be due to a difference of strains. The minimum temperature of 12 C for OA production by *A. ochraceus* almost equalled that determined in corn and wheat by Ciegler (6) and in cured ham by Escher et al. (8). The temperature range for OA production by *P. viridicatum* seems to be dependent on the type of substrate; we found minimum temperatures for OA production of 4 and 12 C on Czapek maize extract agar and barley, respectively, whereas other workers (12) reported a minimum temperature of 5 C on wheat.

Besides environmental conditions, production of OA is influenced by fungal species and strain. Species may have different requirements for production of OA since *A. ochraceus* produced more OA on malt extract agar than on Czapek maize extract agar. This was reversed for *P. viridicatum*. Production of OA by *P. cyclopium* on cheese was low compared with that on Czapek maize extract agar and might be related to type and age of cheese. As far as is known, this is the first report of production of OA in cheese. It indicates a potential risk of contamination of commercial cheese with OA as *P. cyclopium* was found to be the predominant fungal species on hard types of cheese in warehouses, shops and households showing mold growth (23).

Summarizing, it can be concluded that only one strain of *A. ochraceus* and *P. cyclopium* could produce OA, whereas several OA-producing strains of *P. viridicatum*



Figures 4-9. Growth of and ochratoxin A production by one strain of *Penicillium cyclopium* and two strains of *Penicillium viridicatum* on Czapek maize extract sucrose agar (CMS) and Czapek maize extract glycerol agar (CMG) under various conditions of water activity and temperature (N.B. the scales of toxin axis are different: the 1 mm black columns represent amounts of 0.1-2, 0.1-20, and 0.1-10 μ g of ochratoxin A for *P. cyclopium*, *P. viridicatum* RIV 28, and *P. viridicatum* RIV 712, respectively).

were found. Large quantities of OA can be produced at intermediate and high temperatures and high a_w by species belonging to the genera *Penicillium* and *Aspergillus*, whereas at low temperatures the toxin can be produced by penicillia only.

TABLE 2. Growth of and ochratoxin A production by various strains of *Penicillium viridicatum* on Czapek maize extract sucrose agar at unfavorable conditions of a_w and temperature.

Strain no.	0.99 a_w				0.83 a_w
	0 C	4 C	24 C	31 C	(24 C)
RIV 28	G - ^a	G + ^b	G +	G -	NG ^c
RIV 29	G -	G +	G +	G -	NG
RIV 44	G -	G -	G +	NG	NG
RIV 126	G -	G +	G +	G -	NG
RIV 188	G -	G -	G +	NG	NG

^aGrowth and no ochratoxin A detected (< 0.1 μ g per 6 colonies).

^bGrowth and ochratoxin A detected.

^cNo growth.

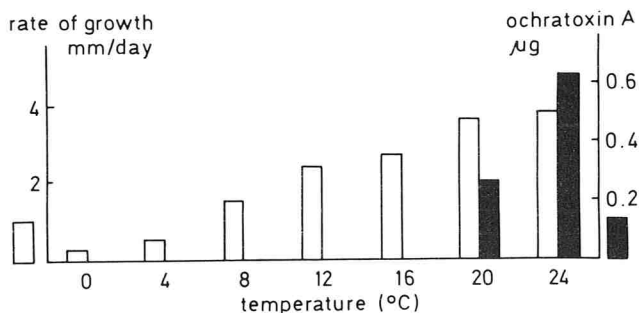


Figure 10. Growth of and ochratoxin A production by *Penicillium cyclopium* NRRL6065 on Edam cheese of 0.95 a_w at different temperatures.

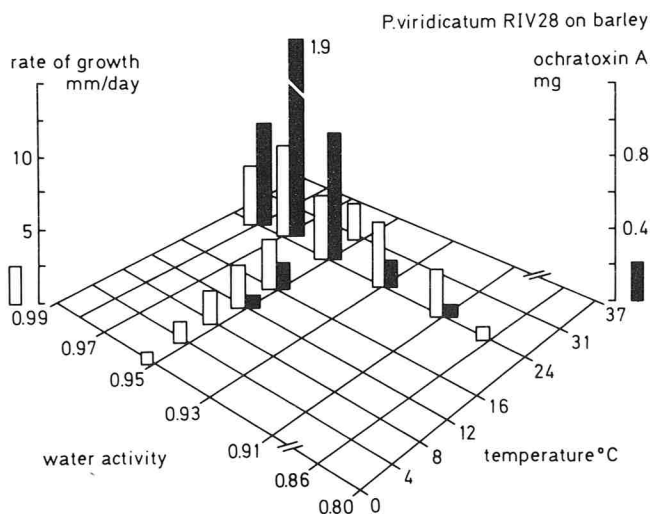


Figure 11. Growth of and ochratoxin A production by *Penicillium viridicatum* RIV 28 on barley meal under various conditions of water activity and temperature (the 1 mm black columns represent amounts of 1-40 μ g of ochratoxin A).

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Depletion of Brilliant Blue F.C.F. and Penicillin G in Milk from Treated Cows

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ABSTRACT

A field trial was set up to compare depletion of Brilliant Blue F.C.F. and penicillin following three infusions of Vetspen cerate 100 into six healthy lactating cows. The presence of dye was noted by milk color at the time of milking, and by the ion exchange resin method in the laboratory. Penicillin residues were determined by a diffusion agar plate microbiological method and by Delvotest-P. Dye extinction times were found to be significantly longer than penicillin extinction times (0.05 level of significance). The proportion of cows requiring more milkings for penicillin extinction than for dye extinction was estimated to be 0.17.

The need for use of intramammary antibiotic preparations for control of mastitis in dairy herds is universally accepted; however, the attendant risk of milk contamination has always been a cause for concern to dairy technologists and health authorities.

The existing methods for detection of antibiotic residues in milk are microbiological assays. While it cannot be denied that these are the most direct and positive methods of regulatory control, it must be realized that these necessitate use of trained personnel and provision of adequate laboratory facilities. Such tests, although in some instances very successful, take a minimum of 2.5 h to do. This, of course, precludes rejection at the farm of contaminated milk and, in addition, these assays may cost several dollars each when sampling costs and laboratory overhead are considered.

With a view to providing a low-cost system for detection of antibiotics in milk, devoid of the above disadvantages, the Drugs Directorate of the Health Protection Branch, Health and Welfare Canada, is investigating the feasibility of the inclusion of tracer dyes in mastitis creams.

It was considered that effective dye-marker systems would provide a quick and inexpensive method for detection of antibiotics in milk. In addition, they would provide a method suitable for testing each batch of milk and rejecting contaminated milk at the farm or creamery following a visual examination or rapid ion exchange assay.

This experiment was designed to compare the depletion times of dye and penicillin following intramammary infusion with dye-marked mastitis cream to healthy cows. The limits of detection for the analytical methods used in this study were as follows: (a) ion

exchange resin method - 0.02 mg/L, (b) cylinder plate method - 0.01 I.U./ml and (c) Delvotest-P - 0.006 I.U./ml.

MATERIALS AND METHODS

Animals

Six healthy cows with normal udders, which had not received any kind of antibiotic therapy in the preceding 3 weeks, were used. Their average production was 17 kg of milk per day, with values ranging from 13.6 - 21.8 (see Table 1). A pre-treatment clinical and bacteriological examination and California Mastitis Test (C.M.T.) were carried out for each quarter. Cows were familiarized with the quarter-milker before commencement of the trial.

Mastitis preparation

One-tenth gram of Procaine Penicillin G (100,000 I.U.) plus 125 mg of Brilliant Blue F.C.F. (C.I. 42090) in a cerate vehicle was used (Vetspen cerate 100, Glaxovet, Australia). With this product the dye concentration of 0.02 mg/liter corresponds to 0.008 I.U. of penicillin per ml of milk.

Method of administration

The two left quarters of all cows were treated, leaving the two right quarters as controls. Three consecutive infusions were done after the morning milking at 24-h intervals, starting 57 h before the first collection of samples. Before infusion, teats were thoroughly cleaned with alcohol-saturated cotton wool. The cap was removed from the tube of medication, the nozzle was inserted into the teat canal and the entire contents were administered. The end of the teat was pinched to prevent escape of the drug and the udder was gently massaged to distribute the cream upwards into it.

Sample collection

Cows were milked at approximately 9- and 15-h intervals. The first sample was collected during the afternoon milking after the last infusion. The quarter-milker, having two containers, was attached in such a manner that milk from treated quarters was collected in partitioned sections of one container, and milk from untreated quarters was collected in similar sections of the other. Yield and color of milk were recorded for each quarter, and individual samples (500 ml) were taken for each treated quarter. The milk from both untreated quarters was emptied into a pail, mixed well, and a pooled sample (500 ml) taken for both quarters. The remainder of the milk was discarded and the milker and pail were washed before milking the next cow. The samples were refrigerated (but not frozen) immediately. Sample collection was continued up to 129 h.

Tracer dye assay in the laboratory

The ion exchange resin column method, developed in our laboratory (2), was used to estimate amount of dye in samples.

Microbiological assay for penicillin residues

Cylinder plate method. Quantitative penicillin residue assays were done according to Kramer et al. (1).

Delvotest-P method. Qualitative assays were done by the Delvotest-P method developed by van Os and co-workers (3).

California Mastitis Test (C.M.T.) and bacteriological examination

C.M.T. and pre-treatment bacteriological examination were done by the Veterinary Services Laboratory, Kemptville, Ontario, Canada.

RESULTS AND DISCUSSION

During a preliminary field trial, comparing relative times of disappearance of dye marker and penicillin, an inverse relation of both to yield was noted. To eliminate variations due to the stage of lactation and yield, this study was set up using cows of similar yield (Table 1).

In this trial the use of commercially available diffusion test for antibiotic residues, Delvotest-P, was introduced. The test was found to give reproducible results in a shorter time with greater sensitivity (0.006 I.U./ml) than the cylinder plate method. The manipulations were simpler, allowing greater numbers of samples to be analyzed in a shorter time, although the results were only qualitative ones.

Penicillin residues (Delvotest-P) were detected, on the average, for 48 h after the last infusion (Table 2), but cow #192 had residues in both treated quarters for 57 h, and another cow (#915) in the left hind quarter for 72 h. Concentration (cylinder plate assay) varied between cows. No penicillin residues were found in milk from untreated quarters.

Color could be detected visually at time of milking in milk from treated quarters for all cows up to 33 h after the last infusion. (Table 3). Cow #192 showed a trace in both treated quarters at 48 h, while #156 and #915 showed a trace in one quarter only at 48 h. No color was detected in milk from untreated quarters.

Measurable amounts of dye were detected in the milk from treated quarters in all cows for 57 h after the last infusion (Table 4) except the hind quarter of #156 and #211 using the column-resin method. Cow #156 showed measurable amounts in the front quarter and #192 showed measurable amounts in both treated quarters at 72 h. Insignificant traces of dye were found in the left hind quarter of cow #184 at 72 h. No dye was detected in samples from untreated quarters.

The C.M.T. and bacteriological examination carried out before the trial indicated that the cows were not infected.

The excretion patterns of dye and penicillin indicated a tendency of the dye residues to remain longer than penicillin when a 0.02 mg/L detection limit is used (Fig. 1). The dye was measurable for up to two milkings more than penicillin in eight quarters, while penicillin residues were detected for one milking more than the dye in only

one quarter. The remaining three quarters had the same extinction time for both penicillin and dye.

The observed numbers of milkings for dye and penicillin extinction are given in Table 5. Analysis of data in this table showed that dye extinction times were significantly longer than penicillin extinction times (0.05 level of significance, one-tailed randomization test). The estimated proportion of "dye-neg" cows is 0.17. The "dye-neg" cow is a cow requiring more milkings for penicillin extinction than for dye extinction. The upper 95% confidence limit on this proportion is 0.58. In the above estimate the assumption that the experimental cows are representative (randomly sampled) of larger populations of cows has been made.

Further results based on this assumption are given in Table 6 (a, b, c). These results may be useful in determining appropriate extinction points of dye and numbers of experimental cows required. Tables 6a, 6b, and 6c were calculated for proportions of "dye-neg" cows of 0.0, 0.10, 0.20, respectively. Tabulated values are upper bounds on the true proportions of "dye-neg" cows for various sample sizes and degrees of certainty. For example, with 20 cows of which two are observed to be "dye-neg", we can be 80% certain that the true proportion of "dye-neg" cows is less than .20 [Table 6 (b)].

If the limit of detection for the ion exchange method is lowered (e.g. 0.04 mg/L), the extinction time for the dye will be reduced and the proportion of "dye-neg" cows will be increased. This will result in more samples having a detectable level of penicillin residues present, when they have been determined as negative based on the dye test.

In conclusion, the Australian preparations were formulated for a 72-h milk-out period and there was a good compliance from a point of view of both dye and penicillin. It should be noted, though, that using the above levels of detection for dye and penicillin, dye was retained for significantly longer periods than penicillin. However, considering the differences which normally occur between the individual quarters and animals, the system is of practical value.

ACKNOWLEDGMENT

The Delvotest-P kits used in this study were obtained through the courtesy of Dr. J. L. van Os of Gist-Brocades NV, Delft, Netherlands. The authors are grateful to Mr. S. Moore for his excellent technical assistance, to Dr. K. Karpinski for the statistical analysis of the results and to Mr. G. Hooper for allowing us to use the Kemptville College barn facilities and animals.

TABLE 1. *Daily milk yield (kg).*

Cow No.	Feb. 6 ^a	Feb. 7	Feb. 8	Feb. 9	Feb. 10	Mean
156	19.6	19.4	18.2	18.2	18.8	18.9
184	16.2	18.9	18.9	18.6	18.3	18.2
192	17.2	13.8	13.6	14.6	14.8	14.8
210	16.2	15.0	17.8	15.9	15.7	16.3
211	20.4	19.3	20.9	20.4	21.8	20.6
915	15.7	15.9	15.7	17.0	17.5	16.3

^aFebruary 6, 1978 - first day of milk samples collection.

TABLE 2. Detection of penicillin in milk from treated cows.

Cow No.	Quarter	Milking (hours after the last infusion)															
		+ 9		+ 24		+ 33		+ 48		+ 57		+ 72		+ 81		+ 96	
		C.P. ^b	D.P.	C.P.	D.P.	C. P.	D. P.	C. P.	D. P.	C.P.	D.P.	C.P.	D.P.	C.P.	D.P.	C.P.	D.P.
156	R.S. ^a	neg	ND	neg	ND	neg	-	neg	-	neg	-	neg	-	neg	-	neg	-
	L.F.	44.5	ND	1.50	ND	0.15	+	neg	+	neg	-	neg	-	neg	-	neg	-
	L.H.	21.3	ND	0.64	ND	0.03	+	neg	+/-	neg	-	neg	-	neg	-	neg	-
184	R.S.	neg	ND	neg	ND	neg	-	neg	-	neg	-	neg	-	neg	-	neg	-
	L.F.	28.7	ND	0.61	ND	0.08	+	neg	+/-	neg	-	neg	-	neg	-	neg	-
	L.H.	13.1	ND	1.64	ND	0.24	+	0.02	+	neg	+	neg	-	neg	-	neg	-
192	R.S.	neg	ND	neg	ND	neg	-	neg	-	neg	-	neg	-	neg	-	neg	-
	L.F.	63.9	ND	1.60	ND	0.48	+	0.09	+	neg	+	neg	-	neg	-	neg	-
	L.H.	152.3	ND	1.64	ND	0.38	+	0.02	+	neg	+	neg	-	neg	-	neg	-
210	R.S.	neg	ND	neg	ND	neg	-	neg	-	neg	-	neg	-	neg	-	neg	-
	L.F.	15.5	ND	2.90	ND	0.12	+	neg	+/-	neg	-	neg	-	neg	-	neg	-
	L.H.	16.7	ND	1.30	ND	0.11	+	neg	+	neg	+/-	neg	-	neg	-	neg	-
211	R.S.	neg	ND	neg	ND	neg	-	neg	-	neg	-	neg	-	neg	-	neg	-
	L.F.	15.3	ND	0.40	ND	0.08	+	neg	+/-	neg	-	neg	-	neg	-	neg	-
	L.H.	7.0	ND	0.50	ND	neg	+	neg	+	neg	-	neg	-	neg	-	neg	-
915	R.S.	neg	ND	neg	ND	neg	-	neg	-	neg	-	neg	-	neg	-	neg	-
	L.F.	17.4	ND	1.80	ND	0.06	+	0.04	+	neg	+	neg	+/-	neg	-	neg	-
	L.H.	28.0	ND	2.40	ND	0.79	+	0.09	+	0.03	+	neg	+	neg	-	neg	-

^aR.S. - Right side, L.F. - Left front, and L.H. - Left hind.^bC.P. - cylinder plate (concentration in µg/ml), D.P. - Delvotest-P, and ND - Not done.

TABLE 3. *Visibility of color in milk at the time of milking.*

Cow No.	Quarter	Milking (hours after the last infusion)					
		+ 9	+ 24	+ 33	+ 48	+ 57	+ 72
156	LF	blue	blue	lt. blue	lt. blue	—	—
	LH	blue	blue	lt. blue	—	—	—
184	LF	blue	blue	lt. blue	—	—	—
	LH	blue	lt. blue	lt. blue	—	—	—
192	LF	blue	blue	lt. blue	lt. blue	—	—
	LH	blue	blue	lt. blue	lt. blue	—	—
210	LF	blue	blue	lt. blue	—	—	—
	LH	blue	lt. blue	lt. blue	—	—	—
211	LF	blue	lt. blue	lt. blue	—	—	—
	LH	blue	lt. blue	lt. blue	—	—	—
915	LF	blue	lt. blue	lt. blue	—	—	—
	LH	blue	blue	lt. blue	lt. blue	—	—

TABLE 4. *Excretion of brilliant blue F.C.F. in milk (µg/ml).*

Cow No.	Quarter	Milking (hours after the last infusion)							
		+ 9	+ 24	+ 33	+ 48	+ 57	+ 72	+ 81	+ 96
156	LF ^a	128	6.4	2.56	0.48	0.12	0.06	—	—
	LH	48	2.4	0.48	0.06	—	—	—	—
184	LF	128	6.4	1.20	0.24	0.06	—	—	—
	LH	48	3.2	1.20	0.32	0.04	—	—	—
192	LF	128	9.6	1.6	0.96	0.16	0.02	—	—
	LH	384	6.4	2.4	0.48	0.12	0.04	—	—
210	LF	96	24	1.6	0.12	—	—	—	—
	LH	48	4.8	1.2	0.06	0.02	—	—	—
211	LF	64	3.2	0.4	0.08	0.03	—	—	—
	LH	32	2.4	1.2	0.08	—	—	—	—
915	LF	48	3.2	1.2	0.16	0.04	—	—	—
	LH	48	6.4	2.4	0.48	0.12	—	—	—

^aLF - Left front, LH - Left hind.

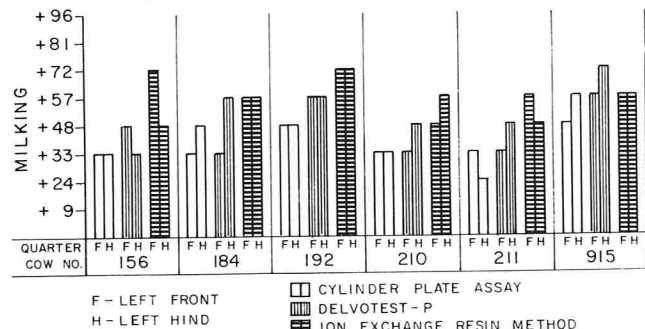


Figure 1. *Excretion patterns for penicillin G and dye marker.*

TABLE 5. *Number of milkings for dye and penicillin extinction.*

Cow No.	Quarter	Dye ^a	Penicillin ^b	Average differences per cow "dye-penicillin"
156	LF	7	5	1.5
	LH	5	4	
184	LF	6	4	1.0
	LH	6	6	
192	LF	7	6	1.0
	LH	7	6	
210	LF	5	4	1.0
	LH	6	5	
211	LF	6	4	1.0
	LH	5	5	
915	LF	6	6	-0.5
	LH	6	7	
Average Difference				0.8

^aIon exchange resin method.

^bDelvotest-P method.

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TABLE 6. *Upper bounds on the proportion of "Dye-neg" cows.*

6 (a) Observed proportion of "dye-neg" cows is 0.0				
Number of cows	Level of confidence:			80%
	95%	90%	90%	
10	.26	.20	.15	
20	.14	.11	.08	
30	.10	.07	.05	
40	.07	.06	.04	
6 (b) Observed proportion of "dye-neg" cows is 0.10				
Number of cows	Level of confidence:			80%
	95%	90%	90%	
10	.39	.33	.27	
20	.28	.24	.20	
30	.24	.21	.18	
40	.21	.19	.16	
6 (c) Observed proportion of "dye-neg" cow is 0.20				
Number of cows	Level of confidence:			80%
	95%	90%	90%	
10	.51	.45	.38	
20	.40	.36	.31	
30	.35	.32	.28	
40	.32	.29	.27	

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Stress Metabolites in Fruits and Vegetables: Introduction

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Various stress conditions, such as temperature adversity, light exposure, mechanical injury, chemical insult and attack by microorganisms, frequently cause formation and accumulation of abnormal metabolites in plant tissues. These metabolites are not ordinarily present in the normal plant tissue which has not undergone trauma. Stress metabolites generally fall into three chemical groupings - isoprenes, phenylpropanoids and isoflavanoids. There appears to be a taxonomic element in phytoalexin biosynthesis. For example, *Leguminosae* produced isoflavanoids, *Solanoceae* produce diterpenes and the *Compositae* produce polyacetylenes. Historically, interest has focused on the role of these metabolites as phytoalexins or antimicrobial agents. Thus, rishitin and related terpenes are related to R-gene resistance of potato to late blight infection, phaseollin and other isoflavanoids appear to play a role in the resistance of beans to various fungi and pisatin and dimethylptero-

carpin are phytoalexins from the garden pea.

Recent evidence points to the importance of stress metabolites from the viewpoint of food safety. Various relatively innocuous conditions of stress may give rise to stress metabolite accumulation in un-infected plant tissues. Evidence now mounting indicates that many stress metabolites are toxic to mammalian cells. Pisatin is known to disrupt mammalian membranes and uncouple oxidative phosphorylation in mitochondria. Phaseollin is highly toxic to embryonated chick eggs. Sweet potatoes may accumulate ipomeamarone which is a hepatotoxin and other terpenes, such as 4-ipomeanol, which causes lung edema in animals. Potato tubers may accumulate the mildly toxic glycoalkaloids solanine and chaconine and a family of terpenes which have been suspect as causal agents of birth defects.

This Symposium brings together information on the chemical identity, biosynthesis, induction by microbial and nonmicrobial stress and toxicology of stress metabolites associated with edible plant tissues.

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Stress Metabolites of Plants — A Growing Concern

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ABSTRACT

For many years it has been known that plants, when subjected to stress, produce "unusual" metabolites in response to that stress. Some of these metabolites are toxic and, consequently are of concern from the standpoint of contamination of foods. Each year additional stress metabolites are isolated and identified from plants and plant products subjected to stress conditions; these conditions include infection of the plant by microorganisms, mechanical damage during processing or storage, exposure to temperature extremes and the like. The need for an intensive research effort into formation, isolation and characterization, and toxicological evaluation of such metabolites in plants used for human food is discussed.

Many plants are susceptible to attack while in the field and during storage by various microorganisms and insects. They are also subjected to mechanical damage during the harvesting operation and various stress conditions during storage and processing. In response to stress situations, the affected tissue of healthy plants produces unusual compounds in an attempt to counteract the stress. Unusual compounds produced by plants in response to various exogenous stimuli are generally referred to as phytoalexins. In a review on phytoalexins by Kuc' (32), it was proposed that "the term phytoalexin should serve as an umbrella under which chemical compounds contributing to disease resistance can be classified whether they are formed in response to injury, physiological stimuli, and the presence of infectious agents or are the products of such agents." In view of this, the term phytoalexin and stress metabolite will be used interchangeably in this discussion. In addition to phytoalexins, there are certain compounds that are natural constituents of plants the concentration of which may increase to toxic levels under various stress conditions. Most of the compounds produced as a result of stress are toxic to certain microorganisms and animals; therefore they may be hazardous to humans. However, we should note that humans have been eating these plants, presumably with their stress metabolites, for longer than recorded history. Their toxic characteristics have not yet emerged through the background of other deliberate or natural insults to our metabolism to be considered as one of the highest priorities in food toxicology.

Stress metabolites produced by several important plant families have already been presented in this symposium. In addition, there are several recent reviews on the subject of phytoalexins (18,27,33,34). The

objectives of this discussion are (a) to account for some of the stress compounds produced in other plant families used for food and (b) to correlate their presence with concerns for the safety of humans consuming these plants and/or their products as food.

LEGUMINOSAE

Many species of plants belonging to the Leguminosae family are included in the daily diets of people throughout the world. It is now well established that many legumes are used as additional protein sources for humans. No attempt will be made to mention all of the species from which stress metabolites have been isolated. The concern here will be only those plants that are consumed directly in this country.

Green beans (Phaseolus vulgaris)

Green beans, also referred to as French beans or snap beans, produce a number of compounds when subjected to stress conditions such as infection by certain fungi, bacteria or viruses and treatment with various chemicals. Some of the compounds that have been isolated and identified are shown in Fig. 1 (6,11,14,21,48,55,65). These compounds are all isoflavonoids. Coumesterol and genistein are known to occur naturally in some leguminous plants; however, their concentration has been shown to increase significantly in stressed beans. All of the compounds except coumesterol have been shown to possess significant antifungal activity. Phaseollinisoflavan and kievitone have been found to be bactericidal in preliminary *in vitro* studies (68). Phaseollin is a phytotoxic agent (17,54); 30 $\mu\text{g}/\text{ml}$ rapidly killed plant cells from beans and bean pods. Phaseollin has also been observed to possess hemolytic properties. It was found that concentrations of 23 $\mu\text{g}/\text{ml}$ and higher rapidly lysed ovine erythrocytes and a concentration of 113 $\mu\text{g}/\text{ml}$ lysed bovine erythrocytes (60,62). The presence of the phenolic hydroxyl group was believed to have influenced this activity. In a preliminary study, it was observed that phaseollin was toxic to embryonated chick eggs (15). No other toxicological data have been reported for these compounds in animals. Coumesterol and genistein have known estrogenic activity; this property will be discussed later.

Lima beans (Phaseolus lunatus)

The roots of lima beans produced coumesterol and other coumestans in response to infection with

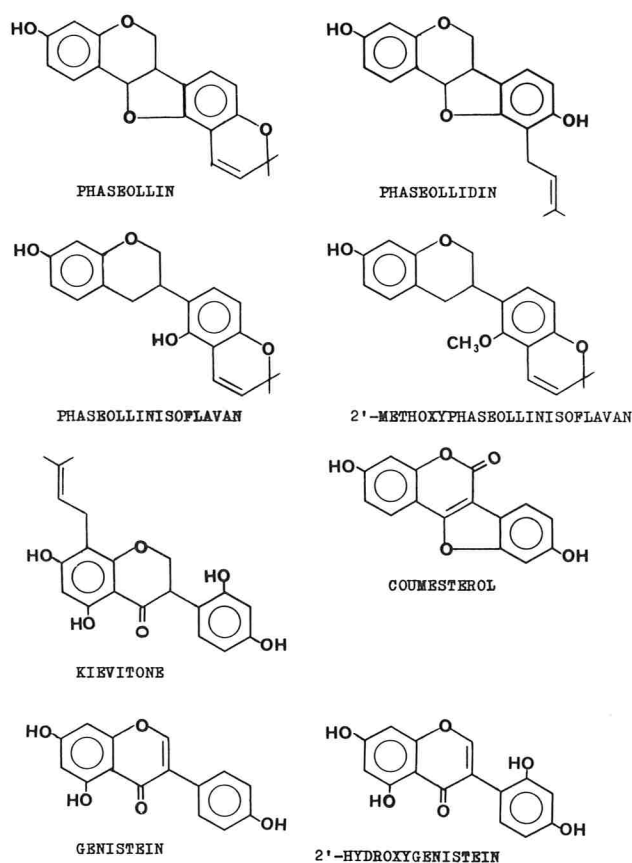


Figure 1. Stress metabolites of green beans.

nematodes (49). These compounds are believed to be active only against nematodes; none exhibited antifungal activity.

Broad beans (Vicia faba)

Isoflavonoid and furanoacetylenic compounds have been isolated from the tissue of infected broad beans (20). The major compounds identified were wyerol, wyerone, wyerone acid, wyerone epoxide and the isoflavonoid medicarpin (Fig. 2). Wyerol exhibited the least antifungal activity and was presumed to be the precursor of the other wyerone derivatives.

Lentil (Lens culinaris)

Wyerone, wyerone epoxide and the isoflavonoid variabilin were isolated from infected lentil seeds (50). Other antifungal compounds were also detected but not yet identified.

Garden peas (Pisum sativum)

A major stress compound identified in garden peas is pisatin (13). This compound is antifungal and can be induced by various fungi, chemicals, UV light and other treatment resulting in cellular injury (47). Pisatin has also been reported as a stress metabolite in other leguminous plants (7,51). Pisatin has been shown to injure plasma membranes of both plant cells and human erythrocytes (40). Other compounds isolated from stressed peas are shown in Fig. 3 (47,57). Inermin (maackiain) is a natural constituent of several legumes and is a more

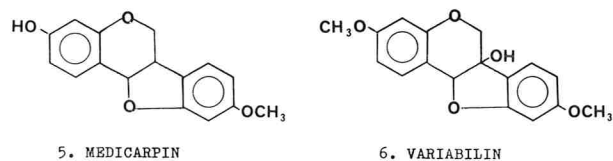
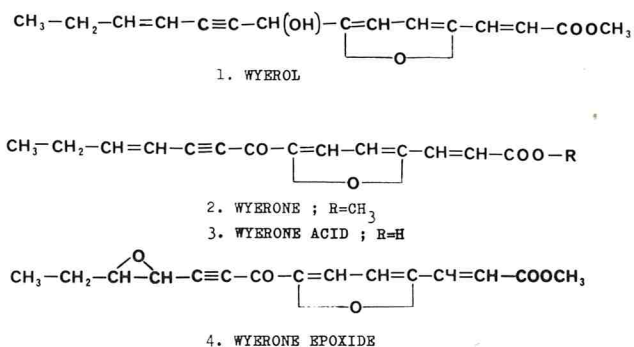


Figure 2. Stress metabolites of broad beans (1-5) and lentils (2,4,6).

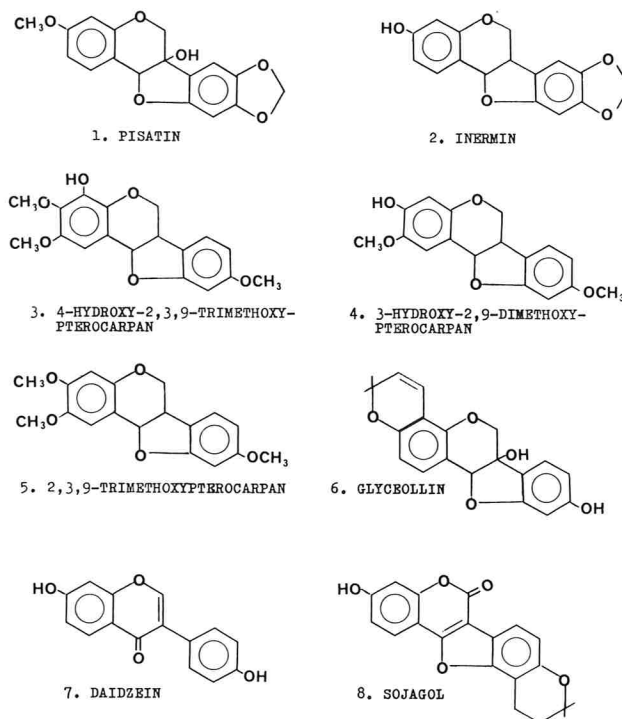


Figure 3. Stress metabolites of peas (1-5) and soybeans (6-8).

potent antifungal agent than pisatin.

Soybeans (Glycine max)

Soybeans are widely used as a protein source for animals. Stress compounds are produced in soybeans as a result of infection by fungi and bacteria or exposure to chemicals and UV light. The compounds identified include glyceollin and its isomers, daidzein, coumestrol and sojagol (10,25,30,31,37). The last three compounds are normal constituents of soybeans; their concentration has been noted to increase also after exposure to air pollutants such as ozone, nitrogen dioxide and sulfur dioxide (29).

Alfalfa (Medicago sativa)

Alfalfa has been used extensively for animal feed; however, in recent years it has been used in various forms for human consumption. At least 11 compounds have been isolated from stressed alfalfa (Fig. 4) (4,41,42,56). All of the compounds except coumesterol, possess significant antifungal activity. Medicarpin at a concentration of 0.35 mM was found to lyse bovine erythrocytes (60).

SATIVAN**SATIVOL****TRIFOLIOL****MEDICAGOL****MEDICARPIN****DAIDZEIN****FORMONONETIN****4',7-DIHYDROXYFLAVONE****3',4',7-TRIHYDROXYFLAVONE****12-O-METHYLCOUMESTEROL****COUMESTEROL**Figure 4. *Stress metabolites of alfalfa.**Groundnuts (Arachis hypogaea)*

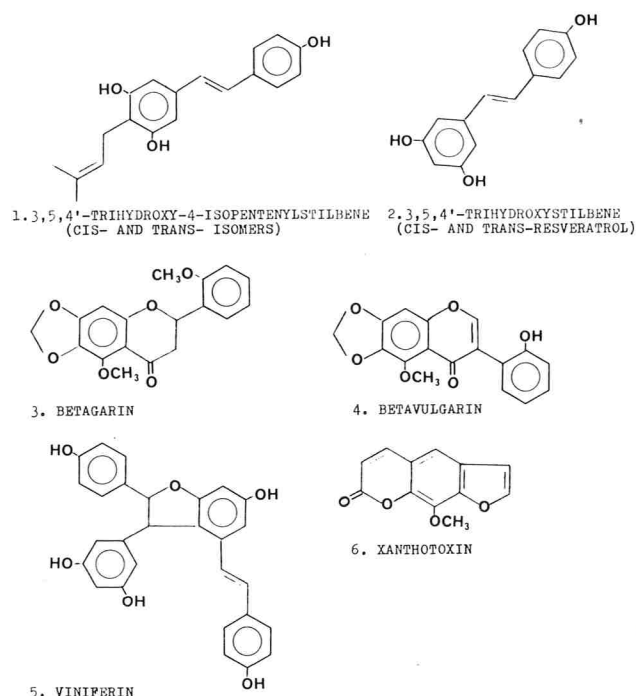
Four antifungal, stilbene-type compounds have been isolated from infected seeds, stems and immature pods of American and African cultivars of peanuts (Fig. 5) (22,28). Before these findings, stilbene compounds had not been reported to occur as stress metabolites in leguminous plants.

Cowpea (Vigna sinensis)

Several isoflavonoids including phaseollin, phaseollidine, kievitone and 2'-O-methylphaseollinisoflavan have been isolated from cowpeas infected by specific fungi or viral agents (2,44) (Fig. 1). A 2-arylbenzofuran compound (vignafuran) has also been isolated (45).

CHENOPODIACEAE

The sugar beet (*Beta vulgaris*) is the only member of this family from which stress compounds have been isolated. Betavulgarin, an isoflavone and betagarin, a flavanone (Fig. 5) were isolated from the leaves of infected beets (16). Betavulgarin exhibited much stronger antifungal activity than betagarin. The results from a study in which the antifungal properties of these compounds were compared with those of other isoflavonoids suggested that both the "iso" structure and

Figure 5. *Stress metabolites of peanuts (1,2), sugar beets (3,4), grapevines (2,5) and parsnips (6).*

the number of substituents on the A ring influence antifungal activity (24). In further studies (38) using whole infected leaves from six different cultivars of sugar beets, it was found that there were distinct differences in the amounts of each stress compound produced, but only the isoflavonoid content could be significantly correlated with a visual rating of the severity of the infection.

VITACEAE

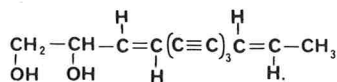
Several compounds are produced in grapevine leaves in response to infection with microorganisms or exposure to UV light (35,36,46). Those identified so far are oligomeric forms of the trihydroxystilbene, resveratrol, which co-occurs with these compounds. The trivial name, viniferins, has been proposed for these compounds. Resveratrol was also isolated from immature grapes.

UMBELLIFERAE

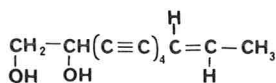
Xanthotoxin (8-methoxypsoralen), a coumarin compound (Fig. 5), is a normal constituent of parsnip tissue. It has antifungal properties and its concentration increases under stress conditions (23). Xanthotoxin and bergapten, which are structurally related, are normal constituents of spring parsley (66). They are reported to be responsible for the phototoxic effect observed when animals consume spring parsley and then remain in the sunlight for several hours (8). Xanthotoxin and 4,5',8-trimethylpsoralen have also been isolated from celery infected with the causative agent of "pink-rot" disease (52).

COMPOSITAE

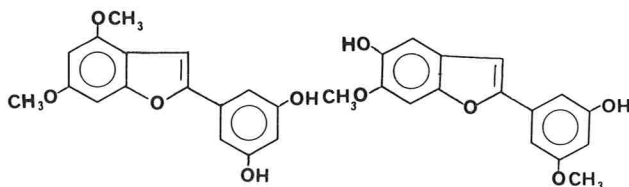
Safynol and dehydrosafynol are acetylenic compounds that are normal constituents of safflower. The levels of these compounds were found to increase significantly in infected plants (1,59). At least 11 other acetylenic compounds have been identified in safflower, but they have not been fully characterized (Fig. 6).



1. SAFYNOLO



2. DEHYDROSAFYNOLO



3. MORACIN A

4. MORACIN B

Figure 6. Stress metabolites of safflower (1,2) and mulberry (3,4).

MORACEAE

Two antifungal compounds designated as moracin A and moracin B were isolated from infected mulberry plants (58). These are believed to be the first stress compounds reported for this plant family (Fig. 6).

CONCERNS FOR THE SAFETY OF FOODS FROM PLANT SOURCES

Production of stress metabolites is not limited to any plant family. A review of the literature reveals that relatively few plant families have been screened for the presence of these compounds. It is significant to note, however, that stress compounds have been either isolated from or suspected of being present in nearly all of the families that have been examined. The discovery that edible seeds of plants like peanuts, peas, green beans and soybeans can produce stress compounds when infected by certain microorganisms (26) should stimulate an increased interest in extending the search for similar compounds in other food plants.

At the present time very little information is available relative to the acute and subacute toxicity of stress metabolites in plants or plant products. It is critical that such information be developed to evaluate the possible hazard to human health posed by the presence of such compounds in foods. It should be noted that of the various compounds referred to in this discussion, no mention has been made of data obtained from detailed

toxicological studies involving these compounds. All of the compounds were reported to exhibit significant antifungal or antibacterial activity (except coumesterol), several caused hemolytic effects in ovine, bovine and human erythrocytes and one was found to be toxic to chick embryos in a preliminary experiment. The need for more toxicological data on these compounds is critical in view of the fact that stress compounds from plants like sweet potatoes (12) and white potatoes (67) have been found to be toxic to several animal species and hence may be hazardous to humans. All of these compounds are not destroyed by normal cooking processes. Concerns about the toxicity of these compounds should not be ignored simply because their concentration may be relatively low in certain foods. Very little information is known about the effects of toxic compounds consumed by humans in low doses for long periods. It has been postulated that some of the nonnutritive minor components in foods may be carcinogenic (39).

The biosynthetic mechanisms involved in production of many stress compounds are not well understood. The progress that has been made in this area on isoflavonoid compounds was reviewed recently by VanEtten and Pueppke (63). It is known that different types of compounds can be produced within the same plant family. This phenomenon has been observed so far in the Leguminosae and Chenopodiaceae families. In cases where detailed studies have been conducted, it has been noted that the metabolite produced depended on the inducing agent. For example, VanEtten and Smith (65) noted a marked contrast in the antifungal compounds produced as a result of infection of bean tissue with *Rhizoctonia solani* and tissue infected with *Fusarium solani*. In both cases, the beans used were from the same seed lot. It is also known from in vitro and in vivo studies that certain fungi are capable of metabolizing stress compounds to give different end products (63). In some cases, the metabolite formed has less antifungal activity than the parent, while in other cases the antifungal activity was found to be equal to that of the parent compound (64). This points out the need for toxicological evaluation of not just one compound, but for as many as possible in each plant family, since no one compound type can be assumed to be the characteristic one for a family.

The question of quantity is a very significant concern with stress compounds. The amount present in a particular plant or plant product will naturally vary depending on the nature and severity of the stress condition and the variety of the plant. In view of this, quantitative methods are needed for these compounds. The level of measurement needed should be based on toxicological considerations. At present, adequate quantitative methodology is not available for most of the stress metabolites.

Several studies have been conducted on certain isoflavonoids to determine the relationship between their structures and their antifungal properties (24,43,61,63).

It was suggested initially that a specific molecular shape was responsible for the antifungal activity of different compounds. This suggestion, however, was not supported by the experimental data obtained; hence further research is needed to establish the relationship. It has also been suggested that the toxic properties of isoflavonoids in the higher plant-fungal interaction may be due to their structural resemblance to steroids (19). Since some fungi are known to require sterols for oospore production, it was suggested that pisatin and phaseollin may prevent the spread of the fungus in the plant by replacing normal sterols in the fungal cell, hence serving as antimetabolites. Coumesterol, genistein, daidzein and formononetin have been shown to be estrogenic. The structures of these compounds are closely related to the natural steroidal estrogen, estradiol. Bickoff et al. (3) determined the relative estrogenic potency of these compounds using a standardized mouse uterine weight bioassay procedure. Although it was found that the relative potency of these compounds was low compared to diethylstilbestrol, they are naturally present in certain plants in sufficient quantities to cause physiological effects. Since the concentration of these compounds can increase under stress conditions, their estrogenic effects will be enhanced. In addition to the above compounds, other isoflavonoids like phaseollin and its congeners have structures similar to diethylstilbestrol; hence their estrogenic potency should be evaluated. Correlation between structure and estrogenic properties of coumesterol and related compounds has been reviewed by Bickoff et al. (5).

The need for genetic improvement in the yield and nutritive value of leguminous plants slated to be used as protein sources for humans has been discussed by Bliss and Hall (9). Plant breeders are continuously developing new varieties of plants as a means of improving their nutritive value, resistance to diseases, texture, market acceptability and other characteristics. It must be kept in mind that genetic alterations to accomplish the above objectives may be accompanied by alterations in the levels of many of the natural components of the plant. It is also possible that the new plant variety may produce stress metabolites quite different from those observed in the original plant. A situation that illustrates this point was reported to have occurred in white potatoes (53). In view of this, it is essential that some toxicological data be obtained on existing plant varieties so that the safety of new varieties can be assessed. These data, along with information on biosynthetic mechanisms involved in forming stress compounds, would be useful to plant breeders and others involved in the development of new plant varieties.

Finally, caution should be exercised in applying new processing, handling and storage techniques indiscriminately to various plant products. Consideration should be given to the fact that certain techniques may subject the product to physiological stress and result in the production of undesirable compounds.

CONCLUSIONS

To ascertain that food from various plant sources is safe for human consumption, it is essential that we have a thorough knowledge of the constituents present. Although progress is being made in the area of isolation and identification of stress compounds from a number of plants, there are many more plants that have yet to be investigated. Very little consideration has been given to the stress imposed on plants from environmental sources like temperature, rainfall, agronomic practices etc. Most of the identified stress compounds have not been evaluated toxicologically. The few toxicological effects that have been noted strongly suggest that more in-depth studies are needed. To obtain all of the information needed as a basis for establishing a monitoring system for stress compounds in foods, there must be a unified effort from many disciplines. A close relationship must be established between plant pathologists and other horticultural scientists, nutritional biochemists, chemists, toxicologists and plant breeders; research obtained from each should be rapidly disseminated. Only through a concerted effort of this type can we hope to obtain the necessary information that will permit a proper assessment of the safety of foods from plant sources.

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Stress Metabolites of the Potato and Other Solanaceous Plants

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ABSTRACT

The effect of stress on the chemical composition of the *Solanaceae*, primarily *Solanum tuberosum* (potato), may be profound and have significant health implications. Changes in glycoalkaloids, steroids, sesquiterpenes and other lipids that result from specific and non-specific stress are discussed. The biochemistry and toxicology of these compounds are reviewed.

The terms "stress metabolite" and "phytoalexin" often have been used interchangeably, especially in discussions of disease resistance in solanaceous plants. For purposes of this review, we classify phytoalexins as a special class of stress metabolites, i.e., compounds that are not normally found in healthy tissue but accumulate in response to a disease situation and have a deleterious effect on the disease organism. Other types of stress metabolites may develop or accumulate in larger amounts than found in healthy tissue in response to nonspecific stress such as mechanical injury or environmental stress. All phytoalexins are stress metabolites but not all stress metabolites are phytoalexins. This paper reviews the chemical characterization of these compounds, their formation, their detection and what is known of their toxicity. We discuss only in passing the relationship of these compounds to disease resistance. This review covers mainly the stress metabolites of the potato (*Solanum tuberosum*), the major edible solanaceous crop on which most of the research on solanaceous stress metabolites has centered.

GLYCOALKALOIDS

Figure 1 shows the structure of α -solanine (I) and α -chaconine (II), the major glycoalkaloids of *S. tuberosum*, the species from which most cultivated potatoes in the United States and Europe are derived. The purpose of initial research on potato glycoalkaloids encompassed the development of methods to isolate, separate, identify and quantify these compounds. From the structure of α -chaconine and α -solanine, it is apparent that this class of compounds presents analytical problems in: (a) isolation due to poor solubility in most solvents and (b) detection by spectroscopy due to lack of a good chromophore. Methods described in the literature were either insensitive or nonspecific (3,9). Figure 2 summarizes the details of a titrimetric method for total

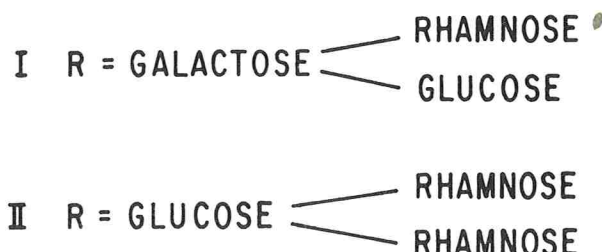
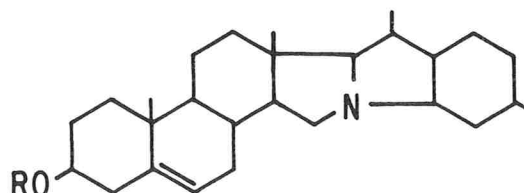


Figure 1. The major glycoalkaloids of *Solanum tuberosum*.

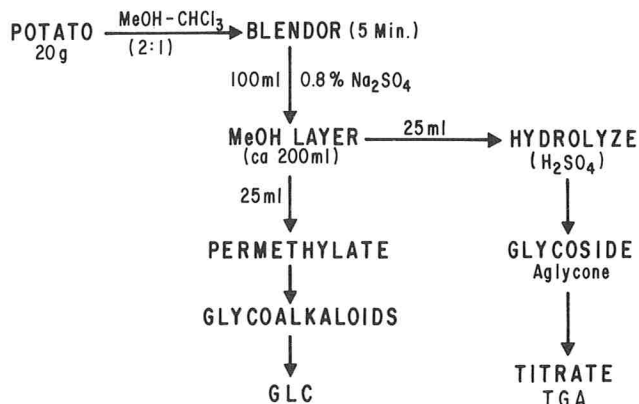


Figure 2. Quantitative and qualitative analytical procedure for potato tuber glycoalkaloids.

glycoalkaloids (TGA) (6) and a gas-liquid chromatographic (GLC) method (12) for qualitative (and relative quantitative) analysis of the individual glycoalkaloids developed in our laboratory. Figure 3 shows a GLC chromatogram of authentic glycoalkaloids. The glycoalkaloids, for qualitative and quantitative analysis, were extracted according to the procedure of Wang et al. (32). The methods we developed are specific for detection of all glycoalkaloids, whereas other methods described in the literature are either specific for a few glycoalkaloids (e.g., base insoluble or $\Delta 5$ unsaturated glycoalkaloids) or are not specific for glycoalkaloids.

We applied our procedures to studies of the glycoalkaloid composition of wild *Solanum* species and

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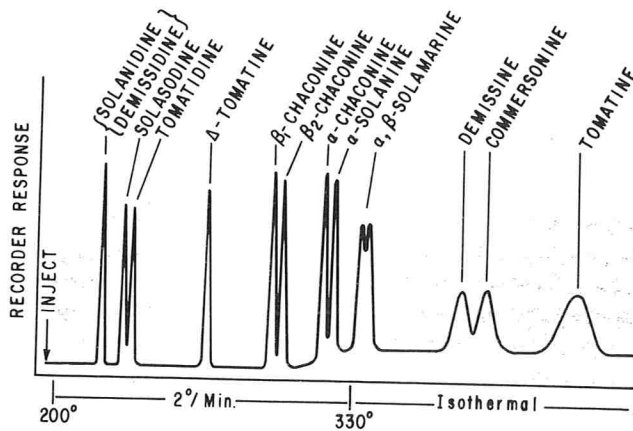


Figure 3. Gas-liquid chromatography of standard glycoalkaloids.

the effect of nonspecific stress on glycoalkaloid composition in potatoes. Our objective was to determine the inter-relationship of breeding stock and stress conditions on the control of glycoalkaloid composition and content of potatoes. The glycoalkaloid compositions of some wild *Solanum* species are summarized in Table 1. Heterogeneity was observed not only between species but also between different clones of the same species.

To dramatize the effects of mechanical stress on glycoalkaloid composition, we used potato slices as a model system. Slicing represents extreme damage that may be reflected to a lesser degree in other types of injury such as incurred in mechanical harvesting. Our investigation (7) of market potatoes revealed a number of tubers that had been cut and subsequently wound-

healed. We chose potatoes that contained only *S. tuberosum* genes and also varieties that had *Solanum demissum* in their parentage; Shih and Kuć (30) had reported the presence of solamarines in slices of cultivars derived from *S. demissum*.

In our experiments (7), facing slices were taken from the center of a potato, one slice was analyzed immediately and the other slice was analyzed after wound-healing (about 4 days at room temperature). Before being sliced, the potatoes were held under typical storage conditions (7 C and 85% relative humidity). Table 2 gives TGA levels for four commercial varieties held for extended storage periods, then sliced and analyzed immediately and after 4 days. As expected, slicing caused dramatic increases in TGA; however, the ability of slices to synthesize glycoalkaloids appears to maximize before 34 weeks of whole-tuber storage. More interestingly, storage times can significantly affect glycoalkaloid composition of damaged tubers (Table 3). After aging, Kennebec slices contained measurable quantities of α - and β -solamarine, and the level of solamarines was higher in aged slices of freshly harvested tubers than in aged slices of tubers stored for 12 or more weeks. These results indicate that damaged tubers of varieties that contain genes from other than *S. tuberosum* can have unexpected glycoalkaloid composition.

The Lenape potato, which was ready for release as a new variety about 6 years ago, rekindled interest in potato glycoalkaloids when tubers from plants grown in a greenhouse caused illness of two persons that ate them.

TABLE 1. Glycoalkaloids of selected *Solanum* species.

Species ^b	Glycoalkaloid						
	β -Chaconine ^c	α -Chaconine	α -Solanine	Solamarines ^a	Demissine	Tomatine	TGA mg/100 g FWE
<i>S. ajanhuiri</i>	3.5	39.0	57.3				
<i>S. curtilobum</i>		34.8	46.4	5.3	13.4		< 0.1
<i>S. stenotomum</i>	5.5	69.8	24.7				< 0.1
<i>S. juzepczukii</i>		14.0	37.8	7.7	40.4		0.7-1.3
<i>S. acaule</i> 1 ^d					95.5		1.7
2					62.1	30.9	1.4-5.0
3					88.2	11.6	1.4-5.0
4					64	34	1.4-5.0

^aCombined value for α - and β -solamarine.

^bAll species are cultivated except for *S. acaule*.

^cValues represent percent of total glycoalkaloids.

^dFour clones of species *S. acaule* were analyzed.

^eFresh weight.

TABLE 2. Total glycoalkaloid content^a of unaged and aged^b tuber slices of four varieties of stored potatoes^c.

Potato variety	Condition	Storage time of tubers before slicing					
		0 time	6 weeks	12 weeks	18 weeks	24 weeks	34 weeks
Wauseon	Unaged	3.16	5.27	4.84	3.01	3.61	5.53
Wauseon	Aged	77.88	126.43	145.85	108.24	92.45	64.38
Katahdin	Unaged	5.61	11.09	7.47	11.48	9.35	9.02
Katahdin	Aged	106.26	117.66	130.21	143.73	137.04	102.31
Houma	Unaged	3.83	6.39	4.93	3.02	3.65	7.30
Houma	Aged	58.97	51.89	34.67	84.20	95.46	61.88
Kennebec	Unaged	9.38	6.84	10.42	7.42	6.39	11.24
Kennebec	Aged	154.56	—	150.50	158.88	163.09	119.52

^aAll values expressed as mg/100 g fresh weight.

^bFour days in the dark at room temperature.

^cControlled storage, 44 F and 85% relative humidity.

Subsequently, the Lenape was found to produce higher levels of glycoalkaloids than did other commercial potato varieties, and it was withdrawn from introduction. This variety was derived from a cross between *Solanum chacoense* and *S. tuberosum*. *S. chacoense* is a heterogeneous species with respect to glycoalkaloid composition; clones have been shown to contain various combinations of α -solanine, α -chaconine, β -chaconine and leptines (28). When we undertook this study, no Lenape potatoes were available (having been withdrawn as a new variety); however, one can speculate that damaged Lenape tubers might have an interesting glycoalkaloid composition.

Although results from many laboratories, including ours, suggest that a possible health problem could result from consumption of damaged potatoes because of high and unusual glycoalkaloid composition, no systematic study has been undertaken to determine the glycoalkaloid composition of damaged market potatoes. We made a cursory study of the glycoalkaloid composition of significantly damaged tubers (8). The TGA's obtained in this experiment are given in Table 4. Higher TGA levels were found in the damaged end (A) of the tuber compared to those found in the equivalent undamaged portion (B) of the same potato; however, these differences were not large and did not appreciably affect average TGA values (C). The effects of stress on the glycoalkaloid composition of other edible *Solanaceae* such as egg plant (*Solanum melongena*) and tomato (*Lycopersicon esculentum*) have not been determined.

Levels of glycoalkaloids toxic to *Helminthosporium carbonium* were found in potato peels (1); however, Deahl et al. (5) found no correlation between levels of glycoalkaloid and late blight resistance.

The toxicity to laboratory animals of the major glycoalkaloids, α -solanine and α -chaconine, has been

measured (20,21). Although these compounds were toxic when administered intraperitoneally, they were not toxic when administered orally to mice at concentrations greater than 1 g/kg of body weight. Cardiac activity measured in a frog heart test for a number of other glycoalkaloids was similar to that of α -solanine (22). Historically, potato toxicity has been associated with glycoalkaloid concentration, although the data supporting this correlation are indirect (2). The potential for producing hazardous levels of some new glycoalkaloid does exist under the right combination of breeding and stress conditions. With the increased use of wild species in breeding programs, especially for increased pest resistance, glycoalkaloid levels and types should be carefully monitored in all parent selections to avoid years of lost research in breeding and associated financial losses.

SESQUITERPENE PHYTOALEXINS

The phytoalexins of *Solanaceae* that are produced in response to microbial infections are a class of stress metabolites that has been extensively examined in the last 10 years. About 20 phytoalexins have been identified to date. These compounds are either sesquiterpene or nor-sesquiterpene derivatives. Figure 4 shows the structures of some of the compounds that have been identified. All are potato phytoalexins except aubergene, which is a stress metabolite of *S. melongena*, and capsidiol, which is a stress metabolite of *Nicotiana* and *Capsicum brutesceus* (goat pepper). All these compounds can be shown to arise from cyclization of farnesyl pyrophosphate to yield the proper intermediate. A likely intermediate leading to most of these structures would be the eudesmane skeleton (Fig. 5) proposed by Stoessl and Ward (31). Sato et al. (27), however, recently demonstrated that the spiro compound oxylubimin (Fig. 4) is

Table 3. Percentages of the major glycoalkaloids in unaged vs. aged tuber slices from potatoes stored at 44 F.

Storage (weeks)	Wauseon				Houma			
	Unaged		Aged ^a		Unaged		Aged ^a	
	α -chac ^b %	α -sol ^c %	α -chac ^b %	α -sol ^c %	α -chac ^b %	α -sol ^c %	α -chac ^b %	α -sol ^c %
0	74.6	25.4	49.6	50.4	69.0	31.0	50.0	50.0
6	68.7	31.3	44.4	55.6	67.2	32.8	48.3	51.7
12	66.2	33.8	40.6	59.4	68.1	31.9	43.2	56.8
18	86.0	14.0	44.8	55.2	74.5	25.5	46.1	53.9
24	80.1	19.9	44.0	56.0	71.8	28.2	42.5	57.5
34	73.3	26.7	43.4	56.6	70.1	29.3	40.1	59.9

Storage	Katahdin				Kennebec					
	Unaged		Aged ^a		Unaged		Aged ^a			
	α -chac ^b	α -sol ^c	α -chac ^b	α -sol ^c	α -chac ^b	α -sol ^c	α -chac ^b	α -sol ^c	β -sola ^d	α -sola ^d
0	64.1	35.9	42.7	57.3	58.9	41.1	24.9	37.1	11.5	26.5
6	58.4	41.6	37.2	62.8	54.9	45.1	26.8	33.3	12.4	27.5
12	60.8	39.6	38.5	61.5	52.3	47.7	24.3	43.4	8.3	24.0
18	65.2	34.8	35.3	64.7	59.8	40.2	28.2	44.6	6.8	20.5
24	67.0	33.0	36.5	63.5	63.9	36.1	26.6	54.0	4.6	14.8
34	65.0	35.0	38.0	62.0	57.7	42.3	23.8	56.0	5.6	14.6

^aFour days in dark room at ambient temperature.

^b α -Chaconine.

^c α -Solanine.

^dSolamarines (found only in aged Kennebec slices).

the precursor of rishitin. Shih et al. (29) suggested that the formation of sesquiterpenes may result at the expense of steroid synthesis. Although the importance of these compounds in disease resistance is still not clear, they do result from what is termed the hypersensitive or incompatible host-parasite interaction. They can be formed also in nonhypersensitive interactions (such as *Erwinia caratovora* var. *atroseptica* vs. *S. tuberosum* (17,18)) in which the typical indicators of hypersensitivity, necrotic lesions, are not observed. Nonspecific inducers of these compounds such as NaF (19) have been reported.

A number of other unidentified stress metabolites arise from fungus- or fungal extract-inoculated potato slices, but these are found in much smaller quantities than the compounds shown in Fig. 4.

The anti-fungal (10) and anti-bacterial activities (16) of rishitin and other potato phytoalexins have been investigated. Although they are fungistatic, these metabolites when sprayed on leaves did not reduce the incidence of late blight, whereas capsidiol sprayed on tomato leaves significantly controlled *P. infestans* development (33).

Stress metabolites were implicated as teratogens when Renwick (25), a well-known British epidemiologist,

proposed a correlation between blighted potato consumption by mothers and the birth defects spina bifida and anencephaly in their offspring. Although some initial experimental evidence (23) supported this hypothesis, more extensive experiments including feeding studies with potato tubers high in the phytoalexins rishitin and phytuberin did not confirm the original conclusion (24). Keeler et al. (13) indicated that potato sprouts may contain materials teratogenic to the golden hamster. The toxicity of the potato phytoalexins has not been investigated comprehensively; however, solavetivone (katahdinone) has been tested in a chick embryo bioassay and found to be nontoxic (4). We intend to determine the toxicity of many of these compounds, if they can be isolated in sufficient amounts for meaningful evaluation.

NONSPECIFIC STRESS COMPOUNDS

While the phytoalexins are generally formed by a specific stress (i.e., fungal infection), nonspecific stress, as already mentioned, also can produce these compounds. Nonspecific stress is responsible for many more chemical alterations in the *Solanaceae* besides changes in glycoalkaloid levels or formation of phytoalexins. Many of these changes, including stimulated phenolic synthesis

TABLE 4. Glycoalkaloid analysis of bruised commercial potatoes.

Cultivar	Sample ^a	Fresh wt (g)	Dry powder (g)	Solids (%)	mg TGA/100 g fresh wt
Katahdin	1-A	112.3	23.8	21	16.5
	1-B	147.7	29.5	19	6.3
	1-C	92.3	18.0	19	13.4
	2-A	65.5	13.6	20	14.5
	2-B	53.8	18.7	34	9.2
	2-C	94.0	11.7	12	8.4
	3-A	97.4	20.2	20	19.5
	3-B	129.8	25.0	19	15.0
	3-C	110.0	22.0	20	11.2
	4-A	61.1	12.4	20	19.3
	4-B	101.0	18.2	18	10.2
	4-C	110.0	20.2	18	10.0
	Russet Burbank	5-A	113.0	28.0	24
5-B		111.0	24.7	22	1.5
5-C		110.0	22.0	20	3.1
6-A		47.0	13.5	28	7.2
6-B		57.0	13.6	23	1.5
6-C		59.0	15.0	25	6.1
7-A		198.5	49.0	24	7.6
7-B		119.6	23.2	19	2.5
7-C		88.1	20.0	22	3.3
8-A		45.2	9.2	20	5.5
8-B		80.0	16.6	20	1.8
8-C		40.0	7.4	18	5.3
Red Pontiac		9-A	114.6	21.5	18
	9-B	123.6	21.5	17	2.3
	9-C	73.4	13.0	17	4.9
	10-A	122.0	21.0	17	6.9
	10-B	95.5	18.0	18	1.8
	10-C	87.5	14.0	16	2.1
	11-A	162.0	35.5	21	7.5
	11-B	133.0	26.0	19	4.6
	11-C	73.0	14.0	19	12.1
	12-A	79.5	14.0	17	6.07
	12-B	73.0	14.0	19	2.58
	12-C	51.0	10.0	19	6.26

^aA = damaged end; B = center section; C = undamaged end.

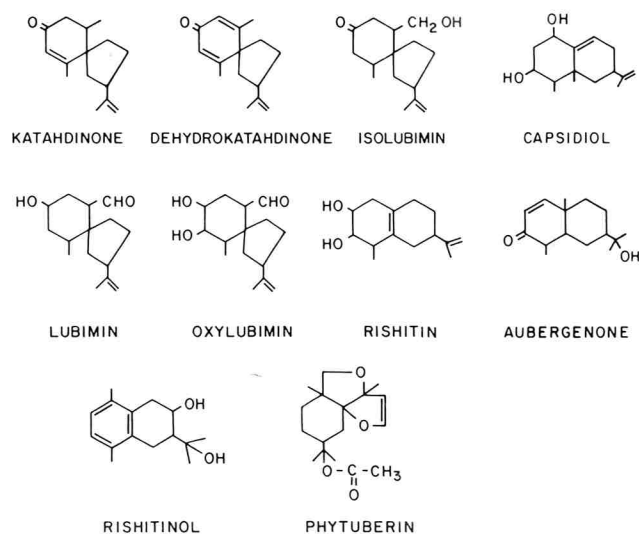


Figure 4. Sesquiterpene phytoalexins.

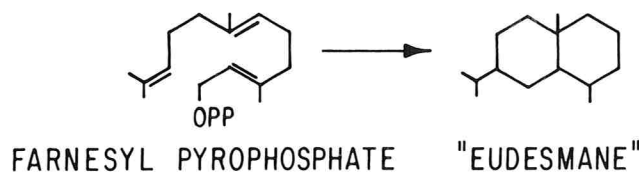


Figure 5. Biosynthetic pathway for phytoalexins.

(such as chlorogenic acid and scopoletin) and suberization, have been defined. Such chemical alterations have been observed because of the ease of detection of these compounds. The extent of damage to fruits and vegetables due to mechanical harvesting and handling necessitates a more comprehensive examination of the chemical composition of these commodities. Toward this end, we analyzed the tuber slice system for compositional changes other than those previously observed. Initially, we examined that fraction of tuber tissue that contains the plant sterols. Hartmann and Benveniste (11) reported the rapid increase of sterols in aged potato slices. This also was observed in our study; however, a much more dramatic chemical distinction was noted when extracts from incubated potato slices were compared with fresh potato extracts. The C-28 alcohol, octacosanol, which could not be found in the fresh extract, was isolated in relatively large amounts from the incubated slices (Table 5). The rate at which octacosanol is formed is shown in Fig. 6. A lesser amount of n-hexacosanol also was isolated, but no other fatty alcohols (C_{16} - C_{30}) could be detected in either fresh or aged slices (15). Suberin, which is rapidly formed during wound healing, contains esters of fatty alcohols in its structure (14). However, only traces of octacosanol were found after deesterification of suberin. Octacosanol may have a physiological function similar to that reported for triacontanol in alfalfa (26). Preliminary results in our laboratory indicate that this compound also may affect host-parasite interactions.

Stress may alter chemical composition via catabolic processes, for example, by stimulating hydrolase activity. In our investigation of the *E. caratovora* var. *atroseptica*-*S. tuberosum* interaction, appreciable amounts of

TABLE 5. Sterol^a content of fresh and aged potato slices.

Sterol	$\mu\text{g/g f.w.}^{\text{b}}$	
	Fresh	4 days aged ^c
Cholesterol	0.1	0.42 ^a
n-octacosanol	< 0.1	3.94
Stigmasterol	0.38	1.50
β -sitosterol	0.56	1.60

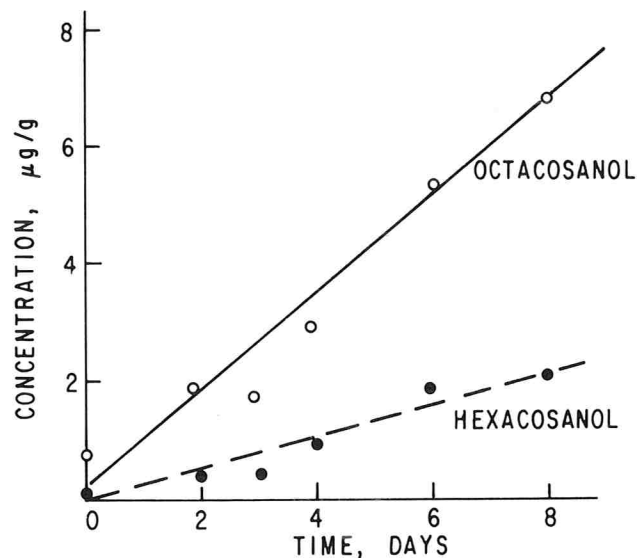
^aMajor sterols.^bFresh weight.^cIn dark.

Figure 6. Production of octa- and hexacosanol in potato slices.

solanidine, the aglycone of solanine and chaconine, were recovered (34). Solanidine is not found in unstressed tuber tissue. The hydrolase activity apparently resides in the tuber, since microwaved slices (that contain solanine and chaconine) do not yield solanidine when treated with this species. Other hydrolytic products of glycoalkaloids also have been identified in nonspecifically disrupted tissue (29).

In this review we attempted to show the complexity of chemical changes that occur when plant tissue is stressed. Although abundant information on the chemical composition of healthy plant tissue is available, we know much less about the composition of this tissue at the time of consumption, when many chemical alterations may have occurred due to stress. These chemical alterations may have significant human health implications and therefore warrant further investigation.

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Jezeski Joins Faculty of the University of Florida

Dr. James J. Jezeski has joined the Faculty of the University of Florida as Extension Dairy Technologist. Jezeski, a well-known microbiologist, served the University of Minnesota for 22 years as Assistant, Associate, and Full Professor of Dairy Bacteriology.

From 1969 to 1973 he was professor and coordinator of environmental studies at Montana State University in Bozeman, MT. From 1973-78 he was Director of Research and Development for the H.B. Fuller Company, Monarch Chemicals Division.

Jezeski was recently selected vice-president of the National Mastitis Council. He is active in IAMFES and is a member of the Applied Laboratory Methods Committee and is chairman of the Farm Sanitation Chemical Residues Advisory Subcommittee.

Active in many other societies and associations, Jezeski is also a member of the American Dairy Science Association, American Public Health Association, American Society for Microbiology, Institute of Food Technologists, National Environmental Health Association, Sigma Xi, Gamma Sigma Delta, and Phi Tau Sigma.

NMC Holds Annual Meeting



The Executive Board of the National Mastitis Council, elected at that group's Annual Meeting in February, are, left to right: President, Boyd Cook, Maryland Cooperative Milk Producers, Inc., Baltimore, MD; Secretary-Treasurer, John B. Adams, National Milk Producers Federation, Washington, DC; and Vice-president, L. H. Schultz, Univ. of Wisconsin, Madison, WI. Approximately 400 persons attended the meeting in Louisville, KY. The NMC summer meeting will follow the IAMFES Annual Meeting Aug. 16 in Orlando, FL. The program for those meetings is included in the May issue of the *Journal*.

Control of Terpenoid Metabolism in the Potato- *Phytophthora infestans* Interaction

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ABSTRACT

Inoculation of tubers with fungi non-pathogenic to potato suppresses glycoalkaloid accumulation and elicits accumulation of norsequi and sesquiterpenoids. Inoculation with compatible races of the pathogen *Phytophthora infestans* suppresses accumulation of terpenoids. Suppressors and elicitors of terpenoid accumulation were isolated from *P. infestans*. Specificity in the *P. infestans* - potato interaction appears controlled by suppressors (17-23 glucose units linked $\beta 1 \rightarrow 3$, $\beta 1 \rightarrow 6$). Ethylene, temperature and aging also markedly influence terpenoid accumulation.

Terpenoids are found in all tissues of potato and, as in all plants and animals, they have vital and varied roles in normal metabolism. Sterols are important constituents of membranes in both plants and animals. In addition, steroids function as hormones and vitamins in animals, whereas sesqui and diterpenoids function as hormones in plants. Two groups of terpenoids, the steroid glycoalkaloids and sesquiterpenoid phytoalexins, have recently received attention because of their possible role in protecting potato against infectious diseases (11-18,28).

The steroid glycoalkaloids are normal constituents in all tissues of potato, but they accumulate to high concentrations around sites of injury. The sesquiterpenoid phytoalexins accumulate only around sites of infection or in response to certain forms of stress. Unlike the steroid glycoalkaloids, their presence in potato is relatively short lived (13). Though steroid glycoalkaloids are found in all tissues of potato at all stages of growth, the sesquiterpenoid phytoalexins are found only in and around infected tissues and are not detected in healthy tubers or foliage. Both the steroid glycoalkaloids and sesquiterpenoid phytoalexins are toxic to many and varied bacteria and fungi, and both may be part of the defense mechanism of potato against infectious disease (17).

Potato tissues carrying R genes for resistance to *Phytophthora infestans* respond hypersensitively when inoculated with incompatible races of the fungus. The hypersensitive reaction is characterized by the loss of electrolytes (21), rapid cell death and tissue browning (10) and accumulation of terpenoids (13,15,16,22,35). Compatible races of the fungus penetrate and develop in host tissue for at least 3 days without causing collapse of host cells (31) or significant terpenoid accumulation (22,35).

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CONTENT AND DISTRIBUTION OF STEROID GLYCOALKALOIDS

The principal steroid glycoalkaloids in potato (*Solanum tuberosum* L.) are α -solanine and α -chaconine. They are found in leaves, stems, flowers, berries, tubers and sprouts. Shih (24) reported the following distribution in potato (expressed as mg/g fresh wt based on α -solanine): leaves, 1.2; stems, 0.11; flowers, 5.6; berries, 1.9; peeled tuber, 0.06; tuber peel, 1.2; sprouts 12.2. Though the steroid glycoalkaloids in tubers are largely concentrated in the peel, peeled potato slices rapidly accumulate the compounds in the upper 1-2 mm of slice surface to levels equal to or greater than that in the peel (1,24). Light is not necessary for accumulation of steroid glycoalkaloids at the slice surface (1,24-26) but sunlight markedly enhances the accumulation in whole tubers. The enhanced content of steroid glycoalkaloids is associated with the greening of tubers (8,24,26), and extends into tissue considerably below the peel (5-10 mm). The high level of steroid glycoalkaloids in foliage, fruit and flowers may be due to the exposure of these parts to light. In sprouts, the steroid glycoalkaloids appear concentrated at the growing point (20) and, as with slices, light is not necessary for their accumulation. Young leaves also contain higher concentrations of steroid glycoalkaloids than old leaves (24). It appears the compounds are localized in the epidermal layers of sprouts (unpublished data) and it would be interesting to determine if they are also localized in outer tissues of foliage. Perhaps they also should be classified as stress metabolites (12,28) as they accumulate in surface tissues of plants as a response to light or oxygen stress.

Recently α -solamarine and β -solamarine were reported as major steroid glycoalkaloids in foliage and in aged tuber slices of the cultivar Kennebec (27). These compounds were not found in 20 other cultivars tested. Kennebec is derived from a cross between a wild Mexican species, *Solanum demissum*, and *Solanum tuberosum*. The glycosylation pattern of α - and β -solamarine is derived from the latter and steroid alkaloid from the former parent.

It is apparent that different tissues of potato differ greatly in the content of steroid glycoalkaloids. Age, light, stress and genetic background can also markedly influence the content.

EFFECT OF INFECTION ON STEROID GLYCOALKALOID CONTENT

Accumulation of steroid glycoalkaloids in potato tuber slices is markedly suppressed when the cut surface is inoculated with either *P. infestans*, a pathogen of potato, or *Helminthosporium carbonum*, a pathogen of corn (26). Cell-free sonicates of compatible and incompatible races of *P. infestans* applied to the surface of potato slices equally suppressed accumulation of steroid glycoalkaloids, but suppression was greater with the living incompatible than compatible race. Marked suppression of steroid glycoalkaloid accumulation is associated with the accumulation of high levels of the norsesquiterpenoid phytoalexin rishitin. The steroid glycoalkaloids apparently do not inhibit rishitin accumulation per se since exogenous α -solanine had little effect on the accumulation of rishitin or the steroid glycoalkaloids. Aging slices for 72 h before inoculation or treatment with sonicates reduced rishitin accumulation, but this effect did not appear to be caused by the steroid glycoalkaloids accumulated during aging before treatment or inoculation.

Accumulation of steroid glycoalkaloids and rishitin in tubers appears due to de novo synthesis via the acetate-mevalonate pathway (25). The branch point in the synthetic pathway which leads to rishitin or the steroid alkaloids appears to be after mevalonate. Uninoculated slices incorporated more ^{14}C from acetate and mevalonate into steroid alkaloids than did slices inoculated with *P. infestans*. Slices inoculated with an incompatible race of the fungus incorporated less isotope into the steroid alkaloids than those inoculated with a compatible race. Incorporation of ^{14}C into rishitin was greater in slices inoculated with the incompatible as compared to the compatible race.

Shih and Ku \bar{c} (25) suggested that the increased accumulation of sesquiterpenoid phytoalexins is due to a diversion of biosynthesis from steroid glycoalkaloids at a branch point beyond mevalonate, probably at farnesyl-pyrophosphate. Ishizaka and Tomiyama (7) suggested that the biosynthesis of α -solanine and α -chaconine are under separate and independent control because the steroid glycoalkaloids accumulate over a much wider area of tissue than do the sesquiterpenoid phytoalexins. Their data do not negate the obvious competition for acetate and subsequent precursors in the acetate-mevalonate pathway, leading to the synthesis of steroid glycoalkaloids, when the sesquiterpenoid phytoalexins accumulate.

It is apparent that accumulation of the sesquiterpenoid phytoalexins is associated with suppressed accumulation of the steroid glycoalkaloids in potato tubers.

ELICITATION AND SUPPRESSION OF SESQUITERPENOID PHYTOALEXINS

Tomiyama et al. (32) isolated rishitin, a norsesquiterpenoid phytoalexin, from potato tubers inoculated with

incompatible races of *P. infestans* and its structure was established by Katsui et al. (9). Using eleven cultivars of potato and three races of *P. infestans*, Varns et al. (35) demonstrated that incompatible but not compatible interactions were associated with rapid, but limited, necrosis of host tissue and the accumulation of 16-18 terpenoids including rishitin and phytuberin. Many of the terpenoids which accumulate have been chemically characterized including rishitin, rishitinol, phytuberin, phytuberol, lubimin, hydroxylubimin, anhydro- β -rotunol and solavetivone (15-17,28). Rishitin and other terpenoid phytoalexins also accumulate in potatoes inoculated with nonpathogens of potato (19,29,32,36). This observation made it evident that susceptibility to *P. infestans* was not determined by the lack of a genetic potential to produce the terpenoid phytoalexins. To complicate matters further, it was demonstrated that cell-free preparations from all races of *P. infestans* elicited accumulation of sesquiterpenoid phytoalexins in all cultivars of potato, even those lacking "R" genes for resistance (22,33,35,36). More recent work in our laboratory indicates that a major elicitor of the hypersensitive reaction in potato is localized in the fungal cell wall. The key to specificity in the potato-*P. infestans* interaction appears to be the ability of compatible races of the fungus to suppress hypersensitive cell death, necrosis and terpenoid accumulation (3,33,34).

Two water soluble glucans containing 17-23 glucose units linked β -(1 \rightarrow 3) and β -(1 \rightarrow 6) were isolated from mycelia and zoospores of race 1234 (compatible) and race 4 (incompatible) of *P. infestans* (4,5). The glucans from both mycelia and zoospores included a nonanionic and an anionic glucan; one or two residues of the latter were esterified with a phosphoryl monoester. Death of host cells, browning and the accumulation of rishitin in tuber slices inoculated with race 4 or treated with an elicitor from the fungus were suppressed by pretreatment of slices with the glucans. The glucans from the compatible race were more active in suppressing the hypersensitive reaction than those from the incompatible race, and the anionic glucan was more active than the nonanionic glucan.

Crude elicitors from race 4 and 1234 lost terpenoid-eliciting activity when mixed with a microsomal fraction prepared from potato tuber tissue. The glucans from the compatible race, but not the incompatible race, markedly reduced the loss resulting from the reaction between crude elicitor and the microsomal fraction. The loss of electrolytes from potato discs treated with elicitors was also suppressed by glucans from the compatible but not incompatible race of the fungus.

The reaction of potato sprouts to compatible and incompatible races of *P. infestans* differs from that of tubers and leaves (20). Zoospores of race 4 and 1234 of *P. infestans* elicited browning and accumulation of the terpenoids rishitin, lubimin, phytuberin and phytuberol in sprouts of Kennebec, Russet Burbank and Red Pontiac potatoes. There was little or no difference in the

quantities of the terpenoids which accumulated in sprouts from potatoes inoculated with a compatible or incompatible race of *P. infestans*. Autoclaved, cell-free sonicates of *P. infestans*, *Pythium aphanidermatum*, *Achlya flagellata*, *Phytophthora parasitica* and *Aphanomyces euteiches* also elicited browning and accumulation of terpenoids. As with spores, there was little or no difference in the extent of browning or quantities of terpenoids that accumulated in sprouts from tubers of a cultivar which lacked R genes for resistance and those from a cultivar with R₁. Kennebec potato sprouts treated with sonicates of *P. infestans* browned more intensely and accumulated more of the terpenoids at 19 C than at 14 or 25 C, but neither browning nor terpenoid accumulation were detected at 30 and 36 C. These data are consistent with the report by Currier and Kuć (2). Sprouts inoculated with zoospores of *P. infestans* browned first closest to the growing point and then over the length of the inoculated area. There was no difference in the appearance of browning of sprouts treated with cell-free sonicates of the fungi. Growth and sporulation of *P. infestans* was not apparent on inoculated sprouts.

Aging of potato slices before inoculation influences the accumulation of rishitin as well as steroid glycoalkaloids. Tomiyama (30) and Sato et al. (23) demonstrated that a 5-24 h period between slicing tubers and inoculating the surface decreased the time required for cell death and rishitin accumulation in incompatible interactions. Aging for more than 24 h markedly reduced the accumulation of rishitin (26), and the reduction might be explained by suberization and phenol accumulation and oxidation at the slice surface. Potato slices aged for 72 h were highly resistant to *P. infestans*, even though rishitin did not accumulate, indicating that suberization and phenol accumulation and oxidation may also be part of the defense mechanism of the potato.

EFFECT OF ETHYLENE ON TERPENOID ACCUMULATION

Potato slices treated with Ethrel (2-chloroethylphosphonic acid) and then inoculated with *Helminthosporium victoriae*, *H. carbonum* or an incompatible race of *P. infestans*, accumulated considerably more phytuberin and a deacetylated derivative of phytuberin, phytuberol, than did slices treated with water followed by inoculation (6). Accumulation of the two terpenoids also was increased in slices treated with Ethrel followed by cell-free, autoclaved sonicates of *P. aphanidermatum* or *A. flagellata*. Ethrel did not elicit consistent changes in the accumulation of rishitin and lubimin in tissue accumulating high concentrations of phytuberin and phytuberol. Ethrel did not increase the accumulation of phytuberin, phytuberol, rishitin or lubimin in noninoculated slices, slices inoculated with compatible races of *P. infestans* or slices treated with sonicates of *H. carbonum*, *H. victoriae* or *Neurospora crassa*. More phytuberin and phytuberol accumulated in slices treated with Ethrel followed by a sonicate of *P. infestans* at 19 C than at 14

or 25 C, and the terpenoids were not detected in tissues incubated at 30 and 36 C. Ethylene (100 µl/l) markedly increased accumulation of phytuberin and phytuberol in slices treated with cell-free sonicates of *P. infestans*, but alone it did not elicit accumulation of the terpenoids. Ethrel did not alter the resistance or susceptibility of the potato cultivars Kennebec or Russet Burbank to race 4 and 1234 of *P. infestans* or the resistance of both cultivars to *H. victoriae* and *H. carbonum*.

DEGRADATION OF SESQUITERPENOID PHYTOALEXINS BY POTATO

Unlike the steroid glycoalkaloids, the sesquiterpenoid phytoalexins are not stable end products of metabolism (7,13,37). They reach a peak in accumulation 96-120 h after infection or treatment with elicitors and their levels decrease until they are barely detectable 7-10 days after treatment. Their accumulation, therefore, may be as much as a result of decreased degradation as a result of increased synthesis.

SUMMARY

Many factors contribute to regulation of terpenoid accumulation in potato tubers infected with *P. infestans*. Incompatible but not compatible races of the fungus strongly elicit accumulation of sesquiterpenoid phytoalexins. Cell-wall constituents from compatible and incompatible races of the fungus have been extracted which elicit terpenoid accumulation in all cultivars of potato. Specificity in the potato - *P. infestans* interaction may be determined by the ability of compatible races of the fungus to produce low molecular weight glucans which suppress terpenoid accumulation and hypersensitive cell death in the host. Temperature, aging, ethylene and mechanisms for degradation within the host also influence accumulation of the terpenoids, and these factors also may influence susceptibility and resistance of potato to the fungus.

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Angevine Award Winners Announced

Winners of the Neil C. Angevine Superior Quality Award were announced at the March 19-21, 1979 American Cultured Dairy Products Institute Kultures and Kurds Klinik in Columbus, Ohio.

The award is presented to the dairy plant with the highest cumulative score for all cultured products evaluated by experts at the national judging contest, held annually in

conjunction with the ACDPI training schools.

First place Angevine Award winner was Borden, Inc., Columbus. Second place finisher in the overall products competition was Smith Dairy Products. Third place was captured by Grocer's Dairy Co.

The 1979 Klinik drew 275 delegates from 30 states, Canada, and Mexico.

Mull Retires from U. of Florida Faculty

Dr. Leon E. Mull retired October 31, 1978 from the Dairy Science Department at the University of Florida following 38 years of service. He and his wife Carol plan to continue living in Gainesville.

Induction of Rishitin and Lubimin Synthesis in Potato Tuber Slices by Non-Specific Elicitors — Role of Gene Derepression

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ABSTRACT

The stress metabolites rishitin and lubimin accumulate at relatively low concentrations (5-20 ppm) in potato tuber slices subjected to various cell-disruptive treatments including heavy metal salts, sulfhydryl reagents, metabolic inhibitors, detergents, ultraviolet light and lysosomal enzymes. Cold-stored (4 C) tubers are more disposed to terpene accumulation than freshly harvested, 25-C stored and conditioned potatoes. Various inhibitors of DNA transcription and mRNA translation block terpene induction by non-specific elicitors when applied at sufficiently high concentration. However, various protein synthesis inhibitors were shown to be potent elicitors of terpene accumulation when applied at lower concentration. Actinomycin D (25 µg/ml) treatment of discs for 30 min elicits higher levels of rishitin than results from *Phytophthora infestans* interaction with potato (> 100 ppm). A mechanism for terpene induction based on derepression of "stress metabolite DNA" is proposed to explain the experimental data.

Early observations by Müller and Börger in 1940 with potato led to the phytoalexin theory of plant immunity. According to the phytoalexin concept, a specific metabolic interaction between a plant host and microorganisms leads to accumulation of chemical compounds which halt infection. These antimicrobial substances generally fall into certain chemical groups - notably isoprenes, phenylpropanoids and isoflavanoids. There appears to be a taxonomic element in phytoalexin biosynthesis (7). Thus, the *Leguminosae* in general produce isoflavanoids, *Solanaceae* diterpenes and the *Compositae* produce polyacetylenes. A given tissue may produce more than one chemical type of phytoalexin under different or even identical conditions of microbial attack. For example, potato tubers will accumulate diterpenes, phenolic substances and glycoalkaloids all of which have been implicated as phytoalexins. The subject of plant immunity has been studied extensively in recent years and is summarized by several authors (2,6,12,14, 16).

In the past few years it has become known that phytoalexin accumulation is not dependent on a specific host-parasite interaction. Rather, a wide range of biological, chemical and physical trauma to the host tissue may incite the injured cells to accumulate anti-microbial agents. The term "stress metabolite" has been adopted by various workers to categorize substances which arise from such varied stimuli or "non-specific elicitors." Some examples of non-specific

stresses known to incite the host tissue to produce these novel compounds are listed in Table 1. Most, if not all, of these treatments lead to a limited disruption of cells at the wounded surface. Tissues differ greatly in their response to such stimuli. Generally, but not always, the amounts of stress metabolites formed as a result of non-specific elicitors are approximately an order of magnitude lower than occurs as a result of interaction with a specific parasite of the host (3).

TABLE 1. *Some trauma known to elicit stress metabolites in plant tissues.*

Physical

Cut-injury, bruising
 Chilling injury
 Ultraviolet light
 Visible light
 Low oxygen tension

Chemical

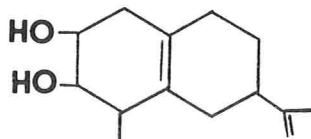
Mercuric and other heavy metal salts
 Sodium lauryl sulfate and other detergents
 Cysteine and various thiol reagents
 Ethylene, cyclic AMP
 DNA intercalating agents, basic proteins and polyamines
 Fungal and insect extracts

Several stress metabolites are now known to be toxic to mammals as well as microorganisms. Stress metabolites are now recognized as an additional concern in the realm of food safety. For this reason, we are investigating the types of trauma which can give rise to these unusual substances during the harvest, postharvest storage, handling, processing and marketing of fruits and vegetables. A basic understanding of the mechanisms underlying the switch triggered by non-specific elicitors will be invaluable to this end.

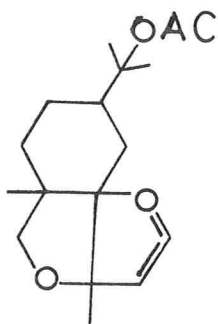
STRESS METABOLITES IN POTATO TUBER

Extensive studies with potato phytoalexins have been in connection with (R) gene resistance of potato to the late blight pathogen *Phytophthora infestans*. One group of phytoalexins accumulating in blighted potatoes are the isoprene derivatives, notably rishitin, phytuberin and lubimin (Fig. 1). Rishitin is first detected when inhibition of *P. infestans* growth occurs and rapidly accumulates to levels many times higher than necessary to prevent fungal growth completely (18,19). Rishitin and other terpenes also accumulate in response to several

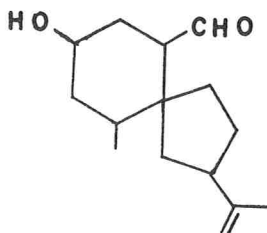
RISHITIN



PHYTUBERIN



LUBIMIN



POTATO TUBER

Figure 1. Examples of terpenes which accumulate in late blight infected potatoes. Approximately sixteen terpenes have been detected on thin layer plates of which eight have been identified.

non-pathogens of potato (15,17). The primary event determining a compatible or incompatible reaction of potato to *P. infestans* appears to be due to an interaction which occurs within hours and probably seconds after penetration (12). There is a paucity of information regarding the induction of these terpenes by non-specific elicitors. Varns et al. (17) and Price et al. (13) indicated that restricted cell death caused by a variety of chemicals and physiological stimuli did not cause rishitin accumulation although cell-free sonicates of *P. infestans* caused rishitin to accumulate with no browning. Tomiyama and Fukaya (15) observed that trace quantities of rishitin (1.4 $\mu\text{g/g}$ of fresh weight) accumulate in tuber discs treated with mercuric chloride after a 4-day incubation period. Similarly, low-level accumulation of rishitin in potato discs was observed after

treatment with a nematocide, DBCP (11). We have recently shown that previous failures in observing significant accumulations of rishitin by non-specific elicitors relate to failure in precisely controlling the storage history of the tubers and experimental conditions of treatment (1).

NON SPECIFIC ELICITORS OF POTATO TERPENES

Freshly harvested tubers do not accumulate appreciable levels of terpenes when subjected to stimuli of the type shown in Table 1. However, after approximately 1 month's postharvest storage, terpene accumulation is readily induced by a wide range of cell-disruptive treatments. The propensity of potato slices to accumulate terpenes increases gradually during the first 3 months of storage and remains intact for at least 1 year. Rishitin accumulation resulting from mercuric acetate treatment of freshly cut slices is shown in Fig. 2. Tubers stored at 4 C are more disposed to terpene accumulation than are those held at 25 C. Conditioning of 4 C tubers for 2 weeks at 25 C resulted in behavior similar to the 25-C stored tubers shown in Fig. 2. Hence the metabolic alterations which occur during 4-C storage are readily reversible. Induction of rishitin accumulation was highly

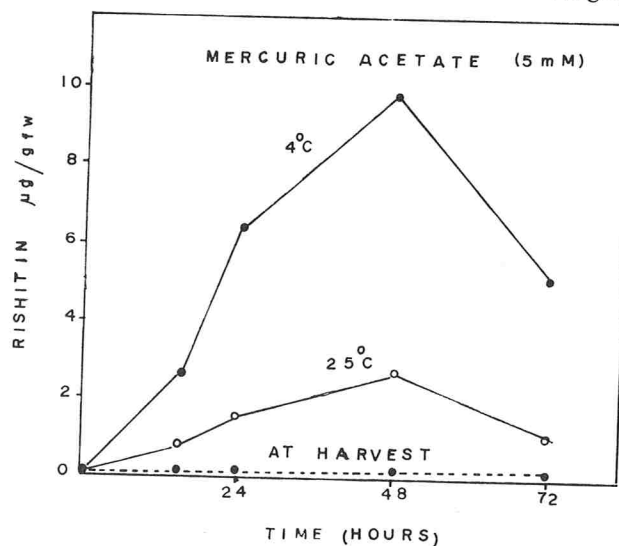


Figure 2. Induction of rishitin by treating freshly prepared tuber slices (5 mm \times 25 mm) with 5 mM mercuric acetate for 3 min followed by incubation at 25 C for up to 72 h. Rishitin was analyzed by gas liquid chromatography as described by Cheema and Haard (1).

dependent on the concentration of mercuric acetate (Fig 3). Optimal induction of rishitin as a function of mercuric acetate concentration was also a function of tissue disc thickness. Thinner discs respond optimally to lower concentrations to mercuric acetate while thicker discs respond best to higher concentrations. Mercuric acetate treatment creates obvious injury and death to surface cells of the discs and rishitin accumulation is dependent on retention of a healthy layer of cells beneath the necrotic zone. Other heavy metal salts are also effective in inducing rishitin formation when the extent of injury is controlled by monitoring metal ion

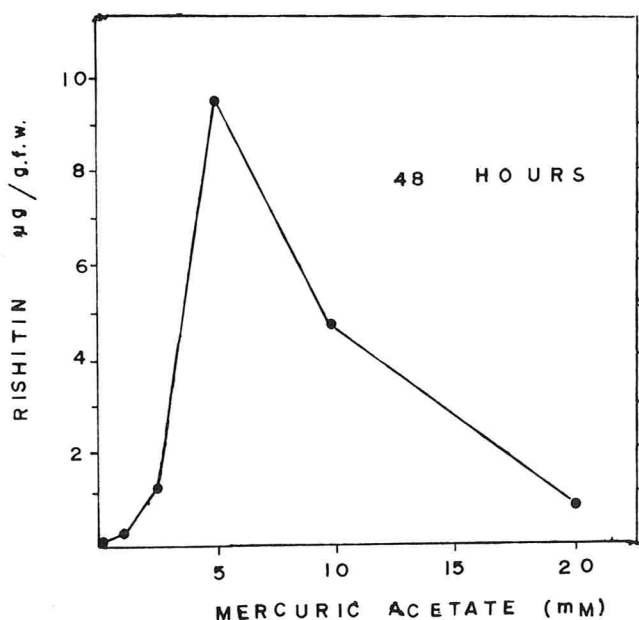


Figure 3. Influence of mercuric acetate concentration on rishitin accumulation by potato slices (5 × 25 mm) after 48 h incubation at 25 C. Slices were treated with mercuric acetate for 3 min immediately after cutting. Data from Cheema and Haard (1).

concentration and disc thickness (Table 2). Ultraviolet light is also an effective elicitor of terpene accumulation in potato discs (Fig. 4). Potatoes stored at 4 C are more disposed to terpene-induction by U.V. light than 25-C stored tubers and freshly harvested tubers are not responsive to this treatment. Ultraviolet light exposure does not cause the obvious tissue browning and necrosis observed with heavy metal treatments. Rishitin accumulation is highly dependent on the exposure time to light which, in turn, is dependent on potato disc thickness (Fig. 5). Long-term exposure to U.V. light, where little rishitin accumulated, results in a loss of cell turgor as judged by the flaccid nature of the discs.

Mercuric acetate and ultraviolet light are conceivably inciting terpene induction by triggering a receptor possessing a thiol group. Therefore an experiment was designed to test the effect of various sulfhydryl group specific reagents on terpene induction. Table 3 shows that influence of these reagents on rishitin and lubimin

TABLE 2. Examples of heavy metal salts which induce rishitin in potato tuber slices.

Inducer (5 mm)	Rishitin (48 h) ¹
Hg Cl ₂	5.2
Hg CH ₂ COOH	11.5
Ag NO ₃	3.6
Cu Cl ₂	13.4
Fe Cl ₃	N.D. ²
Fe Cl ₂	N.D.
Ni Cl ₂	N.D.
Co Cl ₂	N.D.
Mn Cl ₂	N.D.
Ce (NH ₄) NO ₃	N.D.
H ₂ O	N.D.

¹µg/gram fresh weight of discs (5 mm × 25 mm).

²N.D.-not detectable.

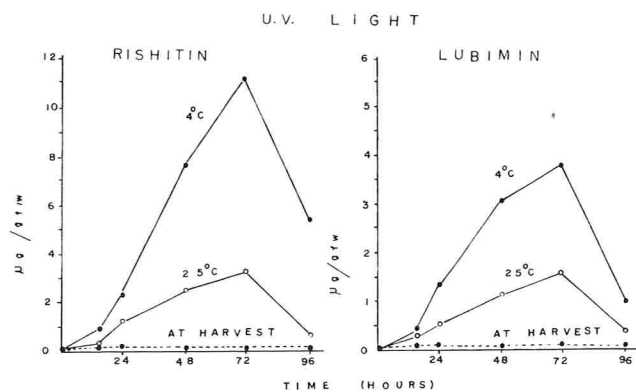


Figure 4. Induction of rishitin and lubimin accumulation in potato discs (5 mm × 25 mm) by ultraviolet light as a function of storage history of the tubers. Discs were irradiated with ultraviolet light at a distance of 20 cm from the source (General Electric G3OT8 bulb) emitting at 254 nm for 2 h and subsequently incubated at 25 C in the dark. Data from Cheema and Haard (1).

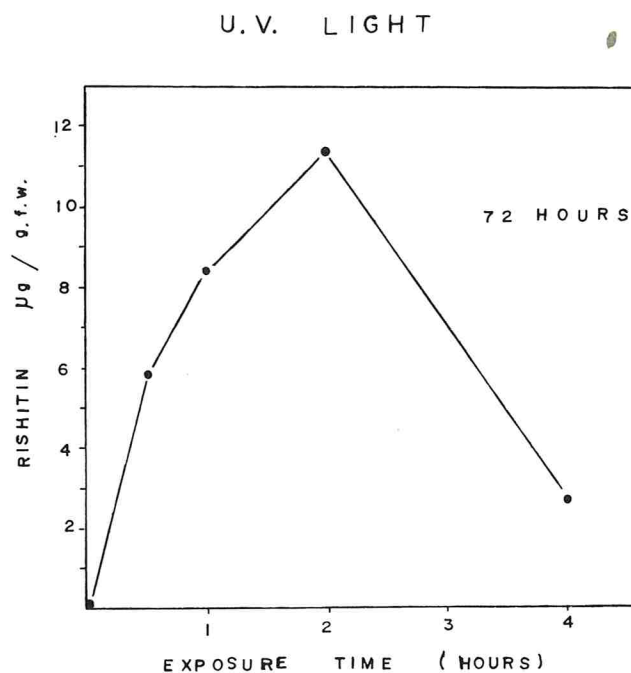


Figure 5. Influence of ultraviolet light exposure time on rishitin accumulation by potato tuber slices. Incubation time after treatment was 72 h at 25 C.

TABLE 3. Induction of terpenes in potato tuber discs by reagents which interact with sulfhydryl groups.

Inducers (5 mm)	4-C stored tubers		25-C stored tubers	
	Rishitin ¹	Lubimin ¹	Rishitin ¹	Lubimin ¹
Mercuric acetate	9.7	0.8	2.1	0.4
Mercuric chloride	5.2	1.1	0.7	0.1
N-ethyl maleimide	0.6	0.3	N.D. ²	N.D.
Iodoacetamide	4.1	2.3	0.2	N.D.
P-Chloromercuribenzoic acid	3.9	3.3	0.1	0.1
H ₂ O	N.D.	N.D.	N.D.	N.D.

¹µg terpene per gram fresh weight 48 hours after treatment (5 mm × 25 mm discs).

²N.D. - not detectable.

(Data from Cheema and Haard, 1978).

accumulation by potato discs from the two storage temperatures. Again, discs from the cold-stored potatoes accumulated more terpenes than those stored at 25 C. All sulfhydryl reagents tested elicited terpene accumulation although their effectiveness was variable. Levels of rishitin and lubimin are similar following pCMB treatment while rishitin concentration is approximately 10 times higher than lubimin in mercuric acetate-treated discs. Iodoacetamide and NEM treatments result in only slight browning of the discs while necrosis from pCMB and mercuric chloride treatments is similar to discs exposed to mercuric acetate.

Various other cell disruptive agents, such as detergents, metabolic inhibitors and lysosomal enzyme preparations, were also somewhat effective in promoting terpene accumulation (Table 4). Hence, a wide range of treatments can trigger cut injured potato tissue to accumulate terpenes. In all instances the predominant terpenes which accumulate are rishitin and lubimin with occasional appearance of other faint zones on thin-layer chromatographic plates after spraying with 50% sulfuric acid. In all cases tested, cold-stored tubers show a greater propensity for terpene accumulation than those stored at 25 C and freshly harvested tubers are insensitive to non-specific elicitors.

REQUIREMENT FOR PROTEIN SYNTHESIS

Various inhibitors of mRNA translation prevent accumulation of rishitin and other terpenes when applied at suitable concentrations before treatment with mercuric acetate or U.V. light (Table 5). These data indicate a requirement for the synthesis of enzymes required for terpene biosynthesis. However, application of actinomycin D, an inhibitor of DNA transcription, did not prevent terpene induction by U.V. light or mercuric acetate. Initially, this indicated to us that potato tubers contain long-lived mRNA for terpene biosynthetic enzymes. However, it was subsequently observed that treatment of discs with actinomycin D alone is effective

TABLE 4. Effect of various treatments on terpene accumulation in discs from 4 C stored in potatoes.

Class	Inducer	Concentration	Rishitin ¹
Metabolic inhibitors	Sodium flouride	5 mm	+
	Sodium azide	5 mm	N.D. ²
	Potassium cyanide	5, 10, 100 mm	N.D.
Detergents	Sodium lauryl sulfate	1%	+
	Triton X-100	1%	N.D.
Oxidants, antioxidants	Hydrogen peroxide	1%	N.D.
	Ascorbate	5-25 mm	N.D.
	BHA	10 mm	N.D.
	TBHQ	10 mm	N.D.
	Na OCl	0.1%	N.D.
Salts	KCl	Saturated	N.D.
	Na Cl	Saturated	N.D.
Rat liver fractions	Sodium acetate	10-25 mm optimal	+
	Homogenate	20 mg/ml	+
	Lysosomes	20 mg/ml	+
	Nuclei	20 mg/ml	+
	Microsomes	20 mg/ml	-
	Cytosol	20 mg/ml	-

¹Rishitin present on thin layer plates at concentrations comparable to mercuric acetate-treated slices. Tubers had been stored at 4 C for at least one month.

²N.D. - not detectable.

TABLE 5. Influence of inhibitors of protein synthesis on mercuric acetate induction of rishitin.

Treatment	Rishitin (μ /g fresh weight)
H ₂ O	N.D.
Mercuric acetate (5 mm)	9.8
Cycloheximide (50 μ m) + mercuric acetate (5 mm)	N.D.
Cycloheximide (50 μ m)	N.D.
Blasticidin S (100 μ m) + mercuric acetate (5 mm)	2.1
Blasticidin S (100 μ m)	N.D.
Puromycin (200 μ m) + mercuric acetate (5 mm)	3.8
Puromycin (200 μ m)	N.D.

¹N.D.-not detectable, data for discs 5 mm \times 25 mm incubated for 48 hours after treatment.

(Data from Cheema and Haard, 1978).

in promoting terpene accumulation when applied at low concentrations (Fig. 6). The time course of rishitin accumulation resulting after actinomycin D treatment differed from that of mercuric acetate treatment in that levels continued to rise up to 96 h (Fig. 7). The

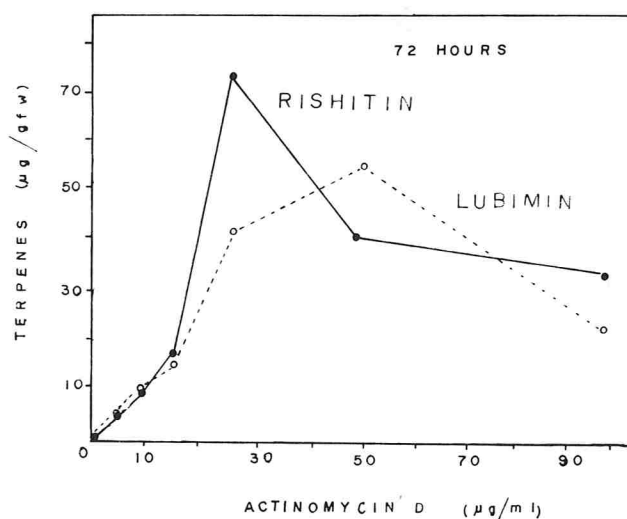


Figure 6. Induction of rishitin and lubimin accumulation in potato tuber slices by treatment with actinomycin D for 30 min. Slices were subsequently incubated at 25 C for 72 h and analyzed for terpenes. Tubers were previously stored at 4 C.

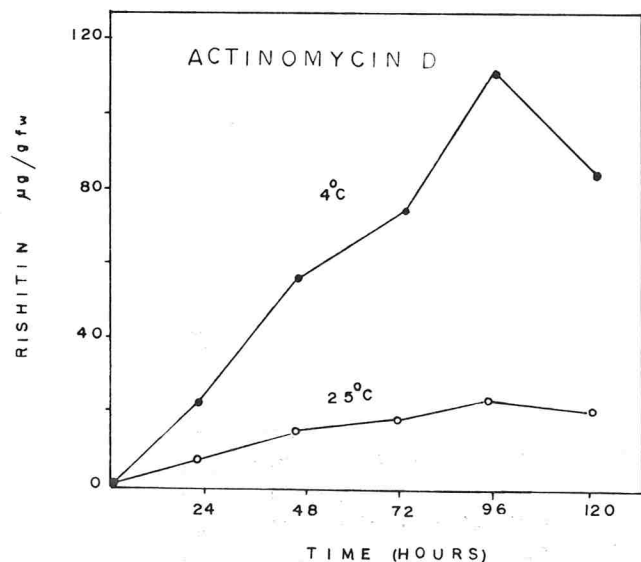


Figure 7. Time course of actinomycin induction of rishitin in slices prepared from potatoes previously stored at 4 or 25 C. Slices were treated with 25 µg/ml actinomycin D/ml for 30 min and subsequently incubated at 25 C for the indicated times.

concentration of rishitin which accumulates is comparable to that observed in *P. infestans*-infected potatoes when optimal concentrations of actinomycin D are applied to freshly prepared discs.

Earlier reports showed that actinomycin D induces accumulation of the isoflavanoid stress metabolite pisatin in pea pods (5). Hadwiger's group has provided evidence that actinomycin D and other inducers of pisatin, such as poly-L-lysine, act by binding to DNA (4). In essence, their theory states that phytoalexin inducers affect multiple segments of nuclear DNA and that the changes in DNA conformation which occur in the globular regions improve the accessibility or "melting in" of polymerase to regions previously inaccessible. The experimental basis for this model is the DNA-complexing properties of inducer compounds, and the induced changes in the template and dye-binding properties of pea chromatin.

We have shown that various DNA binding agents, in addition to actinomycin D, are inducers of rishitin and lubimin (Table 6). These data support Hadwiger's theory of phytoalexin action. However, additional studies have led us to challenge this interpretation of the data and provide an alternate model. First, agents which affect

TABLE 6. Influence of DNA intercalating compounds on rishitin accumulation by discs from 4-C stored tubers.¹

Compound	Concentration	µg/g fresh weight	
		Rishitin	Lubimin
Actinomycin D	25 µg/ml	52.5	30.1
Mitomycin C	100 µg/ml	6.6	N.D.
Distamycin A	25 µg/ml	12.6	5.4
Ethidium bromide	100 µg/ml	7.4	2.8

¹Terpenes were assayed after 72 hours of incubation at 20 C. All treatments were for 30 minutes.

protein synthesis by mechanisms other than DNA-intercalation also induce terpenes in potato discs (Table 7). That is, DNA binding is not a prerequisite for terpene induction. Appropriate low concentration of the translational inhibitor, cycloheximide, is an equally effective inducer of rishitin as DNA intercalating agent (Table 7, Fig. 8). Moreover, induction of rishitin by translational or transcriptional inhibitors does not occur if cut injured tuber discs are incubated for a few hours before treatment (Fig. 9). This indicates that events occurring shortly after cut injury counter the inducer properties of actinomycin D and is not explained by Hadwiger's conception of phytoalexin induction. We have, accordingly, proposed an alternate model to explain these experimental findings (Fig. 10). According to this model, terpene induction by various agents is dependent on the availability of DNA template which contains the necessary information for phytoalexin biosynthesis. Stored tubers do not produce rishitin spontaneously as a consequence of cut injury because the immediate biochemical event following cut injury is the synthesis of repressor proteins which result in a repression of the phytoalexin operon. This is also supported by the work of Kahl and Wielgat (9) who showed that cut injury of tubers results in a dramatic decline in DNA template availability (Fig. 11).

Moreover, potatoes stored at a warm temperature have lower template availability than those stored in the cold. Any agent which perturbs biosynthesis of phytoalexin repressor proteins at the transcriptional or translational level immediately following mechanical wounding, without also inhibiting total protein synthesis, will result in expression of the phytoalexin genome. This is supported by the fact that both transcriptional and translational inhibitors induce terpene accumulation at

TABLE 7. Induction of rishitin by protein synthesis inhibitors which function by mechanisms other than DNA intercalation.¹

Inducer	Action	Concentration	Rishitin	Lubimin
6-Methyl purine	Blocks transcription by mechanism other than intercalation	50 µM	8.2	3.7
Cycloheximide	Blocks translation by interfering with peptide formation	10 µM	17.4	3.5
Puromycin	Blocks translation by mimicking amino-acyl-tRNA	50 µM	12.4	20.6

¹Tubers were previously stored at 4 C; terpenes were assayed after 72 hours of incubation at 20 C. All treatments were for 30 minutes.

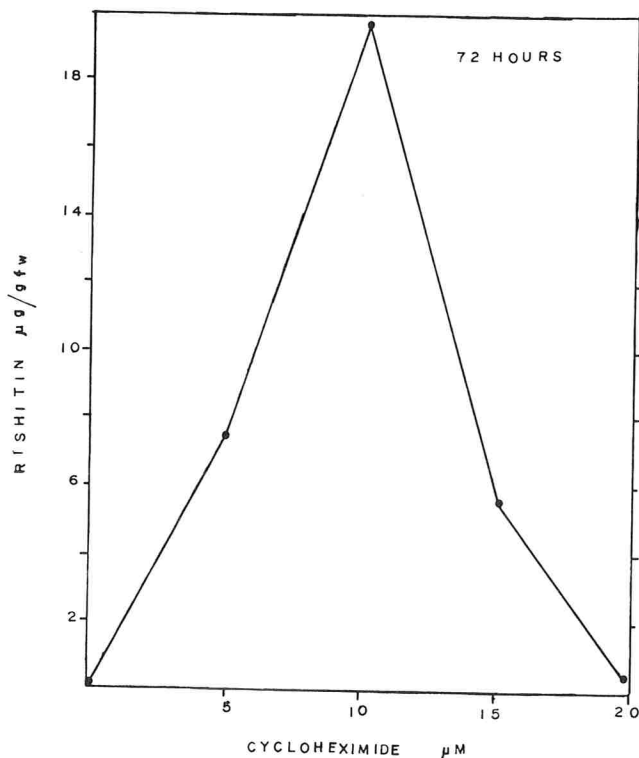


Figure 8. Induction of rishitin accumulation by cycloheximide. Freshly cut discs (5 × 25 mm) were submerged in the indicated concentrations of cycloheximide for 30 min and subsequently incubated at 25 C for 72 h. Higher concentrations of cycloheximide blocked terpene induction by other agents such as actinomycin D.

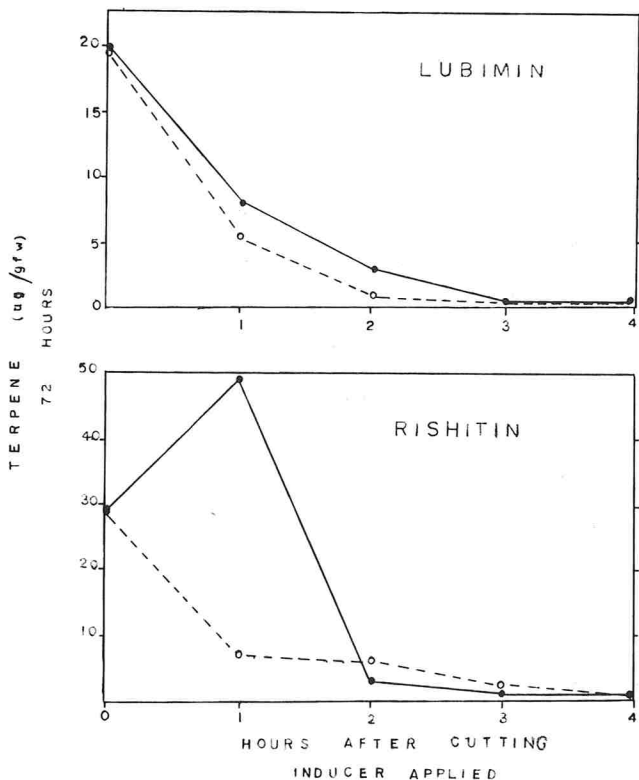


Figure 9. Influence of tissue disc pre-aging in air and terpene induction by 10 µg/ml cycloheximide/ml for 30 min (0---0) and 25 µg actinomycin D/ml for 30 min (●—●).

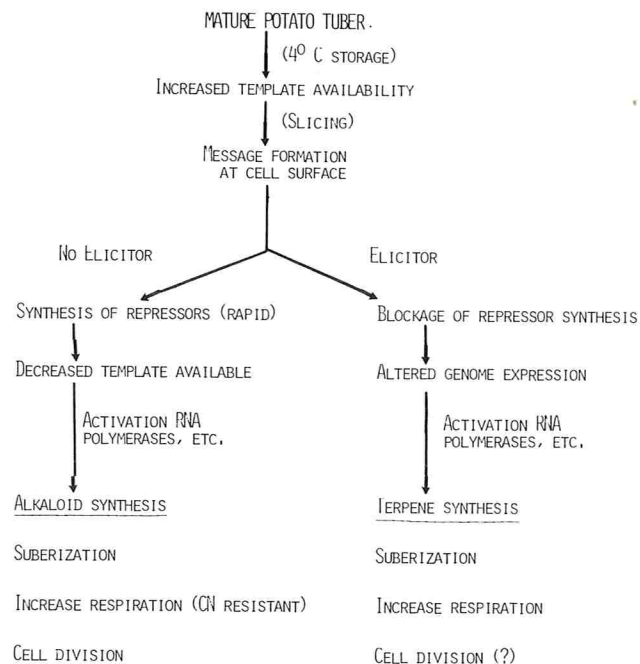


Figure 10. Scheme showing a proposed mechanism of terpene induction by elicitors based on prevention of repressor protein synthesis immediately following cut injury.

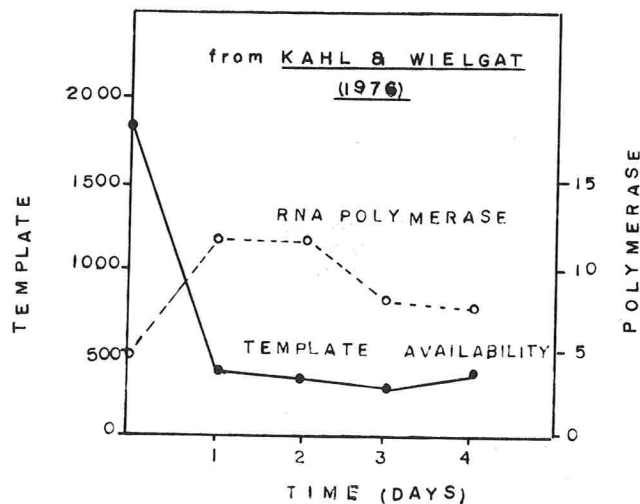


Figure 11. Influence of slicing on DNA template availability and chromatin bound RNA polymerase in potato tubers. Data from Kahl and Wielgat (9).

appropriate low levels but inhibit terpene accumulation at higher levels where it would be expected that total protein synthesis is blocked. Once rishitin induction has been initiated it is clear that continuous protein synthesis is necessary (Fig. 12). Lubimin accumulation is promoted by this treatment. These data indicate that the enzyme systems involved with rishitin biosynthesis are relatively short-lived. It has been proposed that lubimin is a precursor of rishitin (10).

CONCLUSIONS

The phytoalexins rishitin and lubimin accumulate in potato tuber slices as a result of treatment with a variety

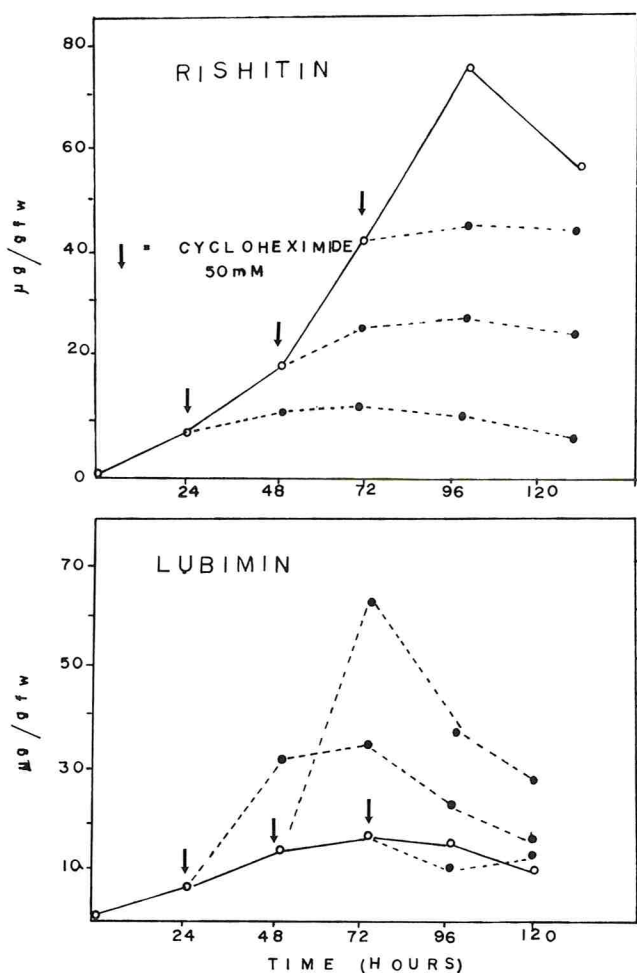


Figure 12. Induction of terpene accumulation in tuber (4-C stored) slices treated with $25 \mu\text{g}$ actinomycin D/ml for 30 min and subsequently incubated at 25°C . Discs were treated with $50 \mu\text{M}$ cycloheximide for 30 min at the times indicated by arrows. Discs treated with cycloheximide ($50 \mu\text{M}$) immediately following actinomycin D treatment did not accumulate terpenes.

of non-specific elicitors. Slices from cold stored potatoes have a greater propensity for terpene induction than freshly harvested or 25-C stored tubers. Evidence is presented which support the view that terpene biosynthesis is dependent on blocking the repression of the phytoalexin operon which occurs shortly after cut injury. The basis of pathogen-incited phytoalexin biosynthesis may reside with certain microbial metabolites, which in sufficiently low concentration, are capable of derepressing the phytoalexin operon by selectively inhibiting the synthesis of repressor proteins. The mechanism may be a direct interaction with translational or transcriptional machinery as appears to be true for actinomycin D, etc.; or the elicitor may trigger a cell wall or membrane site with the resulting formation of an appropriate message (e.g. cyclic AMP) which then directly derepresses the phytoalexin operon.

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Control of Postharvest Glycoalkaloid Formation in Potato Tubers

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ABSTRACT

Light and mechanical injury are the two most important environmental factors which stimulate postharvest glycoalkaloid synthesis in potato tubers. Partial control of light-induced glycoalkaloid formation has been achieved by treating potato tubers with some chemicals as Ethephon, Alar, Phosphon, Phosphon-S, Amchem 72-A42, Amchem 70-334, Nemagon, Telone, detergents, surfactants, control-atmosphere storage and subatmospheric pressure storage. Complete inhibition of light-induced glycoalkaloid formation can only be obtained by hot wax coating, oil coating, vacuum packaging and anoxia water submersion. Treatment with isopropyl-N-(3-chlorophenyl)-carbamate and gamma-irradiation effectively control wound-induced glycoalkaloid formation of potato tubers.

Potatoes (*Solanum tuberosum* L.) have been widely used in many parts of the world as a staple food for man. With an annual production of nearly 290 million metric tons, the potato is also one of the major world food crops (26). Potatoes provide an excellent source of carbohydrate energy and about one-quarter of our daily requirement of vitamin C. Among minerals, phosphorous, potassium, and calcium are significant. Although the protein content of fresh potatoes averages only 2%, the content on a dry weight basis is 10% which brings them relatively close to the protein content in wheat flour. The protein quality of potatoes is high and compares favorably with eggs, which are known as an excellent source of high quality protein. On the basis of total protein production per acre, potatoes are second behind the soybean.

A normal potato contains insignificant amounts of glycoalkaloids which represent a predominant mixture of α -solanine and α -chaconine. However, imposition of certain environmental conditions such as light exposure or mechanical damage will affect normal physiological sequences and induce synthesis of the glycoalkaloids in the potato tuber. A bitter taste and off-flavor have been noticed in such tubers that have developed excessive amounts of the glycoalkaloids. The level of glycoalkaloids depends upon cultivar, state of development, and nature of environmental condition. Considerable losses of potatoes occur between the field and the marketing place owing to the physiological and mechanical damage. Although recent literature does not reveal reports on losses due to light-induced greening, present-day merchandising practices do not eliminate the incidence of potato greening. According to Peterson (20), potato growers lost about 20% of their potential income through potato damage at harvest.

Considerable evidence exists concerning cases of potato-related poisoning in man and farm animals. The poisoning was attributed to the ingestion of large amounts of glycoalkaloids in the green tubers or sprouts. The rapid withdrawal of a potato cultivar "Lenape" because of its high level of steroidal glycoalkaloids has focussed attention on the need for a testing program for all new potato varieties. In the potato plant, most of the tissue, including leaves, shoots, stems, blossoms, tubers, tuber eyes, peels, and sprout, contain the major glycoalkaloids.

PHOTOINDUCTION

When potatoes are exposed to light in field conditions or during post-harvest handling and marketing, a green pigmentation develops at the surface. This condition, known as "greening" indicates formation of chlorophyll which is harmless and tasteless. Green potatoes are usually associated with an increased level of the glycoalkaloids although the processes of chlorophyll and the glycoalkaloid formation are independent of each other.

Conner (2) exposed tubers to different wavelengths of light. The blue end of the spectrum encouraged glycoalkaloid formation the most, while the yellow-red end of the spectrum was most efficient for chlorophyll but did not increase glycoalkaloids. According to the investigations of Zitnak (41), dormant tubers of Netted Gem cultivar responded by a rapid and significant increase of the glycoalkaloid concentration from 5.72 mg% to 11.00, 18.88, 22.42, 21.54, and 23.17 mg% when exposed to the efficient infrared light (18 to 22 C) source for 4, 6, 8, 10, and 16 days. Such tubers developed further increases in glycoalkaloid concentration after 2 months of storage at 4 to 8 C. Greening was noted in all samples, with an intense color after only 4 days of exposure. Ineffectiveness of infrared light on glycoalkaloid synthesis as reported by Conner (2) resulted from defective treatments. In a subsequent experiment (41) tubers that were stored for 3.5 months and then irradiated with ultraviolet light (14 to 18 C) for 4, 6, 8, or 10 days showed a gradual increase in glycoalkaloids over control tubers by 47, 141, 190, and 222%. At 7 to 10 C, however, tubers showed a decrease in glycoalkaloid content when treated for more than 4 days. The effect noted in this treatment was not attributed solely to the

most efficient wavelength at 365 nm because of other wavelengths emitted by the ultraviolet source in the visible spectrum of light. No greening was observed in the tubers irradiated with ultraviolet light.

INDUCTION BY WOUNDING

Production of the glycoalkaloids around sites of wounded potatoes was reported by McKee (15). Although the glycoalkaloids are localized in the peel of whole tuber, mechanical injury caused by slicing could increase their synthesis and accumulation in peeled tubers (1). Injury of tubers sustained by either bruising or mechanical grading after harvesting induced glycoalkaloid synthesis (24). A study conducted by Salunkhe et al. (22) indicated that potato slices (0.3 mg of glycoalkaloids per 100 g) when held in the dark at relatively high temperatures (15 or 23 C) for 2 days synthesized the glycoalkaloids (1.3 and 2.05 mg per 100 g). The rate of alkaloid formation increased (4.94 and 7.4 mg per 100 g) when the slices were stored under high-intensity light (2152 lux) (Fig. 1). In many instances, in potato processing plants, slices, cubes, mash, strings, strips, and shreds are stored at relatively high light intensity and temperature for some time before cooking or processing. This may cause synthesis and subsequent accumulation of the glycoalkaloids.

Wu and Salunkhe (31) studied the relationship between glycoalkaloid content and a type of mechanical injury sustained by potato tubers. They demonstrated that mechanical injuries such as brushing, cutting, dropping, puncturing, and hammering significantly stimulated glycoalkaloid synthesis in both peel and flesh of the tubers. The extent of glycoalkaloid formation depended on cultivar, type of mechanical injury, storage temperature, and duration of storage. High temperature storage stimulated more glycoalkaloid formation than did low temperature. Most of the injury-stimulated glycoalkaloid formation occurred within 15 days after treatments. Mechanical injury caused by cutting of tubers resulted in the highest contents of glycoalkaloids both in flesh and peel.

CONTROL OF POSTHARVEST GLYCOALKALOID FORMATION

Several methods for control of light induced greening, and hence of formation of glycoalkaloids, have been studied. One approach is to protect tubers from light. The use of various packaging (5,6,12,13,14), colored film bags (4,6,38), colored lights and colored-film filters (12,13,38) have been investigated. Controlled atmosphere storage of tubers was explored by Forsyth and Eaves (3) and Patil et al. (19). Application of subatmospheric pressure (hypobaric) is another approach (7). Several workers studied ionizing radiation in relation to greening and glycoalkaloid formation in illuminated potato tubers (4,19,23,33,40) and found it ineffective on the glycoalkaloids (19,33). Mechanically damaged tubers and cut cubes of three cultivars of potato, when subjected to gamma-irradiation,

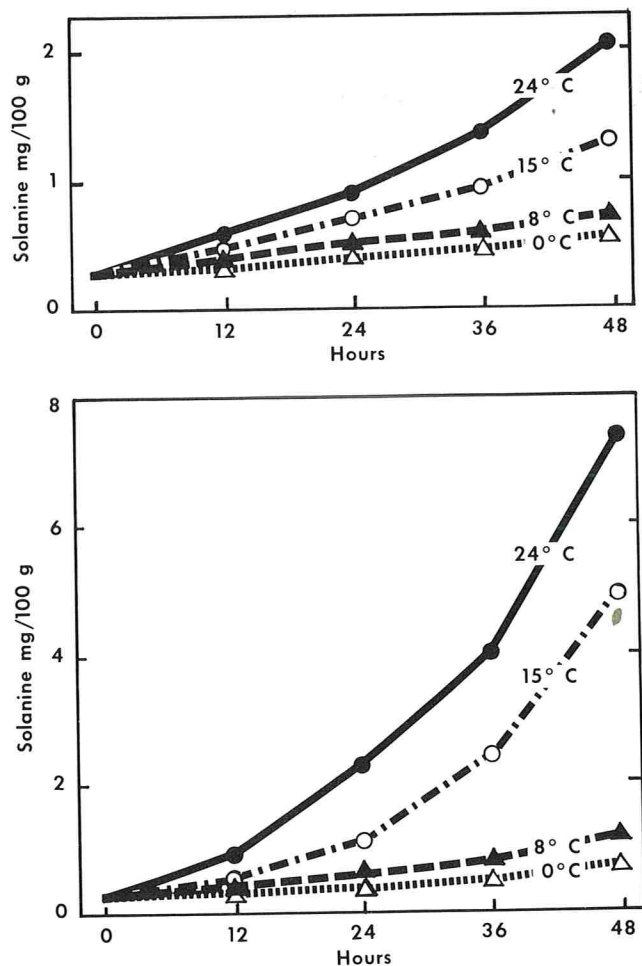


Figure 1. Solanine contents of tuber slices of Russet Burbank cultivar stored in the dark at different temperatures (top) and that of exposed to a fluorescent light (200 ft-c) at different temperatures (bottom).

were shown to produce significantly less amounts of the glycoalkaloids than the control samples under identical storage conditions (33) (Table 1). The inhibitory effect of gamma-irradiation at 25-100 krad was 11-79% in both samples of Russet Burbank cultivar under such a physiological stress. At a dose of 200 krad of gamma-irradiation, inhibition in wound induced glycoalkaloid formation was 81 to 92%. Similar results were reported on White Rose and Red Pontiac cultivars. Gamma-irradiation did not influence the existing glycoalkaloids. Vacuum-packaging with polyethylene bags at 15 and 25 inches of Hg has been shown to inhibit light-induced glycoalkaloid formations of three cultivars of potato tubers (30).

Use of anoxia water treatment to control light-induced greening and glycoalkaloid formation of potato tubers have been recently developed by Wu and Salunkhe (35). Temporary anoxia created by submerging potato tubers in the water completely inhibited light-induced greening and glycoalkaloid formation of Russet Burbank, White Rose and Pontiac cultivar (Table 2 and 3). Preanoxia vacuum treatment of the tuber had the same effect but hastened rotting of the tubers. Anoxia treatment also

inhibited respiration of potato tissue. Partial exposure of the surface of anoxia tubers to air reduced the inhibition. The inhibition of the light-induced greening by the anoxia condition is due to inhibition of tuber respiration. The light absorption by the water used to create anoxia condition did not contribute to inhibition of light-induced greening. The duration which tubers could tolerate anoxia condition depended upon the cultivar and the physical condition of the tubers. Incorporation of 100 to 1000 ppm sodium hypochlorite or daily replacing with fresh water effectively improved storage stability and reduced rotteness. Wu and Salunkhe (36) also found the residual effect of anoxia treatment on potato tubers. The extent of aftereffect of submerging water treatment on light-induced greening and glycoalkaloid formation of potato tubers depended mostly upon the duration of the treatment and less on the temperature and the cultivar of the tubers. Treatments before light exposure all significantly reduced light-induced formation of chlorophylls and glycoalkaloid. For Russet Burbank cultivar treatment at 4 C for 2 days resulted in 48% inhibition of chlorophyll formation and 65% inhibition of

TABLE 2. Effect of partial exposure of the surface of anoxia tubers to air on light induced greening and glycoalkaloid formation of Russet Burbank cultivar.

Approximate percent of tuber surface area exposed to air	Chlorophylls (mg/100 g peel)	Total glycoalkaloids (mg/100 g peel)
Zero time sample	0.102	24.9
0	0.105	25.2
10	0.107	24.6
20	0.224 ^a	29.6 ^a
30	0.514 ^a	41.6 ^a
50	0.733 ^a	44.8 ^a
75	0.846 ^a	49.6 ^a
90	1.153 ^a	53.9 ^a
100 (control)	3.719 ^a	70.1 ^a

^aSignificantly different from zero time sample at 1% level.

glycoalkaloid formation. For White Rose cultivar, the same treatments resulted in 17 and 61% inhibition in chlorophyll and glycoalkaloid formation, respectively. Similar results have also been shown on Pontiac cultivar. Increasing the duration of submerging treatment resulted in more inhibition in both chlorophyll and glycoalkaloid formation. It is noteworthy that in most instances the same treatment resulted in more inhibition

TABLE 1. Effect of gamma-irradiation on wound-induced glycoalkaloid formation of mechanically damaged tubers and cut cubes of Russet Burbank cultivar.

Type of material	Gamma-irradiation (krad)	Days storage at 4 C			
		0	5	10	20
		Total glycoalkaloids mg/100 g dry wt			
Damaged whole tubers	Control	23.0	107.9**	161.5**	190.7**
	25		94.3**	145.0**	171.5**
	50		86.3**	132.4**	160.1**
	100		60.7**	74.5**	83.6**
	200		29.8**	39.6**	46.4**
Cut cubes	Control	1.4	19.6**	25.4**	28.1**
	25		17.2**	22.8**	25.2**
	50		11.5**	18.4**	22.5**
	100		5.3**	6.7**	7.2**
	200		2.9*	3.8**	4.1**

*Significantly different from zero time sample at 0.05 level.

**Significantly different from zero time sample at 0.01 level.

TABLE 3. Effects of anoxia and preanoxia vacuum treatment on light-induced chlorophyll and glycoalkaloid formation of potato tubers.

Cultivar	Treatment	Days storage	Chlorophylls (mg/100 g peel)	Total glycoalkaloids (mg/100 g peel)
Russet Burbank	Control	0	0.104	24.6
		4	1.245 ^a	42.7 ^a
		8	3.736 ^a	69.4 ^a
	Anoxia	4	0.115	23.9
		8	0.110	24.8
	Anoxia plus vacuum	4	0.098	24.4
		8	0.104	24.2
		8	0.186	21.4
White Rose	Control	0	0.186	21.4
		4	1.658 ^a	39.6 ^a
		8	4.863 ^a	58.3 ^a
	Anoxia	4	0.193	22.1
		8	0.189	21.6
	Anoxia plus vacuum	4	0.190	21.9
		8	0.194	21.3
		8	0.235	35.5
Pontiac	Control	0	0.235	35.5
		4	1.350 ^a	56.3 ^a
		8	3.188 ^a	84.5 ^a
	Anoxia	4	0.226	34.6
		8	0.239	35.2
	Anoxia plus vacuum	4	0.231	35.3
		8	0.237	35.0

^aSignificantly different from zero time sample at 1% level.

in formation of glycoalkaloid than that of chlorophyll. For Russet Burbank cultivar, submerging treatment for a minimum of 5 days was necessary for effective control of glycoalkaloid formation, while a minimum of 10 days was needed for both White Rose and Pontiac cultivars. Submerging treatments at elevated temperature (13 C) caused less inhibition of both glycoalkaloid and chlorophyll formation when compared with the same treatment at 4 C. Addition of sodium hypochlorite in the soaking water had slight but no consistent effect on chlorophyll and glycoalkaloid formation of tubers.

Submersion water treatments also had extended inhibitory effect on wound-induced glycoalkaloid formation of potato tuber tissue. Submersion water treatment of three cultivars of potato tubers for 10 days before wounding resulted in 80 to 82% inhibition of wound-induced glycoalkaloid formation, while 20-day anoxia treatment resulted in 97 to 100% inhibition. Incorporation of 200 ppm sodium hypochlorite in the soaking water effectively retarded the microbial population. The surface of potato tubers was the major source of microorganisms in the soaking water. Without sodium hypochlorite, rapid increases in microbial population took place, especially at elevated temperature. With 200 ppm sodium hypochlorite in the soaking water, no microbial count could be detected even after 20 days at 4 C.

Numerous chemicals are known to prevent greening and/or glycoalkaloid synthesis in potato tubers (4,10,16,18,23). A sprout inhibitor, isopropyl-N-(3-chlorophenyl)-carbamate (CIPC), was tested by Wu and Salunkhe (32) on three cultivars of potato tubers as a possible inhibitor of wound-induced glycoalkaloid formation (Table 4). Dipping of mechanically damaged Russet Burbank tubers and cut cubes in 100 and 1000 ppm CIPC-emulsified water solution completely inhibited the

wound-induced glycoalkaloids. A dip concentration at 1 or 10 ppm CIPC was 9-70% effective. Fumigation with 100 and 1000 mg CIPC/m³ caused 6-86% inhibition of the wound-induced glycoalkaloid synthesis. CIPC treatment, however, showed no effect on existing glycoalkaloids and photoinduced glycoalkaloid formation of normal whole potato tubers.

Waxing of potatoes was once a fairly widespread marketing practice because it attracted consumer attention, but it appears to be rapidly declining in importance in the United States. However, the findings of Wu and Salunkhe (27) that hot paraffin wax effectively controls the photo-induced formation of chlorophyll and glycoalkaloids in potato tubers has created a new interest in this area. These authors treated Russet Burbank potatoes with paraffin wax at 60, 80, 100, 120, 140 and 160 C for 0.5 sec and exposed them to fluorescent light (2152 lux) for 10 days at 16 C and 60% R.H. The results indicated no inhibition of chlorophyll and glycoalkaloid synthesis at 60 and 80 C, significant inhibition at 100 and 120 C, and almost complete inhibition at 140 and 160 C (Fig. 2). Heating the tubers at 160 C in air for 3 to 5 min and subsequent exposure to light did not prevent chlorophyll and glycoalkaloid formation. It was the combined treatment of waxing and heating that retarded chlorophyll and glycoalkaloid formation. They further concluded that this treatment is especially useful because paraffin wax does not create problems like most physicochemical treatments and coated wax can be easily removed by peeling the tubers before processing or cooking.

In a later study, corn oil dips were given to potato tubers at 22, 60, 100, and 160 C for 0.5 sec, and excess oil was removed with tissue paper (28). Oiling at 22 C reduced chlorophyll formation by 93 to 100% and glycoalkaloid formation by 92 to 97%. At elevated

TABLE 4. Effect of dipping and fumigation treatments of CIPC on wound-induced glycoalkaloid formation of mechanically damaged tubers and cut cubes of Russet Burbank cultivar.

Type of material	CIPC Treatment	Days Storage at 4 C			
		0	5	10	20
		Total glycoalkaloids mg/100 g dry weight			
Damaged whole tubers	Dipping (ppm)				
	Control	23.2	118.8**	163.0**	194.2**
	1		80.3**	118.4**	130.5**
	10		53.3**	71.6**	84.2**
	100		24.2	24.0	24.8
Cut cubes	1000		23.6	24.4	23.9
	Control	1.5	20.0**	26.3**	28.4**
	1		18.3**	20.1**	21.5**
	10		7.4**	8.9**	9.8**
	100		1.6	1.5	1.6
Damaged whole tubers	1000		1.4	1.5	1.5
	Fumigation (mg CIPC/m ³)				
	10		104.9**	170.3**	190.0**
	100		84.5**	128.4**	133.6**
	1000		38.6**	47.8**	53.9**
Cut cubes	10		21.4**	25.8**	29.3**
	100		18.8**	20.6**	24.3**
	1000		4.0*	4.9**	5.4**

*Significantly different from zero time sample at 0.05 level.

**Significantly different from zero time sample at 0.01 level.

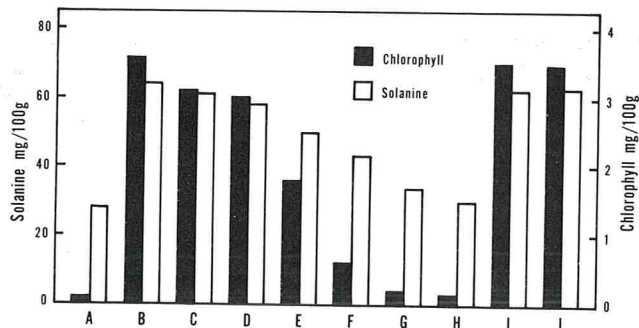


Figure 2. Effects of waxing and heating at different temperatures on chlorophyll and solanine formation in peels of potato tubers after exposure to 200 ft-c light intensity for 10 days at 16 C and 60% R.H. (A) original zero time sample; (B) control, nonwaxed potatoes; (C) waxing at 60 C; (D) waxing at 80 C; (E) waxing at 100 C; (F) waxing at 120 C; (G) waxing at 140 C; (H) waxing at 160 C; (I) heating with air at 160 C for 3 min; (J) heating with air at 160 C for 15 min.

temperatures such as 60, 100 and 160 C, the treatment completely inhibited both chlorophyll and glycoalkaloid synthesis. Wu and Salunkhe (29) (Fig. 3) further reported that treatments with corn oil, peanut oil, olive oil, vegetable oil, or mineral oil at 22 C were equally effective, but the tubers appeared oily. Moreover, oxidative rancidity of the oils and fat might develop in time. To decrease the amount of oil used, corn oil was diluted with acetone (Fig. 4). Treatment with 1/2, 1/4 and 1/8 oil significantly and effectively inhibited formation of chlorophyll and glycoalkaloids. Treatments with 1/16, 1/32, and 1/64 oil caused 95, 72 and 22% inhibition of chlorophyll, and 82, 49 and 28% inhibition of glycoalkaloid synthesis. Tubers treated with acetone alone or with 1/128 oil behaved in the same way as untreated control tubers. A concentration of 1/8 corn oil and 7/8 acetone was the minimum effective dilution. According to their (29) estimate, 1 g of oil in 50 g of acetone is enough to treat 8000 g of potato tubers. The acetone treatment had no apparent harmful effect on the tubers. For practical application, acetone can be recovered by passing the treated tubers through a warm-air chamber and condensing the acetone in the

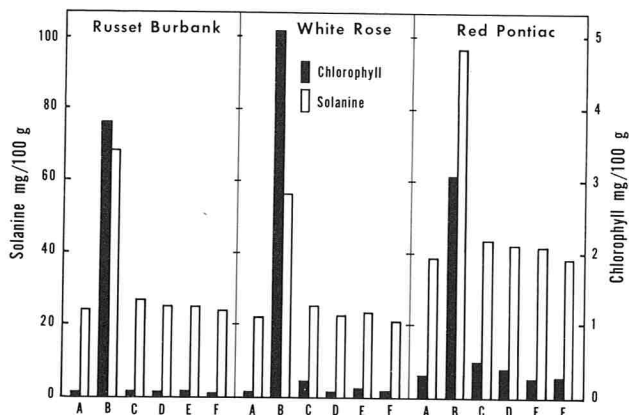


Figure 3. Effect of oil dipping on chlorophyll and solanine development on peel of Russet Burbank, White Rose and Red Pontiac potatoes. After oil dipping, tubers were exposed to light (200 ft-c) for 10 days at 16 C. (A) original, zero time sample; (B) control, non-treated potatoes; (C) oiling at 22 C; (D) oiling at 60 C; (E) oiling at 100 C; (F) oiling at 160 C.

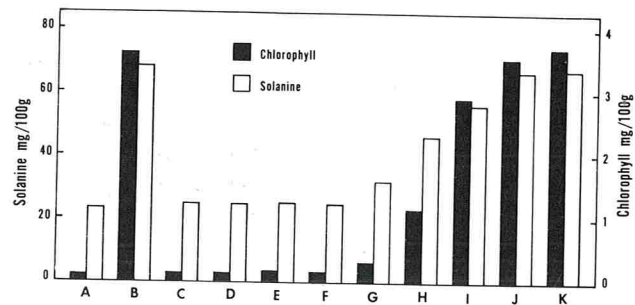


Figure 4. Effects of different dilutions of corn oil with acetone on chlorophyll and solanine development in peels of Russet Burbank potatoes. After treatments, tubers were exposed to 18.5 lux (200 ft-c) for 10 days at 16 C. (A) Original, zero-time sample; (B) control, non-treated potatoes; (C) 100% oil; (D) 1/2 oil and 1/2 acetone; (E) 1/4 oil and 3/4 acetone; (F) 1/8 oil and 7/8 acetone; (G) 1/16 oil and 15/16 acetone; (H) 1/32 oil and 31/32 acetone; (I) 1/64 oil and 63/64 acetone; (J) 1/128 oil and 127/128 acetone; (K) acetone.

warm air by a cooling coil system.

Jadhav and Salunkhe (8) reported the effectiveness of mineral oil at different concentrations. The efficiency of treatment increased with increasing concentration up to 10% (w/v in petroleum ether) and then remained almost constant and maximum up to 100%. Tubers treated with mineral oil up to 10% concentration had an attractive appearance compared with that of untreated and excessively treated ones. At a concentration of 10% mineral oil, tubers did not turn green after exposure to light for 4 weeks, while glycoalkaloid inhibition at the end of the first, second, third, and fourth weeks was 93, 67, 49 and 65% of the control compared with initial values. In general, oil treatment is important because it is a simple, effective, and inexpensive method of controlling greening and glycoalkaloid formation in potato tubers.

Sinden (24) immersed tubers in a 2 or 3% detergent solution at 21.1 C for 10 to 40 min and then rinsed them under tap water. On exposure to light (1291 lux) for 10 days, greening and light-induced glycoalkaloid synthesis were inhibited. Inhibition of chlorophyll synthesis in Russet Burbank tubers was 92% for the first 2 days when treatment was given with 3% detergent solution for 30 min. The inhibitory effect decreased with extended exposure, but inhibition was still more than 50% after 10 days. With Kennebec and Sebago cultivars, both of which green rapidly under ordinary light conditions, the treatment with 2% detergent (Joy) for 2 min resulted in 47% and 33% inhibition. The glycoalkaloid content of the fresh peels of Kennebec was 61% less than in the control. With the cultivar Green Mountain, which greens less rapidly, inhibition was only 14%.

Poapst and Forsyth (21) reported that photoinduced greening of potatoes can be prevented by simply washing or spray-rinsing the tubers with an aqueous solution of an edible surfactant known commercially as Tween 85. An application of 0.04% of the tuber weight prevented chlorophyll formation for 15 days or more while a spray containing 4-5% Tween 85 formed an effective film on the surface of the most susceptible cultivars.

Recently, Wu and Salunkhe (34) recommended an

alternative method of preventing photoinduced greening and glycoalkaloid synthesis by spraying potato tubers with a commercial lecithin spray such as Pam[®], Mazola No Stick[®], Cooking Ease[®], or Griddle Mate[®] (Table 5). These treatments significantly inhibited light-induced glycoalkaloid formation (89-98%) in potato tubers. Additionally, coating of tubers with lecithin (Centromix C[®] or Centrolex F[®]) or hydroxylated lecithin (Centrolene A[®]) resulted in inhibition of light-induced greening and glycoalkaloids when used at 5 to 20% concentration in petroleum ether (37) (Table 6).

TABLE 5. Effects of lecithin spray treatment on light-induced chlorophyll and glycoalkaloid formations in Russet Burbank potato tubers after exposure to 200 ft-c light intensity for 14 days at 10 C and 60% RH.

Treatment	Total chlorophylls (mg/100 g peels)	Total glycoalkaloids (mg/100 g peels)
Original sample (zero time)	0.14**	28.45**
Control (nontreated)	3.83	69.49
PAM [®]	0.41**	33.05**
Mazola No Stick [®]	0.34**	31.93**
Cooking ease [®]	0.26**	29.46**
Griddle mate [®]	0.23**	30.38**

**Significantly different from control (nontreated) tubers at 0.01 level.

ACKNOWLEDGMENT

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TABLE 6. Effects of lecithin and hydroxylated lecithin coating on chlorophyll and glycoalkaloid formation in peripheral zone of potato tubers exposed to 200 ft-c white fluorescent light at 13 C and 80% R.H. for 8 days^a.

Treatment	Concentration (w/v)	Total glycoalkaloid (mg/100 g of peels)			Chlorophyll (mg/100 g of peels)		
		Russet Burbank	White Rose	Pontiac	Russet Burbank	White Rose	Pontiac
Initial		19.38	17.95	24.46	0.104	0.175	0.226
Control		48.04	44.59	50.29	4.218	4.639	3.684
Centrolene A	5%	36.49	36.05	40.17	2.745	2.966	2.227
hydroxylated lecithin [®]	10%	24.10	21.58	28.45	0.693	0.549	0.468
	20%	19.20	18.30	24.80	0.096	0.181	0.211
Centromix C lecithin [®]	5%	31.18	30.46	34.86	1.688	1.245	1.044
	10%	21.09	19.42	26.78	0.390	0.410	0.379
	20%	19.44	17.70	24.24	0.115	0.170	0.230
Centrolex F lecithin [®]	5%	29.45	31.08	33.29	1.498	1.384	0.963
	10%	20.87	19.84	26.08	0.414	0.394	0.402
	20%	19.31	18.15	24.65	0.109	0.178	0.219

^aAll the values of treatments in this table are significantly different from respective controls.

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Kentucky Association Conference Well Attended

A large crowd of approximately 300 persons participated in the 1979 Educational Conference for Fieldman and Sanitarians, sponsored by the Kentucky Association of Milk, Food and Environmental Sanitarians, Inc. The conference was held Feb. 27 and 28 at the Executive West, Louisville, KY.

Highlights of the conference included the following presentations: "State Park Food Service Sanitation," Bennie Hagar, Dept. of Parks, Frankfort; "Food Salvage," Richard Gillespie, Food and Drug Administration Training Facility, Cincinnati, OH; "Radiation Control in Action," Charles Hardin, Radiation Control Branch, Frankfort; and "Fieldmen Relationship with Producer and Regulatory Officials," Charles Turner, Milk Marketing, Inc., Cincinnati, OH.

Those members recognized during the Awards Luncheon were, Outstanding Sanitarian, Bruce Lane, Louisville-Jefferson Co. Health Dept., Louisville; Outstanding Fieldman, John Fitch of Dairymen, Inc., Louisville, and Outstanding Service, Mike Ehrler, Flav-O-Rich, Inc., Louisville.



Officers and Directors of KAMFES include, left to right, John Mattingly, Bland Dorris, Bell McCormich, directors; Leon Townsend, President-elect; Dale Marcum, Sec.-Treasurer; Tommy Coomes, President; Morrell Raleigh, director; Barry Kinslow, Past President; Roger McDonald, director; Bruce Langlois, Vice-president; George Scott, Ellen Ruch, directors.

Williams, con't from p. 538

incumbency on the staffs of two national trade associations. While with the staff of the Int. Assn. of Ice Cream Mfrs. (IAICM), Williams assumed the secretariat of the 3A Committees in 1954.

When he moved to DFISA in 1957 he also became secretary of the DFISA Technical Committee. He coordinated the activities of nearly 40 separate DFISA task committees in preparation of draft standards for more than 30 meetings of the 3A

Committees.

Prior to his affiliation with IAICM, Williams was a dairy technologist from 1947-1951 in the Bureau of Dairy Industry, USDA, where he had responsibility for ice cream research.

A native of Washington, D. C. Williams resides in suburban Maryland. He plans to devote his future energies to long-delayed personal projects in various fields outside the industry.

Naturally-Occurring and Ethylene-Induced Phenolic Compounds in the Carrot Root

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ABSTRACT

Carrots accumulate phenols and often develop off-flavor and color after long periods of storage. To investigate probable causes for such physiological disorder, the effect of ethylene on various aspects of metabolism of carrot roots was studied. Ethylene, when applied at moderate level (100 ppm), caused an increase in total phenol content of the roots. It caused an increased accumulation of the phenols normally present in the tissue, especially isochlorogenic acid. Moreover, relatively longer exposure to a moderate level (100 ppm) and short exposure to high levels (2000 and 50,000 ppm) of ethylene induced formation of new compounds, viz, isocoumarin, eugenin, and two others yet unidentified. Studies with [1-¹⁴C]acetate, [2-¹⁴C]malonate and [3-¹⁴C]acetoacetate indicated that the newly synthesized compounds are probably synthesized via the acetate pathway. Ethylene stimulated the rate of O₂ uptake and CO₂ evolution by carrot slices, indicating probable relationship of glucose metabolism with de novo synthesis of "stress-metabolites". Studies with specifically labelled glucose showed that both the Embden-Meyerhof-Parnas (EMP) and the Pentose Phosphate (PP) pathways operate in carrots, and that ethylene preferentially stimulated the EMP pathway. Like ethylene, dinitrophenol (DNP) induced isocoumarin synthesis in carrots. Methylene blue, an electron acceptor often used for stimulating glucose catabolism via the PP pathway, also induced isocoumarin synthesis in carrots. The effect of cycloheximide, an inhibitor of protein synthesis, suggested that the de novo synthesis of enzyme protein(s) might be required for ethylene-induced isocoumarin synthesis in carrots. In conclusion, it appears that ethylene triggers changes in the metabolism of carrots during storage, which result in, among other things, synthesis of so-called "stress-metabolites," namely isocoumarin and eugenin and related compounds.

Carrot root is an important component of the vegetable portion of our diet. It is a good source of vitamins, minerals and fiber. And it adds rich color and aroma to our food. On the basis of the United States Department of Agriculture statistics for 1970 (1), carrot root has been ranked tenth in terms of nutritional value among 38 other fruits and vegetables, and seventh for its contribution to nutrition.

Current advancement in agriculture has enabled us to produce more carrots than can be marketed as fresh produce, which leaves a large amount of produce to be processed in some form for later use. The time lag between harvest and processing, or future marketing as fresh produce, appears to be crucial in relation to undesirable color and flavor development in carrots (8).

During the early 1950s, processing industries in the eastern U.S. and Canada encountered a problem of bitterness in some carrots that were processed after some

periods of storage (3). The carrots processed soon after harvest were free from this bitter flavor. In an attempt to find a solution to this bitterness problem, investigations into various aspects of carrot production, including cultural and post-harvest handling practices, were initiated by various workers at the Agricultural Experiment Station in Geneva, New York. Atkin (3) reported the results of a survey of various cultural practices of carrot growers. Application of oils (to control weeds), insecticides, fertilizers, and crop rotation were the major management procedures. The results of his survey failed to provide any correlation between above cultural practices and the development of bitterness in carrots. However, Atkin always observed that whenever bitterness occurred, it did so in refrigerated storage.

The nature of the compound(s) responsible for bitterness in carrots was investigated by Sondheimer (24). He suggested that bitter flavor in carrots was caused by the presence of several compounds and that 8-hydroxy-3-methyl-6-methoxy-3,4-dihydroisocoumarin was one of them. He named this compound as 'isocoumarin', and this name has been used ever since.

What causes induction of isocoumarin synthesis in carrots was the subject of further investigation, and various workers suggested that ethylene might be a "triggering factor" (5,6,7).

Although ethylene was suggested as a causative agent for synthesis of isocoumarin in carrots on the basis of isocoumarin's ability to fluoresce under U.V. light (5,6,7), no attempt was made by these workers to isolate and characterize isocoumarin from ethylene-treated carrots to ascertain whether the observed fluorescence was caused only by isocoumarin and not any other compound(s). Further, the question whether isocoumarin is the only compound synthesized in carrots in the presence of ethylene was unanswered until we started our investigation some 12 years later. Our results show that not only isocoumarin but three additional related compounds are synthesized de novo in carrots after exposure to ethylene. Further, the levels of existing phenols, including hydroxy-cinnamic acid derivatives, increased considerably in ethylene-treated carrots.

The cinnamic acid derivatives, namely various phenolic compounds encompassing flavonoids, lignins, and tannins, although classified by plant physiologists as

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secondary metabolites, occupy an important place in food color and flavor. Therefore, it must be recognized by people dealing with foods of plant origin that unless adequate care in handling and storage of fresh produce is taken, undesirable color and/or flavor development may result from various phenolic compounds that are present in these tissues.

We, in this paper, will describe the results of our studies on the effects of ethylene on various phenolic compounds in carrot roots.

EXPERIMENTAL

All the carrots used for experiments were grown at the Horticultural Research Station, Brooks, Alberta. These were stored at 3 ± 1 C and 98% relative humidity, in the absence of light.

The methods of extraction, separation and identification of various phenolic compounds have been published previously (13,17-21), and a detailed description will not be given here. However, it should be noted that we have used intact carrots as well as slices for our studies. The slices were used either for feeding experiments with labelled compounds or to augment the effects of ethylene on carrots over a short time, resulting from an increased surface for exposure to ethylene.

The entire phenol extract of carrots was classified, according to solubility, into ether-soluble and methanol-soluble (or ether-insoluble) fractions. The composition of extracts was analyzed by paper, thin-layer and gas chromatography.

RESULTS AND DISCUSSION

Effect of storage on phenol content of carrots

The total phenol content of carrots stored at 3 ± 1 C increased steadily with time. Figure 1 shows that the amount of phenols in 100 g of carrots increased from 35 to 210 mg during a period of 9 months. Chubey and Nylund (8) observed a similar increase in phenol content of carrot roots during storage at various temperatures. They found that the amount of phenol accumulated was the highest at 10 C. Our laboratory is currently looking at the effect of storage on phenol content of potatoes.

Probable physiological basis for phenol accumulation in carrots during storage will be described later, but from a practical standpoint increased phenol content poses additional problems in post-harvest handling. Carrots richer in phenols are more susceptible to surface browning and other concomitant problems (8).

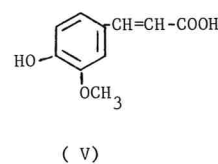
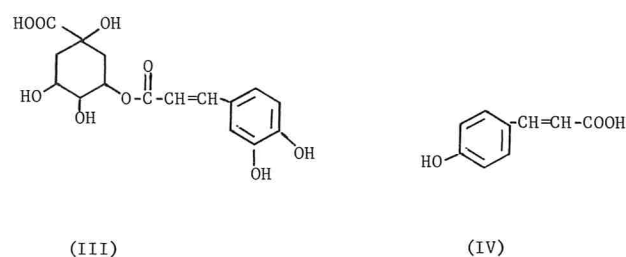
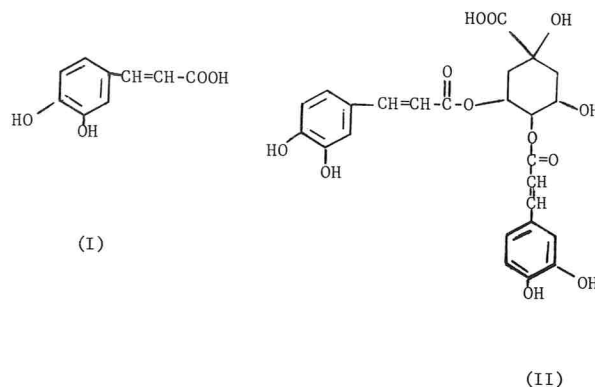
The concern over increased phenol content in carrots in relation to surface browning is heightened because of the existence of a concentration gradient from core to the surface of the carrot root (Table 1). About 85% of phenol is present in the 1-mm thick surface layer of the root. This would easily explain why carrots with bruises on the surface tend to develop brown color (8). Although an increase in phenols in carrots augments the potential for surface browning, no bitterness, as described by Sondheimer (24), was detected.

Composition of phenolic compounds in carrots

The next question that arose was what was the composition of phenolic compounds in carrots, and how is it affected by storage? To answer this question, the aqueous extract of phenols was further extracted with

ether to obtain two fractions: (a) ether-soluble and (b) ether-insoluble. The presence of phenolic compounds in the ether-soluble fraction was not detectable in carrots stored at 3 ± 1 C and ca. 90% relative humidity, and in the absence of ethylene. No isocoumarin or any other related compounds were present in the extract.

The ether-insoluble portion revealed the presence of a number of phenolic compounds on the paper chromatogram (Table 2). Three of these spots were identified by co-chromatography with known compounds; caffeic acid (I), isochlorogenic acid (II) and chlorogenic acid (III). The rest of the spots were not identified as such but



p-coumaric acid (IV), ferulic acid (V) and caffeic acid (I) were identified by thin-layer and gas-liquid-chromatography after acid and alkaline hydrolysis of the original extract (19). We found that under the above storage conditions the levels of hydroxycinnamic acid derivatives increased with storage time.

Effect of ethylene on phenol content of carrots

The situation is quite different, however, if carrots are stored even for a short time in a refrigerated storage in the presence of ethylene. In carrots treated with ethylene (100 μ l/l), the amount of phenol (Fig. 2) increased 5-fold after a 3-day exposure (I) and about 7-fold after a 7-day exposure (II). We studied the effects of various ethylene concentrations (1-2000 ppm) on phenol content of carrot

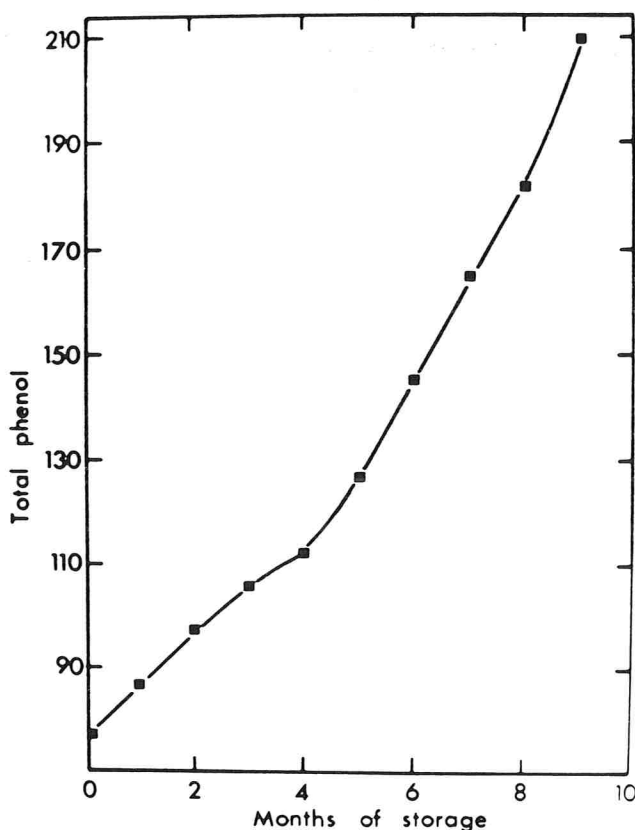


Figure 1. Variation of total phenol content of carrots during storage at 3 ± 1 C. Total phenol is expressed as mg chlorogenic acid per 100 g of fresh weight.

TABLE 1. Ether-insoluble phenols separated by paper chromatography.^{ab}

R _f	Identity
0.82	Caffeic acid
0.76	Isochlorogenic acid
0.64	Chlorogenic acid
0.57	Not identified
0.52	Not identified
0.39	Not identified
0.31	Not identified

^aFrom Sarkar (17).

^bSolvent system was the organic phase of the mixture: n-butanol:acetic acid:water (4:1:5).

TABLE 2. Centripetal distribution of total phenol content in carrots, exposed to air or 100 ppm ethylene for 2 days.^a

Tissue portion	Total phenol content: mg chlorogenic acid per g dry wt.	
	Exposed to air	Exposed to ethylene
Peel	21.87	43.21
Phloem	2.26	4.46
Xylem	1.35	2.88

^aFrom Sarkar and Phan (19).

roots, and found that the higher the concentration the faster the rate of synthesis of all phenols, including isocoumarin and related compounds.

Effect of ethylene on ether-insoluble phenols. When the ether-insoluble phenols were analyzed by paper chromatography, isochlorogenic acid concentration registered a marked increase, whereas chlorogenic acid and caffeic acid concentration declined slightly on

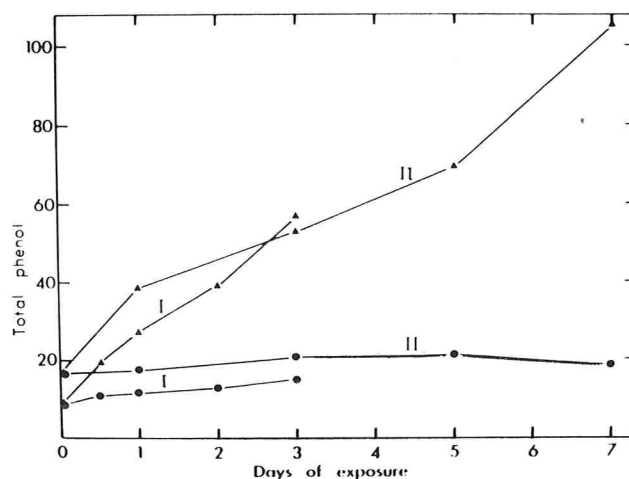


Figure 2. Effect of continued exposure to $100 \mu\text{l/l}$ ethylene on the total phenol content of carrots, expressed as mg of chlorogenic acid per g of dry weight. Total phenol in ethylene-treated carrots (\blacktriangle); total phenol in air-treated carrots (\bullet). I and II refer to two different experiments.

exposure to ethylene (Fig. 3). When the concentrations of isochlorogenic acid and chlorogenic acid were monitored in ethylene-treated carrots over a few days, the former showed a steady increase whereas the latter remained unchanged (Fig. 4). Although such observation was not made previously and therefore cannot be related to any

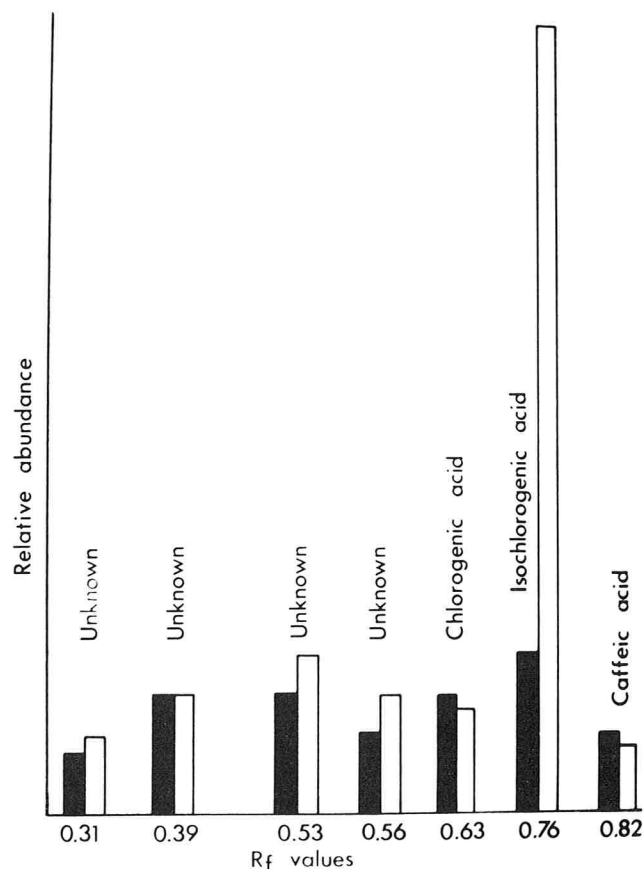


Figure 3. R_f values and relative abundances of ether-insoluble phenols separated by paper chromatography. Relative abundances were estimated visually on the basis of area of fluorescence of different compounds under UV light. Solid bar represents phenols in air-treated, and blank bar represents phenols in 100 ppm of ethylene-treated carrots.

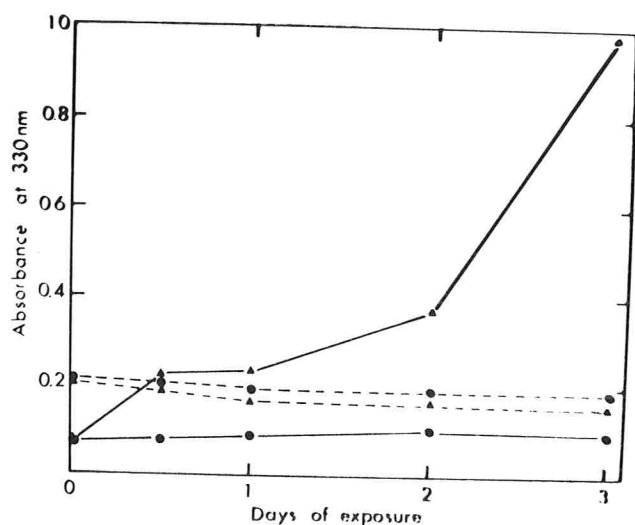


Figure 4. Time course of isochlorogenic acid accumulation in carrots upon exposure to ethylene (100 ppm). Isochlorogenic acid in ethylene-treated (▲), and (●) isochlorogenic acid in air-treated carrots. Broken lines: Chlorogenic acid in ethylene-treated carrots (▲), chlorogenic acid in air-treated carrots (●).

particular physiological event, we have proposed elsewhere that the accumulation of a diester such as isochlorogenic acid probably occurs as a result of ethylene-induced stimulation of the shikimic acid pathway.

Effect of ethylene on ether-soluble phenols. The effect of ethylene on the ether-soluble phenols in carrots was particularly striking. Four more phenolic compounds were synthesized in carrots on exposure to ethylene (Fig. 5). Compounds of band X (R_f 0.69) and band Y (R_f 0.58) were the two most abundant phenols, and were isolated in crystalline form by column chromatography (17). These compounds were characterized unequivocally as 8-hydroxy-3-methyl-6-methoxy-3,4-dihydroisocoumarin, the so-called 'isocoumarin' (band X) and 5-hydroxy-7-methoxy-2-methylchromone or eugenin (band Y) by elemental analysis as well as U.V., I.R., P.M.R. spectral and mass spectrometric data (19). The compounds of band Z and M were not identified but their U.V. spectra and color reactions to phenol detecting reagents were very similar to those of isocoumarin. This would suggest the presence of a similar chromophore in these two compounds.

Why and how more isochlorogenic acid is synthesized in carrot roots in the presence of ethylene is a question that should intrigue plant physiologists and biochemists. To people dealing with post-harvest problems of carrots, increased isochlorogenic acid level makes these roots more susceptible to browning.

The other phenols namely isocoumarin and eugenin, which are synthesized de novo in carrots on exposure to ethylene, pose an altogether different problem. Isocoumarin has been associated with bitter-tasting carrots (24), and there has been some controversy as to the real role of ethylene in the induction of isocoumarin formation in carrots. Condon et al. (9) found that carrot discs inoculated with *Ceratocystis fimbriata* produced

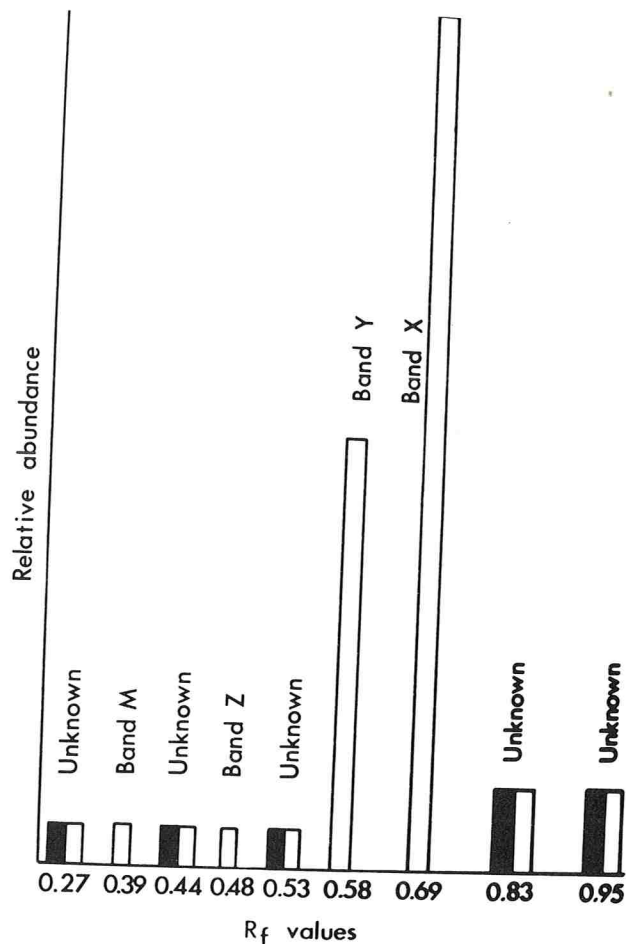


Figure 5. R_f values and relative abundances of ether-soluble compounds separated by thin-layer chromatography. Solid bars represent air-treated, and blank bars represent ethylene-treated carrots.

isocoumarin in carrot disc, and isocoumarin exhibited fungitoxic properties. Further investigation into the fungitoxic properties of isocoumarin led to its inclusion as a phytoalexin (10). Subsequent workers in this area (7,9) found that *C. fimbriata* also produces ethylene and Chalutz et al. (7) correlated isocoumarin synthesis in carrots with ethylene production by *C. fimbriata*. Later, Condon et al. (9) tested four different fungi for their ability to induce isocoumarin synthesis in carrots, and to produce ethylene. They found only one fungus capable of producing ethylene, whereas all four induced isocoumarin formation in carrot roots. Therefore, a strict correlation between ethylene production and isocoumarin synthesis was not found. Things became even more complicated when isocoumarin as well as other closely related compounds were isolated from various fungi, including *Ceratocystis fimbriata* (4,11,12, 25). Thus the time was right to redefine the role of ethylene in isocoumarin synthesis in carrot roots. We have not studied the effects of microorganisms on synthesis of isocoumarin in carrots, but our results clearly show that ethylene is a causative agent for the synthesis of isocoumarin.

Eugenin has not been reported as a normal constituent of carrot root. Its occurrence after ethylene treatment indicates that the gas brings about a profound change in the metabolism of carrot root tissues.

Time course studies on ethylene-induced isocoumarin synthesis

The rate of isocoumarin formation in carrots can be seen in Fig. 6; up to day 2 accumulation of isocoumarin continued rapidly, from day 2 to day 3 it increased at a slower rate and after day 3 it stayed nearly unchanged.

A time-course study of ethylene-induced isocoumarin formation in carrots indicated the presence of a lag period of about 16 h before the presence of isocoumarin could be detected (Fig. 7). Increasing the concentration of ethylene shortened the lag time to 4 h, and increased the initial rate of isocoumarin synthesis but not the final amount accumulated (Fig. 8). The amount of isocoumarin accumulated in 2 days with 100 ppm of ethylene could be attained after a day with 0.2% or 5% ethylene.

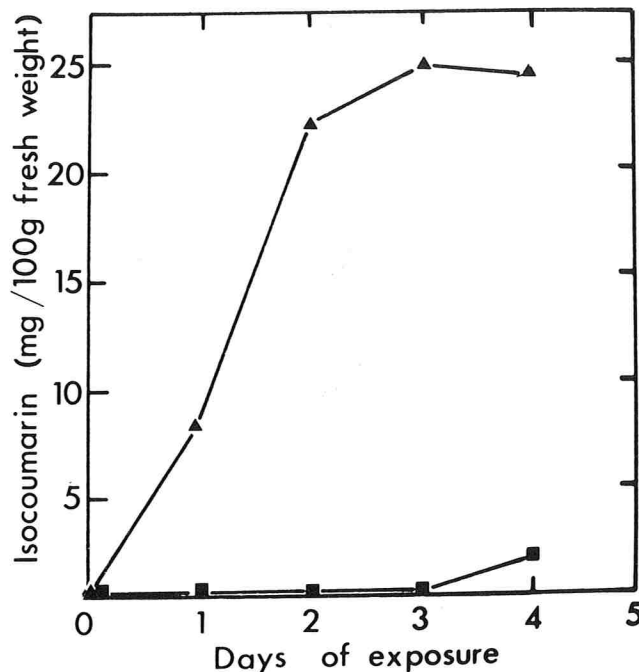


Figure 6. Rate of ethylene-induced isocoumarin synthesis in carrot slices at $25 \pm 0.5^\circ\text{C}$. Ethylene-treated (▲), and air-treated (■) carrots.

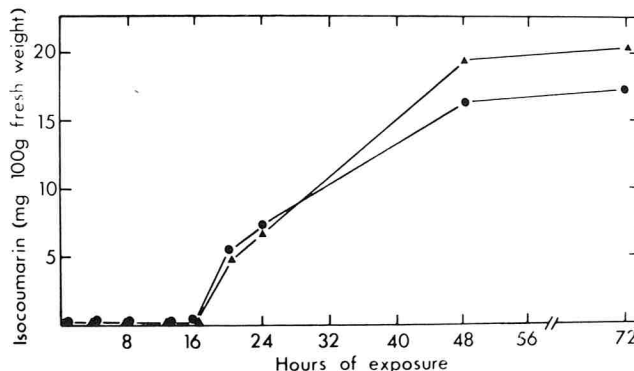


Figure 7. Time course of isocoumarin formation in carrot slices on ethylene treatment. (▲) and (●) represent two different batches of carrots.

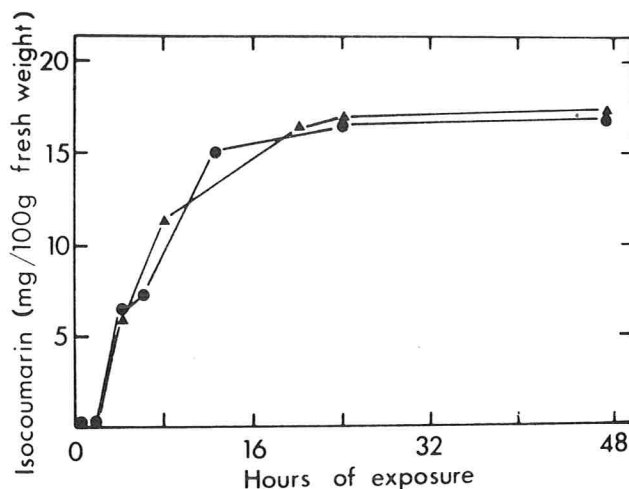


Figure 8. Rate of ethylene-induced isocoumarin synthesis in carrot slices with 0.2% and 5% ethylene. (●) - 0.2% ethylene, and (▲) - 5% ethylene.

Effects of ethylene on the biosynthetic pathways of phenols

From the above data, it is clear that ethylene exerted a pronounced effect on the phenol content of carrot root. This effect is manifested both as an accumulation of existing phenols such as isochlorogenic acid, and as a *de novo* synthesis of at least four phenolic compounds.

It is important to note that the two moieties in isochlorogenic acid (caffeic acid and quinic acid) are synthesized via the shikimic acid pathway. Thus an increased synthesis of isochlorogenic acid must occur as a result of ethylene-induced stimulation of the shikimic acid pathway. One of the key enzymes of this pathway is L-phenylalanine ammonia-lyase (PAL). The effect of ethylene on the activity of this enzyme in carrot roots was investigated. The results (Fig. 9) show an initial increase in PAL activity, which declined to a value less than that of control after 3 days of exposure to ethylene. Ethylene also failed to produce any effect on a cell-free extract from carrot root (20). This would indicate that a direct correlation does not exist between enhanced isochlorogenic acid level and *in vitro* PAL activity. What happens *in vivo* is a matter that needs further investigation.

Structural analysis of 8-hydroxy-6-methoxy-3-methyl-3,4-dihydro-isocoumarin (so-called isocoumarin) and 5-hydroxy-7-methoxy-2-methylchromone (commonly known as eugenin) suggests that these are most likely synthesized via the acetate pathway. Incorporation studies with $[^{14}\text{C}]$ acetate, $[^{14}\text{C}]$ malonate, and ^{14}C acetoacetate were carried out on carrot slices in presence of ethylene (21). The results (Tables 3 and 4) clearly indicated that isocoumarin is synthesized from acetate as suggested earlier by Condon et al. (9).

Similarly, as expected, acetate was readily incorporated into eugenin (Table 5). In this instance, it was further shown that addition of the methyl group on position 7 was probably the last step in the sequence of eugenin synthesis because 5,7-dihydroxy-2-methylchromone (DHMC) was characterized as the immediate

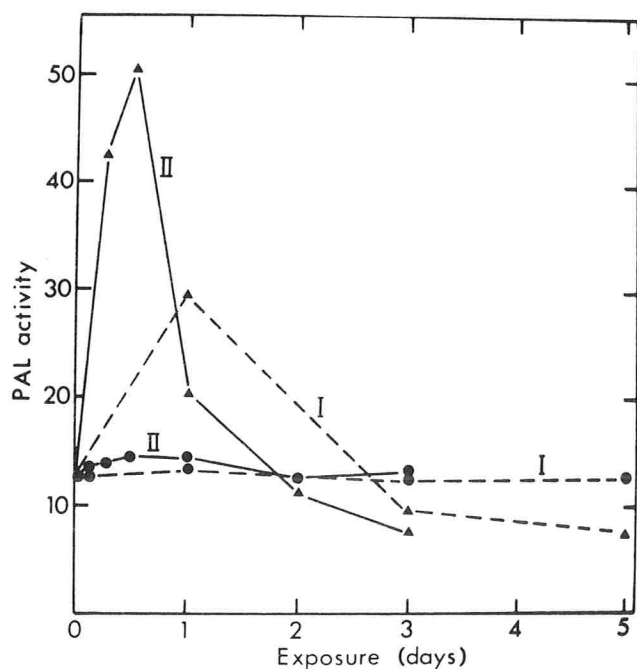


Figure 9. Effect of exposure time on the development of PAL activity in carrots. Ethylene-treated (▲), and air-treated (●) carrots. I and II represent two different experiments.

precursor of eugenin by trapping ¹⁴C-labeled DHMC from carrots fed [¹⁴C]acetate. This is in agreement with the data obtained with other phenolic compounds having

TABLE 3. Incorporation of [¹⁻¹⁴C]acetate and [²⁻¹⁴C]malonate into isocoumarin.^a Substrate: one mm carrot slices were shaken with aqueous solutions (5 ml) of the labelled compounds for 2 days in presence of a continuous stream of 0.2% ethylene. Initial specific activity of acetate and malonate was 10.4 mCi/mmol.

Substrate	Weight of the tissue (g)	Activity added to the tissue (10 ⁻⁶ dpm)	Activity taken up by the tissue (10 ⁻⁶ dpm)	Activity of isocoumarin in the tissue (10 ⁻⁶ dpm)	Conversion (%)	Specific activity of isocoumarin (μCi/mmol)
[1- ¹⁴ C]acetate	3.00	12.7	9.4	0.15	1.6	135
[2- ¹⁴ C]malonate	3.10	12.7	8.8	0.12	1.3	104

^aFrom Sarkar and Phan (21).

TABLE 4. Incorporation of [³⁻¹⁴C]acetoacetate alone or together with unlabelled acetate into isocoumarin.^a [³⁻¹⁴C]acetate together with unlabelled acetate was fed by infiltration into the tissue after 8 h of prior exposure to 0.2% ethylene done for another 40 h at room temperature.

[³⁻¹⁴ C]acetoacetate (in 6.7 ml) (μM)	Weight of the tissue (g)	Activity added to the tissue (10 ⁻⁶ dpm)	Unlabelled acetate (in 6.7 ml) (μM)	Activity taken up by the tissue (10 ⁻⁶ dpm)	Activity of isocoumarin in the tissue (10 ⁻³ dpm)	Conversion (%)
21	3.10	3.5	none added	1.00	17.1	1.70
21	3.20	3.5	210	0.96	6.4	0.67
21	3.05	3.5	420	1.03	7.7	0.75
21	3.00	3.5	630	0.99	18.3	1.86

^aFrom Sarkar and Phan (21).

TABLE 5. Incorporation of [¹⁻¹⁴C]acetate into eugenin and DHMC in the presence or absence of unlabelled DHMC.^a The solution of acetate (5 ml) alone or along with unlabelled DHMC was shaken with 1-mm thick tissue slices after 2 days of prior exposure to 2000 μl/ethylene in the presence of the same concentration of ethylene. The initial specific activity of acetate was 2 mCi/mmol.

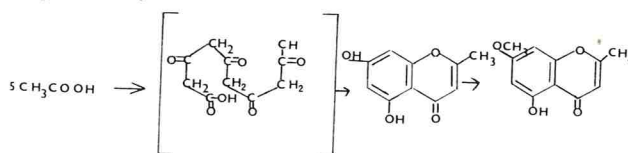
Substrate	Weight of the tissue (g)	Metabolic period (h)	Activity added to the tissue (10 ⁻⁶ dpm)	Activity taken up by the tissue (10 ⁻⁶ dpm)	Conversion %		Activity of eugenin and DHMC in the tissue, 10 ⁻⁶ dpm		Specific activity (μCi/mmol)	
					Eugenin	DHMC	Eugenin	DHMC	Eugenin	DHMC ^b
[1- ¹⁴ C]acetate	8.15	60	39.0	37.4	0.79	—	0.3	—	153	—
[1- ¹⁴ C]acetate + 5 mg of DHCM	6.90	12	39.0	—	0.22 ^c	0.80 ^c	0.1	0.3	18	5

^aFrom Sarkar and Phan (21).

^bThe specific activity of DHMC is after dilution with the carrier.

^cBased on the activity added.

0-methyl groups (15,16). Thus the biosynthetic route for eugenin may be as follows:



Our results from all the above sections bring to light two important observations regarding the effect of ethylene on phenol synthesis in carrots: (a) ethylene stimulates the synthesis of phenols derived via the shikimic acid pathway, and (b) ethylene induces synthesis of four more phenolic compounds that are synthesized via the acetate pathway of origin of aromatic compounds. Therefore, we conclude that ethylene stimulates both the pathways of biosynthesis of aromatic compounds in carrots.

Ethylene-induced phenol synthesis in carrots and its relation to other metabolic activities

Effect of ethylene on respiration and glycolytic pathways. For syntheses of aromatic compounds via either pathway, the required substrates and energy must come from a process or processes that is/are induced or modified by the action of ethylene. One of the main biological functions affected by ethylene is respiration. Although the mode of action of ethylene on the respiratory processes is not yet clear, it is well-established

that ethylene markedly enhances respiration in plant tissues. It has been suggested recently that ethylene-induced rise in respiration might result from a greater participation of cyanide-resistant electron path (23). Our data (Fig. 10) clearly showed that ethylene enhanced the respiratory activity of carrot tissues. Peel (1-mm thick) tissues were more active and responded more noticeably to ethylene treatment than internal tissues (17). Both O_2 uptake and CO_2 output increased, and the R.Q. remained unchanged and was close to one. Thus, carbohydrate appears to be the source of carbon dioxide produced.

To ascertain the distribution of glucose breakdown between the Embden-Meyerhof-Parnas (EMP) and the

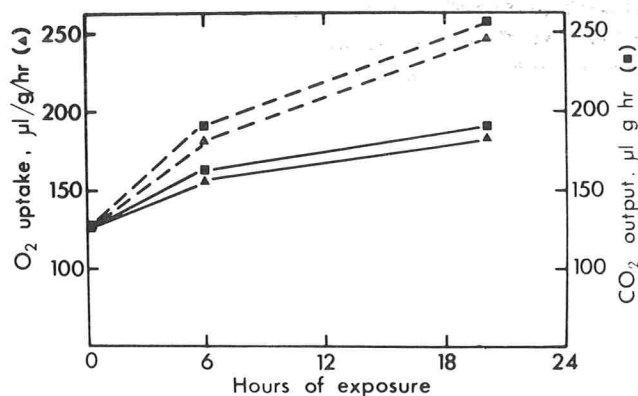


Figure 10. Effect of ethylene on the respiratory activity of carrot root slices. Dotted lines: ethylene-treated. Plain lines: air controls.

Pentose Phosphate (PP) pathways of glucose utilization, specifically labelled glucose was fed to air-treated and ethylene-treated carrot slices. The curves in the upper half of Fig. 11 show that in air-treated samples the rate of CO_2 production from $[1-^{14}C]$ glucose was higher than that from either $[6-^{14}C]$ or $[3,4-^{14}C]$ glucose, and the C-1/C-6 ratios were always greater than one (Table 6).

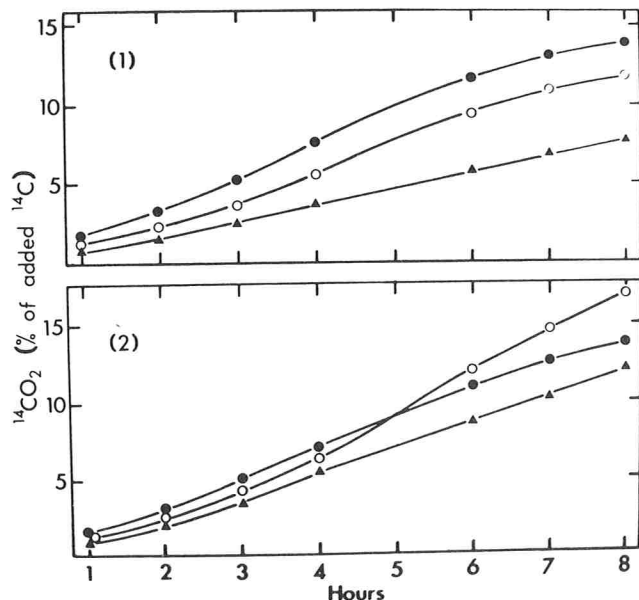


Figure 11. Yields of $^{14}CO_2$ from specifically labelled glucose in air (1) and ethylene (100 ppm) (2). From $[1-^{14}C]$ glucose (▲); from $[3,4-^{14}C]$ glucose (○); from $[6-^{14}C]$ glucose (●).

The pattern of conversion of specifically labelled glucose into isocoumarin is given in Table 7. It will be seen that the yield as well as the specific activity of isocoumarin was much higher when obtained from $[6-^{14}C]$ glucose than those obtained from $[1-^{14}C]$ glucose or $[3,4-^{14}C]$ glucose. This would indicate that the EMP pathway contributes more to isocoumarin synthesis in ethylene-treated carrots. This may have happened because of ethylene-induced shift from the PP to the EMP pathway of carbohydrate dissimilation as discussed above.

Effect of metabolic inhibitors on isocoumarin synthesis in carrots. The effects of DNP and methylene blue on carrot slices are presented in Table 8. At pH 7.1, DNP concentrations of 10^{-5} M and 10^{-4} M were inactive, and only at 10^{-3} M was isocoumarin synthesis induced. The effect of DNP cannot be considered as mediated through ethylene as it depressed ethylene production (14). DNP is known to stimulate respiration and glycolysis, and acts as an uncoupler of oxidative phosphorylation, but ethylene has not been shown to uncouple oxidative phosphorylation.

This indicated that both the PP and the EMP pathways were operative in carrots, as has been reported earlier (2). In ethylene-treated samples (lower half of Fig. 11), the rate of CO_2 production from $[6-^{14}C]$ glucose increased 175% over that what was obtained in air-treated carrots, while the rate of CO_2 production from $[1-^{14}C]$ glucose was slightly depressed. These changes in rates of CO_2 production resulted in lower C-1/C-6 ratios (Table 6), which meant a preferential stimulation of the EMP pathway in presence of ethylene. Such a shift from the PP to the EMP has been reported by Tager (26) in ripening bananas and its concomitant ethylene production.

In view of the studies with DNP, it is reasonable to suggest that acetate, the recognized precursor of isocoumarin and eugenin (21), is formed through an increased activity of the glycolytic pathway, and the energy required for the synthetic processes comes from the operation of the sequence EMP-Krebs cycle and electron transport chain, probably the cyanide resistant path (23).

Methylene blue, too, induced the production of isocoumarin in carrots (Table 8). Although methylene blue is known to stimulate respiration, and enhance the operation of the PP pathway (2), it might be suggested, in this case, that synthesis of isocoumarin followed higher ethylene production; production of ethylene by carrot slices was stimulated by methylene blue (21). Nevertheless, the role of increased respiratory activity (in the presence of methylene blue) in isocoumarin synthesis cannot be ruled out.

The data in Table 9 show that a 10-millimolar solution of arsenite completely inhibited isocoumarin synthesis; a millimolar solution partially inhibited it, while a 0.1-millimolar solution stimulated isocoumarin production. Its stimulatory action at low concentration is intriguing. Could this be caused by a stimulation of

ethylene production by low concentrations of arsenite, as shown by Shimokawa and Kasai (22)? At higher concentration, arsenite is known to function as an inhibitor of keto acid synthesis (27), and its role in inhibition of isocoumarin synthesis may be described by

a decrease in pyruvate oxidase mediated acetyl CoA production.

In light of the above three inhibitor studies, we are proposing that ethylene enhances the production of pyruvate by stimulating glycolysis. The pyruvate thus

TABLE 6. Effect of ethylene on release of ¹⁴C-1 and ¹⁴C-6 of glucose as CO₂.^a

Experiment number	Time (h)	Control			Ethylene (2000 ppm) treated		
		% of C-1 converted to CO ₂	% of C-6 converted to CO ₂	Ratio C-1/C-6	% of C-1 converted to CO ₂	% of C-6 converted to CO ₂	Ratio C-1/C-6
1	0-3	5.23	2.47	2.11	4.80	3.41	1.40
	3-6	6.65	3.40	1.95	6.00	4.80	1.25
2	0-2	3.75	1.56	2.40	2.81	1.61	1.61
	2-4	5.46	2.65	2.06	4.97	3.73	1.33
3	0-2&1/2	2.79	1.28	2.18	2.23	1.40	1.59
	2&1/2-5	3.98	2.13	1.87	3.55	2.54	1.39

^aFrom Sarkar (17).

*C-1 release was determined using [1-¹⁴C]glucose.

**C-6 release was determined using [6-¹⁴C]glucose.

TABLE 7. Incorporation of specifically labeled glucose into isocoumarin.^{a,b}

Substrate	Expt. No.	Activity added (× 10 ⁻⁶ dpm)	Activity taken up (×10 ⁻⁶ dpm)	Isocoumarin (dpm)	% conversion
[1- ¹⁴ C]glucose, specific activity 10.7 mCi/mole	1	18.10	15.33	77,284	0.504
	2	15.32	13.50	75,104	0.556
[6- ¹⁴ C]glucose, specific activity 10.7 mCi/mole	1	18.10	17.29	119,800	0.693
	2	15.32	13.88	107,496	0.774
[3,4- ¹⁴ C]glucose, specific activity 10.7 mCi/mole	1	18.10	16.85	17,025	0.101
	2	15.32	12.99	15,055	0.115

^aFrom Phan and Sarkar (21).

^bPercent conversion was determined on the basis of labeled glucose taken up by the tissue. Average of duplicate determinations.

TABLE 8. Effect of dinitrophenol and methylene blue on isocoumarin^a synthesis by carrot slices.^b

Inhibitor	Days after treatment	Molarity of solutions	Isocoumarin (mg/100 g fresh weight)	
			Control ^c	Treated
Dinitrophenol	1	10 ⁻⁵ M	Not detectable ^d	Not detectable
	1	10 ⁻⁴ M	Not detectable	Not detectable
	1	10 ⁻³ M	Not detectable	10.76 ^e
	2	10 ⁻³ M	Not detectable	22.17
	3	10 ⁻³ M	Not detectable	31.85
Methylene blue	1	10 ⁻³ M	Not detectable	10.08
	2	10 ⁻³ M	Not detectable	31.35

^aNo eugenin was detected.

^bFrom Phan and Sarkar (21).

^cControls were infiltrated with water.

^dLimit of detection: 0.1 μg.

^eAverage of duplicate determinations.

TABLE 9. Effect of sodium arsenite on the synthesis of isocoumarin in carrots.^a

Expt. No.	Ethylene treatment (100 ppm)	Molarity of arsenite solution	Isocoumarin ^b (mg/100 g carrots)	
			Control	Arsenite-treated
1	1 day	None added	11.65	None
	1 day	1 × 10 ⁻² M		
	1 day	1 × 10 ⁻³ M		
	1 day	1 × 10 ⁻⁴ M		
2	2 days	None added	19.85	None
	2 days	1 × 10 ⁻² M		
	2 days	1 × 10 ⁻³ M		
	2 days	1 × 10 ⁻⁴ M		

^aFrom Phan and Sarkar (21).

^bAverage of duplicate determinations.

formed produces more acetyl CoA than can be used by TCA. The excess acetyl CoA is then diverted to the synthesis of acetogenins, namely isocoumarin, eugenin and related compounds. In a similar fashion an increased production of ethanol and acetaldehyde in carrots on DNP treatment has been explained.

The data in Table 10 show that cycloheximide completely inhibited isocoumarin formation only when it was added to tissues that did not receive prior ethylene treatment. Partial inhibition was observed when cyclo-

TABLE 10. Influence of the time of infiltration of cycloheximide ($1 \times 10^{-4}M$) on the isocoumarin content of carrots after 24 hrs of ethylene treatment.^a

Expt. No.	Time of infiltration of cycloheximide (h after ethylene application)	Isocoumarin ^b (mg/100 g carrots)
1	0	Not detectable
	4	7.91
2	No cycloheximide	
	0	11.60
	4	6.15
	No cycloheximide	
		9.80

^aFrom Phan and Sarkar (14).

^bAverage of duplicate determinations.

heximide was added to carrots after 4 h of ethylene treatment. The results indicate that upon ethylene treatment there is an active de novo synthesis of enzyme(s) necessary for isocoumarin synthesis. This would explain the existence of a lag phase preceding the appearance of isocoumarin synthesis in ethylene-treated carrots.

CONCLUSIONS

On the basis of foregoing findings it seems certain that ethylene causes an increased accumulation of existing phenolic compounds in carrots. Ethylene has also been established as a cause for inducing the synthesis of isocoumarin and eugenin in carrots. Although the concentrations of ethylene (100 and 2000 ppm) used in these studies are much higher than the physiological levels, the above findings may be extrapolated to the effects of lower concentrations of ethylene. It is suggested that low levels of ethylene produced by carrots may induce slowly the production of isocoumarin in carrots during storage and render them bitter. What effect(s) eugenin and other phenols (synthesized upon ethylene treatment) may have on the taste and other qualities of carrots will be worth studying.

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E-3-A Sanitary Standards for Continuous Blenders

Number E-3500

Formulated by
International Association of Milk, Food and Environmental Sanitarians
United States Department of Agriculture
Poultry & Egg Institute of America
Dairy and Food Industries Supply Association

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

A. SCOPE

A.1

These standards cover the sanitary aspects of continuous blenders used for combining and/or mixing either wet or dry, (1) egg products or (2) an egg product(s) with an edible non-egg product(s) and includes that portion of any part integral with the blender such as hoppers and valves, which is in contact with the product. It does not pertain to batch-type blenders.

A.2

In order to conform with these E-3-A Sanitary Standards, continuous blenders shall comply with the following design, material and fabrication criteria.

B. DEFINITIONS

B.1

Product: Shall mean the egg or egg product(s) and/or other ingredient(s) which are combined and/or mixed in this equipment.

B.2

Continuous Blenders: (Referred to hereinafter as "blenders") Shall mean equipment in which two or more products are continuously added, the products combined and/or mixed by mechanical means and the blend of products discharged continuously.

B.3

Product Contact Surface: Shall mean all surfaces that are exposed to the product and surfaces from which liquids and/or solids may drain, drop, or be drawn into the product.

B.4

Non-Product Contact Surface: Shall mean all other exposed surfaces.

B.5

Mechanical Cleaning or Mechanically Cleaning: Shall denote cleaning, solely by circulation and/or flowing chemical detergent solutions and water rinses onto and over the surfaces to be cleaned, by mechanical means.

C. MATERIALS

C.1

Product contact surfaces shall be of stainless steel of the AISI 300 series¹ or corresponding ACI² types (See Appendix, Section E.), or metal which under conditions of intended use is at least as corrosion-resistant as stainless steel of the foregoing types and is non-toxic and non-absorbent, except that:

C.1.1

Rubber and rubber-like materials may be used as a coating or covering on rotors and may be used for gaskets, seals, wiping paddles, and parts used in similar applications.

C.1.2

Rubber and rubber-like materials when used for the above specified applications shall comply with the applicable provisions of the E-3-A Sanitary Standards for Multiple-Use Rubber and Rubber-Like Materials Used on Product Contact Surfaces in Egg Processing Equipment, Number E-1800.

C.1.3

Plastic materials may be used as a coating or covering on rotors and may be used for gaskets, seals, wiping paddles, bearings, augers, inspection portcovers, and parts used in similar applications.

C.1.4

Plastic materials when used for the above specified applications shall comply with the applicable

¹The data for this series are contained in the following reference: *AISI Steel Products Manual, Stainless & Heat Resisting Steels, December 1974, Table 2-1, pp. 18-19. Available from: American Iron & Steel Institute 1000 16th Street, NW, Washington, DC 20036.*

²Alloy Casting Institute Division, Steel Founders' Society of America, 20611 Center Ridge Road, Rocky River, OH 44116.

provisions of the 3-A Sanitary Standards for Multiple-Use Plastic Materials Used Product Contact Surfaces for Dairy Equipment, as amended, Number 20-08.

C.1.5

Rubber and rubber-like materials and plastic materials having product contact surfaces that are a coating or a covering, shall be of such composition as to retain their surface and conformation characteristics when exposed to the conditions encountered in the environment of intended use and in cleaning and bactericidal treatment.

C.1.6

Where materials having certain inherent functional properties are required for specific applications, such as seals, carbon and/or ceramic materials may be used. Carbon and ceramic materials shall be inert, non-porous, non-toxic, non-absorbent, insoluble, resistant to scratching, scoring, and distortion when exposed to the conditions encountered in the environment of intended use and in cleaning and bactericidal treatment.

C.1.7

Silver soldered or brazed areas and silver solder or braze material shall be non-toxic and corrosion-resistant.

C.2

Non-product contact surfaces shall be of corrosion-resistant material or material that is rendered corrosion-resistant. If coated, the coating used shall adhere. Non-product contact surfaces shall be relatively non-absorbent, durable and cleanable. Parts removable for cleaning having both product contact and non-product contact surfaces shall not be painted.

D.

FABRICATION

D.1

Product contact surfaces shall be at least as smooth as a No. 4 ground finish on stainless steel sheets free of imperfections such as pits, folds and crevices. (See Appendix, Section F.).

D.2

Permanent joints in metallic product contact surfaces shall be continuously welded. If it is impractical to weld, they may be silver soldered or brazed. Welded areas on product contact surfaces shall be at least as smooth as a No. 4 finish on stainless steel sheets free of imperfections such as pits, folds and crevices.

D.3

Rubber or rubber-like materials and plastic materials having product contact surfaces that are a coating or covering shall be bonded in such a manner that the bond is continuous and mechanically sound, and so that when exposed to the conditions encountered in the environment of intended use and in cleaning and bactericidal treatment, the rubber or

rubber-like material or the plastic material does not separate from the base material.

D.4

Product contact surfaces not designed to be mechanically cleaned shall be easily accessible for cleaning and inspection either when in an assembled position or when removed. Removable parts shall be readily demountable.

D.5

Blenders that are to be mechanically cleaned shall be designed so that all product contact surfaces of the blenders and all non-removable appurtenances thereto can be mechanically cleaned and are accessible for inspection.

D.6

Product contact surfaces of non-removable parts shall be self-draining except for normal clingage.

D.7

Fittings and valves having product contact surfaces that are furnished by the blender manufacturer shall conform to the applicable provisions of the E-3-A Sanitary Standards For Fittings Used on Egg and Egg Products Equipment and Used on Sanitary Lines Conducting Egg and Egg Products, Number E-0800, and/or to the applicable provisions for welded sanitary product-pipelines found in the E-3-A Accepted Practices for Permanently Installed Sanitary Product Pipelines and Cleaning Systems, Number E-60500.

D.8

Instrument connections, when provided, shall conform to the applicable provisions of the E-3-A Sanitary Standards for Instrument Fittings and Connections Used on Egg Products Equipment, as amended, Number E-0900.

D.9

Internal angles of 135° or less on product contact surfaces shall have radii of not less than 1/4 inch, except smaller radii may be used when required for space or functional reasons such as in sealing ring grooves and in the way the rotor fits. When the radius is less than 1/32 inch, the product contact surface of the internal angle must be readily accessible for cleaning and inspection.

D.10

Gaskets shall be removable. Gasket retaining grooves shall be no deeper than their width. The radius of any internal angle in a gasket retaining groove shall be not less than 1/8 inch, except that the radius may be 3/32 inch where a standard 1/4 inch O-Ring is to be used and the radius may be 1/32 inch where a standard 1/8 inch O-Ring is to be used.

D.11

There shall be no threads on product contact surfaces, except where necessary for attaching the rotor to the shaft. When a thread is necessary it shall be an Acme thread, an illustration of which is shown on E-3-A drawing number E-3A-100-30 in the E-3-A

Sanitary Standards for Fittings Used on Egg and Egg Products Equipment and Used on Sanitary Lines Conducting Egg and Egg Products, Number E-0800. The thread shall have (1) not more than 10 threads per inch and (2) basic major diameter of not less than 1/2 inch. The length of the nut shall not exceed three-quarters of the thread basic major diameter and the nut shall be of the open type.

D.12

Coil springs having product contact surfaces shall have at least 3/32 inch openings between coils, including the ends when the spring is in a free position.

D.13

Openings through either hinged or removable covers, to which connections are not permanently attached, shall be flanged upward at least 3/8 inch. All sanitary pipelines and other appurtenances furnished by the blender manufacturer that will enter through the cover shall be fitted with a permanently attached sanitary umbrella deflector that tightly overlaps the edges of the opening. Other openings shall have a removable cover.

D.14

Covers shall pitch to an outside edge(s).

D.15

Agitators, including the complete seal, shall be readily demountable for cleaning. Non-removable parts having product contact surfaces shall be designed so that the product contact surfaces are readily cleanable and inspected from the inside of the blender. Seals for an agitator shaft shall be of a packless type, sanitary in design, with all parts readily accessible for cleaning.

D.16

Supports: The means of supporting a blender shall be one of the following:

D.16.1

With legs: Legs shall be smooth with rounded ends and have no exposed threads. Legs made of hollow stock shall be sealed. Legs shall provide a clearance

between the lowest part of the blender, with the exception of the leg itself, and the floor of at least 4 inches when the blender outlines an area in which no point is more than 12 1/2 inches from the nearest edge or a clearance of at least 6 inches when any point is more than 12 1/2 inches from the nearest edge.

D.16.2

With casters: Blenders which are portable may be equipped with casters. Casters shall be easily cleanable, durable and of a size that will permit easy movement of the blender.

D.17

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

APPENDIX

E.

STAINLESS STEEL MATERIALS

Stainless steel conforming to the applicable composition ranges established by AISI¹ for wrought products, or by ACI² for cast products, should be considered in compliance with the requirements of Section C.1 herein. Where welding is involved the carbon content of the stainless steel should not exceed 0.08 percent. The first reference cited in C.1 sets forth the chemical ranges and limits of acceptable stainless steel of the 300 series. Cast grades of stainless steel corresponding to types 303, 304, and 316 are designated CF-16F, CF-8, and CF-8M, respectively. These cast grades are covered by ASTM³ specifications A296-68 and A351-70.

F.

PRODUCT CONTACT SURFACE FINISH

Surface finish equivalent to 150 grit or better, as obtained with silicon carbide, is considered in compliance with the requirements of Section D.1 herein.

These standards shall become effective May 4, 1979.

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

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Classified Ad — Position Available

The City of St. Joseph, MO (pop. 80,000, mayor-council form of government) is readvertising for a Health Director to administer the public health services of the city, including: landfill, laboratory, animal and insect control, housing, public health nursing and clinic, and general sanitation. Requirements include a BS degree in public health administration, an ability to establish and maintain positive public relations, and five years' public health experience, including two years in a supervisory capacity. Annual salary, \$15,000-\$20,000, commensurate with experience. Excellent fringe benefits. Apply to Mayor Gordon J. Wisner, City of St. Joseph, MO 64501. An Affirmative Action Employer.

News and Events



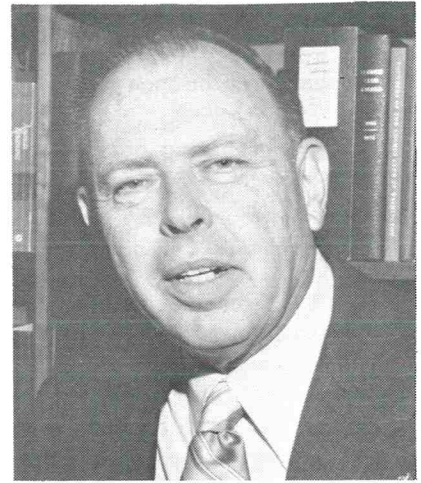
Officers of the new IAMFES affiliate, the Tennessee Association of Milk, Water and Food Protection, include, back row, left to right, President-elect, Harold Rutherford, Dairymen, Inc., Knoxville; Archivist, Ruth Fuqua, Dairymen, Inc., Nashville; Vice-president, Don Spencer, SealTest Foods, Nashville. Front row, left to right, Secretary-Treasurer, Cecil White, Tenn. Dept. of Agriculture, Nashville; and President, Kenneth W. Whaley, Tenn. Dept. of Health, Nashville.

New Affiliate Organized in Tennessee

The Tennessee Association of Milk, Water, and Food Protection was organized during the 27th Annual Tennessee Dairy Institute Conference. The meeting was held March 5-6 at the Plant Science Building of the University of Tennessee at Knoxville.

The first meeting of the newly organized affiliate met at the close of the conference and officers were elected to direct the association business for its first year. The proposed constitution and by-laws for the new association were also accepted.

Williams to Retire from DFISA, 3A Committees



The longtime secretary of the 3A Sanitary Standards Committees, Don Williams, will retire from his position as Technical Director of the Dairy & Foods Industries Supply Assn. on June 30. He has served as 3A secretary for 25 years, during

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PLEASE LOOK FOR additional News and Events items throughout this issue, at the ends of manuscripts. You'll find these items on pages 469, 507, 511, and 525.

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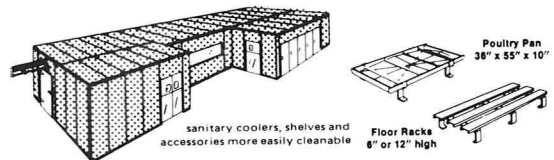
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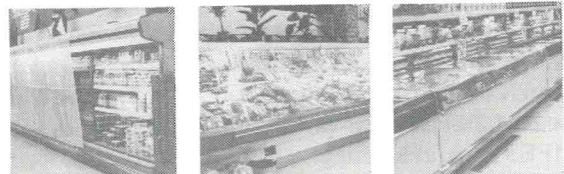
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Coming Events

June 17-21--ASSOCIATION OF FOOD AND DRUG OFFICIALS, 83rd Annual Conference. Hyatt Regency Hotel, Washington, D.C. Contact: Frederick A. Siino, Sec.-Treasurer, Association of Food and Drug Officials, P.O. Box 3, Barrington, RI 02806.

June 18-August 23--PRINCIPLES OF TOXICOLOGY, ten-week graduate course, Massachusetts Institute of Technology. Applicants should have background in chemistry, biology, or related science at Master's level or above. EPA Training Grants will provide stipends and tuition for 24 accepted students who must be U.S. citizens. Tuition: \$3,000, enrollment restricted to 50 persons. For further details or an application, contact: Linda C. Boyar, Program Administrator, Dept. of Nutrition and Food Science, MIT, Room E18-564, Cambridge, MA 02139, 617-253-7023.

June 23-28 -- NATIONAL ENVIRONMENTAL HEALTH ASSOCIATION ANNUAL EDUCATIONAL CONFERENCE. Charleston, SC. Contact: NEHA, 1200 Lincoln St., Suite 704, Denver, CO 80203, 303-861-9090.

June 24-27--AMERICAN DAIRY SCIENCE ASSOCIATION, Annual Meeting, Utah State University, Logan, UT. Contact: Charles V. Morr, Dept. of Food Science, Clemson University, 223 Plant and Animal Science Building, Clemson, SC 29631, 803-656-3397.

June 24-27--AMERICAN SOCIETY OF AGRICULTURAL ENGINEERS, Summer Meeting, Winnipeg, Manitoba, Canada. Contact: Roger R. Castenson, ASAE, 2950 Niles Road, Box 410, St. Joseph, MI 49085, 616-429-0300

July 16-18--BASIC PRINCIPLES OF QUALITY ASSURANCE SEMINAR. American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502. Contact: Darrell Breising, AIB, 913-537-4750.

July 30-Aug. 3--ADVANCES IN FOOD AND APPLIED MICROBIOLOGY. Massachusetts Institute of Technology, Cambridge, MA 02139. Program is under the direction of Anthony J. Sinskey, MIT, Professor of Applied Microbiology. Contact: Director of Summer Session, Rm. E 19-356, Massachusetts Institute of Technology, Cambridge, MA 02139.

July 30-Aug. 10--BAKING FOR ALLIED PERSONNEL. American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502. Contact: Darrell Breising, AIB, 913-537-4750.

Aug. 6-7--PESTICIDE CERTIFICATION COURSE. American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502. Contact: Darrell Breising, AIB, 913-537-4750.

Aug. 12-16--IAMFES ANNUAL MEETING, Sheraton Twin Towers, Orlando, FL. Contact: E. O. Wright, IAMFES Exec. Sec., P.O. Box 701, Ames, IA. 50010, 515-232-6699, or see registration form in this *Journal*.

Aug. 13-17--WORKSHOP ON EDUCATIVE PROCESSES IN FOOD MICROBIOLOGY. Sponsored by the Joint American Society for Microbiology/Institute for Food Technologists Committee on Food Microbiology Education. Quadna Resort, Hill City, MN. Contact: E. A. Zottola, Dept. of Food Science and Nutrition, 1334 Eckles Ave., University of Minnesota, St. Paul, MN 55108.

Aug. 13-Dec. 21--BAKING SCIENCE AND TECHNOLOGY. American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502. Contact: Darrell Breising, AIB, 913-537-4750.

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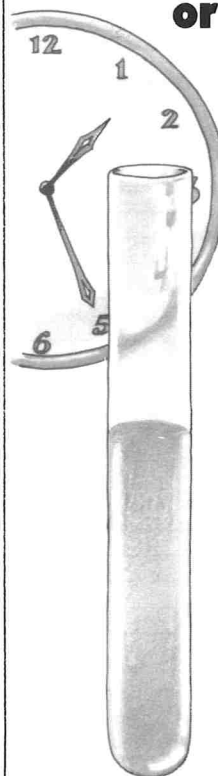
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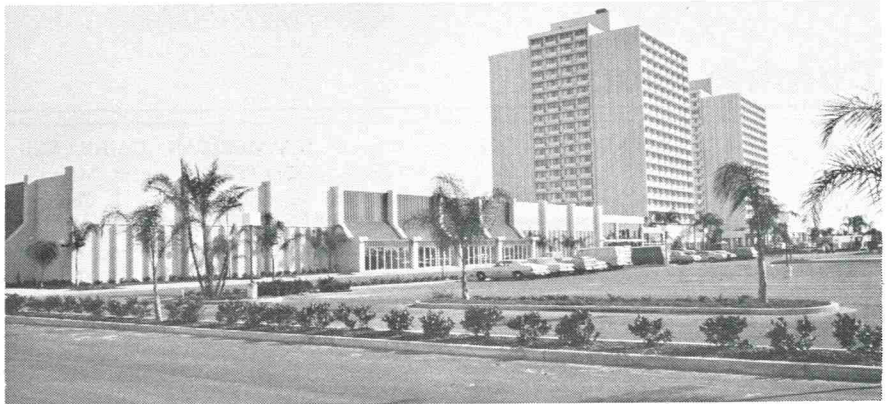
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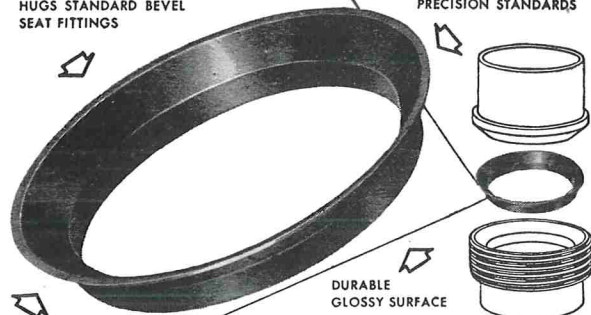
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NEOPRENE GASKET for Sanitary Fittings

Check these **SNAP-TITE** Advantages

Tight joints, no leaks, no shrinkage

Sanitary, unaffected by heat or fats

Non-porous, no seams or crevices

Odorless, polished surfaces, easily cleaned

Withstand sterilization

Time-saving, easy to assemble

Self-centering

No sticking to fittings

Eliminate line blocks

Help overcome line vibrations

Long life, use over and over

Available for 1", 1½", 2", 2½" and 3" fittings.

Packed 100 to the box. Order through your dairy supply house.

THE HAYNES MANUFACTURING CO.
4180 Lorain Avenue • Cleveland 13, Ohio



SIZES 1" - 1½"
2" - 2½" - 3" - 4"

* MADE FROM
TEFLON®

"The Sophisticated Gasket"

THE IDEAL UNION SEAL FOR
BOTH VACUUM AND
PRESSURE LINES

Gasket Color . . .
slightly off-white

SNAP-TITE self-centering gaskets of TEFLON are designed for all
standard bevel seat sanitary fittings. They SNAP into place provid-
ing self-alignment and ease of assembly and disassembly.
HAYNES SNAP-TITES of TEFLON are unaffected by cleaning solu-
tions, steam and solvents. They will not embrittle at temperatures
as low as minus 200° F. and are impervious to heat up to 500° F.
FOR A FITTING GASKET THAT WILL OUT-PERFORM ALL OTHERS...

Specify . . . **HAYNES SNAP-TITES of TEFLON**

• TEFLON ACCEPTED SAFE FOR USE ON FOOD & PROCESSING
EQUIPMENT BY U. S. FOOD AND DRUG ADMINISTRATION

* Gaskets made of DuPont TEFLON® TFE-FLUOROCARBON RESINS

THE HAYNES MANUFACTURING COMPANY
4180 LORAIN AVENUE • CLEVELAND, OHIO 44113

A HEAVY DUTY SANITARY LUBRICANT



Available in both
SPRAY AND TUBE

All Lubri-Film ingredients are
approved additives and can be
safely utilized as a lubricant for
food processing equipment when
used in compliance with existing
food additive regulations.

ESPECIALLY DEVELOPED FOR LUBRICATION OF FOOD
PROCESSING AND PACKAGING EQUIPMENT

For Use in Dairies — Ice Cream Plants — Breweries —
Beverage Plants — Bakeries — Canneries — Packing Plants

SANITARY • NON TOXIC • ODORLESS • TASTELESS

SPRAY — PACKED 6 — 16 OZ. CANS PER CARTON
TUBES — PACKED 12 — 4 OZ. TUBES PER CARTON

THE HAYNES MANUFACTURING CO.
CLEVELAND, OHIO 44113

Total Management Concept: The Team Working For You

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



There is no way the farmer today can operate as a loner. That's why more and more dairymen are looking at their total operations with an eye on Total Management. The Total Management Concept benefits the dairyman because it encourages him to identify and make use of the many

people who have been trained to offer professional advice and service. TMC is teamwork with the dairyman as head coach. He knows who his resource people on the bench are and when to call on them. And, like any winning coach, the dairyman knows the special efforts of all team members must be coordinated to succeed. These resource people are the veterinarians, equipment dealers, nutritionists, agricultural engineers, extension service people, D.H.I.A. or R.O.P., and many others who can provide current, specialized information and help with the business of dairying.

Planning to Avoid Emergencies

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

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

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

The Team in Action

Total Management provides the farmer with

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

• HERD HEALTH

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

• PRODUCTION GOALS

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

• RECORDS

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

• EQUIPMENT

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

• FEEDING PROGRAM

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

Keeping pace with the times offers a rewarding challenge for the dairyman who is willing to use progressive management practices. And, if you face the future by working on Total Management with the help of the many professionals available, you will find a bright future. Today, more than ever, your future depends on having a winning team working for you.



SURGE

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2100 South York Road,
Oak Brook, Illinois 60521

We make your cows worth more.