Tetrahedron. 1967, Vol. 23, pp. 1867 to 1872. Pergamon Press Ltd. Printed in Northern Ireland

STUDIES IN PEROXIDASE ACTION—XVII*

SOME GENERAL OBSERVATIONS

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(*Received* 15 *July* 1966; *accepted for publication* 16 *August* 1966)

Abstract—The peroxidase system oxidizes 2,6-dimethylphenol and 2,6-dimethoxyphenol smoothly to the corresponding tetrasubstituted diphenoquinones; by contrast 2,6-dichlorophenol and 2,4,6-trichlorophenol give intractable products believed to result from the hydrolysis of intermediate chloroquinones.

N,N,2- and N,N,4-trimethylanilines are readily oxidized by the peroxidase system whereas N,N,2,6-tetramethylaniline and N,N,2,4,6-pentamethylaniline are not oxidized. Furthermore *p*-aminobenzonitrile, ethyl *p*-aminobenzoate and *p*-aminoacetophenone are not perceptibly oxidized by hydrogen peroxide and peroxidase. The significance of these negative reactions is considered.

IN continuation of our studies of oxidations by peroxidase systems,[1] we have now examined the enzymic oxidation of three 2,6-disubstituted phenols (the dimethyl-, dimethoxy- and dichloro-compounds) and two further 2,4,6-trisubstituted phenols (trichlorophenol and 2,6-dimethyl-4-iodophenol).

The oxidation of 2,6-dimethylphenol by non-enzymic agents has been studied by many workers. The most common product is 3,5,3′,5′-tetramethyldipheno-4,4′-quinone (I; R = Me); obtained by use of copper sulphate plus $Na_2S_2O_8$ or NaOCl or air,[2] or of dibenzoyl peroxide,[3,4] lead tetra-acetate in benzene or acetic acid,[5] sodium persulphate and silver nitrate,[6] alkaline ferricyanide,[2,7-9] nitric acid,[8] chromic acid,[8] ferric salts,[2,8] potassium dichromate[8] or permanganate,[8] ammonium persulphate,[8] chlorine water,[8] bromine water,[8] silver oxide,[10] argentic picolinate,[11] cupric benzoate,[12] sodium periodate, iodate, bromate, chlorite or hypochlorite,[2] copper periodate,[2] manganese dioxide,[2] and amyl nitrite.[2] The same diphenoquinone (I; R = Me) can also be formed from 2,6-dimethylphenol and hydrogen peroxide in the presence of

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* Part XVI *Tetrahedron* **23**, 1335 (1967).

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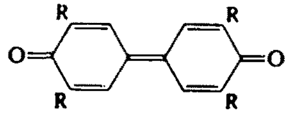
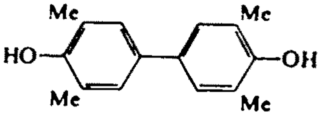
certain salts ($FeSO_4$,[2,13] CuCl, $CuSO_4$, $TiCl_3$, $TiCl_4$, or OsO_4).[2] Such systems can be regarded as "model peroxidases".[1] Recently, enzymes from *Polyporus versicolor* and *Rhus vernicifera* have been shown to catalyse the oxidation of 2,6-dimethylphenol to the diphenoquinone.[14]

The related 4,4′-dihydroxy-3,5,3′,5′-tetramethylbiphenyl (II) can be formed (alone or together with the substituted diphenoquinone) by oxidizing 2,6-dimethylphenol with various agents, e.g. $AgNO_3$ plus $K_2S_2O_8$,[2,6] $CuSO_4$ plus $Na_2S_2O_8$,[2] silver oxide,[10] lead tetra-acetate in benzene,[5] benzoyl peroxide,[3] oxygen in cumene plus ferric stearate,[15] t-butyl peroxide,[16] amyl nitrite,[2] sodium bromate, chlorate, chlorite, or hypochlorite,[2] or hydrogen peroxide in the presence of $FeSO_4$,[13] $TiCl_3$,[2] uranyl nitrate[2] or selenium dioxide.[2] Irradiation of 2,6-dimethylphenol solutions in the presence of oxygen also yields the diphenol (II).[17]



I



II

Attention has recently been paid to the linear polymer "polyphenylene oxide" (PPO) (III; R = Me), formed by oxidation of 2,6-dimethylphenol with oxygen in the presence of copper–amine complex catalysts,[18,19] magnesium–amine complexes,[20] or activated manganese dioxide.[21] In some cases, the diphenoquinone (I; R = Me) is an alternative product, even with these special oxidizing systems.[19,21]

A few cases are known, in which other oxidation products are formed from

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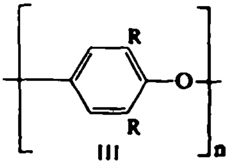
[21] E. J. McNelis, *J. Org. Chem.* **31**, 1255 (1966); Sun Oil Co., U.S. Pat. 3,220,979/1965.

2,6-dimethylphenol, usually in low yields. These products have been summarized recently.[2]



III

The product most often obtained by oxidation of 2,6-dimethoxyphenol is also a diphenoquinone (I; R = OMe). This results from the action of t-butyl perbenzoate,[4] potassium ferricyanide,[7] sodium periodate,[22] lead tetra-acetate,[23] potassium dichromate,[24] chlorine,[24] nitric acid,[24] ferric chloride,[24] sodium nitrite,[25] oxygen in the presence of copper–amine complex catalysts,[26] or certain oxidases.[14] Some 2,6-dimethoxy-*p*-benzoquinone is given by lead tetra-acetate[23] and sodium periodate,[22] (which also forms 3-methoxy-*o*-benzoquinone and 3,8-dimethoxy-1,2-naphthoquinone[22,27]).

We now find that both 2,6-dimethylphenol and 2,6-dimethoxyphenol are oxidized smoothly by peroxidase systems to give the diphenoquinones (I; R = Me or R = OMe). By contrast, we were unable to isolate tetrachlorodiphenoquinone (I; R = Cl) by peroxidase-catalysed oxidation of 2,6-dichlorophenol. This phenol gave a purple product, and chloride ion was liberated. Relatively little work has been carried out on the oxidation of this phenol. Inoue *et al.*[28] isolated 4,4′-dihydroxy-3,5,3′,5′-tetrachlorobiphenyl in low yield when benzoyl peroxide in carbon tetrachloride was used as oxidant, whereas a relatively low-molecular polymer (III; R = Cl) was obtained[29] by the action of oxygen and a copper–pyridine catalyst.

The peroxidase-catalysed oxidation of 2,4,6-trichlorophenol was also investigated. We found that addition of peroxidase and hydrogen peroxide to an aqueous solution of this phenol produced a transient yellow colour, then a persistent red-violet colour, and liberation of chloride ion. The coloured oxidation product was almost completely soluble in water, giving an acidic solution. So far we have been unable to isolate any identifiable compound from this material. It was observed, however, that when 2,6-dichlorobenzoquinone was left for some days in aqueous suspension, a red-violet colour slowly developed similar to that obtained by enzymic oxidation of trichlorophenol. When the two reaction solutions were subjected to circular paper chromatography, they gave similar patterns.

2,6-Dichlorobenzoquinone results from the oxidation of trichlorophenol with lead dioxide[28,30] and certain other inorganic oxidizing agents,[31] whereas various trichlorophenoxy-substituted benzoquinones are formed by the action of benzoyl peroxide,[28] lead dioxide,[30,32] and chromic acid.[32] Polyethers are formed by oxidation with silver

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[31] E. H. Huntress, *Organic Chlorine Compounds* p. 351. Chapman and Hall, London (1948).

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oxide,[30] iodine,[30] lead dioxide,[30] or oxygen in the presence of pyridine plus nitrobenzene.[33]

We have found that 2,6-dimethyl-4-iodophenol is also oxidized by peroxidase and hydrogen peroxide. Iodine was liberated,[34] but after the reaction mixture had been allowed to stand for some time, no free iodine could be detected. If, however, the oxidation was interrupted at an early stage, a small amount of 2,6-dimethylbenzoquinone could be isolated. These observations present no anomaly, as it has been shown[35] that 2,6-dimethylbenzoquinone will give a positive iodoform reaction. Hence the situation is probably that peroxidase liberates iodide from 2,6-dimethyl-4-iodophenol and that the halide is then oxidized to iodine which is subsequently used up in reaction with the dimethylbenzoquinone. Bromide ion was liberated by the action of the peroxidase system on 4-bromo-2,6-dimethylphenol. Various workers have shown that oxidation of 4-bromo- and 4-chloro-2,6-dimethylphenols with inorganic oxidizing agents can lead to halide ion and linear polymer (III; $R = \text{Me}$);[9,36] in addition, a small quantity of 2,6-dimethylbenzoquinone is formed with alkaline ferricyanide or lead dioxide.[9]

The formation of diphenoquinones I ($R = \text{Me}$ or $R = \text{OMe}$) from the 2,6-dimethyl- and 2,6-dimethoxyphenols can reasonably be ascribed to *para-para* C—C coupling of aroxyl radicals,[1,37] followed by oxidation of the resulting 4,4′-dihydroxybiphenyl derivatives. The liberation of halide from 2,6-dimethyl-4-halophenols could perhaps be formulated as involving attack of a HO radical at the *para*-position of the aroxyl radical first formed, with subsequent elimination of hydrogen halide (oxidized then to the halogen, in the case of the iodo-compound). Attack by the HO radical may also be involved in the formation of 3,5-dimethyl-4-hydroxybenzyl alcohol by peroxidase-catalysed oxidation of mesitol.[38] However, as Saunders and Patchett have been unable to detect *free* HO radicals in the peroxidase system[39] it would seem likely that HO radicals (if formed at all) are passed to the hydrogen donor simultaneously with removal of a hydrogen atom. It is of interest that recently it was shown[40] that the action of iodine plus hydrogen peroxide on certain methylphenols (in which the position *para* to the OH group is unsubstituted) forms the corresponding 2,4-di-iodomethylphenols. The iodine atom introduced at the 4-position is then eliminated, to yield 2-iodo-methylbenzoquinones as final products.

By analogy with the oxidations described above, primary products from 2,6-dichloro- and 2,4,6-trichlorophenols should be, respectively, 3,5,3′,5′-tetrachlorodipheno-4,4′-quinone and 2,6-dichlorobenzoquinone (together with chloride ion). The formation of halide from the dihalophenol and our inability to isolate these two haloquinonoid compounds can be attributed to the instability of the chloroquinones (which are vinylogues of acyl chlorides) under the particular reaction conditions (very dilute aqueous solution, long reaction times). This instability was

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[34] B. C. Saunders and B. P. Stark, *Tetrahedron* **4**, 169 (1958).

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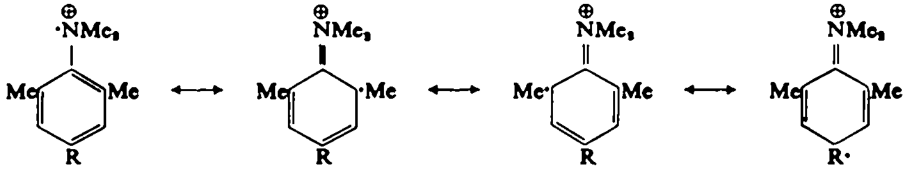
demonstrated with a sample of dichlorobenzoquinone prepared from trichlorophenol by non-enzymic oxidation. The similar lability of a chlorine atom in a related compound, chloranil, has been discussed recently.[41]

The oxidation of two amines (N,N,2,6-tetramethylaniline and *p*-aminobenzonitrile) by the peroxidase system was also attempted. It is known that N,N-dimethylaniline is readily oxidized (by *para-para* C—C coupling of radicals arising by one-electron oxidations) to N,N,N',N'-tetramethylbenzidine.[42] (The latter is then further oxidized[42].) Similar coupling occurs on oxidation of dimethylaniline with dibenzoyl peroxide.[43] Naylor[44] has observed that N,N,2- and N,N,4-trimethylanilines are readily oxidized by the peroxidase system, but that N,N,2,4,6-pentamethylaniline is not. We have now found that N,N,2,6-tetramethylaniline is scarcely oxidized by hydrogen peroxide in the presence of peroxidase. The reluctance of N,N,2,6-tetramethylaniline and N,N,2,4,6-pentamethylaniline to undergo oxidation by the peroxidase system (and by dibenzoyl peroxide[45]) can be attributed to steric strain in the radicals IV (R = H or R = Me); the dimethylamino group in this tetramethylaniline is known to be perpendicular to the ring.[46]

Some aromatic amines which have powerful electron-withdrawing groups (e.g. $—NO_2$, —COOEt, —COMe) in the position *para* to the $—NH_2$ group are known to be unaffected by the peroxidase system.[47] By contrast, aniline and those of its derivatives which have *para*-methyl or methoxy or halide substituent groups are oxidized very easily;[1] the *p*-haloanilines liberate halide ion on oxidation.[1] In the present work, *p*-aminobenzonitrile was recovered unchanged after treatment with peroxidase and hydrogen peroxide. Cyanide ion (which would have "poisoned" the enzyme system) was not liberated, since it was found that addition of mesidine to the reaction mixture immediately produced the characteristic purple 2,6-dimethylbenzoquinone-4-(2',4',6'-trimethyl)anil.[1] Chemical tests for cyanide also gave negative results.



IV

EXPERIMENTAL

Peroxidase. Two preparations of the enzyme were used: one was a concentrated soln (Purpurogallin Number ca. 600) of horse-radish peroxidase, which was diluted with water before use. The other was a less active sample (P.N. ca. 10) of turnip peroxidase.[48]

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Oxidation of 2,6-dimethylphenol. Diluted horse-radish peroxidase (P.N. 10; 27 ml) and H_2O_2 (20-vol; 15 ml) were added intermittently in 1 ml portions during 3 days to a suspension of the phenol (2·0 g) in acetate buffer soln (1000 ml; 0·1M; pH 4·7). The mixture first developed a yellow colour, which was soon succeeded by the precipitation of an orange-coloured solid. The mixture was left for 1 day, and then filtered. The dried ppt (0·85 g) was extracted exhaustively with dry ether, and the undissolved material recrystallized from AcOH; 3,5,3′,5′-tetramethyldipheno-4,4′-quinone (0·47 g, m.p. 205°) was obtained. (Found: C, 79·8; H, 6·4. Calc. for $C_{16}H_{16}O_2$: C, 80·0; H, 6·7%.)

Oxidation of 2,6-dimethoxyphenol. Horse-radish peroxidase (P.N. 10; 27 ml) and H_2O_2 (20-vol; 12 ml.) were added during 2 days to the phenol (1·54 g) in acetate buffer soln (300 ml; 0·1M; pH 4·7). After a further 12 hr the mixture was filtered, and the dried ppt (1·32 g) recrystallized thrice from nitrobenzene to give deep blue needles of 3,5,3′,5′-tetramethoxydipheno-4,4′-quinone (1·15 g, m.p. 293°). (Found: C, 62·8; H, 5·3. Calc. for $C_{16}H_{16}O_6$: C, 63·1; H, 5·3%.)

Oxidation of 2,6-dichlorophenol. Turnip peroxidase (20ml) and H_2O_2 (20-vol; 10 ml) were added during 3 days to 2,6-dichlorophenol (2·5 g) in water (250 ml). The yellow colour first observed was soon succeeded by an orange-red ppt. The mixture was left for 2 days, during which time both solid and soln became purple. The soln now contained chloride ion, and its pH was appreciably lower than at the start of the oxidation.

Oxidation of 2,4,6-trichlorophenol. Turnip peroxidase (49 ml) and H_2O_2 (20-vol; 24 ml) were added during 3 days to a suspension of trichlorophenol (3·93 g) in phosphate-citrate buffer (1000 ml; pH 5·0). At the start of the oxidation, a transient yellow colour was observed; the mixture soon became red-violet. At the end of the reaction time, the mixture was filtered. There resulted 0·17 g of a deep red solid—the colour of this material was discharged by prolonged boiling with Sn and HCl, but soon reappeared when the resulting soln was exposed to air. The deep violet-coloured filtrate from the oxidation mixture was extracted exhaustively for 2 days with ether (125 ml) and the ether evaporated to give a dark red oil which contained some trichlorophenol (volatile in steam). Horizontal chromatograms of this oil on circular Whatman No. 1 filter paper, with a butanol–AcOH–water (5:2:3 v/v) mixture, gave patterns whose most obvious feature was a violet band of *Rf* 0·68. When 2,6-dichlorobenzoquinone was left in suspension in water, a violet material having similar chromatographic behaviour was obtained.

Oxidation of 2,6-dimethyl-4-iodophenol. When horse-radish peroxidase and H_2O_2 were added to a soln of the phenol in water, the soln had a greenish tinge during the earlier stages of the oxidation, and contained free I; no I could be detected after the mixture had been left for some days however. Horse-radish peroxidase (0·5 ml; P.N. 500) and H_2O_2 (5 ml; 20-vol) were added during 2 hr to the phenol (1·42 g) in water (1000 ml). The soln was then extracted with ether (5 × 50 ml), and the ether layers washed with aqueous alkali to remove unchanged phenol, then with water. Evaporation and vacuum sublimation gave 2,6-dimethylbenzoquinone (51 mg; m.p. 72–73°).

Peroxidase and N,N,2,6-*tetramethylaniline.* Turnip peroxidase (15 ml) and H_2O_2 (20-vol; 10 ml) were added during 2 days to the amine (2·98 g) in an acetate buffer soln (3000 ml; 0·1M; pH 4·7). The resulting pale yellow soln was rendered alkaline with 10% NaOHaq, and was then extracted exhaustively with ether (250 ml). The ethereal soln was dried (KOH); removal of solvent gave 2·60 g of unchanged amine.

Peroxidase and p-*aminobenzonitrile.* Turnip peroxidase (9 ml) and H_2O_2 (20 vol; 2 ml) were added during 20 hr to the amine (0·234 g) in acetate buffer (400 ml; 1·0M; pH 4·7). Samples of the mixture were withdrawn at intervals, and a drop of mesidine added; in each case an immediate purple coloration resulted. The main bulk of the mixture was then extracted thoroughly with ether, and the ethereal portions combined and evaporated, to give the unchanged amine (0·213 g after recrystallization from light petroleum).

While this work was being carried out, one of us (B. P. S.) received a grant from the Department of Scientific and Industrial Research.