PHARMACOKINETIC STUDIES ON THE HEPATOTOXICITY
OF LUTEOSKYRIN. (1) INTRACELLULAR DISTRIBUTION
OF RADIOACTIVITY IN THE LIVER OF MICE
ADMINISTERED ^3H -LUTEOSKYRIN

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Abstract—Intracellular distribution of the radioactivity derived from ^3H -luteoskyrin in mouse liver was investigated. It was revealed that luteoskyrin has a high affinity to mitochondria and cell debris of mouse liver cells. This characteristic distribution pattern in the liver cells may be responsible for the mitochondrial impairment and the age and sex differences in the susceptibility of mice to this mycotoxin.

Luteoskyrin, one of the anthraquinoid metabolites of *Penicillium islandicum* Sopp, affects selectively the livers of mice and rats and causes both acute and chronic disorders including centrolobular necrosis, fatty metamorphosis, cirrhosis and hepatoma depending upon the dose and duration of administration (1-3).

In order to elucidate the processes of development of the acute injury into a chronic intoxication, the authors performed toxicological studies of the acute stage of luteoskyrin intoxication, and found that this mycotoxin exhibits varying degrees of toxicity depending on the species, strain, sex and age of experimental animals; the susceptibility of mice is higher than that of rats, and in mice it is higher in males and infants than in females and adults (4).

Pharmacokinetic studies with ^3H -luteoskyrin (5) showed that the hepatotoxicity in mice is ascribable to the selective concentration of the mycotoxin in the liver. Biochemical investigations revealed further that luteoskyrin acts as an inhibitor of the respiratory enzyme system (6) and the coupled phosphorylation (7) of liver mitochondria, and binds to DNA (8-10) with resultant suppression of RNA synthesis (11).

In an attempt to confirm the possibility that luteoskyrin or its metabolites directly attack the cellular components of the liver to cause the above mentioned biochemical alterations in the course of the intoxication, intracellular distribution of the radioactivity derived from ^3H -luteoskyrin in the liver of mice of different ages and sexes was examined. It was found that the large portion of the tritium label taken up by the liver was distribut-

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ed in the fractions of mitochondria and cell debris and the content in these fractions was higher in male and suckling mice than in female and adult animals in accordance with the differences in their susceptibility to this mycotoxin.

MATERIALS AND METHODS

DDD-strain mice bred in the laboratory of Tokyo University of Science were used in all the experiments. Adult animals were housed in groups of two per cage and each group was given 9 g of commercial pellets (CE-2, Nihon Clea Ltd.) a day and water *ad libitum*. Sucklings at 2-weeks-old were housed in a cage with their dam.

³H-luteoskyrin

Luteoskyrin (LS) was extracted from the fungus mat of *P. islandicum* Sopp and purified by Tatsuno's method modified by Ueno and Ishikawa (12), and ³H-labelling of LS was kindly conducted by Dr. T. Komai (National Institute of Health, Tokyo, Japan). Specific radioactivities of the labelled compound are given under the table for each experiment.

Administration of ³H-LS and preparation of the subcellular fractions

³H-LS suspended in olive oil was injected s.c. into the mice. Under ether anesthetic, about 500 mg of the liver was excised and chilled in ice cold saline. The chilled liver tissue was weighed and homogenized in 0.25 M sucrose to make a 10% homogenate. The homogenate was centrifuged at 2,500 rpm for 10 min twice, 6,500 × g for 10 min twice, 15,000 × g for 10 min, and 105,000 × g for 60 min to separate mitochondrial, light mitochondrial, microsomal and supernatant fractions, respectively. The remaining liver was perfused *in situ* via the portal vein with ice cold 0.25 M sucrose containing 3.3 mM CaCl₂ and then removed. The perfused liver was homogenized in twelve volumes of 2.2 M sucrose containing 3.3 mM CaCl₂ in a Potter-Elvehjem type glass homogenizer. The homogenate was filtered through four layers of gauze and centrifuged at 40,000 × g for 90 min in a Hitachi 65P preparative ultracentrifuge. The pellet obtained was suspended in 0.34 M sucrose and centrifuged at 2,000 rpm for 10 min. This pellet was then used as the nuclear fraction.

Measurement of radioactivity

Each fraction was suspended in water and 0.1 to 0.3 ml of the suspension was pipetted into a vial. After 0.1 ml of Hyamine was added to solubilize the sample, 10 ml of the ANPO solution (naphthalene 73.9 g, 2,5-diphenyloxazole 4.6 g, 2-(1-naphthyl)-5-phenyloxazole 0.46 g, xylene 350 ml, dioxane 350 ml, ethanol 210 ml) was added and the radioactivity was counted with a Beckman LS 200 type liquid scintillation counter.

The protein content of each fraction was assayed by the methods of Biuret and Lowry (13).

RESULTS

Adult male mice

Radioactivities detected in the homogenate and subcellular fractions of the livers of male mice injected s.c. with ³H-LS are shown in Table 1. Radioactivities recovered from the li-

TABLE 1. Intracellular distribution of radioactivity in the livers of male mice subcutaneously injected with ^3H -LS

Time after ³ H-LS injection	No. of mice	Radioactivity in liver							% of the injected dose	LS content (μg/g)
		Homogenate	Nuclei	(× 10 ³ dpm/g)						
				Mito-chondria	Light mito-chondria	Micro-somes	Super-natant	Cell debris		
15 hr	1	752.2	3.9	351.6	34.6	34.8	39.2	328.0	1.74	26
	2	589.5	0.6	257.0	28.7	22.2	36.6	245.0	1.64	21
	3	528.0	1.4	230.0	22.5	23.6	43.9	208.0	1.38	18
	(M)	623.2	2.0	279.5	28.6	26.9	39.9	260.3	1.59	22
23 hr	1	815.6	1.4	413.7	33.0	21.4	53.3	318.3	1.82	28
	2	774.7	0.3	407.9	23.0	12.8	57.0	278.2	1.67	27
	3	1,704.4	15.3	779.5	87.9	49.0	85.4	584.0	3.64	59
	(M)	1,098.2	5.7	533.7	48.0	27.7	65.2	393.5	2.38	38
1 week	1	413.5	2.7	149.5	21.9	29.0	49.5	109.5	1.33	14
	2	255.5	2.1	87.9	9.2	9.2	41.9	92.3	0.82	9
	(M)	334.5	2.4	118.7	15.6	19.1	45.7	100.9	1.08	12

2.25 mg/0.3 ml/A of ^3H -LS (Sp. Act. $2.88 \times 10^4 \text{dpm}/\mu\text{g}$) suspended in olive oil were injected s.c. into DDD male mice (10-weeks). M : Mean value.

vers were 1.59% and 2.38% of the total dose administered 15 hr and 23 hr after the injection, respectively. Though the maximum radioactivity detected in the liver was only about 2% of the dose, 1.08% of the dose, corresponding to a half of the maximal level, remained even one week after the injection.

Assuming that all the radioactivities counted were ^3H -LS, the content of LS was 38 μg per g wet liver at the maximum and 12 μg one week after the administration.

Among the subcellular fractions the mitochondrial fraction had the highest radioactivities representing 40 to 50% of the total liver radioactivity. Thirty to 40% of the liver radioactivity was recovered from the cell debris fraction. The microsomal fraction contained only 2 to 6% of the liver radioactivity. The percent radioactivity of the supernatant fraction increased with time after the injection (Table 2).

The specific radioactivity per mg protein calculated from the above data and the protein content is shown in Table 3. The highest specific radioactivity was seen in the mitochondrial fraction followed by the cell debris, nuclear and light mitochondrial fractions.

TABLE 2. Percent radioactivities in the subcellular fractions of the livers of male mice subcutaneously injected with ^3H -LS

Time after ^3H -LS injection	Mitochondria	Light mitochondria	Microsomes	Supernatant	Cell debris
15 hr	43.1	4.6	4.3	6.6	41.5
23 hr	50.5	4.1	2.4	8.5	36.5
1 week	40.1	5.0	5.9	15.5	34.0

Calculated from the mean values in Table 1.

TABLE 3. Specific radioactivities of the subcellular fractions of the livers of male mice subcutaneously injected with ^3H -LS

Time after ^3H -LS injection	Specific radioactivity (dpm/mg protein)					
	Nuclei	Mitochondria	Light mitochondria	Microsomes	Supernatant	Cell debris
15 hr	1645	5298	1132	780	278	1898
23 hr	605	6027	737	1185	165	752
1 week	832	3553	830	488	315	793

Calculated from the values in Table 1 and the protein contents.

The supernatant fraction showed lowest radioactivities, about one tenth to one fortieth that observed in the mitochondrial fraction.

The specific radioactivity in the mitochondrial fraction was highest at 23 hr after the administration and decreased to a half after one week, although it was still higher than those of the other fractions. The highest specific radioactivity of the nuclear fraction was seen at 15 hr after the injection. It corresponded to one third that of the mitochondrial fraction measured at the same time.

Adult female mice

The intracellular distribution of radioactivity in the livers of adult female mice is shown in Table 4. The amount of radioactivity in the livers taken 15 hr and 23 hr after the injection was 0.80% and 0.78%, respectively, of the total dose administered. It decreased to 0.20% one week after the injection. The distribution pattern in the female mice differed

TABLE 4. Intracellular distribution of radioactivity in the livers of female mice subcutaneously injected with ^3H -LS.

Time after ^3H -LS injection	No. of mice	Radioactivity in liver							% of the injected dose	LS content ($\mu\text{g/g}$)
		Homogenate	Nuclei	($\times 10^3$ dpm/g) Mito- chondria	Light mito- chondria	Micro- somes	Super- natant	Cell debris		
15 hr	1	341.3	1.1	78.5	13.4	35.9	78.5	135.0	0.95	12
	2	314.5	2.0	71.5	13.7	33.7	80.6	115.0	0.82	11
	3	205.6	0.7	42.0	13.1	17.8	51.2	81.5	0.62	7
	(M)	287.1	1.3	64.0	13.4	29.1	70.1	110.5	0.80	10
23 hr	1	385.8	3.3	138.8	16.1	20.0	105.1	101.7	0.81	13
	2	517.8	0.5	203.0	14.3	17.8	99.6	215.3	1.19	18
	3	176.6	0.5	41.3	6.3	11.4	68.3	75.3	0.33	6
	(M)	359.9	1.4	127.7	12.2	16.4	91.0	130.9	0.78	13
1 week	1	76.2	0	14.3	4.4	7.9	26.4	22.2	0.19	3
	2	132.5	0	25.2	4.9	10.2	40.3	44.3	0.30	5
	3	40.5	0	7.3	2.6	3.8	15.0	8.3	0.11	1
	(M)	83.1	0	15.6	4.0	7.3	27.2	24.2	0.20	3

2.25 mg/0.3 ml/A of ^3H -LS (Sp. Act. 2.88×10^4 dpm/ μg) suspended in olive oil were injected s.c. into DDD female mice (10-weeks). M: Mean value.

TABLE 5. Percent radioactivities in the subcellular fractions of the liver of female mice subcutaneously injected with ^3H -LS

Time after ^3H -LS injection	Mitochondria	Light mitochondria	Microsomes	Supernatant	Cell debris
15 hr	22.0	4.9	10.0	24.5	38.6
23 hr	31.3	3.3	4.7	25.9	34.6
1 week	19.6	5.6	9.6	35.8	29.0

Calculated from the mean values in Table 4.

TABLE 6. Specific radioactivities of the subcellular fractions of the liver of female mice subcutaneously injected with ^3H -LS

Time after ^3H -LS injection	Specific radioactivity (dpm/mg protein)					
	Nuclei	Mitochondria	Light mitochondria	Microsomes	Supernatant	Cell debris
15 hr	972	1460	235	818	462	657
23 hr	790	1428	323	817	238	258
1 week	136	363	140	185	135	132

Calculated from the values in Table 4 and the protein contents.

from that in the males: the percent radioactivity in the mitochondrial fraction was lower in the females than in the males, whereas the radioactivity in the supernatant fraction was higher in female mice than in male mice at all times (Table 5). As shown in Table 6, highest specific radioactivities were also seen in the hepatic mitochondrial fractions of the female mice, but they were only one fourth those of the males 15 hr and 23 hr after the injection, respectively. The specific radioactivity of the microsomal fraction was higher than those of the cell debris, supernatant and light mitochondrial fractions at all times.

Suckling mice

The radioactivities of the livers from two-week-old mice injected with 0.07 ml of ^3H -LS solution (71 $\mu\text{g}/0.1$ ml) per animal were compared with those of adult mice given 0.3 ml of the same solution per animal. The latter dose corresponded to one tenth the dose administered to the adult mice in the previous experiments. The radioactivities in the livers of the sucklings were 1.62% in male and 1.43% in female mice of the administered dose, and those in the adults were 0.26% in male and 0.19% in female mice (Table 7). The result indicates that, when the dose per kg was the same in sucklings and adults, the amount of radioactivity in the livers of the sucklings was several times higher than that of the adults. In sucklings, the percent radioactivities in the mitochondrial fractions were lower than those in the supernatant fractions. In adult mice, about 30% of the liver radioactivity was distributed in the fractions of mitochondria, supernatant and cell debris. When the specific radioactivities in the subcellular fraction were compared between the sucklings and the adults, the former were up to ten times higher than the latter (Table 7). The highest specific radioactivity was seen in the mitochondrial fraction from both the sucklings and the adults (Table 7). There was no great sex difference between the sucklings and the adults (Table 7).

TABLE 7. Effect of age on accumulation and intracellular distribution of radioactivity in the liver of mice subcutaneously injected with ^3H -LS.

Age (week)	Sex	Radioactivity in liver			Percent of liver radioactivity				
		($\times 10^3$ dpm/g)	LS content ($\mu\text{g/g}$)	% of the injected dose	Mito- chondria	Light mito- chondria	Micro- somes	Super- natant	Cell debris
2	Male	149.6	1.2	1.62	20.3	6.0	5.1	32.4	36.2
	(13)*				820	590	240	447	—
	Female	132.0	1.0	1.43	18.8	5.3	6.2	32.3	37.4
	(9)				660	390	202	306	—
10	Male	72.2	0.6	0.26	32.6	5.9	6.8	27.0	31.0
	(3)				185	78	55	49	—
	Female	52.8	0.4	0.19	26.7	7.2	8.1	32.7	25.3
	(3)				79	52	42	37	—

Bold-type letters : Specific radioactivity (dpm/mg protein).

* () Number of mice used.

Male and female mice of DDD strain (2 and 10 weeks) were used.

An olive oil solution of ^3H -LS (Sp. Act. 1.3×10^5 dpm/ μg , 71 μg /0.1 ml), 0.07 ml for sucklings and 0.3 ml for adults, was injected s.c. 15 hr prior to sacrifice.

DISCUSSION

In agreement with the previous result (5), the low absorption of luteoskyrin in mice was also demonstrated in the present study: the highest content of the ^3H -compounds (^3H -luteoskyrin and its metabolites) in the liver was 2.38% of the dose in the male and 0.80% in the female (Tables 1 and 4). The different percent radioactivities between the male and the female (Tables 1 and 4) or between the suckling and adult (Table 7) indicated that the content of the ^3H -compounds in the liver varied with age and sex.

As revealed in the preceding experiments, the toxic effect of luteoskyrin upon the liver was marked in the young as compared with adult mice and also more marked in the male than the female (4). From both the present and previous observations, it may be concluded that higher contents of the ^3H -compounds are detected in the liver of animals more susceptible to this mycotoxin. Therefore, the high susceptibility of mice to the hepatotoxic mycotoxin may be attributable to the specific concentration of this mycotoxin or its metabolites in the hepatic cells.

As for the distribution of the ^3H -compounds in the hepatic cells, a large portion of the radioactivity was detected in the mitochondrial fraction (Table 1, 4 and 7). The specific radioactivity in this fraction was much higher than that in the other fractions including the microsomes and supernatant (Table 3, 6 and 7). This fact suggests that luteoskyrin or its metabolites have a high affinity to certain mitochondrial components. It was found that more than 80% of the total radioactivity detected in the mitochondrial fraction was derived from ^3H -luteoskyrin itself, therefore it is most likely that the ^3H -compounds described here correspond to ^3H -luteoskyrin (To be published in the succeeding paper). Such being the case, luteoskyrin itself may have a selective, high affinity to mitochondria of the liver. This high affinity of luteoskyrin to mitochondrial components was evident irre-

spective of age and sex (Table 3, 6 and 7).

Based on the electronmicroscopic findings of the swelling of rat liver mitochondria induced by luteoskyrin (14, 15), the light mitochondrial fraction which may include the swollen mitochondria, was anticipated to show a high radioactivity. However, neither the total radioactivity nor the specific radioactivity was high enough to indicate a specific concentration of the ^3H -compounds in this fraction.

According to the previous work, luteoskyrin inhibits activities of the respiratory enzyme system and the coupled phosphorylation of rat liver mitochondria *in vitro*. A suppression of these activities was also demonstrated in mice and rats which had been administered this mycotoxin (6, 7). The concentration of luteoskyrin into the mitochondrial fraction as shown in the present study supports the authors' hypothesis that luteoskyrin impairs the energy producing system of the mitochondria during the course of acute liver injury.

A large part of the liver radioactivity was found in the fraction of cell debris, and the specific radioactivity in this fraction changed in parallel with the susceptibility of mice to this mycotoxin. These findings suggest the important role of some component of the cell debris in the occurrence of the liver cell damage by this mycotoxin. Since this fraction includes the nuclei and the cell membrane, luteoskyrin may have some effect on these cellular components.

As for the effect on the nuclei, an impairment by this mycotoxin had been demonstrated in the pathological and biochemical observations of karyorrhexis (1) and an enhanced incorporation of ^3H -thymidine (16) in the acutely poisoned liver. The binding of luteoskyrin to DNA (8-10) and suppression of the RNA synthesis (11) *in vitro* were further demonstrations of the possible effect on cell nuclei. These observations suggest the high affinity of luteoskyrin to the cell nucleus. Although a relatively high specific radioactivity was obtained in the nuclear fraction, the amounts of the nuclear fraction separated in these experiments were too small for a detailed analysis. More precise experiments are now being carried out.

As for the cell membrane, an inhibitory effect of luteoskyrin on the Na-K-ATPase activity *in vitro* was observed in a previous work (17). Luteoskyrin may also affect the cell membrane, but it is still obscure whether this large amount of the ^3H -compounds accumulates in the cell membrane or in other parts of the cell debris.

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