Biochemical Pharmacology, Vol. 22, pp. 3275–3286. Pergamon Press, 1973. Printed in Great Britain.

EFFECT OF FUNGAL TOXINS ON UPTAKE AND DEGRADATION OF FORMALDEHYDE-TREATED ^{125}I-ALBUMIN IN MOUSE LIVER PHAGOLYSOSOMES

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(*Received* 17 *February* 1973; *accepted* 22 *June* 1973)

Abstract—Aflatoxin, ochratoxin and rubratoxin are hepatotoxic metabolites produced by fungi. These mycotoxins have been evaluated with respect to effects on the reticulo-endothelial function of uptake and degradation of a soluble, denatured protein by phagolysosomes isolated from mouse liver and kidneys. The toxins were dissolved in dimethylsulfoxide and administered intraperitoneally. In addition, rubratoxin B in corn oil was given orally. Particulate fractions isolated from liver and kidney homogenates were incubated at 35° and assayed for proteolytic activity within osmotically active particles (phagolysosomes). Osmotically active particles containing labeled protein were present in liver homogenates 3–4 hr after animals received 2·5 mg/kg of rubratoxin, but these particles exhibited little or no proteolytic activity. Smaller doses of rubratoxin B resulted in increased intralysosomal digestive capacity; however, the relative quantity of osmotically active particles was less than in dimethylsulfoxide-treated controls. No effect was noted on phagolysosome formation or function in the kidney except at high doses. Similar results were obtained when 100 mg/kg of rubratoxin B was administered orally to mice. Rubratoxin B had no effect on proteolytic activity in a crude mouse liver cathepsin preparation. These observations are interpreted as an interference by rubratoxin B in the fusion of lysosomes with phagosomes. Aflatoxin B_1 and ochratoxin had no effect on uptake and degradation of protein in phagolysosomes.

MYCOTOXINS, secondary fungal metabolites, are known hepatotoxins and in the case of aflatoxin, hepatocarcinogens for a number of animal species.[1] For example, in Japan, rice was found to be an excellent growth medium for several molds that produced toxic effects in animal feedings.[2] The toxic syndrome attributed to aflatoxin, a metabolite of *Aspergillus flavus*, was first recognized in 1960 in England.[3,4] Domestic peanut meal contaminated with aflatoxin has since been shown to produce liver carcinoma in rats and other animals.[5,6] In the United States, high incidents of liver cancer in hatchery rainbow trout led to the discovery of aflatoxin-contaminated cotton seed meal in the commercial feed pellets fed to these fish.[7]

Ochratoxin, one of several toxic metabolites produced by *Aspergillus ochraceus*, initially was isolated from sorghum and has been shown to be toxic to a number of animal species.[8] In another study *Penicillium rubrum*, which produces rubratoxin B, was found to be one of several toxigenic fungi isolated from cereal and legume products.[9] The acute toxicity of rubratoxin B in dogs[10] and other laboratory animals[11,12] has been reported.

However, with the exception of aflatoxin, little concerning the mechanism of action of mycotoxins is known. The mechanism of action of aflatoxin B_1 in producing hepatic cell injury has been studied biochemically and morphologically.[13,14] At present there is evidence that aflatoxin B_1 acts at multiple sites but the functional and structural changes are of uncertain significance in the development of lesions. The

early effect of aflatoxin B_1 on hepatic parenchymal cells shows a sequence of change in cytoplasmic organelles. These changes were most marked in the periportal zone, in which necrosis would occur, but also were seen in the centrilobular cells. The disorganization of the reticuloendothelial system correlated with the breakdown of polysomal profiles and the effect of aflatoxin B_1 on polysome binding sites.

Since other cytotoxic agents that induce similar histologic changes have been shown to possess lysosomal-damaging activity, an investigation was undertaken to determine if aflatoxin, ochratoxin and rubratoxin would damage liver and kidney lysosomes. Damage to liver and kidney lysosomes is reflected in a decreased capacity of these organelles to function in the formation of phagolysosomes or in decreased hydrolytic activity. These processes can be evaluated by intravenous injections of formaldehyde-denatured ^{125}I-labeled albumin into experimental animals followed by measurement of the capacity of the liver and kidneys to form phagolysosomes or by assay of proteolysis within such particles.[15]

MATERIALS AND METHODS

Rubratoxin B was prepared by the method of Hayes and Wilson.[16] Aflatoxin B_1 was purchased from CalBiochem, Los Angeles, Calif. Ochratoxin was kindly supplied by Dr. Alex Ciegler, Northern Regional Laboratory, Peoria, Ill., and contained 93% ochratoxin A and 7% ochratoxin B. Compound purity was established by melting point, infrared and mass spectra, thin-layer chromatography and u.v. molar extinction coefficient. After crystallization, the mycotoxins were stored in the dark.

Animals used were adult male and female Swiss–Webster albino mice (25–35 g) which had been originally obtained from Southern Animal Farms, Prattville, Ala. Colonies descendent from these animals are maintained at the University of Alabama. All animals were fed commercial rations and were provided with food and water continuously.

Formaldehyde-treated ^{125}I-albumin (bovine, Sigma Chemical Co., St. Louis, Mo.) was prepared according to Bocci[17] as previously described.[15] This preparation contained 10 mg/ml of protein and approximately $1{\cdot}25 \times 10^7$ cpm/mg.

Mycotoxin solutions were prepared by dissolving the toxins in appropriate amounts of dimethylsulfoxide (DMSO) or in corn oil for the oral study with rubratoxin B. The fungal toxins were injected intraperitoneally (i.p.) in a solvent volume not exceeding 0·1 ml into mice at the dose specified in Results. In the oral dose study, male mice received 50, 100 and 215 mg/kg of rubratoxin B. Unless otherwise stated, the toxins were administered 3·5 hr before ^{125}I-albumin. Labeled protein (0·1 ml) was injected directly into the blood stream via the caudal vein. The animals were killed with ether 30 min after the second injection and the liver and kidneys were rapidly removed, weighed and chilled. Homogenization of organs, isolation of particulate subcellular fractions containing intact phagolysosomes, measurement of proteolytic activities in these particles and estimation of the relative proportion of particle-associated labeled protein releasable by osmotic shock were performed as previously described.[15]

Crude lysosomal cathepsin was prepared from a composite of livers from twelve male mice. The livers were homogenized in cold 0·25 M sucrose and then centrifuged at 500 *g* for 5 min. The supernatant fluid was decanted and recentrifuged at 40,000 g for 20 min. The resulting pellet was resuspended and washed with 40 ml of cold

0·25 M sucrose, suspended in 5 ml of 0·01 M Tris-acetate buffer, pH 8, and dialyzed for 4 hr against 2 l. of cold distilled water. The preparation then was adjusted to pH 5 with 0·5 ml of acetate buffer and centrifuged to remove any precipitates and particulate materials. The resulting clear, pale brown supernatant fluid contained 12·1 mg/ml of protein and was used as the cathepsin preparation.

Acid phosphatase (EC 3.1.3.2) was assayed with *P*-nitrophenyl phosphate as substrate. Free and bound acid phosphatase activity was determined by mixing liver (or kidney) homogenates or suspensions of subcellular particles with 0·1 vol. of 0·25 M sucrose containing 0·25 M Tris-acetate buffer, pH 7·3, or 0·25 M sucrose–0·25 M Tris-acetate containing 1% Triton X-100. Three vol. of both suspensions were incubated at 37° for 5 min with 0·5 ml of 2 mM *P*-nitrophenyl phosphate in 0·25 ml sucrose containing 0·1 M citrate buffer, pH 5, and 3×10^{-3} M EDTA in a total volume of 1 ml. The reactions were stopped and color was developed by 4·0 ml of 0·1 M NaOH. Color density was measured at 410 nm against a reagent blank similarly incubated, without enzyme, in an Acta III Spectrophotometer (Beckman Instruments, Fullerton, Calif.) Bound acid phosphatase was calculated by subtracting the optical density of the sample without Triton X-100 (free acid phosphatase) from the total (Triton X-100), and the resulting data were expressed as a percentage of total acid phosphatase.

RESULTS

Aflatoxin, ochratoxin and rubratoxin were injected i.p. into mice 4 hr before sacrifice because earlier studies had shown the greatest effect on the liver at this time.[18] DMSO had no detectable effect on phagolysosome formation in kidney or liver or on proteolytic activities in these particles 4 hr after i.p. injection. Aflatoxin and ochratoxin at doses of 10 and 20 mg/kg, respectively, had no effect on intralysosomal proteolytic activities in isolated liver phagolysosomes (Figs. 1 and 2). Although liver is more severely affected than kidneys by aflatoxin, aflatoxin B_1 appeared to slightly inhibit digestion in kidney phagolysosomes (Fig. 1). Rubratoxin B (1·0 and 2·5 mg/kg) inhibited proteolysis in liver phagolysosomes between 80 and 100 per cent and inhibited proteolysis 50 per cent in kidney particles at the higher dose (Fig. 3).

The i.p. LD_{50} of rubratoxin B in DMSO for mice has been established as 0·37 mg/kg.[12] The minimal concentration of rubratoxin B eliciting an effect on proteolysis in liver phagolysosomes was 0·25 mg/kg (Table 1). This dose caused no deaths during the experiment. A dose of 0·5 mg/kg, however, caused about 50 per cent mortality within 48 hr after injection. The per cent of particle-bound radioactivity released by osmotic shock in the 500–30,000 *g* particulate fraction from kidney and liver homogenates also is shown in Table 1. In liver phagolysosomes from rubratoxin-injected mice, the released radioactivity ranged from 26 to 42 per cent whereas the released radioactivity in liver phagolysosomes from control animals averaged 59 per cent. However, no correlation was observed between digestive rate and decrease in osmotically releasable radioactivity. For example, in one experiment using liver particles from a mouse injected with 0·5 mg/kg of rubratoxin B, a digestive rate in 40 min of 11·2 per cent protein was obtained, but only 39·5 per cent of the particle-associated labeled protein was released by osmotic shock. In a similar experiment, the per cent of the total protein released by osmotic shock did not differ greatly from this figure but digestion was completely inhibited.

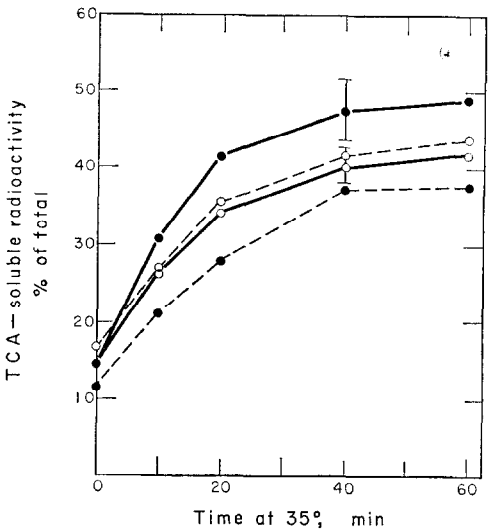

FIG. 1. Digestion of intralysosomal ^{125}I-labeled albumin in liver and kidney phagolysosome suspensions from mice injected i.p. with 10 mg/kg of aflatoxin in DMSO 3·5 hr before labeled protein. Controls are indicated by solid lines and represent the means of twelve animals injected with DMSO. Standard deviations for the controls are indicated at 40 min. Digestive time courses for aflatoxin-injected animals are indicated by dashed lines. Kidney: closed circles; liver: open circles. Total radioactivity in the suspensions from aflatoxin-injected animals was: kidney, 156,990 cpm; liver, 112,350 cpm.

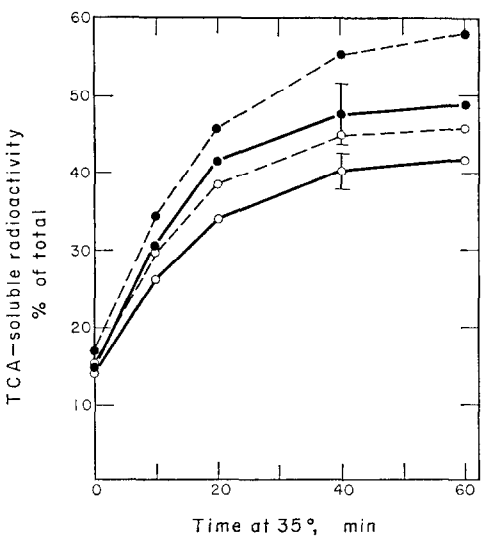

FIG. 2. Digestion of intralysosomal ^{125}I-labeled albumin in liver and kidney phagolysosome suspensions from mice injected i.p. with 20 mg/kg of ochratoxin 3·5 hr before labeled protein. Controls (with standard deviations), solid lines; ochratoxin-injected, dashed lines. Open circles, liver; closed circles, kidney. Total radioactivity in the suspensions from ochratoxin-injected animals was: kidney, 200,830 cpm; liver, 242,345 cpm.

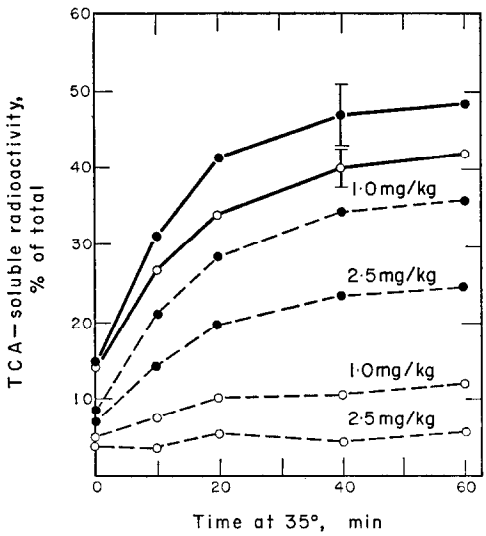

FIG. 3. Effect of i.p. injections of rubratoxin B in DMSO 3·5 hr before labeled albumin on the digestion of the albumin in isolated phagolysosomes. Controls, with standard deviations at 40 min (twelve mice) are indicated by solid lines; dashed lines, rubratoxin-injected animals. Kidney: closed circles; liver: open circles. Total radioactivity in the suspensions from the mouse injected with 1 mg/kg of rubratoxin B was: kidney, 194,412 cpm; liver 32,520 cpm. The suspensions from the mouse injected with 2·5 mg/kg of rubratoxin B contained: kidney, 216,492 cpm; liver, 40,776 cpm.

TABLE 1. EFFECT OF RUBRATOXIN ON PROTEOLYTIC ACTIVITY AND ON THE RELATIVE QUANTITY OF LABELED ALBUMIN IN OSMOTICALLY ACTIVE FORMS IN MOUSE LIVER PARTICLES MATERIAL*

Rubratoxin B (mg/kg)	Digestion (%/40 min)		Osmotically releasable (%)	
	Kidney	Liver	Kidney	Liver
Control (DMSO)	32·3 ± 3·9	25·8 ± 2·8	71·4 ± 6·8	59·3 ± 3·5
0·125	32·6	25·1		
	30·0	18·5		
0·25	33·8	5·1	71·0	30·7
	27·8	8·3		
0·5	22·2	2·9	69·0	36·0
	36·2	11·2	69·0	39·5
1·0	31·1	9·4		26·5
	33·7	6·9	61·0	29·5
	13·0	0·0	43·2	34·5
	25·7	5·6	67·0	41·5
2·5	17·4	0·5	50·2	35·0
	13·9	0·0	47·0	38·0

* Animals were injected i.p. with rubratoxin B in 0·05 ml DMSO 3·5 hr before intravenous injections of formaldehyde-treated ^{125}I-labeled albumin. Total radioactivity in the kidney control experiments ranged from 161,130 to 544,236 cpm with a mean of $3{\cdot}19 \times 10^5$ cpm. In the control liver experiment, the incubated suspensions contained 87,564 to 480,000 cpm with a mean of $2{\cdot}3 \times 10^5$ cpm. Digestion represents the per cent of the total radioactivity in the suspensions converted to a trichloroacetic acid-soluble form during a 40-min incubation of the suspensions at 35°. The standard deviation is shown for the controls (eight experiments).

Experiments were performed to determine the initial onset of the effect of 0·5 mg/kg of rubratoxin on proteolytic activity. The results are summarized in Table 2. Although DMSO had an effect on kidney phagolysosomes approximately 1 hr after injection, there was no solvent effect on digestion in liver particles. The inhibitory effect of DMSO on proteolytic activity in kidney phagolysosomes appeared to be temporary and may have been the result of DMSO accumulation in the kidneys. The effect or rubratoxin on proteolysis in isolated liver phagolysosomes was apparent 3 hr after injection, but proteolytic activity was normal in a surviving mouse 48 hr after injection.

TABLE 2. ALTERATIONS IN DIGESTIVE CAPACITY OF MOUSE LIVER AND KIDNEY PHAGOLYSOSOMES CAUSED BY RUBRATOXIN B*

Time (hr)	Digestion (%/40 min)			
	Dimethylsulfoxide control		Rubratoxin B-treated (0·5 mg/kg)	
	Kidney	Liver	Kidney	Liver
0·5	30·6	24·2	26·0	23·5
1	13·6	24·2	29·8	25·2
	21·2	21·3	23·2	27·5
2	34·1	21·4	34·7	22·5
3	31·1	24·8	32·8	10·5
4	32·3 ± 3·9	25·8 ± 2·8	30·2	10·3
			22·2	11·2
			36·2	2·9
24			20·8	10·1
48			28·4	21·4

* Dimethylsulfoxide (0·05 ml) was injected i.p. into control animals. Standard deviations (eight experiments) are shown at 4 hr for control experiments. Rubratoxin B was dissolved in 0·05 ml DMSO and injected i.p. Intravenous injection of formaldehyde-treated ^{125}I-labeled albumin followed the rubratoxin B injection.

The inhibition of proteolysis in isolated liver phagolysosomes from rubratoxin-treated mice suggested that the labeled protein was taken up by endocytosis but that the capacity of the phagolysosomes to digest the protein was affected. The clearance rate of the labeled albumin from liver was inhibited in rubratoxin-treated animals although clearance was only slightly affected at a dose of 0·25 mg/kg (Fig. 4). The quantity of acid-soluble radioactivity was reduced 40 per cent in animals injected with 1 mg/kg of rubratoxin 4 hr after injection of labeled protein suggesting that little or no proteolysis occurred *in vivo* (Fig. 4).

Rubratoxin apparently had little effect on uptake of labeled protein in the liver (Fig. 4). An experiment was performed to determine if the toxin affected uptake into subcellular fractions relative to solvent controls. Results of a typical experiment are shown in Table 3. Uptake into particulate fractions was not inhibited at the lower rubratoxin doses, but at 2 mg/kg of rubratoxin B there was a significant inhibition of uptake into the 30,000 *g* and 500 *g* fractions.

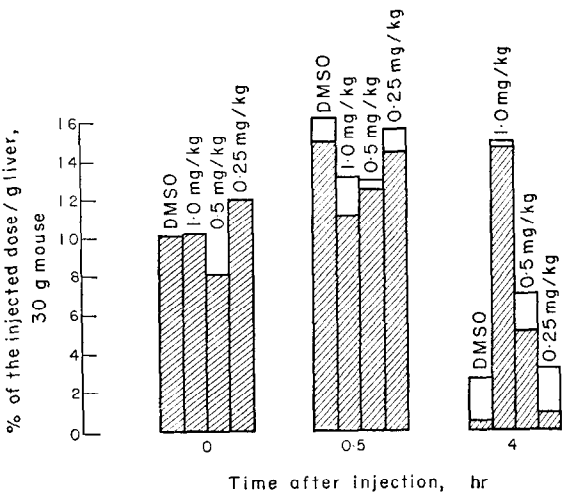

FIG. 4. Effect of i.p. injections of rubratoxin B in DMSO on clearance of radioactivity from livers of mice. Animals were injected with the indicated dose of rubratoxin in DMSO or with DMSO alone (0·05 ml), and 3·5 hr later were injected intravenously with 0·1 ml (1 mg, $1{\cdot}1 \times 10^7$ cpm) formaldehyde-treated ^{125}I-albumin. The mice were sacrificed at the indicated times after these injections, and the livers were removed and homogenized in 0·25 M sucrose. Shaded areas represent trichloroacetic acid-insoluble radioactivity.

TABLE 3. EFFECT OF RUBRATOXIN B (IN DMSO, i.p.) ON UPTAKE OF ^{125}I-LABELED ALBUMIN IN THE LIVER AND ON DISTRIBUTION OF RADIOACTIVITY IN LIVER SUBCELLULAR PARTICULATE FRACTIONS*

	Control	Rubratoxin B (mg/kg)		
		0·25	0·5	2·0
Per cent of injected dose/g liver	12·2	12·1	13·5	10·3
Per cent of total liver radioactivity in				
500 *g* (5 min)	23·6	23·1	15·5	7·9
30,000 *g* (10 min)	14·8	14·8	19·4	5·4
40,000 *g* (1 hr)	1·9	1·1	2·0	1·4

* Formaldehyde-treated ^{125}I-labeled albumin (1 mg, 9×10^6 cpm) was injected 3·5 hr after rubratoxin B. Livers were removed and homogenized in cold 0·25 M sucrose. All particulate fractions were resuspended and washed twice in cold 0·25 M sucrose except the 40,000 *g* fraction which was not washed. Total radioactivity in the livers was as follows: 0·25 mg/kg rubratoxin, $1{\cdot}99 \times 10^6$ cpm; 0·5 mg/kg, $2{\cdot}0 \times 10^6$ cpm; 2·0 mg/kg, $1{\cdot}64 \times 10^6$ cpm; and the control contained $2{\cdot}12 \times 10^6$ cpm.

The data presented thus far suggested that rubratoxin inhibited a step in the process of phagolysosome formation. The presence of osmotically releasable protein in the 30,000 *g* fractions and an absence of proteolytic activity in these particles from rubratoxin-treated animals suggested either the lack of proteolytic enzymes or an inhibition

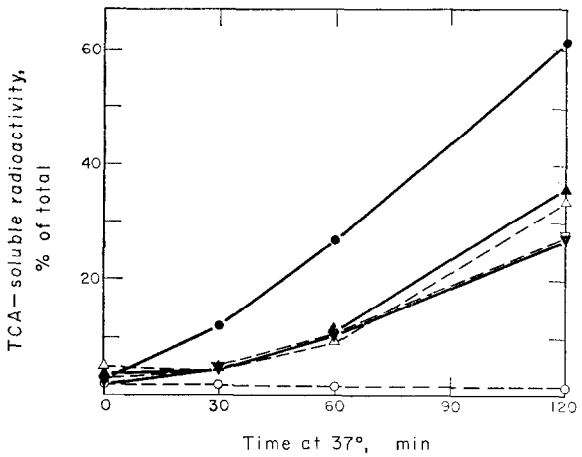

FIG. 5. Effect of rubratoxin B in DMSO or propylene glycol, or the solvents alone, on hydrolysis of ^{125}I-albumin in a crude mouse liver cathepsin preparation. Each reaction mixture contained 0·075 M Tris-acetate buffer, pH 5·0, 0·05 M mercaptoethanol, 10^{-3} M EDTA, 0·5 mg ^{125}I-labeled albumin ($5·8 \times 10^6$ cpm), 3 mg crude mouse liver cathepsin, and 0·05 ml DMSO ▽– – –▽, 0·025 mg rubratoxin in 0·05 ml DMSO ▼——▼, 0·05 ml propylene glycol △– – –△, 0·025 mg rubratoxin in 0·05 ml propylene glycol ▲——▲, or 0·05 ml H_2O (control) ●——● in a total volume of 0·65 ml. A negative control ○– – –○ contained 3 mg ovalbumin instead of enzyme.

by rubratoxin of cathepsin in phagolysosomes. In order to test the latter possibility, the effect of rubratoxin in DMSO was assessed on the ability of a crude lysosomal preparation to hydrolyze labeled protein. Although DMSO and propylene glycol inhibited proteolysis, there was no further inhibition by rubratoxin at a concentration of 4·2 μg/ml (Fig. 5). Furthermore, neither DMSO nor rubratoxin had an effect on proteolytic activity in isolated liver phagolysosomes from an uninjected mouse (Table 4). These results indicated that rubratoxin had no effect on enzyme activity. Therefore, this mycotoxin must have affected some aspect of phagolysosome formation, perhaps the fusion of lysosomes with phagosomes. If rubratoxin caused a disruption of liver lysosomes, phagolysosome formation (fusion of lysosomes with phagosomes) would not be possible. In order to determine if intact lysosomes were present in livers of rubratoxin-treated animals, free and bound acid phosphatase, a lysosomal marker enzyme, was assayed in homogenates and particulate material. The results (Table 5) indicated a decrease in bound acid phosphatase in livers of animals injected with high doses of rubratoxin, but the decrease was not significant near the LD_{50} which previously was shown to cause reduced intralysosomal proteolytic activity.

Similar results were obtained when rubratoxin was administered orally and the data are summarized in Table 6. The oral LD_{50} for rubratoxin in mice is 400–450 mg/kg[12] and the minimal concentration of rubratoxin reducing proteolysis in isolated liver phagolysosomes was 100 mg/kg. A slight decrease in osmotically releasable radioactivity with only 14·8 per cent digestion in 40 min was observed in liver phagolysosomes from mice treated orally with 100 mg/kg of rubratoxin. Osmotically releasable radioactivity and digestion were greatly decreased in both liver and kidney preparations from mice receiving 215 mg/kg of rubratoxin.

TABLE 4. EFFECT OF RUBRATOXIN B IN DMSO OR DMSO ALONE ON PROTEOLYTIC ACTIVITY IN ISOLATED MOUSE LIVER PHAGOLYSOSOMES*

Additions	Digestion (%/40 min)
0·8% (v/v) DMSO	26·1
1·6% (v/v) DMSO	27·1
3·2% (v/v) DMSO	26·2
0·8% DMSO, rubratoxin 0·1%	25·8
1·6% DMSO, rubratoxin 0·2%	25·6
3·2% DMSO, rubratoxin 0·4%	25·2
None (control)	25·8 + 2·8

* Liver particulate preparations obtained from a mouse injected intravenously with 1·5 mg ($1·4\ 10^7$ cpm) formaldehyde-treated ^{125}I-labeled albumin were incubated in total volumes of 12 ml of 0·25 M sucrose—0·05 M mercaptoethanol and additions as noted above. The incubations were carried out for 60 min at 35°. Each incubated preparation contained $1·2\ 10^5$ cpm. Increases in acid soluble radioactivity during the first 40 min are recorded. Controls (eight mice) are shown with the standard deviation.

TABLE 5. EFFECT OF RUBRATOXIN B ON THE PER CENT BOUND ACID PHOSPHATASE ACTIVITY IN MOUSE LIVER HOMOGENATES AND SUBCELLULAR PARTICLES*

Rubratoxin B (mg/kg)	Per cent bound acid phosphatase	
	Homogenate	Pellet (30,000 *g*)
0·0 (10)	36·1 ± 8·0	72·5 ± 5·0
0·125 (2)	35·4	69·4
0·25 (1)	24·0	65·2
0·5 (2)	36·0	73·4
1·0 (1)	20·7	62·9
2·5 (3)	25·9	40·5

* Aliquots of the homogenate and particles sedimented at 500–30,000 *g* from the 0·25 M sucrose homogenate were assayed in the presence of Triton X-100 as described in Materials and Methods. Controls (DMSO alone) are shown with the standard deviation. The number of animals for each dose is shown in parentheses.

DISCUSSION

Of the three fungal toxins tested, only rubratoxin induced an effect on lysosome function as assessed by the capacity of mouse liver and kidneys to form phagolysosomes

TABLE 6. EFFECT OF ORAL DOSES OF RUBRATOXIN B ON DIGESTIVE ACTIVITY AND ON OSMOTICALLY RELEASABLE RADIOACTIVITY IN SUBCELULAR PARTICLES SEDIMENTED FROM MOUSE LIVER HOMOGENATES*

Rubratoxin B (mg/kg)	Digestion (%/40 min)		Osmotically releasable (%)	
	Kidney	Liver	Kidney	Liver
215	21·2	11·9	44·9	32·3
100	30·9	14·8	59·7	49·3
50	40·9	27·2	85·3	53·9
0	32·3 ± 3·9	25·8 ± 2·8	71·4 ± 6·8	53·3 ± 3·5

* Rubratoxin B in corn oil was administered by intubation. Injections of ^{125}I-labeled albumin were made 3·5 hr after rubratoxin B. The standard deviation is shown for the controls (eight experiments).

and to hydrolyze intralysosomal protein. The evidence presented in this communication supports the hypothesis[19] that hepatocellular alterations produced by aflatoxin B_1 are not mediated through damage to lysosomes. Although the mechanism of ochratoxin toxicity has not been studied as extensively as aflatoxin and its potential as a carcinogen is open to question, the results obtained in this study suggest that ochratoxin also has no detectable effect on lysosomes in either liver or kidneys of mice.

The livers of mice treated with rubratoxin B appeared grossly swollen and hemorrhagic. Crude preparations of this toxin have been reported to produce swelling and fragmentation of the endoplasmic reticulum.[20] If acute doses of rubratoxin B affect cells at the capillary wall resulting in cell rupture and subsequent release of blood into the nearby tissues, the hemorrhagic condition could be explained. Furthermore, the most active cells in endocytosis and degradation of macromolecular or particulate materials in the blood stream are those cells near the capillary wall (reticuloendothelial cells). A direct effect of rubratoxin B on the lysosomes of such cells could result in release of acid hydrolases that might produce the gross morphological effect noted above as well as the effect on lysosome function reported in these studies. If lysosomes in parenchymal liver cells are relatively unaffected by rubratoxin B, little or no decrease in bound acid phosphatase would be expected in liver homogenates except at high doses of the toxin. The results described in this study are consistent with these speculations.

If a mouse is injected intravenously with formaldehyde-treated albumin and the livers or kidneys removed and homogenized within 5 min after injection, about 20 per cent of the particle-bound radioactivity can be removed by osmotic shock but no proteolytic activity is detectable in these particles.[15,21] At 10 min after injection, about 40 per cent of the particle-bound label can be removed by suspension of the particles in dilute buffer, and a significant rate of proteolysis is evident during incubation of the fraction at 35°.[15] These observations are consistent with microscopic studies by Straus,[22] Daems *et al.*[23] and others[24,25] which show that phagosomes are formed first and that these particles fuse with lysosomes containing the degradative enzymes. In experiments with rubratoxin-treated mice, large proportions of particle-

associated radioactivity were removed by osmotic shock but in many instances little or no proteolytic activity was detectable. Such data suggest that rubratoxin B interfered with fusion of phagosomes and lysosomes perhaps by inducing lysosomal disruption. Other instances have been described in which fusion of lysosomes with phagosomes is inhibited. In the case of macrophage entrapment of *Mycobacterium tuberculosis*, fusion of lysosomes with phagosomes did not take place if the bacilli were damaged.[26] Colchicine also has been demonstrated to adversely affect degranulation of lysosomal particles after ingestion of bacteria by human leucocytes.[27] The Chediak–Higashi syndrome, a genetically inherited condition affecting mice, mink, cattle and man in which a defect exists in some aspect of lysosome function, may result from a retardation of contact of engulfed proteins with lysosomal enzymes.[28] Although the exact mechanism is unknown, this syndrome could be due to an inhibition of fusion of phagosomes with lysosomes.

The disparity observed in the LD_{50} of rubratoxin when administered i.p. in DMSO or i.p. in propylene glycol is not understood. The difference between oral and i.p. LD_{50} values of rubratoxin B in mice may be the result of low pH in the intestinal tract or microbial degradation. DMSO may facilitate toxin entry into its principle site of action in target organs. However, the same effects were noted on lysosome function in this study, regardless of the route by which the toxin was administered. Such data suggest that DMSO does not react with rubratoxin to form a more active complex.

Similar studies have been carried out with mercuric chloride[29] and with carbon tetrachloride.[30] Intravenous or i.p. injections into mice of mercuric chloride affected phagolysosome formation and function in the kidneys but not in the liver. Unlike the effects produced by rubratoxin B, however, the reduction in proteolytic activity by mercury ions was in many cases proportional to the decrease in osmotically releasable radioactivity in kidney homogenates, and in no case was proteolytic activity completely inhibited. Carbon tetrachloride had no effects on these processes in mouse liver and only a transient effect in the kidney. Carbon tetrachloride is a potent hepatotoxin producing effects on the endoplasmic reticulum much like rubratoxin. The hepatotoxic effects of carbon tetrachloride, unlike rubratoxin, apparently do not involve lysosomal damage.

Acknowledgement—This investigation was supported by U.S. Public Health Service Grants ES 00464 and ES 00591 from The National Institute of Environmental Health Sciences. The authors wish to acknowledge the capable assistance of Mrs. Judith E. Barnes, Miss Judith A. Cain and Mrs. Barbara C. Eller.

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