TOXICITY OF A LIPOPROTEIN AND LIPIDS FROM THE ROE OF A BLENNY, DINOGUNELLUS GRIGORJEWI HERZENSTEIN

TOXICITY OF A LIPOPROTEIN AND LIPIDS FROM THE ROE OF A BLENNY, *DINOGUNELLUS GRIGORJEWI* HERZENSTEIN*

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Introduction

Among the fish roe poisonings which manifest gastrointestinal disorders such as vomiting, abdominal pains and diarrhea, the poisoning caused by *Barbus fluviatilis* L. called "Barben cholera" is the most famous in Europe. The pioneer work by F. H. McCrudden (1) on the toxic principle in the roe of *Barbus fluviatilis* and *Esox lucius* concluded that it belongs to a "Toxalbumin".

The blenny fish, *Dinogunellus grigorjewi* Herzenstein, widely distributed in the northern sea of Japan, has been utilized as the raw materials of Kamaboko (Boiled fish paste) and Soboro (Boiled seasoned and crushed meat) or sold intact for food. The roe is generally taken and discarded, because the toxicity of it has been known by the people in Hokkaido. Howerer, when the roe is taken as a meal by mistake, it often causes several outbreaks of poisonings.

In the present report dealing with the toxic principle of the roe, the authors found that a toxic lipoprotein has a similar nature to "Ichthulin" but does not belongs to a "Toxalbumin" as stated by McCrudden and that a certain lipid has also toxicity, although the relation between both are not yet clarified.

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Experimental

1. Investigations on the Dinogunellus-roe poisoning

Recently the authors obtained two case reports as follows;

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(A) A woman aged 38. She ate the roe (about 20 g) at lunch on May 3rd, 1960. After a while, she was poisoned and obliged to enter a hospital from May 6th to 16th. Her symptoms were headache, diarrhea, nausea, fever (37—37.2°C), sensation of disorders in the abdomen, constriction of chest and dilated pupils. The diagnosis was: Erythrocytes 3.78 millions, Leucocytes 6100. Hemogram of leucocytes: Lymphocytes 40%, Monocytes 2%, Eosinophiles 2%, Neutrophiles 56% (Segment 51%, Stab 5%). Hemoglobin 77% (From Dr. Takayama, Chief of the Mori Town Hospital)

(B) A man aged 65. He ate the roe at supper on May 8th, 1961 (boiled roe at 7:00 p. m.) and in breakfast of the next morning (Miso soup with roe at 5:00 a. m.). About three hours later (8:00 a.m.) while he was working, he was poisoned acutely. His symptoms were abdominal pains, vomiting, dizziness, constriction of the chest, chill, shiver and then coma. He recovered from coma after two hours. Paralysis and cold sensation of body extremities with occasional convulsion, violent thirst. Entered a hospital at 4:30 p.m.. Somewhat indistinct conciousness, respiration normal, thready pulse, somewhat dilated pupils, cyanosis, slight pain in the neighbourhood of the navel when touched, lowering of blood pressure: 75 (max), 73 (min). He was almost in a critical condition when hospitalized. He was treated with heart stimulants and antidote but died after one hour (5:30 p.m.) (From Dr. Inoue, National Sanatorium of Yakumo, Hokkaido).

2. Seasonal changes of gonad weights of *Dinogunellus grigorjewi* and chemical composition of the roe.

The variations in gonad weights of the fish are given in Table 1. In October, the fish ovary is small and immature, while in April, it becomes very large and mature. Therefore, the authors fixed April as the sampling season.

Table 1. Seasonal changes of gonad weights of *Dinogunellus grigorjewi*.

Month	Sample numbers measured	Average body weight and sex	Average gonad weight
October	2	645 g ♀	18.2 g (2.8%)*
〃	2	720 ♂	2.6 g (0.36%)
April	17	757 ♀	172 g (22.7%)
〃	9	743 ♂	9.8 g (1.3%)

* % of gonad weight to total body weight.

Table 2. Chemical composition of Dinogunellus-roe.

Moisture	Crude fat	Crude protein*	Ash
68.93%	5.74%	23.00%	1.25%

* Total nitrogen × 6.25

The general analysis of the chemical composition of Dinogunellus-roe was carried out as shown in Table 2.

3. Characterization of the protein nature of roe poison.

(A) Effects of heating on the toxicity of the roe.

(i) Oral administration to cocks.

The fish roe was divided into two parts. One part was pulverized and dried intact by the electric fan. The other was subjected to autoclaving at 120°C for 30 minutes, pulverized and dried in the same manner. These parts were mixed respectively with commercial diet for rearing chickens and given orally to two groups of White Leghorn cocks (two cocks per one group). The results of feeding test are given in Table 3.

Table 3. Effects of heating upon the toxicity of fish roe by oral administration to cocks.

Fraction	Composition of the diet and duration intervals of leeding	Total weight of the roe given during the experiment	Survival or death
Intact air-dried roe	roe 20 g / diet* 20 g } April 20–21, 1961	360 g (until death)	One cock died on May 1.
	roe 30 g / diet* 20 g } April 22–29, 1961	520 g (until death)	Another cock died on May 6.
	roe 40 g / diet* 20 g } April 30-until death		
Autoclaved air-dried roe (120°C, 30min)	roe 20 g / diet* 20 g } April 20–21, 1961	Over 640 g	Both cocks survived.
	roe 30 g / diet* 20 g } April 22–29. 1961		
	roe 40 g / diet* 20 g } April 30–May 8, 1961		

* Commercial diet for rearing chickens.

(ii) Intraperitoneal injection into mice.

Three hundred grams of the roe were mashed in a mortar, extracted with 300 *ml* of physiological saline solution and centrifuged. Another 300 g of the roe were also mashed in a mortar after autoclaving at 120°C for 30 minutes, extracted with an equal volume of saline solution and centrifuged. One *ml* portions of both supernatants solutions were injected intraperitonerlly into two groups of mice of red strain (rr) respectively. The results obtained are shown in Table 4.

(B) Effects of dialysis upon the toxicity of roe extracts.

The roe was mashed in a mortar, extracted with an equal volume of distilled water or saline solution by shaking for one hour. After removal of the roe residue, the extracts were dialyzed in a refrigerator by the cellophane against distilled water. Dialyzates or dialyzate concentrates were injected

Table 4. Effects of heating upon the toxicity of roe extract by intraperitoneal injection into mice.

Strain of mice	Body weight and sex of mice	Treatment	Volume of extracts injected	Survival or death	Time until death after injection
red(rr)	24.0 g ♀	Autoclaved (120°C, 30 min)	1.0 *ml*	survived	—
〃	25.0 ♀	〃	〃	〃	—
〃	23.0 ♀	〃	〃	〃	—
〃	18.5 ♂	〃	〃	〃	—
〃	21.5 ♀	〃	〃	〃	—
red(rr)	24.0 ♂	Untreated cold extracts	1.0 *ml*	died	Within 23 hours*
〃	25.0 ♀	〃	〃	〃	〃
〃	25.0 ♂	〃	〃	〃	〃
〃	23.0 ♂	〃	〃	〃	〃

Remarks; Mice died within 23 hours.

intraperitoneally into mice of German strain (dd). Both of them showed no toxicity as indicated in Table 5.

Table 5. Effects of dialysis upon the toxicity of the roe extracts.

Strain of mice	Body weight and sex of mice	Dialysates injected	Survival or death
german (dd)	19.5 g ♀	Dialysate concentrates* 0.5 *ml*	Survived
〃	18.5 ♀	〃	〃
〃	22.0 ♀	〃	〃
〃	20.0 ♀	〃	〃
〃	19.5 ♀	〃	〃
german (dd)	21.0 ♀	Dialysates** 0.5 *ml*	Survived
〃	19.5 ♀	〃	〃
〃	23.0 ♀	〃	〃
〃	22.5 ♀	〃	〃
〃	23.0 ♀	〃	〃

Remarks: * Distilled water extracts were dialyzed and the dialyzates concentrated.
** Saline extracts were dialyzed and the dialysates used for injection.

(C) Toxicity of the deproteinized filtrates.

One kilogram of the roe was mashed in a mortar, extracted with 800 *ml* of saline solution by shaking for one hour. The mixture was centrifuged and the supernatant liquid (1150 *ml*) was divided into five equal portions. Each portion was deproteinized with different precipitating agents as described in the following.

The first portion (230 *ml*) was salted out by the addition of saturated ammonium sulfate solution until no more precipitates formed. Then the filtrates were heated to coagulate the remaining protein and refiltered. The filtrates were

dialyzed by cellophane against distilled water. The inner solution (dialyzates) was injected intraperitoneally into mice.

The second and third portions were deproteinized respectively by the addition of basic lead subacetate and mercuric chloride solutions. The filtrates, freed from heavy metals by hydrogen sulfide, were concentrated to about one fifth of its initial volume and neutralized. They were also injected into mice.

The fourth part was deproteinized by addition of 10 per cent trichloracetic acid. The filtrates were shaken with ether containing a small amount of hydrochloric acid to remove the residual trichloracetic acid, then concentrated and neutralized for injection.

The last one was deproteinized by the addition of 30 per cent phosphotungstic acid. To the filtrates, barium hydroxide solution was added to neutralize the remaining acid, and then filtered. From the filtrates, the excess of barium ion

Table 6. Toxicity of the deproteinized filtrates.

Strain of mice	Body weight and sex of mice	Treatment of deproteinization	Volume of injected filtrates	Survival or death
german (dd)	22.5 g ♀	Deproteinized by ammonium sulfate	0.5 *ml*	survived
〃	19.0 ♀	〃	〃	〃
〃	21.0 ♀	〃	〃	〃
〃	22.0 ♀	〃	〃	〃
〃	21.0 ♀	〃	〃	〃
german (dd)	25.0 ♀	Deproteinized by basic lead subacetate	0.5 *ml*	survived
〃	21.0 ♀	〃	〃	〃
〃	22.5 ♀	〃	〃	〃
〃	21.5 ♀	〃	〃	〃
〃	24.0 ♀	〃	〃	〃
german (dd)	22.5 ♀	Deproteinized by mercuric chloride	0.5 *ml*	survived
〃	22.5 ♀	〃	〃	〃
〃	23.0 ♀	〃	〃	〃
〃	24.5 ♀	〃	〃	〃
〃	18.0 ♀	〃	〃	〃
german (dd)	25.0 ♀	Deproteinized by 10% TCA	0.5 *ml*	survived
〃	23.5 ♀	〃	〃	〃
〃	25.0 ♀	〃	〃	〃
〃	21.5 ♀	〃	〃	〃
〃	24.0 ♀	〃	〃	〃
german (dd)	21.5 ♀	Deproteinized by 30% phosphotungstic acid	0.5 *ml*	survived
〃	21.5 ♀	〃	〃	〃
〃	20.5 ♀	〃	〃	〃
〃	23.0 ♀	〃	〃	〃
〃	23.5 ♀	〃	〃	〃

was removed by the addition of a dilute sodium sulfate solution. Then it was concentrated and injected into the animals. Toxicity of these deproteinized filtrates could not be observed as shown in Table 6.

(D) Formation of antigenicity

Two hundred grams of the roe were mashed in a mortar, extracted with 200 *ml* of saline solution by shaking for one hour, and centrifuged. The supernatants (258 *ml*) were divided into three portions. One portion (86 *ml*) was used intact for injection into mice (untreated control). To the second (86 *ml*), formalin solution (reagent grade) was added to make a 0.5 per cent (V/V) formalin mixture and left to stand overnight (Formalin toxoid). To the last portion (86 *ml*), phenol was similarly added to make a 0.5 per cent phenol mixture and left to stand overnight (Phenol toxoid). Five millilitre aliquots of each portions were injected intraperitoneally into three groups of rats of Wistar strain (three rats per one group). The results obtained are shown in Table 7. In this experiment, all rats in the formalin toxoid portion and only one rat in the phenol toxoid portion survived. After seventeen days from the initial injection, untreated cold

Table 7. Formation of antigenicity initial injection.

Strain of rat	No., Body weight and sex of rat	Injected solution	Vol. of injected solution	Survival or death	Time until death after injection
Wistar	(1) 200 g ♀	untreated cold saline extracts	5 *ml*	died	38 hours
〃	(2) 191 ♀	〃	〃	〃	23
〃	(3) 234 ♀	〃	〃	〃	38
Wistar	(4) 202 g ♀	Formalin toxoid	5 *ml*	survived	—
〃	(5) 198 ♀	〃	〃	〃	—
〃	(6) 201 ♀	〃	〃	〃	—
Wistar	(7) 207 g ♀	Phenol toxoid	5 *ml*	survived	—
〃	(8) 193 ♀	〃	〃	died	63
〃	(9) 200 ♀	〃	〃	〃	63
Second injection after 17 days from initial injection					
Wistar	(4) 219 g ♀*	Untreated cold saline extracts	5 *ml*	died	22 hours
〃	(5) 210 ♀*	〃	〃	〃	29
〃	(6) 217 ♀*	〃	〃	〃	46
Wistar	(7) 229 g ♀**	Untreated cold saline extracts	5 *ml*	died	28 hours
Wistar	(10) 217 g ♀***	Untreated cold saline extracts	5 *ml*	died	25 hours

Remarks: * Rats injected Formalin toxoid before 17 days.
** Rat injected Phenol toxoid before 17 days.
*** Rat not injected before (control).

saline extracts were freshly prepared. Five milliltre aliquots of it were re-injected into the survived rats.

4. Isolation of the toxic protein and identification of it as a lipoprotein.

(A) Separation of albumin and globulin fractions by Kekwick's method (2).

Two hundred grams of auhydrous sodium sulfate were dissolved in 500 *ml* of hot water, being kept at over 30°C to avoid crystallization. Three hundred grams of the roe were mashed in a mortar to which an equal volume of sodium sulfate solution was added. The mixture was stirred for a while, and kept for one or two hours. Globulin fraction precipitates were filtered by the filter paper-pulp on the Büchner's funnel. The clear filtrates were adjusted to pH 4.6—4.8 (bromocresolgreen) by careful titration of 0.2*N* sulfuric acid with vigorous stirring.

At final pH 4.6—4.8, sodium sulfate solution was gradually added to the filtrates until the solution appeared to be opalescent.

After standing overnight at over 30°C, the crystallization of albumin could hardly be observed in this case, therefore, the solution was subsequently dialyzed by cellophane against distilled water and this desalted fraction was injected intraperitoneally into the experimental animals. Globulin precipitates on the filter paper-pulp were dissolved in physiological saline solution, then dialyzed in the same fashion. The globulin precipitates in the bottom of cellophane sack were collected by means of centrifuging, dissolved in saline solution and injected into animals. The results obtained are shown in Table 8.

Table 8. Toxicity of albumin and globulin fractions of fish roe.

Animals	Body weight and sex of animals	Volume of injected solution	Fraction of protein*	Survival or death	Time until death after injection
Rat(Wistar strain)	173 g ♀	5 *ml***	Globulin fraction	died	30 hours
〃	185 ♀	〃	〃	〃	24
〃	153 ♀	〃	〃	〃	30
Cock(White Leghorn)	610 g ♂	5 *ml****	Globulin fraction	died	46 hours
Rat(Wistar strain)	202 g ♀	5 *ml***	Albumin fraction	died	71 hours ?
〃	208 ♀	〃	〃	survived	—
〃	226 ♀	〃	〃	〃	—
Cock(White Leghorn)	600 g ♂	5 *ml****	Albumin fraction	survived	—

Remarks: * Precipitating fraction by the addition of saturated sodium sulfate at over 30°C. (Globulin fraction)
The residual protein fraction (filtrate) is regarded as albumin fraction.
** Intraperitoneal injection
*** Intravenous injection

(B) Fractionation of albumin and globulin by McCrudden's method (1).

The roe was mashed in a mortar, extracted with an equal volume of physiological saline solution by shaking for an hour and then filtered. The filtrates were dialyzed overnight by the cellophane in a refrigerator. The globulin precipitates at the bottom of cellophane sack was collected by decantation and centrifugation and redissolved in saline solution. Then it was again precipitated by adding the solution to a large amount of water and passing carbon dioxide gas through the liquid. It was collected by centrifuging. The globulin free filtrate were concentrated by freezing dehydration (Albumin fraction). The results of injection of both fractions are given in Table 9.

Table 9. Toxicity of albumin and globulin fractions of fish roe. (McCrudden's method)

Strain of mice	Body weight and sex of mice	Fraction of protein	Volume of injected solution	Survival or death	Time until death after injection
german(dd)	20.2 g ♀	Albumin fraction	0.5 *ml*	survived	—
〃	21.5 ♀	〃	〃	〃	—
〃	20.5 ♀	〃	〃	〃	—
〃	22.5 ♀	〃	〃	〃	—
〃	20.2 ♀	〃	〃	〃	—
german(dd)	20.5 ♀	Globulin fraction	0.5 *ml*	died	within 27 hours
〃	19.5 ♀	〃	〃	〃	〃
〃	20.0 ♀	〃	〃	〃	〃
〃	18.0 ♀	〃	〃	〃	〃
〃	20.5 ♀	〃	〃	〃	〃
german(dd)	22.0 ♀	Globulin fraction (Heated at 120°C, for 30 min)	0.5 *ml*	survived	—
〃	20.2 ♀	〃	〃	〃	—
〃	18.5 ♀	〃	〃	〃	—
〃	18.2 ♀	〃	〃	〃	—
〃	20.5 ♀	〃	〃	〃	—

(C) Toxicity of lipoprotein fraction of the roe.

Here, the authors suspected that the apparent globulin might be in fact a lipoprotein, therefore, the lipoprotein fraction of the roe was prepared after Chargaff's method (3) used for the preparation of lipovitellin from hen's eggs. Two hundred grams of the roe were mashed in a mortar and mixed with an equal volume of ice-cold saturated sodium chloride solution.

The mixture was shaken with 400 *ml* of peroxide-free ether and stored in a refrigerator for four hours. Then the mixture was centrifuged to remove ether extract as much as possible. The ether extraction of the aqueous emulsion was repeated two times more to eliminate the ether soluble substances in it. The aqueous portion obtained was dialyzed by the cellophane sack against

running tap water for 22 hours. White precipitates were collected by centrifugation, washed twice with 200 *ml* portions of ice-cold distilled water, then dissolved again in 200 *ml* of ice-cold 10 per cent sodium chloride solution. A small amount of insoluble substances were completely removed by centrifuging. The solution was again treated with 200 *ml* of ether. After standing for 24 hours in a refrigerator, the ether layer was removed. The apueous solution was dialyzed in the same manner. Dialysis and ether treatment were repeated twice further. The final protein precipitates were collected (Lipoprotein fraction), dissolved in physiological saline solution as much as possible and injected intraperitoneally into mice. The results are given in Table 10.

Table 10. Toxicity of lipoprotein fraction of fish roe.

Strain of mice	Body weight and sex of mice	Volume of injected solution	Survival or death	Time until death after injection
german(dd)	19.5 g ♀	Lipoprotein fraction (saline sol.) 0.5 *ml*	died	less than 23 hours
〃	21.0 ♀	〃	〃	〃
〃	19.5 ♀	〃	〃	〃
〃	23.0 ♀	〃	〃	〃
〃	17.5 ♀	〃	〃	26 hours

Table 11. Heat tolerance of lipoprotein fraction of fish roe.

Strain of mice	Body weight and sex of mice	Heat treatment	Volume of injected solution	Survival or death	Time until death after injection
german(dd)	19.5 g ♀	Untreated cold aqueous sol.	0.5 *ml*	died	21 hours
〃	19.5 ♀	〃	〃	〃	46
〃	17.5 ♀	〃	〃	〃	46
〃	24.0 ♀	〃	〃	〃	24
german(dd)	20.5 ♀	97°C, 30 min	0.5 *ml*	survived	—
〃	19.5 ♀	〃	〃	〃	—
〃	21.5 ♀	〃	〃	died	72 hours
〃	19.0 ♀	〃	〃	〃	24
〃	18.0 ♀	〃	〃	survived	—
〃	24.0 ♀	〃	〃	〃	—
〃	24.5 ♀	〃	〃	died	66
〃	24.5 ♀	〃	〃	〃	42
〃	23.5 ♀	〃	〃	〃	48
〃	22.5 ♀	〃	〃	〃	42
german(dd)	23.0 ♀	Autoclaved 120°C, 30 min	0.5 *ml*	survived	—
〃	23.0 ♀	〃	〃	〃	—
〃	23.0 ♀	〃	〃	〃	—
〃	23.5 ♀	〃	〃	〃	—

(D) Heat tolerance of lipoprotein fraction.

Lipoprotein was prepared by the same method as in the former section. It was dissolved in physiological saline solution and heated at different temperatures for varying time intervals. After heating, these fractions were injected intraperitoneally into mice. The results are shown in Table 11.

(E) Paper electrophoresis of lipoprotein.

Lipoprotein in the roe was purified as follows; The roe was mashed, extracted with physiological saline solution. The saline extracts were dialyzed by cellophane sack overnight and the protein precipitates in the bottom of

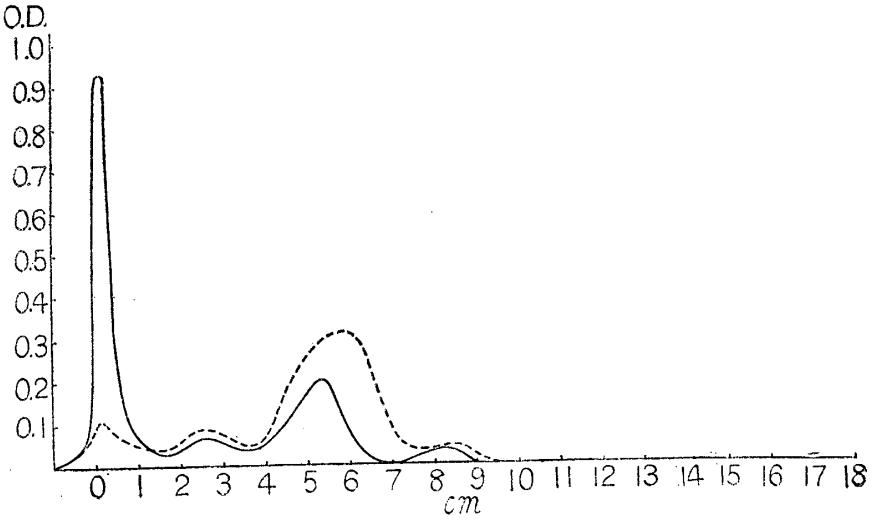
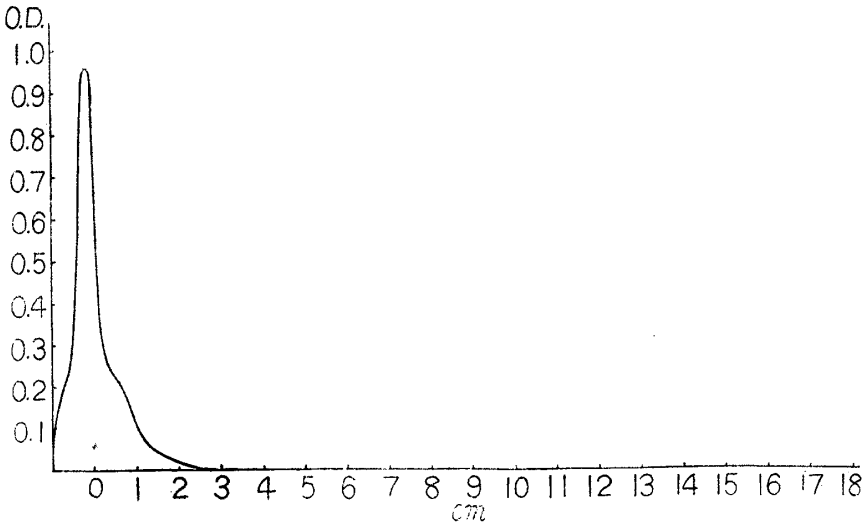


Fig. 1. Paper electrophoresis of saline extracts of the fish roe.
Veronal buffer pH 8.5, u=0.045, 9V/cm, 6 hours.
Amidoschwarz 10B staining.



Fig, 2. Paper electrophoresis of lipoprotein solution.
Veronal buffer pH 8.5, u=0.045. 6V/cm, 2.5 hours.
Amidoschwarz 10B staining.

cellophane sack were collected by centrifugation. The precipitates were used as the starting material in the procedure of Chargaff as in the former section and purified. Initial crude saline extracts and the purified lipoprotein were spotted on the paper, and subjected to electrophoresis using veronal buffer (pH 8.5, μ=0.045) and with potential gradients of 6—9 V/cm.

The results obtained are illustrated in Figures 1—3.

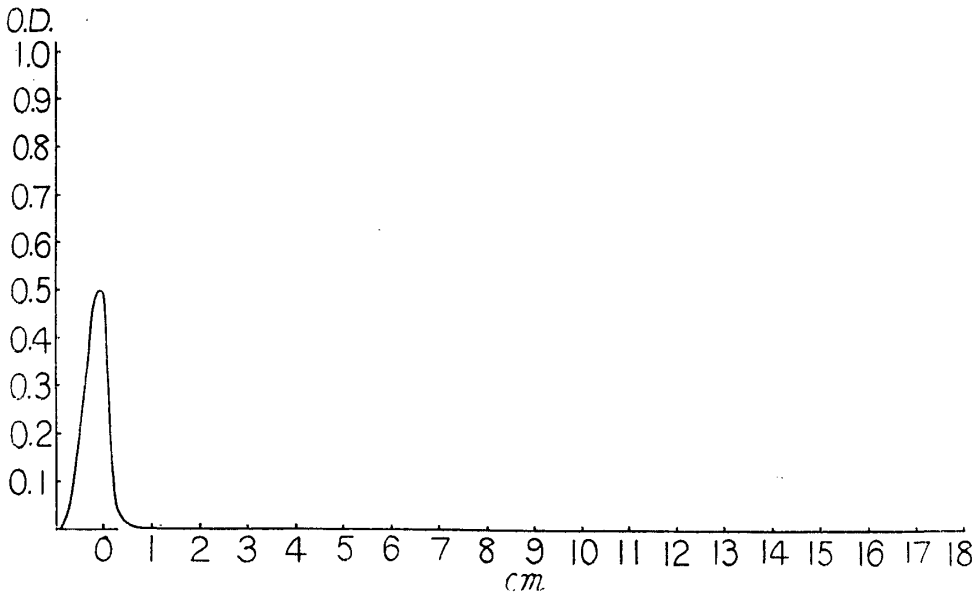


Fig. 3. Paper electrophoresis of lipoprotein solution.
Veronal buffer pH 8.5, u=0.045. 9V/cm, 6 hours.
Sudan Black B staining.

5. Examination on the toxicity of roe-lipids.

(A) Toxicity of ether soluble lipids.

Two hundred grams of the roe were mashed in a homogenizer, extracted twice with ether. The combined ether extracts were concentrated by a current

Table 12. Toxicity of ether soluble lipids of the roe.

Strain of mice	Body weight and sex	Injected solution	Survival or death
Red (rr)	21.2 g ♂	intact lipids 1 *ml*	died after 67 hours
〃	23.0 ♂	〃	〃 43
〃	21.5 ♂	〃	〃 67
〃	18.0 ♂	〃	〃 67
German (dd)	30.0 ♂	intact lipids 0.5 *ml*	died after 67 hours
〃	34.2 ♂	〃	〃 67
〃	41.0 ♂	〃	〃 43
〃	32.7 ♂	〃	〃 67
〃	37.2 ♂	〃	〃 43

of nitrogen. The oil residue was reextracted by ether and the extracts were concentrated in the same way. The yellow liquid lipids were biuret negative and injected intact into mice. The results are given in Table 12.

(B) Toxicity of lipids obtained by Dyer's method (4).

Two hundred grams of the roe were homogenized for a few minutes with a mixture of chloroform-methanol (100 *ml*: 200 *ml*), blended again after the addition of 100 *ml* of chloroform, further blended after the addition of 100 *ml* of water for 30 seconds. The mixture was filtered on the Büchner's funnel using Toyo filter papaer No. 51 with slight suction. The filtrates were poured into a mess cylinder and left for a while. Chloroform layer was siphoned and it was concentrated by a current of nitrogen. Lipid residue was again dissolved in pure chloroform and reconcentrated under nitrogen. Light brown liquid lipids were biuret negative and completely free from chloroform. It was injected intact or after making an emulsion with Tween 60, injected into the animals. The results obtained are shown in Table 13.

Table 13. Toxicity of roe lipids obtained by Dyer's method.

Strain	Boby weight and sex	Injected solution	Survival or death
Rat (Wistar)	159 g ♂	intact lipids 2 *ml*	died within 48 hours
〃	169 ♂	〃 3 *ml*	〃 48
〃	177 ♂	lipids-Tween 60 emulsion 4 *ml*	〃 48
German (dd)	36.0 g ♂	lipids-Tween 60 emulsion 1 *ml*	died within 48 hours
〃	30.0 ♂	〃	〃 48
〃	33.0 ♂	〃	〃 48
〃	36.0 ♂	〃	〃 48
〃	35.5 ♂	〃	〃 48

Tablc 14. Toxicity of ethanol-ether soluble lipids.

Strain of mice	Body weight and sex	Injected solution	Survival or death
Red (rr)	18.0 g ♂	lipid-Tween 60 emulsion 1 *ml*	died after 43.5 hours
〃	17.0 ♀	〃	〃 43.5
〃	18.2 ♂	〃	〃 46
〃	19.2 ♂	〃	〃 43.5
〃	21.0 ♂	〃	〃 27
〃	21.5 ♀	〃	〃 46
〃	17.5 ♀	〃 0.5 *ml*	〃 46
〃	21.5 ♀	〃 〃	〃 27
〃	20.0 ♀	〃 〃	〃 43.5

(C) Toxicity of ethanol-ether soluble lipids.

The roe was homogenized, extracted with a mixture of ethanol-ether (1 : 1) two times. The combined extracts were concentrated under nitrogen and the lipid residue was reextracted with a ethanol-ether mixture. It was again concentrated and yellow brown lipids were emulsified with Tween 60 and injected. The results are shown in Table 14.

(D) Toxicity of lipoprotein-lipid (Dinogunellin-lipid)

Lipid of lipoprotein was extracted by refluxing the protein with ethanol-ether (1 : 1) mixture or by freezing the protein in the presence of ether, followed by extraction with ether after thawing. The obtained lipid was emulsified (Tween 60) and injected into mice. The results are shown in Table 15.

Table 15. Toxicity of lipoprotein-lipid (Dinogunellin-lipid)

Strain of mice	Body weight and sex	Injected solution	Survival or death
Red (rr)	28.0 g ♀	Dinogunellin-lipid Tween 60 emulsion 1 *ml*	died within 42 hours
〃	24.0 ♀	〃	〃 42
〃	23.0 ♀	〃	〃 42
〃	17.5 ♂	〃	〃 18
〃	19.5 ♀	〃	〃 42

6. Autopsy of killed rats by injection of roe lipoprotein or lipids.

Autopsy of dead rats after injection of lipoprotein showed that characteristic microscopic changes occur particularly in their livers, e.g., the section slides of the rats are more intensely and evenly stained by the lipid-dyes such as Sudan III or Sudan Black B than those of normal non-injected rats. It was found by

Table 16. Comparison of crude fat contents of livers of lipoprotein- and lipids injected rats and normal ones.

Treatment	Crude fat contents*	Extraction method of injected solutiod
None (Control)	2.80%	
〃	2.89	
〃	2.46	
Lipoprotein injection	5.79	Chargaff's method
〃	6.29	〃
Lipids injection	10.14	Dyer's method
〃	12.63	EtOH : Et_2O
〃	9.83	EtOH : Et_2O
〃	7.12	Et_2O only

* Crude fat contents are shown on wet basis.

this histochemical observation that such state of livers of injected rats may be regarded as a "fatty liver". Therefore, the authors determined the crude fat contents of rat livers either of injected or non-injected ones. Similarly, the determination was also carried out in the case of the rat livers killed by lipid injection. The results are given in Table 16.

Discussions

Takayanagi and Kitamura (5) investigated firstly the Dinogunellus-roe poisoning which had occurred at Mori in 1952—1953. They investigated the symptoms of the poisoning and successfully reproduced them by oral administration of the roe to human subjects. They also found that the roe poison could not be destroyed by such heating as high as usually used in the home (below or near 100°C, 20–30 minutes) and that 20 or 30 grams of matured roe are enough to cause the poisoning.

To characterize the roe poison, the authors in the first place attempted to examine whether it has a protein nature. The criteria that determine the poison to be a protein, are the following properties.

(1) Loss of toxicity by heating.
(2) Non dialyzability.
(3) Salting out by ammonium sulfate or precipitability by protein precipitants.
(4) Solubility in water and insolubility in hydrophobic organic solvents.
(5) Ability to produce antigenicity.

In the first place, the authors studied on the loss of toxicity after heating the roe. In both cases of oral administration to cocks and of intraperitoneal injection of extracts into mice, the untreated air-dried specimen revealed to be toxic while the autoclaved one lost completely its toxicity. This implies the heat denaturation of the roe poison.

In Hokkaido, some people believe that the roe poison is contained in the egg capsule and that when the roe is heated after removal of the capsule, no food poisonings occur.

However, this is not the case. The authors observed no inherent toxicity in the egg capsule. Thus it is interpreted that the egg capsule interferes heat conduction into the inner part of the egg mass when heated, therefore, resulted in incomplete denaturation of toxic protein. As indicated in Table 11, heating at 97°C for 30 minutes is insufficient for denaturation, and autoclaving at 120°C for 30 minutes just denatures the toxic protein.

The heat tolerance of toxic protein is thought to be fairly stronger as other conjugated protein such as casein.

Secondly, the authors examined whether the roe poison is dialyzable. Dialyzates or dialyzate concentrates showed no toxicity as seen in Table 5.

This suggests that the roe poison is not of low molecular but of high molecular substance.

Thirdly, salting out of poison by ammonium sulfate or precipitation of poison by other precipitants were examined. When injected into mice, deproteinized filtrates by the agents such as ammonium sulfate, basic lead subacetate, mercuric chloride, TCA, and phosphotungstic acid showed no toxicity respectively. The results suggest that the roe poison is precipitated together with other non-toxic proteins, and no toxic substance exists in the filtrates.

Next, the solubility of roe toxic protein was examined. From the results obtained, it was found that the poisonous protein is soluble in neutral salt solution, fairly soluble in ethanol with considerable loss of toxicity, and insoluble in ether and pure water. Preliminary experiments by the usual extraction method for shellfish poison showed the very inconsistent results on the toxicity of the roe. When bioassayed, mice sometimes survived and sometimes died. This suggested that during extraction of roe poison by using polar solvents such as ethanol, some changes in the structure of the roe poison molecule may take place, for example, if the roe poison is a lipoprotein, the dehydration of hydrated water around lipoprotein molecule is caused by the addition of ethanol, therefore, resulted in splitting of lipoprotein molecule into lipid and protein moieties. At any rate, the ethanol extraction method is not available for the present purpose.

Ability to produce antigenicity was also examined. Formalin and phenol toxoids of roe poison could prolong the survival time of rats after injection more than that of untreated extracts. In the case of formalin toxoid, all rats survived and in the case of phenol toxoid, one of three rats survived. However, the second injection of untreated cold saline extracts after seventeen days from the initial injection killed all rats injected. The production of antigenicity in rats by roe poison toxoids appears to be imcomplete yet.

However, the examinations of the criteria above mentioned led the authors to conclude that the roe poison is surely of protein nature. Then, to what class of protein does the roe poison belong?

Separations of albumin and globulin fractions followed by the toxicity tests suggest that the solubilities of toxic roe protein are similar to those of globulins, namely, soluble in sodium chloride solution and precipitable when dialyzed against distilled water. The globulin fraction of the roe protein is responsible for toxicity while albumin fraction is nontoxic.

Fractionation by McCrudden's method also showed that the globulin fraction is responsible for toxicity. However, the authors suspected here that this toxic globulin-like protein might be, in fact, a kind of lipoprotein such as lipovitellin in hen's egg. Thus they isolated lipoprotein fraction from the roe after Chargaff's method for lipovitellin (3). This lipoprotein fraction, when dissolved in physiological saline solution and bioassayed, proved to be toxic. It was fairly

heat resistant and lost its toxicity completely by heating at 120°C for 30 minutes. This lipoprotein, purified by Chargaff's method, were subjected to paper electrophoresis using veronal buffer. After electrophoresis, the lipoprotein band which almost remained firm on the starting line, was stained well both by amido schwarz 10B dye (protein-staining) and by Sudan Black B (lipid-staining). This is an additional evidence of the toxic protein being a lipoprotein. Preliminary analysis of it showed that it consists of protein moiety (about 80%) and the rest moiety (about 20%).

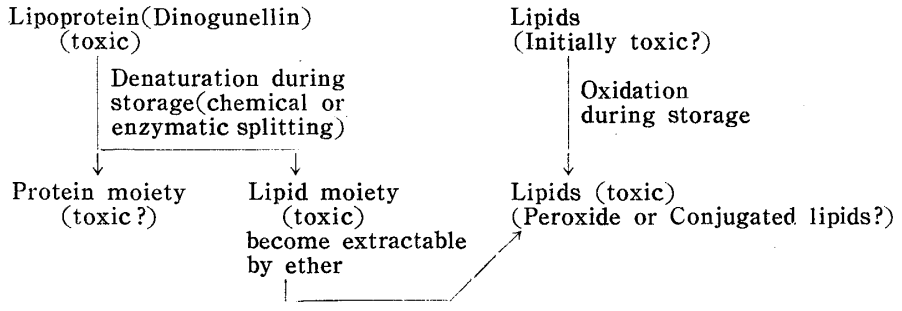
The authors, at the present stage, assumed it to be a new lipoprotein, and tentatively named it as "Dinogunellin". The exact knowledge on both protein- and lipid-moieties awaits further study.

Apart from this lipoprotein, the authors after the opinion of Prof. Sakai, examined the toxicity of lipids. Lipids, obtained by the extractions using ether, chloroform-methanol, ethanol-ether respectively, showed considerable toxicities. It must be noted that the lipid-moiety of Dinogunellin also showed toxicity. There is also a possibility of splitting the bond between both moieties in lipoprotein molecule by using a polar solvent such as alcohol and acetone, and of becoming extractable of lipids by ether. However, in the present case, lipid extracted by ether alone also showed toxicity. Up to present, there is no information concerning ether soluble fish poison except the ciguatera toxin in the tropical reef or inshore fishes. Then, how should the meaning of the toxicity of lipide be interpreted?

In 1955, Kaneda *et al.* (6) confirmed brilliantly the toxicity of the autoxidation products of unsaturated fatty acid in marine animal oils by feeding experiments.

The authors also observed that the roe lipids are remarkably discolored and oxidized even during storage in a refrigerator (−10°~−25°C). At the present stage, the authors are assuming that the inherent poisonous principle of the roe is a lipoprotein (Dinogunellin) and that the toxicity of lipids is probably due to their secondary changes e. g., formation of unsaturated fatty acid peroxide, or splitting of lipid moiety of lipoprotein, although the confirmation is necessary. This assumption is illustrated in Scheme 1.

Scheme 1. Toxic principles in Dinogunellus-roe.



Autopsy of the killed rats by injection of Dinogunellin showed microscopic changes in their livers. The authors stained the section slides of killed rat-livers with lipophilic dyes and found that the histological changes of rat livers may be regarded as a "fatty liver".

The determination of crude fat contents of rat livers support this view. It is worthy to note that the crude fat contents of lipoprotein-injected rat livers doubled that of untreated rat specimens, and in the case of lipid-injected rat livers, it increased as much as four or five times that of the untreated ones. The differences between lipoprotein- and lipid-injected rat livers are now obscure except the microscopic patches produced in the former.

H. Sommer (7) stated that the fish roe poison (perhaps of a species of Cottus) has more longer latent period than that of other marine animal poisons which he had classified and that it caused microscopic changes in the livers of injected animals. However, he did not mention the chemical nature of this poison (P 111). The mechanism of this kind of "fatty liver" is very interesting but nothing is known at present.

Summary

The consumption of the roe of a blenny, *Dinogunellus grigorjewi* Herzenstein, often caused food poisonings in Hokkaido. The symptoms of the poisoning are chiefly gastrointestinal disturbances (vomiting, diarrhea, abdominal pains, lassitude). Two additional case reports were described.

The protein nature of the roe poison was confirmed and this toxic protein belongs to a lipoprotein, named tentatively as "Dinogunellin", and not belongs to a "Toxalbumin". Autoclaving at 120°C for 30 minutes is necessary to destroy the lipoprotein completely. Ordinary boiling of the roe at home (below or near 100°C) is insufficient to denature it. Toxicity of roe lipids was also found, however, whether this toxicity of lipids is inherent even in fresh live fish or is due to secondary change during storage, must be clarified in future.

Autopsy of killed rats by injection of Dinogunellin showed the occurrence of "fatty liver" The crude fat contents of killed rat-livers both by Dinogunellin and by lipids increased remarkably, although the mechanism is unknown.

References

1) McCrudden, F.H. (1921). Arch. exp. path. pharmak., **91**, 46-80.
2) Kekwick, R.A. and R.K. Cannan (1936). Biochem. J., **30**, 232.
3) Chargaff, E. (1942). J. Biol. Chem., **142**, 91.
4) Dyer, W.J. and E.G. Bligh (1959). Can. J. Biochem. Physiol., **37**, 911-917.
5) Takayanagi, F., T. Kitamura and T. Satoh (1953). Lectured at the 5th meeting of the Society of the Public Health of Hokkaido.
6) Kaneda, T., S. Ishii, H. Sakai and K. Arai (1955). Bull. Tokai Regional Fish. Research Lab. No. 12 (Contr. A No. 20).
7) Sommer, H., W. F. Whedon, R.S. Stohler and C.A. Kofoid (1937). Arch. Pathol., **24**, 537.