OCCURRENCE OF TETRAMINE AND CHOLINE COMPOUNDS IN THE SALIVARY GLAND OF A MARINE GASTROPOD, NEPTUNEA ARTHRITICA BERNARDI

OCCURRENCE OF TETRAMINE AND CHOLINE COMPOUNDS IN THE SALIVARY GLAND OF A MARINE GASTROPOD, *NEPTUNEA ARTHRITICA* BERNARDI*

By

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Introduction

In the preceding paper (1) dealing with the food poisoning caused by the consumption of *Neptunea arthritica*, the senior author proved that the poisonous substance is located only in the salivary gland and it belongs to the nerve poison considering from its symptoms of the food poisoning and the animal experiment.

Recently from the subsequent studies on the poisonous substance, the occurrence of tetramine and choline compounds in the salivary gland of the gastropod was confirmed. This paper presents the results of the experiments conducted by the authors on it with reference to the food poisoning.

Acknowledgments

The authors express their gratitude to all who have enabled them to fulfil this study; to Mr. Sangoro SHIBUYA, Chief of the Usu Marine Biological Station and, to the Usu Fisheries Association for collecting the samples; to the Laboratory of Animal Breeding of this Faculty for providing them with a number of mice of pure strain; to the Institute of Applied Microbiology, University of Tokyo, for sending them *Neurospora* mutant for choline assay; to Mr. Aono of the Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, for preparing infrared absorption spectra, to Dr. J. A. Aeschlimann, Hoffmann-La Roche, Inc., for supplying urocanylcholine bromide; to Dr. A. Cohen, Roche Products Ltd., for gifts of β-(3-Indolyl)-propionylcholine iodide. They also wish to acknowledge the valuable criticism of Prof. TSUCHIYA and many suggestions of Dr. Toraichiro KINOSHITA, Hokkaido Regional Fisheries Research Laboratory.

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Experimental

1. Location of the poisonous component in the salivary gland by means of the paper chromatography and the Magnus procedure.

For the paper chromatography of the poisonous component, the spot solution was prepared by following Miyaki's procedure (2). The salivary gland was extracted with methanol acidified with a small quantity of conc. HCL, and the methanol extract was concentrated under reduced pressure. The resulting aqueous solution was defatted with ether and made alkaline with 10 per cent NaOH. After the addition of anhyd. Na_2SO_4, it was shaken with n-Butanol. Then the combined extract was shaken two times with 10 per cent HCL. The acid solution was evaporated to dryness under reduced pressure and the residue was extracted with absolute methanol. The methanol solution was used for the spot solution for the paper chromatography. Namely, it was linearly spotted along the baseline (2.5 cm from the paper end) of the filter paper (Toyo No. 2).

The paper chromatography was carried out using butanol layers of n-BuOH: AcOH : H_2O (4 : 1 : 5) or of n-BuOH saturated with 10 per cent ammonia as the mobile agents. After that, the paper was cut off transversely along the borderlines corresponding to each Rf value. The paper strips were extracted with the known volume of Tyrode's solution respectively. Each extract thus obtained was tested on the ileums of the rabbit or guinea pig according to the usual Magnus procedure. Fig. 1 shows the kymography of the rabbit ileum, held in the perfusing apparatus which was filled with Tyrode's solution as the bathing medium at 37°C with constant bubbling of air. In this case, the paper chromatography was carried out with the solvent system of n-BuOH : AcOH : H_2O. The contraction of ileum was remarkable in the extracts of Fractions 1 (Rf 0–0.2) and 2 (Rf 0.2–0.4).

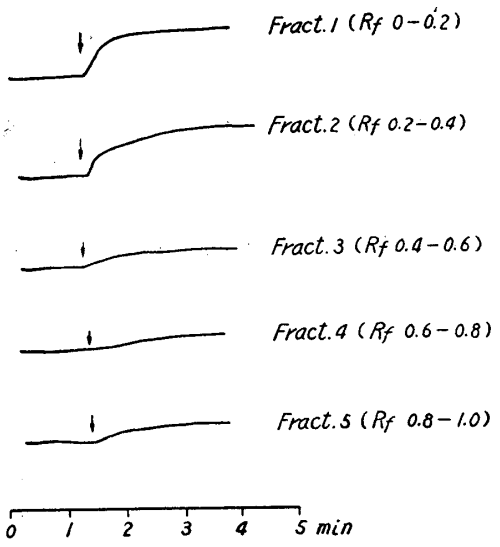


Fig. 1. Kymography of rabbit ileum Magnus method : Tyrode's solution. 37°C, Aeration.

Similar experiments were carried out on the ileums of guinea pigs. (Fig. 2 and 3). In Fig. 2, (solvent system; n-BuOH : AcOH : H_2O) it was found that the contraction was very remarkable in Fraction 2 (Rf 0.15–0.40), and followed by Fraction 3 (Rf 0.4–0.6).

In the experiment of Fig. 3, the solvent system was n-BuOH saturated

with 10 per cent ammonia. The response of ileum was most remarkable in Fraction 3, Rf (0.33–0.66, histamine fraction) and followed by Fraction 2 (Rf 0.17–0.33).

The differences of the kymographical records between the rabbit and

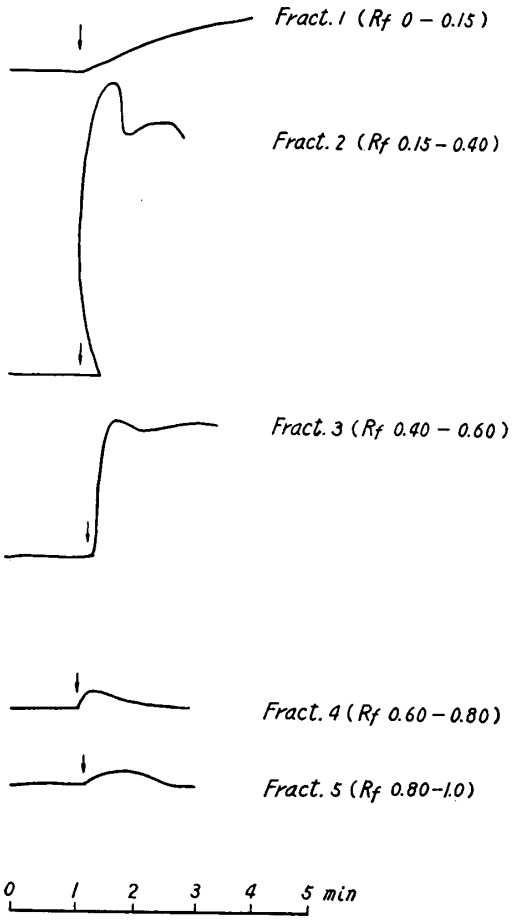


Fig. 2. Kymography of guinea pig ileum. Magnus method : Tyrode solution, 37°C, Aeration.

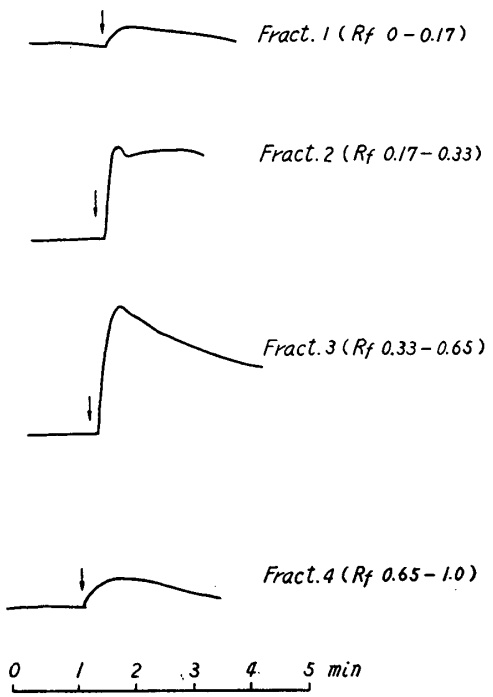


Fig. 3. Kymography of guinea pig ileum. Magnus method : Tyrode's solution, 37°C, Aeration.

guinea pig ileums (Fig. 1 and 2) are due to the dissimilarity of their sensitivity to histamine. The ability of the guinea pig ileum to react to histamine is extraordinary high. The very marked contraction of Fraction 2 seen in Fig. 2 may be explained as the synergic effect of the lethal component and histamine on guinea pig ileum, while in the rabbit or mice ileums they are insensitive to histamine (non-synergic to the lethal component), therefore, the response is due to the lethal component. Although the histamine spot made its appearance in Fraction 2 in the case of n-BuOH : AcOH : H_2O and in Fraction 3 in the case of n-BuOH saturated with 10 per cent ammonia, its content in the salivary gland was far less than the lethal dose of mice, and it acted only as synergist (in case of the guinea pig).

2. Location of the lethal component in the salivary gland by means of paper chromatography and intraperitoneal injection into mice.

The procedures for preparing the spot solution and for the paper chromatography were the same as in the former section. After the chromatography, the paper strips were also made as before, and eluted with distilled water. The eluate was concentrated and made up to 2 ml (original solution). Then 0.5 ml of it was injected intraperitoneally into the mouse (two mice for a fraction) and then the survival time was measured.

From the results of the experiments shown in Tables 1 and 2, it was found that the lethal component was located in the region of Rf 0.38–0.66 of the

Table 1. Location of lethal component in the salivary gland by the paper chromatography and intraperitoneal injection of the paper strip-eluates. (Solvent system; n-BuOH:AcOH:H_2O)

Expt.	Strain of mice	Body wt. & sex of mice	Fract. No.	Rf-interval	Injected eluate	Time until death	Toxicity
(A)	Red (rr)	20.0g ♀	1	0-0.3	0.5 *ml*	4′17″	+
	,,	24.0 ♂	1	0-0.3	,,	5′35″	+
	,,	20.0 ♂	2	0.3-0.65	,,	35″	++
	,,	21.0 ♀	2	0.3-0.65	,,	40″	++
	,,	18.0 ♂	3	0.65-1.0	,,	Survives	—
	,,	17.0 ♂	3	0.65-1.0	,,	Survives	—
(B)	Red (rr)	25.0 ♀	1	0-0.14	0.5 *ml*	Survives	—
	,,	22.0 ♂	1	0-0.14	,,	Survives	—
	,,	21.5 ♀	2	0.14-0.38	,,	1′15″	+
	,,	22.0 ♂	2	0.14-0.38	,,	1′30″	+
	,,	25.0 ♀	3	0.38-0.66	,,	1′30″	+
	,,	24.0 ♂	3	0.38-0.66	,,	1′45″	+
	,,	25.0 ♀	4	0.66-1.0	,,	Survives	—
	,,	24.0 ♂	4	0.66-1.0	,,	Survives	—

paper chromatogram in the case of n-BuOH : AcOH : H_2O solvent system. On the contrary, it was located in the range of Rf 0–0.30 in the case of 10 per cent ammonia saturated n-BuOH.

By narrowing the width of the paper strip (Rf 0.10 interval), a similar toxicity test was repeated for all paper strip-eluates. The results are shown in Tables 3–7, in which the toxicity was expressed as the reciprocal of the survival time in seconds.

In Table 3, the highest toxicity was found in the region of Rf 0.3–0.5. (solvent system: BuOH : AcOH : H_2O). In Table 4, the toxicity peak was found in the region of Rf 0.5–0.8. (solvent system : n-PrOH : HCOOH : H_2O). In Table 5, the toxicity peak was located in the range of Rf 0.5–0.8, especially at Rf 0.6–0.7 (solvent system : n-PrOH : IN AcOH, 3 : 1). Next, the transference

Table 2. Location of lethal component in the salivary gland by the paper chromatography and intraperitoneal injection of the paper strip-eluates into mice. (Solvent system ; 10% ammonia satd. n-BuOH).

Strain of mice	Body wt. & sex of mice	Fract. No.	Rf-interval	Injected eluate	Time until death	Toxicity
Red (rr)	20.0g ♂	1	0-0.15	0.5 *ml*	40″	++
,,	19.0 ♀	1	0-0.15	,,	30″	++
,,	19.5 ♀	2	0.15-0.30	,,	1′15″	+
,,	18.5 ♂	2	0.15-0.30	,,	1′20″	+
,,	20.5 ♂	3	0.30-0.65	,,	Survives	—
,,	18.0 ♀	3	0.30-0.65	,,	Survives	—
,,	17.0 ♂	4	0.65-1.0	,,	Survives	—
,,	19.0 ♀	4	0.65-1.0	,,	Survives	—

Table 3. Location of the lethal component by intraperitoneal injection into mice. (Solvent system ; n-BuOH:AcOH:H_2O)

Rf-interval of paper strips	Toxicity*
0-0.1	1/480
0.1-0.2	1/270
0.2-0.3	1/85
0.3-0.4	1/40
0.4-0.5	1/40
0.5-0.6	1/75
0.6-0.7	1/620
0.7-1.0	Nil

* Remarks ; Toxicity is expressed as the reciprocal of the survival time until death after injection. Red (rr) strain mice were used (Two mice for a fraction).

Table 4. Location of the lethal component by intraperitoneal injection into mice. (Solvent system ; n-PrOH:HCOOH:H_2O, 8:1:1)

Rf-interval of paper strips	Toxicity*
0--0.1	1/670△
0.1-0.2	Nil
0.2-0.3	Nil
0.3-0.4	Nil
0.4-0.5	1/105◎
0.5-0.6	1/45◎
0.6-0.7	1/45◎
0.7-0.8	1/45◎
0.8-0.9	Nil
0.9-1.0	Nil

Remarks ; * Toxicity is expressed similarly in Table 3. dd (albino) strain mice were used (Two mice for a fraction).
△ This fraction was rich in inorganic salts.
◎ These fractions were Dragendorff strong positive.

Table 5. Location of the lethal component by intraperitoneal injection into mice. (Solvent system ; n-PrOH:1N AcOH, 3:1)

Rf-interval of paper strips	Toxicity*
0-0.1	1/730△
0.1-0.2	1/310△
0.2-0.3	Nil
0.3-0.4	Nil
0.4-0.5	Nil
0.5-0.6	1/45#
0.6-0.7	1/40#
0.7-0.8	1/45#
0.8-0.9	Nil
0.9-1.0	Nil

Remarks ; * Toxicity is expressed as before. Red (rr) strain mice were used (Two mice for a fraction).
△ This fraction was rich in inorganic salts.
These fractions were Dragendorff strong positive.

Table 6. Location of the lethal component by intraperitoneal injection into mice. The spot solution was the eluate of Rf 0.3-1.0 portion of the first paper chromatogram using 10 per cent ammonia satd. n-BuOH. Paper chromatography was repeated using the same solvent system.

Rf-interval of paper strips	Toxicity
0-0.1	Nil
0.1-0.2	"
0.2-0.3	"
0.3-0.4	"
0.4-0.5	"
0.5-0.6	"
0.6-0.7	"
0.7-0.8	"
0.8-0.9	"
0.9-1.0	"

Remarks ; Red strain mice were used (Two mice for a fraction).

of the toxic component by changing the solvent systems (repetition of paper chromatography) was examined as follows. At first, the paper chromatogram run with 10 per cent ammonia saturated n-BuOH was cut into two parts of Rf 0–0.3 and the rest. The eluates of the part of Rf 0.3–1.0 were spotted and again paper chromatographed by using the same solvent. As shown in Fig. 6, the eluates of all Rf portions were non-lethal, despite of histamine

Table 7. Location of the lethal component by intraperitoneal injection into mice. The spot solution was the eluate of Rf 0–0.3 portion of the first paper chromatogram using 10 per cent ammonia satd. n-BuOH. Paper chromatography was repeated using n-PrOH:HCOOH:H_2O(8:1:1) as solvent system.

Rf-interval of paper strip	Toxicity
0–0.1	Nil
0.1–0.2	,,
0.2–0.3	,,
0.3–0.4	,,
0.4–0.5	Doubtful*
0.5–0.6	1/165#
0.6–0.7	1/128#
0.7–0.8	1/193#
0.8–0.9	Nil
0.9–1.0	Nil

Remarks; Toxicity was expressed as before.
Red strain mice were used (Two mice for a fraction.)
* In this fraction, one mouse died after longer time.
\# These fractions were Dragendorff strong positive.

fraction was included therein.

On the other hand, the eluates of the lower part (Rf below 0.3) showed a remarkable toxicity in both solvent systems, *i.e.*, in the case of n-BuOH: AcOH: H_2O system, the toxicity peak was located in the region of Rf 0.4–0.5, while in the case of n-PrOH: HCOOH: H_2O, it was located at Rf 0.6–0.7. It is noteworthy that the lethal component reacts strong positive with Dragendorff reagent without exception, suggesting that it may be tertiary- or quaternary ammonium bases.

3. Fraction of lethal component by Vickery Leavenworth's procedure and identification of tetramine.

To separate the lethal component from the contaminants (Diazo-, Dithiocarbamate positive substances), the authors attempted to use the usual but somewhat lengthy procedure of Vickery Leavenworth (3). The flow sheet of the procedure is indicated in Fig. 4.

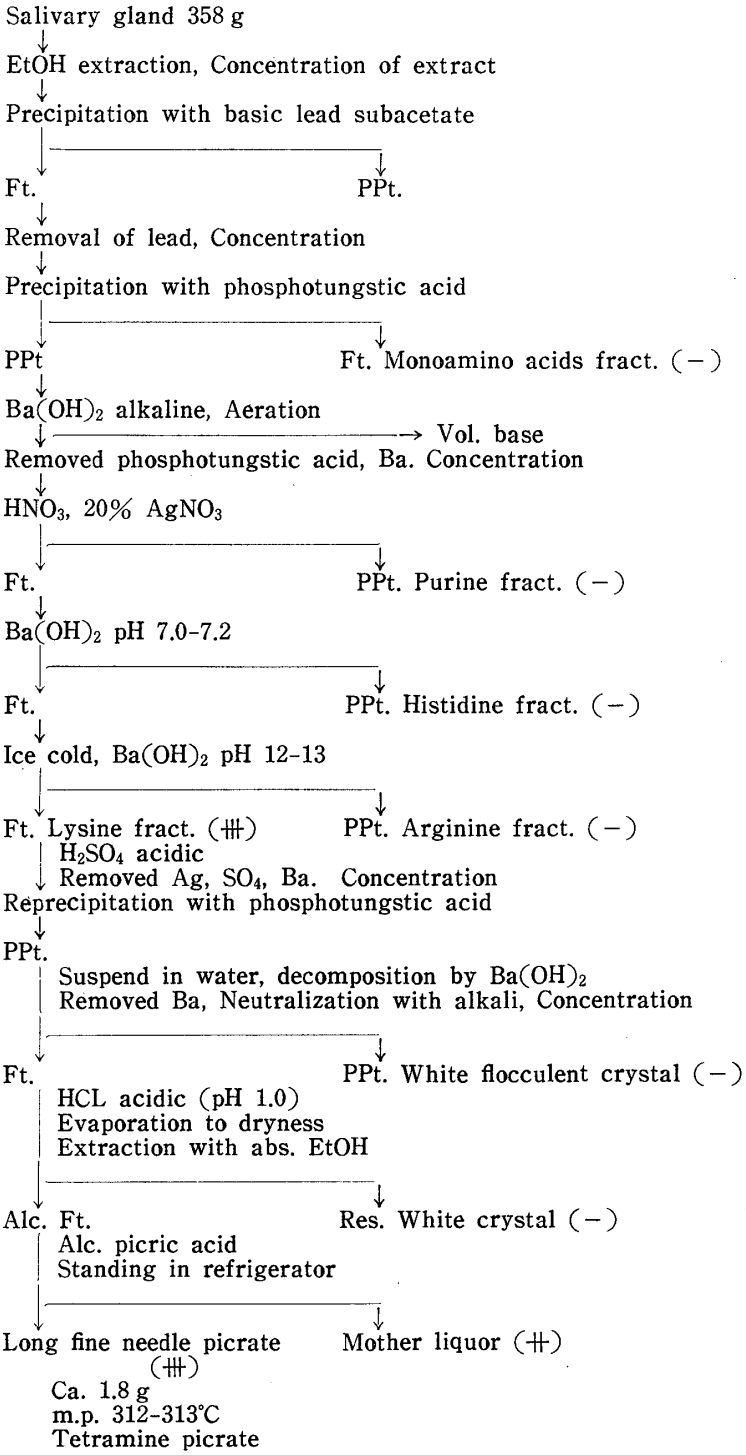
It showed that the toxicity was exclusively found in lysine fraction. All other fractions were non-lethal.

It is well known that the constituents included in the lysine fraction are diamines, diamino carbonic acids, betaines, ω-amino acids, neurine, N-methylpyridiniumhydroxyde, tetramine etc.

Then, starting from 358 g of the salivary gland of the shellfish, the authors

obtained the lysine fraction after the method shown in Fig. 4. Then, Phosphotungstic acid was again added to this fraction and the resulted precipitate was collected, suspended in water and decomposed by addition of $Ba(OH)_2$. After

Fig. 4. Flow sheet for the fractionation of the lethal component by Vickery Leavenworth's method and isolation of tetramine.



filtration of barium phosphotungstate, excess barium was removed as $BaSO_4$. The filtrate was neutralized by NaOH and concentrated to a small volume. White flocculent crystals which were non-poisonous were removed and the residual solution was made acid by HCL (pH 1.0, to Thymol Blue) and evaporated to dryness under reduced pressure. The residue was extracted by absolute ethanol. Alcohol insoluble residue was also non-poisonous. Then the alcoholic picric acid solution was added to the alcoholic extract and the mixture was left to stand overnight in a refrigerator. A long fine needle crystal of picrate was obtained, and alcoholic picric acid solution was repeatedly added to the mother liquor to obtain additional crop. The total yield was about 1.8 g picrate from 358 g of the materials used. (0.5%). The picrate was recrystallized from water. The m.p. (decomposition point) was 312–313°C (4) and it showed no depression when mixed with authentic tetramine picrate. Infrared spectra of the sample picrate and the tetramine picrate were quite identical. (Fig. 5, and 6). From the picrate, picric acid was removed by extraction with ether

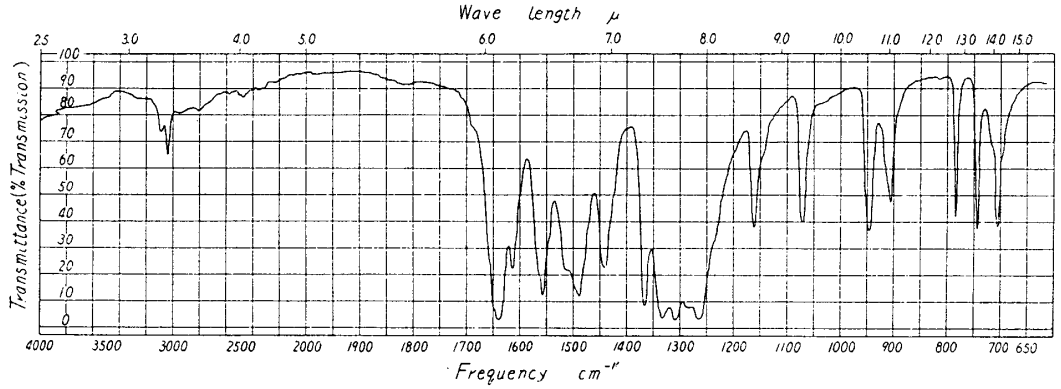


Fig. 5. Infrared spectrum of sample picrate.

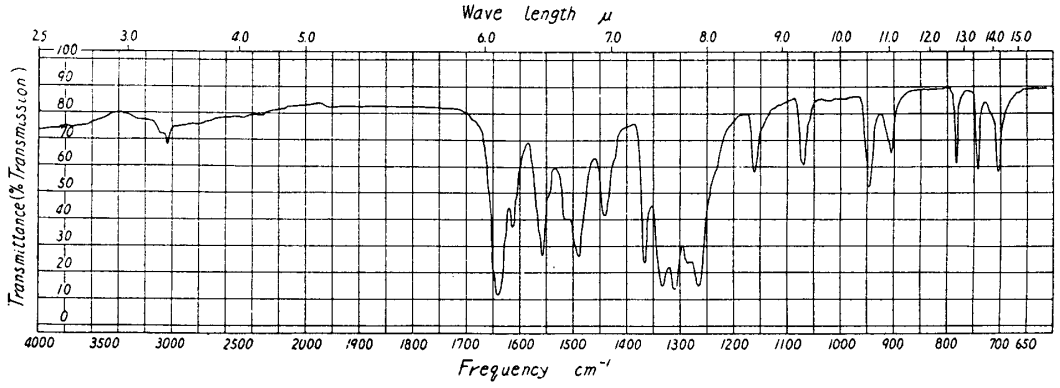


Fig. 6. Infrared spectrum of authentic tetramine picrate.

and HCL. Then 30 per cent of chloroauric acid was added to the remaining solution and tetramine chloroaurate of m.p. 335–336°C (4) was obtained. It also indicated no depression when mixed with the authentic specimen. Infrared

spectra of both chloroaurates were also identical. (Fig. 7 and 8).

The mother liquor was treated with ether and HCL and picric acid was similarly removed. The remaining solution was neutralized with alkali for

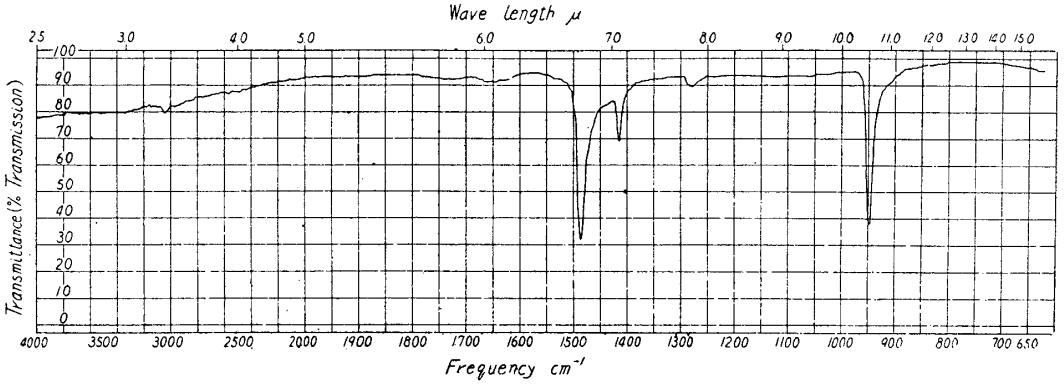


Fig. 7. Infrared spectrum of sample chloroaurate.

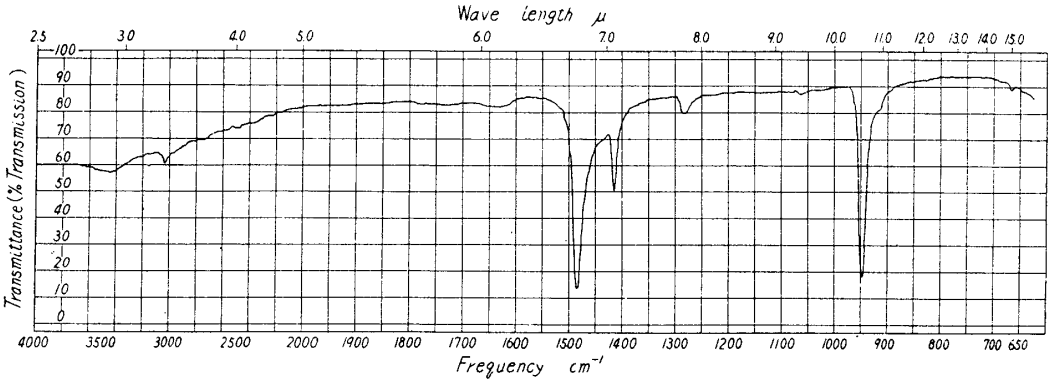


Fig. 8. Infrared spectrum of authentic tetramine chloroaurate.

injection into the mice. It was found that there still remains considerable toxicity in this solution. Therefore, a small quantity of unknown chloroaurate was prepared from it. Its m.p. continued to rise every time of recrystallization until over 300°C, however, the identification failed owing to its small quantity.

4. Paper chromatography of quaternary ammonium bases and choline esters.

As described in Section 2, the lethal component in the salivary gland reacts only positive with Dragendorff-, Chargaff-, and Brante (iodine vapor) reagents. The lysine fraction fractionated by Vickery Leavenworth's method was chosen as the spot solution for the paper chromatography. Paper chromatography of quaternary ammonium bases and choline esters was carried out as follows;

Solvent systems used: (a) n-PrOH : HCOOH : H_2O (8 : 1 : 1, vol)
(b) n-PrOH : IN AcOH (3 : 1, vol)
(c) n-BuOH : AcOH : H_2O (4 : 1 : 5, vol)
(d) n-BuOH : EtOH : AcOH : H_2O (8 : 2 : 1 : 1, vol)
(e) n-BuOH saturated with water

Reference substances (authentic):

Tetramine 10 per cent aq.solution, Neurine bromide, Choline chloride, Acetylcholine chloride, Urocanylcholine bromide, Benzoylcholine chloride, Acetyl-β-methylcholine chloride, Succinylcholine chloride, Myristylcholine chloride, Lauroylcholine chloride, β-(3-Indolyl)-propionylcholine iodide, Trigonelline, Nicotine sulfate

Developer: Dragendorff reagent (5), Chargaff-Levine reagent (6)

The Rf values of the spot solution and the reference substances in the paper chromatograms are listed in Table 8. Considering from the Rf values, it was

Table 8. Paper chromatography of quaternary ammonium bases and choline esters.

Spotted substance	Rf values in paper chromatogram run with solvent system of				
	(a)	(b)	(c)	(d)	(e)
Sample sol. (Free form)	0.66#, 0.50#	0.55#, 0.43#	0.55#, 0.40#	0.47#, 0.35#	0.1-0.3T.
Sample sol. (HCl salt)	0.37, 0.50	0.25, 0.43	0.23, 0.40	0.15, 0.35	
Tetramine	0.66	0.55	0.55	0.47	0.1-0.3T.
Neurine	0.69	0.56	0.60	0.47	0.1-0.2
Choline	0.64, 0.49	0.55, 0.45	0.55, 0.35	0.50, 0.35	0.1
Acetyl-*	0.70, 0.52	0.56	0.59	0.50	0.1-0.3T.
Urocanyl-*	0.27, 0.48	0.52	0.10, 0.48	0.10, 0.47	0.1-0.2
Benzoyl-*	0.86	0.74	0.83	0.75	0.45-0.75T.
Acetyl-β-methyl-*	0.73	0.64	0.76	0.63	0.1-0.4T.
Succinyl-*	0.28	0.27	0.10	0.05-0.1	0
Myristyl-*	—	0.9-0.93	—	—	0.75-0.90T.
Lauroyl-*	—	0.9-0.93	—	—	0.80-0.95T.
β-(3-Indolyl)-propionyl-*	0.84	0.83	0.8-0.85	0.75	0.5-0.65T.
Trigonelline	0.46	0.33	0.26	0.12	—
Nicotine sulfate	0.13, 0.74T.	0.15-0.20T. 0.66T.	0.05, 0.77T.	0.05, 0.70T.	—

Remarks: Solvent systems (a) n-PrOH:HCOOH:H_2O(8:1:1 vol)
(b) n-PrOH:1NAcOH(3:1 vol)
(c) n-BuOH:AcOH:H_2O(4:1:5 vol)
(d) n-BuOH:EtOH:AcOH:H_2O(8:2:1:1 vol)
(e) n-BuOH saturated with water
Overlapping spots, probably of tetramine and choline compounds.
* It indicates choline esters respectively.
T. Tailing of spot

found that the authentic substances whose Rf values were almost the same or closely resembled to that of the lethal component were tetramine, neurine,

choline and acetylcholine. The probability of the lethal component being neurine can be eliminated owing to the other properties such as m.p. of the derivatives or the behavior towards decoloration reaction with N/15 $KMnO_4$.

Urocanylcholine can be distinguished from the lethal componènt by Diazo reaction (yellowish shade). The coexistence of choline and acetylcholine are discussed in a later section.

The spot corresponded to tetramine was obtained without exception in all paper chromatograms. Generally, choline esters exhibit a light yellowish brown color by spraying with the Dragendorff reagent, while quaternary ammonium bases show a dark brown color with the same reagent.

5. The experiment of antagomism between the lethal component and the other pharmacologically active reagents.

Table 9. Antagonism between the lethal component and the other pharmacologically active reagents.

Strain of Mice	Body Wt. & sex of mice	Contents of injected sol.	Survival time after inject.	Average survival time
Red (rr)	18.7 g ♂	Salivary gland extract (about 30 M.U.*) 0.5 *ml*	11′10″	
,,	17.7 ♀	,,	1′20″	9′00″
,,	23.2 ♀	,,	14′30″	
Red (rr)	19.2 ♀	Salivary gland extract 30 M.U. +Adr. 150 μg	38′10″	
,,	17.2 ♂	,,	34′25″	33′32″
,,	16.0 ♀	,,	27′30″	
,,	19.5 ♂	Salivary gland extract 30 M.U. +Adr. 250 μg	54′35″	
,,	21.0 ♂	,,	36′30″	43′17″
,,	20.8 ♂	,,	38′45″	
,,	19.0 ♂	Salivary gland extract 30 M.U. +Adr. 300 μg	56′40″	
,,	17.0 ♂	,,	30′40″	48′10″
,,	18.5 ♂	,,	56′50″	
Red (rr)	15.0 ♀	S.G. Extr. 30 M.U.+Atr. 25 μg	6′ 5″	
,,	16.7 ♂	,,	13′25″	11′ 5″
,,	18.5 ♀	,,	13′45″	
,,	18.5 ♂	S.G. Extr. 30 M.U.+Atr. 250 μg	6′15″	
,,	19.0 ♂	,,	13′ 5″	7′26″
,,	15.0 ♂	,,	3′00″	
Red (rr)	16.0 ♀	S.G. Extr. 30 M.U.+Rest. 75 μg	6′10″	
,,	15.5 ♀	,,	7′25″	7′33″
,,	16.5 ♀	,,	9′ 5″	
,,	19.5 ♂	S.G. Extr. 30 M.U.+Rest. 300 μg	13′10″	
,,	15.5 ♂	,,	5′25″	9′15″
,,	17.5 ♂	,,	9′10″	

Remarks: * M.U. (mouse unit)is defined as the number of gram of mouse (mice) which is (are) killed by intraperitoneal injection of the poison contained in 1g of salivary gland.
Adr. 1-Adrenaline
Atr. Atropine sulfate
Rest. Restamin (Benadryl)
S.G. Extr. Salivary gland extract

To clarify the pharmacological properties of the lethal component in the salivary gland and to estimate its rôle to what extent it plays in total toxicity, the antagonism of a pharmacologically active reagent to the lethal component was examined when both were injected simultaneously into the mice (intraperitoneally).

Table 10. Antagonism between tetramine and the other pharmacologically active reagents.

Strain of mice	Body Wt. & sex of mice	Contents of injected sol.	Survival time after inject.	Average survival time
Red (rr)	24.0 g ♂	Tetr. 1000 μg	1′10″	
,,	25.0 ♀	,,	6′40″	
,,	21.5 ♂	,,	5′40″	7′49″
,,	23.0 ♀	,,	9′40″	
,,	23.0 ♀	,,	15′55″	
Red (rr)	24.5 ♀	Tetr. 1000 μg + Adr. 300 μg	5′25″	
,,	25.0 ♀	,,	76′35″	55′22″
,,	23.0 ♀	,,	84′ 5″	
Red (rr)	20.0 ♀	Tetr. 1000 μg + Eser. sol. 10^{-5}, 0.5 *ml*	20′45″	
,,	19.2 ♀	,,	6′25″	14′35″
,,	18.0 ♂	,,	16′35″	
Red (rr)	22.0 ♀	Tetr. 1000 μg + Atr. 200 μg	11′40″	
,,	23.2 ♀	,,	10′45″	8′34″
,,	22.2 ♀	,,	3′15″	
Red (rr)	20.0 ♀	Tetr. 1000 μg + Rest. 360 μg	12′10″	
,,	18.7 ♂	,,	14′20″	9′14″
,,	19.5 ♀	,,	1′10″	
Red (rr)	20.7 ♂	Tetr. 1000 μg + Nicotine 3.125mg	11′10″	
,,	22.2 ♂	,,	8′55″	
,,	20.0 ♀	,,	7′10″	8′41″
,,	19.0 ♀	,,	7′30″	

Remarks: Tetr. Tetramine
Eser. Eserine sulfate (Physostigmine)
Adr. 1-Adrenaline
Atr. Atropine sulfate
Rest. Restamin
Nicotine. Nicotine sulfate

The following reagents were used as antagonists; 1-Adrenaline, Atropine sulfate, Nicotine sulfate, Restamin (Benadryl) and Eserine sulfate. The results are shown in Tables 9 and 10. Of the reagents tested, 1-Adrenaline was the most effective for prolonging the survival time, probably due to the relaxation of clonic contraction of the bronchial muscle.

The same trend towards 1-Adrenaline was observed when the authentic tetramine was used instead of the lethal component. Therefore, it was confirmed pharmacologically that tetramine is the main active constituent in the salivary gland.

6. The coexistence of choline compounds in the salivary gland.

Besides tetramine, the coexistence of choline and probably acetylcholine was considered according to the following evidences.

a. Acetylcholine equivalence of the salivary gland was determined biologically on the frog rectus abdominis muscle (about 54 γ/g gland).

b. Total choline was estimated microbiologically by the Horowitz-Beadle method (7), using *Neurospora crassa* mutant ATCC 9277 as the test strain. (about 375 γ/g gland).

c. The purified extract by Banister's procedure (8) showed some decreases of toxicity after being subjected to alkaline hydrolysis (pH 12,100°C, 10 min.) suggesting that it is due to the decomposition of choline ester involved.

d. The total choline content in the hydrolysate of the purified extract by Banister's method was much larger than the ester content of equivalent amounts of the purified extract as measured by the ferric hydroxamate method (9). This implies the coexistence of choline and choline ester.

e. In both hydrolysates of the purified extract and the lysine fraction, acetic acid was found as the dominant volatile fatty acid component by the paper chromatography after Fink-Fink's method(10).

Table 11. Toxicity of salivary glands of the related species.

Species	Strain of mice	Body Wt. & sex of mice	Injected solution	Survival time after injection
Neptunea arthritica	Red (rr)	20.0 g ♀	2 *ml*:1 g△ 0.5 *ml*	22 min.
	,,	24.0 ♂	,,	3
	,,	24.0 ♂	,,	19
Neptunea arthritica	Red (rr)	23.5 ♀	1 *ml*:1 g* 0.5 *ml*	1 min.
	,,	22.5 ♀	,,	1.5
	,,	22.5 ♀	,,	1
Neptunea intersculpta	Red (rr)	20.0 ♂	1 *ml*:1 g 0.5 *ml*	1.5 min.
	,,	22.0 ♀	,,	3
	,,	22.0 ♀	,,	1.5
	,,	25.0 ♀	,,	1
Fusitriton oregonensis	Red (rr)	22.5 ♂	1 *ml*:1 g 0.5 *ml*	2 min.
	,,	25.0 ♂	,,	1.5
	,,	24.5 ♀	,,	1.5
Buccinum leucostoma	Red (rr)	18.0 ♀	1 *ml*:1 g 0.5 *ml*	20 min.
	,,	18.0 ♀	,,	Survives after moribund condition
	,,	18.0 ♀	,,	Survives

Remarks: It indicates thal the extractive from 1 g of tissue is dissolved in water, made up to 1 *ml*(*) or 2 *ml*(△).

7. Investigation of toxicity of the related species.

Investigation method of toxicity:

Fresh shellfishes were shucked, and the tissues (salivary gland, hypobranchial gland, gonad plus digestive diverticula) were dissected, weighed and immersed immediately in the alcohol solution (ethanol or methanol).

In the laboratory, each tissue group was extracted two times with neutral alcohol, and the alcohol was distilled off from the combined alcoholic extract. The residue was dissolved in water, made up to 1 ml or 2 ml so as to contain the residue from 1 g of fresh tissue. This final solution was used for injection. As the test animal, brown mice of stain Red (rr) were chiefly used although albino mice of german strain(dd) were sometimes used.

Table 12. Investigation of toxicity of the related species.

Species	Toxicity of tissues					
	Salivary gland	Hypobranchial gland	Gonad	Digestive diverticula	Gonad + Digestive diverticula	Injected solution
Neptunea arthritica	##	—	—	—		1 *ml*:1 g* Usu
,,	#	—	—	—		2 *ml*:1 g△ Onagawa
Neptunea intersculpta	##					1 *ml*:1 g Mori
Buccinum leucostoma	+	—	—	—		1 *ml*:1 g Mori
Volutharpa perryi	—	—			—	1 *ml*:1 g Mori
Babylonia japonica	—	—	—			2 *ml*:1 g Teradomari
Fusitriton oregonensis	#	—	—	—		1 *ml*:1 g Mori
Thais clavigera	—	—			—	2 *ml*:1 g Onagawa
Thais bronni	—	—			—	2 *ml*:1 g Onagawa
Nucella lima	—	—			—	2 *ml*:1 g Onagawa
Ceratostoma burnetti	—	—			—	2 *ml*:1 g Onagawa
Ocenebra spp.	—	—			—	2 *ml*:1 g Onagawa
Natica janthostoma	—				—	1 *ml*:1 g Mori
Tugalis gigas	—		—			2 *ml*:1 g Onagawa

Remarks: + indicates poisonous (lethal for mice).
— indicates non-poisonous.
It indicates that the extractive from 1g of tissue is dissolved in water, made up to 1 *ml*(*) or 2 *ml*(△). 0.5 *ml* of it is used for injection.

Related species investigated were as follows;

Neptunea intersculpta SOWERBY (Buccinidae, Hokkaido), *Buccinum leucostoma* LISCHKE (Buccinidae, Hokkaido), *Volutharpa perryi* JAY (Buccinidae, Miyagi), *Babylonia japonica* REEVE (Buccinidae, Niigata), *Fusitriton oregonensis* REDFIELD (Cymatiidae, Hokkaido), *Thais clavigera* KÜSTER (Muricidae, Miyagi), *Thais bronni* DUNKER (Muricidae, Hokkaido), *Nucella lima* MARTYN (Muricidae, Miyagi), *Ceratostoma burnetti* ADAMS ET REEVE (Muricidae, Miyagi), *Ocenebra spp.* (Muricidae, Miyagi), *Natica janthostoma* DESHAYES (Naticidae, Hokkaido), *Tugalia gigas* MARTENS (Haliotidae, Miyagi)

The results are shown in Tables 11 and 12.

Discussion

In the previous paper(1), the authors confirmed that the poisonous substance in the salivary gland are physiological constituents unrelated to the freshness or postmortem changes of the shellfish.

In the present paper, the lethal component was localized on the paper chromatogram by means of paper chromatography, kymography and toxicity test. The substances which react strong positively for Dragendorff reagent, are very hygroscopic and precipitable with ammonium reineckate or phosphotungstic acid. It was supposed that the substances are tertiary- or quaternary ammonium base or mixture of both.

For the fractionation and purification method of them, Miyaki's procedure (2), used for the isolation of histamine, and the Banister's procedure of choline esters (8) were chosen and examined. However, the fractionation among the lethal component and other accompanying contaminants (Diazo-, Dithiocarbamate-, Nitroprusside positive substances) was unsatisfactory in both procedures.

Fractionation with ion exchange (Amberlite IRC 50) by Kunin's separation method of basic amino acids (11) clarified that the toxicity is exclusively found in the lysine fraction. Ion exchange technique seemed to be more simpler than the Vickery's method described later, however, the drawback was reasonably difficult elution of the lethal component from the resin against the easy adsorption to it. For the complete elution, large amounts of acid solution was necessary, since the ionic bond between the lethal component and the resin particles was very tight.

Then the usual Vickery Leavenworth's method was employed as an alternative. Fractionation by this method followed by the toxicity test of each fraction showed that the toxic component only existed in the lysine fraction. Subsequently the fraction was treated as shown in the flow sheet (Fig.4), and the lethal component in it was identified as tetramine. The evidences of

identification were obtained from the m.p.s of the derivatives (picrate, chloroaurate), no depression of m.p. of mixed specimens, infrared absorption spectra, paper chromatographical behavior and pharmacological tests.

It was Ackermann (1923) who first discovered tetramine in the animal kingdom (*Actina equina*, Coelenterate). He isolated, starting from 33.28 kg of Actinia, 12.4 g of pure tetramine hydrochloride (4).

The authors obtained about 1.8 g of tetramine picrate from 358 g of the salivary gland. (yield 0.5%). Although the isolation was carried out for identification and not for the quantitative purpose, the yield of tetramine in the salivary gland was about five times as much as in Actinia when both are compared as picrate.

Investigation of the toxicity of the related forms proved that the salivary glands of at least three other species (*Neptunea intersculpta*, *Buccinum leucostoma* and *Fusitrition oregonensis*) are poisonous. Kanna and Hirai (1956) reported the similar symtoms (such as visual disturbance, chill and nausea) of the food poisoning caused by the consumption of *Neptunea intersculpta* taken from the Japan sea area and the occurrence of poison only in the salivary gland. (12). Poisonous components in such species are also Dragendorff-positive and likely to be tetramine or related compound.

Owing to the lack of an available determination method for tetramine, the authors estimated tetramine (or tetramine equivalence) by employing the quantitative paper chromatography technique of Miyaki (13) in which the spotted paper strip (7×400 mm) was run with the solvent system of n-PrOH : HCOOH : H_2O and was developed by spraying with Dragendorff reagent. For the more accurate determination of tetramine by this method, further modifications in detail are necessary, however, approximate equivalence contained in the salivary gland of *Neptunea arthritica* was 4–7.5 mg/g gland.

The pharmacological actions of tetramine are consisted of curare like activity and stimulation of parasympathetic system. (14). These actions well accounts for the following symptoms such as motor paralysis, respiratory failure (paralysis of respiratory muscle), salivation, lacrymation, miosis and peristalsis in the actual food poisoning and in the animal experiment.

Tetramine as the paralysing reagent of motor nerve ends resembles tetrodotoxin, however, both are quite dissimilar in chemical nature.

The toxicity of tetramine when administered per os to the warm blooded animal, is 5–10 times weaker than that of tetramine by parenteral injection. Its secretion from the warm blooded animal is considerably rapid, therefore, this agrees with the transient symptoms of the food poisoning.

Toxicity tests of the poisonous principle in the salivary gland and authentic tetramine for fish (*Cyprinus carpio*) were carried out by submuscular injection or by per os administration using polyethylene catheter. After injection or

administration, the following symptoms were observed in both cases; gradual loss of the balance, inversion of the abdomen, delayed respiration rate, clonical convulsion and final death.

Prof. Misawa, in his extensive studies on food allergy, pointed out neurine as one of his "pseudoallergen". (15). The authors report here the actual case of food poisoning chiefly due to tetramine and wish to regard it as one of such "pseudoallergen".

Tetramine is regarded as the major salivary poison, however, the synergic action of coexisting choline compounds must not be overlooked. These choline compounds are considered to potentiate the stimulating action of the parasympathetic system of tetramine.

The choline compounds are widely and abundantly distributed in Mollusca. And recently the occurrence of physiologically active choline esters, e.g., urocanylcholine in four species of Muricidae; β, β-dimethylacrylylcholine in *Thais floridana*, are reported. (16). Such choline compounds and quaternary ammonium bases may play an important rôle in manifesting allergy like symytoms in shellfish poisoning.

Summary

1. Poisonous substance in the salivary gland of a marine gastropod, *Neptunea arthritica* was located by using jointly the paper chromatography, kymography (Magnus method) and the toxicity tests (intraperitoneal injection into mice).
2. The lethal component (Dragendorff-positive) was exclusitively found in the lysine fraction and identified as tetramine (Tetramethylammoniumhydroxyde). The evidences of identification were obtained from m.p.s of the derivatives, infrared spectra, paper chromatographical behavior and pharmacological tests (antagonism tests).
3. 1-Adrenaline reacts antagonistically to the lethal component and was the most effective for prolonging the survival time after injection.
4. Tetramine content in the salivary gland was assayed approximately by the quantitative paper chromatography.
5. Besides tetramine, the coexistence of choline and probably acetylcholine in the salivary gland was discussed.
6. Of 12 related species examined, at least three species (*Neptunea intersculpta*, *Fusitriton oregonensis* and *Buccinum leucostoma*) contain the similar poison in their salivary glands.

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Addendum

Immediately after finishing this report, the senior author received a private communication from Dr. Ragnar Fänge, University of Lund, Sweden, that he is also working on the toxic effects of extracts of the salivary glands of *Neptunea antiqua*. He wrote that the toxic substance corresponds very well with the base neurine (or a compound closely related to neurine). The authors identified the salivary poison of *Neptunea arthritica* as tetramine and assumed that the salivary poison of *N. antiqua* appears to be the same substance.