MATERIALS AND METHODS

A. Animals

Two ages of animals were used in these studies. Animals used throughout these studies were weanling female (35-45 g body weight) and 7-day-old (14-18 g body weight) rats of Fischer derived strain. Rats were supplied by Animal Production Center, Faculty of Science, Mahidol University, Bangkok 4. Weanling female rats were housed in a group of three to eight animals in aluminum tub with galvanized steel wire covers. 7-day-old rats were also housed in a group of four animals and a maximum number of eight animals were kept with their mothers in the aluminum tubs with galvanized steel wire covers. All animals were kept in a room where the temperature was maintained at 25±1^{o}C and humidity at approximately 65%. A schedule provided light for the rats from 6 a.m. to 6 p.m. Animals were fed with regular rat pellets (Gold Coin Ltd., Singapore supplied by Zuellig Company) and water *ad libitum* until sacrificed or fasted. Weanling female rats were usually fasted overnight (14-16 hours).

B. Chemicals

All chemicals and solvents used throughout these experiments were analytical reagent grade. Silica gel G-HR was purchased from Macherey Nagel Co., Germany, whereas Silica gel G (acc. to Stahl) and silica gel (type. 60, 70-230 mesh) were purchased from E. Merck, Darmstaht, Germany. Potato dextrose agar was obtained in dehydrated from Difco Laboratories Inc, Michigan, U.S.A.

Dimethylsulfoxide (DMSO) was purchased from Burdick and Jackson Laboratories, Muskegon, Michigan, U.S.A.

C. Fungi

The fungal isolates used in these experiments were obtained from Mycology and Mycotoxin Laboratory, Department of Physiology, Faculty of Science, Mahidol University, Bangkok 4. These fungal isolates were originally isolated from market foods and foodstuffs in Bangkok (Glinsukon et al, 1975). Stock cultures were kept in sterile soil tubes at 0-4^{o}C. These fungal isolates consists of 26 strains of *Aspergillus niger*, 3 strains of *Aspergillus candidus*, 1 strain of *Aspergillus clavatus*, 4 strains of *Aspergillus fumigatus*, 1 strain of *Aspergillus nidulan* and 2 strains of *penicillium*.

D. Experimental Protocol

Study I

Acute Toxicity of the Crude Toxins from Various Strains of A. *niger* and other species

The objective of this study was to produce the crude toxins (PEI) and PES, and also to determine the potency of the crude toxins (PEI) and PES, clinical symptoms and histopathologic changes in the various organs of the tested animals were also studied.

Experiment I

Production of the Crude Toxins from Various Strains of A. *niger* and other species

This experiment was designed to determine the capability of these fungal isolates to produce the crude toxins (PEI) and

PES on sterile glutinous rice. The fungi isolates from the stock sterile soil tubes were inoculated on potato dextrose agar plates and allowed to grow at room temperature (25°C) for 3 to 4 days. The pure culture was then streaked on potato dextrose agar slant and incubated at room temperature for 5 to 7 days. The spore suspension was obtained by adding 5.0 ml of 0.01% sodium lauryl sulfate (S.L.S.) to each slant to suspend the spores. A portion of 5.0 ml of the spore suspension was then used to inoculate 250 g sterile glutinous rice contained in a 2.8 L Fernbach flask. The mold was grown on steriled glutinous rice (250 g glutinous rice and 200 ml water) at 25°C for 12-14 days which the maximum sporulation was obtained. The moldy glutinous rice was extracted 3 times in a Waring blender (1-gallon) with either chloroform or methylene chloride and methanol (97:3, V:V). The extract was filtered through the cheese cloth and filter paper. The filtrates were pooled and concentrated *in vacuo* by a rotary vacuum evaporator at 40°C until the oily residue was obtained. The oily residue was precipitated by pouring into swirling cooled petroleum ether. The completed precipitation was obtained when it was kept in the refrigerator overnight. The precipitate was harvested by filtering through the filter paper on Buchner funnel by suction. This precipitate is called the crude toxin or petroleum ether insoluble fraction (PEI). The filtrate was evaporated again until the oily like residue was obtained. This oily like residue is called petroleum ether soluble fraction (PES). The crude toxin and petroleum ether soluble fraction (PES) were dried under N_2 gas and *in vacuo*.

Experiment 2

Determination of Aflatoxins in the Crude Toxins

1. Screening for aflatoxins in the crude toxins.

An approximately 5.0 mg of the crude toxins was dissolved in 0.1 ml chloroform and spotted on the thin layer chromatographic plate coated with silica gel G-HR (250 mμ thickness). It was dissolved in equilibrated tank of chloroform:acetone (95:5, v:v) along with standard aflatoxins B_1, B_2, G_1 and G_2.

2. Quantitative determination of aflatoxins in the crude toxins.

Detailed procedures for the quantitative determination of aflatoxins in the crude toxins are given in Appendix I. It is followed the modified method described by Eppley (1968).

The crude toxin which exhibited the characteristics of blue and green fluorescent spots were expected to be aflatoxins. This crude toxin (50 mg) was then dissolved in small volume of chloroform and applied to the silica gel (type 60, 70-230 mesh) column in order to eliminate some compounds out of the aflatoxins. The aflatoxins were eluted in the fraction of chloroform:methanol (97:3, v:v). The amount of aflatoxin was determined by visual comparison with standard aflatoxins B_1, B_2, G_1 and G_2 under Chromato-vue of the long wavelength ultraviolet light. The identity of the aflatoxins was confirmed by the Rf values on thin layer plate after developing in various solvent systems of chloroform: methanol (97:3, v:v) and chloroform:acetone (95:5, v:v).

Experiment 3

Determination of Oxalate in the Crude Toxins

Detailed procedures for the determination of oxalate in the crude toxins are given in Appendix II. It is followed the method described by Hodgkinson and William (1972).

This experiment was designed to estimate the oxalate contents in the crude toxins from various strains of A. *niger* which might be able to produce this compound. The oxalate may be toxic to the tested animals as well as aflatoxins. Thus oxalate and aflatoxin contents should be determined in order to see whether the amounts detected are high enough to be toxic to the tested animals.

A method of oxalate determination in based on the co-precipitation with calcium sulfate and reduced to glycollic acid by boiling with dilute sulfuric acid in the present of zinc pellet. The amount of glycollic acid was then estimated colorimetrically with chromotropic acid. The amount of oxalate is expressed in microgram in 100 mg of the crude toxin.

Experiment 4

Toxicity Testing

This experiment was desiged to estimate the potency of the crude toxins and PES from various strains of A. *niger* and other species in female weanling rats.

Groups of 5 weanling female rats were given intraperitoneal administration of the crude toxins at dose levels equiva-

lent to 50.0, 25.0, 12.5, or 6.25 g moldy glutinous rice per rat in 0.1 ml dimethylsulfoxide. The toxicity of PES was also tested in weanling female rats. The PES was given orally to a group of 3 rats via stomach tube (blunt tip of 2 inches-22gauge needle) at various dose levels according to the total amount of PES but the maximum dose level was in 1.0 ml per rat. The period of observation was 7 days. The clinical symptoms, body weight changes and the time of death were recorded. After 7 days, liver, kidneys, small intestine, spleen, pancreas, adrenal gland, uterus, heart lung of both dead and sacrificed animals were examined and fixed in 10% buffered neutral formalin. The paraffin sections were stained with hematoxylin and eosin (see Appendex III).

Experiment 5

Histopathologic Examinations

In this experiment, it was desired to examine the histopathologic changes in various organs mentioned above from 3 rats treated by particular dose level of the crude toxin or PES. The sections of these organs from those rats that died in 1-3 days after treatment were preference. The severity and the type of histopathologic changes were graded in relative manner in order to classify the crude toxins and PES into neurotoxin, hepatotoxin and nephrotoxin.

Study II

Acute toxicity of a Mycotoxin from A. *niger* (AN-A30-75)

The objective of this study was to produce, prepare, isolate, purify and possibly characterize the mycotoxin from A. *niger* (AN-A30-75).

Experiment 1

Production isolation and purification of a mycotoxin from A. *niger* (AN-A30-75)

Detailed procedures for the production, isolation and purification of a mycotoxins from A. *niger* (AN-A30-75) are given in Appendix IV).

The procedures of productions of a crude toxin from A. *niger* (AN-A30-75) is similar to those described in the production of the crude toxins from various strains of A. *niger* in study I, experiment I.

When the crude toxin was obtained, it was then further purified an one column chromatography (silica gel G type 60, 70-230 mesh) and several steps of thin layer chromatography (silica gel G) using chloroform:benzene:methanol (60:30:10, v:v:v) as sovent system. The crystallized form of the mycotoxin were obtained crystallization in chloroform:petroleum ether (1:10, v:v). The toxicity of each fraction was checked by rat bioassay. Each fraction was dissolved in dimethylsulfoxide (DMSO) are injected intraperitoneally into 7-day-old and weanling rats to screen the toxicity (see Fig. 10).

Experiment 2

1. Acute toxicity of the crude toxin and potassium oxalate

This experiment was designed to determine the comparative potency of the crude toxin and potassium oxalate and also to examine the histopathologic changes in various organs of weanling female rats treated with these compounds in order to make it certain that the crude toxin does not contain enough oxalate which is lethal to the animal. The potency of the crude toxin and potassium oxalate was measured as the LD_{50} in weanling rats.

The animals used in this experiment were weanling female rats (30-45 g BW). The crude toxin was dissolved in dimethylsulfoxide and given intraperitoneally at dose levels of 787, 709, 638, 574, 517, 465 and 419 mg/kg BW. After administration, the clinical sympstoms, and time of death were recorded. The observation period was seven days. The visceral organs were fixed in 10% buffered neutral formalin. Paraffin sections were stained with hematoxylin and eosin. The toxicity of crude toxin expressed in term of LD_{50} was determined and calculated according to the method of Litchfield and Wilcoxon (1949). The acute toxicity of potassium oxalate which was dissolved in normal saline was performed similar to LD_{50} determination of crude toxin A. *niger* (AN-A30-75). The potassium oxalate was administered intreperitoneally at dose level of 360, 324, 291.6, 262.4, 236.2, 212, 191.3, 172.2 mg/kg BW.