THE STRUCTURE OF TETRODOTOXIN

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Certain varieties of puffer fish, especially the tora fugu, or tiger puffer (S. rubripes), and the closely related ma fugu, or common puffer (S. porphyreus), are highly prized as comestibles in Japan. The indulgence of the taste is fraught with some peril, since the livers and ovaries of the fish contain a powerful poison. The presence of this poison has been known through its effects since antiquity, but its labile nature and its extremely low concentration in its natural milieu made the isolation of the toxic principle extraordinarily difficult. Yokoo\(^1\) first succeeded in isolating the crystalline poisonous principle—now known as tetrodotoxin—only fourteen years ago, and shortly thereafter Tsuda and Kawamura\(^2\) independently achieved its isolation in a pure state.

Tetrodotoxin (Figure 1) is a colourless, crystalline, weakly basic substance. Its ultraviolet absorption is not informative in the positive sense, but its infrared spectrum reveals the presence of one or more multiple bonds of some kind. Its toxicity places it in the class of the most powerful poisonous materials known, except for certain bacterial toxins. Although nearly

TETRODOTOXIN

\[ \text{SOURCE: From livers and ovaries of various Spheroides, especially S. rubripes [とら ゆび け] and S. porphyreus [ま ゆび け]} \]

\[ \text{HABIT: Colourless prisms} \]

\[ \text{ULTRAVIOLET ABSORPTION: Transparent above 210 mp} \]

\[ \text{INFRARED ABSORPTION: 1658, 1603 cm}^{-1} \]

\[ \text{DISSOCIATION: Monoacidic base of pK}_a 8.5 \]

\[ \text{TOXICITY: 0.01 g/gram of mouse is fatal} \]

\[ \text{ELEMENTARY COMPOSITION: } C_{10-12} H_{15-19} O_{8-10} N_3 \]

\[ \text{Figure 1} \]

five hundred persons died from its effects in Japan during 1956–58, the toxic dose for man is not known; if the physiologically absurd equation of men with mice be made, it may be anticipated that half a milligram of tetrodotoxin should be sufficient to deprive an average-sized man of his life.

One of the first things which the investigator of a newly isolated natural product wishes to know is the elementary composition of the substance. In

P.A.C.—D
the case of tetrodotoxin, this determination was a matter of very great difficulty. The poison is one of those substances which very tenaciously retains solvents, particularly moisture and other hydroxylic materials, no doubt in consequence of its highly polar nature, and the presence within its molecule of a large number of OH and NH groups. In these circumstances, direct analyses for the elements gave only an approximate idea of its composition, and the best conclusion available until a relatively late stage in the structural investigations was that the composition of tetrodotoxin lay somewhere within the limits defined by the expression C\textsubscript{10-12}H\textsubscript{15-19}O\textsubscript{8-10}N\textsubscript{8}.

Even the early approximate knowledge of the elementary composition of tetrodotoxin revealed that its molecule must be an unusual one, especially in that the number of oxygen and nitrogen atoms was approximately equal to the number of carbon atoms. This unusual circumstance, taken with the relatively low hydrogen content of the molecule, made it difficult to write down trial structures even at an early stage when such attempts were not confined and delimited by any available structural information. It was tempting in these circumstances to alleviate the difficulty by postulating the presence within the tetrodotoxin molecule of one or more bonds directly between nitrogen and oxygen. Consequently, it was of great importance that we were able definitively to eliminate that possibility through the observation that guanidine could be isolated after vigorous oxidative degradation of tetrodotoxin, using concentrated aqueous sodium permanganate at 75° (Figure 2). Clearly this process could not have resulted in the

![Diagram of Tetrodotoxin]

TETRODOTOXIN

[excess aq. NaMnO\textsubscript{4}/75°]

\[
\begin{align*}
\text{NH}_2 \\
\text{HN}=\text{C} & \text{NH}_2 \\
\text{GUANIDINE} \\
\text{Isolated as picrate in >30% yield}
\end{align*}
\]

Figure 2

destruction of any N—O bonds; the experiment was further valuable in that it allowed us to conclude that all three nitrogen atoms of tetrodotoxin are undoubtedly incorporated in the molecule of the poison as an intact guanidine unit.

Our first possible insight into the nature of the fundamental skeleton of the tetrodotoxin molecule was obtained through studies of the degradation of the poison by strong bases (Figure 3). Warm sodium hydroxide was found to bring about deep-seated changes leading to a new base, C\textsubscript{9}H\textsubscript{9}O\textsubscript{2}N\textsubscript{3}, best isolated as the corresponding acetyl derivative, C\textsubscript{15}H\textsubscript{15}O\textsubscript{5}N\textsubscript{3}, here designated as the Singer acetate, after Abraham Singer, who isolated the substance

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while working in Cambridge, Mass., during a period of leave from the United States Army Chemical Center (Maryland) during the academic year 1958/1959. The infrared spectrum of the Singer acetate revealed the presence of a probable phenolic acetoxyl group [5·68 μ], a probable aliphatic acetoxyl group [5·73 μ] and an aromatic acetylamino grouping [2·90, 5·94 and 6·50 μ]. The ultraviolet spectrum was reminiscent of that of naphthalene in its possession of a very intense, sharp, short wavelength band [253 μ], and a broad, weak, long wavelength band [332 μ], and it seemed significant

TETRODOTOXIN

\[
\begin{align*}
2/3 \text{Naq. NaOH} & \\
100^\circ/45 \text{min} & \\
\rightarrow & \\
C_8H_9O_2N_3 & \\
\rightarrow & \\
C_5H_5N/\text{Ac}_2O & \\
25^\circ/17 \text{h} & \\
\rightarrow & \\
\text{SINGER ACETATE} & \\
C_{16}H_{15}O_5N_3 & \\
\text{m.p. 200-201°} & \\
\text{YIELD : 50%} & \\
\end{align*}
\]

SINGER ACETATE

INFRARED SPECTRUM (KBr) :

<table>
<thead>
<tr>
<th>λ</th>
<th>Absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>5·68 μ</td>
<td>ArO·COCH₃</td>
</tr>
<tr>
<td>5·73</td>
<td>AliphO·COCH₃</td>
</tr>
<tr>
<td>2·90</td>
<td></td>
</tr>
<tr>
<td>5·94</td>
<td>ArNH·COCH₃</td>
</tr>
<tr>
<td>6·50</td>
<td></td>
</tr>
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</table>

ULTRAVIOLET SPECTRUM (EIOH) :

<table>
<thead>
<tr>
<th>λ</th>
<th>Absorption</th>
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</thead>
<tbody>
<tr>
<td>253 μ</td>
<td>(58,000) Diazaanaphthalene</td>
</tr>
<tr>
<td>332 μ</td>
<td>(3,200)</td>
</tr>
</tbody>
</table>

Figure 3

that if the atomic groupings —COCH₃, —CH₂OCOCH₃ and —NHCOCOCH₃ were subtracted from the known composition of the Singer acetate, and replaced by hydrogen atoms, the residue, C₈H₆N₂, corresponded in composition to a diazanaphthalene. Examination of the nuclear magnetic resonance spectrum of the Singer acetate (Figure 4) permitted the

SINGER ACETATE

NMR SPECTRUM (CDCl₃) :

<table>
<thead>
<tr>
<th>T</th>
<th>Irel.</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>7·93</td>
<td>3</td>
<td>-COCH₃</td>
</tr>
<tr>
<td>7·63</td>
<td>3</td>
<td>-COCH₃</td>
</tr>
<tr>
<td>7·40</td>
<td>3</td>
<td>-COCH₃</td>
</tr>
<tr>
<td>4·85</td>
<td>2</td>
<td>Ar·CH₂·OAc</td>
</tr>
<tr>
<td>2·48</td>
<td>1</td>
<td>Ar·H</td>
</tr>
<tr>
<td>2·33</td>
<td>1</td>
<td>Ar·H</td>
</tr>
<tr>
<td>0·68</td>
<td>1</td>
<td>Py·H</td>
</tr>
<tr>
<td>0·25</td>
<td>1</td>
<td>Ar·NH·Ac</td>
</tr>
</tbody>
</table>

Figure 4

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verification and amplification of these conclusions. Thus, bands corresponding to the methyl groups of three different acetyl functions were clearly discernible, as was a band for the CH$_2$ group of ArCH$_2$OAc. Furthermore, three low-field bands corresponding to aromatic hydrogen atoms were present; one of them ($\tau$ 0·68) was at such a low-field position that it must arise from a hydrogen atom $\alpha$ to a heterocyclic nitrogen, and the splitting pattern of the other two was such that the hydrogens giving rise to the bands must be non-adjacent. A final band ($\tau$ 0·25) was clearly attributable to the NH group of ArNHAc, since it disappeared from the spectrum of Singer acetate which had been evaporated from deuterium oxide solution. The available structural information could now be summarized in the expression (I). Of the six possible full formulae included within the expression (I), those having $\text{--OAc}$ and $\text{--CH}_2\text{OAc}$ in the adjacent positions 6 and 7 could be dismissed in view of the formation and stability of the parent base, C$_9$H$_9$O$_2$N$_3$, under strongly alkaline conditions; it is well known that ortho-hydroxybenzyl alcohols do not survive such conditions. Among the remaining possibilities, that containing an acetoxy group at C–8 and an acetoxyethyl group at C–6 (Ia, Figure 5) was shown to be correct through the isolation of a beautifully crystalline copper chelate derivative of the Singer acetate (Figure 5). We take much pleasure in pointing out here that entirely independent studies by Tsuda and his collaborators$^3$ led to identical conclusions as to the course of the base degradation of tetrodotoxin. In their studies the structure of the degradation product was proven in a most rigorous way by complete synthesis of a derivative of the C$_9$ base.

We now knew that tetrodotoxin was convertible by base degradation to a quinazoline (II, Figure 6) which contained all of the nitrogen atoms, and by far the major portion of the carbon atoms of the original molecule. In what
measure were we justified at this point in assuming that the quinazoline system found in the degradation product was present in the tetrodotoxin from which it had been obtained? Since it was clear that very deep-seated changes, involving the loss of no less than six to eight oxygen atoms, must necessarily have been involved in the transformation of the one substance into the other, we felt that the structural information implied by the known degradation product must be accepted with very great reservation. It was, in short, entirely possible that the carbocyclic aromatic ring present in (II) might have been constructed from an open-chain precursor by intra-molecular condensation reactions. Our view of the necessity for caution in this matter was only somewhat alleviated when a series of other quinazolines was obtained from tetrodotoxin by alternative methods of degradation. Thus, the Singer acetate (III) (vide supra) was obtained in an alternative and very different fashion when crude fully acetylated tetrodotoxin was simply pyrolysed in vacuo. Further, Tsuda and his collaborators\textsuperscript{4} obtained a simple methylquinazoline (IV) when tetrodotoxin was subjected to vigorous reduction with hydrogen iodide and red phosphorus, and the resulting crude product was oxidized with potassium ferricyanide. Our own reduction experiments with hydrogen iodide led to yet another quinazoline, again isolated in the form of its acetyl derivative (V) (Figure 7). Of very special interest was the isolation by Goto, Kishi, and Hirata\textsuperscript{5} of still a further quinazoline (VI) by the action of concentrated sulphuric acid on tetrodotoxin. It was most striking that this substance differed markedly from the base degradation product in having a phenolic hydroxyl group at C–6 rather than at C–8—a special circumstance which served further to emphasize the possibility that the carbocyclic rings in these quinazoline degradation products could well be artefacts. In order to pursue this important question

Figure 6
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Further, we carried out the alkaline degradation of tetrodotoxin in a medium free of protons (Figure 8). The degradation product, isolated in the usual way as its triacetyl derivative, was found through a combination of mass spectrometric and nuclear magnetic resonance studies to be a mixture of the variously deuterated species (VII), (VIII), (IX), and (X). Of these the most important is the completely undeuterated representative (VII). Since

Figure 7

Figure 8

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the medium in which this substance was produced contained no source of protons, it was clear that the original tetrodotoxin molecule must possess C—H bonds in positions corresponding to those found in the degradation product. While this observation severely limited the number of hypothetical possibilities for the formation of the base degradation product by cyclizations of open-chain precursors, we were still able to elaborate plausible schemes which permitted us to formulate the known facts in terms of tetrodotoxin formulae which contained no carbocyclic ring, and we still did not feel justified in adopting a hydroquinazoline skeleton as the basis of the tetrodotoxin structure.

In order to ascertain whether there was in fact a carbocyclic ring in tetrodotoxin itself, it was imperative to establish the empirical formula of the toxin beyond question. Allusion has already been made to the fact that direct analytical determinations, in the several laboratories concerned with the problem, had not served to define the matter beyond the limits indicated in the expression C₁₀₋₁₂H₁₅₋₁₉O₈₋₁₀N₃. Our own analytical determinations were best interpretable in terms of C₁₁H₁₇O₈N₃ + 0.25 — 0.50 H₂O. We were first able to achieve a definitive solution to the problem through the mass spectrometric examination of the mixture of products obtained by long-continued acetylation of tetrodotoxin under mild conditions (Figure 9).

\[
\begin{align*}
\text{TETRODOTOXIN} & \quad \xrightarrow{C_{5}H_{12}N/\text{Ac}_{2}O} \quad \text{PERACETYLTETRODOTOXIN (S)} \\
C_{10}₋₁₂H₁₅₋₁₉O₈₋₁₀N₃ & \quad \xrightarrow{[C_{5}H_{12}N/\text{Ac}_{2}O]} \quad C_{11}H₁₇O₈N₃ + 0.25 - 0.50 \text{H}_₂\text{O} \\
\end{align*}
\]

<table>
<thead>
<tr>
<th>MASS (Observed)</th>
<th>INTENSITY (Relative)</th>
<th>MASS (Calculated)</th>
<th>IDENTIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>697</td>
<td>10</td>
<td>697</td>
<td>C₁₁H₁₇O₈N₃ + 9 [CH₂CO]</td>
</tr>
<tr>
<td>655</td>
<td>5</td>
<td>655</td>
<td>C₁₁H₁₇O₈N₃ + 8 [CH₂CO]</td>
</tr>
<tr>
<td>637</td>
<td>100</td>
<td>637</td>
<td>C₁₁H₁₇O₈N₃ — H₂O + 8 [CH₂CO]</td>
</tr>
<tr>
<td>595</td>
<td>100</td>
<td>595</td>
<td>C₁₁H₁₇O₈N₃ — H₂O + 7 [CH₂CO]</td>
</tr>
<tr>
<td>553</td>
<td>100</td>
<td>553</td>
<td>C₁₁H₁₇O₈N₃ — H₂O + 6 [CH₂CO]</td>
</tr>
</tbody>
</table>

\[
C_{11}H₁₇O₈N₃ \pm y\text{H}_₂\text{O}
\]

**Figure 9**

It will be observed that the highest mass peak obtained from this mixture corresponds to a nona-acetyl derivative of C₁₁H₁₇O₈N₃, and that all of the observed peaks of lesser mass are derivable from the same C₁₁ expression. We could now formulate tetrodotoxin as C₁₁H₁₇O₈N₃ ± yH₂O with certainty. These initial observations on the crude mixture of acetylated
tetrodotoxins were then confirmed and extended when Inayama was able to isolate two crystalline acetyl derivatives from the crude peracetylated tetrodotoxin (Figure 10). One of these was a hepta-acetylanhydrotetrodotoxin, whose formula, C_{25}H_{29}O_{14}N_{3}, was established beyond question by analysis

\[ \text{PERACETYLTETROTOXIN(S)} \]

\[ \text{INAYAMA ACETATE I} \]

HEPTA-ACETYLANHYDROTETROTOXIN

\[ \text{m.p. 192-194°} \]

\[ C_{25}H_{29}O_{14}N_{3} \]

\[ C_{11}H_{17}O_{8}N_{3}-H_{2}O + 7[CH_{2}CO] \]

MW 595 (MASS SPECTRUM)

\[ \text{INAYAMA ACETATE II} \]

OCTA-ACETYLANHYDROTETROTOXIN

\[ \text{m.p. 214°} \]

\[ C_{27}H_{31}O_{15}N_{3} \]

\[ C_{11}H_{17}O_{8}N_{3}-H_{2}O + 8[CH_{2}CO] \]

MW 637 (MASS SPECTRUM)

\[ C_{11}H_{17}O_{8}N_{3} \pm y H_{2}O \]

*Figure 10*

and mass spectroscopy, while the other was similarly shown to be an octa-acetylanhydrotetrodotoxin of the formula C_{27}H_{31}O_{15}N_{3}. The nature and composition of these acetyl derivatives was further established through their beautiful and highly informative nuclear magnetic resonance spectra (*Figures 11 and 12*). In the case of the hepta-acetyl derivative, bands corresponding to twenty-one hydrogen atoms of the seven acetyl groups were

HEPTA-ACETYLANHYDROTETROTOXIN

\[ [\text{CDCl}_{3}] \]

\[ \begin{array}{ll}
\text{T} & \text{I}_{\text{rel.}} \\
8.03 & 21 \\
7.94 & \text{Complex} \\
7.84 & \\
\end{array} \]

\[ \begin{array}{ll}
\text{Assignment} & \\
\text{CH}_{3}(\text{COR}) \times 7 & \\
\text{CH}(C, C, C) & \\
\text{CH}(C, C, OC_{\text{sat.}}) & \\
\text{CH}(C, C, OC_{\text{sat.}}) & \\
\{\text{CH}(C, C, OC_{\text{sat.}}), \text{CH}_{2}(C, OAc)\} & \\
\text{CH}(C, C, OAc) & \\
\text{CH}(C, C, O, N) & \\
\text{Σ=8 C-H bonds,} & \\
\text{exclusive of CH}_{3}CO \text{ groups} & \\
\end{array} \]

*Figure 11*
STRUCTURE OF TETRODOTOXIN

found, in addition to clearly defined bands corresponding to eight other C—H bonds. The positions of these latter were highly revealing about the situations of the hydrogen atoms from which they were derived; for the present it may be mentioned only that the distribution of bands is such that it was at once clear that only one CH₂ group can be present in the molecule of the hepta-acetyl derivative. Similarly, all thirty-one of the hydrogen atoms of the octa-acetyl derivative could be counted in its nuclear magnetic

OCTA-ACETYLANHYDROTETRODOTOXIN

\[ [\text{CDCl}_3] \]

\[ \begin{align*}
\tau & \quad I_{rel} \quad \text{Assignment} \\
7.99 & \quad 24 \quad \text{CH}_3(\text{COR}) \times 8 \\
7.92 & \quad \text{Complex} \\
7.84 & \\
7.81 & \\
7.73 & \\
5.39 & \text{Closely spaced triplet} \\
\text{[J'=J'=1.8]} & \quad 1 \quad \text{CH}(\text{C, C}, \text{OC}_{\text{sat.}}) \\
4.95 & \text{Quartet} \quad [J=12.1 \text{ c/s}] \\
4.80 & \quad 2 \quad \text{CH}_2(\text{C, OAc}) \\
4.69 & \text{Doublet} \quad [J=1.8 \text{ c/s}] \\
4.51 & \quad 1 \quad \text{CH}(\text{C, C}, \text{OAc}) \\ \\
4.40 & \text{Doublet} \quad [J=1.8 \text{ c/s}] \\
3.15 & \quad 1 \quad \text{CH}(=\text{C, NAc}) \\
\end{align*} \]

Couplings demonstrated by double resonance

\[ \Sigma = 7 \text{ C—H bonds, exclusive of CH}_3\text{CO groups} \]

Figure 12

resonance spectrum (Figure 12). In this case there were in addition to the twenty-four methyl hydrogen atoms, seven others, and it should be noted again that only one CH₂ group can be present. While it seemed clear that the relationship between the two acetyl derivatives could be expressed in a general way as shown in Figure 13, the very fact that the two derivatives

8 CH Bonds

7 CH Bonds

HEPTA-ACETYLANHYDROTETRODOTOXIN

OCTA-ACETYLANHYDROTETRODOTOXIN

\[ \begin{align*}
\text{CH} & \quad \text{C} \quad \text{O} \\
\quad & \quad \text{C}_n \\
\text{n} \gg 0 \\
\text{Figure 13} \\
\end{align*} \]
contain different numbers of CH bonds indicated the danger in attempting to deduce from these observations the number of CH bonds present in the tetrodotoxin molecule itself. The determination of that quantity was a matter of very great moment for our inquiry into the possible presence of a carbocyclic ring in tetrodotoxin, and we had devoted no little effort to attempts to determine the number of CH bonds in tetrodotoxin by direct nuclear magnetic resonance studies. However, the toxin is an extremely insoluble substance, except in acidic media. Further, we were unable to obtain pure crystalline salts from tetrodotoxin, and the measurements which we were able to make on crude acidic solutions were not definitive. It was therefore a signal event when we were able to prepare a new and beautifully crystalline derivative from tetrodotoxin by the action of hydrogen chloride on the base in the presence of acetone and methanol (Figure 14). This new derivative, named after its inventor, Gougoutas, turned out to be an O-methyl-\( O' \), \( O'' \)-isopropylidenetetrodotoxin hydrochloride monohydrate, and its composition was established beyond question through measurement of its density, and the determination of its unit cell dimensions, through

\[
\text{TETRODOTOXIN} \\
[\text{O} \cdot 2 \text{N Hydrogen chloride} \\
\text{Me}_2\text{CO/MeOH: 2/3/5} \\
25^\circ/2 \text{days}]
\]

\[
\text{GOGOUTAS HYDROCHLORIDE} \\
\text{O-METHYL-\( O' \), \( O'' \)-ISOPROPYLIDENETETRODOTOXIN HYDROCHLORIDE MONOHYDRATE}
\]

\[
\begin{align*}
\text{C}_{15} \text{H}_{23} \text{O}_9 \text{N}_3 \cdot \text{HCl} \cdot \text{H}_2\text{O} & \quad \text{Orthorhombic} \\
\text{MW} = 427.64 \quad \text{Space group P2}_1 \text{ 2}_1 \text{ 2}_1 \\
4 \text{ Molecules per unit cell} \\
a = 8.80 \text{ Å} \\
b = 15.02 \\
c = 14.74 \\
\text{MW} = 426 \pm 1 \quad \text{Density} = 1.450 \text{ g/ml} \\
\end{align*}
\]

\[
\text{C}_{11} \text{H}_{17} \text{N}_3 \text{O}_8 \pm y \text{H}_2\text{O} + (\text{CH}_3)_2\text{CO} + \text{CH}_3\text{OH} \xrightarrow{\text{H}_2\text{O}} \text{C}_{15} \text{H}_{23} \text{O}_9 \text{N}_3 + (2+y) \text{H}_2\text{O}
\]

Figure 14

X-ray crystallographic methods. It may be noted first that this determination gave yet another confirmation of the empirical formula \( \text{C}_{11} \text{H}_{17} \text{O}_9 \text{N}_3 \pm y\text{H}_2\text{O} \) for tetrodotoxin. Of even greater importance was the fact that the nuclear magnetic resonance spectrum of the new derivative, and that of tetrodotoxin itself dissolved in mineral acid, were found to be very similar, aside from the bands attributable to the added methyl and isopropylidene groups in the derivative (Figure 15). From this fact, we could be certain that the new substance was formed from tetrodotoxin with minimal structural change. Furthermore, with this pure crystalline derivative in hand,
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we could now make accurate intensity measurements, which in detail were facilitated by the presence of an internal intensity standard, in the shape of the band corresponding to the six hydrogen atoms of the isopropylidene group. We could now be certain that tetrodotoxin contains eight CH bonds. It may also be noted parenthetically, for future use, that these spectra permitted us to conclude that two of the CH bonds are adjacent, and that they are attached, respectively, to carbon bound only to other carbon atoms, and to carbon bearing two electronegative atoms (cf. asterisked bands).

DEUTERATED HYDROCHLORIDES IN D₂O

\[ \Delta = \text{Downfield displacement from external Me}_4\text{Si (c/s)} \]

<table>
<thead>
<tr>
<th>( \Delta )</th>
<th>( \Delta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>88</td>
<td>145</td>
</tr>
<tr>
<td>*140 Doublet ([J = 10 \text{ c/s}])</td>
<td>6</td>
</tr>
<tr>
<td>215</td>
<td>240</td>
</tr>
<tr>
<td>256</td>
<td>244</td>
</tr>
<tr>
<td>268</td>
<td>260</td>
</tr>
<tr>
<td>*322 Doublet ([J = 10 \text{ c/s}])</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>267</td>
</tr>
</tbody>
</table>

\[ \text{O} \text{--C} \text{--C} \]
\[ \text{O or N} \text{--C} \]

\[ \text{Figure 15} \]

Having in hand the hard-won facts that tetrodotoxin (1) possesses the molecular formula \( \text{C}_{11}\text{H}_{17}\text{O}_8\text{N}_3 \pm \gamma \text{H}_2\text{O} \), (2) contains no NO bonds, and (3) contains eight CH bonds, we could now deduce the fundamental nature of the carbon skeleton (Figure 16). Thus, the atoms known to be present could be divided into three groups: (a) a guanidine residue which must be attached to the carbon skeleton by an unknown number of bonds, \( x \), (b) a number of hydroxyl groups, which depends of course upon \( x \), and (c) all other atoms. Now, in a wholly imaginary experiment, the guanidine moiety and the hydroxyl groups were removed from the remaining carbon skeleton and replaced by equivalent numbers of hydrogen atoms. In the resulting expression, each remaining oxygen atom had to be replaced, in yet another logical operation, by two hydrogen atoms. The final residual skeleton was then seen to be \( \text{C}_{10}\text{H}_{20} \). Since tetrodotoxin possesses no olefinic properties, and since there was no reason to suppose that the \( \text{C}_{10} \) carbon skeleton is not continuous, \textit{tetrodotoxin must contain a carbocyclic ring.} This very important conclusion had as its direct result a very great simplification of the task of deducing a structure for tetrodotoxin from the available chemical data. It could now be assumed with a high degree of probability
TETRODOTOXIN

\[ C_{11}H_{17}O_{8}N_{3} \pm \gamma H_{2}O \]  \hspace{1cm} (1)

\[ C_{10}H_{8}O_{4-x} \pm \gamma + CN_{3}H_{5-x}^* + (OH^*)_{4+x} \pm 2\gamma \]  \hspace{1cm} (2)

\( x = \) Number of bonds joining guanidine moiety to the carbon skeleton
\( H^* = \) Active hydrogen

\[ C_{10}H_{6}O_{4-x} \pm \gamma + H_x + H_{4+x} \pm 2\gamma \]  \hspace{1cm} (3)

\[ C_{10}H_{12+2x} \pm 2\gamma O_{4-x} \pm \gamma \]

Replace each remaining \( O \) by two hydrogens

\[ C_{10}H_{12+2x} \pm 2\gamma H_{6-2x} \pm 2\gamma \]  \hspace{1cm} (4)

\[ C_{10}H_{20} \]  \hspace{1cm} (5)

\[ \therefore \text{TETRODOTOXIN MUST CONTAIN ONE CARBOCYCLIC RING} \]

Figure 16

that the carbocyclic ring present in the many quinazolines obtained from degradation was in fact present in the original tetrodotoxin molecule. In particular the many open-chain possibilities, which had had to be kept in mind until this point had been reached, could now be discarded with certainty.

Two of the major quinazoline degradation products from tetrodotoxin are reproduced in Figure 17, and below them are shown two developing part structures for the poison which could be deduced at this time. In (XIII) we see the carbocyclic ring with its branching carbon atoms and its guanidine

\[ \text{Figure 17} \]
grouping placed in accord with the known structure (XI). The further development of (XIII) to (XIV) was easily made when cognizance was taken of the facts, revealed by the nuclear magnetic resonance studies of tetrodotoxin and its acetyl derivatives, that only one HC(C) grouping is present, and that it is adjacent to a HC(ON or O) group. It may be noted that the presence of oxygen adjacent to the \(-\text{CH}_2\text{OH}\) grouping of (XIV) received confirmation from the presence of that same atom at C-6 in the degradation product (XII), and further from the fact that a molecule of formaldehyde was readily obtained from tetrodotoxin by periodate oxidation.

In the expression (XIV) (reproduced in Figure 18) the valency capacity of all of the atoms is fully utilized except those of the carbon atoms at \(a\) and \(b\). Since the composition of tetrodotoxin was now known, it was possible to deduce the number and kind of atoms present in the tetrodotoxin molecule which do not appear in (XIV), and further, it was readily discerned that these hitherto unconsidered atoms could be arranged only in a strictly limited number of ways, as shown beneath (XIV) in Figure 18. Since nuclear magnetic resonance studies had shown that only one CH\(_2\) group, and only one HC(OO or N) are present, all of these possibilities could be excluded except those two—(XV) and (XVI)—in which the new atoms are attached as a glycolic acid residue at one or the other of the only available positions.

Our decision in favour of (XV) was made through a consideration in detail of the course of the degradation of the tetrodotoxin molecule under basic and acidic conditions. The base degradation is shown in Figure 19. The key reaction is the loss of the glycolic acid side-chain by a kind of
R. B. WOODWARD
dealdolization, which is certainly driven forward by the developing aromaticity of the pyrimidine ring in the transition state for the crucial carbon–carbon bond cleavage. It may be noted in particular that the scheme provides for the appearance of either hydrogen or deuterium in the 5 and 7 positions, in accord with experience, as already discussed earlier (cf. Figure 8). The degradation of tetrodotoxin by base was thus easily accommodated to the attachment of the glycolic acid residue at a as in (XV),

DEGRADATION BY BASE

![Diagram of degradation by base](image)

Figure 19

but it must be admitted that the argument is not definitive in this case; a similar scheme can be brought forward to account for the results using the alternative formula (XVI), in which the glycolic acid residue is attached at b.

By contrast, the degradation of tetrodotoxin under acidic conditions could be interpreted plausibly only on the basis of the formula (XV). The scheme is shown in Figure 20, and a key feature is a dealdolization very similar to that just described in the case of the base degradation. A special feature is the incorporation of a retro-Prins reaction which leads to the loss of water and formaldehyde. We were able to discern no way in which the product (XII) can be plausibly formed from a tetrodotoxin structure based upon the alternative (XVI). The central difficulty is the necessity for cleaving both the carbon–carbon and carbon–oxygen bonds to b, and the ultimate introduction of hydrogen rather than oxygen at position 8 in the product.

We had now been able to develop our formula for tetrodotoxin to (XVII) (Figure 21). This expression required further amplification in two directions (asterisks). The aldehyde function shown in (XVII) certainly does not
STRUCTURE OF TETRODOTOXIN

exist free in the tetrodotoxin molecule, nor indeed would it be expected to fail to combine with one of the plethora of nucleophilic atoms present, to give a hemiacetal or a carbinolamine grouping. The formation of many quinazolines by degradation of tetrodotoxin suggested at once that, of the

DEGRADATION BY ACID

![Chemical structures](image)

Figure 20

![Chemical structures](image)

Figure 21

(i) \( J = 10 \text{ c/s for } (C,C) \text{CH} - \text{CH}(O,N \text{ or } O) \) requires antiplanar (dixial) orientation

(ii) Quinazolines by degradation

many possibilities, that shown in (XVIII) is the correct one, but the argument was not strong when one considered the ready reversibility of carbinolamine and hemiacetal formation. Much better evidence that (XVIII) is in fact correct was available from the large splitting constant observed.
for the bands associated with the hydrogen atoms of the (C, C)CH—CH(O, N or O) system in the nuclear magnetic resonance spectra of tetrodotoxin hydrochloride, and of O-methyl-\(O',O''\)-isopropylidenetetrodotoxin hydrochloride. The observed value clearly suggested, for the two CH bonds, a dihedral angle of 180°, which cannot be achieved in the alternatives involving interaction of the aldehyde group with one of the available hydroxyl groups in hemiacetal formation. The presence of the hydroquinazoline system in tetrodotoxin was further deduced from the detailed analysis of the nuclear magnetic resonance spectra of the acetyl derivatives of the poison, but this argument is better presented at a later time (vide infra).

It now remained to remove from the expression (XVIII) (cf. Figure 22) the asterisked carboxyl group, since it was very clear that tetrodotoxin contains

![Structures](image)

Infrared spectra of tetrodotoxin hydrochloride and O'-methyl-\(O',O''\)-isopropyliden hydrochloride possess bands at 5.71 μ, unchanged on deuteration

**Figure 22**

no such function. The simplest manner in which the expression (XVIII) might be disabused of its unwanted carboxyl group was to combine that function with one of the available hydroxyl groups to produce a lactone function. This possibility was clearly in accord with the presence in the infrared spectra of tetrodotoxin hydrochloride, and its O-methyl-\(O',O''\)-isopropyliden derivative, of bands at 5.71 μ which do not change their positions on deuteration. Which of the many hydroxyl groups should be chosen? The position of the just-mentioned infrared bands seemed suggestive, but not conclusive, of a six-membered lactone grouping, as shown in (XIX), and indeed it was possible to establish that choice, again by a detailed analysis of the nuclear magnetic resonance spectra of the acetyl derivatives of the poison (vide infra). At this point, however, the argument was simplified through the obtention of the almost ocular evidence provided by a complete three-dimensional X-ray crystallographic analysis of the structure of O-methyl-\(O',O''\)-isopropylidenetetrodotoxin hydrochloride. We have already indicated that this beautifully crystalline derivative is orthorhombic, and lies in the space group \(P2_12_12_1\). Two thousand and eighty-nine reflections were measured about the \(a\) and \(c\) axes, using a computer-programmed Buerger Automatic Diffractometer. After the experimental data had been corrected in the usual way, the chlorine positions were readily ascertained from a three-dimensional Patterson map,
which was searched using a symmetry minimum function. A first three-dimensional Fourier analysis was then calculated using structure factors weighted, according to Sim, to emphasize those reflections particularly sensitive to the chlorine positions. In this first three-dimensional Fourier analysis the suspected structure (XIX, with added methyl and isopropylidene groups) was clearly discernible, despite much pseudosymmetry, in consequence of the nearness of the chlorine atoms to symmetry axes, and numerous false peaks. Thirteen of the best-defined atoms were chosen as the basis for a second Fourier analysis, in which all of the suspected atoms reappeared and the pseudosymmetry had markedly diminished. A third Fourier based on twenty atoms revealed only atoms of the now very probable structure, and a fourth Fourier, based on all twenty-eight atoms, gave a structure in which all atoms were very well defined, and no false peaks were present. The atomic coordinates were now refined on the basis of the fourth Fourier, and used for a fifth, which gave $R = 0.22$. The structure was now accepted, and $R$ was reduced to 0.12 through least squares refinement of anisotropic thermal parameters and coordinates. The complete structure, as built up directly as the analysis proceeded is shown in Figure 23.

![Figure 23](image)

In Figure 24 the structure (XIX) deduced from degradative and analytical studies may be seen to correspond completely to that established by our X-ray crystallographic analysis of $O$-methyl-$O'$,$O''$-isopropylidenetetrodotoxin hydrochloride (XX). It is with much pleasure that we can refer here also to two beautiful, entirely independent X-ray studies carried out approximately simultaneously with ours by the Japanese groups who have made such outstanding contributions to the study of tetrodotoxin$^6,7$. Tetrodoic acid hydrobromide (XXI), obtained from tetrodotoxin by the long-continued action of hot water, and bromoanhydrotetrodoic lactone hydrobromide
(XXII), obtained by a more complicated series of transformations, are both considerably less closely related to tetrodotoxin itself than is our derivative, but it is clear that all three substances have their origin in the same structure. A special point is that the Tsuda derivative (XXI) differs in configuration at the asterisked carbon atom from the other two, but the possibility of an inversion at the relevant centre in the operations leading to tetrodoic acid is clearly apparent.

![Chemical structures](image)

It might well be imagined that with the availability of no less than three concordant full X-ray crystallographic analyses upon divers derivatives of tetrodotoxin, the full structure of the poison itself would have been immediately apparent. Tetrodotoxin hydrochloride clearly possesses the structure (XXIII) shown in Figure 25, and it might have been presumed that tetrodotoxin itself must simply be the corresponding deprotonated species (XXIV). But it was very obvious from some of the simplest facts about tetrodotoxin that such cannot be the case. Tetrodotoxin is certainly not a lactone, since its infrared spectrum does not contain the short wavelength bands found in its hydrochloride and that of O-methyl-0',0''-isopropylidenetetrodotoxin. Furthermore, the observed basicity of tetrodotoxin is far too low to be associated with a guanidinium system, even if allowance were made for the presence in the molecule of many electron-attracting atoms. A further most significant point is that those bands in the infrared spectra of tetrodotoxin hydrochloride, and O-methyl-0',0''-isopropylidenetetrodotoxin hydrochloride, which must be associated with the guanidinium system appear unchanged in the free base (cf. Figure 26). This observation
STRUCTURE OF TETRODOTOXIN

clearly suggested that the deprotonation of these salts takes place at another site, and this presumption was confirmed by an examination of the dissociation in non-aqueous solutions. The increase in $pK_a$ observed in solvents of low dielectric constant indicated clearly that the dissociation occurs from an hydroxyl group, and the conclusion is inescapable that the

![Tetrodotoxin Hydrochloride](image)

(1) TETRODOTOXIN IS NOT A LACTONE

In the infrared spectrum of tetrodotoxin and 0-methyl-0', 0''-isopropylidene tetrodotoxin, the 5.71 bands found in the hydrochlorides are absent.

(2) THE OBSERVED $pK_a = 8.5$ IS TOO LOW FOR A GUANIDINIUM SYSTEM

Figure 25

IONIZATION OF
0-METHYL-0', 0''-ISOPROPYLIDENETETRODOTOXIN HYDROCHLORIDE

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$pK_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>8.3</td>
</tr>
<tr>
<td>Dioxan/water</td>
<td>9.2</td>
</tr>
</tbody>
</table>

$\text{OH} \rightarrow -\text{O}^- + \text{H}^+$

INFRARED BANDS ASSOCIATED WITH GUANIDINE MOIETIES

<table>
<thead>
<tr>
<th>Salt</th>
<th>Free base</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrodotoxin</td>
<td>6.03, 6.22</td>
</tr>
<tr>
<td>0-methyl-0', 0''-isopropylidene tetrodotoxin</td>
<td>6.04, 6.24</td>
</tr>
</tbody>
</table>

The free bases are zwitterions!

![Zwitterion](image)

Figure 26
free bases are zwitterions. But from which hydroxyl group does the dissociation occur? If we examine again the expression (XXIII) (cf. Figure 27) we cannot discern in it an hydroxyl group which might be expected to dissociate with the relatively strongly acidic $pK_a$ 8.5. The solution to this dilemma was discerned through a detailed examination of the nuclear magnetic resonance spectrum of hepta-acetylhexahydrotetrodotoxin (Figure 11). If the molecule (XXIII) were to undergo acylation, no less than three hydrogen atoms attached to carbon bearing acyloxy groups should appear in the product, *viz.*, those at C-5, C-7, and C-8 (cf. XXVI). In fact, the nuclear magnetic resonance spectrum of the hepta-acetyl derivative shows clearly that there can be only one such hydrogen atom, since the resonances associated with hydrogen of that type must necessarily appear at lower field than those associated with the hydrogen atoms in the system—CH$_2$OAc. Consequently, it was clear that in some way, two of the three groups shown in (XXV) must co-operate to form a new system. The only possibility is that shown in Figure 28, in which one of the hydroxyl groups is added to the lactone grouping to create a new hemilactal system. The acetylation product (XXVII) now contains, as required, only one hydrogen bound to acyloxy-lated carbon. The other two relevant hydrogen atoms are now attached to carbon which bears ethereal oxygen, and the resonances associated with them appear at higher field. Furthermore, the double resonance experiments summarized in Figure 11 showed clearly that it is the C-5 hydroxyl group which co-operates with the lactone function to form the new hemilactal group. If now we inspect a three-dimensional representation of tetrodotoxin hydrochloride (XXVIII) (Figure 29), it is at once apparent that the C-5 hydroxyl group is admirably placed in space for interaction with the lactone grouping. The rigidity of the entire skeleton of the open-chain tautomer is
undoubtedly a factor which favours the establishment of equilibrium with the hemilactal form (XXIX) which, it is interesting to note, incorporates the feature of a dioxa-adamantane nucleus. It is also worthy of note at this time that the lactone band in the infrared spectrum of (non-crystalline) tetrodotoxin hydrochloride is relatively weak, and the implication is clear that
in this material both open-chain and hemilactal ring-chain tautomers co-exist. By contrast, the crystalline $O$-methyl-$O',O''$-isopropylidenetetrodotoxin hydrochloride exists entirely in the lactone form (Figure 30). However, this same substance is equilibrated to a greater or lesser degree with the isomeric hemilactal form, depending upon the environment. In deuterium oxide solution the carbonyl band in the infrared spectrum of this hydrochloride has all but vanished, and it may be estimated roughly that 90 per cent of the molecules are in the hemilactal form in that medium.

**HYDROXYLACTONE / HEMILACTAL EQUILIBRIUM**

in

$$\textit{O-METHYL-}O',O''\textit{-ISOpropYLDENETETROTOXIN HYDROCHLORIDE}$$

<table>
<thead>
<tr>
<th>State</th>
<th>Percent hemilactal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystalline</td>
<td>0</td>
</tr>
<tr>
<td>$\text{Me}_2\text{SO}$ solution</td>
<td>$\sim 70$</td>
</tr>
<tr>
<td>$D_2O$ solution</td>
<td>$\sim 90$</td>
</tr>
</tbody>
</table>

*Figure 30*

If the already rigid system of the tetrodotoxin molecule is further rigidified by introduction of yet another element of constraint in the form of a new ring, the equilibrium just discussed between hydroxylactone and hemilactal forms is strongly displaced in favour of the hemilactal tautomer. These are the circumstances which obtain in the so-called anhydro series (Figure 31). When tetrodotoxin was treated with hydrogen chloride in acetone for a short time, a beautifully crystalline $O,O'$-isopropylideneanhydrotetrodotoxin hydrochloride was produced. This substance was easily formulated as (XXX), since it was clear that the carbinolamine hydroxyl group should share the ready capacity for ether formation which is characteristic of its class, and since the only hydroxyl group with which ether formation is possible is that at C-11. Infrared spectra of the new hydrochloride, either as crystalline solid or in a variety of solvents, display no lactone band whatsoever, and it is clear that the derivative exists in the hemilactal form under all conditions. It is of much interest that hepta-acetylanhydrotetrodotoxin is also a member of this anhydro series (Figure 32). Its full structure (XXXI) is entirely in accord with its detailed nuclear magnetic resonance spectrum (Figure 11). In particular, it may be noted that with change in configuration at C-4, the carbon–hydrogen bond at that position makes a dihedral angle with the adjacent bridge-head carbon–hydrogen bond of approximately 90°, and that consequently, the coupling constant is very nearly 0. Further,
STRUCTURE OF TETRODOTOXIN

Double resonance experiments show the expected coupling between the resonances associated with the hydrogen atoms at C-7 and C-8, as well as the relationship already alluded to between those at C-5 and C-10. A final point of interest is the observation of the expected 1,3 coupling between the equatorial hydrogens at C-5 and C-7, by contrast with the absence of

THE ANHYDRO SERIES

TETRODOTOXIN

\[ \text{Hydrogen chloride / Me}_2\text{CO} \]

25% 2 h

RAJAPPA HYDROCHLORIDE

\[ O,O'-\text{ISOPROPYLDENEANHYDROTETRODOTOXIN HYDROCHLORIDE} \]

\[ \text{C}_{14}\text{H}_{19}\text{O}_{7}\text{N}_{3} \cdot \text{HCl} \cdot \text{H}_{2}\text{O} \]

Figure 31

HEPTA-ACETYLHANHYDROTETRODOTOXIN

OCTA-ACETYLHANHYDROTETRODOTOXIN

coupling between the axial hydrogens at C-8 and C-10. It may also be noted that the complete analysis of the nuclear magnetic resonance spectrum of this acetyl derivative permitted the complete derivation of the tetrodotoxin structure, except for the stereochemistry at C-6, without recourse to the independent evidence from our X-ray crystallographic study of O-methyl-O',O''-isopropylidenetetrodotoxin hydrochloride. Finally, we may note
briefly that the full structure of octa-acetylanhydrotetrodotoxin (XXXII), which may be deduced readily from the relationship already earlier adumbrated (Figure 13), is also in full accord with a detailed analysis of its nuclear magnetic resonance spectrum (Figure 12).

It is now clear that the free base, tetrodotoxin, is a zwitterion (XXXIII) (Figure 33), convertible on protonation into a hemilactal (XXXIV), which may exist in equilibrium with a corresponding hydroxylactone tautomer (XXXV), to an extent depending upon environmental and detailed structural factors. It is of special interest (Figure 34) that we were able to bring

\[
\begin{align*}
\text{FREE BASE} & \quad \text{(XXXIII)} \\
\text{SALT} & \quad \text{Hemilactal tautomer} \\
\text{SALT} & \quad \text{Hydroxylactone tautomer}
\end{align*}
\]

\[pK_a = 8.5\]

forward a classical chemical demonstration of the zwitterionic nature of a tetrodotoxin derivative when it was found that isopropylideneanhydro-
tetrodotoxin (XXXVI) was converted by methyl iodide in methyl sulphate to a beautifully crystalline methosulphate (XXXVII). The infrared spectrum of the new derivative possesses the characteristic guanidinium bands as usual. Its nuclear magnetic resonance spectrum possesses a new band at a position characteristic of the hydrogen atoms of a methoxyl group, and finally, the normal basic function of the tetrodotoxin series is now masked; the new derivative possesses only a very strongly basic guanidinium function.

We may now summarize our knowledge of the structure of tetrodotoxin in the final structure (XXXVIII) (Figure 35). In the light of our present position, it is interesting to comment on the outcome of the special problem which was apparent at the very outset of the work, when, although even the empirical formula of tetrodotoxin was but imperfectly known, it was clear
that the number of oxygen and nitrogen atoms was approximately the same as the number of carbon atoms. We see that in the final structure every carbon atom but one bears at least one oxygen or nitrogen atom, while one carbon atom bears two, and two carbon atoms are attached to no less than three hetero atoms. Further, one of the latter arrays—the hemilactal function—is entirely unique, not having been observed before in the structure of any organic molecule, whether of natural or purely synthetic origin.

RAJAPPA BASE
IR: 6·02, 6·33 μ
pK<sub>a</sub> = 7·5

METHOSULPHATE
IR: 6·02, 6·31 μ
pK<sub>a</sub> ~ 11
NMR: new band at δ = -207 c/s
[D<sub>2</sub>O/external Me<sub>4</sub>Si]

Figure 34

TETRODOTOXIN
(XXXVIII)

Figure 35

The appearance of the array in the tetrodotoxin molecule presents a clear lesson for the future in its intimation that if normally non-interacting groups are appositely attached to a rigid skeleton, or otherwise brought into forced proximity, they may be expected to co-operate in the formation of structural groupings which are not observed in simpler systems. It is worthy of note that tetrodotoxin is yet another in the long series of natural products whose study has time and again turned up for the first time new and unique systems,
and provided stimulating insights into the fundamental behaviour of organic chemical systems.

This is not the place to speculate upon the relationship between the structure of tetrodotoxin, and the striking physiological activity of the poison, nor upon the biogenesis of the molecule; though in the latter respect, the wild surmise might be briefly made that the molecule could be constructed by a variant of the familiar polycarbonate scheme, with added branching carbon, along lines somewhat similar to those used in the construction of scleroticin and its relatives.

No account of this work would be complete without special acknowledgement of the skill and devotion of the men who brought it to a successful conclusion. Tetrodotoxin is not a substance which yielded easily the crystalline derivatives which were the indispensable cornerstones of our work. From time-to-time in this lecture I have given these latter eponymous designations which permitted me to express in a concise way my affection both for the compounds, and for the men who played the major roles in the adventure I have described here—Mr Jack Gougoutas, Dr Seiichi Inayama, and Dr Srinivasachari Rajappa. The very valuable early work of Mr Abraham Singer has also very deservedly been eponymized, and all of us who have participated in the final exciting stages of the work should acknowledge our debt to Drs Erach Talaty and Faizulla Kathawala, whose careful and painstaking work during a much less dramatic phase of the investigation laid a solid foundation for the later definitive advances. Our investigation was facilitated a great deal through help which we are glad to acknowledge from Professor Klaus Biemann of the Massachusetts Institute of Technology, who carried out numerous mass spectrometric measurements for us, and from Professor William N. Lipscomb and Dr Iain Paul of this Department, whose wise and ready counsel was of the very greatest value in our first foray into X-ray crystallography. We should like further to express our very warm appreciation to Dr George Nelson and his associates of Loma Linda University who performed the Herculean labour of extracting from many tons of puffer fish entrails the pure tetrodotoxin which was used in our work. Finally, we are indebted to the United States Army Chemical Center Procurement Agency and the United States Army Research Office, Durham for their generous support of our work, and in particular to Drs Thomas Simmons of the Army Chemical Center and Robert Ghirardelli of the Army Research Office, who have unfailingly given us help and encouragement.

References