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Biochemical Studies on Oral Toxicity of Ricin. I. Ricin Administered Orally Can Impair Sugar Absorption by Rat Small Intestine

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Rats intoxicated orally with ricin (30 mg/kg) showed clear signs of sickness and all died within 36 h. Dying animals shivered and lost body weight. The animals suffered from severe diarrhea within 5—10 h and profuse watery purging. In all intoxicated animals, large amounts of watery fluid were seen in the small intestine, but no significant changes in other organs were observed on gross examination.

The effect of oral administration of ricin (30 mg/kg) on D-glucose absorption by rat small intestine was examined by the *in vitro* everted sac method. The amount of D-glucose absorbed by the small intestine derived from ricin-treated rats was 61.8 μg per 100 mg of tissue per hour or 30% of that of normal rats (206 μg/100 mg of tissue/h) at 5 h after intoxication. Absorptions of D-galactose, D-mannose, and 3-*O*-methylglucose by the small intestine of ricin-treated rats were 66, 46, and 80% of those of the normal intestine, respectively.

Light microscopic examination revealed significant changes such as loss of villi and delay of the regeneration of absorptive epithelials of the small intestine at 5 h after administration.

From these results, it was inferred that ricin acts primarily on the intestinal mucosa and impairs sugar absorption.

Keywords——ricin; phytotoxin; oral toxicity; sugar absorption; everted sac method; D-glucose

Ricin is a highly toxic protein that occurs in the seeds of *Ricinus communis* L. (Euphorbeaceae). The toxin has been purified and studied extensively and the mechanism of action at the molecular level is now understood.[1,2] Ricin is a potent inhibitor of protein synthesis by interference with ribosomal function. It is a phytotoxin which in many respects resembles bacterial toxins such as tetanus and diphtheria toxins.[3] There has been no report on the oral toxicity of ricin in animals, however. It is of interest to know why ricin exerts its toxic action after oral administration even though it is a proteinous substance which would be degraded in the stomach and intestine. We have therefore investigated the effect of ricin administered *p.o.* on the absorption of sugars by rat small intestine *in vitro*.

Experimental

Ricin was prepared from the seeds of castor bean (*Ricinus communis* L.; large grains, imported from Thailand) according to the method described by Hara *et al.*[4] The ricin preparation thus obtained was shown to be homogeneous by polyacrylamide gel disc electrophoresis and its intraperitoneal minimal lethal dose (MLD) was 0.6—1.2 μg/kg of body weight in mice.

Male albino rats of the Wistar strain (body weight, 200—250 g) and male mice of ddY strain (20—25 g) were used. Animals were fed with a commercial chow *ad libitum* before initiation of the experiments. Thereafter they were fasted for 24 h but allowed water *ad libitum*.

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Everted sacs from the jejunal portion of the small intestine were prepared according to the original method of Wilson and Wiseman.[5] Briefly, an animal was killed by a blow on the head, the abdomen was opened, and the intestine was washed out *in situ* with 0.15 M sodium chloride. The small intestine, next, was stripped off its mesentery while being pulled from the abdomen. After the freed intestine had been everted over a long stainless steel probe, segments of jejunum were filled with approximately 1 ml of fluid medium (Krebs–Ringer bicarbonate solution, KRBS, pH 7.4) and each end was tied off to form an individual sac 2—4 cm long and 0.5 to 1 cm in diameter. Sacs were incubated singly in 30 ml Ehlenmeyer flasks containing additional medium. Since it is in contact with the mucosal epithelium, this medium in the flask is termed mucosal fluid, while that in the lumen of the everted sac is termed serosal fluid. After being gassed with 5% CO_2 and 95% O_2, the sacs were incubated at 37 °C for 1 h.

Determination of D-Glucose Absorbed——D-Glucose absorbed was determined colorimetrically by the *o*-toluidine-boric acid method[6,7] and the results are expressed as μg D-glucose absorbed per 100 mg tissue per hour. In some cases, D-glucose was determined enzymatically with Glucose C-Test (Wako Chemicals, Japan), and the accuracy of the colorimetric method was confirmed. Determinations of D-galactose, D-mannose and 3-*O*-methylglucose were also carried out by the *o*-toluidine-boric acid method. If color intensity of D-glucose is taken as 100, those of D-galactose, D-mannose and 3-*O*-methylglucose were 150, 90 and 87, respectively. In the case of 3-*O*-methylglucose, sugar concentrations in both the serosal and mucosal fluids were determined as described above and the results were analyzed in terms of S/M ratio, where S and M are the sugar concentrations of the serosal and mucosal fluids, respectively. In the everted sac method employed throughout this study, the volumes of the serosal solution before and after the incubation were 0.57 ± 0.01 ml in the case of the normal intestine ($n=30$) and 0.52 ± 0.02 ml in the case of ricin-intoxicated intestine ($n=30$).

Absorption of Ricin by the Small Intestine——The amount of ricin transported from the mucosal to the serosal fluid in the *in vitro* everted sac method was assayed by radioimmunoassay employing rabbit anti-ricin antiserum (a kind gift from Professor Toshitaka Koga, School of Dentistry, Kyushu University) and ^{125}I-labeled ricin. Antigen-antibody complex was separated by the double antibody method utilizing goat anti-rabbit 7S-gamma-globulin (Hyland Corp.). We also assayed the amount of ricin absorbed by rats *in vivo*. In this case, ricin (30 mg/kg) was administered orally to rats, and the serum was obtained after 5 h and analyzed by radioimmunoassay as described above.

Light Microscopic Examination——The removed organs were fixed in 10% formalin. The segments taken from the small intestinal loops for histological study were opened carefully and also fixed in formalin. Thin sections were stained with hematoxylin–eosin and periodic acid-Schiff stain.

Results

Study in Mice and Rats

Groups of 8 mice received *p.o.* various doses of ricin (0.01—0.06 mg/g body weight of mouse) and the animals were observed every 12 h for clinical signs of toxicity and death. Animals began to die after 20—36 h; MLD was about 30 mg/kg body weight of mouse, which is 1/500 of the toxicity after *i.p.* or *i.v.* injection. Even after a supralethal dose no animal died before about 15 h. During the first few hours after administration of the toxin, the animals appeared unaffected. Then, after a time that depended on the dose, they showed clear signs of sickness. The animals shivered and appeared to have reduced body temperature. After ricin treatment, the animals lost appetite and stopped drinking water. All the animals suffered from severe diarrhea within 5—10 h. In all intoxicated animals, the intestine was filled with a large amount of watery fluid, but no other significant changes were seen on gross examination.

Effect of Ricin on Absorption of Sugars by the Small Intestine *in Vitro* (Everted Sac Method)

Glucose absorption, one of the major functions of the small intestine, was markedly impaired, as shown in Fig. 1. Although no effect was seen when the sac from normal rat was incubated with 0.5% glucose in the presence of ricin (Fig. 1, I), about 70% impairment of glucose uptake was observed when the small intestine derived from ricin-intoxicated rat was employed (Fig. 1, II) (206.3 ± 8.3 *vs.* 61.8 ± 4.1 μg/100 mg tissue/h). Figure 2 shows the effect of time after ricin poisoning on glucose absorption by rat small intestine. The decrease in glucose absorption became significant at 5 h and reached maximum at 10 h, and did not recover until death. Galactose and mannose absorptions were also impaired, as shown in Fig.

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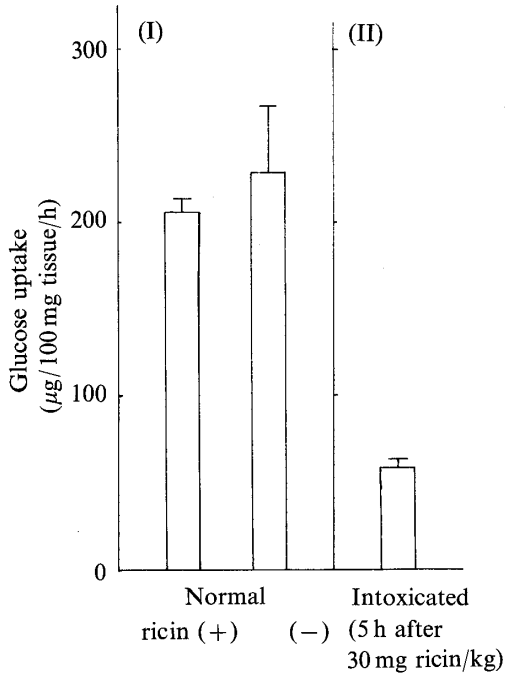

Fig. 1. Effect of Ricin on Glucose Absorption *in Vitro* by Rat Small Intestine

The effect of ricin on glucose transport was investigated by the *in vitro* everted sac method. The sacs were prepared from normal rats (Experiment I) and from rats treated with 30 mg ricin/kg, *p.o.* (Experiment II). Normal sacs were placed in 5 ml of mucosal solution [0.5% D-(+)-glucose in KRB solution, pH 7.4] containing either 0.05% ricin (+) or not (−). Sacs from the intoxicated rats were incubated with 0.5% glucose in KRB solution. All sacs were incubated singly at 37 °C for 1 h under gassing with 5% CO_2 and 95% O_2. Glucose in the serosal solution was determined colorimetrically as described in the text. Each value is the mean ± S.E. of 6 experiments.

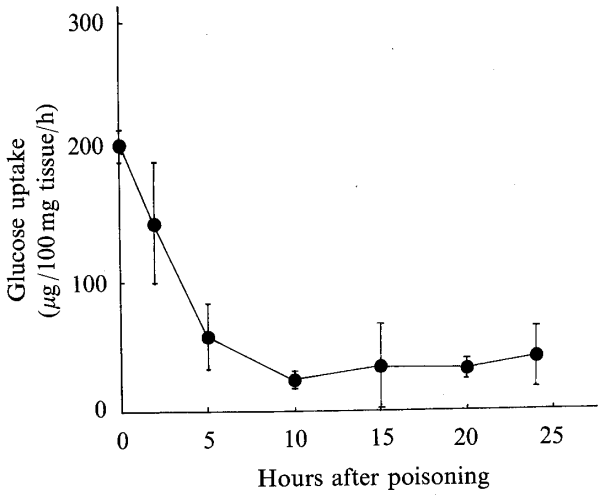

Fig. 2. Effect of Ricin Poisoning on Glucose Absorption

Eight groups of rats were given ricin (30 mg/kg) *p.o.*, and killed at 0(6), 2(4), 5(6), 10(2), 15(3), 20(2), and 24(2) h, respectively. (numbers in parentheses are numbers of rats killed). The everted sacs were prepared from the jejunum portion of the small intestine, and tested for glucose absorption as described in the text. Each value is the mean ± S.E.

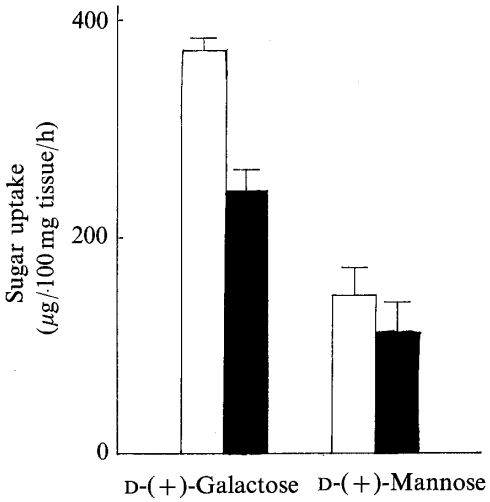

Fig. 3. Effect of Ricin Poisoning on Sugar Absorption

The everted sacs were prepared as described in the legend to Fig. 1. The sacs were incubated separately with either 0.5% D-(+)-galactose or 0.5% D-(+)-mannose as the mucosal solution. Each value is the mean ± S.E. of 2 experiments. □, normal; ■, ricin-treated.

3 (galactose 373.9 ± 11.9 μg→245.6 ± 20.6 μg; mannose 146.6 ± 24.9 μg→67.6 ± 28.0 μg).

The effect of ricin intoxication on absorption of 3-*O*-methylglucose is summarized in Table I. The results were analyzed in terms of *S*/*M* ratio *versus* the initial concentration, as shown in Fig. 4. In the normal intestine, in the presence of Na^+, the transport of this sugar was dependent on the initial concentration up to 0.05%, but not at 0.2, 0.5 and 1%. In the ricin-intoxicated intestine, the concentration dependency was not seen. In the presence of K^+, the transport through both normal and ricin-intoxicated intestinal membranes was independent of the initial concentration. However, the amount transported through the ricin-intoxicated membrane appeared to be slightly decreased.

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TABLE I. Transport of 3-*O*-Methylglucose through Rat Intestine[a]

Initial concn.	Intestine used[b]	No. of exp.	Sugar concentration (mM)		Sugar transported (μg/100 mg tissue/h)	*S*/*M*
			Mucosal (*M*)	Serosal (*S*)		
[Na^+] System						
0.01% (0.51 mM)	N	2	10.1±0.5	5.25±0.75	12.2±2.3	0.53
0.05%	N	4	44.0±2.5	21.6±1.1	51.1±3.4	0.49
(2.57 mM)	R	3	37.1±0.3	12.8±1.5	11.5±1.4	0.35
0.2%	N	5	168.4±2.3	54.3±3.3	144.7±11.8	0.32
(10.3 mM)	R	3	177.8±3.8	55.7±1.3	153.0±6.2	0.31
0.5%	N	4	480.0±5.9	146.3±7.0	335.3±26.6	0.30
(25.7 mM)	R	4	486.6±5.3	142.0±1.6	290.2±7.5	0.29
1.0% (51.5 mM)	N	2	931.0±25.0	241.5±18.5	544.8±93.8	0.26
[K^+] System						
0.05%	N	2	43.5±2.0	7.25±0.75	18.6±2.0	0.17
	R	3	44.8±1.2	3.67±0.19	6.38±0.35	0.082
0.2%	N	3	176.7±3.7	20.0±5.1	46.7±16.4	0.10
	R	3	192.4±1.0	19.1±0.3	40.1±2.2	0.10
0.5%	N	3	489.2±25.2	50.9±8.0	102.7±18.5	0.10
	R	3	440.8±10.6	33.6±8.1	78.8±22.2	0.076

a) Values are means±S.E.
b) N and R represent normal and ricin-treated intestines, respectively.

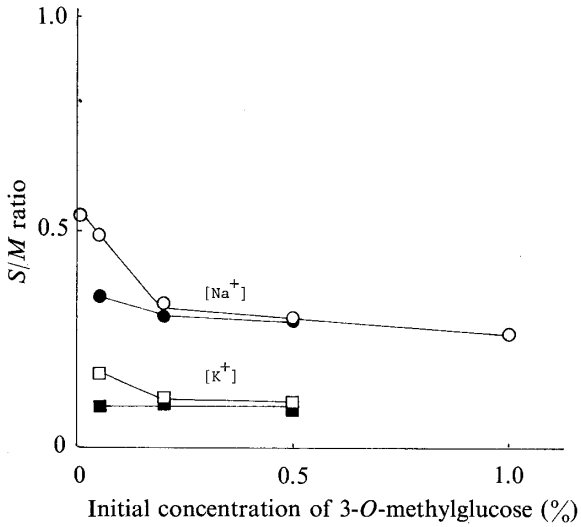

Fig. 4. Effect of Ricin Poisoning on 3-*O*-Methylglucose Transport

The everted sacs were prepared as described in the legend to Fig. 1. The sacs were incubated separately with various concentrations of 3-*O*-methylglucose. After 1 h at 37 °C, sugar concentrations of the mucosal and serosal solutions were determined colorimetrically (the results are shown in Table I). Incubation was carried out in [Na^+] and [K^+] systems; in the latter case Na^+ was replaced by K^+.

Absorption of Ricin by the Small Intestine *in Vitro*

The jejunum segment of the small intestine of normal rat was everted and filled with KRB solution. The sac was incubated with 10 ml of ricin-KRB solution (3.0 mg ricin/flask). The ricin content in the serosal solution after 60 min at 37 °C was measured by radioimmunoassay and found to be 0.006%. Ricin-intoxicated rats (30 mg ricin/kg, *p.o.*) were sacrificed after 5 h and the content of immunoreactive ricin in sera was determined by radioimmunoassay to be 0.017%.

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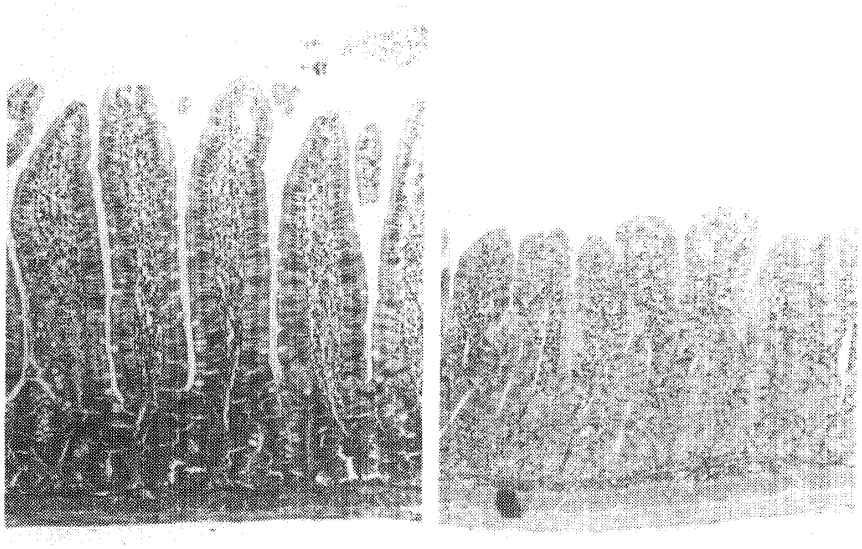

Normal Ricin-intoxicated (5 h)

Fig. 5. Photomicrographs of Rat Small Intestine

Rats given ricin (30 mg/kg), *p.o.*, were killed at 0, 2, 5, 10, and 15 h, and segments taken from the small intestinal loops were opened and fixed in 10% formalin. Thin sections were stained with hematoxylin–eosin and periodic acid-Schiff stain. The villous structures of the small intestine of the normal rat (left) and intoxicated-rat at 5 h after administration (right) are shown (× 100).

Pathological Examination

When the abdomen was opened, the serosal surface of the stomach and the intestine appeared congested. The intestinal loops were distended with watery and occasionally bloody contents. Other organs were unremarkable on gross examination. Microscopically, the mucosa of the small intestine showed various degrees of villous atrophy and inflammatory reaction. At 5 h, the villi were somewhat shortened and the glandular layers were longer. The goblet cells were empty. The number of mononuclear cells in the lamina propria seemed to be increased (Fig. 5, right). At 10 to 15 h, the atrophy of the villi became severe and desquamation of the villous epithelium occurred. The mucosa of the stomach was almost normal except for occasional superficial erosions. Other organs were also unremarkable microscopically.

Discussion

Ricin is one of the very few proteinous toxins which exert toxic action on oral administration. Numerous cases of intoxication resulting from the ingestion of castor bean seeds have been described in humans and animals.[8] Efforts have also been made to utilize ricin as an anti-tumor agent. However, no systematic studies on the oral toxicity or biodegradation of ricin have been reported.

In this study, we sought to identify which organ of the rat is the primary site of action of ricin. When rats were treated with ricin (30 mg/kg, *p.o.*), change in the villous structure of the intestinal mucosa was seen at 5 h after administration. No significant changes were seen in other organs, especially the stomach and liver. Moreover, intoxicated rats suffered from severe diarrhea and died within 36 h. These observations resemble those obtained by Koga *et al.*,[9] who reported a pathological study on the toxic action of ricin in rats. They concluded that the reticuloendothelial system of rats was selectively destroyed at 10 h after intra-

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peritoneal injection of ricin. They also noted hemorrhage and congestion in the mucous membrane of the small intestine and the lymph node, while no remarkable changes were observed in pancreas, kidney and adrenals.

These findings led us to investigate the effect of ricin on sugar absorption by rat small intestine *in vitro*. As described above, glucose absorption was affected by ricin poisoning from 5 h after oral administration. The inhibition reached maximum at 10 h, and remained until death. Absorption of D-galactose and D-mannose was also impaired.

Analysis of the results obtained with 3-*O*-methylglucose, which is known not to be metabolized during transmembrane transport, suggested that ricin inhibited the initial concentration-dependent transport but did not inhibit the concentration-independent transport. In the presence of K^+, where no active transport was expected to occur, only concentration-independent transport was observed with the normal and ricin-treated intestine. However, the amount of sugar transported through the ricin-treated membrane was slightly decreased. This is probably due to the destruction of the villous structure of the small intestine.

It is interesting to note that the time when the light microscopical changes in villous structure of jejunum appeared coincided with the time when the impairment of sugar absorption of ricin-treated small intestine was noticed.

The amount of ricin absorbed by the small intestine was determined by radioimmunoassay to be 0.006% or 180 ng ricin/rat *in vitro*, and 0.015% or 510 ng ricin/rat *in vivo* (in the latter case the content of sera was assumed to be 5% of the body weight). The amount of ricin absorbed could account for the death of the animals if it were transferred to the circulatory system as intact toxin. The nature of the absorbed ricin, however, remained unclear.

These results suggest that ricin administered orally interacts primarily with the small intestine and impairs sugar absorption.

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