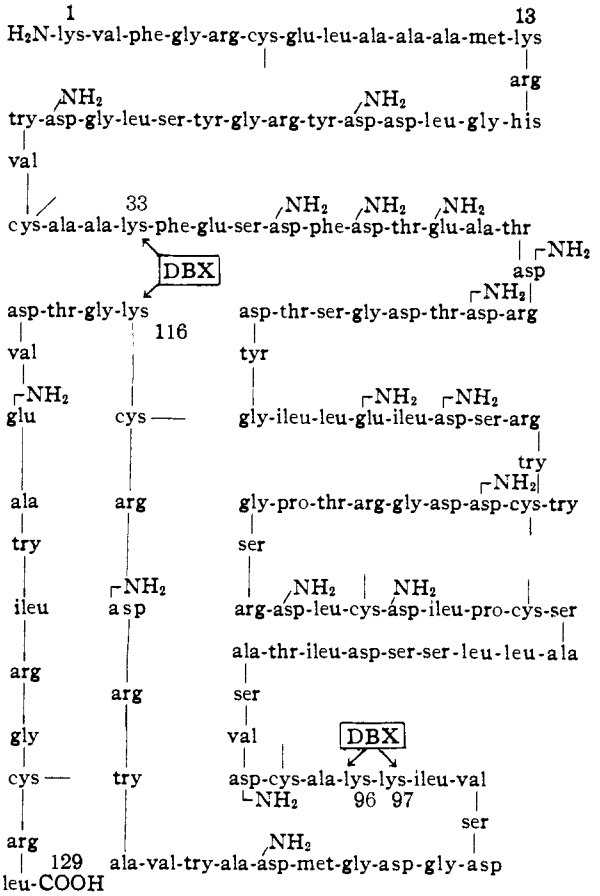

Fig. 1.—Schematic representation of points of attachment of α,α'-dibromo-*p*-xylenesulfonic acid.

Further purification of the labeled peptides was then followed by removal of the cross-linking residues under conditions used to hydrogenolyze benzyl amines.[16]

TABLE I

QUALITATIVE ANALYSIS OF PEPTIDES INVOLVED IN CROSS LINKAGES

Peptide	Cross linkage,[a] lysine residue	Amino acids
A_{11}	96 → 97	Calcd.: ala, arg, asp, gly, leu, met, ser, val
		Found: ala, arg, asp, gly, ileu,[b] met, ser, val
A_{12}	96 → 97	Calcd.: lys
		Found: lys
A_{13}	96 → 97	Calcd.: ala, asp, cys, ileu, leu, lys, pro, ser, thr, try, val
		Found: ala, asp, cys, ileu, leu, lys, pro, ser, thr, . . .,[c] val
B_{11}	33 → 116	Calcd.: ala, asp, cys, gly, leu, lys, ser, try, tyr, val
		Found: ala, asp, cys, gly, leu, lys, ser, . . .,[c] tyr, val
B_{12}	33 → 116	Calcd.: ala, arg, asp, glu, phe, ser, thr
		Found: ala, arg, asp, glu, phe, ser, thr
B_{21}	33 → 116	Calcd.: cys, lys
		Found: cys, lys
B_{22}	33 → 116	Calcd.: ala, arg, asp, glu, gly, ileu, thr, try, val
		Found: ala, arg, asp, glu, gly, ileu, thr, . . .,[c] val

[a] According to Canfield's sequence.[16] [b] Analysis ambiguous with respect to differentiating between leucine and isoleucine. [c] Acid hydrolysis left no detectable tryptophan residues.

(16) L. Birkofer, *Ber. deut. chem. Ges.*, **75**, 429 (1942).

Peptide A afforded only one nonradioactive peptide (A_1) upon hydrogenolysis and peptide B gave two nonradioactive peptides (B_1 and B_2). Peptide C was present in such small amounts that it was neglected. Peptide A_1 upon trypsin digestion yielded three new peptides (A_{11}, A_{12}, and A_{13}) the analysis of which (Table I) showed that they were derived from amino acids 74 to 112 in the primary sequence announced by Canfield[17] (*cf.* Fig. 1). The three tryptides in this region correspond to Canfield's T_{11}, T_{12}, and T_{13}. Peptides B_1 and B_2 upon tryptic digestion each led to two new peptides (B_{11}, B_{12}, B_{21}, and B_{22}), respectively. These were analyzed and shown to correspond to Canfield's tryptides T_6, T_7, T_{15}, and T_{16}, respectively (see Fig. 1 and Table I). It follows, then, that DBX introduced cross-links into lysozyme and in particular between the ϵ-amino groups of lysine residues 96–97 and 33–116. The link between residues 33 and 116 corresponds to an assignment made by Herzig, *et al.*,[12] for phenol-2,4-disulfonyl chloride, a reagent of nearly identical span. The possible significance in the difference in patterns of cross-linking by reagents of nearly identical dimensions of greatly different reactivities will be discussed elsewhere.[18]

(17) R. E. Canfield, *J. Biol. Chem.*, **238**, 2698 (1962).

(18) We acknowledge the generous support provided by the National Science Foundation (G-9794 and G-16161) and the American Cancer Society (In-79 and P-343) for this research.

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RECEIVED AUGUST 21, 1964

Radical Scavenging in the Radiolysis of Liquid Methanol–Benzene Mixtures

Sir:

Radiolysis of liquid methanol containing increasing concentrations of benzene results in a sharp decrease in both hydrogen[1] and ethylene glycol[2] yields similar to that observed for the hydrogen yield in cyclohexane–benzene mixtures.[3] This phenomenon has been attributed to radical scavenging[1] or to energy transfer.[3] An examination of the radiolysis products of the cyclohexane–benzene system[4] has shown that phenylcyclohexane is formed with a *G*-value which reaches a maximum at ≈10% v./v. benzene, and then falls linearly with benzene concentration. It was also found that the *G*-values of both cyclohexylcyclohexene and bicyclohexyl show a sharp initial decrease with benzene concentration. Burr and Goodspeed have[5] recently discussed the formation of phenylcyclohexane and "polymers" from the radiolysis of benzene–cyclohexane mixtures and have indicated the importance of radical-scavenging mechanisms.

We now wish to report analogous observations for the methanol–benzene system. A number of radiolysis products have been separated and identified including

(1) J. H. Baxendale and F. W. Mellows, *J. Am. Chem. Soc.*, **83**, 4720 1961).

(2) W. G. Brown and M. K. Eberhardt, "Radiolysis of Liquid Methanol. Inhibitory Effects of Additives," ARL Report 90, contract No. AF 33(616)-3875, The University of Chicago (1961).

(3) J. P. Manion and M. Burton, *J. Phys. Chem.*, **58**, 421 (1954).

(4) T. Gaumann, *Helv. Chim. Acta*, **44**, 1337 (1961).

(5) J. G. Burr and F. C. Goodspeed, *J. Chem. Phys.*, **40**, 1433 (1964).

biphenyl, anisole, and what is possibly a benzyl alcohol derivative. Changes in concentration of these products with benzene concentration have been evaluated. This work is of current interest because of pulse radiolysis studies with aliphatic alcohols, particularly in the presence of aromatic additives.[6]

In a typical experiment solutions of methanol (purified by triple distillation from DNP–H_2SO_4) and benzene were carefully outgassed, sealed under high vacuum, and irradiated to a total dose of 1×10^7 rads with Co^{60} at 2×10^5 rads/hr. Analysis was carried out on a Perkin-Elmer Model 800 v.p.c. unit, using a column of 1.5% silicone gum on chromosorb, operated at 200 and 130°. Glycol analysis was carried out according to Jackson.[7]

A typical chromatogram of an irradiated solution contained five major peaks as well as six minor ones (relative intensity, major/minor = 10:1). The retention times for the major peaks and for some of the expected products measured under identical conditions are given in Table I.

TABLE I

V.P.C. RETENTION TIMES OF MAJOR PRODUCTS FROM IRRADIATED BENZENE–METHANOL MIXTURES

Peak no.	Retention time, min. 200°	130°
1	...	1.30
2	0.60	1.65
3	1.05	3.15
4	3.85	...
5	4.06	...
Anisole	0.60	1.65
Benzyl alcohol	1.05	3.25
Biphenyl	4.06	...

Peaks 1 and 4 have not been identified to date, but as 4 appears in the chromatogram of pure irradiated benzene, it is a radiolysis product of benzene, possibly a hydrogenated biphenyl.[4] Peaks 2 and 5 were identified as anisole and biphenyl, respectively.

Peak 3, although having similar retention times to benzyl alcohol both at 200 and 130°, could not be positively identified. However, the peak exhibits considerable "tailing," characteristic of hydroxy compounds, and, by analogy with cyclohexylcyclohexadiene found in cyclohexane–benzene mixtures,[8] may well be partially hydrogenated benzyl alcohol. Since the $\cdot CH_2OH$ radical has been observed in e.s.r. studies of irradiated methanol,[9,10] the absence of benzyl alcohol would be surprising. The anisole is undoubtedly formed by reactions of methoxy radicals with either benzene or with phenyl radicals. This would suggest that methoxy radicals are not a precursor to the yield of formaldehyde, since the formaldehyde yield is linear with benzene concentration.[1]

The effect of benzene on the peak area of the scavenging products (Fig. 1) shows that small concentrations of benzene result in a sharp increase in the concentration of scavenged products and that this is compensated by a sharp decrease in glycol yield. When the benzene concentration exceeds 10% by volume, the increase in concentration of the scavenging products is reduced and becomes linear with benzene concentration to approximately 90%.

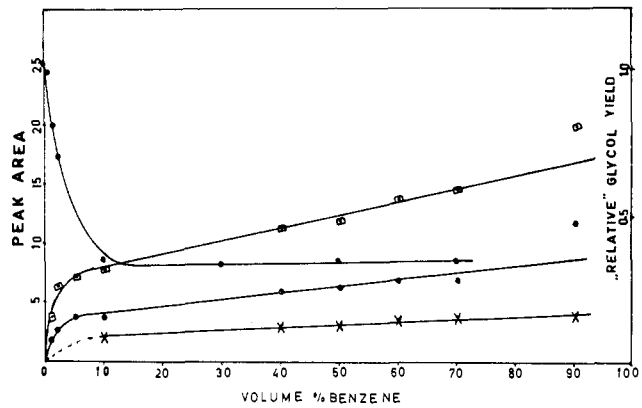

Fig. 1.—Yield of products from irradiated benzene–methanol mixtures: × biphenyl, corrected for dilution of benzene by methanol; ⊙ anisole, corrected for dilution of methanol by benzene; ⊡ peak 3 (see Table I), corrected for dilution of methanol by benzene; ● ethylene glycol, corrected for dilution of methanol by benzene, normalized to $G = 1$.

This behavior is similar to that of the ethylene glycol yield which becomes independent of additive concentration above 10%, thus indicating a "molecular" yield of glycol, possibly from hydrogen-bonded methanol dimers, or alternatively indicating that approximately 20% of the glycol precursors are not capable of being scavenged by benzene.

At low concentrations of benzene (<1%) it was found that most of the benzene decomposed during radiolysis. This result is consistent with the data obtained for dilute methanolic solutions ($<10^{-3}$ M) of polycyclic aromatic hydrocarbons (biphenyl, naphthalene, azulene, phenanthene, pyrene, and benz[*a*]anthracene), where it has been shown that these compounds decompose with high G-values, and that the decompositions is not linear with concentration, but corresponds to a decrease in glycol yield.[11] The latter results are important with respect to the observation of the biphenyl and naphthalene negative ions during pulse radiolysis of dilute solutions of the above additives in methanol.[6]

The interesting feature of the present results is the consistency with the benzene–cyclohexane system.[4,5] The data indicate that radical scavenging is a major contributing process in the radiolysis of methanolic solutions.

Acknowledgment.—The authors thank Mr. M. Withers for v.p.c. assistance, the Australian Institute of Nuclear Science and Engineering for the irradiations, the Australian Atomic Energy Commission for financial

(6) I. A. Taub, M. C. Sauer, and L. M. Dorfman, *Trans. Faraday Soc.*, in press.
(7) E. L. Jackson, *Org. Reactions*, **2**, 341 (1944).
(8) G. R. Freeman, *J. Chem. Phys.*, **33**, 71 (1960).
(9) B. Smaller and M. S. Matheson, *ibid.*, **28**, 1169 (1958).
(10) P. J. Sullivan and W. S. Koski, *J. Am. Chem. Soc.*, **85**, 384 (1963).
(11) A. Ekstrom and J. L. Garnett, unpublished results.

support (A. E.), and Mr. B. O'Leary for technical assistance with the irradiation.

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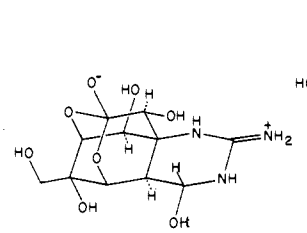
RECEIVED JULY 13, 1964

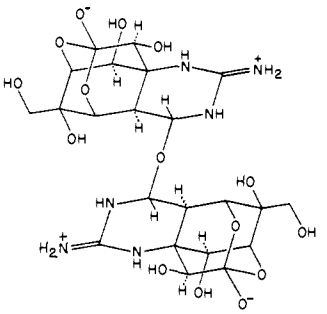
The Structure of Tetrodotoxin

Sir:

Recently we presented the evidence which led us to deduce the structure I for the *Spheroides* poison tetrodotoxin.[1,2] The very interesting alternative suggestion[3] that the toxin possesses the structure II, in which two units of the structure I are joined together by an



I



II

ethereal oxygen atom, deserves serious consideration. The problem of differentiating between the two structures is not accessible to study by the usual methods of solution chemistry, since tetrodotoxin is all but completely insoluble in all solvents except acids, and the latter might well effect cleavage of the ether link in II, whose lability would have to be accepted in view of the fact that all transformation products of established constitution are derived from the C_{11} structure I rather than a C_{22} molecule. We now wish to present evidence which permits an unequivocal decision in favor of the structure I for tetrodotoxin.

Tetrodotoxin is ordinarily crystallized by addition of ether and ethanol or methanol to solutions of the toxin in dilute aqueous acetic acid.[4] The aggregates of very small crystals prepared in this way are not suitable for fundamental crystallographic studies. However, when material thrice recrystallized by the standard procedure was dissolved in dilute aqueous acetic acid, the solvent removed *in vacuo*, the residue taken up in a minimum quantity of pure water, and the solution allowed to stand for three weeks, completely transparent, beautifully formed, individual parallelopipeds separated. This material was shown to be tetrodotoxin through the identity of its infrared spectrum (KBr) and Debye–Scherrer diagram with those of an authentic sample. Although the crystals were still very small (maximum extension $\leqslant$ 0.06 mm.), it was possible to mount several of them for single crystal X-ray diffraction studies, using Cu Kα radiation.

The crystals were found to be monoclinic through observation of C_{2h} diffraction symmetry in oscillation and precession photographs, and to belong to space group C_2^2-$P2_1$, since tetrodotoxin is optically active, and systematic extinctions were found only for $0k0$, k odd ($k \leqslant 13$), in Weissenberg photographs. Precession photographs taken of the $h0l$ and $h1l$ reciprocal lattice planes established the axial lengths a = 6.72 ± 0.04 Å., c = 6.49 ± 0.02 Å., and the interaxial angle β = 113°56′ ± 22′. The length of the unique axis, b = 14.66 ± 0.02 Å., was determined through observation of the $0k0$ reciprocal lattice line, after suitable reorientation of the same crystal which provided the above information. The standard deviations were ascertained through superimposing lead nitrate powder diagrams upon the precession photographs. Satisfactory confirmatory values were obtained for all three axial lengths from oscillation photographs taken on different crystals mounted along each of the crystallographic axes. The density of the crystals was found to be 1.792 ± 0.006 g./cm.^{3} by the flotation method; this is almost certainly a minimum value, since observations were made on aggregates of the parallelopipeds, rather than on the exceedingly small and very difficultly observable single crystals.

The volume of the unit cell of the tetrodotoxin crystal is therefore 585 ± 4 Å.^{3}, and the weight is 632 ± 6 on the atomic scale. Further, for a nonpolymeric substance, *the crystal symmetry requires the presence within the unit cell of an even number of complete, discrete, unconnected molecules.* These conditions are fully met by a cell containing two molecules of the structure I (2 × mol. wt. = 638), and cannot in any way be reconciled with the alternative ether structure II, which would require a minimum cell weight of 1240.

Acknowledgment.—We are pleased to acknowledge generous support by the National Institutes of Health and the Army Research Office/Durham, and the kindness of Professor William N. Lipscomb, who placed his counsel and equipment freely at our disposal.

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RECEIVED SEPTEMBER 17, 1964

(1) Lecture presented on April 14, 1964, in Kyoto at the 3rd International Symposium on the Chemistry of Natural Products; R. B. Woodward, *Pure Appl. Chem.*, **9**, 49 (1964). The structure there presented is actually the mirror image of I. Professor Nitta and his colleagues reported at the same symposium that extension of their X-ray crystallographic studies on bromoanhydrotetrodoic lactone hydrobromide [Y. Tomiie, A. Furusaki, K. Kasami, N. Yasuoka, K. Miyaki, M. Haisa, and I. Nitta, *Tetrahedron Letters*, **30**, 2101 (1963)] had enabled them to deduce the absolute configuration of that derivative, in the sense corresponding to I for tetrodotoxin itself.

(2) For independent studies leading to consideration of the same structure, *cf.* T. Goto, Y. Kishi, S. Takahashi, and Y. Hirata, *ibid.*, 779, 1831 (1964), and K. Tsuda, C. Tamura, R. Tachikawa, K. Sakai, O. Amakasu, M. Kawamura, and S. Ikuma, *Chem. Pharm. Bull.* (Tokyo), **12**, 634 (1964). It is also of much interest that tarichatoxin, from the California salamander *Taricha torosa*, has recently been shown to be identical with tetrodotoxin; H. S. Mosher, F. A. Fuhrman, H. D. Buchwald and H. G. Fischer, *Science*, **144**, 1100 (1964).

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

(4) K. Tsuda and M. Kawamura, *J. Pharm. Soc. Japan*, **72**, 771 (1952); *cf.* also H. Kakisawa, Y. Okumura, and Y. Hirata, *J. Chem. Soc. Japan*, **80**, 1483 (1959).

Concerning "Bond-Fixation" in Olefin–Iron Tricarbonyl Complexes

Sir:

The unusual nuclear magnetic resonance spectrum of cyclooctatetraene–iron tricarbonyl (**1**) has been reconciled with its structure, as determined by X-ray