694. *Studies in Peroxidase Action. Part V. The Oxidation of Dimethylaniline.*

By F. T. Naylor and B. C. Saunders.

Dimethylaniline is readily oxidised at room temperature (in dilute acetic acid at pH 4·5) by peroxidase and hydrogen peroxide oxidase. A transient yellow colour soon gives place to a deep green colour, and a solid separates. Gradually the colour of the mixture changes from green to blue. The reaction is very complex and is not yet completely elucidated, but sufficient evidence has been obtained to suggest the main course of the oxidation. *NNN'N'*-Tetramethylbenzidine has been obtained in a pure condition, and structures are assigned to certain coloured products.

In earlier work on peroxidase action, the detailed investigation was recorded of the oxidising action of the system, hydrogen peroxide and the enzyme peroxidase, on aniline (P. J. G. Mann and Saunders, *Proc. Roy. Soc.*, 1935, *B*, **119**, 47), *p*-toluidine (Saunders and P. J. G. Mann, *J.*, 1940, 769), and mesidine (Chapman and Saunders, *J.*, 1941, 496). This work showed that the oxidation was relatively less complex with the *C*-substituted anilines, owing to the blocking of the " free points " by methyl groups.

The work has now been extended to dimethylaniline as substrate, to discover the effect of replacing the *N*-hydrogen atoms by methyl groups. The literature does not contain detailed examination of the peroxidase oxidation of this amine. Bach (*Arch. Sci. phys. nat.* 1916, 42) observed that the amine was attacked by peroxidase, but did not examine the product chemically. Casolari (*Biochem. terap. Sp.*, 1929, **16**, 252) recorded that a mixture of *p*-phenylenediamine hydrochloride and dimethylaniline, dissolved in aqueous acetic acid, gave a green colour with hydrogen peroxide and milk peroxidase. He assigned a formula to the compound, which cannot now be considered correct.

For most of our experiments an enzyme preparation obtained from turnips (see F. G. Mann and Saunders, " Practical Organic Chemistry," Longmans, p. 372) was used. In the summer months horseradish was used instead of turnips, and in a few experiments we used a highly purified specimen of the enzyme, kindly supplied by Professor Keilin (see Keilin and Mann, *Proc. Roy. Soc.*, 1937, *B*, **122**, 119). Similar results were obtained with each type of preparation. Control experiments were carried out with the enzyme in the absence of hydrogen peroxide and with hydrogen peroxide alone. In the former case no action was observed, whilst with the hydrogen peroxide alone a very faint magenta colour developed only after several hours. Therefore it may be concluded that the oxidations described below were brought about by peroxidase action.

For the oxidations a 2% solution of dimethylaniline in dilute acetic acid was used, the pH being 4·0—4·7. An aqueous solution of the enzyme preparation (P.N.* *ca.* 100) was added to the amine solution and then hydrogen peroxide was added in small quantities. In order to discuss the mechanism it is necessary to indicate briefly the colour changes which took place. The reaction mixture became transiently yellow (stage 1), and quickly changed to intense green (stage 2). A light blue precipitate then slowly separated and the green colour gradually changed to blue-green and finally to blue-purple. During the early stages of the oxidation, the green colour reappeared on each addition of hydrogen peroxide. When a small portion of the green solution (stage 2) was removed and treated with a large excess of hydrogen peroxide and peroxidase it became orange (stage 3), but rapidly changed back to green when kept.

At the conclusion of the oxidation, the solid was filtered off. The main constituent of this was found to be *NNN'N'*-tetramethylbenzidine which was obtained pure by chromatography. This procedure showed the presence of traces of many coloured materials which could not be obtained crystalline, *e.g.*, a very small amount of a violet dye of the triphenylmethane type, resembling both methyl-violet and crystal-violet, but apparently identical with neither, and a substance that, from its colour reactions, resembled a phenazine. The filtrate at the end of the oxidation did not contain dimethylaniline oxide—an observation of some interest. It gave, however, an unidentified picrate which was neither that of dimethylaniline nor that of *NNN'N'*-tetramethylbenzidine.

The formation of *NNN'N'*-tetramethylbenzidine (II) from dimethylaniline involves dehydrogenation in the *para*-position. It may be that free radicals (I) are produced which immediately combine in pairs to produce the substituted benzidine (II), some of which separates as a solid,

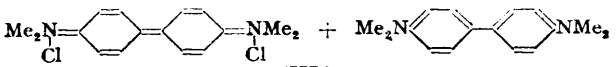
* P.N. = Purpurogallin number. For definition see Saunders and Watson, *Biochem. J.*, 1950, **46**, 629.

the remainder being further oxidised. [Alternatively the first stage may be the removal of an electron from dimethylaniline giving the ion-radical (I*a*). Such ion-radicals would combine in pairs to give (II) with the simultaneous elimination of two protons.] It is possible that the transient yellow colour (stage 1) is concerned with this initial process.

We next examined the action of peroxidase and hydrogen peroxide on *NNN'N'*-tetramethylbenzidine in dilute acetic acid. A transient greenish-yellow colour quickly gave way to an intense green. Further oxidation, carried out quickly, gave an orange-coloured solution which slowly reverted to green. When dilute hydrochloric acid was added to the green solution, the orange stage was immediately reached. Dilute sodium hydroxide solution on the other hand seemed to act as a reducing agent by converting the orange-coloured solution to green, and then causing the green colour to disappear with the separation of tetramethylbenzidine. There seems little doubt that the green and the orange colour here recorded correspond with those observed in the oxidation of dimethylaniline—stages 2 and 3 respectively. The final colour when tetramethylbenzidine was oxidised was, however, reddish-purple.

The oxidation of *NNN'N'*-tetramethylbenzidine by other agents was also investigated. Oxidations carried out in the presence of mineral acids gave no green colour, an orange-coloured solution being formed immediately. The best method of preparing the green compound in the solid state was by oxidising tetramethylbenzidine with bromine water, a green amorphous solid separating. A green compound had previously been obtained by Willstätter and Kalb (*Ber.*, 1904, **37**, 3761) by the action of ferric chloride on *NNN'N'*-tetramethylbenzidine, which like our green compound gave an unstable orange salt on treatment with excess of hydrochloric acid.

Willstätter and Piccard (*Ber.*, 1908, **41**, 3252) believed that their green compound had the structure (III). It is now suggested that the intensely green compound (in solution) is a free



(III.)

radical (IV), analogous to a Wurster salt, formed by the removal of one electron from the *NNN'N'*-tetramethylbenzidine molecule.

It is clear that the cation (IV) will be a resonance hybrid of a number of unsaturated structures (*e.g.*, one in which the second nitrogen atom has only seven electrons in its outer orbit). In some of the structures a carbon atom of the benzene rings will be " unsatisfied." The intense colour of the compound is readily accounted for on this view, and it owes its stability to resonance among the several participating structures.

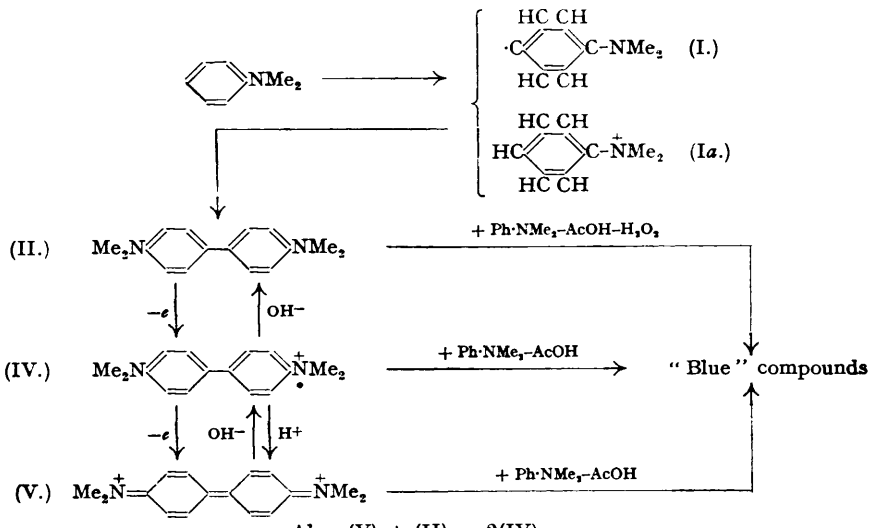
Further oxidation will result in the formation of the quinonoid structure (V) by the loss of a second electron. This will be only feebly coloured because of the reduced possibility of resonance, and it is considered to be the structure of the compound responsible for the orange colour previously described. In support of this we have shown by quantitative experiments that the amount of bromine water required to oxidise *NNN'N'*-tetramethylbenzidine completely to the orange compound is in accordance with the constitution (V).

We do not propose at the moment a mechanism for the conversion of (V) into (IV) and thence into *NNN'N'*-tetramethylbenzidine by dilute aqueous sodium hydroxide. However, it may be noted that helicorubin, a pigment of the intestine of snails and crayfish, is oxidised in acid solution, but when the pH is raised above 7·3 reduction automatically takes place (Roche and Morena, *Compt. rend. Soc. Biol.*, 1936, **123**, 1218). The fact that no intensely green solution is obtained during the oxidation of *NNN'N'*-tetramethylbenzidine in the presence of mineral acid is discussed later. Frequently during the oxidation of dimethylaniline, the orange stage (3) was reached and then subsequently reverted to the green stage (2). This was probably caused by (V) reacting by electron transfer with unchanged tetramethylbenzidine. The absence of stage (3) when the oxidation of tetramethylbenzidine was carried out slowly suggests that the quinonoid compound (V), as soon as it is formed, takes part in further reactions, with the formation of coloured compounds. It seems likely also that in the oxidation of dimethylaniline many products are formed *via* this intermediate quinonoid compound (V). In support of this we have shown that (V) and dimethylaniline react in dilute acetic acid to give a blue substance, and also that dimethylaniline and (IV) react in dilute acetic acid solution, especially in the presence of hydrogen peroxide, to give similar blue compounds. Some, at least, of these compounds may perhaps be of the electron-transfer type (cf. Weiss, *J.*, 1942, 245).

The formation of traces of a triphenylmethane dye is not surprising in view of the fact that dimethylaniline is oxidised by a variety of reagents to dyes of this type (cf. Hofmann, *Ber.*, 1873,

6, 357). Other possible reactions must not be overlooked, such as the formation of *p*-hydroxydimethylaniline and its oxidation products.

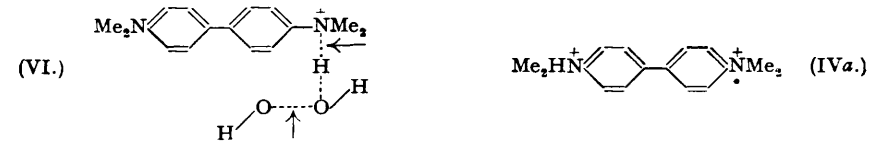
The main changes can therefore be shown in outline as in the chart.



Also, (V) + (II) = 2(IV).

There is as yet no definite evidence as to the mechanism of the removal of an electron in the above enzymic processes. It may be noted, however, that tetramethylbenzidine (II) could presumably exist in acid solution as the salts (II*a*) and (II*b*). It is possible that (II*a*) might form an activated complex (VI) (cf. Saunders and Watson, Part IV, *Biochem. J.*, 1950, **46**, 629) with hydrogen peroxide (or with a hydrogen peroxide–peroxidase compound). This on the elimin-

ation of water would give the free ion-radical (IV) and a hydroxyl radical (which may combine in pairs, or may immediately undergo further reactions with other substances present). By a similar process (II*b*) would pass directly into (V). This would explain why the orange stage 3



is rapidly reached in the presence of excess of acid. In strongly acid solutions, resonance in (IV) is destroyed by the formation of the " salt " (IV*a*) which *via* a hydrogen peroxide complex will also give (V).

Turning now to the initial stage of the oxidation, a hydrogen peroxide complex of the salt of dimethylaniline could similarly give (I*a*). There is, however, very little decisive evidence on these points at present.

Experimental.

Oxidation of Dimethylaniline.—Dimethylaniline (11·3 g.) was dissolved in acetic acid (*ca.* 15 ml.) and diluted with water (500 ml.). More acetic acid was added, to give a clear solution of pH approx. 4·5. To this solution were added hydrogen peroxide (2 ml.; 20-vol.) and a small quantity of an aqueous peroxidase solution. A transient yellow colour was produced which immediately changed to green. More hydrogen peroxide, at the rate of 1 ml. every 45 minutes, was added until 40 ml. had been added. At intervals of about 4 hours further small additions of the enzyme solution were made. A light-blue solid gradually separated and the solution slowly became bluish-purple. The green colour was noticeable on the first five or six additions of hydrogen peroxide. (When a few ml. of the green solution were removed and large excesses of hydrogen peroxide and peroxidase quickly added, an orange-coloured solution was obtained, which quickly reverted to green.) At the end of the oxidation the solid (3—4 g.) was filtered off and dried.

The solid oxidation product. Extraction with light petroleum (b. p. 40—60°) in a Soxhlet apparatus dissolved 1·5 g. of solid. The red extract, when kept, deposited a grey solid, a solution of which in light petroleum (b. p. 40—60°) was examined chromatographically with aluminum oxide. A lower colourless band, on elution with light petroleum and subsequent concentration, yielded white needles of *NNN'N'*-tetramethylbenzidine, m. p. and mixed m. p. 194° (Found : C, 80·3; H, 8·4; N, 11·9. Calc. for $C_{16}H_{20}N_2$: C, 79·9; H, 8·4; N, 11·7%). Unidentified bands were also obtained. Chromatographic analysis of the mother-liquor gave yellow and green bands.

The residue from the petroleum extraction was next extracted with ether in a Soxhlet apparatus, 1·2 g. of solid being dissolved. The brown ethereal extract, on storage, deposited a trace of an impure bluish-green solid. The mother-liquor was examined chromatographically. A lower colourless band, on elution with ether and subsequent concentration, deposited needles of *NNN'N'*-tetramethylbenzidine. When exposed to the air, in the presence of ether, they slowly assumed a green colour. Purple and green bands were also obtained.

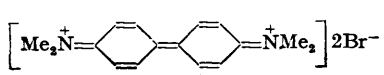
The residue from the previous extraction was completely soluble in methanol, giving a reddish-violet solution, which was examined chromatographically. The lower violet band, on elution with methanol and subsequent evaporation, gave a brown non-crystalline solid. Its behaviour recalled that of a triphenylmethane dye (Found : C, 75·2; H, 7·6; N, 11·4. Calc. for the carbinol base of *NNN'N'N''*-pentamethylpararosaniline, $C_{24}H_{29}ON_3$: C, 76·8; H, 7·7; N, 11·2%). It should be noted that " methyl-violet " is a mixture of this pararosaniline with other partly methylated derivatives. The second band was dull violet and, on elution with methanol and subsequent evaporation, yielded a brownish-violet solid which resembled a phenazine in its behaviour. Other dark bands were also obtained.

The filtrate. The original purple filtrate from the oxidation product was made alkaline and the dark tarry solid which formed was filtered off. The filtrate was concentrated by distillation, unchanged dimethylaniline being removed in steam. A hot saturated aqueous solution of picric acid was then added; the picrate which separated recrystallised from chloroform as yellow leaflets, m. p. 140—141° (Found : C, 57·5; H, 5·4; N, 15·6%). The picrate developed a red surface sheen on storage, but this disappeared on agitation.

Another portion of the filtrate was extracted with chloroform, both layers being violet. A dark tarry solid was formed by evaporation of the dry chloroform extract. A portion of this tar was soluble in water, giving an intense violet solution, which on evaporation yielded a very small quantity of bluish-violet solid (acetate). The violet solution became successively blue, green, and greenish-yellow by the addition of very small quantities of dilute hydrochloric acid. The acetate was converted, by evaporation with dilute hydrochloric acid, into a deliquescent violet hydrochloride, which resembled the acetate in its behaviour. The acetate and the chloride did not possess the lustre of either methyl-violet or crystal-violet in the solid state.

Peroxidase Oxidation of NNN'N'-*Tetramethylbenzidine.*—A solution of tetramethylbenzidine (0·5 g.) in acetic acid (10 ml.) was diluted with water to 300 ml. The oxidation was carried out as described for dimethylaniline. A transient greenish-yellow colour was produced on the first addition of hydrogen peroxide (in the presence of peroxidase). This was immediately replaced by an intense green colour (stage 2) which gradually became reddish-purple. When the oxidation was performed quickly by frequent addition of hydrogen peroxide and peroxidase, the green solution changed to orange (stage 3), but this reverted to green on storage. Addition of dilute sodium hydroxide to the orange solution (stage 3) reversed the colour changes observed during the oxidation, and *NNN'N'*-tetramethylbenzidine separated. The green solution (stage 2) became orange on the addition of dilute hydrochloric acid, and became colourless on the addition of alkali, *NNN'N'*-tetramethylbenzidine then separating.

Oxidation of NNN'N'-*Tetramethylbenzidine by Other Agents.*—(i) *By bromine.* Tetramethylbenzidine (0·062 g.) was dissolved in dilute acetic acid (20 ml.; 1 : 1), and bromine water slowly added from a burette. The solution first became intensely green, then orange-coloured, and finally a red precipitate began to form when 0·0443 g. of bromine had been added. On the assumption that the red precipitate [which is probably the perbromide $(C_{16}H_{20}N_2)^{++}2Br_3^-$] begins to form when all the substituted benzidine has been converted into the quinonoid compound (*A*), 0·062 g. of tetramethylbenzidine would require 0·0413 g. of bromine.

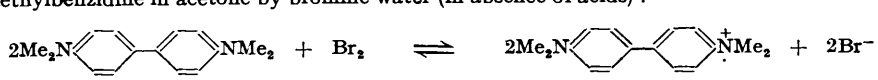


(*A*).

When the oxidation was performed in the presence of hydrochloric acid, instead of acetic acid, an orange colour was produced immediately and further oxidation produced a red precipitate. The addition of dilute sodium hydroxide to the orange-coloured solution produced a transient green colour and then an almost colourless precipitate of (II).

(ii) *By ferric chloride.* When ferric chloride solution was added to a solution of *NNN'N'*-tetramethylbenzidine in dilute hydrochloric acid an orange-coloured solution resulted. When the oxidation was performed in dilute acetic acid solution a green colour was first produced, which changed to orange on further oxidation. When, in both cases, the orange-coloured solution was treated with very dilute sodium hydroxide a transient green colour preceded the separation of a pale yellow solid (II).

The green colouring matter. This was best prepared in the solid state by the oxidation of *NNN'N'*-tetramethylbenzidine in acetone by bromine water (in absence of acids) :



The unstable green solid gave the test for a bromide, and, when heated, changed sharply at 100° to a light-brown solid, which was shown to contain *NNN'N'*-tetramethylbenzidine. Its green aqueous

solution gave, with dilute sodium hydroxide solution, tetramethylbenzidine, and with dilute hydrochloric acid an orange-coloured solution.

The green aqueous solution was treated with a solution of dimethylaniline in dilute acetic acid. A blue colour was slowly produced, but faded on storage. If hydrogen peroxide was also present a blue colour was again produced, but this later became violet and finally dark red.

The aqueous solution of the green solid was oxidised by bromine water just to the orange-coloured stage, and then a solution of dimethylaniline in dilute acetic acid was added. An immediate green colour was produced which quickly became blue and then faded slightly on long storage.

Our thanks are offered to the D.S.I.R. for a maintenance grant to one of us (F. T. N.). The above work, essentially as described above, appeared in a Report dated March 31st, 1943, to the D.S.I.R., but war-time circumstances caused delay in publication. We are grateful to Professor Sir John Lennard-Jones, F.R.S., and to Dr. G. W. Kenner, with whom we have had valuable discussions.

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