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PART I
STUDIES ON THE CHOLAGOGUE SUBSTANCES OF *SETA-SHIJIMI* (*CORBICULA SANDAI* REINHARDT)

BY

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ABSTRACT

It was shown that the extract of Seta-Shijimi (*Corbicula sandai* Reinhardt) has a stimulatory effect on biliverdin excretion from liver cells in rabbit, and a method of biological determination of the cholagogue activity was established for the investigation of this stimulatory effect. Using this method, the active component(s) were shown to be heat-stable, alkaline-labile, and dialyzable in their nature. Purification of the active component(s) was carried out and finally two fluorescent substances were isolated. Injections of 10 to 100γ of these active substances caused considerable stimulations of biliverdin excretion in adult rabbit.

Introduction

From ancient times, it has been well known in Japan that "*Shijimi*" (*Corbicula* Sp.), a kind of fresh-water shell widely distributed in this country, is useful as a cure of jaundice. The shell-fish has not merely been used as a popular cure, but adopted in hospitals for the cure of hepatitis[1].

In 1933, the first experimental approach on the curing effect of *Shijimi* was made by Kayaba[2]. A cannula was inserted in the bile duct of a rabbit without anaesthesia. When an acid extract of the shell-fish was injected subcutaneously or intravenously, the volume of excreted bile increased to a level of 150 to 180% of the normal over 7 hours. In 1934, Horie reported that a similar result was obtained using the dog operated with ether-chloroform anaesthesia[3]. In both cases, the experiments were carried out within short periods after the operations. To exclude any undesirable effect of the surgical operation, Kitani[4] devised the following technique. A cannula was inserted in the gall-bladder of a dog and the bile duct was tied and shut with a thread. The dog was kept for three weeks, then the *Shijimi* extract was administrated orally. Increase of the volume and the dry weight of bile was also observed as well as the above cases. Rather recently, Hori[5] showed the increase in bile pigment concentration by *Shijimi* extract using a rabbit operated without anaesthesia.

Since jaundice is caused by the obstructions of the excretion mechanism of bile

pigment from blood into bile, it is most probable that a substance causing the stimulation of bile pigment excretion is capable of curing jaundice. The author repeated the Hori's experiment and examined further the following possibilities; determination of the cholagogue activity in terms of the increase in bile pigment concentration; purification of the active component(s). A semi-quantitative comparison between activities of different samples is found to be attainable. This biological test made it possible to fractionate the crude extract and to purify active component(s). The active factor was adsorbed on active charcoal from the crude extract and eluted with aqueous ethanol. Preliminary surveys of the nature of the active factor indicated that the factor is heat-stable, alkaline-labile, and dialyzable.

Experimental

Animal

Adult rabbits weighing 1.8 to 2.5 kg. were used throughout the experiments. They were fasted for 24 hours prior to the operation.

Surgical Operation

To a fasted rabbit 0.4 ml. of 5% pentobarbital solution per kg. of body weight was injected intraperitoneously. After 15 minutes, the animal fell into anaesthesia and was fixed on an operation-table on its back. Pelage was cut away in its abdomen and the bare skin was sterilized with tincture of iodine. As the local anaesthesia, 10 ml. of 1% procaine solution was injected subcutaniously along the median line from the end of breast bone to near navel, in which a 10 to 12 cm. section of skin was made and then the abdominal walls were cut carefully without injuring viscera and vessels. Stomach was separated from liver and bile duct was exposed. At the nearest point to duodenum, a small cut in the bile duct was made along the tube using ophthalmic scissors. A vinyl cannula was inserted in the duct and was fixed with a thread tightly. Another small cut was made in duodenum and a vinyl cannula was inserted and fixed as above. Open ends of the two cannulae were connected. In some cases, the duct leading to gall-bladder was tied with a thread and concentrated bile contained in it was removed by a syringe. The abdominal walls and the skin were sutured successively.

The vinyl cannula used in this operation was made as follows. In a vinyl tube (inner diameter 1 mm., length 20 mm.) a tightly fitted glass capillary was inserted and both ends of the vinyl tube were heated with a microburner to make a small node. The glass capillary was removed from the vinyl tube and a rubber tube (inner diameter 2 mm., length 150 mm.) was connected to one end of the vinyl cannula.

After the operation the animals were kept warm. Upon awakening from anaesthesia, rabbits started to shiver with cold and weakened.

Analysis of Bile

The sampling of bile was started 6 hours or later after the operation. Before this time, the excreted bile showed abnormally high concentrations of bile pigment and bile acids. After that, the concentrations of these components gradually decreased and settled at constant levels. In some cases, gradual increases in the concentration of bile pigment were observed within several hours after the operation and lasted over 24 hours. These rabbits were not used for the biological test.

When the pigment concentration of excreated bile settled, it was usually maintained for at least 48 hours. After this period, the pigment concentration and the turbidity of excreted bile increased.

The samplings of bile were carried out at intervals of a half hour. At each time, the excreted bile for a certain length of time (usually 1 to 2 ml. of bile was obtained in 5 to 10 minutes) was collected and its volume, pigment and bile acid concentrations were determined.

Bile pigment was determined spectrophotometrically as follows. As shown in *Fig.* 1, the spectrum of rabbit bile shows a maximum at 675 mμ (corresponding to the

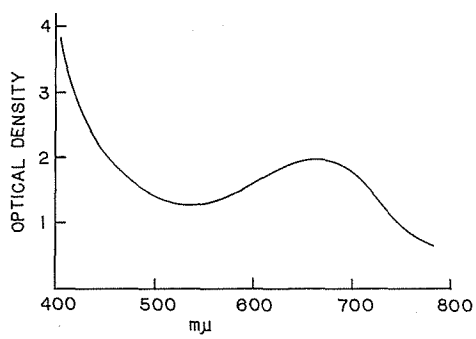

Fig. 1. Absorption spectrum of rabbit bile.

absorption maximum of biliverdin), but not at 450 mμ (corresponding to that of bilirubin). The pigment contained in rabbit bile, therefore, is biliverdin. Usually 1 ml. of bile was diluted to 4 ml. with distilled water and the absorbancy at 675 mμ was measured. Biliverdin concentration was expressed in terms of optical density at this wave length.

Cholic and deoxycholic acids were determined colorimetrically by the method of Singer *et al.*[6].

Injection of Sample

Samples were injected into muscle of femur.

Preparation of Crude Extract of Shijimi

Seta-Shijimi (*Corbicula sandai* Reinhardt) was obtained commercially. It was

boiled in an enamelled pot for 1 hour with a half weight of water. The extract was concentrated to a small volume (usually 20 ml./kg. of the shell-fish) over a small bare flame. Resulting insoluble materials were centrifuged off at 10,000 X g for 15 minutes. The brownish yellow supernate showing weak fluorescence was obtained.

Chemicals and others

Active charcoal used is Shirasagi A of the Wako Pure chemicals. Pentobarbital solution (Nembutal) was obtained from Abbott Laboratories. Procaine solution (Omnicain) was purchased from the Dai-ichi-seiyaku.

Tap water was used in all the experiments for purification. Procedures were carried out at room temperature unless otherwise specified.

Results

Effect of Shijimi Extract

When physiological NaCl solution was injected to an operated rabbit, the excreted bile showed no significant change in its biliverdin concentration as shown in Fig. 2. On the contrary, when the crude *Shijimi* extract was injected, a gradual increase in biliverdin concentration was observed in a half hour after the injection and lasted for several hours as shown in *Fig.* 2. The volume of bile remained unchanged through the increase in pigment concentration as shown in *Fig.* 3, and concentrations of bile

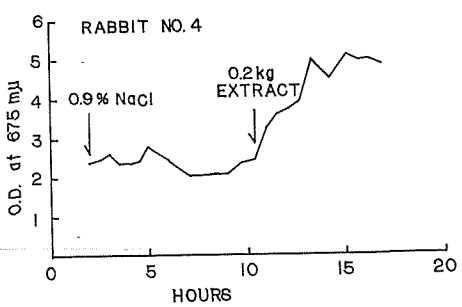

Fig. 2. Effect of the *Shijimi* extract on biliverdin concentration of rabbit bile. Eighteen ml. of 0.9% NaCl and 5 ml. of the extract (equivalent to 0.2 kg. of the shell-fish) were injected.

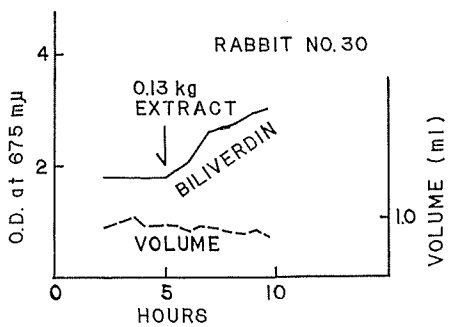

Fig. 3. Effect of the *Shijimi* extract on the volume of excreted bile of rabbit. Two ml. of the extract (equivalent to 0.13 kg. of the shell-fish) was injected.

acids also showed no appreciable change as shown in *Fig.* 4. These data indicate that the increase in bile pigment concentration is not due to a discharge of reserved bile in gall-bladder, but to a specific stimulation of biliverdin excretion from liver cells. It was confirmed by the fact that the effect of *Shijimi* extract could be observed even after the removal of gall-baldder as shown in *Fig.* 5.

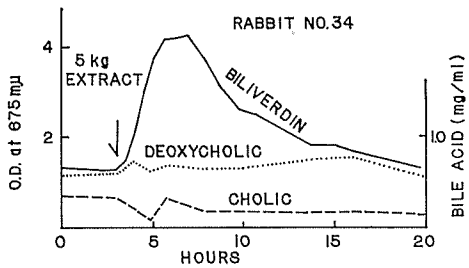

Fig. 4. Effect of the *Shijimi* extract on bile acids concentration of rabbit bile. Six ml. of the extract (equivalent to 5 kg. of the shell-fish) was injected.

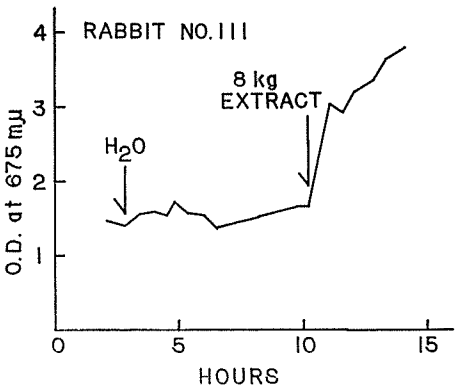

Fig. 5. Effect of the *Shijimi* extract on biliverdin concentration of rabbit bile after the removal of gall-bladder. Five ml. of distilled water and 5 ml. of the extract (equivalent to 8 kg. of the shell-fish) were injected.

Table 1 Difference in Sensitivity of Rabbits to *Shijimi* Extract
Experimental conditions are as described in the text.

Animal No.	Dose (kg. equivalent)*	Increase in biliverdin concentration (Units)
8	0.2	8.8
19	0.04	0
	0.2	1.0
20	0.1	0
	0.2	3.0
21	0.4	10
28	0.64	50
29	0.064	7.5
30	0.13	20
32	3.9	0
34	0.85	0
	2.3	17
35	0.05	0
	0.1	7.5
49	0.4	21

* Dose was expressed in terms of the weight of the original shell-fish.

As shown in Table 1, the variation in the rate of the increase in biliverdin concentration caused by a certain dose of the *Shijimi* extract was too large to make comparisons among data obtained by different animals. Limiting to an individual, however, the higher the rate of the increase was observed, the higher the dose to be injected as shown in *Fig.* 6. Therefore, a semi-quantitative comparison between activities of two samples was found to be attainable.

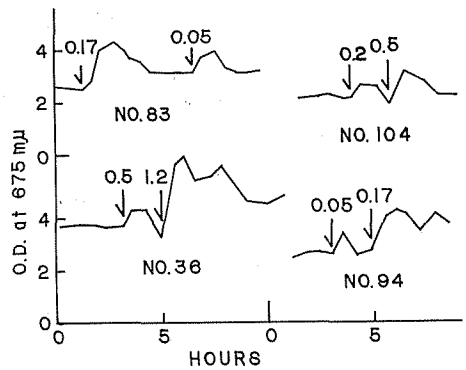

Fig. 6. Relation of the rate of increase in biliverdin concentration to the amount of the injected extract. The indicated amount (kg. equivalent: see foot-note in Table 1) of the extract was injected at the time pointed by an arrow.

For the convenience of expression of the biological data, 10% increase in biliverdin concentration lasted for 1 hour was defined as 1 unit. Thus the number of unit corresponds to the increased area on the figures. This assay method is capable of detecting significant difference only when there are 3-fold changes in dose of active factor as shown in Fig. 6.

Charcoal Fractionation of Crude Extract

One g. of active charcoal was well suspended in 20 ml. of crude extract (equivalent to 0.8 kg. of *Shijimi*) and kept for 15 minutes. The suspension was centrifuged at 3,600 X *g* for 15 minutes and the supernatant solution was saved (charcoal supernate fraction). The charcoal was washed twice with 50 ml. of water and the washings were combined and concentrated on a boiling water bath to 20 ml (charcoal washing fraction). The washed charcoal was suspended in 40 ml. of 50% aqueous ethanol and kept for 15 minutes with occasional stirring. The charcoal was separated by centrifuging at 3,600 X *g* for 15 minutes and the supernatant was evaporated to dryness. The resulting brown syrupy residue was dissolved in 20 ml. of water (charcoal eluate fraction). In another experiment, ammoniac ethanol (1 part of 28% NH_4OH was added to 100 parts of 50% aqueous ethanol) was used instead of neutral aqueous ethanol. The eluate was evaporated and redissolved in 20 ml. of water (ammoniac eluate fraction). Biological activities of these fractions were tested, the results are shown in Table 2. The experimental results indicate that the active component(s) contained in the crude extract were adsorbed on charcoal and were eluted with aqueous ethanol. When ammoniac ethanol was used in place of neutral ethanol, no activity was appeared in the eluate. This data consists with the instability of the active component(s) under

Table 2 Charcoal Fractionation of Crude Extract
Experimental conditions are as described in the text.

Animal No.	Fraction	Dose (kg. equivalent)*	Increase in biliverdin concentration (units)
8	Charcoal supernate	0.2	0
	Charcoal eluate	0.2	5.6
51	Charcoal washing	1.0	0
	Charcoal eluate	0.2	16
	Ammoniac eluate	0.46	0

* See foot-note in Table 1.

Table 3 Properties of Active Component (s)
Experimental conditions are as described in the text.

Animal No.	Treatment	Dose (kg. equivalent)*	Increase in biliverdin concentration (units)
51	None	0.2	16
	2% NH_4OH, 100°C	0.5	0
	50% AcOH, 100°C	0.25	12
	50% AcOH, boiling	0.25	0
50	Dialysis {Dialyzate	1.0	10
	Dialysis {Outer solution	1.0	27

* See foot-note in Table 1.

alkaline condition presented below.

Properties of active component(s)

The fact that the activity can be extracted with boiling water and be reserved throughout the concentration process at high temperature indicates the high stability of active component(s) in neutral aqeous solution.

To test the stabilities in acid and alkaline conditions, the following samples were prepared and their biological activities were examined. (1) To 5 ml. of charcoal eluate (equivalent to 0.5 kg. of the shell-fish) 4 ml. of 28% NH_4OH and 200 ml. of water were added and the mixture was evaporated at 100°C. The residue was redissolved in 2.5 ml. of water and neutralized to pH 7 with 1 N acetic acid. (2) To 5 ml. of charcoal eluate 200 ml. of 50% acetic acid was added. A half of the mixture was evaporated at 100°C and the other half was evaporated at its boiling temperature. The residues were redissolved in each 2.5 ml. of water and neutralized to pH 7 with 1 N NaOH. As shown in Table 3, the activity is more unstable in alkaline condition than in acidic one.

Charcoal fraction (3.4 ml., 1 kg. equivalent) was dialyzed against 600 ml. of distilled water for 24 hours in the cold using Visking cellophane tube. The dialyzate and the outer solution were concentrated to 3 ml. and their cholagogue activities were

tested. As shown in Table 3 the outer solution showed higher activity than the dialyzate. This indicates that the active component(s) have rather low molecular weight.

Discussion

Kayaba[2], Horie[3], and Kitani[4] reported that the amount of excreted bile increased upon injection of the *Shijimi* extract to muscle. In addition to these, Kitani[4] found an increase in the dry weight of bile. Nevertheless, concentration of the bile pigment was not examined by these authors. Hori[5] reported that the concentration of pigment increased upon injection of the *Shijimi* extract, although the volume of excreted bile did not show any significant change. The author's experiments confirmed the Hori's results, and no increase in volume was observed in all cases. The discrepancy still remains unclarified.

Kayaba[2] showed that the cholagogue effect of *Shijimi* could not be observed in rabbits which both vagus nerves were cut at their necks. This result is quite interesting, because it suggests that the cholagogue factor does not act on liver cells directly, but acts on vagus nerve, which controls the formation and excretion of bile in liver by as yet unknown mechanisms.

It is a traditional knowledge that a bathing in the extract of *Shijimi* is effective for the cure of jaundice as well as the oral use. This suggests that the active factor is absorbable into body through skins and may be a substance with a low molecular weight. The dialyzability of the activity showed in the present experiment agrees with the above tradition.