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STUDIES IN PEROXIDASE ACTION—XV*

MECHANISM OF THE PEROXIDASE OXIDATION OF MESIDINE

B. C. SAUNDERS and J. WODAK†
University Chemical Laboratory, Lensfield Rd., Cambridge

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Abstract—Mesidine is a key "substrate" in investigating peroxidase action. The reaction is almost quantitative and a single crystalline product is obtained. Oxidations by the peroxidase system have now been carried out on mixtures of (i) mesidine and 4-amino-3,5-dimethylbenzyl alcohol and (ii) mesidine and 4-amino-3,5-dimethylbenzaldehyde. From the results obtained certain deductions have been made regarding the mechanism of the oxidation of mesidine alone.

THE major product from the peroxidase-catalysed oxidation of mesidine is the quinone anil I.[1] There are two interesting features of this reaction. The first of these is the fact that I is obtained in high yield (ca. 95%), with either no or very little azomesitylene; the second is that the carbon atom which is eliminated appears as formaldehyde (there was some doubt about this in the original work, but it has since been confirmed[2]). The implication of these observations is that the oxidation probably does not proceed essentially *via* the radical ArNH, but perhaps by nucleophilic attack at the *para* carbon atom of a mesidine residue. This is somewhat similar to the oxidation of the corresponding phenol, mesitol, which gives 2,6-dimethylbenzoquinone (II) as an end-product, and 4-hydroxy-3,5-dimethylbenzyl alcohol (III) and 4-hydroxy-3,5-dimethylbenzaldehyde (IV) as intermediates.[3]

It was because of this formal resemblance between the end-products of the two reactions that we decided to investigate whether the corresponding amino-alcohol (V) and aldehyde (VI) might be intermediates in the mesidine oxidation, by carrying out mixed oxidations of mesidine with these two compounds. Previous investigation of the mixed oxidation of two amines[4] had given little precise information. Nevertheless it was hoped that the results of the proposed mixed oxidation would not be too difficult to interpret. Another problem was the synthesis of 4-amino-3,5-dimethylbenzyl alcohol (V) and 4-amino-3,5-dimethylbenzaldehyde (VI), neither of which had previously been reported. The alcohol was obtained in 68% yield by the LAH reduction of methyl-4-amino-3,5-dimethylbenzoate, which was itself prepared by the method used by Saunders and Watson.[5] The aldehyde, which proved to be unstable and was therefore not purified, was prepared by oxidation of the alcohol with

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† Present address: Department of Biochemistry, University of Alberta, Canada.

[1] N. B. Chapman and B. C. Saunders, *J. Chem. Soc.* 496 (1941).

[2] Y. Avi-Dor and K.-G. Paul, *Acta Chem. Scand.* **7**, 444 (1953).

[3] H. Booth and B. C. Saunders, *Nature, Lond* **165**, 567 (1950); *J. Chem. Soc.* 940 (1956).

[4] G. M. K. Hughes and B. C. Saunders, *J. Chem. Soc.* 3814 (1956).

[5] B. C. Saunders and G. H. R. Watson, *Biochem. J.* **46**, 629 (1950).

N-chlorosuccinimide,[6] after attempts to make it in one step from nitromesitylene (cf. preparation of *p*-aminobenzaldehyde[7]) had failed.

Because of the difference in purity of the two compounds, slightly different procedures were adopted for each mixed oxidation. In the case of the amino-alcohol (V), almost equimolecular proportions of it and mesidine were oxidized together, and the effect of the former simply assessed by examination of the product. For the aldehyde, however, two identical solutions of mesidine were prepared, in one of which a sample of VI was dissolved, and the two solutions were then oxidized in parallel. In all these reactions the crude product was worked up by extraction with chloroform followed by chromatography on alumina. The bands were eluted, and the yields of I estimated either spectroscopically or by evaporation.

The results of these experiments were quite unambiguous. From the oxidation of mesidine with V, a far higher yield of I was obtained than could possibly have come from the mesidine alone. On the other hand, identical yields of I were obtained from the oxidation of mesidine in the presence of VI and the "control" containing mesidine alone. These findings appear to indicate that whereas V is either an intermediate in the oxidation of mesidine, or is related to one, VI is not, and the relation between the oxidations of mesidine and mesitol is therefore not as close as was suspected. This tends to confirm our previous findings, when we isolated an intermediate (IMC) from incomplete oxidations of mesidine, for which the imine structure VII was proposed.[8] If this is correct, then II cannot be an intermediate, and the coupling of the two residues must take place before all the oxidation steps have been completed.

The effect of V on the rate of the oxidation was also investigated. This was done by plotting progress curves for the oxidations of mesidine alone, and mixtures containing 4:1 and 3:2 molar ratios of mesidine: amino-alcohol. The total molarity in the three sets of reactions was constant, and, as this was low, the yields of I were conveniently estimated from the optical densities of n-hexane extracts. The low concentration of amines also meant that sufficient hydrogen peroxide could be introduced at the start of the reaction without inhibiting the enzyme.[9] The progress curves obtained in this way (Fig. 1) clearly show that increasing the proportion of the amino-alcohol increases the rate of the reaction without greatly affecting the yield (ca. 90%) of anil.

Two further enzymic oxidations were carried out. These were: (a) of the amino-alcohol (V), and (b) of mesidine itself, to see whether further confirmation of the above results could be obtained. The first of these gave a red compound in 60% yield which was probably[10] VIII, the hydroxymethyl analogue of I, an equivalent quantity of formaldehyde (identified as the dimedone derivative), and 10% of the amino-aldehyde VI. The oxidation of mesidine gave, in addition to the expected high yield of I, a quantity of red-brown oil, which appeared to consist mainly of the amino-aldehyde VI.

In interpreting the above results the first point to be made concerns the effect

[6] M. F. Hebbelynck and R. H. Martin, *Experientia* **5**, 69 (1949).
[7] H. G. Beard and H. H. Hodgson, *J. Chem. Soc.* 4 (1944).
[8] A. G. Holmes-Siedle and B. C. Saunders, *Chem. & Ind.* 164 (1959); and unpublished work.
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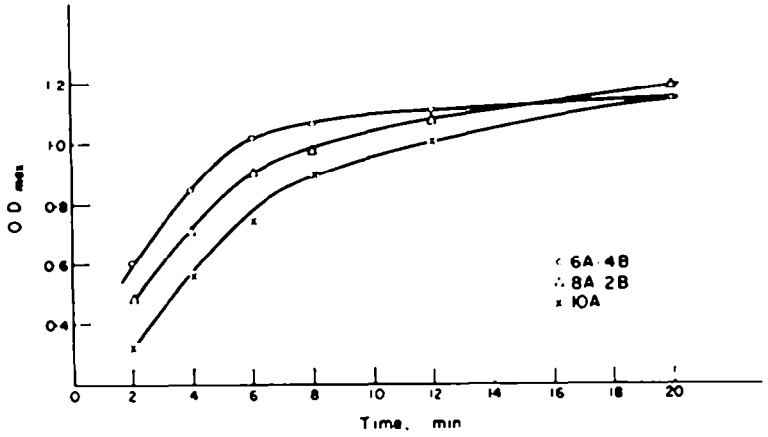

FIG. 1

of V on the rate of the reaction. Such an effect would be observed if the formation of V is the slowest step in the reaction sequence, and a consequence of this is that the concentration of amino-alcohol in an oxidation of mesidine is probably never sufficiently high to interfere with mesidine itself in the coupling reaction. The second point for discussion is the mechanism by which V is formed. Now an intermediate which has previously been proposed in this reaction[1,2] is the imine methide (IX, R = NH) which could be formed by loss of a proton from the ion X. Comparison of this species with the isoelectronic quinone methide (IX; R = O) suggests[11] that in an aqueous medium its characteristic reaction would be a 1,6 addition of water to give V directly. The ion X, which would be obtained from mesidine by the removal of two electrons and a proton (catalysed by peroxidase) is also a satisfactory intermediate, in that it avoids the concept of a free radical in solution. Thirdly, if VI is not an intermediate, then the oxidation of V must be accompanied by the coupling reaction which introduces the second mesidine residue. If this reaction is a nucleophilic attack of the free amine on the ion XI (which would be obtained from a second oxidation step), then it is possible to write a mechanism for the elimination of formaldehyde which gives as the other product the aminodiphenylamine derivative XII. And oxidation of this last compound followed by hydrolysis should give VII, and then I, in high yield.

As a final point, we would explain why we tentatively suggest that the peroxidase oxidation of amines may perhaps involve a two electron change in the face of strong evidence[12] that the oxidations of phenols and ascorbic acid proceed *via* the derived free radicals. In the first place, the products obtained from the oxidations of amines[13] (particularly the proportions of azo derivatives) are not characteristic of free radical intermediates, and secondly, amines are easier to oxidise than phenols (nitrogen being less electro-negative than oxygen[14]), and might therefore perhaps utilise the

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[12] I. Yamazaki, H. S. Mason and L. Piette, *J. Biol. Chem.* **235**, 2444 (1960).

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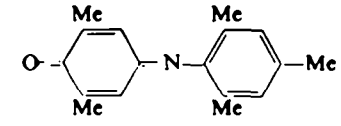
[14] L. Pauling, *The Nature of the Chemical Bond* (3rd Edition) p. 93. Cornell University Press (1960).



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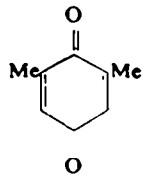

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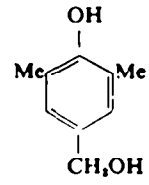

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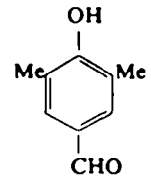

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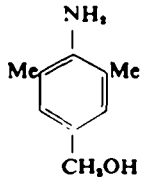

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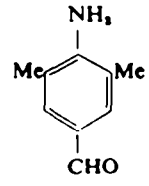

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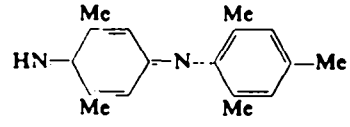

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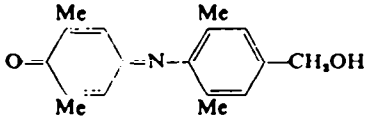

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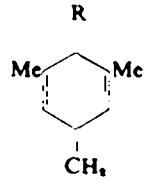

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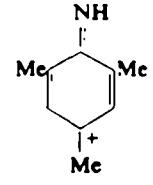

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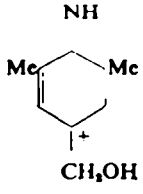

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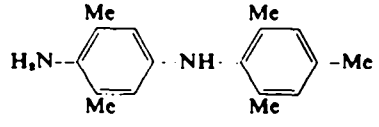

XII

two oxidising equivalents of Peroxidase Complex I without dissociation at the one-electron (Complex II) oxidation level.

EXPERIMENTAL

Materials and equipment. Peroxidase was obtained as a soln, PN 142, a gift from the late Professor D. Keilin, to whom we were grateful. Working solns of the enzyme were prepared by diluting this × 50.

Hydrogen Peroxide: "20 volume" H_2O_2 soln was used, except where otherwise indicated.

All chromatograms were carried out on alumina columns, using a mixture of chf and CCl_4 (b.p. 66–68°) as eluant. The proportion of chf was increased for eluting strongly adsorbed bands.

The IR spectra—Perkin-Elmer "Infracord" Spectrophotometer, and the qualitative and semi-quantitative UV spectra—Perkin-Elmer Model 137 UV spectrophotometer. Quantitative UV spectra Cary UV Recording Spectrophotometer, Model 14.

4-Amino-3,5-dimethylbenzyl alcohol

Methyl-4-amino-3,5-dimethylbenzoate (3·1 g, 0·017 mole) was dissolved in dry ether (100 ml) and added, with stirring, to a soln-suspension of LAH (0·91 g, 0·024 mole) in dry ether (150 ml), during 1½ hr. Stirring was continued for a further 2 hr, giving a light yellow suspension which coagulated when a small quantity of AcOEt followed by a small quantity of water were carefully added. The precipitate was filtered off, and washed with hot MeOH. Evaporation of the filtrate and washings gave 2·7 g of solid which, after recrystallization from chlorobenzene gave colourless needles of *4-amino-3,5-dimethylbenzyl alcohol* (2·0 g, 68%), m.p. 94·5°. (Found C, 71·4; H, 8·9; N, 9·7, 9·1. $C_9H_{13}NO$ requires: C, 71·5; H, 8·7; N, 9·3%.) UV (EtOH): 240 mμ and 286 mμ (log ε 3·99 and 3·305).

4-Amino-3,5-dimethylbenzaldehyde

4-Amino-3,5-dimethylbenzyl alcohol (302 mg, 0·002 mole) was dissolved in chlorobenzene (13·5 ml), and N-chlorosuccinimide (267 mg, 0·002 mole) were added. The mixture was gently warmed, and left to stand overnight. The soln was then dark brown in colour, and a quantity of tarry solid had been deposited. The soln was washed with 1% NaOHaq and then extracted with dil HCl. Basification of this extract, followed by extraction with chf and evaporation gave a viscous red oil (ca. 150 mg, 50%) which did not crystallize. The IR spectrum of this oil had the expected peaks for an amino group (triplet 3450–3250 cm $^{-1}$, and 1640 cm^{-1}), and for an aromatic aldehyde (2700 cm^{-1} and 1675 cm^{-1}), and it gave a *2,4-dinitrophenylhydrazone*, which after recrystallization from EtOH had m.p. 244° (dec).

Mixed oxidation of mesidine and 4-amino-3,5-dimethylbenzyl alcohol

Mesidine (140 mg, $1·03 \times 10^{-3}$ mole) and 4-amino-3,5-dimethylbenzyl alcohol (154 mg, $1·02 \times 10^{-3}$ mole) were dissolved in 1% AcOH (100 ml), giving a soln of pH 4·5. H_2O_2 ($8 \times 0·5$ ml) and peroxidase solution ($10 \times 1·0$ ml) were added over a period of 72 hr., after which further additions of enzyme and peroxide caused no further turbidity in the mixture. The mixture was then extracted with chf until colourless, and the extract, after concentration, was chromatographed. Two bands were observed, of which the major one was rapidly eluted. Evaporation of the eluate gave 209 mg (81% based on total amine present) of a red solid having an IR spectrum identical in every respect with that of the anil obtained from the oxidation of mesidine alone, and which, after recrystallization from aqueous EtOH had m.p. 95° (lit. 96°).

Mixed oxidation of mesidine and 4-amino-3,5-dimethylbenzaldehyde

Mesidine (133 mg, $1·0 \times 10^{-3}$ mole) and 4-amino-3,5-dimethylbenzaldehyde (ca. 50 mg, $0·33 \times 10^{-3}$ mole) were dissolved in 100 ml of 0·1 M acetate buffer, pH 4·7. A second soln was prepared, containing only mesidine (138 mg). These two solutions were oxidized separately and the products examined exactly as described in the preceding section. Results: from mesidine + aldehyde, 103 mg anil (77%), 0·9 mg azomesitylene; from "control", 106 mg anil (77%), 1·2 mg azomesitylene. It must be noted that azomesitylene has not previously been reported as a product of this reaction; its formation may possibly be due to the presence of acetate ion (cf. effect of alcohols and sugars on peroxidase reactions[18]).

Progress curves for the mesidine oxidation

The solutions used were as follows:

A. Mesidine (11·6 mg, $8·6 \times 10^{-5}$ mole/l.) in 0·1M acetate buffer, pH 4·7.

B. 4-Amino-3,5-dimethylbenzyl alcohol (13·0 mg, $8·6 \times 10^{-5}$ mole/l.) in 0·1M acetate buffer, pH 4·7.

[18] B. Matkovics, E. Kovacs and G. Buzas, *Naturwiss.*, **45**, 491 (1958); B. Z. Siegel and S. M. Siegel, *Nature, Lond.* **186**, 391 (1960); B. Z. Siegel, S. M. Siegel and N. S. Goodman, *ibid.* **191**, 180 (1961).

Enzyme. A solution of peroxidase having an optical density of 0·30 at 400 mμ, was diluted accurately ten times. H_2O_2: "20 volume", diluted × 1000.

Method. All reactions used a total of 10 ml (A + B), and 1 ml of peroxide solution. The three ratios of A:B were 10:0, 8:2 and 6:4, and the reactions were carried out in sets of three, one for each ratio, so that the conditions were constant. At time t = 0, 1 ml of enzyme solution was added, with shaking. During the course of the reaction, 10 ml of n-hexane were carefully added to the reaction tube, so that no mixing occurred. At the end of the reaction the tube was securely corked and vigorously shaken so that complete mixing occurred; 5 ml of the organic layer were withdrawn, and its absorption spectrum in the region 260–290 mμ measured. The position and intensity of the absorption maximum was noted.

Precautions. The following points were checked:

(a) The solvent had no absorption above 260 mμ.

(b) In the absence of enzyme, a control experiment gave an optical density of 0·08 at 273 mμ, showing that there was little interference from unchanged mesidine.

(c) The optical densities of the extracts did not change with time.

Results. Optical densities of the absorption maxima:

Time (min)	2	4	6	8	12	20
10 ml A:0 ml B	0·32[a]	0·56[c]	0·74[d]	0·89	1·01	1·15
8 ml A:2 ml B	0·49[b]	0·72[d]	0·91	0·99	1·09	1·19
6 ml A:4 ml B	0·61[c]	0·85[e]	1·02	1·06	1·11	1·15

Positions of the maxima (in mμ): [a] 286; [b] 280; [c] 278; [d] 276; [e] 275.
All other maxima at 273 mμ.

For 2,6-dimethylbenzoquinone-4-(2′,4′,6′-trimethyl)-anil in n-hexane, absorption maxima are at:

272 mμ (log ε 4·49)

485 mμ (log ε 3·12)

Optical density for 100% yield in above conditions: 1·26.

Oxidation of 4-amino-3,5-dimethylbenzyl alcohol

4-Amino-3,5-dimethylbenzyl alcohol (500 mg, $3·3 \times 10^{-3}$ mole) was dissolved in 1% AcOH (100 ml), and oxidized with peroxidase and H_2O_2. At the end of the reaction the supernatant liquid was filtered from the red precipitate, and treated with a 10% solution of dimedone in EtOH. A precipitate was obtained (284 mg), which, after recrystallization from aqueous EtOH, had m.p. 195° (lit. 189–192°)[9] and mixed m.p. with sample of dimedone derivative of formaldehyde: 193°.

The residue from the reaction was dissolved in chf, which was washed with dil HCl and chromatographed. Four bands were observed on the chromatogram, from the fourth of which 300 mg of a red solid were obtained; the IR spectrum of this solid had a strong peak at 1640 cm^{-1} (quinonoid C=O), and peaks at 3250 cm^{-1} and 1140 cm^{-1} (—C–O–H). The UV spectrum had maxima at 277 mμ (high intensity) and 486 mμ (low intensity) in EtOH soln. The other three bands produced insignificant quantities of red solids. The acid extract gave 48 mg of an oil whose IR spectrum was identical with that of 4-amino-3,5-dimethylbenzaldehyde; as this compound is slightly soluble in water, the total yield was probably greater.

Oxidation of mesidine

Mesidine (0·5 g, $3·7 \times 10^{-3}$ mole) was oxidized with peroxidase and H_2O_2 as described in the preceding section. At the end of the reaction the whole of the reaction mixture was extracted with chf (the usual method of separation being filtration) and chromatographed. In addition to the usual quinone anil in 80% yield, a strongly adsorbed red band was observed, which, on elution and evaporation, gave 32 mg of red-brown oil whose IR spectrum again indicated that it consisted mainly of the amino-aldehyde.

One of us (J. W.) thanks the D.S.I.R. and the Trustees of the Dowager Countess Peel Trust for maintenance allowances.