Production of Rubratoxin B by *Penicillium purpurogenum* Stoll

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Culture filtrates and chloroform extracts from *Penicillium purpurogenum* isolated from foodstuffs were subjected to preliminary survey for toxic effects on HeLa cells and mice. The toxic metabolite was isolated in a crystalline form from *P. purpurogenum* and was identified as rubratoxin B. Pathological findings observed in HeLa cells and mice treated with the metabolite are briefly described.

In view of the increasing significance of naturally occurring carcinogens, especially as related to food contaminants, we have conducted a study for the detection and isolation of mycotoxin-producing fungi from the foodstuffs (14). A parallel epidemiological investigation has been in progress with regard to the regional characteristics of cancer mortality in Japan. During the course of the extensive screening of molds, several fungi were noticed to have toxic properties. Among them, *Penicillium purpurogenum* and allied species were frequently isolated from polished rice, wheat, various kinds of cereal products, and miso (fermented paste of soybeans) (14). Of these isolates, one isolate of *P. purpurogenum* has been shown to be considerably toxic to HeLa cells and mice.

Early work on moldy-corrn poisoning of swine in the United States was conducted by Burnside et al. (4), who found *P. rubrum* to produce a mycotoxin in feed. Shortly after this study, Forgacs et al. (6) fed grains infected with *P. rubrum* and *P. purpurogenum* to chicks and observed a hemorrhagic syndrome. In 1962, Wilson and Wilson (17) reported preliminary results of chemical and pathological studies on the toxic metabolite isolated from *P. rubrum*. Townsend et al. (16) carried out further chemical studies of the culture broth of *P. rubrum* (MR-043) and postmortem examinations on infected mice, guinea pigs, and dogs and subsequently isolated two toxic metabolites, designated rubratoxin A and B. As a result of extensive chemical studies, Moss et al. (9-12) proposed the structures I and II for rubratoxin A and B, respectively, as nonadrides with anhydride and lactone rings (Fig. 1). Hayes and Wilson (7) reported the bioproduction of rubratoxin B and pointed out that the highest yield of rubratoxin B was obtained by using stationary cultures of the fungus on Mosseray's simplified Raulin solution enriched with 2.5% malt extract. Carlton et al. (5) investigated toxic effects in mice fed certain species of molds belonging to *Penicillium*. They discovered marked liver lesions in mice fed with the *P. purpurogenum* diet, but chemical study of its toxic metabolite was not carried out.

**MATERIALS AND METHODS**

Culture and culturing conditions. *P. purpurogenum* NHL-6124 was isolated from wheat grain which was collected at Minamikushiyama Village, Nagasaki Prefecture, Japan, August 1967. The fungus was grown in 900-ml Roux flasks containing 200 ml of modified Czapek-Dox medium [glucose (50.0 g), NaNO$_3$ (2.0 g), KH$_2$PO$_4$ (1.0 g), MgSO$_4$·7H$_2$O (0.5 g), KCl (0.5 g), FeSO$_4$·7H$_2$O (0.001 g), ZnSO$_4$·7H$_2$O (0.01 g), CuSO$_4$·5H$_2$O (0.005 g), malt extract (Difco; 0.5 g), yeast extract (0.2 g), deionized water (1 liter)]. The inoculated flasks were incubated at 25 C for 3 weeks under stationary conditions. The growth in each flask was removed by filtration and later extracted. The filtrates were initially screened for toxins.

**Isolation and identification of the toxic metabolite.** The culture filtrate was treated as shown in Fig. 2. Melting points were determined in a Yanagimoto melting point apparatus and are not corrected; the ultraviolet spectra were taken on a Hitachi EPU-2A spectrophotometer; the infrared (IR) spectra were measured on a Nihon Bunko DS-301 spectrophotometer; the nuclear magnetic resonance (NMR) spectra were run on a JEOL JNM-6-60 HL spectrometer by using tetramethylsilane as the internal standard.

**Tissue culture experiment.** A modification of the plastic panel technique by Toplin (15) using HeLa cells was employed for toxicity bioassay of the fungal culture filtrate, the separated fractions, and rubra-
toxin B at various concentrations. The fractions, or rubratoxin B, were dissolved in dimethyl sulfoxide and diluted in the medium. The cells in experimental media were grown on round cover glasses placed in each cup of plastic panels (Disposo-tray, FB-54) for 3 days, fixed with Carnoy’s fixative, and stained with hematoxylin and eosin for toxicity assay and cytomorphological examination. For the observation of chromosomal changes, cells exposed to the filtrate or rubratoxin B were hypotonically treated, fixed with acetic acid and methanol mixture, spread on slides, and stained with Giemsa.

Animal experiment. The male mice of DDD strain (Institute of Medical Science, Tokyo, Japan) were used throughout the experiment (Table 1). For the purpose of screening the toxicity, mice, in groups of three, were injected intraperitoneally with 0.4, 0.2, and 0.1 ml/20 g of body weight of the culture filtrate diluted with distilled water by four times. Suspensions of rubratoxin B were prepared at concentrations ranging from 20 to 200 μg/0.1 ml in lecithin-water solution. In the first experiment, mice, in groups of two, were injected subcutaneously with 30, 50, 80, 130, and 200 μg of rubratoxin B per 10 g of body weight. In the second experiment, mice, in groups of six, were injected with 42, 50, 60, 72, 87, and 100 μg of rubratoxin B per 10 g. The control animals received solvent alone.

![Chemical structure of rubratoxin A and B](image)

FIG. 1. Structures of rubratoxin A and B.

| TABLE 1. Acute toxicity of the culture filtrate of Penicillium purpurogenum and rubratoxin B on DDD male mice |
|---------------------------------------------------------------|
| Culture filtrate<sup>a</sup> | Mortality | Rubratoxin B<sup>b</sup> | Mortality |
|-----------------------------|-----------|-------------------------|-----------|
| 0.4                         | 3/3       | 200                     | 2/2       |
| 0.2                         | 1/3       | 130                     | 2/2       |
| 0.1                         | 1/3       | 80                      | 1/2       |
|                             |           | 50                      | 2/2       |
|                             |           | 30                      | 0/2       |
|                             |           | 100                     | 3/3       |
|                             |           | 87                      | 3/6       |
|                             |           | 72                      | 4/6       |
|                             |           | 60                      | 1/6       |
|                             |           | 50                      | 2/6       |
|                             |           | 42                      | 1/6       |

<sup>a</sup> Diluted by four times. Values expressed in milliliters per 20 g of body weight; intraperitoneal injection.

<sup>b</sup> Values expressed in micrograms per 10 g; subcutaneous injection.

RESULTS AND DISCUSSION

Cytotoxic effects on HeLa cells. The culture filtrate of P. purpurogenum NHL-6124 at 3.2% concentration affected HeLa cells lethally. Morphological examination at this concentration revealed an increase of abnormal mitotic cells and the appearance of polynuclear cells. Mitotic cells were composed of short chromosomes dispersed in the whole cytoplasm. Chromosome preparations showed degenerative changes of treated cells, including chromosome breaks or shortening or complete destruction of the chromosomes.

Toxicity bioassay by plastic panel technique was useful for monitoring fractions containing toxic metabolites. Isolated rubratoxin B was subjected to further study and proved to produce cytological changes at 32 to 100 μg/ml, consistent with those of the filtrate. In this condition, the chromosomes showed destructive changes including breaks, rod-shaped chromosomes, or completely degenerated round particles.

Identification of the toxic metabolite. The culture filtrate of the fungus was examined by the separation procedure shown in Fig. 2. From the ether extract, a large amount of colorless crystals was separated. Each fraction was checked by the toxicity bioassay, and the crystals obtained from the ether extract proved to be the main toxic metabolite. The mycelium of the strain was also extracted successively with hexane, benzene, ether, chloroform, and methanol, but none of the extracts showed toxicity. Mannitol was isolated from the methanol extract and identified by melting point and IR spectrum.

The toxic crystalline substances thus obtained
were combined and further purified by recrystallization from acetone or acetone-hexane to colorless needles of melting point 185–186°C (decomposition, $[\alpha]_D + 68.30$ (c = 2.0, acetone). The metabolite showed the following spectral data: IR maxima in Nujol were 3,550, 1,860, 1,821, 1,784, 1,761, 1,719, 1,693, 1,255, 926, 719 cm$^{-1}$; IR maxima in KBr were 3,300–3,550, 1,842, 1,822, 1,768, 1,701, 1,690, 1,258, 948, 740 cm$^{-1}$; UV maxima in ethyl alcohol were 207, 249 nm ($\log \varepsilon 4.15, 3.89$); and maxima in CH$_3$CN 204, 251 nm ($\log \varepsilon 4.13, 3.88$); NMR (Fig. 3). IR and UV spectra indicated the presence of acid anhydride group(s) similar to those in gluconic acid isolated from the same fungus (2, 3; G. Ferguson, G. A. Sim, and J. M. Robertson, Proc. Chem. Soc., 1962, p. 385) and an $\alpha,\beta$-unsaturated lactone. IR and NMR spectra also showed the presence of hydroxyl groups and of an alkyl (probably hexyl) side chain. At this stage of the work, the structures of rubratoxin A and B (10, 11), isolated from P. rubrum, were proposed by the British workers as shown in Fig. 1. All the physical data of our toxin agree well with those of rubratoxin B. The direct comparison with the authentic sample of rubratoxin B, kindly supplied by M. O. Moss, University of Surrey, Surrey, England, showed identity by thin-layer chromatography (TLC) [$R_F 0.43$ on a Silica Gel G plate treated with 3% oxalic acid; solvent, benzene-ethyl acetate (1:5)], IR, and a mixed fusion. These results indicate that the main biologically active metabolite of the fungus is rubratoxin B.

Although rubratoxin A has not been obtained in a crystalline state, TLC of the crude deposits clearly shows contamination by a compound having the same $R_F$ value as rubratoxin A. It might be worthwhile to mention that the overall yield of rubratoxin B from the filtrate is as high as 3.5 g from 1 liter of the medium under the conditions employed.

**Histological findings.** The lethal effect of the culture filtrate and rubratoxin B is summarized in Table 1. The subcutaneous $LD_{50}$ of rubratoxin B was estimated as 6.8 mg/kg in DDD mice. Histological findings of lesions in organs of mice
treated with culture filtrate as well as rubratoxin B showed a great variety of toxic effects, which can be summarized as combinations of hepatotoxic and nephrotoxic effects and also as mitosis disorders as described below.

Most of the mice injected with doses above the LD₅₀, i.e., 20 and 13 mg of rubratoxin B per kg, died within several hours. In these cases the liver was markedly congested with subcapsular hemorrhage. In some the duodenum was dilated and the kidneys were slightly swollen and anemic. Histologically, portal and central veins of the liver were engorged and sinusoids were dilated. Irregular areas of coagulation necrosis suggesting infarctions were located in the intermediary and periportal regions of the lobules. The kidneys showed mild change: a few hyaline casts in the dilated distal convoluted tubules with mild vacuolic degeneration. In the ileum, mucosa necrosis of a few crypt cells was seen.

With lower doses, the course to death was longer, 23 to 48 hr. The liver was enlarged and mottled in appearance. In a few mice, the duodenum was dilated with fluid content. Histologically, the liver cells showed acidophilic necrosis, vacuolic degeneration, and ballooning of the cytoplasm scattering in the intermediary as well as peripheral zones of the lobules. Occasionally, liver cell nuclei with irregular shape and pyknotic change were found in the lobules. Bile ductules and capillaries were invariably dilated. In the kidneys, the tubular epithelial cells of the proximal convoluted tubules showed hydropic degeneration and occasionally coagulation necrosis with desquamation. These changes were more severe in the distal part of the convoluted tubules.

Mitotic arrest and subsequent necrotic changes of the actively dividing cells were observed in the gastrointestinal tract, thymus, lymph nodes, spleen, and bone marrow. In the intestinal mucosa, a few crypt cells were necrotic and desquamated in the glandular lumen. The lesions were similar to those observed with HeLa cells in culture. The brains of the mice administered well above the lethal dose of the toxin revealed hydropic degeneration of the nerve cells.

This is the first report concerning the production of rubratoxin B in P. purpureogenum, although the toxin has previously been found in P. rubrum in the United States and Britain. Up to the present, only one strain of P. purpureogenum has been recognized as a rubratoxin-producing strain.

During the last 3 years, fungi which are capable of producing aflatoxins and ochratoxins were isolated from certain foodstuffs in Japan from our mycotoxicological studies (8; S. Natori et al., Annu. Meeting Pharm. Soc. Jap. (Nagoya), 1969, p. 500). These findings and our discovery of a fungus producing rubratoxin further indicate the importance of the mycotoxin problem in the field of food hygiene in Japan. In addition to these facts, our histological study on toxicity of rubratoxin B in mice revealed the characteristic polyfunctional effects of the metabolite. Hepatotoxic and nephrotoxic damage was observed by Burnside et al. (4), Forgacs et al. (6), Wilson and Wilson (17), and Carlton et al. (5) by use of contaminated cereals or crude toxins. However, the cytotoxic effects of the toxin on the actively dividing cells in mice as well as HeLa cells have not been reported before. A detailed investigation...
should be conducted on the effect of rubratoxin B on chromosomes of proliferating HeLa cells. In view of the remarkable toxic effects of this metabolite, further studies on chronic toxicity in animals and its mechanism of action are now being carried out in our laboratory. Additional work is also in progress on the possibility of rubratoxin contamination in Japanese foodstuffs, especially fermented foods.

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