

86. Studies in Peroxidase Action. Part III. The Oxidation of Mesidine.

By NORMAN B. CHAPMAN and BERNARD C. SAUNDERS.

The system, dilute aqueous hydrogen peroxide and the enzyme peroxidase, has been shown to oxidise mesidine at room temperature and at p_H 4.0—4.7 (dilute acetic acid solution). A reddish-purple coloration is produced immediately, and in the course of time a solid of similar colour separates. From this solid a highly crystalline purple compound has been isolated in high yield, and has been shown to be 2 : 6-dimethylbenzoquinone-4-(2' : 4' : 6'-trimethyl)anil. Its formation involves the loss of a methyl group, and the oxidation may throw some light on the mechanism of peroxidase action. The compound has also been synthesised chemically.

The action of hydrogen peroxide and ferrous sulphate (in place of the enzyme) on mesidine dissolved in dilute acetic acid is different from the reaction described above: only traces of the purple compound are obtained, the remainder of the product being ill-defined amorphous material.

IN Part I (P. J. G. Mann and Saunders, *Proc. Roy. Soc.*, 1935, B, **119**, 47) and Part II (Saunders and P. J. G. Mann, J., 1940, 769) the detailed investigation was recorded of the action of the enzyme peroxidase in the presence of dilute aqueous hydrogen peroxide on aniline and on *p*-toluidine respectively. The nature of the oxidation in each of these cases was necessarily complex because of the large number of "free points" in the molecule at which oxidation and condensation could take place: aniline gave rise to five compounds and *p*-toluidine to eight.

This work has now been extended to mesidine (2 : 4 : 6-trimethylaniline) as substrate. Since this compound has only two free nuclear hydrogen atoms, it seemed likely that only a very restricted number of compounds could be produced, and that a knowledge of their constitution might throw some light on the mechanism of peroxidase oxidation.

There is no reference in the literature to the action of the enzyme on this amine. For most of our experiments an enzyme preparation obtained from turnips (see F. G. Mann and Saunders, "Practical Organic Chemistry," p. 372) was used. In a few experiments we used a highly purified specimen of the enzyme prepared from horseradish (see Keilin and T. Mann, *Proc. Roy. Soc.*, 1937, B, **122**, 119). Similar reactions took place with each type of preparation, thus proving that the crude enzyme obtained from turnips is satisfactory for these oxidations. Control experiments were carried out with the enzyme in the absence of hydrogen peroxide, with the heat-inactivated enzyme in the presence of hydrogen peroxide, and with hydrogen peroxide alone. In all these cases no reaction was detectable, and therefore it may be concluded that the oxidations described below were brought about by peroxidase action.

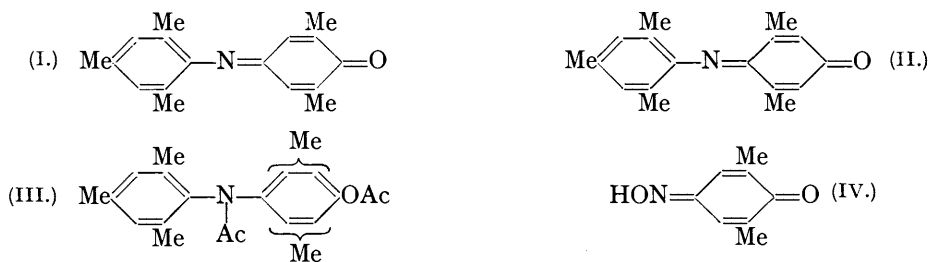
For most of the oxidations a 2% solution of mesidine in dilute acetic acid was used, the p_H being 4.0—4.7. An aqueous solution of the enzyme preparation was added to the amine solution, and hydrogen peroxide was added in small quantities at a time, since excess of it inhibits peroxidase action. The reaction mixture soon became deep reddish-purple, and gradually a reddish-purple solid separated.

When crude enzyme preparations were used, this solid gave 85% of a purple crystalline compound, the remainder consisting of ill-defined amorphous material. With the more highly purified enzyme preparation no amorphous material was formed, and the yield of the purple crystalline compound rose to 95%. Neither nitroso- nor nitro-compounds were detected, an observation which falls into line with our previous investigations on the enzymatic oxidations of aniline and of *p*-toluidine. The absence of 2 : 4 : 6 : 2' : 4' : 6'-hexamethylazobenzene is noteworthy (see below). Chromatographic analysis of the crude oxidation product confirmed the absence of other compounds.

The enzymatic oxidation was also carried out in a dilution of one part of amine in 2,000 parts of solution with the same results, and this probably by no means represents the limit of dilution at which the reaction can take place.

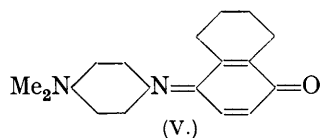
Elementary analysis and molecular-weight determinations showed that the purple

crystalline compound had the molecular formula $C_{17}H_{19}ON$, indicating that it was formed from two mesidine nuclei with the elimination of a carbon atom (as a methyl group). The conjecture was confirmed by hydrolysing the compound with 10% sulphuric acid, mesidine and 2:6-dimethylbenzoquinone being produced. Thus the constitution



of the purple compound appeared to be either 2:6-dimethylbenzoquinone-4-(2':4':6'-trimethyl)anil (I), or the corresponding 3:5-dimethyl compound (II), for both would give the above hydrolysis compounds. The purple compound liberated iodine from acidified potassium iodide solution and was decolorised by aqueous-alcoholic sulphur dioxide. Reductive acetylation gave the colourless neutral diacetyl derivative of 4-hydroxy-2:6:2':4':6'- (or 3:5:2':4':6')-pentamethyldiphenylamine (III).

We synthesised the purple compound in good yield by condensing mesidine with 2:6-dimethylbenzoquinone, thus definitely establishing the constitution as (I) or (II). We were also able to show that mesidine would not condense with duroquinone (2:3:5:6-tetramethylbenzoquinone), undoubtedly because of the influence of the *o*-methyl groups. Hence it is extremely probable that it is the >CO group at position 4 of 2:6-dimethylbenzoquinone which reacts with mesidine, and therefore the purple compound has the constitution (I). Furthermore, the fact that 2:3:6-trimethylbenzoquinone reacts with only one molecule of hydroxylamine (Kehrmann, *Ber.*, 1888, **21**, 3315; *J. pr. Chem.*, 1889, **39**, 392) indicates that 2:6-dimethylbenzoquinone must react at position 4 and not at position 1. Finally, we showed that mesidine would not condense with 5-nitroso-*m*-2-xylenol, and, the latter being assumed to be tautomeric with the monoxime form (IV), further evidence is provided in support of formula (I).



The only recorded oxidation of mesidine is that by chromic anhydride and sulphuric acid (Nölting and Baumann, *Ber.*, 1885, **18**, 1151), dimethylbenzoquinone being produced. Lastly, we were able to produce evidence of a different kind in favour of formula (I). It is known (Köchlin and Witt, *Ber.*, 1883, **16**, 2851; 1885, **18**, 2931) that *p*-aminodimethylaniline will condense with α -naphthol in the presence of certain oxidising agents (*e.g.*, potassium dichromate), giving the compound (V). Employing similar conditions of oxidation, we carried out a series of experiments with the following results:

Oxidation of	Yield of purple compound, %.
(1) Mesidine alone	8
(2) „ + <i>m</i> -2-xylenol (equimol. quantities)	26
(3) „ + <i>m</i> -5-xylenol (equimol. quantities)	0—1

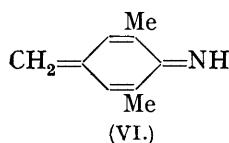
It follows then that, although mesidine gives rise to some of the purple compound, *m*-2-xylenol* is necessary for the formation of the compound in increased yield. The negligible yield in the presence of *m*-5-xylenol is significant, and all these facts suggest that formula (I) should be assigned to the oxidation product of mesidine by peroxidase.

The product from the oxidation of mesidine with dilute hydrogen peroxide solution and ferrous sulphate as catalyst was shown to contain only traces of (I) and consisted mainly of ill-defined amorphous material.

* The xylenol alone is oxidised by chromic anhydride to 3:5:3':5'-tetramethyldiphenquinone (4:4') (Auwers and Markovits, *Ber.*, 1905, **38**, 226). Hence in the reaction with mesidine, coupling probably takes place before oxidation.

With reference to the possible mechanism of the peroxidase oxidation of mesidine, the first point of interest is the absence of azomesitylene among the products. If the free radical $C_6H_2Me_3 \cdot NH$ or $C_6H_2Me_3 \cdot N <$ is the primary product formed by the dehydrogenating action of the peroxidase system, then one might expect the production of the azo-compound in high yield, especially as the *p*-position in mesidine is blocked. The fact, however, that the free radicals would exhibit resonance and might not therefore couple up symmetrically (*i.e.*, through the nitrogen atoms) must not be overlooked. We have, however, shown that mesidine in ethereal solution is oxidised in the cold by lead peroxide with the production of azomesitylene: these being the conditions used by Goldschmidt (*Ber.*, 1920, 53, 35) in his experiments on amines which led him to formulate his free-radical hypothesis of oxidation.

As we have seen, a methyl group is removed during the peroxidase oxidation of mesidine, a reaction which can be carried out at room temperature and at a dilution as high as one part of the amine in 2000 parts of solution. Therefore one explanation, though perhaps not very probable, is that dehydrogenation first produces the methylenequinone-



imine (VI), followed by its conversion into 2:6-dimethylbenzoquinone. In support of this we have shown that the condensation between 2:6-dimethylbenzoquinone and mesidine is a very facile one, and even takes place at dilutions corresponding to those employed in the enzymatic oxidation. The hydrolysis of the imino-group of (VI) presents no difficulty, as it was shown that during the oxidation exactly 1 mol. of ammonia was produced from 2 mols. of mesidine. The more difficult problem is to account for the loss of the methylene group, which might be removed as formaldehyde by the hydrogen peroxide acting alone. Against this, however, it has not been possible to obtain any definite evidence of its formation during the reaction, and furthermore, we have shown that formaldehyde is destroyed only very slowly by hydrogen peroxide, even in the presence of peroxidase. A very tentative alternative view is that methane may be produced by the direct hydrolysis of the $CH_2:C <$ group, but we have no evidence for this. In any case the methylenequinone, which does not appear to have been described, would undoubtedly be a very reactive compound.

EXPERIMENTAL.

The Enzyme Preparation.—(A) A crude product (containing some ammonium sulphate) was prepared as indicated on p. 496, and 0.3 g. was ground with 20 c.c. of water, and the suspension centrifuged. (B) A purer product (of purpurogallin number about 120), free from ammonium sulphate and colloidal matter, was prepared according to the method of Keilin and T. Mann (*loc. cit.*), and 0.05 g. dissolved in 20 c.c. of water for use.

Oxidation of Mesidine by Peroxidase.—Mesidine was prepared from nitromesitylene ("Organic Syntheses," 14, 68) and had b. p. 232–233°. Mesidine (10 g.) and glacial acetic acid (11.5 c.c.) were mixed with water (500 c.c.), and the mixture thoroughly shaken. Glacial acetic acid was then added drop by drop until a clear solution was just formed, the p_H being approximately 4.7. To this solution were added hydrogen peroxide (1 c.c., 20-vol.) and enzyme solution A (4 c.c.), and the mixture well shaken. A pale red coloration was produced immediately and gradually deepened to a dark red. Hydrogen peroxide was then added at the rate of 1 c.c. per $\frac{3}{4}$ hour until in all 40 c.c. had been added. At 3-hourly intervals further quantities (of ca. 2 c.c.) of enzyme solution were added. Ultimately a reddish-purple solid separated, and when reaction was complete this was filtered off, washed free from acetic acid, and dried. Yield 9 g.

Identical colour changes were observed on using enzyme preparation B, but the solid which separated was crystalline. Control experiments showed that no coloration or precipitate was produced by treating mesidine with hydrogen peroxide (20-vol.) alone or together with boiled enzyme solution or ammonium sulphate solution, or with enzyme solution alone.

The Solid Oxidation Product.—The crude product (5 g.) was steam-distilled, and a residue (0.75 g.) of non-volatile, ill-defined amorphous material remained (when preparation A was used, see p. 496). The steam-volatile portion condensed to a purple solid (4.25 g.) which recrystallised from aqueous alcohol in glistening purple plates, m. p. 97° (azomesitylene has m. p. 75°).

The compound (I) was readily soluble in most organic solvents, sublimed under reduced

pressure, and became colourless on reduction either with aqueous-alcoholic sulphur dioxide or with zinc dust and dilute acetic acid. It liberated iodine from acidified potassium iodide solution, and with nitrous acid gave neither a diazonium compound nor a nitrosoamine (Found : C, 80.3; H, 7.2; N, 5.6; M, semi-microbullioscopic in 2.81% chloroform solution, 240. $C_{17}H_{19}ON$ requires C, 80.7; H, 7.5; N, 5.6%; M, 253).

Examination of the Filtrate.—Enzyme preparation B was used. (a) Aliquot portions of the filtrate (1155 c.c.) were boiled with sodium hydroxide, and the ammonia evolved absorbed in *N*-sulphuric acid (Found : NH_3 , 0.25 g. Calc., on assumption of 100% yield of purple compound : NH_3 , 0.26 g.). (b) In the case of incomplete oxidations, an oil was precipitated by alkali, and consisted of unchanged mesidine and some of the oxidation product; in the most complete oxidations the filtrate contained only traces of organic matter. (c) The Schiff-Elvove reagent (Snethlage, *Chem. Weekblad*, 1929, 26, 611) gave a positive reaction for formaldehyde, whereas 2 : 4-dinitrophenylhydrazine and dimedone failed to give the corresponding derivatives of formaldehyde.

Reductive Acetylation of 2 : 6-Dimethylbenzoquinone-4-(2' : 4' : 6'-trimethyl)anil (I).—The compound (0.2 g.) was dissolved in excess of acetic anhydride and 1 drop each of pyridine and acetic acid and 0.5 g. (excess) of zinc dust were added. After being heated under reflux for 20 minutes, the mixture was decolorised; it was then filtered hot from the excess of zinc, and poured into ice-water. An oil separated which, on standing and scratching, solidified. The solid so obtained was recrystallised from a concentrated solution in methyl alcohol until pure, colourless leaflets of the ON-diacetyl derivative of 4-hydroxy-2 : 6 : 2' : 4' : 6'-pentamethyl-diphenylamine (III) were obtained : m. p. 143° (Found : C, 74.2; H, 7.4; N, 4.9. $C_{21}H_{25}O_3N$ requires C, 74.3; H, 7.4; N, 4.1%). The compound was neutral, and not readily hydrolysed by *N*-sulphuric acid.

Hydrolysis of the Anil (I).—The anil (0.5 g.) was heated under reflux with sulphuric acid (30 c.c., 10%) for 30 minutes. An oil distilled in the steam and solidified as yellow crystals on the condenser walls. The solid was washed out with acetone, the acetone evaporated, and the resultant solid recrystallised from light petroleum, forming bright yellow needles, m. p. 72°. It sublimed readily, decomposed on standing, and gave a mixed m. p. of 72.5° with an authentic specimen of 2 : 6-dimethylbenzoquinone, m. p. 72°, prepared according to the method of Nölting and Baumann (*loc. cit.*).

The residual acid liquid did not contain any steam-volatile substance, but when it was basified and steam-distilled, an oil was obtained; this was extracted with ether, the extract dried (potassium hydroxide), and the ether distilled off. A pink oil remained and was acetylated by 50 mins.' boiling with excess of acetic acid and anhydride (equal vols.), pouring into water, and recrystallisation of the resulting solid from alcohol; colourless needles, m. p. 217—218°; mixed m. p. with authentic acetylmesidine (m. p. 217—218°), 218°. Hence the products of hydrolysis were mesidine and 2 : 6-dimethylbenzoquinone.

Condensation of 2 : 6-Dimethylbenzoquinone with Mesidine.—(i) *Under the conditions of the enzymatic oxidation.* Mesidine (0.1 g.) was dissolved in a mixture of a water (10 c.c.) and glacial acetic acid (0.2 c.c.). Dimethylbenzoquinone (0.1 g.; ca. 1 mol.) was added with shaking, a trace of acetone being added to ensure complete solution of the quinone. The mixture was kept for 2 days, and the supernatant liquid then poured off, and the residual solid recrystallised from aqueous alcohol; it formed lustrous purple plates, m. p. 96°; mixed m. p. with the anil (I) prepared by enzymatic oxidation, 95°.

(ii) *Without a solvent.* On addition to mesidine of an equal weight of 2 : 6-dimethylbenzoquinone, an immediate purple coloration was produced; it was not destroyed by the addition of solvents, thus showing that a "true" compound and not a molecular compound had been formed. The product on crystallisation from aqueous alcohol gave purple crystals, m. p. 96°; mixed m. p. with (I), 96°.

Reaction between Mesidine and Duroquinone.—Equimolecular quantities of the two reactants were melted together, and a red coloration was produced. This was, however, discharged on addition of light petroleum, showing that only a molecular compound had been produced (cf. Jackson and Clarke, *Amer. Chem. J.*, 1905, 34, 441).

*Oxidation of a Mixture of Mesidine and *m*-2-Xylenol by Chromic Acid.*—Mesidine (1.35 g., 0.01 mol.) and *m*-2-xylenol (1.22 g., 0.01 mol.) were suspended in water (136 c.c.), and sodium hydroxide (0.45 g.) dissolved in water (4 c.c.) was added. The mixture was well shaken and then a solution of potassium dichromate (0.85 g.) in water (30 c.c.) was added. The whole was vigorously shaken and kept at room temperature for 45 minutes, during which there was no change of colour. The mixture was acidified with glacial acetic acid and shaken at room

temperature for 12 hours. The product was made strongly alkaline and extracted with ether. The ether was dried and distilled, a deep red oil (A) remaining. The aqueous layer was acidified with dilute sulphuric acid and extracted with ether, which on distillation left a brown oil (B).

The oil (A) was distilled in steam, and a purple solid obtained (0.65 g. ; 26%) which crystallised from aqueous alcohol in reddish-purple plates, m. p. 96°; mixed m. p. with (I), 97°. The oil (B) was shaken with sodium carbonate solution to remove acetic acid, and the aqueous layer extracted with ether. The ether was distilled, and on steam-distilling the residue a small quantity (about 0.1 g.) of unchanged *m*-2-xylenol was obtained. Some tarry material remained after the steam-distillation.

A mixture of mesidine and *m*-5-xylenol was similarly oxidised; the oil (A) gave only 0.025 g. of steam-distilled purple solid (1% yield). When mesidine (2.7 g.) was oxidised in this manner it gave an oil (A) as above, from which 0.19 g. of steam-distilled, purple solid was obtained (8% yield).

Oxidation of Mesidine by Hydrogen Peroxide and Ferrous Sulphate.—To a solution of mesidine (3.3 g.) in water (100 c.c.) and glacial acetic acid (11.8 c.c.) were added hydrogen peroxide (0.3 c.c. ; 20-vol.) and ferrous sulphate (1 c.c. of 3% aqueous solution). A reddish coloration was produced and hydrogen peroxide was added at the rate of 0.3 c.c. per $\frac{3}{4}$ hour until in all 15 c.c. had been added. Further quantities of about 0.5 c.c. of ferrous sulphate were added at 3-hourly intervals. The product consisted of a mixture of a dark red solid and a considerable amount of tarry matter. These were worked up separately and found to contain only traces of the purple anil (I) together with much brown, amorphous material.

Oxidation of Mesidine in Ethereal Solution by Lead Peroxide.—Mesidine (3 g.), anhydrous ether (220 c.c.), glacial acetic acid (9 c.c.), and dry lead peroxide (30 g.) were mixed and shaken in a stoppered bottle for 4 hours. The mixture was then filtered from inorganic matter, and the ethereal solution washed with sodium carbonate solution, dried, and evaporated; the partly crystalline mass obtained was dissolved in light petroleum and submitted to chromatographic analysis upon aluminium oxide, the continuous elution apparatus of Mann and Saunders (J., 1940, 772) being used. A lower red band on elution with light petroleum and subsequent concentration gave pure crystalline azomesitylene, m. p. 75°. An upper yellow band, eluted with benzene, gave on evaporation only traces of an unidentified crystalline material.

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THE UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE.

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