Horseradish Peroxidase-catalysed Oxidation of Aromatic Tertiary Amines with Hydrogen Peroxide

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The horseradish peroxidase-catalysed oxidation of aromatic tertiary amines with hydrogen peroxide leads to a secondary amine and an aldehyde. With alkylmethylanilines demethylation is preferred to dealkylation. Evidence is given for the coexistence of the usual peroxidation reaction and a free radical chain reaction which depends upon oxygen concentration.

HORSERADISH peroxidase (HRP, EC 1.11.1.7, donor–H_2O_2 oxido-reductase) catalyses the oxidation of a great number of compounds by hydrogen peroxide. The reactions have been studied mainly in terms of reactants and enzyme intermediates, but not in terms of products.

Most work in product analysis has been performed by Saunders *et al.*, a considerable amount of which deals with the oxidation of aromatic amines.[1] Primary aromatic amines have been found to yield polycondensation products, but non-enzymic oxidation of donors such as aniline or p-toluidine in a system made up by hydrogen peroxide and iron(II) sulphate gave different products than when the reactions were performed in the presence of HRP.[2]

In the field of aromatic tertiary amines, Saunders reported the oxidation of NN-dimethylaniline in the presence of relatively large amounts of both enzyme and hydrogen peroxide.[3] The reaction products were NNN'-N'-tetramethylbenzidine and products deriving from further oxidation.

The complexity of this and other results and the discrepancies in the behaviour of these substrates in different conditions prompted us to investigate whether and how it was possible to establish a connection between the mechanism of the oxidation of aromatic amines by the peroxidase–hydrogen peroxide system and by inorganic oxidants.

The reaction of dimethylanilines in chloroform–acetic anhydride with the acetates of Pb^{IV}, Mn^{III}, Co^{III}, and Tl^{III} to yield demethylated acetylated amines and formaldehyde provides a useful model for comparison. In such a system some mechanistic conclusions could be drawn.[4,5] Furthermore, the study of the reaction of alkylmethylanilines with the same oxidants led to the conclusion that demethylation is preferred to dealkylation.[6]

RESULTS AND DISCUSSION

Here we report the results of an investigation on the products and modes of reaction of some dialkylanilines in the HRP-catalysed oxidation with hydrogen peroxide.

The experimental conditions are much milder than those reported by Saunders *et al.* in order to avoid the formation of compounds deriving from further oxidation of

TABLE 1

Reaction yields in the oxidation of NN-dimethylaniline with hydrogen peroxide in the presence of HRP

Compound	Demethylated (%)	Starting material (%)	Reaction time
4-MeOC$_6$H$_4$NMe$_2$	76	12	15 min
4-MeC$_6$H$_4$NMe$_2$	40	17	30 min
C$_6$H$_5$NMe$_2$	20	14	1 h
4-FC$_6$H$_4$NMe$_2$	46	19	2 h
3-MeSC$_6$H$_4$NMe$_2$	42	24	8 h
4-ClC$_6$H$_4$NMe$_2$	49	16	17 h
4-IC$_6$H$_4$NMe$_2$	70	18	24 h
3-ClC$_6$H$_4$NMe$_2$	59	15	7 h
4-CNC$_6$H$_4$NMe$_2$	No reaction		
3-NO$_2$C$_6$H$_4$NMe$_2$	No reaction		
4-NO$_2$C$_6$H$_4$NMe$_2$	No reaction		

TABLE 2

Reaction yields in the oxidation of N-alkyl-N-methyl-anilines with hydrogen peroxide in the presence of HRP

Compound	Demethylated (%)	Dealkylated (%)	Starting material (%)	Reaction time
4-MeOC$_6$H$_4$NMeBu^n	58	16	8	15 min
C$_6$H$_5$NMeBu^n	21		21	1 h
4-ClC$_6$H$_4$NMeBu^n	75		9	17 h
4-ClC$_6$H$_4$NMeEt	24	9	5	17 h

TABLE 3

Reaction yields in the oxidation of NN-dibutylanilines with hydrogen peroxide in the presence of HRP

Compound	Dealkylated (%)	Starting material (%)	Reaction time (h)
4-MeC$_6$H$_4$NBu$_2$^{n}	16	16	1
C$_6$H$_5$NBu$_2$^{n}	35	14	2
4-FC$_6$H$_4$NBu$_2$^{n}	12	26	6
4-ClC$_6$H$_4$NBu$_2$^{n}	19	29	17

the primary products. The yields obtained from the oxidation of three groups of amines, respectively NN-dimethyl-, N-alkyl-N-methyl- and NN-dibutylanilines, are shown in Tables 1—3. Some of these data have

[1] B. C. Saunders, A. S. Holmes-Siedle, and B. P. Stark, ' Peroxidase ', Butterworths, London, 1974.
[2] D. G. Daniels, F. T. Naylor, and B. C. Saunders, *J. Chem. Soc.*, 1951, 3433.
[3] F. T. Naylor and B. C. Saunders, *J. Chem. Soc.*, 1950, 3519.
[4] B. Rindone and C. Scolastico, *Tetrahedron Letters*, 1974, 3379.
[5] G. Galliani, B. Rindone, and P. L. Beltrame, *J.C.S. Perkin II*, 1976, 1803.
[6] G. Galliani, B. Rindone, and C. Scolastico, *Tetrahedron Letters*, 1975, 1285.

been reported in a preliminary communication.[7] An hydrogen peroxide concentration $(4.8 \times 10^{-5}M)$ lower than the concentration of the substrate $(3 \times 10^{-4}M)$ in the pH 6 buffer was used in order to obtain highest yields. The reaction products were a secondary amine and an aldehyde, by analogy with the results obtained from these substrates and the aforementioned metal oxidants. Control experiments showed that both HRP and hydrogen peroxide are necessary for the reaction. The data shown in Tables 1—3 indicate the amount of material recovered based on the amount of starting material used. The low recovery noted in some instances has to be attributed to the difficulty of extraction of some of the amines. Traces of coloured tars were
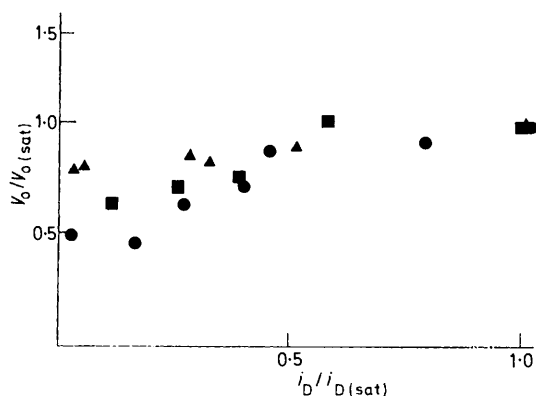

FIGURE 1 Plots of $(v_0$ for different oxygen concentrations)/ $(v_0$ in a saturated solution) versus $i_D/i_{D \text{ (standard)}}$ in the reaction of 4-fluorodimethylaniline (▲), 4-chlorodimethylaniline (●), and 3-chlorodimethylaniline (■) with HRP and H_2O_2. Kinetic runs allow the correspondence between $i_D/i_{D(\text{standard})}$ and $[O_2]/[O_2]_{(\text{standard})}$

formed only with the more reactive 4-methoxy-, 4-methyl-, and unsubstituted amine. For alkylmethylanilines demethylation was strongly preferred to dealkylation, in accord with the results obtained with high valence metal oxidants. In no case was there the evidence for the formation of N-oxides. When a stoicheiometric amount of hydrogen peroxide was used according to equation (1), the main products derived from polycondensation. The yields in dealkylated

$$RC_6H_4NMe_2 + H_2O_2 \xrightarrow{\text{HRP}} RC_6H_4NHMe + CH_2O + H_2O \quad (1)$$
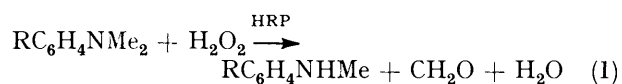
amine were generally higher than would be expected according to the stoicheiometric relation between the reactants. This fact suggested the intervention of another oxidant, besides hydrogen peroxide. The radical nature of the reaction was apparent from the fact that the presence of a radical trapping reagent such as 4-t-butylcatechol resulted in strong inhibition.

A more detailed study of the reaction was performed choosing three substrates which had sufficiently high oxidation rates without yielding any detectable troubling trace of coloured tars. These were 4-fluoro-, 4-chloro-,

and 3-chloro-dimethylaniline. The ratio of tertiary to secondary amine in the reaction was inspected by measuring the change in absorption at 290, 275, and 270 nm respectively. At these wavelengths there was the maximum difference between the absorption of the secondary and the corresponding tertiary amine. The ratio of tertiary to secondary amine thus obtained was used in a polarographic study on the dependence of the reaction rate for these three substrates upon the concentration of oxygen. The results obtained are reported in Figure 1. These results indicate that the reaction rate, measured through the degree of conversion, was increased when increasing the initial oxygen concentration. This fact suggested that the second oxidant involved in the reaction was oxygen. Moreover, in Figure 1 it is apparent that the dependence of the reaction rate upon the concentration of oxygen is slightly different for the three substrates tested.

TABLE 4

T 25 °C; [HRP] 50 μg l^{-1}; S = 4-fluorodimethylaniline; $[H_2O_2]$ in μmol l^{-1}; v_0 in μmol l^{-1} min^{-1}

[S] 300 μmol l^{-1}		[S] 400 μmol l^{-1}		[S] 500 μmol l^{-1}	
$[H_2O_2]$	v_0	$[H_2O_2]$	v_0	$[H_2O_2]$	v_0
16.4	2.73	9.8	0.53	32.8	6.37
26.3	3.23	24.6	4.60	39.4	6.90
32.9	3.76	32.8	5.17	46.0	8.49
36.2	3.92	53.4	5.91	72.3	9.85
49.3	4.17	65.7	5.91	111.0	8.49
65.7	4.17	90.3	5.17	118.0	8.49
72.3	4.21	98.6	4.87		
82.2	4.21	102.7	4.87		
98.6	3.74				

TABLE 5

T 25 °C; [HRP] 50 μg l^{-1}; S = 4-chlorodimethylaniline; $[H_2O_2]$ in μmol l^{-1}; v_0 in μmol l^{-1} min^{-1}

[S] 300 μmol l^{-1}		[S] 400 μmol l^{-1}		[S] 500 μmol l^{-1}	
$[H_2O_2]$	v_0	$[H_2O_2]$	v_0	$[H_2O_2]$	v_0
3.3	8.51	16.4	17.72	13.1	10.11
6.2	9.63	24.6	19.43	19.7	11.03
16.4	17.03	82.1	24.53	32.9	20.78
32.8	19.08	143.7	25.42	49.3	22.06
49.3	19.30				

TABLE 6

T 25 °C; [HRP] 50 μg l^{-1}; S = 3-chlorodimethylaniline; $[H_2O_2]$ in μmol l^{-1}; v_0 in μmol l^{-1} min^{-1}

[S] 300 μmol l^{-1}		[S] 500 μmol l^{-1}	
$[H_2O_2]$	v_0	$[H_2O_2]$	v_0
26.3	1.94	21.9	1.61
49.3	1.96	49.3	1.90
82.3	2.02	60.2	2.00
		98.6	2.02
		131.5	1.91
		137.0	1.87

The effect of the concentration of hydrogen peroxide on the reaction rate is shown in Tables 4—6. Inspection of the values obtained indicates that an increase of the

[7] G. Galliani, B. Rindone, and A. Marchesini, *J.C.S. Chem. Comm.*, 1976, 782.

hydrogen peroxide concentration increases the rate up to a maximum value. These data indicate also that the effect of the organic substrate concentration on the
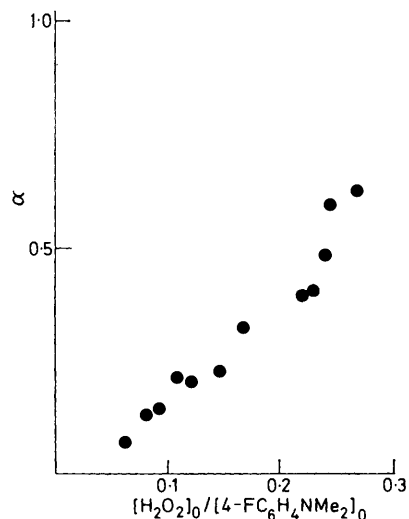

FIGURE 2 Plot of α (defined as [4-FC$_6$H$_4$NHMe]$_{\text{final}}$/[4-FC$_6$H$_4$-NMe$_2$]$_0$) versus [H$_2$O$_2$]$_0$/[4-FC$_6$H$_4$NMe$_2$]$_0$. Data are referred to substrate concentrations ranging from 300 to 500 μmol l^{-1}

reaction rate is well evident in the case of 4-fluoro-dimethylaniline, whereas the reaction rates for 3- and 4-chlorodimethylaniline at different substrate concentrations are nearly the same. This could be accounted for by the different affinity (or apparent K_M) of each substrate toward the enzyme.
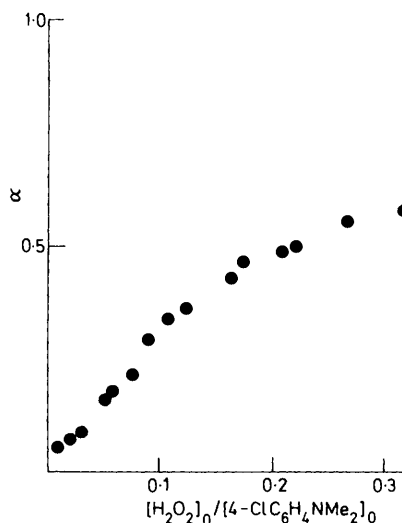
Experiments in which both hydrogen peroxide and

FIGURE 3 Plot of α (defined as [4-ClC$_6$H$_4$NHMe]$_{\text{final}}$/[4-ClC$_6$H$_4$-NMe$_2$]$_0$) versus [H$_2$O$_2$]$_0$/[4-ClC$_6$H$_4$NMe$_2$]$_0$. Data are referred to substrate concentrations ranging from 300 to 500 μmol l^{-1}

amine concentrations varied were performed in order to establish the stoicheiometry of the reaction. The results are reported in Figures 2—4. From these data it is

apparent that the degree of conversion of the tertiary amine into the demethylated product depends upon the ratio between the concentrations of hydrogen peroxide and amine. Control experiments showed a complete consumption of hydrogen peroxide in these reactions. Therefore, the role of hydrogen peroxide is stoicheio-metric and not catalytic.

The consumption of oxygen ($2\Delta O_2$) was measured with a Clark electrode in runs in which both hydrogen peroxide and amine concentrations ([P] and [S] respect-ively) were varied. Tables 7—9 report the comparison between the oxidation equivalents consumed * (hydrogen
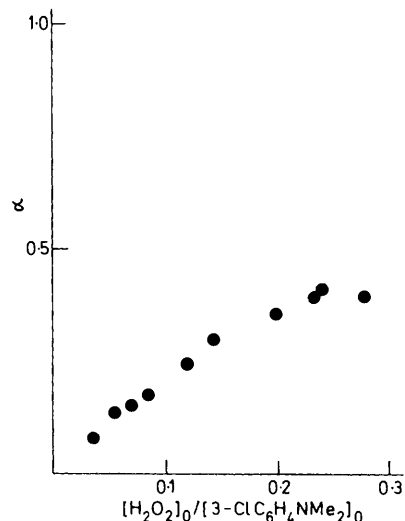

FIGURE 4 Plot of α (defined as [3-ClC$_6$H$_4$NHMe]$_{\text{final}}$/[3-ClC$_6$H$_4$-NMe$_2$]$_0$) versus [H$_2$O$_2$]$_0$/[3-ClC$_6$H$_4$NMe]$_0$. Data are referred to substrate concentrations ranging from 300 to 500 μmol l^{-1}

TABLE 7

S = 4-Fluorodimethylaniline]

[S]	[P]	[P]/[S]	ΔO_2	[P] + $2\Delta O_2$	([P] + $2\Delta O_2$)/[S]	α
300	49.3	0.164	24.1	97.5	0.32	0.33
400	49.3	0.123	24.1	97.5	0.24	0.22
500	49.3	0.099	24.1	97.5	0.19	0.17
500	32.8	0.066	18.6	70.0	0.14	0.10
500	65.7	0.131	29.2	124.1	0.25	0.23
500	98.6	0.197	39.9	178.4	0.36	0.35

TABLE 8

S = 4-Chlorodimethylaniline

[S]	[P]	[P]/[S]	ΔO_2	[P] + $2\Delta O_2$	([P] + $2\Delta O_2$)/[S]	α
175	65.7	0.375	18.8	103.9	0.59	0.59
350	65.7	0.187	30.8	127.1	0.36	0.42
475	65.7	0.142	26.8	119.5	0.25	0.30
475	54.8	0.115	34.2	123.2	0.25	0.30
475	82.2	0.173	46.2	174.6	0.36	0.42
475	109.5	0.230	38.4	186.3	0.39	0.50

peroxide plus oxygen {([P] + $2\Delta O_2$)/[S])} and the experi-mental value of α (ratio between the final concentration

* The experimental decrease of the concentration of oxygen (Δ[O$_2$] is correlated with the consumption of oxygen equivalents in this reaction (2Δ[O$_2$]) by assuming that every atom of oxygen accepts two electrons in this reaction.

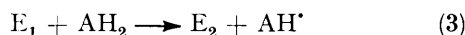of secondary amine and the initial concentration of tertiary amine). The values thus obtained are in good accord. These results were obtained at 25 °C. [HRP]
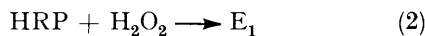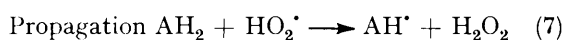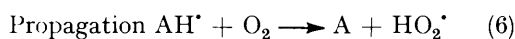
TABLE 9

S = 3-Chlorodimethylaniline

[S]	[P]	[P]/[S]	ΔO_2	[P] + 2ΔO_2	([P] + 2ΔO_2)/[S]	α
210	27.4	0.130	20.0	67.5	0.32	0.28
350	27.4	0.078	33.5	94.4	0.26	0.18
550	27.4	0.050	25.0	77.4	0.14	0.14
550	54.8	0.099	33.5	121.8	0.22	0.20
550	82.2	0.149	45.8	173.8	0.31	0.32
550	109.5	0.199	39.5	188.5	0.34	0.35

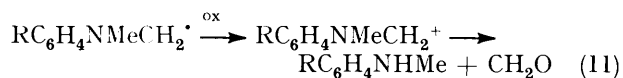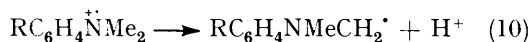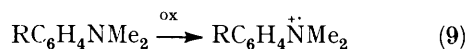was 50 μg l^{-1}. The concentrations are expressed as μmol l^{-1}.

The peroxidase-catalysed one electron oxidation of an organic substrate AH_2 is generally described as shown in equations (2)—(4).[8-11] Compounds E_1 and E_2 are intermediates in the oxidation of AH_2 to the radical $AH^{\bullet}$.

$$HRP + H_2O_2 \longrightarrow E_1 \qquad (2)$$

$$E_1 + AH_2 \longrightarrow E_2 + AH^{\bullet} \qquad (3)$$

$$E_2 + AH_2 \longrightarrow HRP + AH^{\bullet} \qquad (4)$$

Moreover, peroxidase catalyses the aerobic oxidation of several compounds such as indoleacetic acid, triose reductone, and reduced nicotinamide–adenine dinucleotide. An interpretation of this peroxidase oxidase reaction has been suggested by Yamazaki et al.[12,13] The reaction is started by the peroxidation reaction [equations (2)—(4)] which transforms the substrates into free radicals. A chain reaction is then started by intervention of oxygen [equations (5)—(8)].

$$\text{Initiation } AH_2 \xrightarrow{\text{H}_2\text{O}_2,\text{HRP}} AH^{\bullet} + H_2O \qquad (5)$$

$$\text{Propagation } AH^{\bullet} + O_2 \longrightarrow A + HO_2^{\bullet} \qquad (6)$$

$$\text{Propagation } AH_2 + HO_2^{\bullet} \longrightarrow AH^{\bullet} + H_2O_2 \qquad (7)$$

$$\text{Net } AH_2 + O_2 \longrightarrow A + H_2O_2 \qquad (8)$$
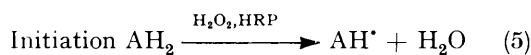
The HRP-catalysed oxidation of dialkylanilines here reported occurs through complete consumption of the hydrogen peroxide available and consumption of oxygen. This suggests that peroxidation and oxidation processes coexist. If one considers that aromatic tertiary amines undergo oxidative dealkylation with most oxidants with the mechanism shown in equations (9)—(11)[5,14] the
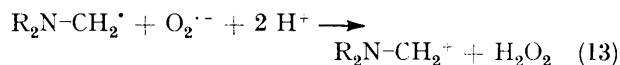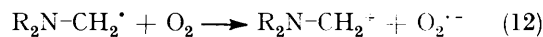
$$RC_6H_4NMe_2 \xrightarrow{\text{ox}} RC_6H_4\overset{+\cdot}{N}Me_2 \qquad (9)$$

$$RC_6H_4\overset{+\cdot}{N}Me_2 \longrightarrow RC_6H_4NMeCH_2^{\bullet} + H^+ \qquad (10)$$

$$RC_6H_4NMeCH_2^{\bullet} \xrightarrow{\text{ox}} RC_6H_4NMeCH_2^+ \longrightarrow RC_6H_4NHMe + CH_2O \qquad (11)$$
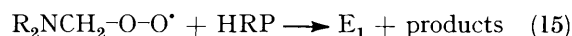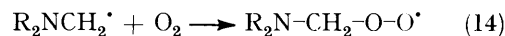
[8] B. Chance, Arch. Biochem. Biophys., 1949, 38, 224.
[9] B. Chance, Arch. Biochem. Biophys., 1952, 41, 416.
[10] B. Chance, Arch. Biochem. Biophys., 1952, 41, 235.

radicals occurring in this process could be formed via the peroxidation reaction. The peroxidation reaction could coexist with an oxidatic process involving the transfer of one electron from the organic radical to oxygen.[13,15] This reaction is depicted in equations (12) and (13) for a α-dialkylamino carbon radical. Alternatively, an oxidation process involving bond formation

$$R_2N-CH_2^{\bullet} + O_2 \longrightarrow R_2N-CH_2^+ + O_2^{\bullet -} \qquad (12)$$

$$R_2N-CH_2^{\bullet} + O_2^{\bullet -} + 2 H^+ \longrightarrow$$
$$R_2N-CH_2^+ + H_2O_2 \qquad (13)$$

between the organic radical and oxygen [equations (14) and (15)] could operate.[16] A similar reaction has been

$$R_2NCH_2^{\bullet} + O_2 \longrightarrow R_2N-CH_2-O-O^{\bullet} \qquad (14)$$

$$R_2NCH_2-O-O^{\bullet} + HRP \longrightarrow E_1 + \text{products} \qquad (15)$$

proposed for the peroxidase-catalysed oxygenation of 2′,4,4′-trihydroxychalcone,[16] and operates in many autooxidation reactions.

Further work is needed in order to distinguish between these two oxidation processes involving chain propagation steps.

EXPERIMENTAL

Products Studies.—A solution of amine (3 × 10^{-4} mol) in buffer (1 l; 5.4 g l^{-1} of NaH_2PO_4,H_2O and 2.7 g^{-1} l of $Na_2HPO_4,12H_2O$) was added to HRP solution (50 ml) and 30% H_2O_2 (5 μl). After the time needed for each substrate, the solution was extracted with ethyl acetate (3 × 200 ml) (continuous extraction was used for dibutylanilines). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure and the residue was treated overnight with acetic anhydride. The residue after the evaporation of acetic anhydride under reduced pressure was separated by silica gel chromatography using silica gel G. Merck 0.05—0.2 mm (R 100) eluting with chloroform.

Kinetic and Stoicheiometric Studies.—Working curves of both dimethyl- and monomethyl-anilines were drawn at those wavelengths where the u.v. spectra of each couple showed the largest difference in absorption and were not

[11] P. George, Nature, 1952, 169, 612.
[12] I. Yamazaki, K. Yokota, and R. Nakajima, ' Oxidases and Related Redox Systems,' New York, 1965, vol. 1, pp. 485 et seq.
[13] I. Yamazaki and L. H. Piett, Biochim. Biophys. Acta, 1963, 77, 47.
[14] C. A. Audeh and J. R. Lindsay Smith, J. Chem. Soc. (B), 1971, 1741.
[15] I. Yamazaki, H. Mason, and L. H. Piett, J. Biol. Chem., 1960, 235, 2444.
[16] I. Yamazaki, H. Mason, and L. H. Piett, Biochem. Biophys. Res. Comm., 1959, 1, 336.

modified by ageing of the solutions. The wavelengths for 4-fluoro-, 4-chloro-, and 3-chloro-dimethylaniline were 290, 275, and 270 nm respectively. Solutions of the amines and HRP were thermostatted before the experiments. The HRP concentration was held constant and was 0.5 μg ml⁻¹. In all the runs the appropriate amount of *ca.* 2M solution of hydrogen peroxide was added to the solution of the amine and HRP. The determination of the degree of conversion (α) for different initial concentrations of substrate and hydrogen peroxide was performed following the change in absorbance at the appropriate wavelength against time in samples (3 ml) until a constant value (D_{fin}) was reached. The degree of conversion (α) is given by equation (16) where D_{dimethyl} and $D_{\text{monomethyl}}$ are the absorb-

$$\alpha = (D_{\text{fin}} - D_{\text{dimethyl}})/(D_{\text{monomethyl}} - D_{\text{dimethyl}}) \quad (16)$$

ances of the tertiary and the secondary amine in the reaction conditions. The results obtained are reported in Figures 2—4. The plot of absorbance against time was also used to obtain graphically the initial rate (v_0) for different concentrations of substrate and hydrogen peroxide. The results are shown in Tables 4—6. Partially deoxygenated solutions of amine and HRP were obtained by bubbling nitrogen through solutions (100 ml) of amine and hydrogen peroxide and the concentration of oxygen was measured polarographically (the diffusion current was measured at the first oxygen reduction wave). Samples (3 ml) of these partially deoxygenated solutions were used for measuring the plot of absorbance against time. The initial rate in these runs could be obtained graphically. The results are shown in Figure 1. The concentration of oxygen in the partially deoxygenated solutions did not vary appreciably with time if shaking was avoided. Samples (3 ml) of solutions containing HRP and different concentrations of substrate and hydrogen peroxide were used for the determination of oxygen consumption. The decrease of oxygen concentration with time was obtained with a Clark electrode. The results are shown in Tables 7—9. The consumption of hydrogen peroxide was tested for each substrate by adding known amounts of an aqueous solution of guaiacol and monitoring changes in absorbance at 475 nm.