Tetrahedron, 1965, Vol. 21, pp. 2059 to 2088. Pergamon Press Ltd. Printed in Northern Ireland

TETRODOTOXIN[1,2]

T. GOTO, Y. KISHI, S. TAKAHASHI and Y. HIRATA
Chemical Institute, Faculty of Science, Nagoya University,
Chikusa, Nagoya, Japan

(*Received* 20 *February* 1965)

Abstract—A new procedure of extraction of tetrodotoxin (I) is described. The structures of three quinazoline derivatives, tetrodoic acid (VIII) and anhydrotetrodoic acid (XII) obtained from I have been elucidated. Based on these structures, the structure and the absolute configuration of the toxin (I) are assigned as Ib. It is also demonstrated that I is a C_{11}-compound and not a dimeric C_{22}-form. The toxin (I) and anhydroepitetrodotoxin (II) are mutually convertible by treatment with acids. Three acetyl derivatives of II are also described. Aminodesoxytetrodotoxin has the structure XXa and is not a dimeric C_{22}-form.

TETRODOTOXIN is one of the most toxic compounds among the poisons having low molecular weight.[3] This toxin is found mostly in the ovaries and livers of puffer fish (*Spheroides rubripes, S. vermicularis*, etc.; Japanese name *fugu*). Poisoning due to the toxin has long been a serious problem in Japan since puffer fish is highly esteemed for the table. The structural studies of the toxin have been carried out in our laboratory and the results reported in several papers. A summary of these studies together with some unreported observations are now described.

Isolation[4]

Since the end of last century many workers[5] have investigated the isolation of the puffer-fish toxin, tetrodotoxin, but only in 1950, did Yokoo[6] succeed in obtaining the toxin in crystalline form. Later, Tsuda and Kawamura[7] also obtained it in a pure state. Then, Nagai,[8] and Kakisawa, Okumura and one of us (Y.H)[9] reported modified methods of isolation of the toxin. All of the procedures, however, are lengthy and troublesome, and the yields usually very low.

[1] Part XI. For the preceding contribution, T. Goto, Y. Kishi, S. Takahashi and Y. Hirata, *J. Chem. Soc. Japan* **85**, 667 (1964).

[2] Preliminary communications appeared in *Tetrahedron Letters* 2105, 2115 (1963); 779, 1831 (1964), and were also presented at the *3rd IUPAC Symposium on the Chemistry of Natural Products*, 13 April, 1964 (Kyoto, Japan). Independent work leading to the same conclusion were presented by Professor Tsuda and his coworkers and Professor Woodward at this same Symposium. K. Tsuda *et al., Chem. Pharm. Bull., Japan* **11**, 1473 (1963), **12**, 634, 642, 1357 (1964); R. B. Woodward, *Pure Appl. Chem.* **9**, 49 (1964), *J. Amer. Chem. Soc.* **86**, 5030 (1964). Professor Mosher reported at the Symposium an interesting observation that tarichatoxin obtained from the California Salamander *Taricha torosa* is identical with tetrodotoxin, and he also reported its structural studies: H. S. Mosher *et al., Science, Lond.* **140**, 295 (1963), **143**, 474, **144**, 1100 (1964).

[3] Only the shellfish toxin, saxitoxin (E. J. Schantz, *Annals New York Acad. Sci.* **90**, 843 (1960)) and the frog toxin, kokoi venom (F. Märki and B. Witkop, *Experientia* **19**, 329 (1963)) were reported to have comparable toxicity with tetrodotoxin.

[4] T. Goto, S. Takahashi, Y. Kishi and Y. Hirata, *J. Chem. Soc. Japan* **85**, 508 (1964).

[5] For example, Y. Tahara, *J. Pharm. Soc. Japan* **29**, 587 (1909); *Biochem. Z.* **30**, 255 (1911). He gave his very crude toxin the name of tetrodotoxin.

[6] A. Yokoo, *J. Chem. Soc. Japan* **71**, 590 (1950); *Proc. Japan Acad.* **28**, 200 (1952). He named the toxin spheroidin.

[7] K. Tsuda and M. Kawamura, *Pharm. Bull. Japan* **1**, 112 (1953).

[8] J. Nagai, *Fukuoka Igaku Zasshi* **45**, 1 (1954).

[9] H. Kakisawa, Y. Okumura and Y. Hirata, *J. Chem. Soc. Japan* **80**, 1483 (1959).

Our improved isolation procedure[10] is shown in Fig. 1. A key step in this procedure is to boil the aqueous extract of the ovaries in order that a large portion of protein and enzymes, which cause autolysis, can be removed, and, after filtration, the filtrate is suitable for treatment on a column of ion-exchange resin. Otherwise, a large quantity of amino acids produced from the protein by autolysis prevents absorption of the

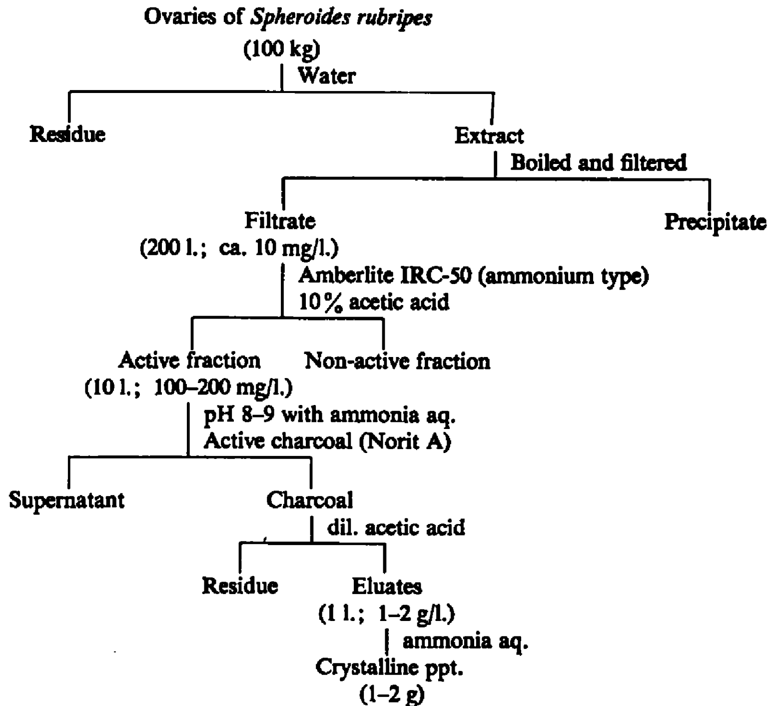


FIG. 1. Diagram of the isolation procedure.
Concentration of the toxin was calculated from the LD_{100} of the solution against mice (LD_{100} of the pure toxin is 0·01 γ/g mouse[6,7])

toxin on the resin. Since the content of the toxin is estimated at about 0·001[11] to 0·002%[9] of the ovaries, the yield of this procedure exceeds 50% of the original contents.

Purification[4]

Since tetrodotoxin does not dissolve in any solvents except acids and since in acidic solution it is partly epimerized to anhydroepitetrodotoxin (*vide infra*), purification of the toxin is very difficult. The usual methods which have been reported are the precipitation from acidic solutions by aqueous ammonia or ethanol–ether,[7] but the product thus obtained is not pure as shown below.

We found that it can be purified through its crystalline picrate. After recrystallization from hot water, the picrate is converted to the pure toxin by treatment with aqueous ammonia. Crystalline tetrodotoxin hydrobromide can only be obtained from the toxin purified by this method.

[10] Y. Hirata and T. Goto, Japanese patent No. 290717 (1961).
[11] K. Tsuda, *Shizen, Japan* **18**, 51 (1963).

As there are no known methods for an accurate estimation of the purity of the toxin (it does not show a sharp m.p. and the IR and NMR spectra as well as the toxicological tests are not very accurate) an examination of the titration curves gives the best results. Tetrodotoxin (I) and anhydroepitetrodotoxin (II) differ in p*Ka* values; the former being p*Ka'* 8·76 whereas the latter is 7·95. Thus, the pure toxin (I) obtained from the picrate shows a typical titration curve corresponding to one dissociation group, but the curve of an impure sample obtained by the usual precipitation method does not fit the theoretical curve calculated from the p*Ka'* 8·76 (Fig. 2).

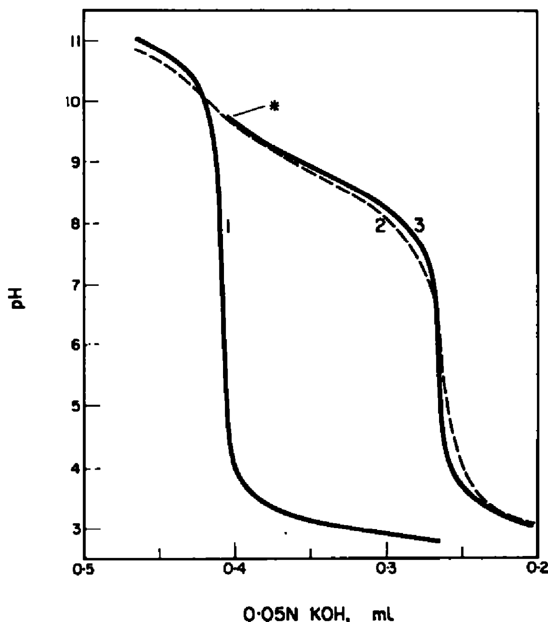


FIG. 2. Titration curves of tetrodotoxin.

Curve 1: blank; curve 2: tetrodotoxin purified by reprecipitation method; curve 3: tetrodotoxin purified through the picrate (pptd, at mark*). ca. 2·3 mg of sample in 2 ml of 0·01 N HCl was titrated with 0·05 N KOH.

The amount of the contaminating anhydroepi compound (II) can be measured as follows: Tetrodotoxin (I) dissolves slowly in aqueous potassium hydroxide with decomposition and the p*Ka'* 8·76 disappears but the anhydroepi compound (II) which does not decompose retains its p*Ka'* 7·95. If an impure sample dissolved in dil. hydrochloric acid, is made alkaline by addition of excess potassium hydroxide and the solution back-titrated with acid, then the consumption of the acid is due to the presence of anhydro (II) and occurs around pH 7·95. By this method it was shown that a sample obtained by the precipitation method contains about 15% II (Fig. 3).

Molecular formula[12]

The molecular formula of tetrodotoxin (I) was said by Yokoo[6] in 1952 to be $C_{12}H_{17}O_{10}N_3$. Later, Kakisawa *et al.*[9] and Tsuda *et al.*[13] gave a molecular formula of $C_{12}H_{19}O_9N_3$.

[12] T. Goto, S. Takahashi, Y. Kishi and Y. Hirata, *Bull. Chem. Soc. Japan* **37**, 283 (1964).
[13] K. Tsuda, M. Kawamura and R. Hayatsu, *Chem. Pharm. Bull. Japan* **8**, 257 (1960).

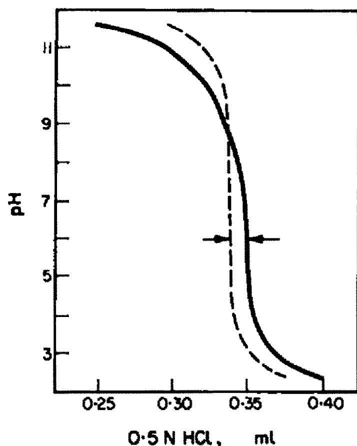


FIG. 3. Titration curve of impure tetrodotoxin.

To a solution of a sample (6·596 mg) in 0·0333 N HCl was added 0·100 N KOH (2 ml) and the mixture was titrated with 0·50 N HCl. (dotted line: blank curve). Difference between two curves at pH 6 corresponds to ca. 15% anhydroepitetrodotoxin (II) contaminated in I.

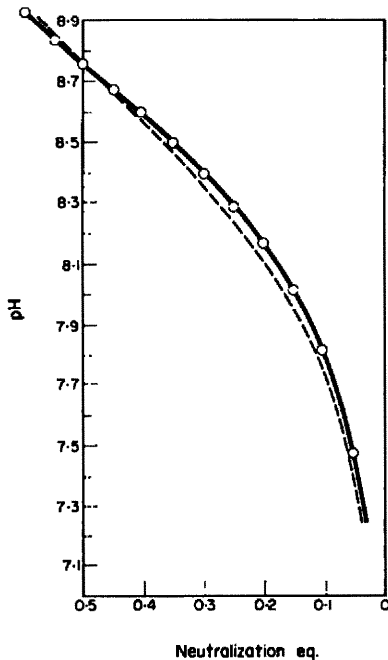


FIG. 4. Titration curve of pure tetrodotoxin.

Sample (1·242 mg) in 0·01 N HCl (2 ml) was titrated with 0·025 N KOH. Circle: observed value; solid line: calc. curve as having one dissociation group of p*Ka* 8·76; dotted line: calc. curve as having two dissociation groups of pKa_1 8·46 and pKa_2 9·06.

The purified toxin described above has a p*Ka'* 8·76 and the equivalent values corresponding to this dissociation were determined by several titrations to be between 311 and 324. The mol. wt. of a crystalline acetyl derivative of I, pentaacetylanhydroepitetrodotoxin (XVI) *p*-toluenesulphonate (*vide infra*), which is converted to the toxin (I) by hydrolysis to II and epimerization of II, was found to be 673 $\pm$ 10 (corresponding to 311 $\pm$ 10 for the free toxin I) by means of the osmometer method in a chloroform solution.[14]

These results, coupled with the analytical values of I, the picrate, the hydrobromide and the acetate (XVI) led to the molecular formula $C_{11}H_{17}O_8N_3$ (mol. wt. 319·27; the free toxin (I) exists in a hydrate form $C_{11}H_{17}O_8N_3 \cdot \frac{1}{2}H_2O$, mol. wt. 328·28) for I, instead of the C_{12} formula reported previously. The possibility of the toxin (I) existing as a dimeric C_{22}-form (or polymeric form) suggested by Tsuda *et al.*[15] is excluded by careful examination of the titration curve.[16] Thus, pure toxin (I) obtained through its picrate gives a typical titration curve for a *mono*basic acid or a *mono*acidic base, and the p*Ka* values obtained from several points on the titration curve fall within a range of 8·76 $\pm$ 0·01 (Fig. 4). Theoretically, two p*Ka* values of a dibasic acid or a diacidic base must differ from each other at least $\Delta pKa = 0{\cdot}6$ unit.[17]

Quinazoline derivatives[18]

Drastic alkaline degradation of tetrodotoxin (I) affords a yellow compound III (C_9-base)[19], the structure of which was established as 2-amino-6-hydroxymethyl-8-hydroxyquinazoline by Tsuda *et al.*[20] and later confirmed by us.[21]

Another quinazoline derivative, C_8-base (IV), is obtained from the toxin (I) by treatment with conc. sulphuric acid and its structure was assigned from its UV and NMR spectra as 2-amino-6-hydroxyquinazoline[22] which was proved by synthesis of its methyl ether (V)[18].

III

IV, R = H
V, R = CH_3

[14] A Mechrolab. Inc. Osmometer, Model 301A, was used. We thank Takeda Chemical Industries, Ltd., for the measurement.

[15] K. Tsuda, R. Tachikawa, K. Sakai, C. Tamura, O. Amakasu, M. Kawamura and S. Ikuma, *Chem. Pharm. Bull. Japan* **12**, 642 (1964).

[16] Possibility that the toxin (I) might be a dimeric form in a *solid state*[15] was also excluded by R. B. Woodward and J. Z. Gougoutas (*J. Amer. Chem. Soc.* **86**, 5030 (1964)).

[17] H. C. Brown, D. H. McDaniel and O. Häfliger, *Dissociation Constants* in E. A. Braude and F. C. Nachod, *Determination of Organic Structures by Physical Methods* p. 623. Academic Press, New York (1955).

[18] Y. Kishi, H. Taguchi, T. Goto and Y. Hirata, *J. Chem. Soc. Japan* **85**, 564 (1964).

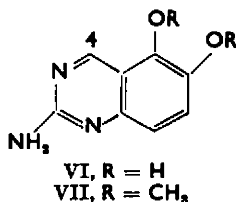
[19] M. Kawamura, *Chem. Pharm. Bull. Japan* **8**, 262 (1960).

[20] K. Tsuda, S. Ikuma, M. Kawamura, R. Tachikawa, I. Baba and T. Miyadera, *Chem. Pharm. Bull. Japan* **10**, 247 ,856, 865 (1962).

[21] T. Goto, Y. Kishi and Y. Hirata, *Bull. Chem. Soc. Japan* **35**, 1045 (1962).

[22] T. Goto, Y. Kishi and Y. Hirata, *Bull. Chem. Soc. Japan* **35**, 1244 (1962).

By treatment with acid, nortetrodoic acid (IX) (*vide infra*) gives another quinazoline, oxy-C_8-base (VI), $C_8H_7O_2N_3$. From its mode of formation, there should be an oxygen function at the C_6-position. This inference coupled with the examination of its NMR spectrum, which shows the presence of two protons vicinal in an aromatic ring (−0·05 and −0·70 ppm, doublets, J = 9 c/s)[23] and a proton at the C_4-position (−2·50 ppm, singlet), suggests the structure VI. This structure was confirmed by synthesis of its dimethyl ether (VII).[18]



Mechanism of the formation of these quinazolines will be discussed later.

Permanganate oxidation of the toxin I (as a mixture of acetates) affords guanidine which was identified as its picrate. These results indicate that the three nitrogen atoms in the toxin (I) exist as a guanidine moiety.

Tetrodoic acid[24]

When heated with water in a sealed tube at 110°, the toxin (I) affords a crystalline compound, tetrodoic acid[25] (VIII), which exhibits no UV absorption and does not consume bromine; hence it is a saturated compound. Unlike the case of tetrodotoxin itself, examination of tetrodoic acid gives clear-cut results and hence the acid was used for the determination of the fundamental skeleton.

Its analytical values agree with the composition $C_{11}H_{19}O_9N_3$. It is a zwitterionic compound having pKa_1 below 2·5 and pKa_2 ca. 11·8, indicating the presence of a carboxylate and a guanidine group. The existence of the carboxylate group was also demonstrated by its IR spectra; whereas the spectrum of VIII has a carboxylate band at 1590 cm^{-1}, its hydrochloride shows a band at 1720 cm^{-1} corresponding to a free carboxyl group. Negative Sakaguchi reaction of the acid (VIII) indicates that the guanidine group is not a monosubstituted one; the guanidinium bands[26] in its IR spectrum (1660 and 1573 cm^{-1} in VIII hydrochloride) correspond to an N,N′-disubstituted guanidine moiety.[27]

The NMR spectrum[23] of the acid (VIII) hydrochloride recorded in a deuterium oxide solution (Fig. 5) shows well resolved signals corresponding to eight protons which cannot be exchanged with deuterium.[28] Since four of the remaining eleven

[23] NMR spectra were measured in D_2O containing HCl. Chemical shifts were represented in ppm from an external benzene and coupling constants in c/s.

[24] *a* T. Goto, Y. Kishi, S. Takahashi and Y. Hirata, *Tetrahedron Letters* 2105 (1963); *b* Y. Kishi, T. Goto and Y. Hirata, *J. Chem. Soc. Japan* **85**, 572 (1964).

[25] This compound should be identical with tetrodonic acid reported by Tsuda *et al.*[19]

[26] T. Goto, K. Nakanishi and M. Ohashi, *Bull. Chem. Soc. Japan* **30**, 723 (1957).

[27] S. Takahashi, T. Goto and Y. Hirata, *J. Chem. Soc. Japan* **85**, 586 (1964).

[28] Possibility of overlapping of the proton signals with the strong DOH signal was eliminated by the addition of HCl to the sample solution; the DOH signal was shifted to a lower field, but no signal appeared in the region previously covered by the DOH signal.

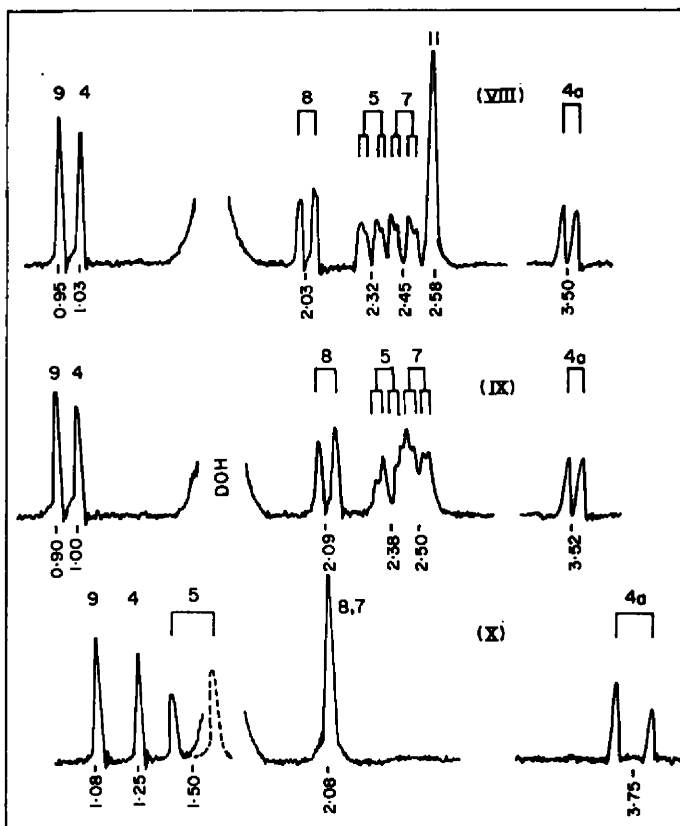
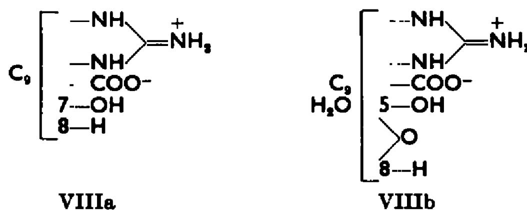


FIG. 5. The NMR spectra of tetrodoic acid (VIII), nortetrodoic acid (IX) and seconortetrododioic acid (X).[28]

exchangeable protons are included in the disubstituted guanidinium cation, seven protons must be attached to seven oxygen atoms out of nine, and the remaining two oxygen atoms are in the carboxyl group. We at first[24a] considered that the seven protons and the seven oxygen atoms must exist as seven hydroxyl groups (formula



VIIIa), but Tsuda *et al.*[29,30] showed that the acid (VIII) actually contains one mole water of crystallization i.e. the molecular formula must be $C_{11}H_{17}O_8N_3 \cdot H_2O$, and

[29] K. Tsuda, C. Tamura, R. Tachikawa, K. Sakai, O. Amakasu, M. Kawamura and S. Ikuma, *Chem. Pharm. Bull. Japan* **11**, 1473 (1963)

[30] K. Tsuda, S. Ikuma, M. Kawamura, R. Tachikawa, S. Sakai, C. Tamura and O. Amakasu, *Chem. Pharm. Bull. Japan* **12**, 1357 (1964).

hence it must contain five hydroxyl groups, an ether oxygen and a molecule of water as expressed by the formula VIIIb.

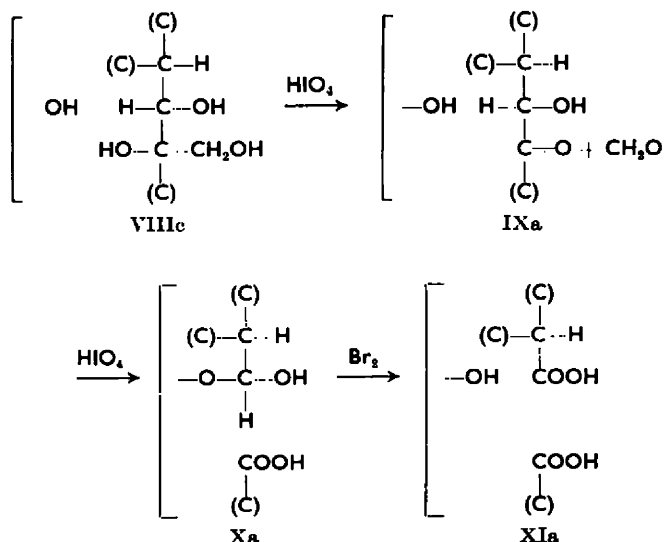
In either case, the number of free bonds in the parentheses of the formulae is eighteen which must be attached to the nine carbon atoms. The acid (VIII), therefore, must have at least one carbocyclic ring. If a tri- or more substituted guanidine group were present, the number of non-exchangeable protons should exceed eight.

The acid (VIII) consumes 2 moles of periodic acid in 0·1 N sulphuric acid at 4°. When one mole of the reagent is added, it yields formaldehyde and crystalline nortetrodoic acid (IX). The formation of formaldehyde suggests the presence of a grouping C(OH)—CH_2OH in VIII. The NMR signal corresponding to the —CH_2OH group is a singlet at 2·58 ppm, and hence no proton is attached at the carbon atom bearing it. Indeed, only this singlet disappears in the NMR spectrum of the nor acid (IX).

The nor acid (IX) shows a positive reaction with the Tollens and tetrazolium tests, indicating the presence of an α-ketol group.[31] It consumes another mole of periodic acid, yielding seconortetrododioic acid (X) which is an acidic substance; pKa_1 ca. 2 (carboxylate), pKa_2 3·3 (carboxylic acid) and pKa_3 above 10·5 (guanidine); and consumes no periodic acid.

In the NMR spectrum of the seconor acid (X) a doublet among the signals at 2·0–2·5 ppm, which was present in that of IX, disappears and a new doublet appears at 1·50 ppm. The latter signal is attributed to a proton attached to a hemiacetal carbon atom which is produced from a secondary alcoholic carbon atom by periodic acid cleavage with formation of a hemiacetal linkage.

Seconordioic acid (X) was oxidized with ca. 1 mole of bromine in water in the presence of strontium carbonate giving a crystalline product (XI) which is considered to be a strontium salt of seconortetrodotrioic acid. This reaction also supports the presence of the hemiacetal group in the seconor acid (X). Thus, the transformation



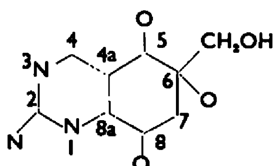
[31] Since no IR band is present between 1700–1800 cm^{-1}, the ketonic carbonyl group of IX would exist in an enol or a hydrated form.

of tetrodoic acid (VIII) to the seconortrioic acid (XI) can be represented by the reactions in the preceding page.

The signal at 1·50 ppm in the NMR spectrum of X is spin-coupled (J = 9 c/s) with a doublet at the highest field (3·75 ppm) and hence the hemiacetal proton in X must be vicinal to the proton attached to a tertiary carbon atom which does not link with oxygen or nitrogen atom.

The acid (VIII) contains the grouping —CHX—CHY—, where X and Y are electronegative atoms, as demonstrated by the NMR signals at 2·05 and 2·45 ppm coupled with each other J = 4 c/s. From the coupling constant it is suggested that this group is included in a cyclic structure, since if it were in an open chain structure the coupling constant must be of a magnitude of 6–7 c/s.[32] This inference can be also applied to the case of the grouping discussed above, in which the coupling constant between the two protons attached to the vicinal carbon atoms is 4 c/s (signals at 2·32 and 2·45 ppm in the spectrum of VIII).

That tetrodoic acid (VIII) as well as tetrodotoxin (I) can be transformed into the C_9-base (III) and the C_8-base (IV) by treatment with base and acid, respectively, and nortetrodoic acid (IX) by treatment with acid into the oxy-C_8-base (VI), and that both a carbocyclic ring and an N,N'-disubstituted guanidine moiety are present in VIII suggests that the acid (VIII) has the perhydroquinazoline nucleus having a hydroxymethyl group at the C_6-position and that oxygen atoms must be attached at least at the 5,6 and 8-positions. The perhydroquinazoline ring with the hydroxymethyl

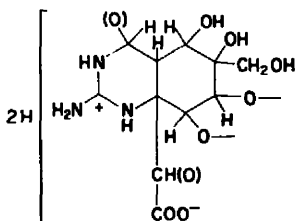


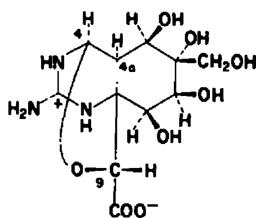
group contains nine carbon atoms; the remaining two carbon atoms, one of which is the carboxyl group, must be linked together since Kawamura[19] isolated oxalic acid along with the C_9-base during the alkaline degradation of the toxin (I). These observations can only be accommodated in the formula VIIId. An alternative formula having the two-carbon side chain at the C_7-position is ruled out since the signal of H_{4a} does not couple with the proton in the group —CHX—CHY—, instead the C_5 proton shows a small coupling (1 c/s) with one of the protons in the group possibly by a long-range effect.[33]

Tsuda *et al.*[29,30] established the structure of tetrodoic acid (VIII) by means of X-ray analysis as shown by the formula VIIIe. Then, the structures of the nor-acid (IX) and seconordioic acid (X) can be represented by the formulae IXb and Xb, respectively. The angle between the C_4—H and the C_{4a}—H in the formulae VIIIe, IXb and Xb is nearly 90° which coincides with the observation that the coupling constant between the two protons is nearly zero.

[32] J. D. Roberts, *Nuclear Magnetic Resonance* p. 53. McGraw-Hill, New York (1959).

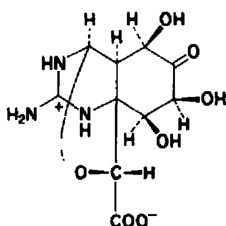
[33] F. A. L. Anet, *Canad. J. Chem.* **39**, 789 (1961); K. B. Wiberg, B. R. Lowry and B. J. Nist, *J. Amer. Chem. Soc.* **84**, 1594 (1962); J. Meinwald and Y. C. Meinwald, *Ibid.* **85**, 2514 (1963); N. S. Bhacca and D. H. Williams, *Applications of NMR Spectroscopy in Organic Chemistry* p. 108. Holden-Day, San Francisco (1964).

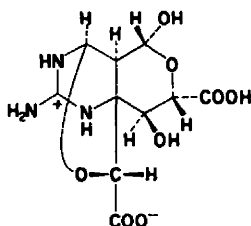




VIIId

VIIIe





IXb

Xb

Anhydrotetrodoic acid[34]

Treatment of the toxin (I) with 5% barium hydroxide solution at room temperature affords anhydrotetrodoic acid (XII), $C_{11}H_{17}O_8N_3 \cdot nH_2O$ (n = 0·5–2·5). It is a zwitterionic compound having pKa_1 2·5 (carboxylate) and pKa_2 10·9 (guanidine), and exhibits an absorption maximum in the UV region (Fig. 6), indicating the presence of an unsaturated group. It reacts smoothly with 1 mole of bromine in water to form in almost quantitative yield bromoanhydrotetrodoic lactone (XIII) hydrobromide, $C_{11}H_{14}O_7N_3Br \cdot HBr$, which shows no UV absorption maximum and is stable to acids but extremely unstable to bases. It has a γ-lactone group (IR band at 1795 cm^{-1}) which can be opened reversibly by the addition of 1 mole of sodium hydroxide and reclosed by acidification. An apparent pKa value of this lactone group is 6·7 and no hysteresis was observed.

The bromo lactone (XIII) hydrobromide forms big prisms suitable for X-ray crystallographic analysis and its structure was established by Nitta *et al.*[35] as XIIIa. Hence the structure XIIa can be assigned for the anhydro acid (XII) without ambiguity, though the composition of XII could not be accurately determined by elemental analysis due to indefinite amounts of water of crystallization.

The mechanism of the formation of the bromo lactone (XIII) is as follows: an electrophilic attack of bromonium ion at the C_{4a} is accompanied by the addition of

[34] T. Goto, S. Takahashi, Y. Kishi and Y. Hirata, *Tetrahedron Letters* 2115 (1963); S. Takahashi, T. Goto and Y. Hirata, *J. Chem. Soc. Japan* **85**, 579 (1964).

[35] Y. Tomiie, A. Furusaki, K. Kasami, N. Yasuoka, K. Miyake, M. Haisa and I. Nitta, *Tetrahedron Letters* 2101 (1963); also presented at the *3rd IUPAC Symposium on the Chemistry of Natural Products* April 13, Kyoto (1964).

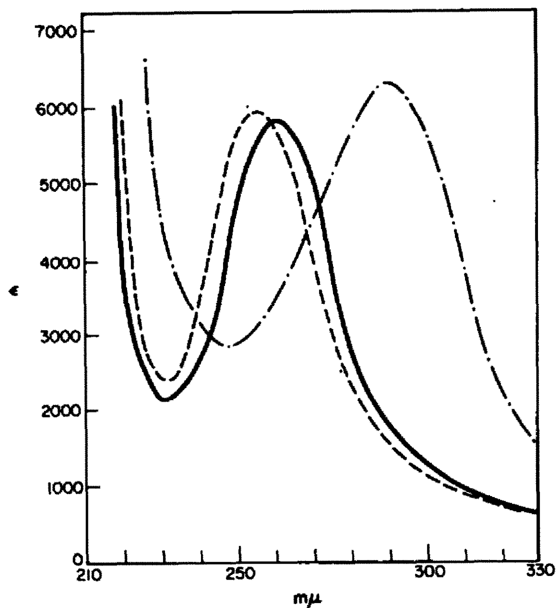


Fig. 6. Ultra-violet spectra of anhydrotetrodoic acid: ——— neutral; - - - - - - acidic; — – — alkaline.

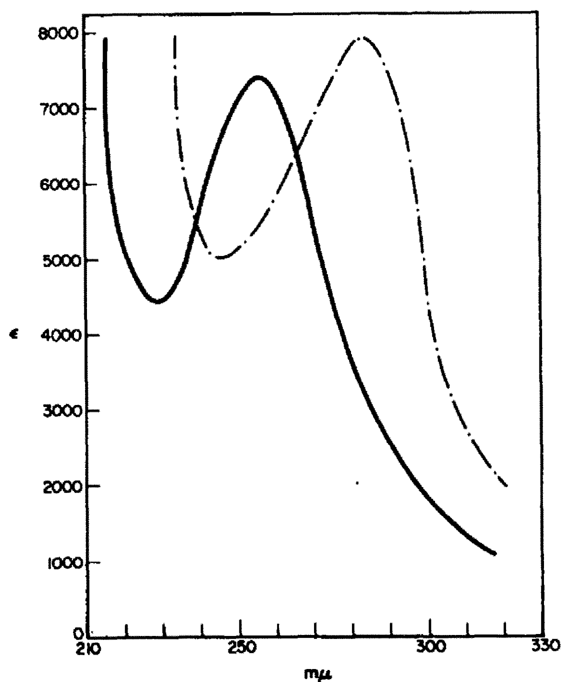
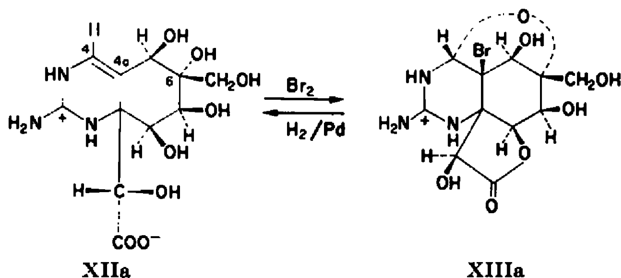


Fig. 7. Ultra-violet spectra of 2-amino-1,6-dihydropyrimidine hydrochloride (XIV): ——— neutral; — – — alkaline.



the C_6-hydroxyl group at the other end of the double bond, and simultaneous formation of the lactone ring occurs by the assistance of the acidity of hydrogen bromide formed. That the free hydroxyl group is present at the C_6 position in the acid (XII) but not in the lactone (XIII) is apparent from the fact that the acid yields formaldehyde by treatment with periodic acid whereas no formaldehyde was detected from the lactone (as the nitrate) by the same treatment. The bromo lactone (XIII) can be converted to the acid (XII) by hydrogenation with Pd–C in the presence of calcium carbonate.

That the UV absorption of the acid (XII) comes from the α,β-unsaturated guanidine group is evident from a model compound, 2-amino-1,6-dihydropyrimidine (XIV),[36] which shows a similar absorption spectrum to that of XII (Fig. 7).

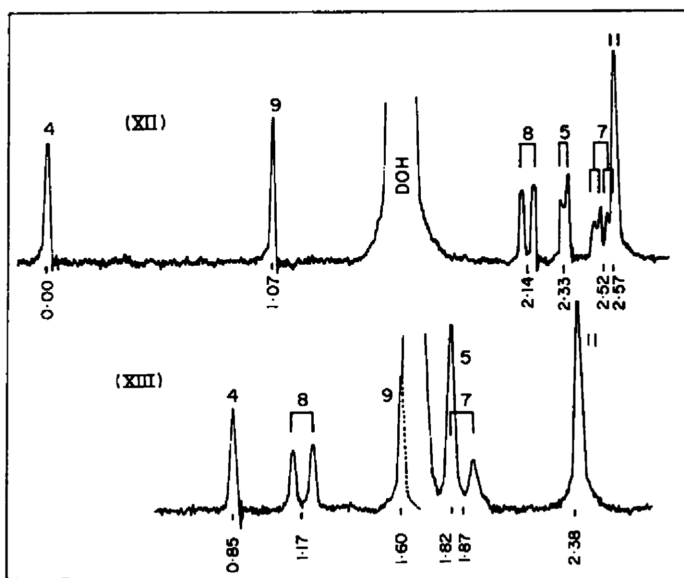


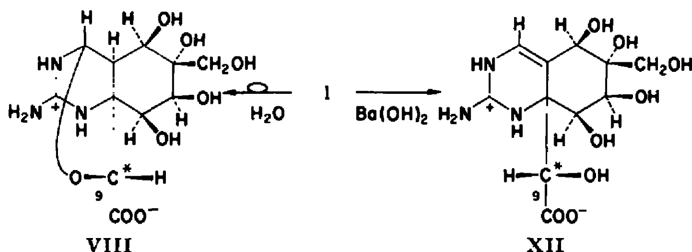
Fig. 8. The NMR spectra of anhydrotetrodoic acid (XII) and bromoanhydrotetrodoic lactone hydrobromide (XIII).[23]

The NMR spectrum of the acid (XII) is also consistent with the formula XIIa. A singlet at the lowest field (O ppm) corresponds to H_4 and no signal is present above 2·6 ppm indicating no proton at C_{4a}. Chemical shifts of other signals are in accord with that of tetrodoic acid (VIII).

[36] S. Inoue, E. Suzuki and T. Goto, unpublished.

Configuration at the C_9-position[37,24b]

The configuration at the C_9-position in tetrodoic acid (VIII) differs from that of anhydrotetrodoic acid (XII). This means that the configuration must be inverted during either the reaction which affords the anhydro acid (XII) or the reaction by



which tetrodoic acid (VIII) is formed. This inversion takes place during the hydrolysis of the toxin (I) to the acid (VIII) as shown by the following experiments.[38]

Both acids (VIII) and XII were prepared from the toxin (I) in deuterium oxide and their NMR spectra measured. Whereas the spectrum of the anhydro acid (XII) thus obtained is identical in all respects with that of the anhydro acid (XII) prepared in water, in the spectrum of the acid (VIII) the signal at the lowest field (0·95 ppm), which can be assigned to the proton at the C_9-position, has disappeared. This suggests that the reaction giving the acid (VIII) involves epimerization at the C_9-position through an enol intermediate. Such epimerization of α-hydroxy acids has been observed in the reaction of saccharic acids.[39]

Structure of tetrodotoxin[37,40]

Tetrodotoxin (I) is a white crystalline solid, darkens above 220° without melting, and exhibits no UV absorption. As it is completely insoluble in all solvents except acids, it is apparently a weak base (pKa′ 8·76), and forms a crystalline hydrobromide soluble in water, from which the toxin (I) is recovered by treatment with ammonia.

Since both the anhydrotetrodoic acid (XII) and tetrodoic acid (VIII) are obtained from the toxin (I) by mild treatments and have the same molecular formula as that of the toxin, it is reasonable to assume that the carbon-nitrogen skeleton of the toxin is the same as that of the acids. This assumption will be further verified by evidence presented later and especially by examination of the anhydroepi derivatives discussed in the following chapters.

The toxin (I) has a proton at the C_{4a} as in the case of the acid (VIII) since there is an NMR signal at 4·07 ppm. The signal is spin-coupled with one at the lowest field (1·00 ppm) with a coupling constant 10 c/s[41]; the latter signal being attributable to

[37] T. Goto, Y. Kishi, S. Takahashi and Y. Hirata, *Tetrahedron Letters* 779 (1964).
[38] Tsuda *et al.* also reported the same observation, see Ref. 30.
[39] M. Steiger, *Helv. Chim. Acta* **19**, 189 (1936).
[40] T. Goto, Y. Kishi, S. Takahashi and Y. Hirata, *J. Chem. Soc. Japan* **85**, 661 (1964).
[41] This coupling constant was further confirmed by recording the NMR spectrum at 100 Mc. We thank Dr. K. Nukada, Basic Research Institute, Toyo Rayon Co., Ltd., for the measurement.

the proton at the C_4. The magnitude of the coupling constant suggests that the dihedral angle between the C_4—H and the C_{4a}—H bonds would be ca. 0° or 180°.

The toxin (I) has no p*Ka* value below 8 and hence unlike the acids (VIII and XII) it can not have a free carboxyl group. Instead, a lactone, a lactam or an orthoester group must be considered as a potential carboxyl group. The existence of a lactone group in the toxin is excluded since no band is observed between 1700–1800 cm^{-1} in its IR spectrum. Structures containing an amide linkage such as formula Ia were

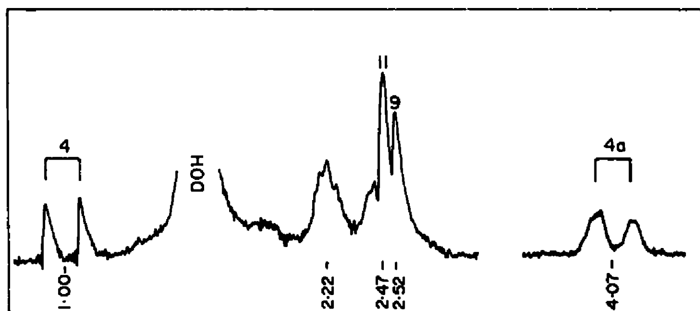


Fig. 9. The NMR spectrum of tetrodotoxin (I).[28]

attractive since the toxin consumes 1 equivalent of acid to form a salt and the p*Ka* value (8·76) seems to correspond well to an acylguanidine derivative,[42] but this was abandoned for the following reason.[37]

The p*Ka*′ value of the toxin (I) in water is 8·76, whereas in 50% aqueous ethanol it is 9·4. This solvent effect clearly indicates that the dissociation group is not basic but acidic in nature.[43] Therefore, the toxin (I) must be a zwitterionic compound. Similar solvent effects were also observed with some other derivatives of I listed in Table 1.

Table 1. p*Ka*′ values (20°)

	in water	in 50% ethanol	in water containing CH_2O*
Tetrodotoxin (I)	8·76	9·4	8·7
Anhydroepi-	7·96	8·8	7·95
tetrodotoxin (II)	11·5	—	10·8
Diacetyl-II (XVIII)	7·7	8·2	
β-Alacreatinine	7·05	7·05	6·3
4-Hydroxytetra-			
hydropyrimidine	11·8		10·5
Glucosamine	7·6	7·5	

* Formalin (40%, 0·1 ml) was added.

This conclusion is also supported by the fact that, although the titration of the toxin (I) above pH 9·6 is prevented by the insolubility of the toxin in a basic medium, closely related anhydroepitetrodotoxin (II) (*vide infra*) does not precipitate during

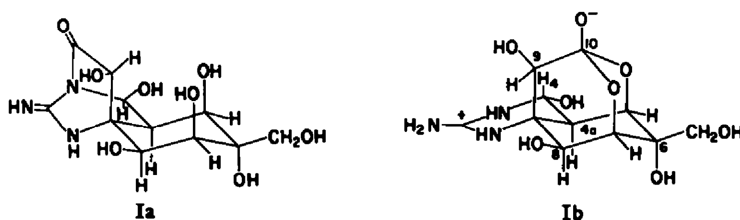
[42] See Ref. 34. Examples of the p*Ka* values of acylguanidines are: acetylguanidine, 8·33; benzoylguanidine, 6·8; creatinine, 3·55; β-alacreatinine, 7·05.

[43] T. H. Jukes and C. L. A. Schmidt, *J. Biol. Chem.* **105**, 359 (1934).

titration and clearly shows two p*Ka*'s 7·95 and 11·5; the lower p*Ka* corresponds to an acidic group as in the toxin (I) and the higher one to a basic group (guanidine).

Further evidence was obtained by the following experiments. Previously we reported[44] that the p*Ka* value of the guanidine group is lowered about 1 p*Ka* unit by the addition of formalin. The results of titration of some of tetrodotoxin derivatives in the presence of formalin are shown in Table 1. This shows that the p*Ka* around 8–9 of the toxin (I) and anhydroepi compound (II) are not associated with the guanidine group, whereas the higher p*Ka* (11·5) of the latter is clearly lowered by the addition of formalin, indicating the guanidine group.

Thus, the structure of tetrodotoxin (I) must be represented by the orthoester formula (Ib); the C_{10}-hydroxyl group must be associated with the lower p*Ka* which is



acidic. No such acidic two-thirds orthoester group has been reported in the literature. The hydroxyl group at the C_4-position cannot be involved in the formation of the orthoester linkage, since the C_4—H and the C_{4a}—H must be *trans*-diaxial (NMR: $J_{4,4a} = 10$ c/s). An alternative possibility involving the hydroxyl group at C_8 in the orthoester linkage is not favoured by inspection of molecular models, and is eliminated by the following evidence.

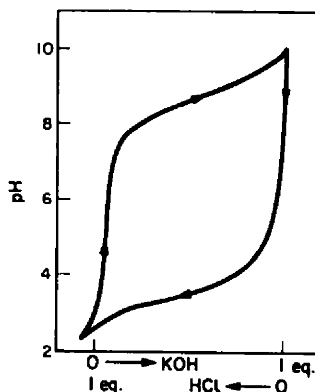
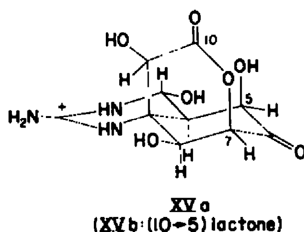


FIG. 10. Titration curve of nortetrodotoxin (XV) sulphate.

When treated with one mole of periodic acid in 0·1 N sulphuric acid at 0°, the toxin (I) gives in addition to formaldehyde, amorphous nortetrodotoxin (XV) sulphate in a good yield. It is evident from a band at 1745 (KBr disc) and at 1750 cm^{-1} (in dimethyl sulphoxide) in its IR spectra that the nor compound (XV) has a carbonyl group. It consumes 1 mole of sodium hydroxide at pH 8–9, and the product shows

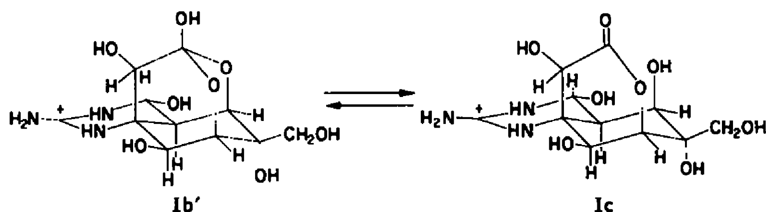
[44] T. Goto, Y. Hirata, S. Hosoya and N. Komatsu, *Bull. Chem. Soc. Japan* **30**, 729 (1957).

a carboxylate band at 1600 cm^{-1} and no band between 1700–1800 cm^{-1}. Back titration gives a p*Ka* 3·35 corresponding to a free carboxyl group (Fig. 10). This suggests the presence of a lactone group in XV. Since the carbonyl stretching band of the γ-lactone group of the bromolactone (XIII) appears around 1800 (KBr) and 1795 cm^{-1} (in DMSO), the carbonyl band of the nor lactone (XV) can be assigned to the δ-lactone group in the formula XVa or XVb—XVa being sterically favoured.



XVa
(XVb: (10→5) lactone)

Whereas tetrodotoxin itself shows no carbonyl band between 1700–1800 cm^{-1} (KBr), its salts exhibit a weak band at 1747–1750 cm^{-1} in a dimethyl sulphoxide solution (sulphate, hydrobromide and picrate) and at 1730–1745 cm^{-1} in a KBr disc (amorphous sulphate and amorphous hydrochloride). These phenomena are explained in terms of equilibrium between the orthoester (Ib′) and the lactone (Ic) structures.



Ib′ Ic

If there were an ether bridge between C_{10} and C_8 in the orthoester structure, i.e. (10 → 5,8) or (10 → 7,8) orthoester, the (10 → 8) γ-lactone structure would be more favoured in the equilibrium than the δ-lactone (Ic) or (10 → 5) δ-lactone structure. The toxin (I) and its crystalline hydrobromide show no carbonyl absorption in a solid state (KBr disc), suggesting that they exist completely in the orthoester form Ib or Ib′ (protonated form of Ib).

It is also deduced from the NMR spectrum of the pentaacetate (XVI), in which the orthoester linkage is still retained, that in the acetate, one of the hydroxyl groups at C_7 or C_8 is acetylated (*vide infra*) and hence the (10 → 7,8) orthoester structure is not possible for the toxin (I). Further support for the orthoester structure (Ib) are obtained from the anhydroepi compounds (*vide infra*). They have an ether linkage between C_4 and C_9, which can be formed only in the structures derived from Ib.

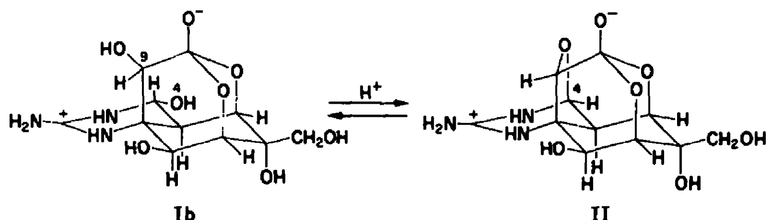
Absolute configuration of tetrodotoxin[40]

Absolute configuration of the bromolactone (XIII) was established by Nitta *et al.*[45] with assistance of the X-ray crystallographic technique in the sense corresponding to the formula XIIIa. Then, it is no doubt that the absolute configuration of tetrodotoxin and its derivatives are represented as formulated in this report.

[45] Y. Tomiie, A. Furusaki, K. Kasami, N. Yasuoka, K. Miyake, M. Haisa and I. Nitta, reported at the *3rd IUPAC Symposium on the Chemistry of Natural Products*, April 13, Kyoto (1964).

Anhydroepitetrodotoxin[37,40]

Anhydroepitetrodotoxin (II), $C_{11}H_{15}O_7N_3$, is obtained by hydrolysis of the acetyl derivatives of tetrodotoxin (I) such as penta- (XVI), tetra- (XVII) or di-acetylanhydroepitetrodotoxin (XVIII). A major difference of the compound II from the toxin (I) is the coupling constant between H_4 and H_{4a}; the former shows J = 0 c/s whereas the large coupling (10 c/s) is observed in the NMR spectrum of the latter. This is explicable only by assuming that the configuration at C_4 in the toxin (I) is epimerized and forms an ether linkage with the hydroxyl group at C_9. Since the toxin (I) and anhydroepi compound (II) are interconvertible with acid, dehydration must be associated with the epimerization at C_4. By the formation of the five-membered ring the dihedral angle between the C_4—H and the C_{4a}—H bonds becomes nearly 90°. This is consistent with the observed coupling constant $J_{4,4a}$ which is nearly zero c/s.



Both the toxin (I) and II, when dissolved in mineral acids, epimerized slowly and afford an equilibrium mixture of I and II in ca. 4:1 ratio (NMR). Crude tetrodotoxin may contain the anhydroepi compound II (*vide supra*).

Whereas tetrodotoxin is decomposed with base, the anhydroepi compound (II) is stable in dilute alkali at room temperature and exhibits p*Ka*'s 7·95 and 11·5. The difference in the stability towards alkali may be attributable to the ether linkage between C_4 and C_9 which stabilizes the orthoester linkage and prevents hydrolysis.

Acetyl derivatives of tetrodotoxin[37,46]

Acetylation of tetrodotoxin (I) with pyridine–acetic anhydride affords a complex mixture, from which a hexaacetate (XIX) may be isolated in very low yield. On the other hand, acetylation of the toxin (I) with acetic anhydride in the presence of *p*-toluenesulphonic acid affords, in good yield, amorphous tetraacetylanhydroepitetrodotoxin *p*-toluenesulphonate (XVII), which is further acetylated with acetic anhydride and pyridine to the crystalline pentaacetylanhydroepitetrodotoxin *p*-toluenesulphonate (XVI). Diacetylanhydroepitetrodotoxin (XVIII) can be obtained from either the tetraacetate (XVII) or the pentaacetate (XVI) by hydrolysis with a limited amount of aqueous ammonia or trimethylamine at room temperature; when an excess of ammonia is used the anhydroepi compound (II) is obtained.

In the NMR spectra of the latter three acetates (Fig. 11) the signal of H_{4a} appears around 3 ppm as a doublet with a coupling constant of 3 c/s, whereas the signal of H_4, which appears in the lowest field, is a singlet and the position is almost the same as that of anhydroepi compound (II), indicating that the C_4-position in the acetates is not acetylated. These acetates, therefore, belong to the anhydroepi series with an ether linkage between C_4 and C_9.

[46] T. Goto, Y. Kishi, S. Takahashi and Y. Hirata, *J. Chem. Soc. Japan* **85**, 667 (1964).

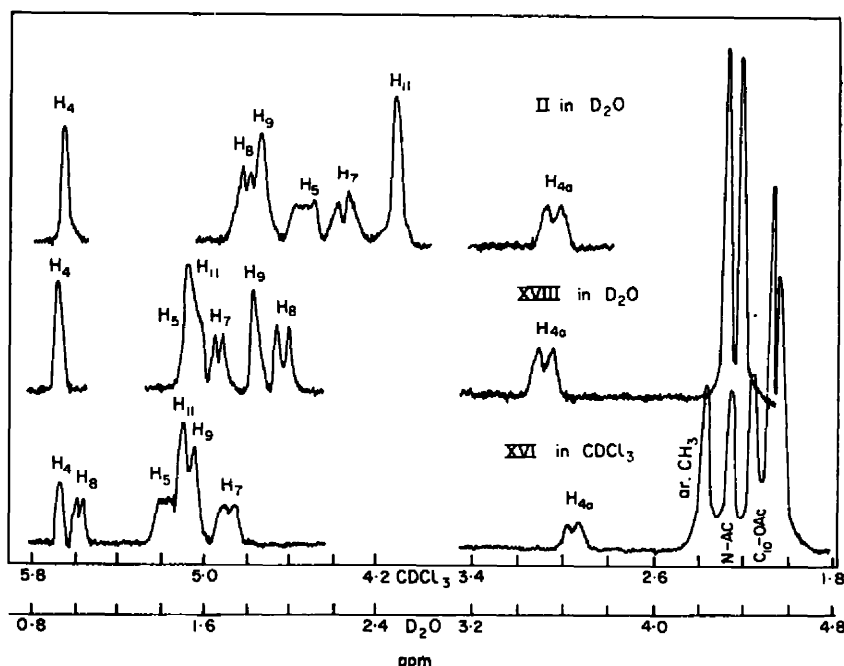
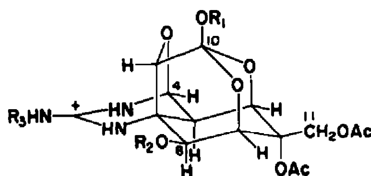


FIG. 11. The NMR spectra of anhydroepitetrodotoxin (II), diacetylanhydroepitetrodotoxin (XVIII) and pentaacetylanhydroepitetrodotoxin *p*-toluenesulphonate (XVI): upper scale;* ppm from internal tetramethylsilane, lower scale;* ppm from external benzene.

A p*Ka*′ of the pentaacetate (XVI) is 5·1 in water and 4·9 in 50% ethanol. This solvent effect indicates the presence of a weakly basic group. No such p*Ka* is observed in the case of the tetraacetate (XVII). Hence the fifth acetyl group introduced in the pentaacetate is attached at the guanidine group.

Structures of the penta-, tetra- and diacetate can be assigned as XVIa, XVIIa and XVIIIa, respectively. No acetyl group is attached at the C_{10} in the diacetate (XVIII) since it has a p*Ka*′ 7·7 in water and 8·2 in 50% ethanol; the acidic orthoester group



* Both scales can not be strictly compared, but, for the rough comparison of the spectra, they are arranged so that both H_{11} protons in XVIII and XVI possess the same chemical shift. Chemical shift of the external benzene is taken as 6·6 ppm from the internal tetramethylsilane.

being present. Moreover, the diacetate has no acetyl group at the C_8 since the NMR signal of H_{11} of the diacetate appears ca. 1 ppm lower field than that of anhydroepi compound (II) owing to the introduction of an acetyl group at this position but the signal of H_8 is moved rather upfield. On the other hand, presence of an acetyl group on C_8 of the pentaacetate (XVI) is evident from the chemical shift of the signal which is ca. 1 ppm lower field than that of the diacetate.

Although a rapid titration of the tetraacetate (XVII) reveals an apparent p*Ka* around 8·5, this is not true dissociation but is caused by hydrolysis of the acetyl group

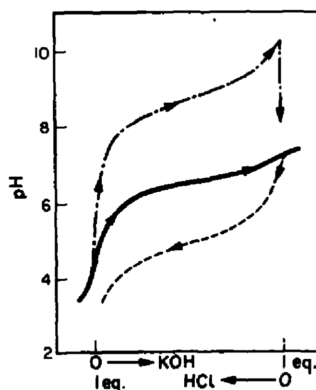


FIG. 12. Titration curves of XVII.

– – –Rapid titration (ca. 10 min) with 0·1 N KOH.

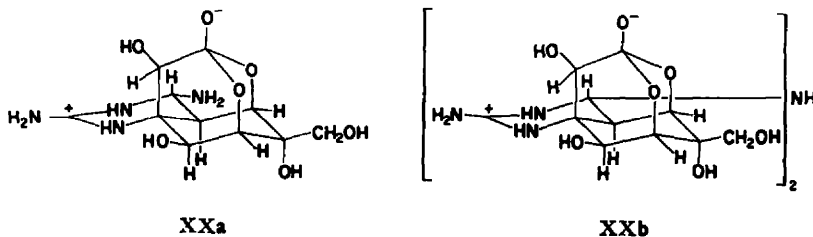
——— Slow titration (ca. 2 hr) with 0·1 N KOH.

- - - - - Back titration with 0·1 N HCl after 1 eq of KOH has been consumed.

attached at the orthoester group. When it was titrated very slowly, one mole of alkali is consumed at pH 6–7·5, and back-titration exhibits a p*Ka* 4·74 corresponding to one mole of acetic acid liberated (Fig. 12). Thus, the acetyl group attached at the orthoester group is hydrolysed with exceptional ease.

Aminodesoxytetrodotoxin[47]

Aminodesoxytetrodotoxin (XX), $C_{11}H_{18}O_7N_4$, can be obtained from the acetates (XVI, XVII or XVIII) by treatment with conc. ammonia aq. It has pKa_1 4·01 and pKa_2 8·66 in water and 3·86 and 9·30 in 50% ethanol showing that there is a weakly basic group as well as the acidic orthoester moiety. This result coupled with the similarity of IR and NMR spectra to those of tetrodotoxin, especially the coulping constant $J_{4,4a}$ (10 c/s), suggest structure XXa.



[47] T. Goto, S. Takahashi, Y. Kishi and Y. Hirata, *Tetrahedron Letters* 1831 (1964).

Contrary to our results, Tsuda *et al.*[48] suggested that tetrodaminotoxin, which must be identical with our aminodesoxytetrodotoxin, possibly exists in a dimeric form (XXb). However, the aminodesoxy compound has two p*Ka* values as mentioned above and the titration equivalents of these two groups are equal (ca, 340), indicating that

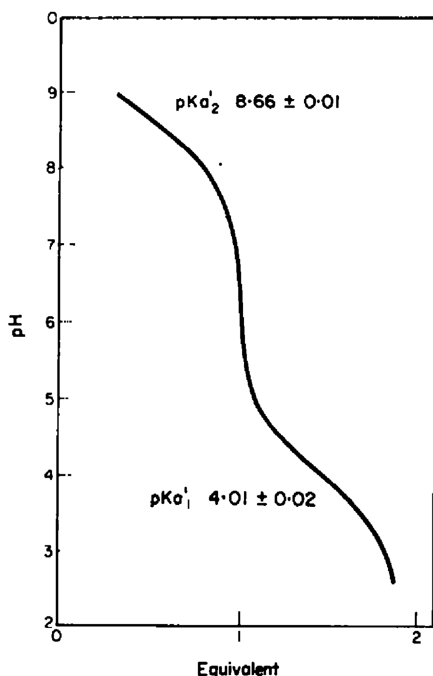


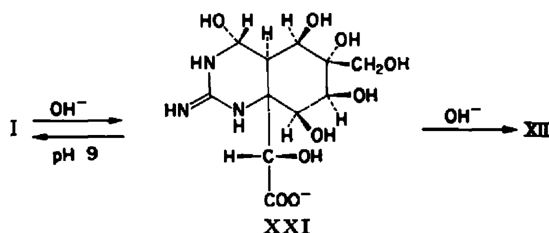
Fig. 13. Titration curve of aminodesoxytetrodotoxin (XX).
XXA (3·201 mg) in 0·020 N HCl (2·00 ml) was titrated with 0·100 N KOH and a blank value was subtracted. Equivalents were calculated as mol. wt. 327·29. XXB showed a curve identical with that of XXA.

the compound (XX) is the monomeric C_{11}-compound (mol. wt. 327·29) and not the dimeric C_{22}-form, since if it is the dimeric form, the titration equivalent corresponding to the p*Ka*′ 8·66 must be twice as much as that for the p*Ka*′ 4·01 (Fig, 13). Furthermore, the titration curve is in agreement with a theoretical curve for the monomeric structure (XXa).

Tetrodotoxinic acid[37,40]

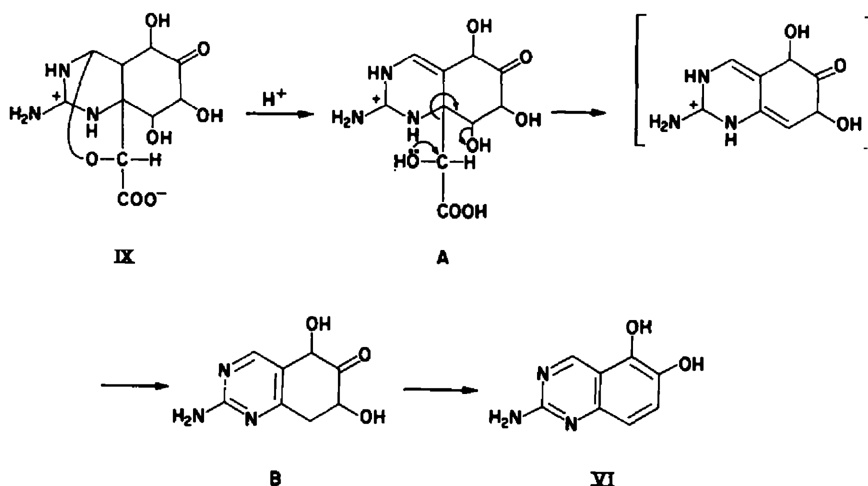
Tetrodotoxin (I) is not soluble in alkaline solutions, but when excess sodium hydroxide solution is added at once to an acidic solution of the toxin (I) a clear solution is obtained. The solution has no toxicity against mice and exhibits two p*Ka*'s 2·3 and ca. 10·8 and shows no inflection in the titration curve around 8·7 (p*Ka* of I). Adjustment of the alkaline solution to pH 9 causes gradual precipitation of the toxin (I), suggesting a reversible formation of a new compound, tetrodotoxinic acid (XXI), from the toxin (I). When the alkaline solution is allowed to stand at room temperature, the intensity of its UV absorption at 293 mμ gradually increases owing to the irreversible formation of anhydrotetrodoic acid (XII).

[48] K. Tsuda, R. Tachikawa, K. Sakai, C. Tamura, O. Amakasu, M. Kawamura and S. Ikuma, *Chem. Pharm. Bull. Japan* **12**, 642 (1964).

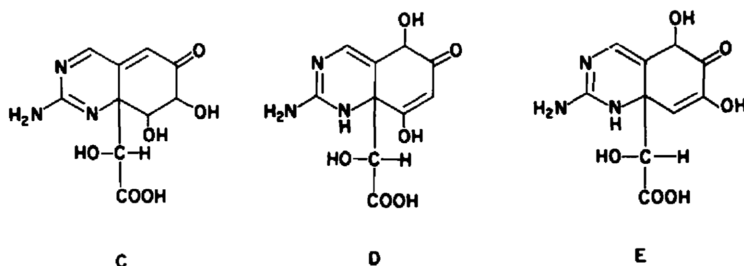


Mechanisms of formation of the quinazoline derivatives[18]

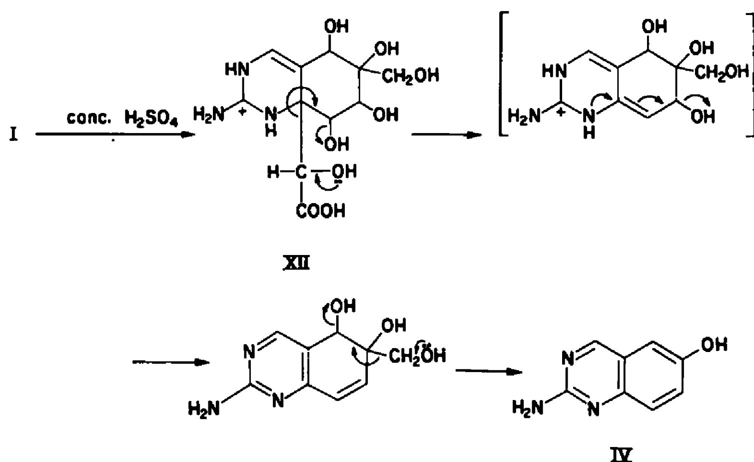
The most probable route of the formation of the oxy-C_8-base (VI) from nor-tetrodoic acid (IX) is as follows:



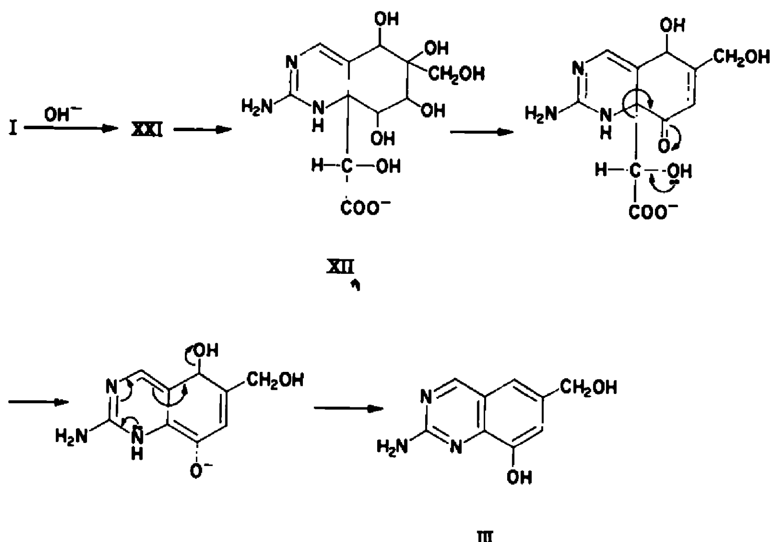
The first step is the formation of A, which is not isolated but in the initial stage the reaction mixture shows an UV spectrum similar to that of anhydrotetrodoic acid (XII). Then, the elimination of the two-carbon side chain must occur to form B since all other possible intermediates, such as C, D or E, cannot account for the formation of the final product (VI).



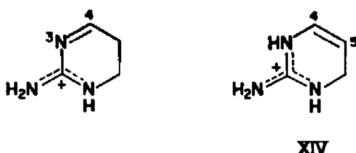
Assuming that a mechanism similar to that above is operating also in the case of the formation of the C_8-base from the toxin (I), the following scheme may be considered. Tetrodoic acid (VIII), anhydrotetrodoic acid (XII) and anhydroepitetrodotoxin (II) also afforded the C_8-base by treatment with conc. sulphuric acid. These compounds can also be converted to the C_9-base by heating with potassium hydroxide.



In the case of the formation of the C_9-base from the toxin (I), tetrodotoxinic acid (XXI) and anhydrotetrodoic acid (XII) must be intermediates. Although the next step is not clear, the following scheme is suggested:



The mechanism involving intermediates containing 3,4-double bonds in the perhydropyrimidine ring is less probable since the 3,4-double bond inhibits a full resonance of guanidinium group and makes the ring unstable. Indeed, the double bond in a model compound, 2-aminodihydropyrimidine (XIV), is located at the 4,5-position (NMR).[36]



EXPERIMENTAL

All m.ps are uncorrected. Tetrodotoxin and most of its derivatives exhibit no definite m.p. (carbonize) and hence IR spectra are usually used for identification.

The spectra were recorded on the following instruments: IR spectra, Nihon-Bunko DS-402G and IR-S; UV spectra, Beckman DK-2; NMR spectra, Nihon-Denshi JNM-3 spectrometer (60 Mc). The p*Ka*'s were measured by dissolving a few mg of sample in 0·01 N HCl (2 ml) and titrating it with 0·1 N KOH by means of Radiometer TTT-1 pH meter with an automatic recorder. For measurements of very high (above 10) or very low (below 3) p*Ka* values, 0·05 N HCl and 0·5 N KOH were used. After subtracting a blank value, the pH value of the half-neutralized point was taken as the p*Ka*'.

Unless otherwise noted the analytical samples were dried over P_2O_5 at 60–80° for 6–12 hr *in vacuo*.

Extraction of tetrodotoxin (I). Choped fresh ovaries (100 kg) of *Spheroides rubripes* were soaked in water (100 l.) with occasional stirring and, after 2 days, the supernatant was collected by decantation. The residue was extracted repeatedly with water (100, 50 l.) and the combined aqueous extract (200 l.) were boiled for a very short time causing a large quantity of protein to coagulate. The slurry

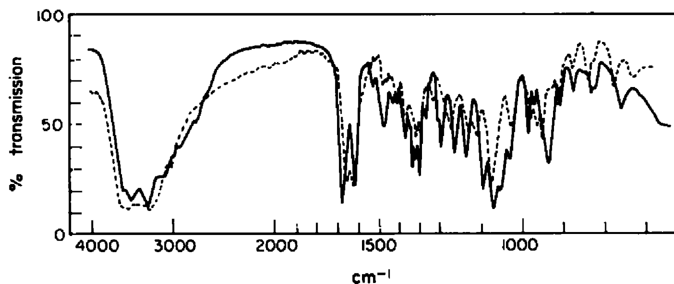


FIG. 14

was filtered and the filtrate poured onto a column (8 cm diam. 2 m, length) packed with Amberlite IRC-50 resin (ammonium type, 8 l.). The column was washed with water and eluted with 10% acetic acid (13 l.) and water (10 l.), successively. The first 3 l. of the eluates had no toxicity against mice and were discarded. The next fraction (10 l.) was strongly toxic and used for the subsequent steps. The last fraction (10 l.) contained a large amount of acetic acid and hence it was used for the preparation of 10% acetic acid solution to be used subsequently.

The toxic fraction was brought to pH 8–9 by the addition of NH_4OH, Norit A (100 g) added, and the mixture shaken for 2 hr. The charcoal was collected by filtration and washed with water. This procedure was repeated 3 times and the combined charcoal extracted with 20% EtOH containing 0·5% acetic acid (250 ml × 3). After evaporation to approx. 50 ml, the combined extracts were brought to pH 9 by the addition of NH_4OH resulting in the separation of white precipitates which were collected by filtration, dissolved in dil. acetic acid and reprecipitated by the addition of NH_4OH, to give crude tetrodotoxin (1–2 g).

Tetrodotoxin (I). Crude tetrodotoxin (3·2 g) and picric acid (2·3 g) were dissolved in boiling water (20 ml) and the solution filtered while hot. On cooling the filtrate, a crystalline precipitate separated which on recrystallization (thrice) from hot water gave *tetrodotoxin picrate* as yellow needles (4·8 g). It darkened over 200° without melting. An analytical sample was dried at 80–100° for 20 hr *in vacuo*. (Found: C, 36·25, 36·52; H, 3·95, 4·03; N, 14·24, 14·40. $C_{11}H_{17}O_8N_3{\cdot}C_6H_3O_7N_3{\cdot}H_2O$ requires: C, 36·05; H, 3·92; N, 14·84%.)

To a solution of the picrate (4·7 g) in hot water NH_4OH was added to pH 9 and, after cooling, the precipitated solid was filtered off and washed with water. It was dissolved in a minimum amount of dil. acetic acid and precipitated by addition of NH_4OH; yield of pure tetrodotoxin was 2·6 g. UV, end absorption $\varepsilon_{230\,m\mu}^{0.1\,N\,HCl}$ 75; IR, Fig. 14 (——); p*Ka*' 8·76 (in water). (Found: C, 40·58, 40·87; H, 5·63, 5·94; N, 12·61, 12·60. $C_{11}H_{17}O_8N_3{\cdot}\frac{1}{2}H_2O$ requires: C, 40·24; H, 5·53; N, 12·80%.)

The toxin (I: 500 mg) obtained from the picrate was dissolved in a minimum quantity (ca. 0·9 ml) 2 N HBr and the solution filtered. After dilution with EtOH (10 ml) the solution was allowed to stand a few days, when crystals separated. Recrystallization from aqueous EtOH gave *tetrodotoxin hydrobromide* as prisms (230 mg); no definite m.p.; IR, Fig. 14 (– – –). (Found: C, 32·95, 33·10; H, 4·84, 4·67; N, 10·60, 10·71. 10·55, 10·63. $C_{11}H_{17}O_8N_3{\cdot}HBr$ requires: C, 33·09; H, 4·29; N, 10·53%.)

When dissolved in water (5 ml) and treated with NH_4OH, the hydrobromide (100 mg) afforded the free toxin (I; 70 mg).

C_9-Base (III). A suspension of tetrodotoxin (500 mg) in aqueous EtOH (60%, 10 ml) containing KOH (1 g) was heated under reflux for 3 hr. The reaction mixture was brought to pH 3–4 by the addition of dil. HCl aq. and extracted with n-butanol (5 times). After evaporation of the solvent, the residue was washed with ether and sublimed at 150° *in vacuo* (10^{-2} mm) to give *C_9-base hydrochloride* as yellow crystals (195 mg), m.p. above 250° (sealed tube); $\lambda_{max}^{H_2O}$ mμ (log ε) 232 (4·26), 260 (4·37); $\lambda_{max}^{0.1\,N\,NaOH}$ 274 (4·38); $\lambda_{max}^{0.1\,N\,HCl}$ 228 (4·26), 264 (4·38); ν_{max}^{KBr} cm^{-1} 3300, 3000, 1660, 1590, 1505; p*Ka'* 4·45, 8·90; NMR (D_2O containing NaOD, ppm from ext. C_6H_6) −1·27s (1H), −0·07s (1H), 0·35s (1H), 2·20s (2H). (Found: C, 47·86; H, 4·47; N, 18·30. $C_9H_9O_2N_3$·HCl requires: C, 47·48; H, 4·42; N, 18·46%.)

The C_9-base was obtained from the above hydrochloride by dissolving in water and neutralizing with NaOH. It was crystallized from water as yellow needles, m.p. 200–201°; ν_{max}^{KBr} cm^{-1} 3300–3100, 1660, 1640, 1600, 1580; and could be converted to the hydrochloride by treatment with HCl.

The C_9-base was also obtained from tetrodotoxin by heating with 5% $Ba(OH)_2$ in a sealed tube at 100° for 3 hr. On cooling the C_9-base separated from the reaction mixture as yellow needles.

A mixture of the C_9-base hydrochloride (40 mg), acetic anhydride (2 ml) and pyridine (4 ml) was heated at 70–80° for 3 hr and then evaporated to dryness. The residue was extracted with $CHCl_3$ and the extract dried (Na_2SO_4) and evaporated. The residual solid on crystallization from MeOH gave *C_9-base triacetate* as colourless needles (35 mg), m.p. 196–198°; λ_{max}^{EtOH} mμ (log ε) 255 (3·95), 294 (3·63); ν_{max}^{KBr} cm^{-1} 3200, 1770, 1740, 1690, 1600, 1250, 1200; NMR ($CDCl_3$, ppm from int. TMS) 8·00br (NH), 7·80s (3H), 7·50s (3H), 7·30s (3H), 4·70s (2H), 2·40s (1H), 2·25s (1H), 0·69s (1H). (Found: C, 56·71; H, 4·62; N, 13·15. $C_9H_6O_2N_3(CH_3CO)_3$ requires: C, 56·78; H, 4·77; N, 13·24%.)

An ether solution of diazomethane (250 mg) was added to a MeOH solution (30 ml) of the C_9-base hydrochloride (100 mg). The mixture was allowed to stand overnight at room temp and then evaporated to dryness. Crystallization of the residual solid from MeOH afforded *C_9-base methyl ether* as yellow needles (65 mg), m.p. 220–222°; λ_{max}^{EtOH} mμ (log ε) 235 (4·42), 261 (4·15); ν_{max}^{KBr} cm^{-1} 3480, 3300–2850, 1625, 1595, 1560. (Found: C, 58·60; H, 5·28; N, 20·73. $C_{10}H_{11}O_2N_3$ requires: C, 58·53; H, 5·40; N, 20·48%.)

C_8-Base (IV). A mixture of the toxin (I; 500 mg) and conc. H_2SO_4 (5 ml) was stirred at room temp for 3 days, then diluted with ice-water, neutralized with $Ba(OH)_2$ aq and filtered. The filtrate was brought to pH 4 by addition of dil. HCl and evaporated to dryness. The residue was washed with ether, dried and sublimed at 150° *in vacuo* (10^{-2} mm) to give *C_8-base hydrochloride* as yellow crystals (58 mg), m.p. above 250° (sealed tube); $\lambda_{max}^{H_2O}$ mμ (log ε) 233 (4·34); $\lambda_{max}^{0.1\,N\,NaOH}$ 245 (4·47); ν_{max}^{KBr} cm^{-1} 3400. 3120, 1680, 1510, 1480, 1320; p*Ka'* 4·10, 8·50 (H_2O); NMR (D_2O containing NaOD, ppm from ext. C_6H_6) −1·82s (1H), −0·57s (2H), −0·05s (1H). (Found: C, 48·89; H, 4·50; N, 21·15. $C_8H_7ON_3$·HCl requires: C, 48·62; H, 4·00; N, 21·26%.)

Acetylation of C_8-base hydrochloride (36 mg) with acetic anhydride (3 ml) and pyridine (3 ml) at 70–80° for 3 hr gave, on crystallization from MeOH, *C_8-base diacetate* as colourless needles (25 mg), m.p. 198° (sealed tube); ν_{max}^{KBr} cm^{-1} 1750, 1680, 1210, 1190. (Found: C, 58·64; H, 4·55; N, 17·31. $C_8H_5ON_3(CH_3CO)_2$ requires: C, 58·77; H, 4·52; N, 17·14%.)

Treatment of C_8-base hydrochloride (27 mg) with an ether solution of diazomethane (100 mg) afforded *C_8-base methyl ether* (V) as yellow needles (21 mg), m.p. 198–199° (sealed tube); $\lambda_{max}^{H_2O}$ mμ (log ε) 236 (4·66), 260 (4·06); $\lambda_{max}^{0.1\,N\,HCl}$ 241 (4·21), 251 (4·21); ν_{max}^{KBr} cm^{-1} 3340, 3160, 1665, 1590, 1575, 1485, 1385, 1220, 1030, 825. (Found: C, 61·70; H, 5·48; N, 23·50. $C_9H_9ON_3$ requires: C, 61·70; H, 5·18; N, 23·99%.)

Synthesis of 2-amino-6-methoxyquinazoline (V). A solution of 2-nitro-5-methoxybenzaldehyde[49] (300 mg) in EtOH (30 ml) was catalytically hydrogenated in the presence of PtO_2 (6 mg) at room temp. After removal of the catalyst by filtration, the reaction mixture was evaporated at below 50° and the residue refluxed with a mixture of decalin (5 ml), guanidine sulphate (350 mg) and Na_2CO_3 (130 mg) for 30 min. The reaction mixture was filtered and cooled, when a yellow crystalline solid separated. The solid was dissolved in 10% HCl (140 ml) and the solution neutralized with NH_4OH giving a precipitate which on crystallization from EtOH gave yellow needles, m.p. 199–199·5°

[49] I. M. Heilbron, F. N. Kitchen, E. B. Parkers, G. D. Sutton, *J. Chem. Soc.* **127**, 2167 (1925).

(sealed tube). This is identical with the C_8-base methyl ether (V) by mixed m.p. determination and comparison of UV and IR spectra.

Hydroxy-C_8-base (VI). A solution of nortetrodoic acid (IX; 124 mg) in HCl (1 N, 6·0 ml) was heated on a water bath for 45 min and then concentrated *in vacuo*. The yellow crystalline solid which had precipitated from the solution was filtered and crystallized from 0·2 N HCl giving *hydroxy-C_8-base hydrochloride* as yellow needles (37 mg): no definite m.p.; $\lambda_{max}^{0.1\,N\,HCl}$ mμ (log ϵ) 310 (3·85), 265 (4·28), 228 (4·14); $\lambda_{max}^{0.1\,N\,NaOH}$ 309 (4·09), 228 (4·13); after acidification the solution showed a different spectrum from that recorded above: $\lambda_{max}^{0.1\,N\,HCl}$ 298 (4·06), 220 (3·96); ν_{max}^{KBr} cm^{-1} 3060, 1690, 1290; NMR (D_2O, ppm from ext. C_6H_6) −2·50s (1H), −0·70d (1H, J = 9 c/s), −0·05d 1H, J = 9 c/s); p*Ka'* 4·9, 8·0 (H_2O). (Found: C, 41·52; H, 4·50; N, 17·25. $C_8H_7O_2N_3 \cdot HCl \cdot H_2O$ requires: C, 41·48; H, 4·35; N, 18·4%.)

Methylation of the hydroxy-C_8-base hydrochloride (21 mg) with diazomethane in MeOH–ether afforded, on crystallization from MeOH, *hydroxy-C_8-base dimethyl ether* (VII) as yellow needles (14 mg), m.p. 183–186° (sealed tube); λ_{max}^{EtOH} mμ (log ϵ) 238 (4·51), 257 (4·30); $\lambda_{max}^{0.1\,N\,HCl-EtOH}$ 252 (4·19), 296 (3·52); ν_{max}^{KBr} cm^{-1} 3330, 3160, 1657, 1580, 1480, 1447, 1410, 1355, 1235, 1075, 815. (Found: 58·07; H, 5·11; N, 20·42. $C_{10}H_{11}O_2N_3$ requires: C, 58·53; H, 5·40; N, 20·48%.)

Synthesis of 2-amino-5,6-dimethoxyquinazoline (VII). 2,3-Dimethoxy-6-nitrobenzaldehyde[50] (500 mg) in EtOH (50 ml) was reduced with PtO_2 (10 mg) and the product condensed with guanidine as described in the preparation of V to give yellow needles (80 mg), m.p. 186–187° (sealed tube). This is identical with hydroxy-C_8-base dimethyl ether by mixed m.p. determination and comparison of UV and IR spectra.

Tetrodoic acid (VIII). A suspension of I (1 g) in water (20 ml) was heated in a sealed tube at 120–130° for 2–3 hr. When the solid was completely dissolved, the solution was cooled and the crystalline precipitate which separated was crystallized from water to give colourless needles, which darken above 260°; Sakaguchi and Dragendorff tests were negative; UV, end absorption $\epsilon_{max}^{0.1\,N\,HCl}$ 410; IR, Fig. 15 (——); p*Ka'* below 2·5, ca, 11·8. (Found: C, 39·14, 38·87; H, 5·96, 5·75; N, 12·33, 12·25. $C_{11}H_{17}O_8N_3 \cdot H_2O$ requires: C, 39·17; H, 5·68; N, 12·46%.)

Evaporation of a solution of VIII in 1 N HCl gave *tetrodoic acid hydrochloride* as colourless needles which darken above 250° without melting; IR, Fig. 15 (- - -). (Found: C, 37·11, 36·99; H, 5·28, 5·47; N, 11·41, 11·74. $C_{11}H_{17}O_8N_3 \cdot HCl$ requires: C, 37·14; H, 5·10; N, 11·81%.)

The hydrochloride was converted to the free acid VIII by neutralization with NH_4OH.

9-*Deuteriotetrodoic acid* was obtained by heating I (345 mg) in D_2O (3 ml) as described in the preparation of VIII; yield 112 mg. Its NMR spectrum was recorded in D_2O containing H_2SO_4. The spectrum was identical with that of VIII except a signal at 0·95 ppm (from ext. C_6H_6) which was completely absent in the former.

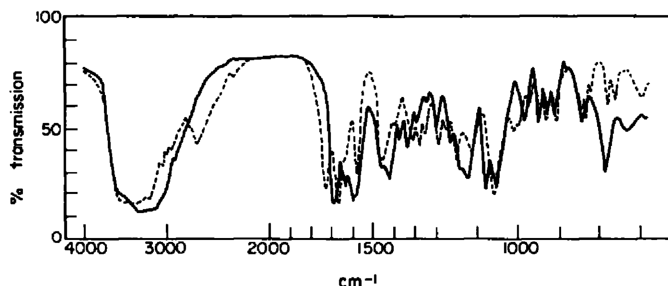


Fig. 15

Nortetrodoic acid (IX). A solution of VIII (206·2 mg) in H_2SO_4 (0·109 N, 6·0 ml) and water (50 ml) was treated at 0° with a solution of periodic acid dihydrate (146·5 mg, 1·05 eq) in water (20 ml) for 4·5 hr, and then allowed to stand overnight at room temp. A small quantity of HI was added to the reaction mixture and the liberated I_2 extracted with CCl_4. After repetition of this treatment until no I_2 was liberated, the solution was brought to pH 4·8 by careful addition of

[50] W. H. Perkin, R. Robinson, F. W. Stoyle, *J. Chem. Soc.* **125**, 2355 (1924),

Amberlite IR4B (free base) and distilled under red. press. The distillate was treated with a saturated solution of 2,4-dinitrophenylhydrazine in 1 N HCl and the yellow needles formed were identified as formaldehyde 2,4-dinitrophenylhydrazone by mixed m.p. determination and comparison of IR spectra; yield 53 mg (0·4 eq).

The distillation was continued until 10 ml was left. This residual solution was diluted with EtOH (10 ml) and the precipitate collected by filtration and crystallized from water to give IX as colourless needles (140 mg), which darkened above 250° without melting. Sakaguchi and Dragendorff tests were negative, but positive reactions were observed on the Tollens and tetrazolium tests. UV, end absorption $\varepsilon_{210\,m\mu}^{H_2O}$ 2300, $\varepsilon_{210\,m\mu}^{0\cdot1\,N\,HCl}$ 3000; IR, Fig. 16; p*Ka'* ca. 2·9, ca. 11·3. (Found: C, 38·81, 38·01; H, 5·35, 5·47; N, 13·60. $C_{10}H_{13}O_7N_3 \cdot 1\frac{1}{2}H_2O$ requires: C, 38·21; H, 5·13; N, 13·37%.) It consumes one mole of periodic acid in 0·1 N H_2SO_4 at 4°.

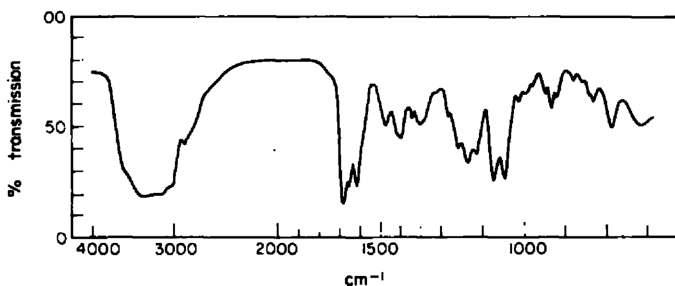


FIG. 16

Seconortetrododioic acid (X). A solution of VIII (199 mg) in H_2SO_4 (0·1 N, 30 ml) was treated at 4° with a solution of periodic acid dihydrate (339 mg, 2·66 eq) in water (5 ml) for 3 hr. Excess periodic acid was removed by addition of HI and extraction with CCl_4 as described above and the solution thus obtained was brought to pH 3 by treatment with Amberlite IR4B (free base). After concentration of the solution to 10 ml, EtOH (20 ml) was added and the precipitate crystallized from water giving X as colourless prisms (51 mg).

The same compound could be obtained from IX (68 mg) by treatment with periodic acid dihydrate (63 mg); yield 30 mg. It darkens above 250° without melting and shows no reaction with Sakaguchi and Dragendorff tests, but a weak reaction with Tollens test. UV, end absorption $\varepsilon_{210\,m\mu}^{H_2O}$ 2500, $\varepsilon_{210\,m\mu}^{0\cdot1\,N\,HCl}$ 2800, $\varepsilon_{210\,m\mu}^{0\cdot1\,N\,NaOH}$ 2200; IR, Fig. 17 (——); p*Ka'* below 2, 3·3, above 10·5. (Found: C, 36·75, 37·00, 36·84; H, 4·96, 5·09, 4·94; N, 13·21, 13·05, 13·18, 13·15. $C_{10}H_{13}O_8N_3 \cdot H_2O$ requires: C, 37·39; H, 4·71; N, 13·08%.) It consumed no periodic acid in 0·1 N H_2SO_4 or at pH 4·4 at 4°.

A solution of X (50 mg) in water (10 ml) was treated with Ag_2CO_3 (102 mg) and the mixture stirred overnight at room temp. The solid was filtered and the filtrate evaporated to dryness *in vacuo*. The residue was crystallized from water (1 ml) to give *the silver salt of X* as colourless needles (35 mg), no definite m.p. and soluble in water; IR, Fig. 17 (- - -).

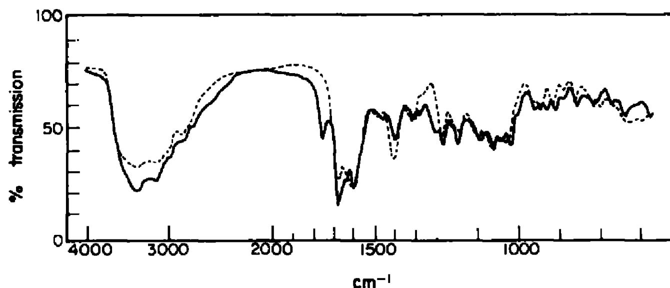


FIG. 17

Oxidation of X *with* Br_2 *aq in the presence of* $SrCO_3$. To a solution of X (102 mg) in water (20 ml), $SrCO_3$ (350 mg) and then Br_2 aq were added dropwise at room temp. The solid which remained was filtered off and a stream of air passed through the filtrate to eliminate excess Br_2.

Ag_2CO_3 (200 mg) was added to the solution and the mixture stirred for 2 hr and filtered. The filtrate was freed from silver by treatment with H_2S and concentrated *in vacuo* to give *strontium seconortetrodotrioate* (XI) as white micro-crystals (112 mg), which darkened above 200° without melting. (Found: Sr, 17·0 by EDTA titration. $C_{10}H_{13}O_{10}N_3Sr{\cdot}5H_2O$ requires: Sr, 17·5%.)

Anhydrotetrodoic acid (XII). A suspension of I (1 g) in $Ba(OH)_2$ aq (5%, 50 ml) was stirred under N_2 atm for 36 hr. The pale-yellow clear solution thus obtained was treated with CO_2 and the $BaCO_3$ which was precipitated was removed by filtration. Evaporation of the filtrate *in vacuo* gave a crystalline residue. Recrystallization of the residue from water gave XII as colourless scaly crystals (1 g), darkened above 240° without melting; $\lambda_{max}^{H_2O}$ mμ (log ε) 261 (3·77), $\lambda_{max}^{0\cdot1\,N\ NaOH}$ 290 (3·81), $\lambda_{max}^{0\cdot1\,N\ HCl}$ 257 (3·78); IR, Fig. 18; p*Ka'* 2·5, 10·9. Found (dried at 50–70° *in vacuo* for 24 hr): C, 36·63, 36·48, 36·02; H, 6·40, 6·42, 6·12; N, 11·24, 11·31, 11·65, 11·59. $C_{11}H_{17}O_8N_3{\cdot}2\frac{1}{2}H_2O$ requires: C, 36·26; H, 6·09; N, 11·54%. Found (dried at 80–90° *in vacuo* for 24 hr): C, 39·29, 39·17; H, 6·13, 6·09; N, 13·04, 13·22. $C_{11}H_{17}O_8N_3{\cdot}H_2O$ requires: C, 39·17; H, 5·68; N, 12·46%. Found (dried at 100° *in vacuo* for 24 hr): C, 40·16, 39·94; H, 6·02, 5·96; N, 13·32, 13·26. $C_{11}H_{17}O_8N_3{\cdot}\frac{1}{2}H_2O$ requires: C, 40·24; H, 5·53; N, 12·80%.

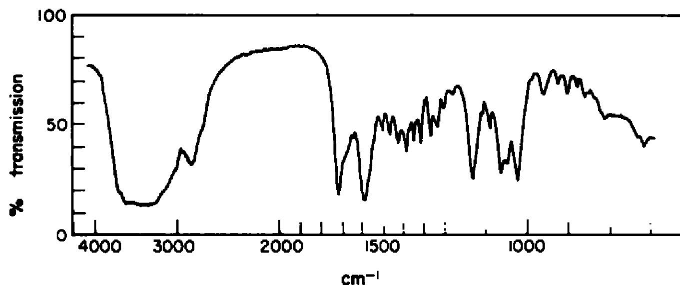


Fig. 18

Bromoanhydrotetrodoic lactone (XIII). A solution of XII in water was treated with an excess Br_2 aq and concentrated *in vacuo* giving in a quantitative yield of XIII *hydrobromide* as colourless prisms, m.p. 195° (dec); UV, end absorption $\varepsilon_{215\,m\mu}^{H_2O}$ 2250; IR, Fig. 19. (Found: C, 28·69, 28·74; H, 3·17, 3·33; N, 8·93, 8·85; O, 25·35, 25·05; Br, 33·62, 33·66. $C_{11}H_{14}O_7N_3Br{\cdot}HBr$ requires: C, 28·65; H, 3·28; N, 9·11; O, 24·29; Br, 34·66%.)

An aqueous solution of $AgNO_3$ was added to a solution of the lactone hydrobromide in water and the precipitate removed by filtration. After removal of excess silver by treatment with H_2S, concentration of the solution gave XIII *nitrate* as colourless cubic crystals, which were again recrystallized from aqueous EtOH, m.p. ca. 180° (dec). It did not consume periodic acid.

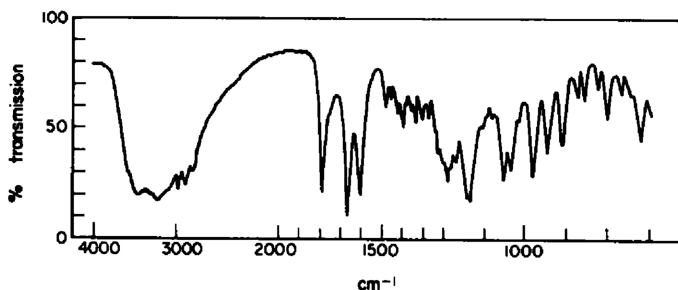


Fig. 19

Reduction of XIII *to* XII. A solution of XIII hydrobromide (320 mg) in water (30 ml) containing $CaCO_3$ (500 mg) was hydrogenated in the presence of Pd–C catalyst (10%, 300 mg). After 1·5 mole H_2 has been absorbed (3 hr), the solution was filtered and evaporated *in vacuo*. The residual semi-solid product was washed with EtOH, dissolved in water, and the solution diluted with EtOH to give crystals of XII (160 mg).

Nortetrodotoxin (XV). To a solution of I (184·4 mg) in H_2SO_4 (0·109 N, 50·0 ml), a solution of periodic acid dihydrate (134·3 mg, 1·05 eq) in water (10 ml) was added and the mixture allowed to stand at 2·5° for 6 hr and then overnight at room temp. Excess periodic acid was removed by the procedure described in the preparation of IX and the solution brought to pH 5 by careful addition of Amberlite IR4B (free base), concentrated to 2 ml and then diluted with EtOH (60 ml), whereupon the product precipitated. It was collected by filtration, washed with EtOH and ether, and purified by precipitation with EtOH from its aqueous solution; yield 170 mg; IR, Fig. 20.

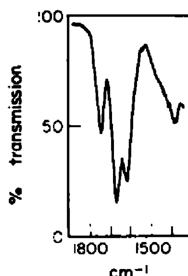


FIG. 20

Tetraacetylanhydroepitetrodotoxin p-*toluenesulphonate* (XVII). A suspension of I (100 mg) in a solution of *p*-toluenesulphonate hydrate (100 mg) in acetic anhydride (2 ml) was stirred overnight at room temp. The resultant clear solution was evaporated to dryness at 55° *in vacuo*, the residue was treated with ice-water containing sodium acetate (200 mg) and then extracted with $CHCl_3$ (3 times). The combined extracts were washed with water, dried (Na_2SO_4) and evaporated to dryness. The residue was dissolved in $CHCl_3$ and precipitated by addition of pet. ether. The precipitate (110 mg) was further purified by dissolving in benzene and reprecipitation with ether; amorphous powder, m.p. 162–164°; $\lambda_{max}^{H_2O}$ mμ (log ε) 220 (4·08), 260 (2·96); IR, Fig. 21 (——). (Found: C, 46·97; H, 5·27; N, 6·44. $C_{11}H_{11}O_7N_3(CH_3CO)_4{\cdot}C_7H_8O_3S{\cdot}H_2O$ requires: C, 47·34; H, 5·04; N, 6·37%.)

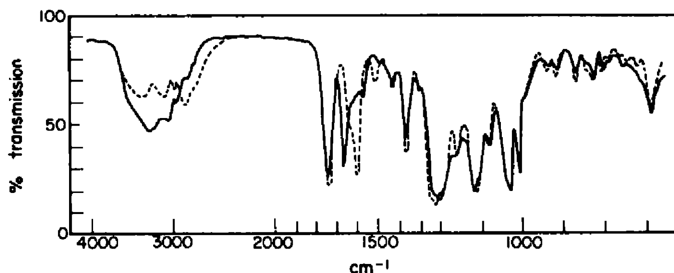


FIG. 21

Pentaacetylanhydroepitetrodotoxin p-*toluenesulphonate* (XVI). A mixture of XVII (500 mg), pyridine (2 ml) and acetic anhydride (2 ml) was allowed to stand overnight at room temp, then evaporated at 55° *in vacuo* and the residue dissolved in $CHCl_3$. Ether was added to the solution to precipitate the product, which was then crystallized from EtOH giving white needles (200 mg), m.p. 208–210°; IR, Fig. 21 (- - -). (Found: C, 48·83, 48·53, 48·80, 48·77; H, 5·20, 5·03, 5·40; N, 5·81, 6·32, 6·13; O, 35·42. $C_{11}H_{10}O_7N_3(CH_3CO)_5{\cdot}C_7H_8O_3S$ requires: C, 49·19; H, 4·87; N, 6·15; O, 35·11%.)

Diacetylanhydroepitetrodotoxin (XVIII). The tetraacetate XVII (300 mg) was dissolved on a minimum amount of MeOH and then diluted with water (10 ml). When excess NH_4OH was added, the solution immediately afforded needles (140 mg), which were collected and washed successively with water, MeOH and ether. It darkened above 200° without melting and showed no UV absorption maximum; IR, Fig. 22; p*Ka*′ 7·7 (H_2O). (Found: C, 44·53; H, 5·33; N, 10·34. $C_{11}H_{13}O_7N_3(CH_3CO)_2{\cdot}H_2O$ requires: C, 44·66; H, 5·25; N, 10·42%.)

Anhydroepitetrodotoxin (II). A methanolic solution (10 ml) of XVII (300 mg) was diluted with water (20 ml), evaporated to 20 ml and treated at 60° with conc. NH_4OH (5 ml) or trimethylamine. On standing at room temp for 1–2 hr, the solution deposited needles, which, after standing overnight were collected by filtration and washed with water, EtOH and ether, successively; yield 120 mg. It was purified by dissolving it in 0·1 N H_2SO_4 and reprecipitation by addition of trimethylamine. The same compound was obtained from XVII by treatment with slight excess NaOH aq and also from XVIII by dissolving in dil. H_2SO_4 and treatment with conc. NH_4OH or trimethylamine at 60°.

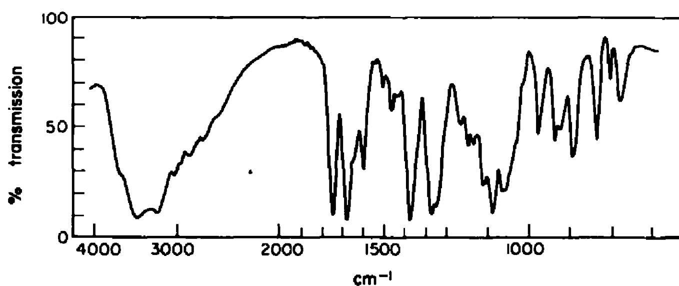


FIG. 22

It gave two polymorphic forms which were interconvertible by reprecipitation as mentioned above. It darkened above 220° without melting; IR, Fig. 23; p*Ka*′ 7·95, 11·5 (H_2O). (Found: C, 43·37; H, 5·57; N, 13·23. $C_{11}H_{15}O_7N_3$ requires: C, 43·85; H, 5·02; N, 13·92%.) Periodic acid consumption of II in 0·1 N H_2SO_4 or at pH 4·6 at 5°: 1·5 mole after 30 min; 2 moles after 2 hr; 2 moles after 24 hr.

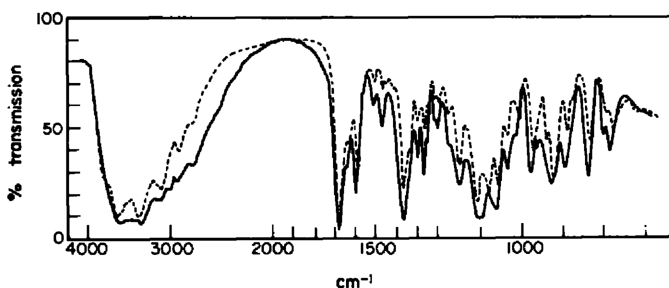


FIG. 23

Aminodesoxytetrodotoxin (XX). To a solution of XVIII (600 mg) in 0·1 N H_2SO_4 (10 ml), conc. NH_4OH (5 ml) was added with immediate formation of a crystalline precipitate. The mixture was stirred at 30° for a week, and the precipitate collected by filtration and washed with water, MeOH and ether, successively. The solid was then dissolved in dil. H_2SO_4 and precipitated by addition of NH_4OH; 370 mg of XX was obtained. The same compound was also obtained from II, XVI and XVII by treatment with ammonia.

It gave two polymorphic forms which were interconvertible by reprecipitation as mentioned above and could be distinguished by their IR spectra, one of which (XXA) being very similar to that of tetrodotoxin (I). IR, Fig. 24 (A: ——; B: - - -); p*Ka*′ 4·01, 8·66 (H_2O). (Found: (XXA) C, 40·46, 40·77; H, 5·93, 5·95; N, 17·20, 17·14; (XXB) C, 41·02, 41·01; H, 5·97, 5·88; N, 16·79, 17·08. $C_{11}H_{18}O_7N_4 \cdot \frac{1}{2}H_2O$ requires: C, 40·36; H, 5·85; N, 17·12%.)

Tetrodotoxinic acid (XXI). To a solution of I (4·233 mg) in 0·0200 N HCl (1·00 ml), 0·100 N KOH (2·00 ml) was added at once and the mixture immediately titrated with 0·50 N HCl. The titration curve showed pKa_1′ 2·36 ± 0·04 and pKa_2′ 10·8 ± 0·1; p*Ka*′ 8·76, which was possessed by I, having completely disappeared. The alkaline mixture (containing 0·5 γ XXI) showed no toxicity against a mouse (10 g).

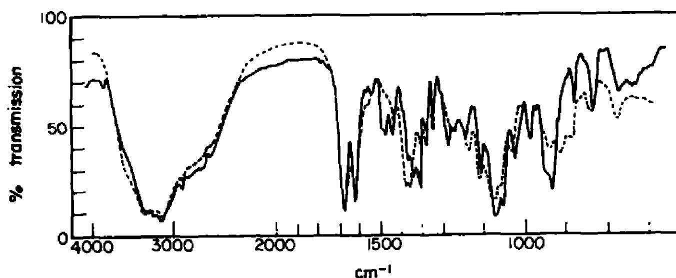


FIG. 24

To a solution of I (101 mg) in 0·109 N H_2SO_4 (5 ml), 0·2 N NaOH (5 ml) was added and the mixture filtered. The filtrate was brought to pH 8–9 by addition of 0·1 N H_2SO_4 and the solution allowed to stand overnight to give crystalline precipitate (50 mg), which was identified as I by comparison of IR spectra and the toxicity against mice.

To a solution of I (2·20 mg) in 0·02 N HCl (1 ml) at room temp, 0·10 N KOH (2 ml) was added and the absorption intensity of the mixture at 290 mμ measured; $\varepsilon = 1000$ after 5 min and $\varepsilon = 1800$ after 4 hr. In an alkaline medium the molecular extinction coefficient (ε) of anhydrotetrodoic acid (XII) is 6420.

Acknowledgements—The authors thank professors I. Nitta, Y. Tomiie and H. Kakisawa for valuable discussions and Miss I. Nakagami for technical assistance. Acknowledgement is made to the Fujisawa Pharmaceutical Co., Ltd. for collecting ovaries of puffer fish, and the Public Health Service, National Institutes of Health, U.S.A., for support of this work under Research Grant RG-7969.