(15) G. J. Durant, G. M. Smith, and R. G. W. Spickett, *ibid.*, **8**, 598(1965).
(16) G. Szilagyi, E. Kasztreiner, L. Vargha, and J. Borsy, *Acta Pharm. Hung.*, **39**, 66(1969).
(17) P. B. Dews, *Brit. J. Pharmacol. Chemother.*, **8**, 46(1953).
(18) E. A. Swinyard, W. C. Brown, and L. S. Goodman, *J. Pharmacol. Exp. Ther.*, **106**, 319(1952).
(19) V. G. Vernier, H. M. Hanson, and C. A. Stone, in "Psychosomatic Medicine," J. H. Nodine and J. H. Moyer, Eds., Lea & Febiger, Philadelphia, Pa., 1962, p. 683.

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* Present address: Department of Pharmacognosy-Pharmacology, School of Pharmacy, University of Illinois, Chicago, IL 60680

Structure and Activity in Molluscicides III: Enzymatic Peroxidation of the Molluscicidal Agent Pentachlorophenol

I. NABIH and J. METRI

Abstract □ Pentachlorophenol, a widely used molluscicidal and herbicidal agent, is biologically oxidized through the catalytic effect of the protoporphyrin enzyme peroxidase, detected within the snail's body, to give 2,2′,3,3′,5,5′,6,6′-octachlorobiphenylquinone. This compound showed potent molluscicidal activity. The reaction goes through the symmetrical pairing of the initially formed chlorophenoxy radical, accompanied by the rupture of the *para*- (C—Cl) link. The eliminated chlorine ion was determined in the reaction medium.

Keyphrases □ Pentachlorophenol—enzymatic peroxidation, structure–activity relationships of molluscicides □ Molluscicidal agents —pentachlorophenol enzymatic peroxidation, structure–activity relationships □ Structure–activity relationships—molluscicidal agents

A previous communication (1) explained the structure–activity relationship in molluscicides of the phenolic type. Biological findings reported by various investigators (2, 3) showed the existence of the structural specificity that governs the biological activity as molluscicides within these types of compounds. The activity depends on the nature of substituents on the aromatic ring. Those bearing bulky groups were of least activity, while those carrying smaller substituents were of significant activity. This specificity in structure with relation to the biological activity parallels the ability of the phenoxy radical, the intermediate stage in the chemical oxidation of phenols, to undergo subsequent transformation to the corresponding quinoid system, which is similarly dependent on the nature of the substituent on the ring. Compounds belonging to this system are biologically active as molluscicides.

Previous studies revealed the presence of the protoporphyrin enzyme, peroxidase, in the snail's body. The biochemical assay for this enzyme was determined in some species of snails of economic importance (4).

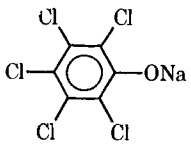
MECHANISM

The effect of the enzyme peroxidase on some types of phenols and amines has been studied. Of these, 2,6-dimethylphenol could be oxidized by this system to give 3,5,3′,5′-tetramethyldipheno-4,4′-quinone; 2,6-dimethoxyphenol could be similarly changed to the corresponding diphenoquinone: 3,5,3′,5′-tetramethoxy-4,4′-quinone (5). Oxidation may also proceed to give a mesomeric quinoid structure as 2-hydroxy-1,3,5-trimethylbenzene, with which the reaction gives 2,6-dimethyl-1,4-benzoquinone (6, 7). With polysubstituted alkylphenols such as 1-hydroxy-2,3,5,6-tetramethylbenzene, the corresponding 2,3,5,6-tetramethyl-1,4-benzoquinone was obtained. With *para*-halogenated amines, such as *p*-chloroaniline, elimination of the chlorine ion takes place, thus causing a drop in the pH of the reaction medium and resulting in the formation of 2-amino-5-(*p*-chloroanilino)-benzoquinone-di-*p*-chloranil (8). Other aromatic amines with halogens as *para*-substituents could be oxidized in a similar fashion to give quininoid systems with the displacement of the halogen atom at the *para*-position. In the case of fluorine, the reaction is toxified through the hydrofluoride which acts as an enzyme poison.

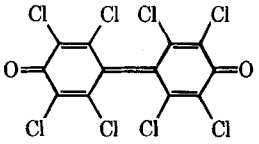
Mechanistically, it has been suggested that the oxidation through peroxidase involves free radical formation either by direct loss of a hydrogen atom or by an electron removal followed by a loss of a proton; this may be followed by the symmetrical pairing of two such radicals (9).

The phenoxy radical is a monovalent oxygen radical species, which can be formed through the homolysis of the O—H bond. The delocalization of the unpaired electron over the aromatic ring and its substituents was proved (10, 11); thus the radical is stabilized by resonance. For the radical to exist other than as a transient intermediate, it is necessary that the ring be substituted with bulky groups, which would give steric hindrance to block further decomposition of the radical by slow dimerization or disproportionation. The polyhalophenoxy radical usually undergoes dimer formation readily. The stability of these radicals is due to the mesomeric contribution of halogen on the ring. The pentachlorophenoxy radical has been studied (12, 13); carbon–carbon dimerization of the radical may proceed with the loss of the *para*-substituent. The formation of such dimers was reported for a large number of these phenols (14, 15).

In this work, the catalytic oxidation of the enzyme peroxidase and hydrogen peroxide on pentachlorophenol (I), a widely used molluscicide and herbicide, was investigated. It was submitted, as its sodium salt, to the action of the enzyme in a water–phosphate buffer solution at pH 7 and 37°. The hydrogen peroxide solution was added to the sodium pentachlorophenate in the phosphate buffer, followed by the addition of the enzyme peroxidase solution; the temperature of the mixture was kept constant. The isolated product was identified as 2,2′,3,3′,5,5′,6,6′-octachlorobiphenylquinone (II). IR spectral analysis showed an absorption band at 1610 cm.$^{-1}$ referring to the quinoidal structure (16). Oxidation of II



I



II

with lead dioxide in dilute sulfuric acid gave 2,3,5,6-tetrachlorobenzoquinone (chloranil). Oxidation of diphenoquinone by the same method usually gave the free *p*-benzoquinone (17).

An alternative synthesis for II was reported as a patent (18). This included the oxidation of 4,4′-dihydroxy-2,2′,3,3′,5,5′,6,6′-octachlorobiphenyl obtained by oxidation of decachlorobiphenyl in presence of excess alkali. Compound II was reported to be useful as an insecticide, fungicide, herbicide, fire-proofing compound, stabilizer for dielectric compounds, additive for oils, pigment for plastics and coating compounds, and dye intermediate (19).

In this biological oxidation, an initial formation of the polyhalophenoxy radical apparently took place, accompanied with the repulsion of the *p*-located chlorine followed by the dimerization of the radical. The liberated chlorine ion was quantitatively determined (20), and it corresponded to the calculated value.

EXPERIMENTAL[1]

Reagents—*Sodium Pentachlorophenate Solution* (7×10^{-3} M)—Five grams was dissolved by warming in 2500 ml. of 0.1 *M* phosphate buffer of pH 7.

Phosphate Buffer Solution (0.1 M pH 7)—Stock Solution A consisted of 0.2 *M* solution of monobasic sodium phosphate (55.6 g. of NaH_2PO_4) in 2000 ml. bidistilled water. Stock Solution B consisted of 0.2 *M* solution of dibasic sodium phosphate (107.3 g. of $Na_2HPO_4 \cdot 7H_2O$) in 2000 ml. bidistilled water. Then 487.5 ml. of Solution A and 762.5 ml. of Solution B were diluted to 2500 ml. with bidistilled water.

Hydrogen Peroxide Solution (3×10^{-2} M)—Hydrogen peroxide (30% w/v), 0.33 ml., was diluted to 100 ml. with bidistilled water.

Peroxidase[2] *Solution*—Thirty-five milligrams of the enzyme was dissolved in 50 ml. 0.05 *M* phosphate buffer.

Procedure—To 2500 ml. of sodium pentachlorophenate solution, 1 ml. of hydrogen peroxide solution was added in 15-min. intervals, followed by the addition of 1 ml. of the enzyme solution. Addition of both reagents to the phenate solution was continued until 50 ml. of each reagent was added. The temperature of the reaction mixture was kept at 37° during the addition period. A yellow precipitate was formed. This was left overnight at 37°, filtered, washed with bidistilled water several times, and dried. Upon recrystallization from dilute alcohol, the product melted at 154–156° and weighed 2.6 g. (65% yield).

Anal.—Calc. for $C_{12}Cl_8O_2$: C, 31.34; Cl, 61.69. Found: C, 30.78; Cl, 61.10.

Chlorine-Ion Determination in Reaction Medium (20)—The phosphate buffer was precipitated with concentrated nitric acid and ammonium molybdate solution and removed by filtration. To 100 ml. of the filtrate was slowly added 0.1 *N* silver nitrate solution during stirring. A slight excess of silver nitrate was added until no precipitate was formed. The mixture was heated to coagulate the precipitate; the solution was allowed to stand in the dark for 2 hr. and filtered in a weighed crucible. The precipitate was then washed with 0.02 *N* nitric acid solution until 3–5 ml. of the washing gave no turbidity with a drop of 0.1 *N* hydrochloric acid. It was dried at 130–150° for 1 hr. and weighed to give 0.044 g. of silver chloride.

The calculated amount of chlorine present in that portion equaled 0.011 g. The total amount of chlorine present in the whole filtrate and washings was:

$$\frac{0.011 \times 3250}{100} = 0.3575 \text{ g.} \qquad \text{(Eq. 1)}$$

The theoretical value of chlorine was 0.3630 g.

Oxidation of II to Produce 2,3,5,6-Tetrachlorobenzoquinone (Chloranil)—To a hot solution of II (0.5 g.) in 100 ml. (20%) sulfuric acid was added 2 g. lead dioxide in small portions. The mixture was refluxed for 2 hr. and filtered; then the precipitate was recrystallized from alcohol, m.p. 280° dec. (no depression in mixed melting point with 2,3,5,6-tetrachlorobenzoquinone). UV for both compounds in *n*-hexane showed identical maxima absorption at 286 nm.

Biological Screening—Five snails were placed with 200 ml. of the solution. Compound II was dissolved in the smallest possible amount of alcohol and diluted with bidistilled water to form the solution. All the snails died after the exposure period of 24 hr. (21).

[1] Melting points were uncorrected and were taken in open capillary tubes by the use of a Gallenkamp electric melting-point apparatus. UV spectra were determined in *n*-hexane and a Unicam spectrophotometer, model 500. The IR spectra were recorded with a Carl Zeiss Infra-cord spectrophotometer, model "UR 10." Microanalyses were performed by the microanalytical laboratory, National Research Centre, Cairo, U.A.R., and the Spang Microanalytical Laboratory, Ann Arbor, Mich.

[2] Horse-radish peroxidase type II, provided by Sigma Chemical Co., St. Louis, Mo., approximately 110 purpurogallin units/mg.

REFERENCES

(1) I. Nabih and M. El-Wassimi, *J. Pharm. Sci.*, **57**, 1202(1968).

(2) M. O. Nolan and E. R. Mann, *Amer. J. Trop. Med. Hyg.*, **2**, 716(1953).

(3) E. J. Batte and L. E. Swanson, *J. Parasitol.*, **38**, 65(1952).

(4) I. Nabih, M. El-Wassimi, and J. Metri, *J. Pharm. Sci.*, **57**, 1609(1968).

(5) B. Stark, Ph.D. dissertation, University of Cambridge, Cambridge, England, 1958.

(6) H. Booth and B. C. Saunders, *J. Chem. Soc.*, **1954**, 940.

(7) H. Booth and B. C. Saunders, *Nature*, **165**, 567(1950).

(8) D. G. H. Daniels and B. C. Saunders, *J. Chem. Soc.*, **1953**, 822.

(9) C. S. Bernard, P. Jocelyn, and J. Mann, *ibid.*, **1940**, 769.

(10) J. K. Becconsall, S. Clough, and G. Scott, *Trans. Faraday Soc.*, **56**, 459(1960).

(11) E. Muller, H. Eggensperger, A. Rieker, K. Scheffler, H. D. Spanagel, H. B. Stegmann, and B. Teissier, *Tetrahedron*, **21**, 227 (1965).

(12) E. Muller, A. Ricker, and W. Beckert, *Z. Naturforsch.*, **176**, 567(1962).

(13) R. Read, *J. Amer. Chem. Soc.*, **80**, 219(1958).

(14) R. Barton, A. M. Deflorin, and O. E. Edwards, *J. Chem. Soc.*, **1956**, 530.

(15) H. S. Blanchard, *J. Org. Chem.*, **25**, 264(1960).

(16) L. J. Bellamy "Infra-red Spectra of Complex Molecules," 2nd ed., Wiley, New York, N. Y., 1964, p. 152.

(17) R. Willstätter and K. Ludwig, *Chem. Ber.*, **38**, 1235(1905).

(18) D. Franklin and O. Smith, U. S. pat. 2,449,088 (Sept. 14, 1948).

(19) C. Dermer, *Chem. Abstr.*, **43**, 813(1949).

(20) A. Vogel, "Quantitative Inorganic Analysis," Longmans Green and Co., London, England, 1951, p. 399.

(21) M. O. Nolan, W. Howard, and R. Elizabeth, *Amer. J. Trop. Med. Hyg.*, **2**, 716(1953).

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