Studies in Peroxidase Action

4. THE OXIDATION OF 4-METHOXY-2:6-DIMETHYLANILINE. SUGGESTIONS FOR A MECHANISM

BY B. C. SAUNDERS AND G. H. R. WATSON
Chemical Laboratory, University of Cambridge

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In Part 3 of this series (Chapman & Saunders, 1941) it was shown that mesidine (2:4:6-trimethylaniline) was oxidized at pH 4.5 by hydrogen peroxide and peroxidase, with the facile loss of a methyl group to a purple crystalline compound which proved to be 2:6-dimethylbenzoquinone-4(2':4':6'-trimethyl)-anil. The yield, using highly purified peroxidase, was 95%. It has now been shown manometrically that mesidine is not oxidized by indophenol oxidase, catechol oxidase or laccase (Keilin & Saunders, unpublished work).

In the reaction mentioned above the elimination of a methyl group at room temperature was unexpected. Attempts to ascertain the fate of the methyl group were not very conclusive. The main purpose of the present investigation was to examine the peroxidase oxidation of some related amine in which the *para* substituent was again likely to be eliminated, but was of such a nature that it might be possible to trace the products of fragmentation.

For this purpose we selected 4-methoxy-2:6-dimethylaniline, the synthesis of which is described later. Using the purified enzyme, and carrying out the oxidation at pH 4.5, a crystalline purple compound of m.p. 87–88° was obtained. The compound had an empirical formula $C_{17}H_{19}O_4N$ and contained a methoxyl group. On hydrolysis it gave 4-methoxy-2:6-dimethylaniline (I) and 2:6-dimethyl-*p*-benzoquinone (II). Reductive acetylation gave the

mono-acetyl derivative of a 4-hydroxy-4'-methoxy-tetramethyl-diphenylamine (III), the precise constitution of which is discussed below.

It seemed possible that the oxidation product might be synthesized from the hydrolysis products (I) and (II). Accordingly these two compounds were fused together in equimolecular quantities, a dark red product being obtained. This was recrystallized

from aqueous ethanol as dark purple needles (darker than the actual oxidation product) of m.p. 36°. It was therefore not identical with the oxidation product. Analysis indicated that it was an *addition*

compound of 1 molecule of (I) and 1 molecule of (II). This was confirmed by the fact that it could be separated chromatographically into its components, and, moreover, the solution in light petroleum became progressively lighter in colour on dilution. This addition compound was probably of the 'quinhydrone' type.

In a second experiment the amine (I) was dissolved in dilute acetic acid and the pH adjusted to 4.5. One molecular proportion of 2:6-dimethyl-*p*-benzoquinone dissolved in ethanol was then added slowly, and on standing, fine deep purple needles separated. When recrystallized from ethanol these had m.p. 78.5°. The analysis corresponded with a molecular compound (IV) of an anil $C_{17}H_{19}O_4N$ with the quinone (II). Chromatography of this new compound gave two well defined components, viz:

2:6-dimethyl-*p*-benzoquinone and a compound identical in every respect with the oxidation pro-

duct from the peroxidase reaction. Furthermore, reductive acetylation of (IV) gave the substituted diphenylamine (III). These facts showed that the oxidation product was either 2:6-dimethylbenzoquinone-4-(4'-methoxy-2':6'-dimethyl) anil (V) or 3:5-dimethyl-benzoquinone-4-(4'-methoxy-2':6'-dimethyl)anil (VI). At first sight, however, it

seemed puzzling that reductive acetylation of the original oxidation product and of (IV) gave only the monoacetyl derivative (III) and not the *ON*-diacetyl derivative. Steps were then taken to determine whether the monoacetyl derivative was the *O*- or the *N*-compound. When (III) was treated with concentrated sulphuric acid and a drop of very dilute nitric acid, a transient red coloration was obtained, which on standing changed to yellow. The production of a coloration with this oxidizing agent is consistent with the diphenylamine structure shown. Attempts to acetylate (III) further by acetic anhydride, acetic anhydride-acetic acid mixtures and acetyl chloride-pyridine failed. This resistance to acetylation led us to examine the acetylation of 4-methoxydiphenylamine itself. Using an acetic anhydride-acetic acid mixture, a red gum was obtained, but no crystalline acetyl derivative was isolated under the conditions used. The formation of a red colour indicated that a certain amount of oxidation was probably taking place. It is not therefore surprising that the reductive acetylation of the anil should tend to stop with the formation of the *O*-acetyl derivative.

The choice between formulae (V) and (VI) for the oxidation product did not prove difficult for we

found that 2:3:5:6-tetramethyl-*p*-benzoquinone (VII) would not condense with the amine (I) although it would form an addition compound.

Evidently the methyl groups cause steric hindrance. This suggests that formula (V) is correct and that its synthesis is effected as follows:

It is known that *p*-nitrosodimethylaniline will condense with phenol (acting in the tautomeric quinonoid form). We have shown that 3:5-dimethylphenol (VIII) will not condense with *p*-nitroso-

dimethylaniline, thus providing further evidence of the steric effect of the *o*-methyl groups, and hence giving additional support in favour of formula (V).

In the peroxidase oxidation of mesidine (Chapman & Saunders, 1941) a methyl group was lost, but the fate of the methyl group was obscure. In the present experiment with 4-methoxy-2:6-dimethylaniline, the filtrate after the removal of the solid oxidation product was made alkaline and treated as described in the Experimental section. Besides ammonia, the presence of methanol was established by the isolation of a small quantity of its 3:5-dinitrobenzoyl derivative. The formation of (V) may therefore be represented thus:

It should be emphasized that the first stages shown above represent only the overall oxidation of (I). If the primary product were the free radical $\text{MeO} \cdot \text{C}_6\text{H}_2\text{Me}_2 \cdot \text{NH} \cdot$ we should expect the azo compound to be produced in good yield, but this compound was not produced in detectable quantity.

A very tentative explanation of this peroxidic conversion of (I) into (II) is now made as follows. It is suggested (cf. Saunders, 1949) that an intermediate (X) might be formed. This is both a hemiketal and a ketone-ammonia and such groupings are known to be very unstable, particularly in aqueous solution. The facile elimination of methanol and ammonia with the simultaneous production of (II) would therefore be the expected course of the reaction. The formation of (X) might be explained by assuming an initial transfer of two π electrons from (I) and their localization as p -electrons giving the diradical (IX). The contribution of (IX) to the

the production of a hydroxyl radical. A transition state may also be formulated with the hydrogen peroxide attached to the p -substituent.

Other alternative mechanisms for the conversion of (I) into (II) are clearly possible such as, for example, the initial removal of an electron from (I) giving (XII), which in the form (XIIa) would, after reaction with hydrogen peroxide, pass subsequently to (II).

We would emphasize that the above suggestions are intended merely to serve as convenient working hypotheses for this type of oxidation. In this connexion, however, it is interesting to note that methyl 4-amino-3:5-dimethylbenzoate (XIII) failed to give a coloration when treated with hydrogen peroxide and peroxidase at pH 4.5. Such a compound containing a strong electron-attracting substituent might perhaps be less likely either (a) to react directly with hydrogen peroxide as above, or (b) to permit the removal of an electron for the

real structure of 4-methoxy-2:6-dimethylaniline must be very small, and to achieve the conversion $\text{I} \rightarrow \text{IX}$ the resonance energy of the aromatic system must be supplied. The diradical may then react with the hydrogen peroxide giving (X). Such a reaction does not imply the presence of hydroxyl radicals in the system, but merely suggests that (IX) may cause a homolytic split of hydrogen peroxide into hydroxyl radicals (cf. Mackay & Melville, 1949) which then immediately react with (IX).

suggested alternative mechanism. In fact the oxidizability of a compound by the peroxidase system may depend primarily upon the tendency to such electronic changes. *p*-Nitroaniline, for example, does not appear to be oxidized by peroxidase.

Any mechanism along the above lines for the oxidation of mesidine (Chapman & Saunders, 1941), would seem to require the loss of ammonia and methane in giving rise to the quinone. Chapman & Saunders obtained a quantitative yield of ammonia,

It is not unlikely, however, that the reaction of (I) with hydrogen peroxide actually involves an activated complex such as (XI), but this again implies

but were unable to decide the fate of the methyl group and did not detect formaldehyde which might result from a simple dehydrogenation mechanism.

To test the above suggestion, we propose to select as substrate a similar compound in which, however, the *p*-methyl group is replaced by a group which might give rise to a more readily detectable hydrocarbon.

Our substrate, 4-methoxy-2:6-dimethylaniline, had hitherto been prepared by laborious methods and accordingly we devised a new and more efficient synthesis as follows. 3:5-Dimethylphenol was coupled with benzenediazonium chloride and the phenolic hydroxyl group of the resulting azo compound methylated with dimethyl sulphate. The methoxyazo compound was then reduced with hydrogen and Raney nickel giving 4-methoxy-2:6-dimethylaniline.

An improved synthesis of methyl 4-amino-3:5-dimethylbenzoate was also effected by first oxidizing nitromesitylene with chromic anhydride in glacial acetic acid to 4-nitro-3:5-dimethylbenzoic acid. This on catalytic reduction gave the corresponding amino-acid, which was then esterified with methanol and hydrogen chloride giving methyl 4-amino-3:5-dimethylbenzoate (m.p. 104–106°). Meyer (1904) gave m.p. 93°. His analysis, however, was not good and so his specimen was probably impure.

EXPERIMENTAL

All m.p.'s are uncorrected.

4-Hydroxy-2:6-dimethyl-azobenzene (cf. Auwers & Michaelis, 1914). 3:5-Dimethylphenol (10 g.) was dissolved in aqueous NaOH (100 ml., 10%, w/v) and the solution cooled. Aniline (7.8 g.) was diazotized (NaNO_2 6.5 g., 6*N*-HCl 30 ml.) and added slowly to the cold phenol solution, with stirring. The resulting mixture was allowed to stand at 5° for 30 min. and the solid product filtered off as a pale orange feathery mass (24.6 g.). This was digested with a mixture of 400 ml. of glacial acetic acid and 400 ml. of methanol, the mixture filtered and the filtrate poured into water (2.5 l.). On standing, 4-hydroxy-2:6-dimethyl-azobenzene separated and was recrystallized from light petroleum (b.p. 80–100°) as deep-red needles: m.p. 100–101°. Yield 12 g. (Found: N, 12.7. Calc. for $\text{C}_{14}\text{H}_{14}\text{ON}_2$: N, 12.4%.)

By this process 3:5-dimethylphenol-2:4-bisazobenzene (Auwers & Michaelis, 1914) was also produced, but this remained in solution when the reaction product was treated with 1:1-acetic acid-methanol mixture.

4-Methoxy-2:6-dimethyl-azobenzene. The foregoing mono-azo-compound (6.4 g.) was shaken with dimethyl sulphate (4.5 g.) and NaOH (15 ml., 10% w/v) for 30 min. The product was filtered and recrystallized from aqueous ethanol as deep red plates: m.p. 53°. Yield 5.5 g. (Found: N, 11.8. Calc. for $\text{C}_{18}\text{H}_{18}\text{ON}_2$: N, 11.7%.)

4-Methoxy-2:6-dimethylaniline. 4-Methoxy-2:6-dimethyl-azobenzene (38 g.) was hydrogenated in ethanol at 100–110 atm. for 1 hr. using Raney Ni (30 g.) as catalyst. The product was filtered, the ethanol distilled off and the residue fractionated giving the amine of b.p. 135°/12 mm. This solidified to colourless plates which were recrystallized from light petroleum (b.p. 40–60°) and had m.p. 41°. (Found: N, 9.38. Calc. for $\text{C}_9\text{H}_{13}\text{ON}$: N, 9.42%.)

Oxidation of 4-methoxy-2:6-dimethylaniline. (a) On a small scale. The amine (0.41 g.) was dissolved in water (20 ml.)

containing acetic acid (0.5 g.). The pH was about 4. A dilute solution of peroxidase (0.5 ml. of P.N. 100)* was added, followed by H_2O_2 (0.2 ml., 20 vol.). Further similar quantities of H_2O_2 were added every 20 min. and the enzyme (0.1 ml.) every 30 min. The following changes were noted. On the addition of H_2O_2 a reddish purple coloration was produced immediately. This gradually deepened and after about 30 min. purple solid began to separate. When the addition of H_2O_2 (3 ml.) was complete, the mixture was allowed to stand for 18 hr. and then filtered giving the crude crystalline product (0.255 g.). The 2:6-dimethylbenzoquinone-4-(4'-methoxy-2':6'-dimethyl) anil was recrystallized from aqueous ethanol: m.p. 87–88°. (Found: C, 76.2; H, 6.85; N, 5.42; MeO, 10.9%. Mol.wt. cryoscopic in benzene, 259. $\text{C}_{17}\text{H}_{19}\text{O}_2\text{N}$ requires C, 75.8; H, 7.05; N, 5.2; MeO (one), 11.4%. Mol.wt. 269.)

(b) On a larger scale. The amine (6.8 g.) was dissolved in glacial acetic acid (8.2 ml.) and diluted with water (350 ml.). The whole was divided into 5 equal parts, and to each was added the enzyme solution (1 ml.). Hydrogen peroxide (1 ml.) was added every half hour until 6 ml. had been added (i.e. 30 ml. for the complete batch). Further small quantities of enzyme were also added at intervals. After standing for 18 hr., the solutions were combined and filtered giving the crude oxidation product (4.99 g.).

Hydrolysis of the oxidation product. The substance (1 g.) was heated under reflux with H_2SO_4 (60 ml., 10%, w/v), when a yellow sublimate collected on the cooler parts of the condenser. After 45 min. heating, the sublimate was washed out of the condenser with ethanol which, on concentration, gave 2:6-dimethyl-*p*-benzoquinone, m.p. and mixed m.p. with authentic specimen, 70.5°.

The acid solution was made alkaline with NaOH solution, extracted with ether, dried (Na_2SO_4), the ether distilled off and the residue fractionated giving pure 4-methoxy-2:6-dimethylaniline, b.p. 134°/12 mm.

Reductive acetylation of oxidation product. The compound (0.5 g.) was heated under reflux with acetic anhydride (60 ml.), pyridine (0.1 ml.), acetic acid (0.1 ml.) and zinc powder (about 0.5 g.). After 0.5 hr., the product was filtered and poured into ice water. On scratching, a solid separated which was recrystallized from aqueous methanol. 4'-Acetoxy-4-methoxy-2:6:3':5'-tetramethyldiphenylamine was thus obtained as colourless needles, m.p. 127.5°. (Found: C, 73.0; H, 7.34; N, 4.7. $\text{C}_{19}\text{H}_{23}\text{O}_3\text{N}$ requires C, 72.9; H, 7.35; N, 4.63%.)

Fusion of 4-methoxy-2:6-dimethylaniline and 2:6-dimethyl-*p*-benzoquinone. The amine (0.5 g.) and the quinone (0.5 g.) were fused and allowed to cool. The product was recrystallized from aqueous ethanol as very dark purple glistening plates, m.p. 36°. The solution in light petroleum was red. (Found: C, 71.5; H, 7.17; N, 5.21. The addition compound of the amine and quinone, $\text{C}_9\text{H}_{13}\text{ON}$, $\text{C}_6\text{H}_6\text{O}$ requires C, 71.4; H, 7.31; N, 4.89%.)

Synthesis of 2:6-dimethylbenzoquinone-4-(4'-methoxy-2':6'-dimethyl) anil. 4-Methoxy-2:6-dimethylaniline (0.224 g.) was dissolved in glacial acetic acid (0.2 ml.) and water (1 ml.) added. One drop of acetic acid was then added to clarify

* Purpurogallin number (P.N.) is defined as the number of mg. of purpurogallin formed by 1 mg. of peroxidase preparation, when allowed to act upon 5 g. of pyrogallol in 2 l. of water for 5 min. at 20° in presence of 10 ml. of 0.5% H_2O_2 .

the solution. 2:6-Dimethyl-*p*-benzoquinone (0.2 g.) was dissolved in the minimum volume of ethanol and added drop by drop during a period of 30 min. (thus imitating the conditions of the peroxidic oxidation of the amine). A purple coloration resulted, but no solid separated. After standing, however, for 48 hr. in a refrigerator, violet needles separated and were recrystallized from aqueous ethanol, m.p. 78.5°. (Found: C, 74.1; H, 6.85; N, 4.11. The addition compound of the anil and quinone, $C_{17}H_{19}O_2N$, $C_8H_8O_2$ requires C, 74.0; H, 6.77; N, 3.5%.)

This addition compound was dissolved in light petroleum (b.p. 80–100°) and examined chromatographically on alumina. Two bands were obtained: (A) pale yellow; (B) deep purple. (A) travelled quickly through the alumina and proved to be 2:6-dimethyl-*p*-benzoquinone. Elution of (B) with ethanol followed by concentration gave a purple solid which, on recrystallization from aqueous ethanol, gave purple crystals of m.p. 87–88°. Mixed m.p. with peroxidase oxidation product, 87.5°. (Found: C, 75.7; H, 7.1; N, 5.3. Calc. for $C_{17}H_{19}O_2N$: C, 75.8; H, 7.05; N, 5.2%.)

Attempted condensation of p-nitrosodimethylaniline with 3:5-dimethylphenol. (i) The phenol (1.7 g.) was dissolved in NaOH solution (20 ml., 10%, w/v), and powdered *p*-nitrosodimethylaniline (1.5 g.) and Zn dust (3 g.) were added. The mixture was shaken for 2 hr. and allowed to stand for 24 hr. A dark blue coloration developed and the product was shaken with ether. On dilution with more ether, the blue coloration disappeared indicating that a molecular compound had been formed. On concentrating the ether and cooling, crystals of unchanged *p*-nitrosodimethylaniline were obtained.

(ii) The phenol and *p*-nitrosodimethylaniline were heated together in xylene solution, under reflux, for 2 hr. A dark tarry material was obtained which appeared to contain decomposition products of the nitroso compound.

Examination of the filtrate from the oxidation product. The filtrate was allowed to stand for 7 days to complete the oxidation, and then filtered. A small quantity of a crystalline purple compound was obtained. It had m.p. 79° and was proved to be the addition compound of the anil and the quinone.

The filtrate was made alkaline and filtered free from traces of a red amorphous material. It was then fractionated using a Fenski column and 2.5 ml. of a liquid of b.p. 69–78° were obtained. On shaking this liquid with powdered 3:5-dinitrobenzoyl chloride and NaOH solution, a solid was obtained which was recrystallized from light petroleum. It had m.p. 184° and was proved, by mixed m.p. and by its general reactions, to be 3:5-dinitrobenzamide thus showing that NH_3 was a product of the peroxidase reaction. The mother liquor from the amide was concentrated, and colourless needles were obtained which, after four recrystallizations, had m.p. 103° and the mixed m.p. with authentic methyl 3:5-dinitrobenzoate was 103°. Thus the presence of methanol in the original filtrate was proved.

4-Nitro-3:5-dimethylbenzoic acid. Nitromesitylene (10 g.) was dissolved in glacial acetic acid (25 ml.) and heated in a boiling water bath. CrO_3 (7 g.) in glacial acetic acid (100 ml.) and water (20 ml.) was slowly added during 1 hr. and the mixture stirred for a further 5 hr. The product was poured into ice water, made alkaline with NaOH solution and steam distilled. The residual liquid was acidified with HCl and cooled, when the acid separated. It was recrystallized as colourless prisms from aqueous ethanol and had m.p. 215°. Yield, 1.6 g. (Found: C, 55.6; H, 4.94; N, 7.45. Calc. for $C_9H_7O_4N$: C, 55.4; H, 4.67; N, 7.62%.)

Methyl 4-amino-3:5-dimethylbenzoate. The foregoing nitro acid (1.6 g.) was dissolved in ethanol (75 ml.) and a small quantity of Adams's PtO_2 catalyst added. The mixture was hydrogenated at 75 atm. and 100° for 1 hr., then filtered and evaporated to dryness *in vacuo* yielding the crude amino-acid (5 g.). (This could be recrystallized from ethanol in the form of long needles, m.p. 248°, decomp.). The crude acid was dissolved in methanol and heated under reflux for 2 hr. with a stream of dry HCl passing through. The solution was concentrated, poured into water, neutralized with NaOH solution, and the amino-ester separated after standing for 2 hr. It was recrystallized from aqueous methanol and had m.p. 104–106°. Yield 3.2 g. Meyer (1904) claimed that the ester had m.p. 93°. (Found: C, 66.48; H, 6.48; N, 7.62. Calc. for $C_{10}H_{13}O_2N$: C, 67.0; H, 6.53; N, 7.83%.)

Attempted oxidation of methyl 4-amino-3:5-dimethylbenzoate. Considerable difficulty was encountered because of the low solubility of the compound in dilute acid, but a series of experiments was carried out on a test-tube scale at pH 4–6 and at concentrations of about 0.01–0.05%. The addition of peroxidase and H_2O_2 produced no coloration, and the amino-ester was recovered unchanged in each case.

SUMMARY

1. Peroxidase and hydrogen peroxide oxidize 4-methoxy-2:6-dimethylaniline to a crystalline purple compound which by degradation and unambiguous synthesis has been proved to be 2:6-dimethylbenzoquinone-4-(4'-methoxy-2':6'-dimethyl) anil. Ammonia and methanol are also produced during the oxidation.

2. Hypothetical intermediate compounds have been postulated to explain the reaction.

3. It appears that amines containing powerful electron-attracting groups are not readily oxidized by the peroxidase system.

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