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STUDIES IN PEROXIDASE ACTION—XXV

STEREOSPECIFICITY OF PEROXIDASE ACTION

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Abstract—The oxidations of some 4-n-alkyl substituted aromatic amines by peroxidase have been studied. 4-n-Decylaniline inhibits the enzyme as do amines higher in the homologous series while amines lower in the series are oxidised normally although the rate of oxidation is slower as the length of the chain increases.

INTRODUCTION

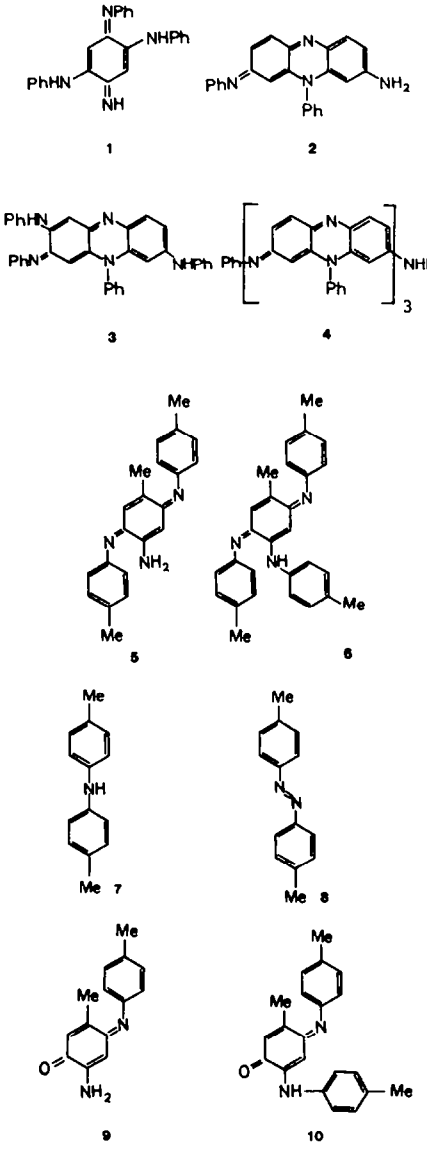
The oxidation of aniline by the peroxidase system has been studied in detail by Mann and Saunders[1] and the products have been isolated and characterised as follows: 2,5-dianilino-*p*-benzoquinone imide-anil (**1**), pseudo-mauveine (**2**), induline (**3**) and aniline black (**4**).

An investigation into the oxidation of *p*-toluidine has also been carried out,[2] and the following products have been isolated: 4-amino-2,5-toluquinonebis-*p*-tolylimine (**5**), 4-*p*-toluidino-5, 2-toluquinonebis-*p*-tolylimine (**6**), 4,4'-dimethyldiphenylamine (**7**), 4,4'-dimethylazobenzene (**8**), 4-amino-2,5-toluquinone-2-*p*-tolylimine (**9**) and 4-*p*-toluidino-2,5-toluquinone-2-*p*-tolylimine (**10**). The last two compounds are found only in small quantities and arise from the hydrolysis of the first two compounds. No further references to the peroxidase oxidations of this series of aromatic amines could be found in the literature.

In order to attempt to understand further the mechanism of peroxidase action, amines in the homologous series beginning with aniline and *p*-toluidine have been oxidised by the peroxidase system. The following amines have been prepared in the laboratory: 4-n-propylaniline, 4-n-hexylaniline, 4-n-octylaniline, 4-n-nonylaniline, 4-n-decylaniline and 4-n-undecylaniline and the following were obtained from commercial sources: 4-n-ethylaniline, 4-n-hexylaniline and 4-n-dodecylaniline. The oxidations were carried out under similar conditions to those of mesidine.[3] At the end of each oxidation the solid matter was either filtered off or extracted into ether for examination by TLC. It was found that the oxidation of all the amines, up to and including 4-n-nonylaniline, took place in the normal manner. However, it was found that amines higher in the homologous series were not oxidised by peroxidase and hydrogen peroxide.

Several factors could be put forward to explain the failure of the enzyme to oxidise the amines higher in the homologous series. There are: (i) the basicity of the amine: (ii) the solubility of the amine; (iii) steric factors.

The pK_a is of critical importance in determining whether the amine can be oxidised by the peroxidase system. Saunders *et al.* have cited a pK_a of 3·5 as the critical level for peroxidase action.[4] Although the pK_a's of 4-n-decylaniline and the higher members of the series are not known, they are certain to be above this critical level. The pK_a of aniline is 4·61 and that of *p*-toluidine is 5·09. The pK_a of 4-n-decylaniline will certainly be very close to that of *p*-toluidine.



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The solubility of 4-n-decylaniline in pH 4·8 acetate buffer is approx. 50 mg l^{-1} (2×10^{-4} moles l^{-1}). This concentration is high enough for oxidation by peroxidase to take place since aniline has been shown to react with peroxidase, producing its characterisic purple colour in dilutions as low as 10 mg l^{-1} (10^{-4} moles l^{-1}).[5]

Thus, in the absence of any other reasonable alternative, steric factors must be responsible for the failure of the enzyme to oxidise 4-n-decylaniline and the higher members of the series. The case of N,N,2,4,6-pentamethylaniline has been considered,[6] and the failure of this amine to react with peroxidase is undoubtedly due to the inability of the lone pair on the N atom to interact with the π electrons on the benzene ring. This is caused by steric interactions between the Me groups on the nitrogen and those in the *ortho*-positions on the ring, and no such interaction can take place in the case under consideration. Holland has also cited the case of N-acetyl-2-aminofluorene[7] which does not react with peroxidase, whereas 2-aminofluorene is very readily oxidised. This is due to electrophilic removal of the lone pair from the N atom by the acetyl group thus preventing conjugation. Once again, this cannot be applied to the amines in question.

In view of the fact that none of the normal steric considerations can account for the failure of 4-n-decylaniline to react with the peroxidase system, the reason must lie in the size of the amines, and, more particularly, in the length of the side-chain. There are two possible places in the peroxidase reaction sequence where steric considerations could be of importance. If the amine fails to form a ternary complex with the initial peroxidase/hydrogen peroxide compound (Compound I[8]), then no reaction can take place and no oxidation products would be observed. Alternatively the Compound I/amine complex could be held in such a configuration that the formation of any reactive intermediate is unfavourable. In this second case, also, no oxidation products would be observed.

If the failure to react is simply due to the large size of the amine, then any spatially-large amines should fail to react with peroxidase and the phenomenon should be a common one. However, this is not so, and many sterically bulky amines have been shown to react with peroxidase.

However, should the complex be formed between Compound I and the amine with no resultant reaction owing to the unfavourable enzyme complex configuration, this would be the first recorded failure of the enzyme to react with an amine after the formation of the initial complex. Experiments were carried out with the intention of showing that Compound I, had, in fact, formed a complex with the amine and that the overall effect was and of the enzyme by the amine.

Addition of peroxidase and H_2O_2 to a solution of 4-n-decylaniline in acetate buffer results in no colour change and no oxidation products are produced. The addition of a dilute solution of aniline in the same acetate buffer to this unchanged solution causes an exceedingly slow colour change to the purple colour of the aniline oxidation products. However, addition of an identical aniline solution to an identical solution of peroxidase and hydrogen peroxide, but containing no 4-n-decylaniline results in an extremely rapid generation of the purple products.

Thus, it appears that 4-n-decylaniline is reversibly inhibiting Compound I. Addition of aniline results in slow formation of the aniline/Compound I complex and production of the oxidation products of aniline in the usual way. This scheme of inhibition is illustrated in Fig. 1.

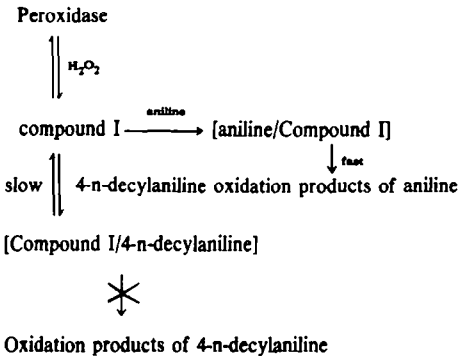

Fig. 1. Scheme of inhibition of peroxidase by long-chain alkylanilines.

The oxidation products .

Examination. The oxidation products of the amines were extracted from the aqueous solution and examined by TLC on alumina. Azo-compounds were isolated from each reaction mixture and characterised by comparison with an authentic sample, prepared by the method of Chapman and Saunders.[3]

The other products of oxidation of higher amines were not obtained in large enough quantities for characterisation. In general, they were not stable to recrystallisation and underwent considerable decomposition.

EXPERIMENTAL

Materials and equipment. The enzyme preparation was a purified horseradish peroxidase supplied by Seravac Laboratories Limited and had RZ 0·3. Column chromatography was carried out on Spence "H" alumina (100–200 mesh) activated by heating for 4 hr at 350°. The H_2O_2 soln was approx approx 20 vol. TLC was carried out on plates prepared with Merck alumina "G" and the amines were distilled or recrystallised immediately before use.

Preparation of the amines. Two methods were used for the preparation. 4-n-Propylaniline and 4-n-hexylaniline were prepared by nitration of the parent n-alkylbenzene,[9] separation of the pure *para* isomer by column chromatography and reduction of the nitro-compound to yield the amine.[10] The other amines were prepared by the method of Anahara *et al.*[11] In this preparation, aniline, zinc chloride and the appropriate alcohol were heated together. The amine was finally distilled from the mixture. The purity of the amines was carefully checked by TLC and by NMR spectroscopy. The *ortho*-isomers of the lower amines were removed by fractional distillation as previously mentioned. The preparation of the higher amines was by a specific reaction forming the *para*-isomer. NMR spectra in each case showed a perturbed A_2B_2 pair of doublets integrating for four protons in the aromatic region indicating only a *para*-disubstituted compound. Furthermore a 1:2:1 triplet integrating for the two benzylic protons in the side chain showed that no rearrangement of the side chain was taking place in the course of the alkylation reaction. All other spectral data confirmed the structure of the compounds.

Oxidation of the amines. All the amines were oxidised under similar conditions. The quantity of amine introduced into the mixture decreased as the length of the side chain increased, owing to decreasing solubility. The amine (1 g for 4-ethylaniline, 50 mg for 4-n-decylaniline) was dissolved in glacial AcOH (1 ml) and added to a solution of peroxidase (5 mg) and H_2O_2 (2 ml) in pH 4·8 acetate buffer (1 l). Thereafter peroxidase (2 mg) and H_2O_2 (1 ml) were added every 4 hr for 24 hr. At the conclusion of the reaction, the soln was made basic with NaOH soln (10%) and extracted into ether. In all cases, a control soln was kept under identical conditions to the peroxidase oxidation, but no enzyme

Table 1.

Amine	Colour of Oxidation Reaction
4-ethylaniline	Red
4-n-propylaniline	Red
4-n-butylaniline	Orange-red
4-n-hexylaniline	Orange
4-n-octylaniline	Yellow-brown
4-n-nonylaniline	Yellow-brown
4-n-decylaniline	None
4-n-undecylaniline	None
4-n-dodecylaniline	None

Table 2.

Azo-compound	M.P.	Mixed m.p. with an authentic specimen
4,4'-diethylazobenzene	63° (lit. 63°)	62°
4,4'-di-n-butylazobenzene	36°	36.5°
4,4'-di-n-hexylazobenzene	28°	27°
4,4'-di-n-octylazobenzene	46°	45°
4,4'-di-n-nonylazobenzene	36°	35°

was added to it before addition of the amine or later. This soln was also made basic and extracted into ether after 24 hr.

In order to determine the action of peroxidase and H_2O_2 on a given amine, the colour change on addition of the amine to the enzymic soln was noted (if it occured). The extracts from the enzymic oxidation and the control solution were compared by TLC.

It was found that 4-n-propylaniline, 4-n-butylaniline, 4-n-hexylaniline, 4-n-octylaniline and 4-n-nonylaniline gave coloured solutions on addition of the amine to the enzymic solution, while 4-n-decylaniline, 4-n-undecylaniline and 4-n-dodecylaniline gave no colour (Table 1). Furthermore, while examination by TLC of the oxidation of the former amines showed several compounds, the latter amines showed only one compound with R_f value identical with the starting material. In all cases, only starting material was present in the control solns after 24 hr.

The inhibition of peroxidase by 4-n-decylaniline. 4-n-Decylaniline (25 mg) in glacial AcOH (1 ml) was added to a soln of peroxidase (5 mg) and H_2O_2 (2 ml) in pH 4·8 acetate buffer (500 ml). No coloration developed. Aniline (100 mg) in glacial AcOH (1 ml) was added to the above soln and also to a similar control soln, but containing no 4-n-decylaniline. A purple colour was immediately produced in the latter soln, but appeared only very slowly in the former soln. Addition of fresh peroxidase (1 mg) to the former soln immediately resulted in the production of a deep purple colour which is characteristic of the peroxidase oxidation of aniline.

The products of peroxidase oxidation. 4,4'-Dialkylazobenzenes were isolated from the mixtures by column chromatography and by preparative TLC using light petroleum (b.p. 60-80Bp) as eluent in both cases. These were characterised by comparison with authentic speciments prepared by the method of Chapman and Saunders.[3] The results are summarised in Table 2. The remaining oxidation products were not isolated in a sufficiently pure condition for full characterisation. However, analysis by TLC showed marked similarities between the unidentified oxidation products and those of *p*-toluidine.

The preparation of the azobenzenes. These compounds were prepared according to the method of Chapman and Saunders[3] for the preparation of azomesitylene from mesidine. The amine (0·022 moles) was added to ether (220 ml) containing glacial AcOH (9 ml) and lead dioxide (20 g). The mixture was shaken for 4 hr and filtered. The ethereal soln was washed with Na_2CO_3 soln, followed by water, dried (Na_2SO_4) and evaporated. The dark oil obtained after evaporation was dissolved in benzene (5 ml) and light petroleum b.p. 40–60° (15 ml) and chromatographed on aluminina. The same mixture of solvents was used as eluent. The fast-running yellow band was eluted, evaporated to dryness and recrystallised (EtOH) to yield the pure substituted azobenzene in yields of approx. 20%.

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