

Potential Production and Detoxification of Penicillic Acid in Mold-Fermented Sausage (Salami)

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About 10% of 346 *Penicillium* cultures isolated from mold-fermented sausage synthesized the toxic metabolite penicillic acid on liquid media. Five of these producing cultures inoculated onto sausage failed to produce this toxin in up to 70 days of ripening. Several amino acids normally occurring in meat (cysteine, glutathione, arginine, histidine, and lysine) were found capable of readily reacting with penicillic acid. The adducts formed by the reaction between cysteine or glutathione with penicillic acid were identified and found to be non-toxic to mice, quails, and in the rabbit skin test but exhibited toxicity to the chick embryo. Hypotheses accounting for this residual toxicity are advanced.

Mold-fermented sausage (salami), although not an item of importance in the American food trade, represents a high percentage of the total sausage produced in many European countries. Mintzloff and Leistner (Zentralbl. Veterinärmed., *in press*) estimated the following production figures (in percent): Rumania, 100; Italy, 95; Bulgaria, 90; Hungary, 80; Switzerland, 70; Spain, 50; Austria, 30; lesser percentages ranging from 1 to 5 were noted for France, Belgium, Yugoslavia, West Germany, Poland, and the United States.

The compositions of these sausages vary widely but there are only two general types, Hungarian in which the meat is smoked lightly during the ripening period and Italian in which no smoke is used. The mold growth, which is confined to the outer casing, strongly influences the appearance, taste, odor, and even the keeping qualities of the final product.

In commercial production pure culture technique generally is not practiced, the molds developing on the casing depending in great part on the fungal floras of the ripening chambers. Various investigators have reported that members of the genus *Penicillium* appear to predominate on these sausages (7, 11, 15, 20). Leistner and Ayres (15) isolated, in particular, *P. simplicissimum*, *P. miczynskii* and *P. expansum*.

Fermentations are usually initiated at 22 C, 95% relative humidity, followed by a gradual

decrease over a five-day period to 15 C, 75% relative humidity. Fungal growth on the casing is essentially complete by this time, and the sausages can then be stored up to 70 days at 14 C, 75% relative humidity; an approximate 40% loss in weight takes place over this time span so that the final product is rather dry and firm in texture.

This combination of high moisture, low temperature, and the predominance of penicillia are conditions previously found conducive to the production of penicillic acid (PA) on various grains (3, 14). PA is known to be toxic to mammals and has proved carcinogenic to rats (5).

The initial purpose of this investigation was to determine the capability of the various *Penicillium* strains found on fermented sausage to produce PA. Subsequently, the scope of the investigation was widened to include the study of a reaction(s) that resulted in disappearance of PA from raw sausage.

MATERIALS AND METHODS

Cultures. *Penicillium* cultures were isolated from commercial mold-fermented sausages that had been produced in 11 different European countries. Isolation procedures were those suggested by Raper and Thom (16) and identification of isolates, where made, was based on their manual. Isolates were maintained on Difco potato dextrose agar (PDA) slants.

Fermentation. Several media including Difco potato dextrose broth, modified Raulin-Thom broth (1), and yeast extract sucrose (YES) broth (4), and two growth temperatures (15 and 25 C) initially were used to screen the various cultures for production. However, experience indicated that no advantage was gained by the use of multiple conditions over use of YES broth alone incubated at 25 C. Two hundred milliliters of medium, in 450-ml Schott-Mainz-manufactured small Fernbach flasks, were inoculated from 1- to 2-week-old PDA slants and incubated as still cultures for 12 days.

Extraction and analyses. Culture broths were adjusted to pH 1.5 with HCl and extracted with an equivalent volume of CHCl_3 . The solvent, after being dried with anhydrous Na_2SO_4 , was removed by flash evaporation and the residual solids were dissolved in 2 ml of CHCl_3 . Ten-microliter portions were spotted on Brinkmann silica gel N-HR thin-layer chromatography (TLC) plates, along with a PA reference sample, and the plates developed in toluene, ethyl acetate, formic acid (6:3:1). After development, plates were sprayed with *p*-anisaldehyde reagent (17) and heated at 105 C for about 5 min; PA gives a dark-green spot at an R_f of about 0.45.

For confirmation two additional solvent systems were used: chloroform-ethyl acetate-formic acid (60:40:1, R_f 0.5) and chloroform-ethanol (90:10, R_f 0.75). Exposure to ammonium fumes gave a blue fluorescent derivative with an emission at 440 nm as determined with an Aminco-Bowman spectrofluorometer (2). A more intense blue fluorescent derivative could be obtained by spraying the plates with diphenylboric acid-ethanolamine (19). In ultraviolet and after isolation by preparative TLC, PA showed a single peak at 221 nm with a shift to 293 nm on addition of 0.02 N NaOH.

The PA used for standards and for animal studies was produced and isolated in crystalline form by the procedure of Bentley and Keil (1). Identity and purity of the crystalline product was established by melting point, ultraviolet (UV) molar extinction coefficient, mass spectra, and TLC.

Sausage fermentation. Sausages of approximately 200 g in weight were prepared from a commercially produced meat mixture consisting of one part each of pork, pork fat, and beef; the meat was not smoked. Spore suspensions were made by washing 1- to 2-week-old YM agar (Difco) slants with a 0.3% Tween 80 solution to give a concentration of about 10^6 spores/ml. The sausages, after being sprayed with these spore suspensions, were incubated under the following regimen: 22 C, 95% relative humidity for 40 hr; 20 C, 90% relative humidity for 24 hr; 18 C, 85% relative humidity for 10 hr; and 14 C, 75% relative humidity for 70 days. Analyses of duplicate sausages for PA were carried out after 2, 4, and 10 weeks of incubation.

Sausage analysis. Each sausage was homogenized for 3 min in a Waring Blendor with 400 ml of acetonitrile-water (9:1) plus 100 ml of hexane. The solvent was recovered by Büchner funnel filtration and the hexane layer was discarded. The acetonitrile-water layer, after being washed with a second 100-ml volume of hexane to remove residual lipids,

was added to 200 ml of water plus 200 ml of chloroform. The solvents were shaken in a separatory funnel, the layers were permitted to separate, and the recovered chloroform, after drying with anhydrous Na_2SO_4 , was flash evaporated. The residual solids were dissolved in a small amount of chloroform and analyzed by TLC for PA.

The adequacy of the analytical method was tested by extracting sausages to which known amounts of PA were added.

Penicillic acid reaction with amino acids. Penicillic acid and a given amino acid were dissolved in distilled water in a 1:1 or 1:2 molar ratio. The pH of the solution was adjusted to 7.0 to 7.3 by addition of either 10% NH_4OH or 1.0 N HCl. The solution was flash evaporated to near dryness and the final volume adjusted with distilled water to give a desired compound concentration. The percent of completion of the reaction was determined by quantitative TLC of residual PA (2).

Analyses of the reaction products were made on preparations lyophilized at -20 C immediately following completion of a reaction between 1:1 molar ratios of PA and the amino acid under study.

Animal toxicity tests. Animals used included mice of both sexes (strain NMRI/HAN), 4-week-old 50-g Japanese quail (*Coturnix coturnix japonica*), adult white laboratory rabbits, and 4-day-old chick embryos (strain HNL, Lohmann Cuxhaven). The various compounds tested were dissolved in water and administered as noted in the text. Animals dying from acute toxicity or sacrificed at completion of an experiment were examined for gross pathology, and the kidneys and livers were prepared for histological examination.

RESULTS

Survey. About 10% of the *Penicillium* isolates grown on YES broth produced PA with yields between 1 to 100 mg/100 ml of medium (Table 1). Although less than half of the producing cultures were identified to species, all of the known strains of *P. janthinellum* and *P. viridicatum*, plus the one identified strain of *P. cyclopium*, synthesized PA.

Sausage fermentation. Sausages in duplicate were analyzed after 2, 4, and 10 weeks of incubation. Growth of the five cultures examined (*P. janthinellum*, *P. simplicissimum*, *P. cyclopium*, *P. viridicatum*, and an unidentified *Penicillium* sp.) was excellent, completely coating the sausage casings. However, PA was not detected even though all of the cultures used could produce this toxin in mycological media.

Penicillic acid addition to sausage. Prior to inoculating the toxin-producing strains onto sausage, we had tested our analytical methods by PA addition to the meat. In three trials, essentially 100% of the toxin was recovered when solvent extraction immediately followed PA addition. However, if a time lapse of 24 hr

or more intervened, only a small percentage of the toxin added could be detected (Table 2).

Penicillic acid reaction with sulfhydryl-amino acids. Penicillic acid has been shown to be capable of reacting with cysteine (CSH) although the product of the reaction has not been identified (6, 9). Because meat is known to contain CSH as well as glutathione (GSH), we reexamined this reaction.

The effect of a pH value of between 5 to 7 on the reaction rate between PA and CSH or GSH was negligible from a practical standpoint. Representative data for the reaction between GSH and CSH are shown in Tables 2 and 3. However, the buffer composition did affect the reaction, e.g., the use of NH_4OH to adjust the reaction solution pH to 7 resulted in a reaction rate too rapid to measure, whereas, when PO_4 buffer at pH was used, the rate was considerably lower. Tris(hydroxymethyl)aminomethane buffer was unsatisfactory, for PA reacted with this substance prior to amino acid addition.

Attempts to crystallize the products of the reactions from various solvents were unsuccessful, for only white amorphous powders resulted. However, these exhibited only a single spot in various TLC systems.

TABLE 1. *Penicillic acid (PA) production by Penicillium species isolated from mold-fermented sausage*

Species	PA producers per no. of cultures examined
<i>P. janthinellum</i>	3/3
<i>P. simplicissimum</i>	2/9
<i>P. miczynskii</i>	0/3
<i>P. cyclopium</i>	1/1
<i>P. expansum</i>	1/11
<i>P. viridicatum</i>	7/7
<i>P. spp.</i>	20/312
Total cultures	34/346

TABLE 2. *Recovery of added penicillic acid (PA) from raw sausage incubated at 25 C*

Recovery time (days)	PA recovered (mg)				
	0 ^a	2	5	10	25
1	0	0	0.5	0.15	0.45
2	0	0	0	0.10	0.20
3				0.05	0.15

^a Milligrams of PA added.

TABLE 3. *Reaction between penicillic acid (PA) and glutathione (1:1 M ratio) at several pH values*

Time (hr)	PA reaction (%)			
	pH 5 ^a	pH 6 ^b	pH 7 ^b	pH 7 ^c
0.0	38	46	41	100
0.5	75	86	85	
1.0	81	92	93	
2.0	83	94	94	
3.0	88	95	95	
5.0	88	95	98	
7.0	90	97	100	

^a Citrate acetate buffer, 0.1 M.

^b Phosphate buffer, 0.1 M.

^c The pH was adjusted with 10% NH_4OH .

The UV absorption spectra in water of the amino acid reaction products with PA at pH 7 were: PA + CSH, 228 nm ($\epsilon = 4.2 \times 10^3$); PA + GSH, 228 nm ($\epsilon = 3.3 \times 10^3$); PA, 226 nm ($\epsilon = 2.3 \times 10^3$).

The reaction products were more polar than PA but on exposure to NH_4OH fluoresced the same intense blue as had PA alone. However, the adducts, unlike PA, did not fluoresce blue on reaction with diphenylboric acid-ethanolamine. Both adducts were soluble in water and methanol but insoluble in other solvents, including ethanol.

Both reaction products gave a negative nitroprusside test for sulfhydryl but a positive ninhydrin reaction, indicating that addition of the amino acids via the sulfhydryl group to PA had occurred. Although cysteine is a trifunctional compound, the rate data of Friedman et al. (8) lends further support to preferential attack by the thiol group in the preceding reactions.

The nuclear magnetic resonance (NMR) spectra give evidence that both glutathione and cysteine add, in the same manner, to the isolated double bond of PA. The important features in the NMR spectrum of PA are the absorptions assigned to the methylene protons on the isolated double bond and the olefinic methyl protons (Fig. 1). The former exhibits resonance at $\delta 5.51$ and $\delta 5.61$ whereas the latter absorption is found at $\delta 1.78$. These signals do not appear in the spectra of the reaction products indicating that addition occurs to the isolated double bond. A further indication that the reaction takes place at the isolated double bond is given by the virtually unchanged chemical shift of the olefinic proton on the conjugated double bond. If addition had occurred to the conjugated double bond, the resonance would have shifted upfield and would no

longer appear as a one-proton singlet. The spectra of the reaction products provide information on the mode of addition to the double bond. If hydrogen were added to the terminal methylene carbon, an additional methyl group would result. Inspection of the spectra and the integrated intensities indicate that there is no additional methyl absorption. The NMR data together with chemical analysis establish the structures of the reaction products as shown in Fig. 1.

Penicillic acid reaction with other amino acids. Because primary amines are known to be Michael addition reaction nucleophiles (12), the reactivity of three additional amino acids with PA was investigated. The reaction between lysine and histidine with PA on a 2:1 molar ratio proceeded slowly to completion over a 9-day period, but arginine ceased to react after 4 days and only went to 60% of completion (Table 4). The mechanism of the reaction was not investigated although a positive ninhydrin test for the new products formed indicated an addition reaction had occurred.

Toxicity tests. The S-alkylated adducts formed by the reaction of PA with CSH or GSH were nontoxic to mice or quail even though the amount of substance administered contained a concentration of toxin equivalent to that which previously had been determined to be about the lethal dose (LD₁₀₀; Tables 5 and 6). Test animals were sacrificed 2 to 3 weeks after dosage but no gross or histopathological abnormalities were observed.

Gross pathology of quails administered lethal doses of PA revealed only fatty liver degeneration; no other organs appeared to be affected. Histological examination of the liver showed a generalized fatty degeneration of the parenchyma, plasmogranulation, and enlarged vacuolated nuclei.

Histological preparations of mice liver, after administration of PA, showed enlarged sinusoids, histocytic infiltration, and a generalized cell necrosis.

The chicken embryo test indicated that the GSH adduct of PA was about 40 to 50% as

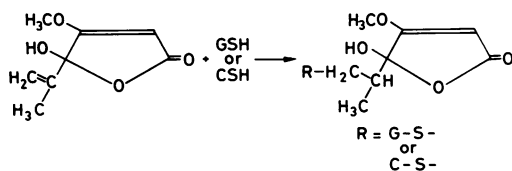


FIG. 1. Reaction between penicillic acid and cysteine (CSH) or glutathione (GSH).

TABLE 4. Reaction of penicillic acid (PA) with three amino acids^a

Time (days)	PA reaction (%)		
	Arginine	Histidine	Lysine
1	33	50	60
2	38	66	70
3	50	86	86
4	60	90	90
7	60	95	95
9	60	100	100

^a In aqueous solution adjusted to pH 7.0 with 1.0 N HCl, 10⁻² M PA reacted with 2 × 10⁻² M amino acid.

TABLE 5. Toxicity tests of penicillic acid (PA) reacted with cysteine (CSH)

Test system	Equivalent toxin dose per unit of body weight	Administration route ^a	Trial time (days)	Deaths per no. of test animals	
				PA	PA + CSH
Mouse	8.5 mg/20 g	ip	7	5/5	0/5
Mouse	15.0 mg/20 g	po	7	5/5	0/5
Quail	50.0 mg/100 g	po	7	5/5	0/5
Chicken embryo	0.85 mg/egg	air sac	7	29/43	29/42
Chicken embryo	0.85 mg/egg	air sac	6	39/49	39/50
Chicken embryo	0.85 mg/egg	air sac	17	60/63	52/63

^a Abbreviations: ip = intraperitoneal; po = by mouth.

TABLE 6. Toxicity tests of penicillic acid (PA) reacted with glutathione (GSH)

Test system	Toxin dose per unit of body weight	Administration route ^a	Trial time (days)	Deaths per no. of test animals	
				PA	PA + GSH
Mouse	8.5 mg/20 g	ip	14	15/15	0/15
Mouse	15.0 mg/20 g	po	14	14/20	0/20
Quail	50.0 mg/100 g	po	7	6/6	0/6
Chicken embryo	0.85 mg/egg	air sac	7	50/50	25/50
Chicken embryo	0.85 mg/egg	air sac	14	40/40	18/40
Chicken embryo	0.85 mg/egg	air sac	17	58/63	26/63

^a Abbreviations: ip = intraperitoneal; po = by mouth.

toxic as a comparable dose of PA; the CSH adduct of PA was as toxic as PA itself.

Application of 10 mg of PA suspended in olive oil to a 4-cm² area of rabbit skin resulted within 2 hr in a severe edema that was sharply demarcated by 24 hr and showed superficial

skin necrosis by 48 hr. Application of an equivalent amount of PA reacted with CSH or GSH gave no visible reaction (Fig. 2).

DISCUSSION

Our finding that 10% of the *Penicillium* cultures isolated from mold-fermented sausage are capable of producing PA is not surprising for the ability to produce this compound is widespread among molds (18). However, we believe that this may represent the first report of PA production by *P. janthinellum* and *P.*

simplicissimum. The amount of toxin produced by the various isolates on YES medium generally was not large and averaged between 4 to 10 mg/100 ml of medium. However, no attempt was made to determine fermentation conditions that probably could have given higher yields, for this was beyond the purpose of our experiments.

Of more practical importance was the question of the ability of these isolates to produce PA on sausage during a mold-ripening process. We did not detect any PA on sausages that had been inoculated with five PA-producing strains even after incubation periods of up to 70 days.

A previous investigation on fungi isolated from grains had shown that natural products high in protein, e.g., soybeans, peanuts, and cottonseed, do not support PA production (3). This seemed to indicate that meat might also be a poor substrate for synthesis of this secondary metabolite. However, this hypothesis was weakened by our finding that amino acids normally occurring in meat are capable of reacting with PA to form highly polar adducts that would not be detected by our assay methods and which, in fact, were not sought during our sausage analyses. A reexamination of the sausage extracts by TLC did not reveal the presence of any adducts that might have been formed between PA and CSH or GSH. However, these adducts could have been present in small amounts which would have been readily masked by the very large concentration of highly polar compounds present in the extracts. Hence, the possibility remained that PA could have been produced in the sausage but had undergone a reaction with amino acids.

Interest then shifted to the potential toxicity of such PA-amino acid reaction compounds. Because our data indicated that PA reacted most rapidly with amino acids containing a sulfhydryl moiety, we examined the toxicity of the S-alkylated adducts formed by the reaction between PA and CSH or GSH. These adducts exhibited no toxicity to the animals investigated but showed 40 to 50% (PA plus GSH) or 100% (PA plus CSH) toxicity, based on comparable quantities of PA administered, to the chick embryo. These data differ from those of Hofmann et al. (10) who found that patulin in reaction with GSH possessed no toxicity to the chick embryo. An examination of the structures of the two S-alkylated adducts (Fig. 1) indicated a potential cause of this residual toxicity and, in addition, revealed that the mechanism of the reaction involved in

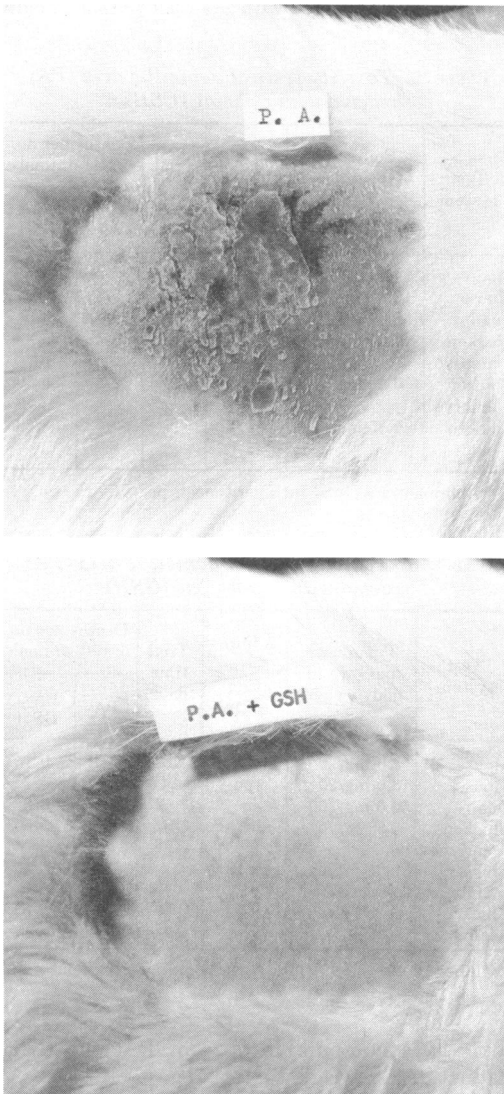


FIG. 2. Rabbit skin test illustrating nontoxicity of the penicillic acid (PA) plus glutathione (GSH) adduct.

the formation of these substances was somewhat unexpected.

Jones and Young (13) have shown that lactones possessing a double bond in conjugation with the lactone carbonyl readily undergo Michael addition of the nucleophilic thiol group in cysteine to the conjugated double bond to form S-alkylated adducts. An equally rapid attack by the cysteine thiol group resulted with those lactones that possessed an unconjugated double bond; however, addition in this case occurred at the lactone carbonyl and gave rise to S-acylated intermediates which rearranged rapidly in neutral solution to give N-acylated cysteine.

Penicillic acid possesses two double bonds, one in conjugation with the carbonyl moiety of the lactone and the second out of conjugation. On the basis of Jones and Young's data (12), nucleophilic attack by the CSH thiol group to the isolated nonconjugated double bond of PA was unexpected. However, because a 1:1 molar ratio of CSH or GSH to PA was used in our reactions, there remained in the S-alkylated adducts a highly reactive conjugated double bond and a carbonyl group capable of attack by nucleophiles. Rapidly metabolizing embryonic systems such as the chick embryo are probably more susceptible than are adult animals to perturbations of their metabolism and possibly accounts for the toxicity of the PA plus CSH adduct. Similar residual toxicity of a PA plus CSH adduct was recorded by Geiger and Conn (9) who used bacteria for their test system. Bacteria in their growth phase may be regarded as akin to the chick embryo in that both possess high metabolic rates.

The 40 to 50% toxicity of the PA plus GSH adduct can be accounted for if one assumes a spatial hindrance by the large attached GSH moiety to nucleophilic attack on the conjugated double bond of the PA fragment. These concepts are currently under investigation in our laboratory.

Based on our inability to detect production of PA on mold-fermented sausage and the rapidity with which amino acids normally found as meat constituents can react with PA to produce adducts that are nontoxic to adult animals, we tentatively and cautiously conclude that consumption of mold-fermented sausage (from the standpoint of PA presence) does not represent a potential health hazard. This conclusion is somewhat weakened by our finding that adducts of PA plus CSH or GSH retain toxicity to the chick embryo. Long-term feeding trials of these adducts to adult laboratory animals are planned to determine poten-

tial chronic toxicity and carcinogenicity.

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LITERATURE CITED

- Bentley, R., and J. G. Keil. 1962. Tetrionic acid biosynthesis in molds. II. Formation of penicillic acid in *Penicillium cyclopium*. J. Biol. Chem. **237**:867-873.
- Ciegler, A., and C. P. Kurtzman. 1970. Fluorodensitometric assay of penicillic acid. J. Chromatogr. **51**: 511-516.
- Ciegler, A., and C. P. Kurtzman. 1970. Penicillic acid production by blue-eye fungi on various agricultural commodities. Appl. Microbiol. **20**:761-764.
- Davis, N. D., U. L. Diener, and D. W. Eldridge. 1966. Production of aflatoxins B₁ and G₁ by *Aspergillus flavus* in a semisynthetic medium. Appl. Microbiol. **14**:378-380.
- Dickens, F. 1964. Carcinogenic lactones and related substances. Brit. Med. Bull. **20**:96-101.
- Dickens, F., and J. Cooke. 1965. Rates of hydrolysis and interaction with cysteine of some carcinogenic lactones and related substances. Brit. J. Cancer **19**:404-410.
- Farkas, J. 1958. Untersuchung für die Ausbildung der äusserlichen Mikroflora bei ungarischer Salami. Husipar **7**:225-230.
- Friedman, M., J. F. Cavins, and J. S. Wall. 1965. Relative nucleophilic reactivities of amino groups and mercaptide ions in addition reactions with α, β -unsaturated compounds. J. Amer. Chem. Soc. **87**:3672-3682.
- Geiger, W. B., and J. E. Conn. 1945. The mechanism of the antibiotic action of clavacin and penicillic acid. J. Amer. Chem. Soc. **67**:112-116.
- Hofmann, K., H.-J. Mintzlaff, I. Alperden, and L. Leistner. 1971. Untersuchung über die Inaktivierung des Mykotoxins Patulin durch Sulphydrylgruppen. Fleischwirtschaft **51**:1534-1536, 1539.
- Jirkovsky, M., and J. Galgoczy. 1966. Die Untersuchung der Schimmelpilzflora der ungarischen Wintersalami. Fleischwirtschaft **46**:128, 131.
- Hones, J. B., and J. M. Young. 1966. Carcinogenicity of lactones. I. The reaction of 4-methylbuteno- and 4-methylbutano- γ -lactones with primary amines. Can. J. Chem. **44**:1059-1068.
- Jones, J. B., and J. M. Young. 1968. Carcinogenicity of lactones. III. The reactions of unsaturated γ -lactones with L-cysteine. J. Med. Chem. **11**:1176-1182.
- Kurtzman, C. P., and A. Ciegler. 1970. Mycotoxin from a blue-eye mold of corn. Appl. Microbiol. **20**:204-207.
- Leistner, L., and J. C. Ayres. 1967. Schimmelpilze und Fleischwaren. Fleischwirtschaft **47**:1320-1326.
- Raper, K. B., and C. Thom. 1949. A manual of the penicillia. The Williams & Wilkins Co., Baltimore.
- Scott, P. M., J. W. Lawrence, and W. van Walbeek. 1970. Detection of mycotoxins by thin-layer chromatography: application to screening of fungal extracts. Appl. Microbiol. **20**:839-842.
- Shibata, S., S. Natori, and S. Udagawa. 1964. List of fungal products. Univ. of Tokyo Press, Tokyo.
- Tauchmann, F., L. Tóth, and L. Leistner. 1971. Eine halbquantitative Methode für die Bestimmung von Patulin in Fleisch und Fleischwaren. Fleischwirtschaft **51**:1079-1080.
- Vas, K., and G. Pulay. 1955. Note on the microflora of salami and on its controlled development. Acta Microbiol. Acad. Sci. Hung. **3**:109-115.