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The Rubratoxins: Causative Agents in Food/Feedborne Disease[1]

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ABSTRACT

The rubratoxins, toxic metabolites elaborated by *Penicillium rubrum* and *Penicillium purpurogenum*, have long been implicated in livestock disease. Because of this and renewed interest in these metabolites, this review was prepared. The following topics are discussed: rubratoxin occurrence; animal and microbial toxicity; morphology; isolation, identification and analysis; physical and chemical properties; new analytical methodologies; biosynthesis and toxin synthesis under controlled conditions.

Many common fungi regularly inhabit stored grains and feeds. Because of the regularity with which certain mold species, especially aspergilli and penicillia, inhabit these feeds, they are often referred to as "storage fungi." Certain of these molds, particularly *Aspergillus flavus* and *Penicillium rubrum*, are known to elaborate toxic metabolites (aflatoxins and rubratoxins, respectively) into the medium on which they are growing. Commonly, the term "mycotoxin" is applied to these toxic fungal metabolites. They may either be excreted into the medium on which they are growing or retained within the mold cells and released only with the breaking of the mycelium. Primary factors involved in mold growth and mycotoxin production are (*a*) moisture content of the substrate, (*b*) temperature, (*c*) aeration, (*d*) substrate and (*e*) genetics. Hesseltine (*27*) provided an excellent discussion and review of this subject.

OCCURRENCE OF RUBRATOXINS

As early as 1940, investigators reproduced moldy corn poisoning in horses by feeding samples of corn collected from a field case; however, these fungi were never identified. "Moldy corn toxicosis" has been defined as the poisoning of a host, manifested in various ways, following entrance into the body of toxic fungal metabolites (*22*). This disease affects both man and animals. As with other mycotoxicoses, severe outbreaks are likely to recur whenever climatic conditions become optimal for both fungal growth and biotoxin formation.

Like most other naturally occurring mycotoxicoses, moldy corn toxicosis occurs in both acute and chronic form. Clinical signs between species range widely, with the young being particularly susceptible. Under natural field conditions, in the acute form, swine develop anorexia, depression, staggering in the hind quarters and paleness of the mucous membranes, followed by death within 2 to 5 days. Body temperature and blood counts remain normal throughout, although the plasma vitamin A level frequently drops. Before death, swine exhibit salivation, alternating with periods of hyperirritability and depression (*22*).

In the chronic form (transpiring over two-thirds of the animal's lifetime), if physiologic damage is severe enough, it is considered economically correct to dispose of the animal. Pathology of this toxicosis in swine shows profuse hemorrhage in many tissues. In the acute form, a fatty degeneration and necrosis of the liver cells occur, while in the chronic form, lesions of subacute toxic hepatitis are generally observed (*22*).

TOXICITY OF RUBRATOXINS

Animal toxicity

In the Fall of 1952, swine and cattle disease(s) were widespread in the southwest United States. These outbreaks were apparently the result of moldy soft corn contaminated by certain biotoxin-producing fungi (*40*). In these outbreaks acute and chronic hepatitis were the major clinical signs. Later, Burnside et al. (*3*) isolated and screened 13 possibly toxic fungal cultures from moldy soft corn and fed swine pure mold cultures with their regular feed. The moldy soft corn, in suspension, was administered to pigs via a funnel attached to a stomach tube. Toxicological evaluations were made using mice as the test animals. Of the 13 molds isolated, only two produced toxins, *A. flavus* Link and *P. rubrum* Stoll. When the toxicity of the *P. rubrum* toxin was tested, resulting lesions were most prominent in the liver and kidney. There was also a direct correlation between the severity of the kidney lesions and the time interval from the first dose of moldy corn until death; however, this same relation was not observed with liver lesions. Liver changes were slight to moderate, indicating intoxicative hepatitis. Over a period of time, however, the liver was able to detoxify small amounts of this toxin. Here a "threshold effect" was believed to be in operation, allowing partial excretion of the toxin substance so as not

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to allow sufficient build up (increased injury) during this particular dose/time interval.

The data of Burnside and co-workers (*3*) also indicated that *P. rubrum* produced the most toxic of the mold metabolites investigated. The toxicity of the (autoclaved) mold corn substrate remained unchanged after being heated to 60-70 C for 26 h or during storage in a cool dry place for 61 days. The toxic metabolite(s) produced by *P. rubrum* Stoll was toxic to goats, swine and horses.

Wilson and Wilson (*46*) showed *P. rubrum* P-13 to be extremely toxic to dogs, rabbits, guinea-pigs and mice. The LD_{50} values for white mice were 0.2-0.4 mg intravenously (i.v.) and 0.1-0.2 mg intraperitioneally (i.p.). Dermal application to rabbits failed to induce any inflammatory reaction. Dead animals consistently showed a uniformly pale or mottled liver. The liver also showed lesions, extreme congestion, hemorrhage and cellular degeneration. This work suggests that this toxic substance may have caused both the moldy corn toxicosis in swine and cattle and the hepatitis-x in dogs (*46*).

In 1962, Wilson and Wilson (*47*) extracted and partially characterized a hepatotoxic substance from *P. rubrum*. This toxic acidic substance was extracted from cultures of *P. rubrum* P-13 grown on a corn-sucrose medium. Administration of the toxin to laboratory animals produced hepatotoxic and hemorrhagic syndromes; this strain (P-13) proved to be markedly toxic to mice. The toxic factor was water-extractable and heat-stable and possessed antibiotic properties (*5,47*). In this study, toxin was recovered as early as 5 days, although the largest quantity was present after 20 days of growth on moist cracked corn. Midway through this growth, mycelia characteristically developed a brilliant orange color with small amounts of red pigment, neither of which has been shown to be toxic (*2,6*). Initial chemical studies of the residues revealed properties of an organic acid. The extracts were only slightly soluble in water but were readily soluble in n-alcohols through octanol.

Since hepatotoxic fungi occur frequently on foods and feeds, a number of fungi were isolated from randomly selected cereal and legume products by Scott (*39*). Of the fungi isolated, five strains of *Penicillium purpurogenum* var. *rubrisclerotium* Thom were examined, but none was toxic. *P. rubrum* was also secured and tested for toxicity in Pekin ducklings. Understandably, both strains of *P. rubrum* produced toxic effects in the day-old ducklings.

Townsend et al. (*41*) showed that with sublethal doses of rubratoxin, liver tissue in mice was able to regenerate within 1 week. Similar findings have been shown for various laboratory animals. Moreover, the compounds described here were distinct from the pigments produced by *P. rubrum* and did not contribute to the toxicity of the mold.

Carlton et al. (*4*) isolated several species of penicillia from moldy corn; these cultures were incubated at either 16 or 24 C. In toxicological studies on mice, the cultures incubated at 24 C were generally more toxic. *Penicillium oxalicum* and *Penicillium viridicatum* were clinically toxic as evidenced by weight loss and death in mice. Of the various tests performed, only *P. viridicatum* toxin consistently produced gross and microscopic lesions. (*P. rubrum* was not used in this study.) Testing with *P. purpurogenum* revealed zero weight gain in one instance, and all animals in the test group showed liver lesions. It was speculated that in a few instances the liver metabolized the fungal substance(s) into a more toxic material (bioactivation) resulting in ductal destruction in laboratory animals (*4*).

Madhavikutti and Shanmugasundaram (*29*) tested *A. flavus, Aspergillus oryzae, P. rubrum,* and *P. purpurogenum* for toxicity in mice. Of the four cultures examined, only *A. flavus* and *P. rubrum* toxins were hepatotoxic; neither *A. oryzae* nor *P. purpurogenum* had any growth-retarding effect in mice. In the affected animals, degeneration of the liver cells was the most noticeable feature.

Hayes and Wilson (*24*) found that a single dose of rubratoxin B (0.67 mg/kg, i.p.) in propylene glycol caused a transient inhibition of leucine incorporation in liver protein in mice. Initially there was a 12-h suppression; this was followed by a stimulation of hepatic protein synthesis that persisted for 2 days after dosing. This effect was observed both i.p. and p.o. (by mouth), although in the latter case an 80-fold increase in dose was required to produce similar effects. The highest doses (1.67 and 2.67 mg/kg, respectively) decreased total glycogen and protein levels.

After 24 h, total liver lipids, especially triacylglycerols, were increased at the 2.67 mg/kg dose level, whereas at an intermediate dose (1.67 mg/kg) total liver lipids decreased. Other experiments indicated that the mouse is able, at least in part, to metabolize the toxic component to a less harmful product and/or eliminate the parent compound from the system within 72-96 h (*24*).

Natori et al. (*33*) isolated *P. purpurogenum* from foodstuffs and then tested the purified toxic metabolite on HeLa cells and mice. A toxic metabolite identified as rubratoxin B was isolated from this mold species. This metabolite was shown to be considerably toxic to HeLa cells and mice at levels of 32-100 μl. Here chromosomes showed destructive changes, including breakage. From culture filtrates these investigators were able to attain toxin yields as high as 3.5 g/l. In mice, a wide array of toxic signs were observed; hepatotoxic and nephrotoxic effects, however, were the most common. Most of the mice injected with doses greater than the LD_{50} died within a few hours (*33*).

Wogan and co-workers (*48*) found rubratoxin B to have the following LD_{50} values (i.p.) when dissolved in dimethyl sulfoxide (DMSO): mice 0.27 mg/kg, rats 0.35 mg/kg, guinea pigs 0.48 mg/kg, and cats 0.20 mg/kg. These and other data are depicted in Table 1. In the rat, oral administration via a stomach tube increased LD_{50} values to 400-450 mg/kg. They suggest there is

probably some toxin decomposition taking place in the stomach due to the highly acidic condition. Another factor found to influence toxicity was the particular carrier solvent used. These experiments indicated that rubratoxin B was not carcinogenic to rats.

In another experiment (*48*), rats were exposed to rubratoxin B, aflatoxin B_1, or both. While neither biotoxin alone was found to be responsible for growth suppression when rats were exposed simultaneously to both toxins, a synergistic effect was evident since nearly 50% of the animals died during the treatment period. These findings (*4a,27,30,38,* and *49*) have obvious implications with respect to food and feed contaminated by various toxigenic storage fungi. Because such multiple contaminations probably occur with some frequency, Wogan et al. suggested that such contaminations might be related to previous outbreaks of disease.

Hood et al. (*28*), investigating the effects of rubratoxin B, found that single i.p. injections, at all dose levels, significantly increased embryonic mortality. These deaths also correlated with dose levels and were day-dependent where treatment on day 8 (dose level = 0.4 mg/kg) resulted in 100% mortality. In general, rubratoxin B also decreased weights significantly with surviving fetuses averaging only one-half that of control groups. These data suggest that single sublethal doses produce retardation of growth and development, death, and congenital malformations in fetuses of mice (*28*). Moreover, the authors reported that this mycotoxin is a potent embryocide and suggested that consuming mold-contaminated feeds could result in abortion or deformed offspring in farm animals.

Watson and Hayes (*43*) reported disaggregation of liver polyribosomes into single ribosomal subunits in mice treated with rubratoxin B. Polyribosomal profiles clearly indicated progressive degradation of mouse liver polysomes at 1.0 mg of rubratoxin B/kg; test animals treated with 2.0 mg of rubratoxin B/kg did not survive. This ribrosomal disaggregation indicates a possible basis for altered protein synthesis in mice. The effect of the toxin at sublethal doses appeared to be reversible since no effect was observed at 48 h. Rubratoxin B also caused a shift in ultra-violet absorption spectra of DNA and RNA in vitro, showing that this toxin binds nucleic acids (*43*).

In 1975, Davis et al. (*8*) reported on a screening program in which fungi were isolated from a variety of human foods. These isolates were identified and tested for toxicity to brine shrimp (primary bioassay) and chicken embryos (secondary bioassay). Penicillia were the most frequently isolated fungi. Of the 17 *Penicillium* spp. isolated, 41% were found to be toxic, and these data are representative of other investigations; Penicillia are the most prevalent toxigenic fungi found in food and feed stuffs, probably because of their ability to grow at low temperatures in high moisture foods.

Microorganism toxicity

Hayes and Wyatt (*25*) tested 133 microorganisms to rubratoxin B sensitivity. *Tetrahymena pyriformis* and *Volvox aureus* were the most sensitive, being inhibited at 25 and 50 μg/ml, respectively. In that study rubratoxin B had no effect at 100 and 1000 μg of toxin/disc on algae, fungi or gram-negative bacteria. Four species of bacilli (including *Bacillus subtilis*), two species of micrococci, and *Staphylococcus aureus* were inhibited by 1000 μg of toxin/disc.

Unlike aflatoxins, which appear to be toxic to a wide range of biological systems, the toxicity of the rubratoxins seems to be better defined; they appear to be significantly toxic toward a number of higher organisms but relatively nontoxic toward many microorganisms. For example, rubratoxin B strongly inhibited germination of *Lepidium sativum* seeds, even more so than aflatoxin B_1 (*35a,51*), but it did not influence growth of *Chlorella*. Furthermore, when certain fungi were exposed to concentrations of 100 μg/disc, sporulation was

TABLE 1. *Lethal potency of rubratoxin B in several species*[a].

Species	Sex	Weight	No. of animals	Dosing route	Vehicle	LD_{50} (mg/kg)
Rat	M	58 g	60	ip	PG[b]	0.36 (0.27-0.49)[c]
Rat	F	60 g	50	ip	PG	0.36 (0.28-0.46)
Rat	F	59 g	70	ip	DMSO[d]	0.35 (0.28-0.45)
Rat	M	60 g	25	po[e]	DMSO	ca. 400
Rat	F	58 g	25	po	DMSO	ca. 450
Mouse	F	25 g	25	ip	DMSO	0.27 (0.22-0.34)
Mouse	F	25 g	30	ip	PG	2.6 (2.0 -3.1)
Guinea pig	M	565 g	18	ip	DMSO	0.48 (0.41-0.56)
Cat	M	3 kg	3	ip	DMSO	ca. 0.2
Cat	M, F	3 kg	8	ip	PG	1.0-1.5
Dog	M	3 kg	7	ip	PG	> 5.0
Chicken	M	500 g	6	ip	PG	> 4.0

[a]Wogan et al. (*48*).
[b]Propylene glycol.
[c]95% confidence interval; calculated by the method of Litchfield and Wilcoxon.
[d]Dimethyl sulfoxide.
[e]By stomach tube.

inhibited. In particular, this toxin severely damaged the vegetative hyphae of *Aspergillus niger*, *A. flavus*, and *Rhizopus nigricans*, where hyphal tips were often ruptured, swollen and greatly septate, or transformed into giant cells. It was assumed that this toxic metabolite interfered with cell wall synthesis. On the other hand, concentrations up to 10 μg/ml enhanced the fermentative activity of *Saccharomyces cerevisiae*.

MORPHOLOGY OF *PENICILLIUM RUBRUM*

In 1930, *P. rubrum* Stoll, species NRRL 1062, was isolated from "mildewed" currency (*35*), and later from cotton duck exposed in Panama. Colonies on Czapek's agar attained a diameter of 1-2 cm in 12 to 14 days at room temperature. The colonies were generally velvety, with colored aerial hyphae ranging from yellow to orange or red-purple. Massed conidial structures from deep yellow to green-gray were produced; interestingly, the reverse sides of the plates were generally deep cherry red or purplish. Colonies on steep agar grew somewhat faster, attaining a diameter of 3.0 cm in 2 weeks, and when grown on malt extract, a diameter of 6-6.5 cm was reached within 14 days.

P. rubrum and *P. purpurogenum*, along with a few other penicillia, are grouped in the *P. purpurogenum* series of the biverticillata-symmetrica by Raper and Thom (*35*). *P. rubrum* is distinguished from *P. purpurogenum* primarily by its smooth-walled conidia, but some individual strains are intermediate between the two species. Raper and Thom suggest they be regarded as representing different aspects of the same species. While both organisms are widely distributed in nature (*7*), they are easily isolated from soil or various organic materials (*45*). The species is readily identified by the reddish pigmentation it produces or by a fragrant aromatic odor (apple or walnut-like) when cultured on malt agar.

ISOLATION, IDENTIFICATION AND ANALYSIS OF THE RUBRATOXINS

P. rubrum M.R. 043 grown on Raulin-Thom medium, enriched with 2.5% malt extract (ME), for 11 to 13 days produced a complex of toxins that were isolated in good yields (*41*). Two distinct, highly toxic components were isolated from the crude toxin. It was suggested that the more easily obtainable toxin be referred to as rubratoxin A, while the second and more toxic component be called rubratoxin B.

Hayes and Wilson (*23*) found that large quantities of crystalline rubratoxin B could be produced for chemical and biological analyses by growing *P. rubrum* P-13 in Mosseray's simplified Raulin solution, but only when malt extract (ME) was provided. While ME contains one or more ingredients essential for rubratoxin biosynthesis, the essential component(s) was not supplied by either yeast extract or corn steep. These investigators (*23*) found that cultures could be maintained several months on moistened corn without detectable loss of ability to produce rubratoxin. The pH of the medium decreased, from an initial 6.7 to about 3.8 by the third day, and remained fairly constant throughout the incubation period. Around the seventh day, orange-red pigmentation correlated well with the first detection of toxin, and sporulation commenced after 25 to 30 days of incubation. Although the toxin concentration decreased during this period, toxin was not found in cultures where mycelia remained white with occasional green colored areas, nor was toxin obtained in shake cultures or in fermentation chambers with restricted aeration (*23*). Growth and toxin production are graphically illustrated in Fig. 1.

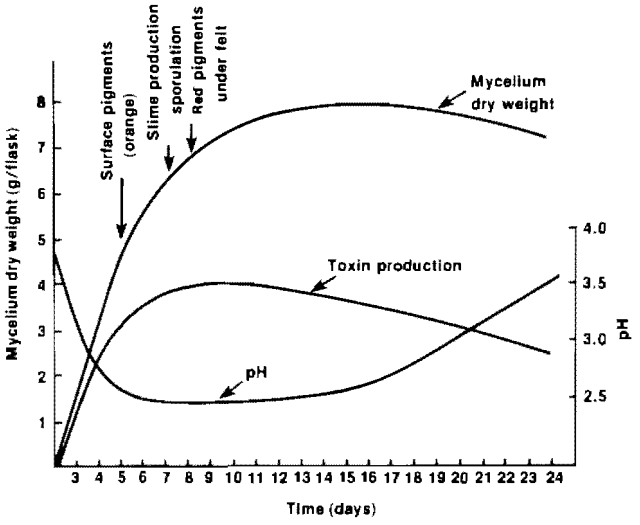

Figure 1. *Growth and toxin production by* P. rubrum *growing in stationary culture on Raulin-Thom medium enriched with 2.5% malt extract. Moss (32).*

Emeh and Marth (*13*) describe the extraction and purification of the rubratoxins (Figure 2). Extracts were screened for rubratoxins by thin layer chromatography (TLC). Most rubratoxin B was recovered when the different media were extracted successively with diethylether, ethylacetate-benzene, and diethylether. Ethylacetate and acetonitrile were considered poor extracting solvents. Although diethylether is a satisfactory extraction solvent, it carries pigments through the analysis causing interferences which may reduce toxin yields. Large amounts of rubratoxins A and B were obtained using a combination of preparative thin layer and column chromatography. Here, a pH of 1.5 was optimum for rubratoxin recovery.

Moss and Hill (*31*) studied a number of *P. rubrum* isolates for their ability to produce toxic metabolites. Certain strains produced large quantities of rubratoxin A; other strains produced mainly rubratoxin B, and some of the isolates did not produce any toxic metabolites. Since absolute R_f values proved to be sensitive to a number of factors when TLC was used to isolate the toxins, the authors suggested that markers of the pure compound always be run with materials to be analyzed. This is in agreement with the findings of other

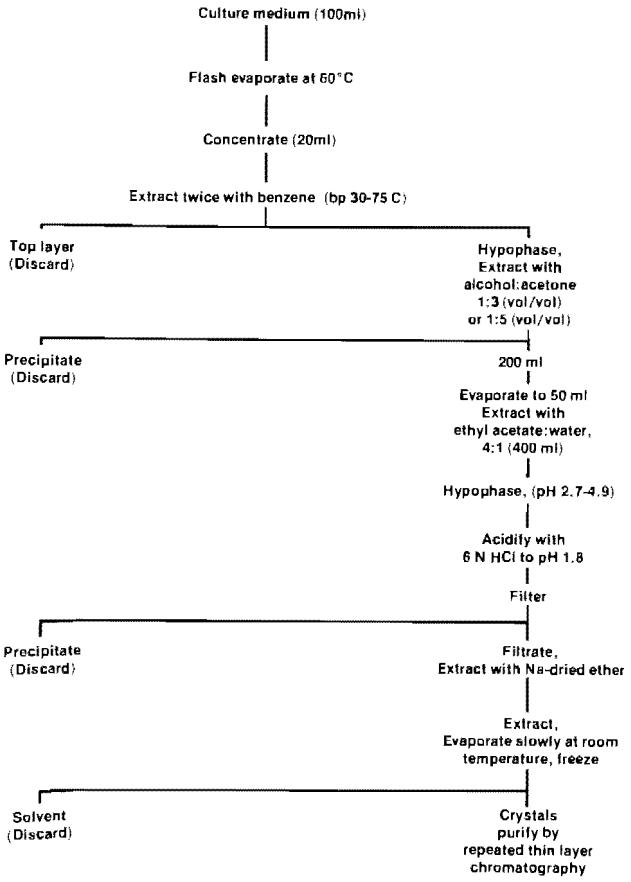

Figure 2. *Procedure for extraction of rubratoxin A and B from broth cultures of* P. rubrum. *Emeh and Marth (13).*

authors involved in isolating and purifying the rubratoxins.

Moss (*32*) and Hayes (*26*) provide excellent reviews of the rubratoxins, including methods of isolation and detailed discussion of their physical and chemical properties.

PHYSICAL AND CHEMICAL PROPERTIES

Investigations by Moss on the structure of the rubratoxins indicated that only carbon, hydrogen and oxygen were present. In 1969, Moss et al. (*30*) elucidated the structure of rubratoxin A, a minor metabolite of *P. rubrum*. NMR spectral data showed that the two rubratoxins had common features (Fig. 3). Rubratoxin A differs from rubratoxin B by the addition of two hydrogen atoms to the former compound.

Pure rubratoxins are only slightly soluble in water. They are fairly soluble in alcohols and esters and extremely soluble in acetone; however, they are completely insoluble in nonpolar solvents. The two are also distinguishable by their solubility in ethanol; rubratoxin A is significantly more soluble. Both decompose on melting, and it has been shown, by means of mass spectroscopy, that pyrolytic decomposition of rubratoxin B results in the loss of one mole of carbon dioxide. Heating rubratoxin A in 2 N sodium hydroxide resulted in formation of a mixture of volatile aldehydes,

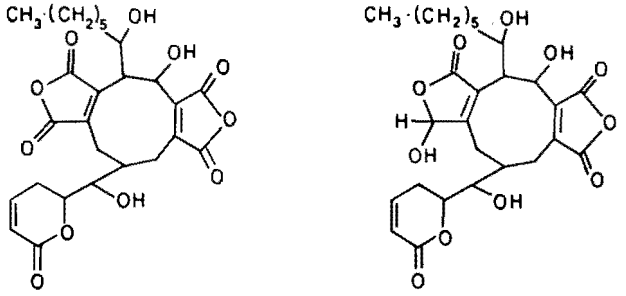

Figure 3. *The rubratoxins. Moss (32). (B, left; A, right).*

including heptaldehyde. Their physical and chemical properties are compared in Table 2.

ANALYTICAL PROPERTIES AND METHODOLOGIES

Wyllie and Morhouse (*50*) indicated that a colorimetric assay had been developed for rubratoxin A but not rubratoxin B. Rubratoxin A heated in sodium hydroxide gives a yellow color, whereas rubratoxin B under the same conditions does not change color. An alternative assay, however, estimating rubratoxin B in corn and using a fluorescent derivative has been developed (*39a,49*). The detection limit in corn was 70-100 ppm. These same authors reported that a specific bioassay system that would distinguish the rubratoxins from other hepatotoxicants has not been described in the literature. More recently, however, several high performance liquid chromatography (HPLC) techniques have been developed (*21,42*).

TABLE 2. *Comparison of some properties of rubratoxins.*[a]

Property	Rubratoxin A	Rubratoxin B
Formula	$C_{26}H_{32}O_{11}$	$C_{26}H_{30}O_{11}$
Molecular weight	520	518
Melting point	210-214 C	168-170 C
a_D^{20} (= acetone)	+ 84°	+ 67°
λmax (CH_3CN)	252 nm (4430)	251 nm (9700)
LD_{50} i.p. (propyleneglycol in mice)	6.6 mg/kg	3.0 mg/kg

[a]Moss (*32*).

Engstrom (*21*) developed an HPLC procedure to resolve a mixture of seven mycotoxins, including rubratoxin B. A reversed phase μBondapak/C18 column, a ternary mobile phase (acetonitrile/water/ethylacetate; 55/45/2:v/v/v), and a flow rate of 1.0 m./min was used to resolve these mycotoxins. Rubratoxin B was resolved in 7 min with detection limits as low as 5 ng. Using a μBondapak C18 column and a ternary solvent system, Unger and Hayes (*42*) developed an HPLC method to resolve and quantify rubratoxins A and B. Results were highly reproducible for both toxins and sensitivity was at the nanogram level. A typical chromatogram is shown in Fig. 4.

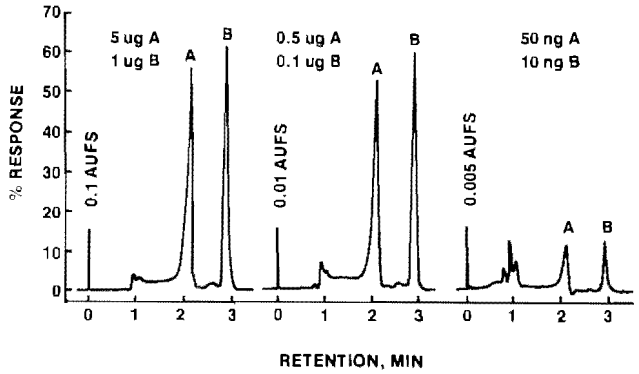

Figure 4. *HPLC resolution of rubratoxins A and B on μBondapak C_{18}; elution solvent, acetonitrile-water-ethyl acetate (11:9.9:3); flow rate, 1.7 ml/min. Unger and Hayes (42).*

Neely et al. (*34*) reported on the analytically useful spectral properties of rubratoxin B. Included are corrected fluorescence excitation and emission spectra, calculated fluorescence life time and quantum yield, useful concentration ranges for ultraviolet absorption measurements and phosphorescence, which was not observed. More recently, Whidden (*44*) developed a procedure to screen for rubratoxin B and other mycotoxins in corn; three new confirmatory tests for rubratoxin B are described. In this report, rubratoxin B was detected and quantitated in corn spiked with 10 mg of toxin/kg of corn.

BIOSYNTHESIS

In general, because of their highly complex biochemical characteristics, fungi are capable of synthesizing a multitude of chemical compounds under various conditions. These metabolites are quite often highly complex chemically, and often they are structurally unrelated with diverse physiological properties. Moss and Hill (*31*) suggested that the time of production and the relatively large toxin yield may indicate that the production of these metabolites (the rubratoxins) could be related to reactions essential to growth of the organism.

There is a direct biochemical relationship between the chemical structures of the rubratoxins and the nonadrides, glauconic and glaucanic acids, produced by *P. purpurogenum*. Baldwin et al. (*1*) described, in great detail, the constitutions of these two acids, which like certain other fungal metabolites are derived from acetate or its equivalent malonate. Byssochlamic acid produced by *Byssochlamis fulva* is also said to be involved in this relationship. According to Moss (*32*), rubratoxin B could be a substituted higher homolog of byssochlamic acid.

It has been postulated and now generally accepted that the rubratoxins (C26 compounds) are produced by the condensation of two C13 moieties derived from the coupling of decanoic and oxaloacetic acid, with a resulting decarboxylation (*20,26,32*). The two C13 units are believed to be the result of head-to-tail, head-to-tail coupling. But it is not clear at what point(s) in biosynthesis the oxygen is incorporated.

According to Moss (*32*), the carboxyl group of the *a,β*-unsaturated *δ*-lactone probably originates from the methyl carbon of acetate. Moss also reported the presence of a disubstituted maleic anhydride, and an *a,β*-unsaturated *δ*-lactone in rubratoxin B. Patulin, a fungal toxin produced by several species of the storage fungi, also has this *a,β*-unsaturated lactone moiety. Many investigators believe that this part of the molecule is definitely related to the toxicity, at least in part, since saturating or removing the ring reduces the toxicity considerably. These compounds are distinct from the nontoxic colorful pigments produced by these fungi (*2,6*).

Roberts and Thompson (*36*) correctly established the molecular formula of purporogenone ($C_{29}H_{20}O_{11}$), the primary red pigment produced by *P. purpurogenum*. They suggest that this metabolite was also of acetate-malonate origin. In another paper, Roberts and Thompson (*37*) described a secondary coloring material, deoxypurpurogenone, isolated from *P. purpurogenum* by protracted chromatography. This pigment had nearly the same U.V. and I.R. spectra as purpurogenone.

Studying the synthesis of rubratoxin and total lipid, as measured by $2^{14}C$ acetate incorporation, Emeh and Marth (*18*) found that maximum toxin label incorporation occurred at 72 h, while lipid synthesis increased throughout the incubation period. The occurrence of yellow-orange pigmentation coincided with a decline in labeled acetate incorporation. In another paper (*20*), incorporation of labeled small molecules into rubratoxin was studied. An overall, low incorporation efficiency was reported. The authors suggested that this may have resulted from incorporation of the labeled precursors into lipid. Looking at the relationship between primary metabolism and rubratoxin biosynthesis (*15*), both protein and RNA were synthesized rapidly during 108 h of incubation. Synthesis decreased rapidly thereafter. The close parallel between maximum protein synthesis (66 h) and maximum toxin formation (72 h) was discussed.

FACTORS AFFECTING RUBRATOXIN FORMATION UNDER CONTROLLED CONDITIONS

Investigating cultural and nutritional factors controlling rubratoxin formation (*10*), it was found that maximum mold growth and toxin production were favored by 20% glucose in the medium. The greatest yields appeared after 6 days for rubratoxin A, and 12 days for rubratoxin B. Both toxins disappeared from the cultures after the third week. Rubratoxin degradation, as affected by nature of the culture, length of incubation and type of rubratoxin, was discussed in another paper by these authors (*19*).

In a series of articles (*12,14,16*), Emeh and Marth described the nutrient requirements for rubratoxin formation. When the medium was fortified with organic acids and intermediates of the TCA cycle, the TCA intermediates (except malonate and succinate) caused a

reduction in accumulation of dry weight and toxin formation. Acetyl CoA (10^{-5}M/flask) caused an 80% increase in toxin yield (*12*). The presence of sucrose, glucose, fructose, maltose, galactose and starch resulted in good yields of rubratoxin B, with maximum fungal growth and toxin production occurring when the broth contained 20% sucrose. Addition of asparagine and aspartic acid enhanced both toxin formation and mold growth, whereas oleic acid did not support growth or toxin production (*14*). Interestingly, single strains of the mold (P-13, P-3290) produced more toxin than did mixed cultures.

Nutritional supplements, including potassium sulfite and sodium metabisulfite, caused moderate reductions in toxin formation, while sodium acetate enhanced rubratoxin production. Sodium arsenite and iodoacetate, at test concentrations, blocked mold growth and toxin formation, whereas sodium azide and 2,4-dinitrophenol caused a reduction in mold growth while inhibiting toxin formation completely (*16*).

With the increasing importance of soya-based foods, a study was made of rubratoxin production in a soy whey-malt medium (*9*). Six *P. rubrum* strains were evaluated for toxin production. Rubratoxin was not produced in shake cultures but when cultured quiescently, both rubratoxins were synthesized, except one strain (P-13) which only produced rubratoxin B. Malt extract stimulated toxin formation, with a 2.5% addition favoring maximum rubratoxin production.

In another study (*11*), several sterile soybean substrates were investigated for rubratoxin formation on natural and synthetic media. While ME and glucose favored toxin production, neither unsupplemented soy-whey nor soy-milk supported rubratoxin formation. Investigating rubratoxin formation on various natural substrates (coconut, soybean, rice and corn), Emeh and Marth (*9*) found that toxin yield was dependent on the strain of mold and the substrate used. Rice and corn supported maximum mold growth, with the cultures grown on corn favoring toxin formation. Rubratoxin A production ranged from 25-33% that of rubratoxin B.

SUMMARY

The rubratoxins, along with a host of storage fungi, should be of concern in feed production, since molds have long been suspected of causing livestock disease. Available information also indicates that many toxic metabolites produced by molds are relatively stable in storage; therefore, one should take necessary measures to inhibit mold growth in stored grains (animal feed and human food). Large numbers of molds in feeds, however, do not regularly or necessarily indicate biotoxin activity. It should be emphasized also that many complex interactions, possibly toxin potentiating, may be taking place in these stored products.

This possibility, coupled with the widespread occurrence of hepatotoxic fungi on agricultural products and little if any idea of how much is there--let alone the amount we are ingesting--should certainly warrant concern. For further information on mycotoxins, a definitive trilogy has been prepared by Wyllie and Morehouse (*49-51*).

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