OCCURRENCE OF CHOLINE IN THE SHELLFISH, CALLISTA BREVISIPHONATA CARPENTER

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By

Motokazu ASANO

Department of Fisheries, Faculty of Agriculture,
Tohoku University, Sendai, Japan
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Introduction

Shellfish poisoning from *Callista brevisiphonata* Carpenter is, as we have already reported (1), of allergic nature, and its clinical symptoms are the complex of allergic urticaria, bronchitic asthma and gastro-intestinal disturbances. Concerning the mechanism of allergic manifestation based on antigen antibody reaction, there are two theories, namely, physical and chemical ones. As the allergen in the latter theory, a number of investigators have attributed it to two active principles, the one is a group of histamine and so-called 'H-like substances' and the other is a group of acetylcholine.

In this paper, the author has tried to determine whether one of these compounds is originally present in the shellfish or another allergen is responsible for the secondary occurrence of these active substances in vivo. Furthermore, he has analysed whether this food poisoning is genuine allergy or is an apparent one caused by the "pseudoallergen" according to Misawa et al (2). The correlation between the poisonous principles and its seasonal prevalences was also analysed.

This study was undertaken as a part of the cooperative study 'On the cause and mechanism being poisonous of shellfish and its prevention from food poisoning'. The expense for this study was partly defrayed by the research funds of Ministry of Education for which the author expresses his cordial thanks. He also expresses his gratitude to the Mori Health Center for the samples and many conviniences received throughout this study. Technical assistance of Mr. Takayanagi, Miss T. Kitamura and S. Kohama is also acknowledged.

Experimental

1. Acetylcholine and choline compounds.

A. Determination of lecithine content in visceral organs of the shellfish.

Lecithine content of the visceral organs (ovary, testes and digestive diverticula) were determined by Lintzel's method (3). Fresh organs were weighed, minced in a mortar with sea sand and extracted by boiling several times with alcohol, alcohol-ether mixture (3 : 1) and ether respectively. The combined extracts were evaporated to dryness under reduced pressure and the residues were dissolved in small amounts of ether. (lecithine samples). Definite volume of the samples were taken and evaporated. Then they were saponified by alcoholic-KOH. The hydrolysates (solution of choline formed) were neutralized by the addition of hydrochloric acid, washed several times with petroleum ether. Then they were gently decomposed by three per cent potassium permanganate solution. Trimethylamine generated was absobred in $N/50$ sulfuric acid solution after passing through the trapping bottle of alkaline formaldehyde. Then the acid solution was titrated by $N/50$ trimethylamine solution, using phenolphthalein as an indicator. Lecithine content = Trimethylamine-N × 50.

The results are shown in Table 1.

Table 1. Lecithine content of the visceral organs of the shellfish.

Organ	Lecithine content	average weight of each organs per one shellfish	average lecithine content per one shellfish. (calculated)
Ovary	24.3mg/Ig fresh organ	20.0g	486 mg
Digestive diverticula	20.1	3.5	70
Testes	9.7	17.5	170

B. The estimation of lecithase in visceral organs of shellfish.

The estimation of lecithase was made as follows.

Enzyme solution : Each organs (Ig) were minced in a mortar and suspended in 100 ml. of water.

Substrate : Two grammes of lecithine was dissolved in the smallest amounts of ethanol and made up to 100 ml. with water.

The composition of reaction medium consist from 2 ml. of citrate buffer (pH 6.0). These reaction media were incubated at 37°C for 48 hours and titrated with 0.0225 N NaOH. The results are indicated in Table 2.

C. The determination of choline ester-splitting enzyme in the visceral organs of the shellfish.

Choline ester-splitting enzyme was determined after Stedman's method (4).

Enzyme solution : 10 g of each organs were minced in a mortar and suspended in 100 ml. of water to which was added 20 g of ammonium sulphate. Then they were centrifuged. To the supernatant, 23 g of ammonium sulphate and

Table 2. Lecithase in the visceral organs of the shellfish.

Fraction No.	Lecithase activity expressed as the titer of 0.0225N NaOH.		
	ml.	(1)ml.	(2)ml.
1. (D)+(L)+(B)	0.40	0.25	0.10
2. (D)+(D.W.)+(B)	0.15		
3. (O)+(L)+(B)	0.25	0.15	0
4. (O)+(D.W.)+(B)	0.10		
5. (T)+(L)+(B)	0.20	0.15	0
6. (T)+(D.W.)+(B)	0.05		
7. (C)+(L)+(B)	0.30	0.30	0.15
8. (C)+(D.W.)+(B)	0		
9. lecithine emulsion (control)		0.15	

Remarks : Fraction Nos. 2, 4, 6, 8, are fraction controls.

   (1) Figures in this column were obtained by substracting the values of fraction controls (Nos. 2, 4, 6, 8) from the corresponding values of Nos. 1, 3, 5, 7 respectively.

   (2) Figures in this column were obtained by substracting No. 9 from the values of the second column respectively.

Abbreviations :

(D)	Digestive diverticula	(T)	Testes
(L)	Lecithine emulsion	(C)	Crystalline style
(B)	Citrate buffer		
(O)	Ovary		
(D. W.)	Distilled water		

8 ml. of N/2 acetic acid were added (slightly acid to lithmus). After allowing to stand for 30 minutes, the mixed solution was centrifuged. The precipitate remaining in the centrifuge was mixed with an equal volume of ammonium sulphate solution (35 g per 100 ml.) and left overnight and then centrifuged. The supernatant was dialysed with water in a cellophan bag for a night and a clear enzyme solution was obtained.

Procedure : One hundred ml. of water was heated to 30°C to which the acetylcholine solution of known concentration (2 ml) was added. After adding 5 drops of B. T. B. indicator, the reaction medium was adjusted to pH 7.4 by the addition of 0.0225N NaOH. 10 ml. of the tissue enzyme solution was added to the reaction medium and incubated at 37°C for varying time intervals. Due to the enzymic liberation of acetic acid, the reaction of the digest medium shifts more to the acid side, therefore, increased acidity can be titrated by 0.0225N NaOH until initial pH 7.4. Titers of 0.0225N NaOH indicate the choline ester-splitting enzyme activity. The results are shown in Table 3.

D. Isolation of acetylcholine and choline.

Isolation of acetylcholine and choline was attempted after Kapfhammer-

Table 3. Choline ester-splitting enzyme in the visceral organs
of the Shellfish.

Organ	Acetylcholine 11.1 mg/ml.		Acetylcholine 7.0 mg/ml
	After 5 min.	After 10 min.	After 1 hour
Ovary	4.60	5.35	3.60
Digestive diverticula	3.20	4.80	3.00
Crystalline style	3.10	4.75	3.00
Testes	—	—	3.60
Control	0	0	0

Bischoff's method (5). Extracts of the visceral organs were made acid by the addition of oxalic acid and were extracted with 3 vol. of 96 per cent ethanol. After standing for a few hours, the combined extracts were evaporated to a small volume at low temperature (below 40°C), and then an equal volume of 20 per cent trichloracetic acid were added to the residue and left overnight in an ice box. Then the deproteinized solution was placed into the separating funnel and shaken with anhydrous ether to remove the fatty substance and trichloracetic acid. The aqueous fraction was filtered and concentrated under reduced pressure to remove the remaining ether. To the aqueous solution, the solution of reinecke salt (4g/100 ml.) was added and left overnight in an ice box. A rose colored precipitate of reineckate was freed from the solution by centrifugation and then rinsed in a centrifuge tube once with iced water, thrice with anhydrous alcohol, and again once with anhydrous ether. Washed reineckate was dried over anhydrous P_2O_5 in a vacuum dessicator. Dried pulverized reincekate was dissolved in the smallest possible volume of acetone and filtered. Dark reddish acetone solution was evaporated and the residue was extracted with anhydrous ether. The residue (crude reineckate) was dried in the P_2O_5 dessicator. 2.000 g of crude reineckate thus obtained was dissolved in 100 ml. of acetone-water mixture (1 : 1), and 123.40 ml. of silver sulphate solution (6.00 g silver sulphate was dissolved in a liter of water.) was further added to it. After filtering off the silver reineckate, 56.64 ml. of $BaCl_2$ solution (2.5626 g $BaCl_2.2H_2O/250$ ml. distilled water) was added to the colorless filtrate and $BaSO_4$ thus formed was filtered off. The filtrate was concentrated under reduced pressure until 10 ml. 10 ml. of 10 per cent $HAuCl_4$ solution was added to the concentrate and left for over 12 hours. Dark yellow colored chloraurate was recrystalized three times from water and the needle crystal was identified as choline chloraurate as indicated in Tables 4 and 5.

E. Determination of choline content in the ovary extract.

As the presence of choline in the ovary was confirmed in the former section,

Table 4. Melting point of chloraurates recrystalized thrice from water.

Melting point of recrystallized choline chloraurates	Organ	Remarks
Ovary	249°C	} These chloraurates seems to be chloraurates of the substances other than choline.
Testes	194-196°C	
Digestive diverticula	215-217°C	
Acetylcholine chloraurate	166-168°C 154-155°C	DUDLEY (1931) NOTHNAGEL (1894)

Table 5. Identification of choline chloraurate from ovary extract with prepared chloine chloraurate.

Preparation	Melting point	Au%	N%	Remarks
Choline chloraurate from ovary extract	249°C	43.60	3.26	
Prepared choline chloraurate	251°C	44.02	3.25	
Calculated as				Melting points reported by
$C_5H_{14}ON \cdot Cl \cdot AuCl_3$	244-243°C } 249°C 250-252°C	44.50	3.18	SMORODINZEW (1912)
	267-270°C 257°C			LOHMANN (1908) REUTER (1912)

choline content was determined after Shaw's method (6). The sample solution was prepared by dissolving the extract into 10 per cent trichloracetic acid (Ig per 10 ml. of trichloracteic acid). The filtered solution was extracted by shaking once with ether, twice with petroleum ether and again twice with ether. Then it was evaporated at 40°C until 1 ml. being equivalent to 1-2 g of the organ. A standard reincekate suspension was prepared by adding 0.3 ml. of ammonium reincekate to 1 ml. of a solution of choline containing 20 μg. It was centrifuged and then the precipitate was suspended in 1 ml. of water. To 1-5 ml. of the organ extract was added half the volume of ammonium reineckate. 0.1-0.5 ml. of IN NaOH was added for 1-5 ml. of sample solution. Then the mixed solution was shaken. It was centrifuged for 2 minutes at 3000 r.p.m., the liquid was poured off and the tube was drained as much as possible, care being taken that none of the precipitate was lost. Water was now added, 1 ml. at a time, with shaking, till a suspension of the same density as the standard reineckate previously prepared was obtained. The volume added was noted and the tube was placed in a water bath at 60°C. After 2 minutes, it was centrifuged for 2 minutes and the fluid was poured into another tube; 0.3 ml.

of the iodine reagent was added and the developed color in the sample tube was compared with that of a standard in Duboscq colorimeter. 0.3 ml. of alcohol was added to the unknown and was again compared. The correct value was obtained by substraction. The standard was prepared by taking 1 ml. of a solution containing 25 μg of choline to which was added 0.5 ml. of ammonium reineckate. It was shaken and centrifuged, the fluid was poured off and 1 ml. of water is added to the precipitate which was now heated to 60°C, cooled and 0.3 ml. of iodine was added as before. The method does not serve to distinguish between choline and acetylcholine, so strictly speaking, the total choline was estimated.

The determination method of choline by Glick (7) was also applied ; this depend upon the extraction of choline containing lipids from natural sources, liberation of choline by hydrolysis, neutralization, formation of choline reineckate, dissolution of choline reineckate with acetone, spectrophotometric determination at 526 mμ. The results are shown in Table 6.

Table 6. Choline content in the ovary extract.

Method	Choline content	Remarks
SHAW's method GLICK's method	1.065 mg/Ig ovary extract 5.35 mg/Ig ovary extract	On dry basis. It indicates total choline. On dry basis. It indicates choline content as cholinechloride after hydrolysis by baryta.

2. Histamine and relating compounds.

A. Color reaction of histamine.

After Vickery-Leavenworth's procedure (8), the ovary extract was fractionized. Color reaction of imidazole compounds after Koessler-Hanke (9) was applied to both histidine- and arginine fractions, since histamine distributes in both fractions. The results of color reaction were negative for histamine- and arginine fractions, although positive for histidine fraction.

B. Isolation of histamine from visceral extracts of shellfish.

Isolation of histamine from the visceral extract was attempted after Abderhalden's method (10). However, the author could not obtain the picrate of histamine from all fractions. Therefore he carried out the paper partition chromatography, using butanol-acetic acid- water mixture (4 : 1 : 5) as the mobile phase. After developing, sodium carbonate was sprayed and next diazobenzene sulfoninc acid-sodium carbonate mixture was sprayed on the paper. However, no spot appeared at the position where should correspond to the

position of histamine, although appeared spots were much lower ones, corresponding to the histidine position, and the higher ones corresponding to the tyrosine position. (Figure 1).
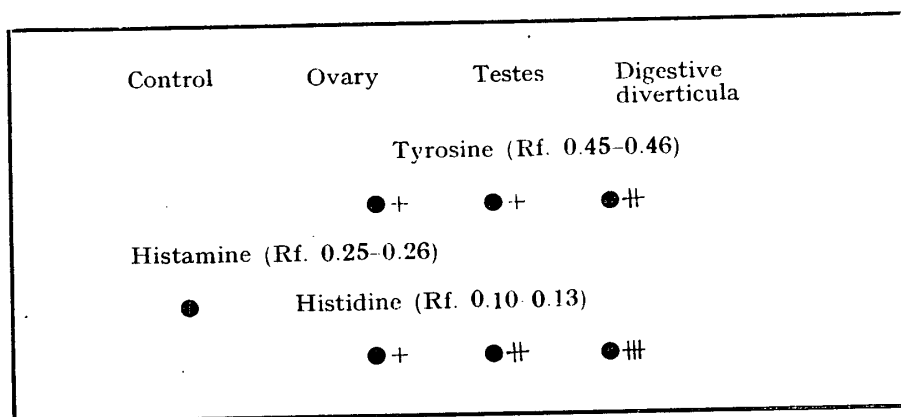
Control	Ovary	Testes	Digestive diverticula
	Tyrosine (Rf. 0.45-0.46)		
	● +	● +	● ++
Histamine (Rf. 0.25-0.26)			
●	Histidine (Rf. 0.10 0.13)		
	● +	● ++	● +++

Figure 1. Papergramm of visceral extracts by Pauli's reaction.

C. Changes of histamine content in blood plasma of guinea pigs sensitized by the shellfish allergen.

From the experiments mentioned above, the author could not perceive free histamine in the ovary extract, although the possibility of secondary occurrence of histamine in vivo still remains, being released from the bound histamine in blood plasma by the reinjection of the shellfish allergen. Shellfish allergen was prepared as already described (1), followed by the filtration with Seitz filter. The allergen was injected subcutaneously to guinea pigs, after three weeks, reinjected intravenously to sensitized guinea pigs. If the shellfish poisoning is genuine allergy based on antigen antibody reaction, an anaphylactic shock should accompany and the symptoms of hypersensitiveness such as decrease of body temperature (2-3°C subnormal), rubbing nose and shiver, excitation, uneasiness, characteristic convulsion, defecation, respiratory failure should accompany. However, in the experiment, observed symptoms were only shiver and slight decrease of body temperature (within 1°C).

The guinea pigs were killed at once to collect blood specimens. The change of histamine content in blood plasma was estimated after Lubschez's method (11). The result was the decrease of histamine content in blood plasma of reinjected guinea pigs as compared with that of the control animals, opposing the anticipation.

3. Kymographic observation of pharmacological action of the shellfish allergen on the isolated intestine of guinea pigs.

Kymographic observation of pharmacological action of the shellfish allergen,

by means of perfusion through the isolated intestine (ileum) of guinea pigs, was carried out in comparison with those of related compounds such as acetylcholine, histamine and betaine. About 2 cm length of the intestinal strip of a guinea pig was dissected and placed into the perfusion apparatus described in the report of Chen & Clarke (12). The apparatus was filled with a constant flow of Tyrode solution supplied with oxygen bubbles through a capillary and kept at 38°C to observe the response (chiefly contraction) of the intestinal strip for a variety of test solutions. The kymographic curves drawn by the intestinal strip are shown schematically in Figure 2. The response for acetylcholine and histamine, as is well known, showed severe contraction and that for betaine was slight contraction. On the contrary, the response for the sample solution (shellfish allergen and extract) indicated relaxation.
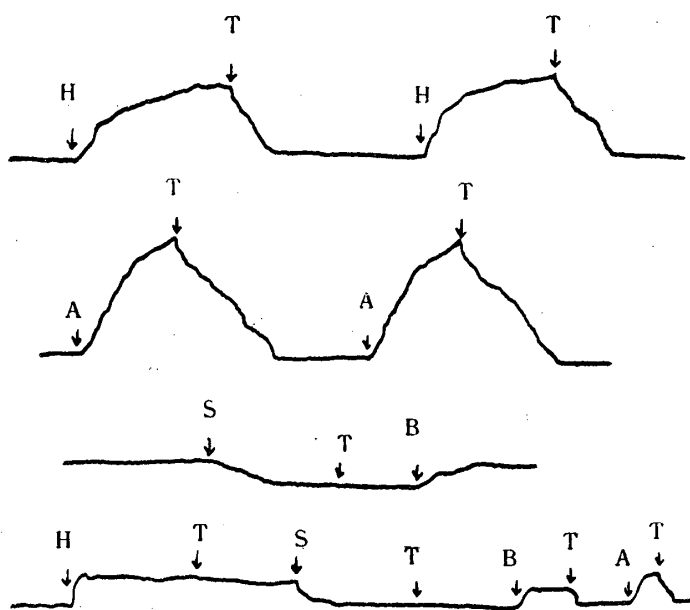

Figure. 2. Kymographic curves of the sample solution and other chemicals for isolated intestinal strip of guinea pigs. (513g female and 450g female) Concentration of acetylcholine, histamine and betaine are Img/ml. and Iμg/ml. Sample solutions are shellfish allergen and extract purified ater MC Tires method. Rate of rotation of the drawing drum is ca. Imm per second. (5.5:I) A..Acetylcholine, B..Betaine, H..Histamine, S..Sample solution T..Tyrode solution.

4. Phenolphthalein reaction in relation to seasonal variation of toxicity.

Phenolphthalein reaction, found by Akiba & Hattori (13), was applied to the

fresh tissues of the shellfish.

Preparation of phenolphthalein reagent : 2g of phenolphthalein is dissolved into 100 ml. of water to which is added 20 g of KOH and 10 g of zinc dust, this was heated until decolorized and then small granules of zinc were added. This was kept in colored bottle.

Procedure : A definite volume of the visceral organs (1g) was minced in a mortar and 1 per cent of the original sample solution was made by the addition of water. The solution was diluted stepwise to the proper concentration. To 10 ml. of each aliquot in the test tube was added 5 drops of phenolphthalein reagent, 2 drops of 30 per cent acetic acid and 3 drops of 3 per cent hydrogen peroxide solution. After allowing to stand for 10 minutes at room temperature a pink color developed. The smallest concentration (degree of dilution of original solution) in the series of aliquots which indicates a positive color reaction as compared with control, was defined as the critical concentration. Critical concentration (%) ×1000 value was also defined as P (1) value. Then, after boiling the original solution for one hour, the same procedure was repeated as before and the second critical concentration (%) ×1000 was defined as P (2) value. The results of the test are shown in Table 7.

Table 7. Phenolphthalein reaction in relation to seasonal variation of toxicity.

Sample	P(1) value	P(2) value	Remarks
Dried shellfish gonad	1000	1000	Non poisonous
Fresh shellfish Digestive diverticula	0.01–0.1	1000	10th July 1951
Testes	0.1–1.0	100	〃
Testes	10	1000	24th May 1952
Ovary	0.1–1.0	1.0	〃
Digestive diverticula	0.1–1.0	10–100	〃
Ovary	0.1–1.0	1.0–10	23th June 1952
Digestive diverticula	0.001	10	24th June 1952
Ovary	0.001–0.01	0.001–0.01	〃
Digestive diverticula	0.1–0.01	10–100	15th June 1953
Ovary	1.0–10	10	〃
Testes	1.0	100	〃
Ovary	1.0–10	1000	28th June 1953
Digestive diverticula	1.0	100–1000	〃
Testes	1.0–10	1000	〃
Ovary	0.001	1000	22th Sept 1953
Testes	0.01	1000	〃
Digestive diverticula	0.001	1000	〃

Discussion

As already mentioned, our attentions are focussed on whether an active substance occurs originally in the shellfish or occurs secondarily in vivo from inert mother substance induced by the shellfish allergen. There are many investigators who insist the cause of allergy to be due to the free histamine released from the bound histamine into plasma. With regard to histamine, the author could not, as far as examined, find out its participation in this allergy, against the anticipation and the results of many investigators. Negative results of color reaction and isolation of histamine, the decrease of histamine content in blood plasma of guinea pigs sensitized by shellfish allergen, and kymographic observations all agreed with this conclusion. Apparently effective application of antihistaminics to the subjects for the diagnosis of this poisoning is contradictory to the experimental evidence just described. This complicity is seen in the following experiment, carried out by TAKAYANAGI. He ascertained the antagonizing effect of antihistaminics upon the skin test of ovary allergen. Commercial antihistaminics (Restamin for injection) was mixed with the ovary allergen (1 : 9) and injected intracutaneously to the subjects as before. The effect of antihistaminics upon the ovary allergen was the decrease of redness and much less itching of the skin. (Table 8) It is interesting to note that Restamin displays its pharmacological action as anti-acetylcholine rather than as antihistamincs.

Table 8. Antagonizing effect of antihistaminics upon the skin test reaction of the shellfish allergen. (After TAKAYANAGI)

Fraction	Age & sex of subjects	Skin test reaction of ovary allergen after		
		5 min.	10 min.	30 min.
Ovary allergen +Antihistaminics (9 : 1)	30 ♂	20×25 mm °10×10	22×22 mm °13×10	40×45 mm °30×15
	25 ♀	35×30 °27×12	36×30 °28×13	40×28 °31×15
Ovary allergen alone (control)	30 ♂	45×35 °15×8	50×48 °26×15	50×55 °30×16
	25 ♀	53×36 °24×11	58×42 °30×14	50×40 °33×15

Remarks : Figures with mark (o) indicate the induration or papule.

The occurrence of choline has been ascertained. Melting point of a mixture of chloraurate from the ovary extract and of authentic choline chloraurate did not decrease.

As to the origin of choline, the lecithine-lecithase system is most responsible, according to Contardi & Ercoli (14). However in this experiment, the lecithine

content was much less than that of chicken eggs and lecithase activity was feebler than expected. The moderately stronger activity and its wide distribution of choline ester-splitting enzyme suggested that the occurrence of choline may be largely due to the degradation of choline ester by the latter enzyme. Considering from the steam distillation curves of the visceral organs after Duclaux, it is evident that acetic- and propionic acids are present in the distillate, therfore, it is supposed that these acid residues combine to form choline ester in the shellfish, although these choline esters could not be obtained in the experiment. From the results of the test causing hypersensitiveness and agglutination test to guinea pigs using the ovary allergen (Takayanagi), it is evident that this shellfish allergy is not genuine allergy based on antigen antibody reaction. The clinical pictures of the symptoms of this allergy can be called "cholinergic". Consulting literature (15), pharmacological action of choline is noted as follows : Stimulating action to the peripheral nervous system and autonomic system (decrease of blood pressure), weak nicotine action (primary decrease following increase of blood pressure, irritation of gangliones), muscarine action (respiratory paralysis, increased secretion of autonomic nerved glands), very slight miosis, accerelation of tonus, dilatation of isolated vessels, increase of lymphocytes. In comparing the symptoms of the shellfish allergy with those above described, it is clear that choline or choline ester (acetylcholine and pro-pionylcholine) play a chief role in allergic manifestation. The distribution of free choline in the animal kingdom is fairly universal, however, its content in the shellfish, *Callista brevisiphonata* CARPENTER, is reasonably abundant (100–500 mg choline per 100 g dry material.).

The toxicity of choline is not so severe as acetylcholine and histamine and this is the reason why the experimental animal such as mice are insensitive to the active substance in this allergy. However the initial quantities of choline in the shellfish may be small, it is still possible that it stimulates, when introduced, the parasympathetic nerve (Vagus) of sensitive subjects to release more active substance (acetylcholine) secondarily into the blood plasma. According to Misawa (2), apparent allergy caused by "pseudoallergen" such as neurine, choline, spinacin and trimethylamine must be distinguished from the genuine allergy. This allergy belongs to this category.

Akiba & Hattori have found phenolphthalein reaction to determine the toxicity of *Venerupis semidecussata* REEVE (Venerupin) in Hamana lake instead of biological assay, since moderately good correlation between the toxicity and the results of the reaction have been observed. Application of the test to our case in connection with seasonal variation of toxicity seems to have been successful. The reactions of the ovary and digestive diverticula show positive results, i.e., low P (1) value for both during spawning season, although P (2)

value of digestive diverticula after boiling for one hour indicates that this peroxidase activity is due to enzymic action. Whereas P (2) value of the ovary remained constant, indicating that it is due to the heat resistent substance. When the spawning season is over, these values increase in all cases showing the bivalves to be nontoxic. However, application of the test to our case seems to be less reliable, since there are too many variable factors to obtain consistent results. The significance of the relation between the results of phenolphthalein reaction and allergic nature awaits further investigation.

Summary

Occurrence of choline in the ovary of the shellfish, *Callista brevisiphonata* CARPENTER, has been ascertained. The content of choline ranges 100–500 mg choline per 100 g of dry material. Choline is presumably formed from choline esters by choline easter-splitting enzyme rather than by the degradation of lecithine by lecithase. When consumed, choline in the shellfish is supposed to be responsible for the secondary release of acetylcholine in the human plasma. This allergy belongs to the apparent allergy caused by the "pseduallergen" after MISAWA. As far as the experiment was concerned, histamine took no part in this allergy. As a measure of seasonal variation of toxicity, the skin test is more reliable than the phenolphthalein reaction.

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