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.

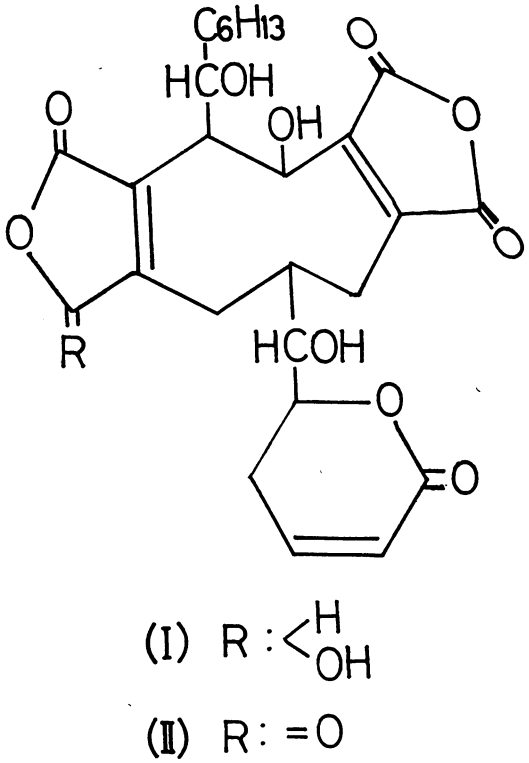

FIG. 1. *Structures of rubratoxin A and B.*

TABLE 1. *Acute toxicity of the culture filtrate of Penicillium purpurogenum and rubratoxin B on DDD male mice*

Culture filtrate[a]	Mortality	Rubratoxin B[b]	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

[a] Diluted by four times. Values expressed in milliliters per 20 g of body weight; intraperitoneal injection.

[b] Values expressed in micrograms per 10 g; subcutaneous injection.

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.

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

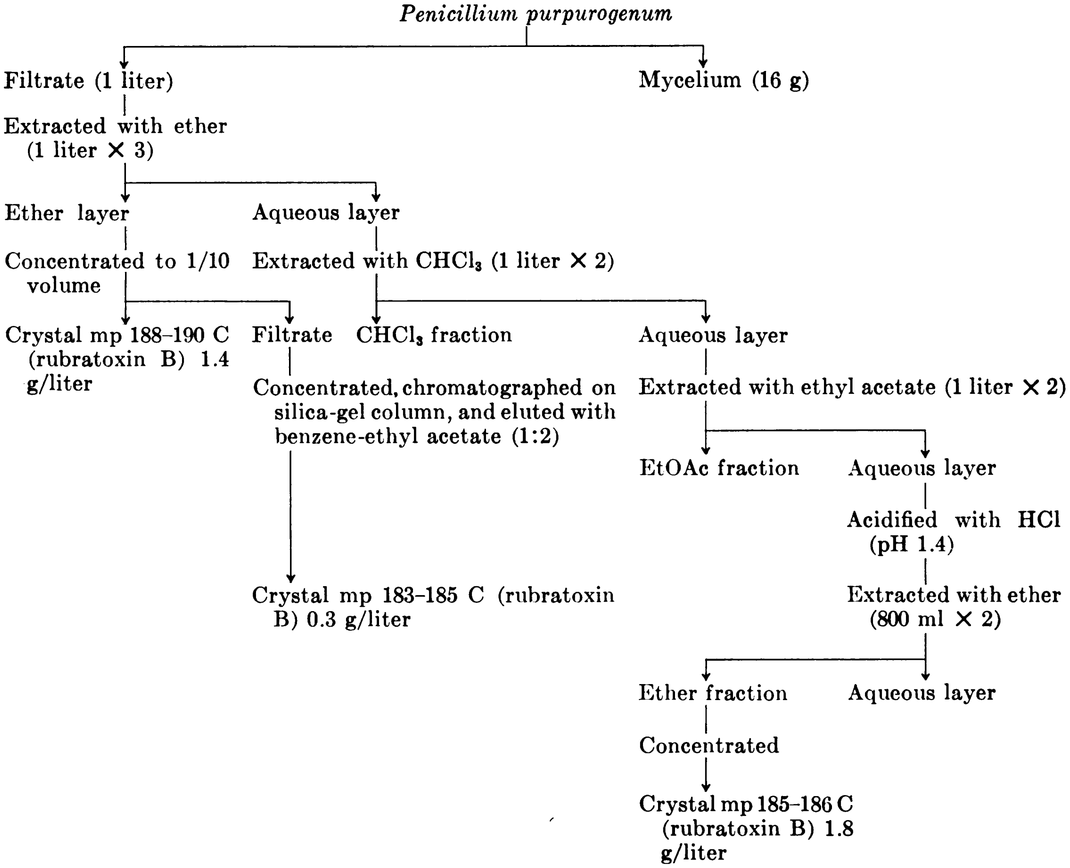

FIG. 2. *Isolation of rubratoxin B.*

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 ϵ 4.15, 3.89); and maxima in CH_3CN 204, 251 nm (log ϵ 4.13, 3.88); NMR (Fig. 3). IR and UV spectra indicated the presence of acid anhydride group(s) similar to those in glauconic 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 α,β-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

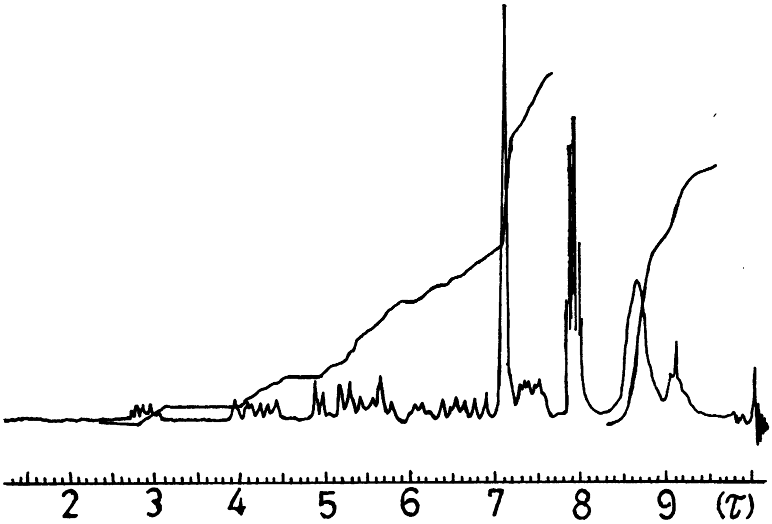

FIG. 3. *Nuclear magnetic resonance spectrum of rubratoxin B in d_6-acetone (60 mc).*

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 LD_{50}, 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. purpurogenum*, 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. purpurogenum* 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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LITERATURE CITED

1. Bailey, W. S., and A. H. Groth, Jr. 1969. The relationship of hepatitis-x of dogs and moldy corn poisoning of swine. J. Amer. Vet. Med. Ass. 134:514–519.
2. Baldwin, J. E., D. H. R. Barton, J. L. Bloomer, L. M. Jackman, L. Rodriguez-Hahn, and J. K. Sutherland, 1962. The constitutions of glauconic, glaucanic and byssochlamic acids. Experientia 18:345–352.
3. Bloomer, J. L., C. E. Moppett, and J. K. Sutherland. 1968. The nonadrides. Part V. Biosynthesis of glauconic acid. J. Chem. Soc. (London), p. 588–591.
4. Burnside, J. E., W. L. Sippel, J. Forgacs, W. T. Carll, M. B. Atwood, and E. R. Doll. 1957. A disease of swine and cattle caused by eating moldy corn. II. Experimental production with pure cultures of molds. Amer. J. Vet. Res. 18: 817–824.
5. Carlton, W. W., J. Tuite, and P. Mislivec. 1958. Investigations of the toxic effects in mice of certain species of *Penicillium*. Toxicol. Appl. Pharmacol. 13:372–387.
6. Forgacs, J., H. Koch, W. T. Carll, and R. H. White-Stevens. 1958. Additional studies on the relationship of mycotoxicoses to the poultry hemorrhagic syndrome. Amer. J. Vet. Res. 19:744–753.
7. Hayes, A. W., and B. J. Wilson. 1968. Bioproduction and purification of rubratoxin B. Appl. Microbiol. 16:1163–1167.
8. Kurata, H., H. Tanabe, K. Kanota, S. Udagawa, and M. Ichinoe. 1968. Studies on the population of toxigenic fungi in foodstuffs. IV. Aflatoxin-producing fungi isolated from foodstuffs in Japan. J. Food Hyg. Soc. Jap. 9:29–34.
9. Moss, M. O., F. V. Robinson, and A. B. Wood. 1968. Rubratoxin B, a toxic metabolite of *Penicillium rubrum*. Chem. Ind. (London) p. 587–588.
10. Moss, M. O., F. V. Robinson, A. B. Wood, and A. Morrison. 1967. Observations on the structure of the toxins from *Penicillium rubrum*. Chem. Ind. (London) p. 755–757.
11. Moss, M. O., F. V. Robinson, A. B. Wood, H. M. Paisley, and J. Feeney. 1968. Rubratoxin B, a proposed structure for a bis-anhydride from *Penicillium rubrum* Stoll. Nature (London) 220:767–770.
12. Moss, M. O., A. B. Wood, and F. V. Robinson 1969. The structure of rubratoxin A, a toxic metabolite of *Penicillium rubrum*. Tetrahedron Lett. 367–370.
13. Roberts, J. C., and C. W. H. Warren. 1955. Studies in mycological chemistry. IV. Purpurogenone, a metabolic product of *Penicillium purpurogenum* Stoll. J. Chem. Soc. p. 2992–2998.
14. Saito, M., M. Umeda, K. Ohtsubo, H. Kurata, S. Udagawa, and S. Natori. 1968. Studies on the detection of carcinogens in natural products. I. Toxic effects of fungi isolated from foodstuffs, p. 59. Proc. Jap. Cancer Assoc., 27th Annual Meeting, Tokyo.
15. Toplin, I. 1959. A tissue culture cytotoxity test for large-scale cancer chemotherapy screening. Cancer Res. 19: 959–965.
16. Townsend, R. J., M. O. Moss, and H. M. Peck. 1966. Isolation and characterization of hepatotoxins from *Penicillium rubrum*. J. Pharm. Pharmacol. 18:471–473.
17. Wilson, B. J., and C. H. Wilson. 1962. Extraction and preliminary characterization of a hepatotoxic substance from cultures of *Penicillium rubrum*. J. Bacteriol. 84:283–290.