26. Kyosuke Tsuda* and Masaaki Kawamura**: The Constituents of the Ovaries of Globefish. VIII.[1] Studies on Tetrodotoxin. (1).

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In the previous paper[1], it was reported that the purification of the toxic principle extracted from the ovaries of globefish yielded a crystalline substance which could further be purified by recrystallization from diluted acetic acid, alcohol, or ether.

By exactly the same procedure, 855 mg. of purified poison, tetrodotoxin, was obtained and the following experiments were carried out.

Using once-crystallized tetrodotoxin as the material, recrystallization was repeated five times, and elemental analyses of carbon, hydrogen, and nitrogen, and determination of potency were carried out with the sample from each recrystallization. Infrared absorption spectrum, specific rotation, and neutralization equivalence by titration with 0.01*N* HCl were also determined with some of the samples. The results are shown in Table I and Fig. 1.

As these data show, the six samples shown as Nos. 0 to 5 gave almost constant values, so that it can be assumed that this method of purification gives tetrodotoxin of definite quality.

TABLE I. Test Results of Samples obtained by Five Recrystallizations of Tetrodotoxin

No.	Yield mg.	Elemental Analyses[a]			Potency[b] γ	$[\alpha]_D$[c]	Equivalence[d]	Infrared Spectrum
		C%	H%	N%				
0	855	41.22	5.07	12.78	0.014～0.013	−8.64	346	Fig. 1A
1	500	40.33	5.55	12.82	0.013～0.012			
2	390	40.93	5.25	13.28	0.011～0.010			
3	349	40.23	5.41	12.85	0.010～0.009		363	
4	286	41.14	5.36	12.65	0.010～0.009		352	Fig. 1B
5	210	40.92	5.88	13.00	0.010～0.009		355	Fig. 1C

a) Samples were dried for 48 hours over phosphorus pentoxide, in a vacuum desiccator. All samples gave negative reactions for phosphorus (Iversen method), halogen (Beilstein test), and sulfur (alkali fusion method). No ash.
b) Minimun lethal dose by subcutaneous injection into mouse, indicated as γ/g. wt.
c) Determined on 855 mg. of sample dissolved in 10 cc. diluted acetic acid, c=8.55, at 25°.
d) Five to ten mg. of sample dissolved in 5 cc. 0.01*N* HCl and back-titrated with 0.01*N* NaOH. Equivalence of the sample calculated from the 0.01*N* HCl required. Indicator, methyl red.

Various qualitative estimations of tetrodotoxin were carried out which gave strong positive result for Molisch reaction (polyhydroxylacyl compounds, saccharides), weak positive result for Tarugi Lenci reaction[2] (primary and secondary amines, acid amides), and weak positive result for silver mirror reaction. Initial distillate obtained by the distillation of a mixture of the sample and 10% sodium hydroxide gave a positive reaction to the Nessler reagent.

The sample gave negative results to orcinol-hydrochloric acid reaction (pentose), Pauly reaction (pyrimidine, imidazole), Ehrlich reaction, Fehling reagent, Sakaguchi reaction, Ninhydrin reaction, xanthoprotein reaction, and Biuret reaction.

The infrared absorption spectra shown in Fig. 1 indicate following facts: The stretching bands for OH or NH appear at 3.0 μ and the peak of stronger absorption band at

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1) Part VII: J. Pharm. Soc. Japan, 72, 771 (1952); C. A., 46, 9733 (1952).
2) Tarugi Lenci: The Merck Index, 5th Ed., 931 (1940).

3.12 μ is due to the stretching of the hydrogen bond in the hydroxyl radical. Two strong absorption bands appear in the double-bond region, and the band at 6.0 μ is due to the stretching of the amide carbonyl -CO-N< or of C=N, probably of the former. The band at 6.23 μ is uncertain but it is assumed to be the one due to the stretching of the double bond conjugated to the amide carbonyl. The fact that the region of 5.7~5.9 μ lacks any absorption clearly shows that the molecule does not contain any ester, lactone, lactam, ketone, or aldehyde-type carbonyl.

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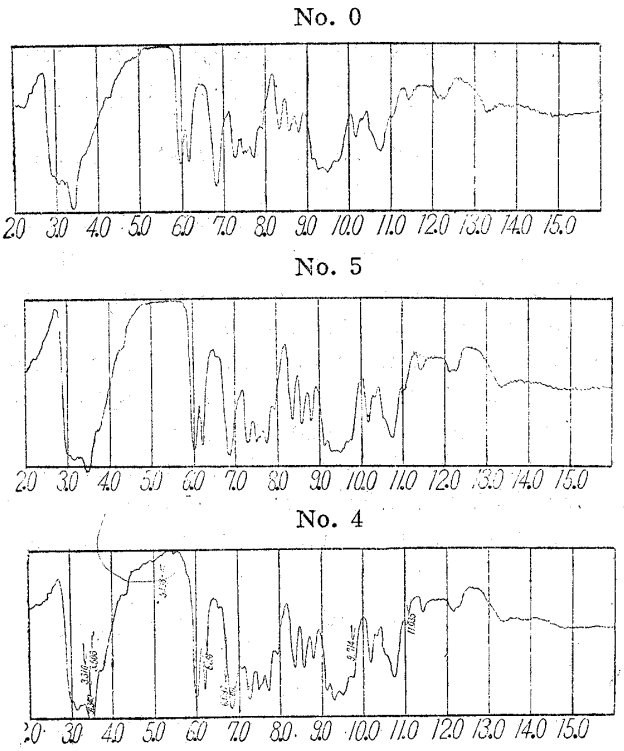

Fig. 1. Infrared Spectra of Tetrodotoxin (Nujol)

Experimental

Molisch Reaction—To a solution of 2 mg. of the sample dissolved in a drop of AcOH, 1 drop of 5% alcoholic solution of α-naphthol was added, and conc. H_2SO_4 superimposed over the mixture by carefully introducing it along the wall of the vessel. A distinct reddish brown ring appeared at the zone of contact.

Tarugi Lenci Reaction—To 5 mg. of the sample, crystals of freshly distilled phenol were added, and 3% NaOCl solution dropped in by which faint yellow color appeared. The hydrolyzate of tetrodotoxin (by HCl) gives bluish-green color.

Infrared absorption spectra were determined by the Baird Double-beam Infrared Spectrophotometer.

Summary

Tetrodotoxin crystals were obtained by the purification method given in the previous paper[1] and repeated recrystallizations were carried out to determine whether the purity could be increased by such a measure. Determinations were, therefore, carried out on elemental analyses, potency, neutralization equivalence, specific rotation, and infrared absorption spectrum. The results obtained showed that the crystals of definite purity had been obtained by the recrystallizations. Examinations of infrared absorption spectra indicated the presence of OH, (NH) and -CO-N< groups in the molecule. Qualitative estimations indicated the possibility of the presence of an acid amide group of a polyhydroxylacyl compound.

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