STUDIES IN PEROXIDASE
ACTION—XXIII[a]

THE OXIDATION OF POLY-METHYLATED ANILINES

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Abstract—2,4,5-Trimethylaniline, 3,4,5-trimethylaniline, 2,3,5,6-tetramethylaniline and 2,3,4,5,6-pentamethylaniline have been oxidised by the peroxidase system and the reactions have been studied in detail. The mechanism of peroxidase action is discussed in the light of these findings.

The oxidations of certain di- and tri-methylated[1,2] anilines by the peroxidase system have already been studied and mechanisms have been suggested for the action of the enzyme on these compounds.[1,3] It is important however that further carefully chosen key compounds be studied in order to verify the proposed mechanisms.

There is strong evidence that the oxidation of phenols by peroxidase and hydrogen peroxide[4] proceeds through a free radical intermediate. It is probable, however, that the oxidation of aromatic amines proceeds mainly by an alternative route. Firstly, the nature of the products obtained by the peroxidation of aromatic amines, and in particular the proportions of azo- compounds formed, are not characteristic of free radical intermediates. Secondly, since nitrogen is less electronegative than oxygen, amines are more easily oxidised than phenols. Therefore, it is to be expected that amines will utilise the two oxidising equivalents of the initial peroxidase/hydrogen peroxide complex (Compound I).[5] The action of the peroxidase system on aromatic amines may be summarised (Fig 1).

$$\text{Ar—NH}_2 \xrightarrow{-e\ -\text{H}^+} \text{Ar—NH}_2 \xrightarrow{-e} \text{ArNH}^+$$
$$\downarrow \qquad\qquad\qquad\qquad \downarrow$$
$$\text{Products} \qquad\qquad \text{Products}$$
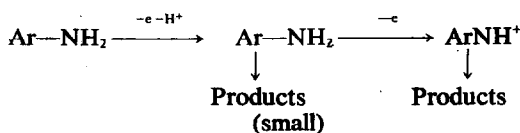$$\text{(small)}$$

Fig 1

We have now found that the oxidation of 3,4,5-trimethylaniline by peroxidase gives the azo-compound (1; 6% yield). No other compound could be isolated from the oxidation product which is

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very tarry and probably polymeric. In contrast, 2,4,5-trimethylaniline gives the corresponding azo-compound (2) in 25% yield, i.e. higher than from any amine yet studied. No electronic or steric factors within the amine will account for this unexpected result and it must be assumed that the complex between the amine and compound I is unusually well-suited sterically for nitrogen to nitrogen coupling between two 2,4,5-trimethylanilino radicals. Three further compounds from 2,4,5-trimethylaniline have been isolated and identified. The formation of the mono-anil (3) and the di-anil (4) can be explained on the basis of mechanisms suggested by previous workers.[1,3] Phenazines are rarely isolated from peroxidase oxidations[6] since further oxidation to polymeric compounds usually readily takes place. This particular phenazine (5) is however sterically hindered and therefore further oxidation will take place only very slowly. The compound is produced in an analogous manner to the phenazine isolated from the peroxidase oxidation of 2-naphthylamine.[6]

The oxidation of 2,3,5,6-tetramethylaniline by peroxidase gives three compounds, the anil (6), the mono-imine (7) and the bis-imine (8). The anil is the expected oxidation product from the peroxidase oxidation of this amine, but steric hindrance at the 4-position prevents its formation in higher yield and the formation of the more unusual type of product is favoured. The two compounds (7 and 8) are formed in approximately equal quantities and the following mechanism is proposed.

Enzymic oxidation of the amine to the radical (9), a mesomer of which undergoes 4-carbon to 4-carbon coupling, followed by rearomatisation gives octamethylbenzidine (9). This is a similar mechanism to that put forward for the oxidation of N, N-dimethylaniline.[7] Benzidines are usually very rapidly oxidised by the peroxidase system. However, in the case of octamethylbenzidine, steric in-

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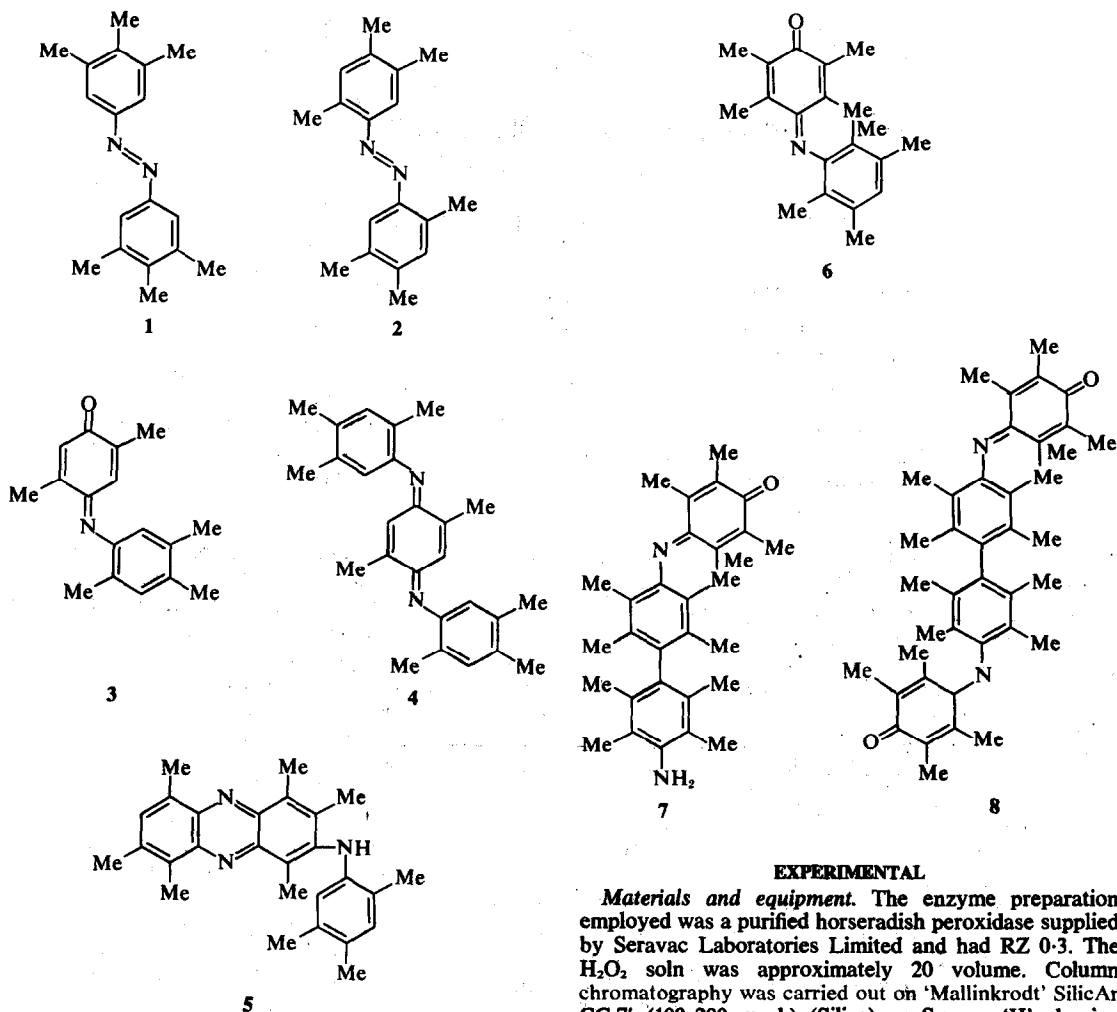
teraction between the Me groups on the adjacent benzene rings prevents planarity, and no reaction takes place when octamethylbenzidine alone is treated with peroxidase and hydrogen peroxide. However, in the presence of the cation (11), oc-tamethylbenzidine acts as a nucleophile with resultant formation of the monoimine (7). Attack by the second amino group on a second cation may also take place with formation of the bis-imine (8) (Fig 2). 4-Cation to 4-carbon radical coupling is favoured not only by the hindered nature of the duridine amino group, but also by the lack of a substituent at the 4-position on the nucleus.

Oxidation by the peroxidase system of 2,3,4,5,6-pentamethylaniline gives a single compound in high yield, namely the anil (12). The mechanism is analogous to that put forward for the peroxidase oxidation of mesidine.[3]

This study on the peroxidation of polymethylated anilines gives adequate support to our current theories on the mechanism of peroxidase action.

EXPERIMENTAL

Materials and equipment. The enzyme preparation employed was a purified horseradish peroxidase supplied by Seravac Laboratories Limited and had RZ 0·3. The H_2O_2 soln was approximately 20 volume. Column chromatography was carried out on 'Mallinkrodt' SilicAr CC-7' (100-200 mesh) (Silica) or Spence 'H' alumina (100-200 mesh) activated by heating.

Preparation of the amines. 3,4,5-Trimethylaniline was prepared by the method of Beringer and Ugelow.[8] 2,3,5,6-Tetramethylaniline was prepared by bromination of the parent hydrocarbon[9] followed by nitration,[10] and finally reduction of bromonitrotetramethylbenzene gave the amine.[11] 2,3,4,5,6-Pentamethylaniline was prepared by the method of Birtles and Hampson.[11] The method of Dimroth *et al.*[12] gave a lower yield and that of Dyson and Hunter gave no yield at all.[13]

The oxidation of 3,4,5-trimethylaniline. The oxidation of 3,4,5-trimethylaniline (2·1 g) was carried out under similar conditions to those for mesidine.[2] A yellow colour rapidly appeared, changing to brown, and finally giving a purple ppt. The solid was filtered off and dried (1·93 g) and subjected to column chromatography on alumina using 25% benzene in light petroleum (b.p. 40–60°) as eluent. A single yellow solid was eluted which, on evaporation and recrystallisation (EtOH), gave yellow prisms of 3,4,5,3',4',5'-hexamethylazobenzene (118 mg, 6% of the total oxidation product), m.p. 202°. (Found: C, 81·2; H, 8·2; N, 10·4. $C_{18}H_{22}N_2$ requires: C, 81·2; H, 8·3; N, 10·5%). NMR spectrum (CCl_4) showed singlet (6 Me protons) at 7·80τ; singlet (12 Me protons) at 7·68τ and singlet (4
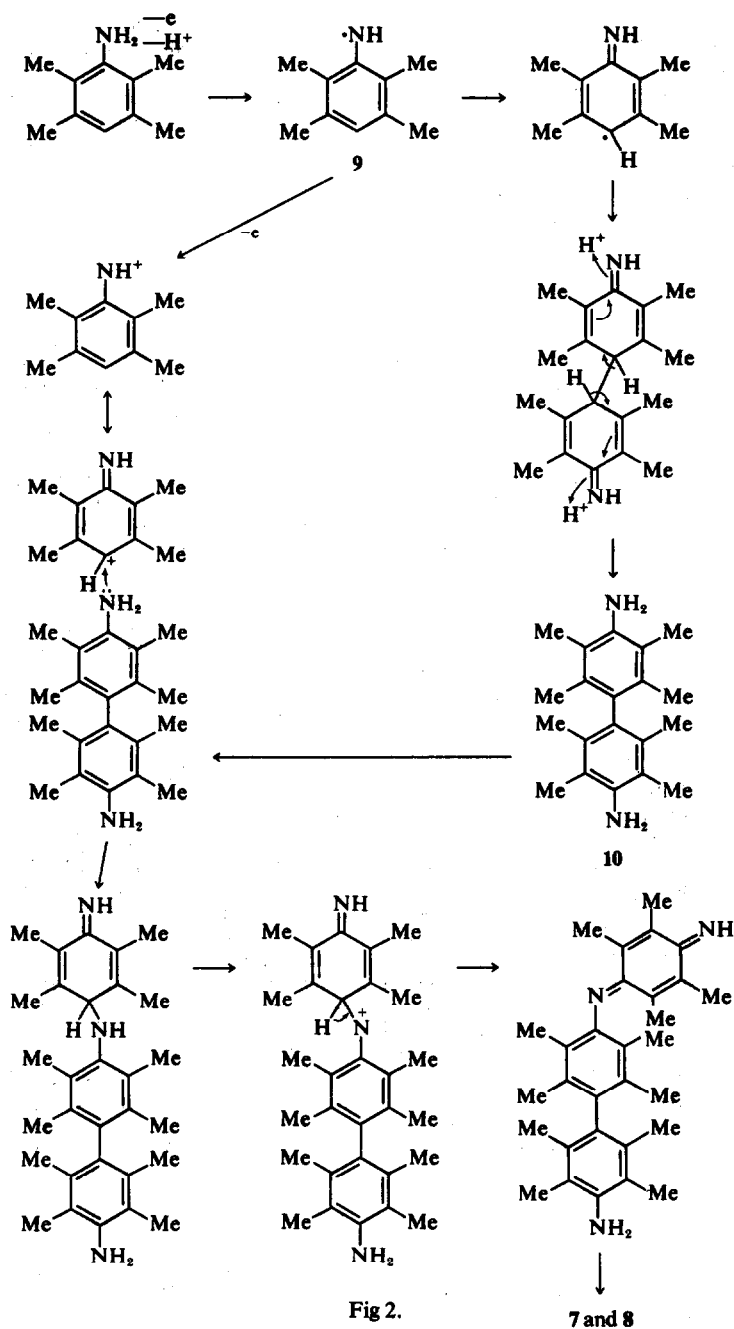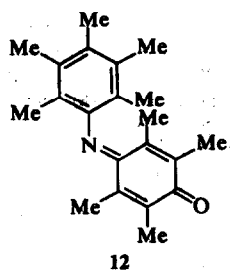
Fig 2.

aromatic protons) at $2 \cdot 53\tau$. IR spectrum (KBr disc) showed prominent bands at 2980, 2930, 2870, 1600, 1590, 1480, 1320, 1295, 1130, 895, 720 cm^{-1}. Mass spectrum: m/e 267, (% of base peak 6); 266, (29); 223, (5); 208, (4); 194, (5); 147, (7); 120, (24); 119, (100); 118, (4); 117, (8); 116, (2); 115, (7). M.W. = 266. UV spectrum (95% EtOH): λ_{max} 242 mμ, (log$_{10}\epsilon$ 4·164); 345 mμ, (4·388); 440 mμ, (3·169); λ_{min} 229 mμ, (4·079); 272 mμ, (3·518); 405 mμ, (3·057).

Further elution of the column with highly polar solvents gave dark coloured tarry products. No trace of quinone anils could be found.

The oxidation of 2,3,5-trimethylaniline. Similar conditions were employed for the oxidation of 2,3,5-trimethylaniline (2·2 g). At the conclusion of the reaction, a red solid was filtered off from the soln and dried (2·0 g). The solid was dissolved in light petroleum, b.p. 40–60° (15 ml) and benzene (5 ml) and chromatographed on alumina in the same mixture of solvents. After development, three bands were observed:
(a) fast running yellow; (b) slow running red; (c) dark brown which remained at the top of the column.

The eluate from band (a) gave yellow needles (490 mg, 25% of the total oxidation product) after evaporation and recrystallisation (ethanol). These were 2,4,5,2',4',5'-hexamethylazobenzene, m.p. 173° (lit. 173–174°). (Found: C, 81·2; H, 8·2; N, 10·7. Calc. for C$_{18}$H$_{22}$N$_2$: C, 81·2; H, 8·3; N, 10·5%). NMR spectrum (CCl$_4$) showed singlet (12 Me protons) at $7 \cdot 71\tau$; singlet (6 Me protons) at $7 \cdot 33\tau$; singlet (2 aromatic protons) at $3 \cdot 00\tau$ and singlet (2 aromatic protons) at $2 \cdot 65\tau$. UV spectrum (ethanol): λ_{max} 242 mμ (log$_{10}\epsilon$ 4·008); 356 mμ, (4·258); 450 mμ (3·029); λ_{min} 228 mμ (3·882); 270 mμ, (3·417); 415 mμ, (2·895).

The eluate from band (b) was subjected to further chromatographic analysis on silica, using the same eluent. Two red compounds were eluted and evaporated to give red solids. Recrystallisation (n-hexane) of the faster running compound gave red crystals of 2,5 - dimethyl - p - benzoquinonebis - (2',3',5' - trimethyl)anil (402 mg, 20% of the of the oxidation product), m.p. 199·5°. (Found: C, 84·5; H, 8·0; N, 7·3. C$_{26}$H$_{30}$N$_2$ requires: C, 84·3; H, 8·1; N, 7·5%). NMR spectrum (CCl$_4$) showed singlet (6 Me protons) at $8 \cdot 00\tau$; doublet (6 Me protons) at $7 \cdot 90\tau$ (J = 1 c/s); singlet (12 Me protons) at $7 \cdot 80\tau$: singlet (2 aromatic protons) at $3 \cdot 70\tau$; doublet (2 aromatic protons) at $3 \cdot 50\tau$ (J = 1 c/s) and singlet (2 aromatic protons) at $3 \cdot 10\tau$. IR spectrum (nujol mull) showed prominent bands at 1620, 1590, 1275, 1200, 1165, 1050, 1005, 910, 895, 885 cm^{-1}. Mass spectrum: m/e 373, (% of base peak 23); 372, (82); 371, (31); 370 (100); 369, (22); 356, (22); 355, (60); 341, (21); 340, (65); 339, (37); 247, (5); 237, (16); 236, (26); 224, (10); 222, (10); 186, (10); 184, (14); 177, (27); 169, (25). M.W. = 370. UV spectrum (EtOH): λ_{max} 302 mμ (log$_{10}$ ϵ 4·542); 474 mμ, (3·782); λ_{min} 241 mμ, (3·782); 379 mμ, (3·199).

The second red compound was recrystallised (EtOH/water) to give red plates of 2,5-dimethylbenzoquinone-4-(2',4',5'-trimethyl) anil (42 mg, 2%), m.p. 105°. (Found C, 80·7; H, 7·7; N, 5·3. C$_{17}$H$_{19}$NO requires: C, 80·6; H, 7·6; N, 5·4%). IR spectrum (nujol mull) showed prominent peaks at 1640, 1600, 1245, 1160, 1000, 920, 895 cm^{-1}. Mass spectrum: m/e 255, (% of base peak 20); 254, (20); 253, (100); 252, (27); 239, (19); 238, (92); 224, (28); 223, (10); 210, (19); 209 (13); 208, (10); 195, (15); 130, (10); 91, (17); 77, (14). M.W. = 253. UV spectrum (EtOH): λ_{max} 275 mμ, (log$_{10}\epsilon$ 4·292); 500 mμ, (3·426); λ_{min} 239 mμ, (3·701); 400 mμ, (2·786).

Further column chromatography on silica and elution with benzene showed a fourth compound in very low

yield. This was identified as 3-(2', 4', 5'-trimethylanilino)-1, 2, 4, 6, 7, 9-hexamethylphenazine (LIV) (2·9 mg, 0·1%), m.p. 220°, recrystallised from EtOH. The compound gave a fluorescent yellow soln in benzene and a green colour with concentrated mineral acids. (Found: C, 81·8; H, 8·1; N, 10·3. C$_{27}$H$_{31}$N$_3$ requires: C, 81·6; H, 7·8; N, 10·6). IR spectrum (nujol mull) showed prominent peaks at 3440, 1700, 1630, 1575, 1510, 1300, 1210, 1175, 1140, 1000, 890, 870 cm^{-1}. Mass spectrum: m/e 400, (% of base peak 6); 399, (25); 398, (11); 397, (34); 396, (8); 381, (25); 380, (45); 297, (13); 264, (38); 263, (20); 262, (100); 261, (15); 246, (76); 232, (26); 218, (20); 216, (11); M.W. 397. UV spectrum (ethanol): λ_{max} 270 mμ, (log$_{10}$ ϵ 4·623); 400 mμ, (3·725); λ_{min} 230 mμ, (4·297); 332 mμ, (3·137); λ_{infl} 249 mμ, (4·431); 430 mμ (3·490).

The remainder of the oxidation product was removed from the column with MeOH and was shown by TLC to be an intractable mixture of many dark coloured components.

A sample of the phenazine (5 mg) was subjected to peroxidase oxidation under the usual conditions. Analysis of the product by thin-layer chromatography revealed traces of polymeric compounds.

The oxidation of 2,3,5,6-tetramethylaniline. The amine (2 g) was dissolved in glacial AcOH (2 ml) and added to pH 4·8 acetate buffer (1 L) containing peroxidase (2 mg) and H$_2$O$_2$ (2 ml). A purple colour was immediately produced, which gradually deepened, giving a deep purple solid. Peroxidase (1 mg) and H$_2$O$_2$ (1 ml) were added at intervals of 2 hr for 12 hr. Finally the mixture was allowed to stand for 24 hr to complete the oxidation. The purple solid was filtered off and dried. Further addition of peroxidase and H$_2$O$_2$ produced no coloration in the residual soln.

The purple solid (1·7 g) obtained from the oxidation was subjected to column chromatography, using dichloromethane as eluent. Three bands were eluted which gave the following compounds on evaporation.

(a) Fast running red compound (595 mg, 35%). The solid was recrystallised (light petroleum, b.p. 60–80°) to give red plates of 2,3,5,6-tetramethylbenzoquinone-4-(2', 3', 5', 6'-tetramethyl)anil m.p. 163°. (Found: C, 81·15; H, 8·4; N, 4·85. C$_{20}$H$_{25}$NO requires: C, 81·4; H, 8·5; N, 4·7%). NMR spectrum (CCl$_4$) showed singlet (broad, 12 Me protons) at $8 \cdot 20\tau$; singlet (6 Me protons) at $8 \cdot 02\tau$; singlet (6 Me protons) at $7 \cdot 80\tau$ and singlet (1 aromatic proton) at $3 \cdot 48\tau$. IR spectrum (KBr disc) showed prominent peaks at 2920, 2860, 1625, 1600, 1575, 1450, 1375, 1290, 1265, 1225, 1145, 1030, 875, 785, 690 cm^{-1}. Mass spectrum: m/e 297 (% of base peak 11); 296, (22); 295, (100); 281, (11); 280, (53); 252, (33); 237, (9); 236, (9). M.W. = 295. UV spectrum (EtOH): λ_{max} 279 m, (log$_{10}$ ϵ 4·452); 500 mμ, (2·954); λ_{min} 243 mμ, (2·743); 410 mμ. (2·462).

(b) Slow running purple compound (500 mg, 30%), m.p. 272° (decomp.). The compound could not be recrystallised satisfactorily from any solvent, but degradative and spectral data confirmed the structure as 2,2', 3,3', 5,5', 6,6' - octamethyl - N,N' - bis - (2, 3, 5, 6 - tetramethyl - 4 - oxocyclohexa - 2,5 - dienyldiene) biphenyl - 4,4' - diamine (8). NMR spectrum (CDCl$_3$) showed 3 peaks (broad, aliphatic Me protons) at $8 \cdot 19\tau$, $8 \cdot 11\tau$, $8 \cdot 00\tau$. No aromatic protons were present. IR spectrum (KBr disc) showed prominent peaks at 2920, 2860, 1630, 1580, 1450, 1380, 1290, 1260, 1225, 1145, 1060, 1040, 870, 760, 690 cm^{-1}. Mass spectrum: m/e 592, (% of base peak 4); 591, (12); 590, (34); 589, (55); 588, (100); 576, (6); 575, (10); 574, (20); 564, (6); 560, (5); 545, (10); 530, (5); 515, (5); 437, (6); 409,

(6); 393, (5); 279, (8); 265, (9); 175, (8); 136, (22); 121, (38); 95, (36); 91, (28). M.W. = 588. UV spectrum (ether): λ_{max} 279 mμ (log$_{10}$ ϵ 4·366); 287 mμ (4·355); 510 mμ, (4·169); λ_{min} 241 mμ, (4·141); 283 mμ, (4·355); 390 mμ, (2·433).

(c) Slower running purple band (490 mg, 29%), m.p. 284° (with decomp.). This compound could not be recrystallised satisfactorily from any solvent system, but degradative and spectral data confirmed its structure as 2,2', 3,3', 5,5', 6,6'-octamethyl-N-(2, 3, 5, 6-tetramethyl-4-oxocyclohexa-2, 5-dienylidene) biphenyl-4,4'-diamine (7). NMR spectrum (CDCl$_3$) showed 4 singlets (36 Me protons) at 8·21τ, 8·10τ, 8·00τ and 7·94τ; singlet (broad, 2 N–H protons at 6·56 and no peaks in the aromatic region. IR spectrum (NaCl disc) showed prominent peaks at 3450 (broad), 2920, 2880, 1630, 1585, 1450, 1375, 1290, 1260, 1230, 1145, 865, 690 cm^{-1}. Mass spectrum: m/e 444, (% of base peak 8); 443, (42); 442, (100); 428, (10); 427, (30); 383, (10); 276, (10); 261, (17); 250, (10); 221, (17); 220, (12); 215, (22); 214, (66); 207, (20); 206, (17); 199, (22); 198, (17); 197, (22); 192, (22); 191, (22). M.W. = 442. UV spectrum (ethanol); λ_{max} 281 mμ, (log$_{10}$ ϵ 4·197); λ_{min} 251 mμ, (4·136).

Examination of the filtrate. The filtrate was made strongly acid with 5M H$_2$SO$_4$ (100 ml) and steam-distilled. A yellow solid present in the distillate was identified as duroquinone. The residue after steam-distillation was made alkaline with 20% NaOH aq and extracted with ether. Analysis by preparative TLC on silica using dichloromethane as eluent showed the presence of 2,3,5,6-tetramethylaniline and octamethylbenzidine. The identity of both compounds was ascertained by mixed m.p.'s with authentic samples.

The hydrolysis of the monoimine (7). 7 (50 mg) was heated under reflux for $\frac{1}{2}$ hr with 3M H$_2$SO$_4$ (10 ml) and the soln steam-distilled. Duroquinone was identified as the only compound in the distillate.

The residual soln after steam-distillation was made alkaline with 10% NaOH aq and extracted with ether. The extracts were dried (Na$_2$SO$_4$) and evaporated. The white solid remaining was recrystallised (EtOH, water) and dried. White needles of *octamethylbenzidine*, m.p. 238°, were obtained. (Found: C, 81·2; H, 9·4; N, 9·2. C$_{20}$H$_{28}$N$_2$ requires: C, 81·1; H, 9·5; N, 9·5%). NMR singlet (12 Me protons) at 7·93τ and singlet (broad, 4 N–H protons) at 6·65τ. IR spectrum (KBr disc) showed prominent peaks at 3460, 3390, 3000, 2020, 2880, 1615, 1575, 1460, 1405, 1370, 1310, 1280, 1225, 1100, 920 cm^{-1}. Mass spectrum: m/e 298. (% of base peak at 4); 297, (22); 296, (100); 281, (4); 266, (14); 251, (8); 148, (14); 133, (14); 126, (5); 125, (5). M.W. = 296. UV spectrum (ethanol): λ_{max} 210 mμ, (log$_{10}$ ϵ 4·605); 291 mμ, (3·217): λ_{min} 271 mμ, (3·157).

The hydrolysis of the bisimine (8). 8 (50 mg) was hydrolysed in an analogous manner to 7. The products were isolated in a similar manner and shown to be identical.

The oxidation of 2, 3, 5, 5, 6 - pentamethylaniline. 2,3,4,5,6-Pentamethylaniline (102 mg) was oxidised with peroxidase and H$_2$O$_2$ in the usual way. A red-purple colour developed slowly, finally resulting in a purple ppt. The

ppt was filtered off and dried (96 mg). Recrystallisation (EtOH) gave purple crystals of 2, 3, 4, 5 - *tetramethyl-benzoquinone - 4 - (2', 3', 4', 5', 6' - pentamethyl) anil* (87 mg, 90%), m.p. 152°. (Found: C, 81·4; H, 8·8; N, 4·5. C$_{19}$H$_{27}$NO requires: C, 81·6; H, 8·7; N, 4·5%). NMR spectrum (CDCl$_3$) showed singlet (9 Me protons) at 8·12τ; singlet (6 Me protons) at 8·00τ and singlet (12 Me protons) at 7·69τ. IR spectrum (KBr disc) showed prominent bands at: 2930, 1635, 1445, 1370, 1290, 1270, 1150, 1060, 1040, 875, 845, 790, 690 cm^{-1}. Mass spectrum m/e 310, (% of base peak 9); 309, (36); 294, (12); 163, (72); 162, (100); 161, (32); 148, (66); 136, (30); 121, (22). M.W. = 309. UV spectrum (EtOH): λ_{max} 278 mμ, (log$_{10}$ ϵ 4·169); 515 mμ, (2·929); λ_{min} 224 mμ (3·361); 400 mμ, (2·231).

The filtrate after removal of the solid oxidation product was made alkaline with Na$_2$CO$_3$aq and extracted with ether. Examination of the extracts by TLC revealed a very small quantity of 2, 3, 4, 5, 6-pentamethylaniline.

Structural confirmations. The structures of azocompounds from peroxidase oxidations were confirmed by lead dioxide oxidation of the parent amines.[2] The structures of the quinone anils were confirmed by syntheses from the parent amines and quinones according to the method of Holland *et al.*[1] The structures of the two imines and the anils were also confirmed by acidic hydrolysis to the parent compounds.

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