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STUDIES IN PEROXIDASE ACTION—XIV*

INTERMEDIATE COMPOUNDS IN THE OXIDATION OF *p*-TOLUIDINE

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Abstract—Previous work has shown that the peroxidation of *p*-toluidine gives a complex mixture of six products one of which is 4,4′-dimethyldiphenylamine. It has also been suggested[2] that 4-methyl-N^2-*p*-tolyl-*o*-phenylenediamine is an intermediate compound in the oxidation. Experiments have now been carried out to demonstrate that both these compounds are possible reaction intermediates.

It has also been established that the relative yields of the oxidation products depend upon the initial concentration of the *p*-toluidine. TLC and column chromatography have been extensively used in the separation and identification of the products.

SAUNDERS and Mann recorded[1] the major products of the oxidation of *p*-toluidine by peroxidase–hydrogen peroxide as:
4-*p*-toluidino-2,5-toluquinonebis-*p*-tolylimine (Perkin's base) (I)
4-amino-2,5-toluquinonebis-*p*-tolylimine (Barsilowsky's base) (II)
4,4′-dimethyldiphenylamine, (III)
4,4′-dimethylazobenzene, (IV)
hydrolysis products of I and II; (V and VI respectively).

We have now shown that the products I, II and IV are stable to oxidation by peroxidase–hydrogen peroxide, but that III is not. Experiments in which the initial dilute solution of *p*-toluidine contains 4,4′-dimethyldiphenylamine show considerably increased yields of I over those obtained from similar solutions containing no 4,4′-dimethyldiphenylamine. The base I is therefore believed to be produced by oxidative coupling of a molecule of 4,4′-dimethyldiphenylamine with a molecule of *p*-toluidine giving the intermediate NN′-di-*p*-tolyl-4-methyl-*o*-phenylenediamine, (VII), which itself undergoes oxidative coupling with a molecule of *p*-toluidine to give I. The low solubility of 4,4′-dimethyldiphenylamine results in its removal from the reaction by precipitation thereby reducing the yield of I.

The action of peroxidase–hydrogen peroxide on 4,4′-dimethyldiphenylamine alone gives an intractable black solid which is not produced when *p*-toluidine is present. The addition of 4,4′-dimethyldiphenylamine during the oxidation of *p*-toluidine does not produce a significant quantity of any product which is not present in the oxidation products of *p*-toluidine.

It is interesting to note that the production of 4,4′-dimethyldiphenylamine is not an oxidation step, but is produced from two molecules of *p*-toluidine with the elimination of a molecule of ammonia. This is an example of peroxidase acting in a non-oxidative capacity.

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* Paper XIII. B. C. Saunders and J. Wodak, *Tetrahedron* **22**, 505 (1966).

[1] B. C. Saunders and P. J. G. Mann, *J. Chem. Soc.* 769 (1940).

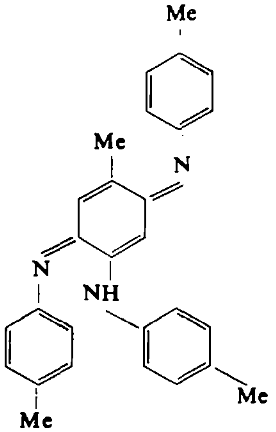
[2] B. C. Saunders, A. G. Holmes-Siedle and B. P. Stark, *Peroxidase—The Properties and Uses of a Versatile Enzyme and of Some Related Catalysts* p. 30. Butterworths, London (1964).

Evidence for the presence of VII is given by the observation that if a solution of *p*-toluidine containing 4-methyl-N^2-*p*-tolyl-*o*-phenylenediamine (VIII), (the mono-*p*-tolyl analogue of VII) is oxidized, a considerably increased yield of II is obtained over that produced by the oxidation of a similar solution not containing VIII.

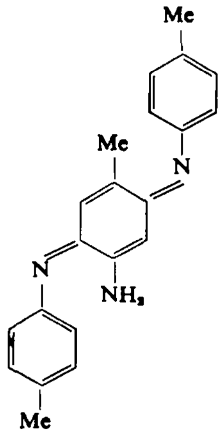
The action of peroxidase–hydrogen peroxide on VIII alone gives an intractable deep purple solid, which is not produced when *p*-toluidine is present. The addition of VIII to the oxidation of *p*-toluidine does not produce a significant quantity of any product which is not present in the oxidation products of *p*-toluidine.

Another interesting feature of the reaction is the dependence of the ratio of the yields of I and II on the initial concentration of *p*-toluidine. Experiments carried out on solutions of *p*-toluidine in which the initial concentration of the amine ranged from 0·1 to 25·0 g/l. show that I (and its precursor 4,4′-dimethyldiphenylamine), is not produced in detectable amounts unless the concentration of *p*-toluidine is 1 g/l. or greater. The production of 4-methyl-N^2-*p*-tolyl-*o*-phenylenediamine and 4,4′-dimethylhydrazobenzene, (the latter being the precursor of 4,4′-dimethylazobenzene), does not, however, depend on the initial amine concentration.

There is no evidence that 4,4′-dimethylhydrazobenzene undergoes an ortho-semidine rearrangement under the conditions of peroxidase oxidation. Under weakly acid conditions (pH = 4·5) in the presence of hydrogen peroxide 4,4′-dimethylhydrazobenzene is rapidly oxidized to the azo-compound and does not rearrange to 4-methyl-N^2-*p*-tolyl-*o*-phenylenediamine.



I



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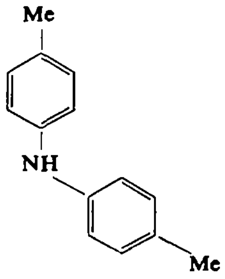

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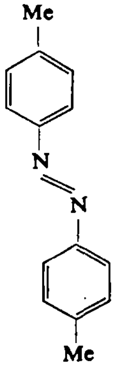

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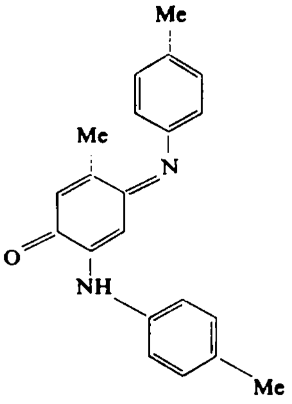

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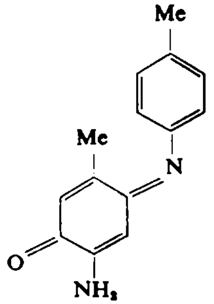

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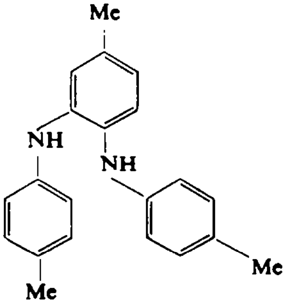
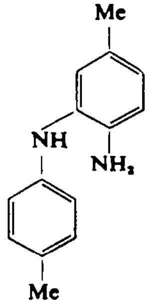


VII



VIII

EXPERIMENTAL

Materials and equipment. All oxidations were carried out using a purified horse-radish peroxidase (RZ = 0·3), supplied by Seravac Laboratories Ltd., and 20 volumes H_2O_2. All column chromatograms were carried out on alumina using 40% chf in light petroleum (b.p. 60–80°) as eluant. TLC plates were prepared using alumina 'G' supplied by E. Merck AG.

Peroxidase system and compounds I, II and IV. Treatment of well stirred suspensions of I, II and IV in solns of AcOH at pH 4·5 (the compounds having been prepared as described by Saunders and Mann[1]), with peroxidase–hydrogen peroxide resulted in each case in no reaction. Each suspension was filtered after 6 hr and the filtered solids analysed by TLC. No spots other than those of the starting materials were detected.

Mixed oxidations

(a) *Oxidation of a mixture of* p-*toluidine and* 4,4′-*dimethyldiphenylamine. p*-Toluidine (0·053 g, 0·0005 mole) and 4,4′-dimethyldiphenylamine (0·049 g, 0·00025 mole) were dissolved in 0·25 M acetate buffer (50 ml) at pH 4·5. Over a period of 6 hr the following additions were made to the stirred solution:

(a) a solution of *p*-toluidine (0·159 g, 0·0015 mole) and 4,4′-dimethyldiphenylamine (0·147 g, 0·00075 mole) in dil AcOH (10%, 10 ml).

(b) 20 vol H_2O_2 (1 ml) every ½ hr.

(c) peroxidase (1 mg) every 2 hr.

A red colouration appeared on addition of the enzyme and after ½ hr a red precipitate formed. After 6 hr the reaction mixture was extracted with chf and evaporated to dryness, yield of red solid, 0·351 g. The solid was dissolved in 40% chf–light petroleum and chromatographed. The main product appeared as a fast running red band which was eluted, evaporated and dried *in vacuo*. Wt of red solid, 0·177 g m.p. and mixed m.p. with an authentic sample of 4-*p*-toluidino-2,5-toluquinone-bis-*p*-tolylimine, (I), 182°. *Rf* on alumina TLC (0·4) identical with that of authentic I. Percentage by wt in the crude oxidation product = 50·4.

Oxidation of p-*toluidine alone under the same conditions.* *p*-Toluidine (0·212 g, 0·002 mole) was treated as above, except that no 4,4′-dimethyldiphenylamine was added. After evaporation of the chf extract and drying, 0·160 g of the red solid oxidation product was obtained. After chromatography on alumina, elution and evaporation of the fast running red band the dry wt of the red solid was 0·051 g, m.p. and mixed m.p. with an authentic sample of 4-*p*-toluidino-2,5-toluquinonebis-*p*-tolylimine, (I), 182°. *Rf* on alumina TLC (0·4) identical with that of authentic I. Percentage by wt in the crude oxidation product = 31·9.

Analysis by alumina TLC of the two crude oxidation products obtained above showed no difference in the number, nature nor intensities of the spots, other than a relatively more intense spot at *Rf* 0·4 in the former oxidation product, indicating the increased yield of I.

(b) *Oxidation of a mixture of* p-*toluidine and* 4-*methyl*-N^2-p-*tolyl*-o-*phenylenediamine.* *p*-Toluidine (0·214 g, 0·002 mole) was dissolved in glacial AcOH (1 ml) and diluted to 20 ml with 0·25 M acetate buffer. The solution had pH 4·5. Over a period of 6 hr the following additions were made to the stirred solution:

(a) 4-methyl-N^2-*p*-tolyl-*o*-phenylenediamine (0·212 g, 0·001 mole) (prepared as described by Yamada *et al.*[8] from 4,4′-dimethylhydrazobenzene, m.p. 108°), in dil AcOH (10 ml).

(b) hydrogen peroxide (1 ml) every ½ hr.

(c) peroxidase (1 mg) every 2 hr.

A red coloration appeared on addition of the enzyme and after ½ hr a red precipitate formed. After 6 hr this was filtered off and the red solid washed, dried and weighed (0·295 g). The solid was chromatographed and the main product, appearing as a slower running red band, was eluted, evaporated and dried *in vacuo*. Wt of red solid 0·142 g, m.p. and mixed m.p. with an authentic sample of 4-amino-2,5-toluquinonebis-*p*-tolylimine, (II), 234°. *Rf* on alumina TLC (0·2) identical with that of authentic II. Percentage by wt in the crude oxidation product = 48·1.

Oxidation of p-*toluidine alone under the same conditions.* *p*-Toluidine (0·212 g, 0·002 mole) was treated as above, except that no 4-methyl-N^2-*p*-tolyl-*o*-phenylenediamine was added. After filtration and drying the red solid oxidation product weighed 0·157 g. Chromatography elution and evaporation of the slower running red band gave after drying 0·451 g of a red solid, m.p. and mixed m.p. with an authentic sample of 4-amino-2,5-toluquinonebis-*p*-tolylimine, (II), 234°. *Rf* on alumina TLC (0·2) identical with that of authentic II. Percentage by wt in the crude oxidation product = 28·7.

Analysis by alumina TLC of the two crude oxidation products obtained above showed no difference in the number, nature nor intensities of the spots, other than a relatively more intense red spot at *Rf* 0·2 in the former oxidation product, indicating the increased yield of II.

Oxidation of p-*toluidine solutions at different concentrations.* Solutions of *p*-toluidine at the following concentrations in 0·5M acetate buffer (200 ml) at pH 4·5 were prepared: 0·1, 0·5, 1·0, 5·0, 10·0, 25·0 g/l. To each was added H_2O_2 (1 ml) and peroxidase (1 mg) every 2 hr. Each soln developed a red colouration and then a red precipitate appeared. Each soln was extracted with chf (10 ml) and the extract washed with water. Samples of each soln were analysed by alumina TLC, using 25% benzene–light petroleum (b.p. 60–80°), and the following chromatograms were obtained:

Initial concentration of *p*-toluidine g/l.	Intensity of spot *Rf*										
	0	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9	1·0
0·1	w		m							w	
0·5	w		m							w	
1·0	w		m		vw					w	
5·0	w		s		s			w		w	
10·0	w		s		s			w		w	
25·0	w		s		s			w		w	
	purple		red		red			purple (after spraying with conc. HNO_3)		yellow	

vw = very weak, w = weak, m = medium, s = strong.

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[8] S. Yamada, I. Chibata and R. Tsurui, *Pharm. Bull*, Japan **2**, 59 (1954).

Initial experiments with the compounds I to VI gave the following spots on alumina TLC using the same eluant:

		R_f
I	red	0·4
II	red	0·2
III	purple	0·75 after spraying with conc. HNO_3.
IV	yellow	0·9
V	purple	0
VI	purple	0

One of us (V. R. H.) is indebted to the Morrell fund for a maintenance allowance.