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Production and Biological Activity of Patulin and Citrinin from *Penicillium expansum*

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Penicillium expansum isolated from meat and apples produced both patulin and citrinin. Toxin identity was confirmed by spectroscopic and physical methods. The mean lethal dose in chicken embryos was determined for toxins administered both singly and in various ratios. Data from simultaneous administration of mycotoxin combinations plotted as isobolograms showed an additive effect. Both toxins were teratogenic in chicken embryos.

Mycotoxicoses may represent a more complex manifestation of interactions between susceptible hosts and etiological agents than was initially surmised. In addition to the physiological variables inherent to the host, more than a single toxin may be involved, resulting in potential synergistic toxicity. Additionally, a synergistic response may result from interaction of a mycotoxin with nontoxic substances, e.g., aflatoxin with cyclopropenoid fatty acids (6). The subject of mycotoxin synergism has recently been reviewed in extenso (E. B. Lillehoj and A. Ciegler, *Microbiology–1975*, p. 383-403, American Society for Microbiology, 1975). During an analysis of the mycotoxin-producing potential of molds isolated from mold-fermented sausage (2), we isolated three strains of *Penicillium expansum* capable of producing the two mycotoxins patulin and citrinin. Harwig et al. (4) also isolated strains of *P. expansum* from natural rots of apples capable of synthesizing these two toxins; however, only patulin was recovered and crystallized, citrinin being present in only trace amounts.

As part of our program on studying secondary fungal metabolites that may effect mycotoxicoses, we proved the capability of *P. expansum* to produce both patulin and citrinin and then determined the action of these two toxins upon simultaneous administration to 4-day-old chicken embryos.

TABLE 1. *Citrinin and patulin production in YES medium*[a]

Time (days)	Citrinin (mg/500 ml)	Patulin (mg/500 ml)
5	15	10
7	35	33
9	50	55
17	63	3

[a] Cultures incubated at 25°C, no agitation. YES: yeast extract, 2%; sucrose, 15%; 500 ml of medium per 2.8-liter flask.

One of the isolates, *P. expansum* NRRL 6069, was inoculated into YES broth (2% yeast extract, 15% sucrose) and incubated statically at 25°C for 14 days. Twenty-five liters of supernatant was concentrated to 1.5 liters, and the concentrate was extracted twice, each successively, with chloroform and ethyl acetate. The solvent extracts were combined and divided in half for separate recovery of the two toxins. Patulin was isolated and crystallized by the method described by Harwig et al. (4) (*m/e*, 154; mp, 109 to 110°C; literature value, 111°C). Purified citrinin was obtained by column chromatography on silica gel (elution with $CHCl_3$), extraction of the eluate with 0.2 M $NaHCO_3$, acidification of the bicarbonate solution, and several crystallizations of the precipitated yellow solids from absolute ethanol (*m/e*, 250; mp, 175 to 176°C; literature value, 175°C). Infrared spectra were obtained from films on KRS-5

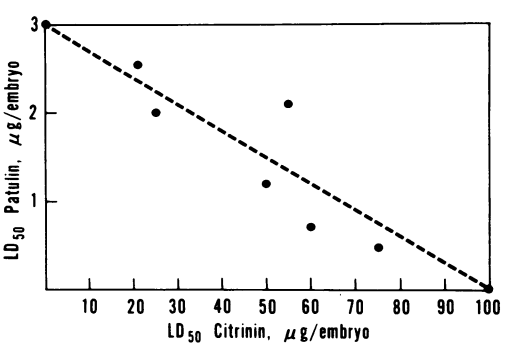

FIG. 1. *Isobologram of LD_{50} values in 4-day-old chicken embryos injected with various ratios of patulin plus citrinin.*

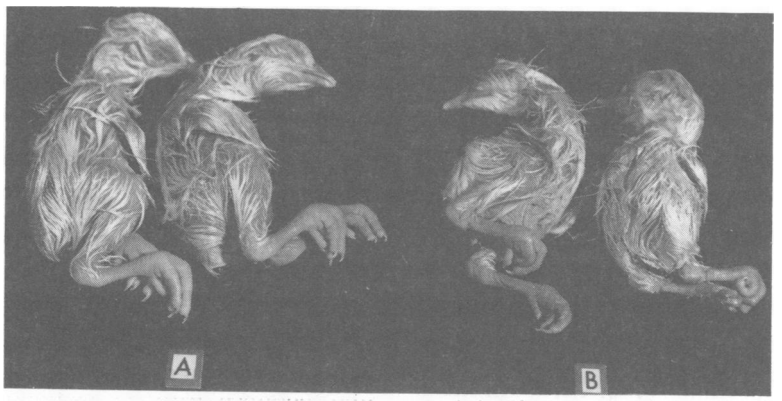

FIG. 2. *Teratogenic effects in chicken embryos from citrinin administration. (a) Control; (b) citrinin (note heads twisted to left).*

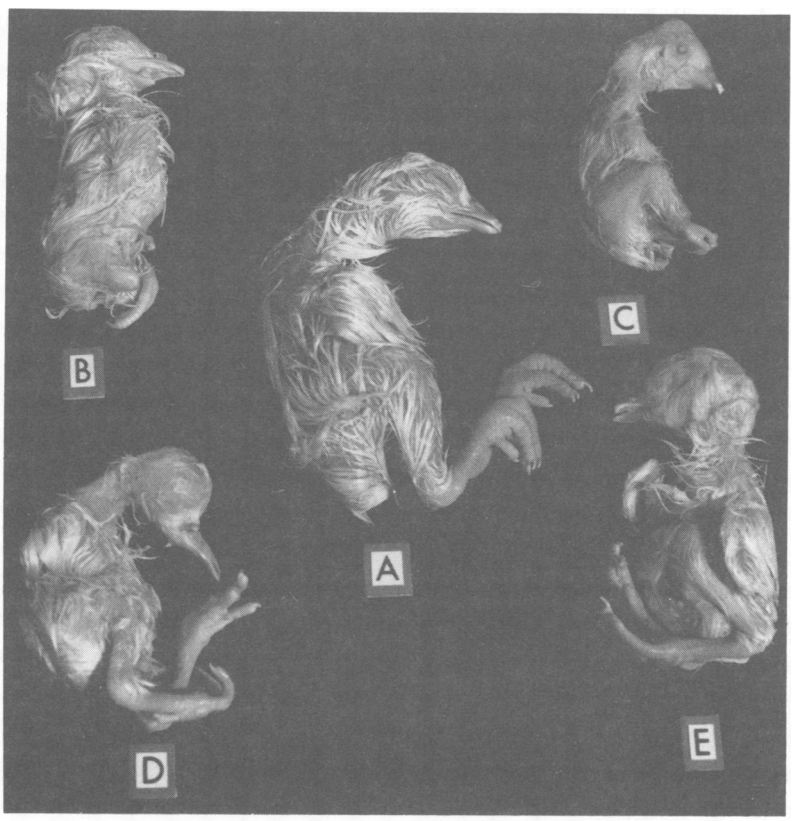

FIG. 3. *Teratogenic effects in chicken embryos from citrinin administration. (a) Control; (b–e) citrinin treated.*

plates and were superimposible on those obtained from standards.

Nuclear magnetic resonance signals for citrinin in $CDCl_3$ matched those of an authentic standard: δ 1.2 d ($C\underline{H}_3CH$), 1.35 d ($C\underline{H}_3CH$), 2.0 s ($\underline{CH}_3$—C=C), 3 q ($CH_3C\underline{H}$), 4.8 q ($\overline{CH}_3$—C—$\underline{H}$), 8.23 s (C=C$\underline{H}$), 15.11 s (C=C—O$\underline{H}$), 15.85 s (C=C—COO$\underline{H}$).

The nuclear magnetic resonance spectrum taken in $CDCl_3$ is consistent with the reported data for patulin: δ 4.5(m), 6.0(m, AB portion of an ABX system), 3.24 (d, J = 5.3 Hz) (7).

TABLE 2. *Teratogenicity in chicken embryos surviving citrinin dosing*[a]

Citrinin (μg/egg)	No. of teratogenic chicks/surviving embryos			Teratogenic (avg %)
	Expt 1	Expt 2	Expt 3	
10	1/22	1/23	2/22	6
50	3/6	10/16	4/15	46
100	6/12	7/13	3/8	48
150	4/5	5/7	2/3	73

[a] Twenty-five eggs were dosed at each toxin level.

Production of the two toxins was followed in YES medium (500 ml/2.8-liter Fernbach flask) incubated in static culture at 25°C (Table 1). Flask contents were extracted twice with ethyl acetate, the solvent extract was dried with anhydrous Na_2SO_4 and concentrated, and toxin content was determined quantitatively by thin-layer chromatography in comparison to a graded series of standards (patulin: Silica Gel 60 F_{254} [Brinkmann, Des Plaines, Ill.], benzene-acetic acid-methanol, 90:5:5; citrinin: Silica Gel G, chloroform-methanol-acetic acid, 19:10:2). Plates were observed under short-wave ultraviolet light (254 nm) for quantitation. The decrease in patulin concentration noted in Table 1 need not represent toxin degradation but may involve adduct formation with other compounds in the menstruum (1); this possibility, as well as determining the toxicity of potential adducts, is under investigation.

The mean lethal dose (LD_{50}) for patulin and for citrinin in the 4-day-old chicken embryo was determined by the computer program of Daum (3), based on three replicate experiments (five dose levels per replicate, 25 eggs per dose level): LD_{50} for citrinin—80.5 μg/egg, upper limit of 131 μg, lower limit of 54.3 μg; LD_{50} for patulin—2.4 μg/egg, upper limit of 2.8 μg, lower limit of 2.0 μg. Data obtained from simultaneous administration of varying ratios of the two mycotoxins were plotted as an isobologram according to the method of Hewlett (5). The straight dashed line represents the expected LD_{50} of toxin combinations based on a simple additive response. Data from two experiments fall along this line, indicating an additive effect from patulin-plus-citrinin dosing (Fig. 1).

Citrinin as well as patulin, which was previously noted to be teratogenic (1), caused teratogenic effects in chicken embryos, with malformations primarily in the extremities; however, exencephaly, exophthalmia, and crossed beaks were also noted, with an occasional chick showing the head and neck twisted to the left instead of the right as normally occurs (Fig. 2 and 3). A dose response relationship was noted (Table 2).

Additional strains of *P. expansum* as well as taxonomically closely related cultures of *P. crustosum*, isolated from various sources, were examined for their ability to produce both toxins. Patulin synthesis occurred with both species, but only cultures of *P. expansum* isolated from apples produced both patulin and citrinin. However, we have noted that cultures isolated from meat appear to be unstable with respect to phenotypic and genotypic characteristics, but the basis for this instability has not been investigated.

Data from the above experiments emphasize the need to determine the complete toxin-producing capabilities of cultures isolated from foods and feeds and their potential interaction before a proper evaluation can be made of the role they play in mycotoxicoses.

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