143. *Studies in Peroxidase Action. Part II. The Oxidation of* p-*Toluidine.*

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The enzyme peroxidase, derived either from horseradish or from turnips, readily oxidises *p*-toluidine, at room temperature, in the presence of dilute hydrogen peroxide at p_H 4·5 (dilute acetic acid solution). A red coloration is first produced and then a red-brown solid gradually separates. This has been shown to consist of 4-amino-2 : 5-toluquinonebis-*p*-tolylimine, 4-*p*-toluidino-2 : 5-toluquinonebis-*p*-tolylimine, 4 : 4′-dimethyldiphenylamine, a small quantity of 4 : 4′-dimethylazobenzene, and very small quantities of 4-amino-2 : 5-toluquinone-2-*p*-tolylimine, 4-*p*-toluidino-2 : 5-toluquinone-2-*p*-tolylimine, and a trace of a substance of m. p. 167°.

The action of hydrogen peroxide and ferrous sulphate (in place of the enzyme) on *p*-toluidine dissolved in dilute acetic acid is different from the reaction described above.

The adaptation of Irvine's filter for the continuous elution of a chromatogram is described.

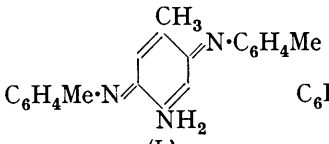
In Part I (Mann and Saunders, *Proc. Roy. Soc.*, 1935, *B*, **119**, 47), an account was given of the action of the enzyme peroxidase in the presence of dilute aqueous hydrogen peroxide on aniline. The brown oxidation product was shown to be a mixture of 2 : 5-dianilino-*p*-benzoquinoneimineanil, 2-amino-8-anil-*N*-phenyldiphenazine (pseudo-mauveine), an induline, and an aniline-black which did not turn green. Carter, Moulds, and Riley (J., 1937, 1307) later oxidised aniline by heating it with graphitic oxide, and obtained 2 : 5-

dianilino-*p*-benzoquinonedianil (not obtained in the peroxidase oxidation) and "a dye of the mauveine type."

In continuing our investigation of the action of peroxidase on relatively simple organic compounds, we selected *p*-toluidine as substrate. The only reference to the action of the enzyme on *p*-toluidine appears to be that by Casolari (*Biochim. terap. sper.*, 1929, **16**, 5203), who stated that an orange-red coloration was produced with subsequent turbidity but did not investigate the nature of the product.

For our experiments, the enzyme was obtained either from horseradish or from turnips by the methods of Willstätter and Stoll (*Annalen*, 1918, **416**, 21) and Elliott (*Biochem. J.*, 1932, **26**, 128). Identical oxidation products were obtained with each type of preparation. Control experiments were carried out with the active enzyme preparation in the absence of hydrogen peroxide and also with the heat-inactivated enzyme in the presence of hydrogen peroxide. No action at the dilution employed was observed in either series of experiments, so we conclude that the oxidations recorded below were the result of peroxidase action.

The experiments were carried out at room temperature, a 2% solution of *p*-toluidine in dilute acetic acid at p_H 4·5 being used. A few mg. of the peroxidase preparation were added, and hydrogen peroxide solution was run in at intervals: this intermittent addition is essential, since excess of hydrogen peroxide inhibits the reaction. The first addition of peroxide produced a red coloration, and a reddish-brown solid gradually separated. At the conclusion of the reaction, the solid was filtered off, and was found to be a mixture of 4-amino-2 : 5-toluquinonebis-*p*-tolylimine (I), 4-*p*-toluidino-2 : 5-toluquinonebis-*p*-tolylimine (II), 4 : 4′-dimethyldiphenylamine (V), a small quantity of 4 : 4′-dimethylazobenzene (VI), and traces of 4-amino-2 : 5-toluquinone-2-*p*-tolylimine (III), 4-*p*-toluidino-2 : 5-toluquinone-2-*p*-tolylimine (IV), and a substance of m. p. 167° (VII). [Formulæ are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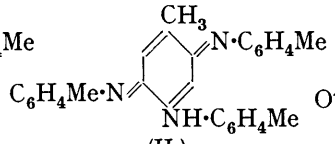
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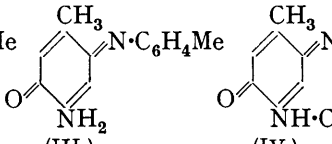
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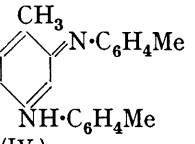
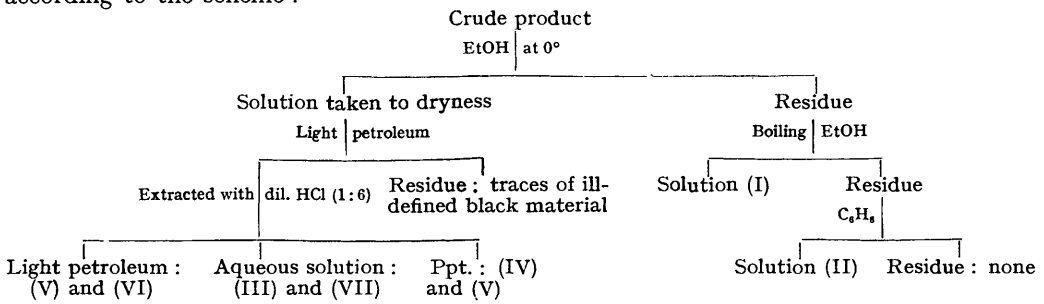

(IV.)

not given for (V), (VI), and (VII).] The isolation of these products was accomplished according to the scheme:



It is not improbable that traces of other substances may have been produced during the oxidation, but a chromatographic analysis of appropriate extracts of the original crude material did not reveal any well-defined substances other than those obtained by purely chemical means. The absence of *p*-nitrotoluene is noteworthy and falls into line with the absence of nitrobenzene from the products of the oxidation of aniline by peroxidase.

The conversion of 4-amino- into 4-*p*-toluidino-2 : 5-toluquinonebis-*p*-tolylimine proceeds presumably with the elimination of ammonia, as also must the conversion of *p*-toluidine into 4 : 4′-dimethyldiphenylamine. Whether this process is really an oxidation or a true deamination, brought about by a deaminase associated with the peroxidase, is uncertain.

It is obvious that 4-amino- and 4-*p*-toluidino-2 : 5-toluquinone-2-*p*-tolylimine are produced by hydrolysis of the corresponding bis-*p*-tolylimines. The method of working up the crude product (shaking with cold dilute hydrochloric acid for a few minutes) may

be responsible for some of this hydrolysis, but it is also likely that hydrolysis takes place to some extent during the oxidation process, especially as these products can sometimes be detected by chromatographic analysis of the crude product before the treatment with dilute hydrochloric acid.

A purely chemical oxidation of *p*-toluidine by warming its acetic acid solution with hydrogen peroxide was recorded by Leeds (*Ber.*, 1881, **14**, 1383), who obtained 4 : 4′-dimethylazobenzene. Perkin (J., 1880, **37**, 546) oxidised *p*-toluidine with potassium dichromate, and thereby was the first to obtain compounds (I) and (II), but their structures were only determined later by other workers.

Mann and Saunders (Part I, *loc. cit.*) found that when aniline was oxidised by hydrogen peroxide and ferrous sulphate, azobenzene, aminoanilino- and 2 : 5-dianilino-benzoquinone-monoanil were produced. The corresponding oxidation of *p*-toluidine under the conditions of the enzymatic reaction, but with ferrous sulphate instead of the enzyme, is now being examined, and will be recorded later. As anticipated, the reaction is different from the peroxidase oxidation, for (I) and (II) are not among the products; so far, 4-*p*-toluidino-2 : 5-toluquinone-2-*p*-tolylimine, 4 : 4′-dimethylazobenzene, and a compound not yet identified (and not produced by peroxidase action) have been isolated.

Among recorded oxidations of *p*-toluidine, the one most resembling the enzymatic oxidation is that by lead peroxide (Börnstein, *Ber.*, 1901, **34**, 1274).

In Part I it was suggested that *p*-benzoquinonephenyldi-imine, $NPh{:}C_6H_4{:}NH$, represented a stage in the enzymatic oxidation of aniline (a view which seems to be generally accepted), and at the time we were inclined to think that this di-imine was produced *via* phenylhydroxylamine and 4-aminodiphenylamine in accordance with the classical views of Bamberger and Tschirner (*Annalen*, 1899, **311**, 79). Work in progress confirms our previous observation that 4-aminodiphenylamine is readily oxidised by the enzyme system to emeraldine. We now find, however, that the oxidation of phenylhydroxylamine depends upon several factors, and that azoxybenzene is normally the principal product, although a quantity of ill-defined material is also usually produced. The observed production of emeraldine was no doubt dependent upon one of the very facile changes which phenylhydroxylamine is known to undergo in solution; *e.g.*, Nover (*Ber.*, 1907, **40**, 290) obtained 4-aminodiphenylamine and emeraldine as well as azoxybenzene by shaking it with aqueous sodium bisulphite solution, and Bamberger and Brady (*Annalen*, 1899, **311**, 84) obtained 4-aminodiphenylamine from it by the action of aluminium sulphate.

Although there is, as yet, no direct experimental evidence, it seems probable that the phenyldi-imine might be produced in these enzymatic oxidations directly from the free radical PhNH· or PhN< as in several purely chemical oxidations (Sidgwick, "Organic Chemistry of Nitrogen," p. 54).

Experimental.

p-Toluidine (10 g.) was dissolved in glacial acetic acid (11·5 c.c.) and diluted with 500 c.c. of water, giving a solution of approximately p_H 4·5. To this solution were added hydrogen peroxide (2 c.c., 20-vol.) and a suitable quantity of the enzyme preparation (*e.g.*, about 20 mg. of a preparation of purpurogallin number about 100, dissolved in a few c.c. of water). A red coloration was produced immediately, and a red precipitate gradually separated. Amounts of hydrogen peroxide of the order of 1 c.c. were added at intervals of about 45 mins., until in all 40 c.c. had been added. At intervals of about 4 hours further small additions of the enzyme preparation were made. At the conclusion of the reaction, the solid was filtered off and dried. Yield 7—8 g.

4-*Amino*-2 : 5-*toluquinonebis*-p-*tolylimine*.—The crude product (10 g.) was extracted with ice-cold alcohol (100 c.c.), and filtered. The filtrate (A) was retained and worked up as described later. The insoluble residue (5·15 g.) was boiled with alcohol (about 150 c.c.), from which bright red crystals (0·7 g.) separated on cooling. The residue was again boiled with alcohol (100 c.c.), and a further crop of red crystals (0·5 g.) was obtained. By working up the mother-liquors, a total of 1·6 g. of the pure crystalline material was obtained, m. p. 236°. Green (*Ber.*, 1893, **26**, 2774) gave 227°, and Börnstein (*loc. cit.*) gave 235° (Found : C, 80·0; H, 7·0; N, 13·5. Calc. for $C_{21}H_{21}N_3$: C, 80·0; H, 6·7; N, 13·3%).

The identity of the substance was further established as follows : (*a*) Addition of concentrated sulphuric acid gave a blue coloration, which on heating rapidly changed to red. (*b*) The sub-

stance was hydrolysed by alcoholic hydrogen chloride at 25° to 4-amino-2 : 5-toluquinone-2-*p*-tolylimine, which crystallised from aqueous alcohol in red-brown needles, m. p. 149°. Green (*loc. cit.*) gave m. p. 143—145° (Found : C, 73·9; H, 6·4; N, 12·2. Calc. for $C_{14}H_{14}ON_2$: C, 74·1; H, 6·2; N, 12·4%); it dissolved in concentrated sulphuric acid, giving a violet-red coloration which faded through brown to yellow.

4-p-*Toluidino*-2 : 5-*toluquinonebis*-p-*tolylimine*.—The residue (3·1 g.) after treatment with boiling alcohol was recrystallised from benzene (in which it dissolved completely) and formed red needles, m. p. 183° (Found : C, 82·8; H, 6·3; N, 10·5. Calc. for $C_{28}H_{27}N_3$: C, 82·95; H, 6·6; N, 10·4%). Its identity was further established as follows : (*a*) It dissolved in concentrated sulphuric acid, giving a violet coloration, which rapidly faded to green. (*b*) Hydrolysed by alcoholic sulphuric acid at room temperature, it afforded 4-*p*-toluidino-2 : 5-toluquinone-2-*p*-tolylimine, which crystallised from alcohol in red-brown needles, m. p. 179—180° (Found : C, 79·6; H, 6·7; N, 8·6. Calc. for $C_{21}H_{20}ON_2$: C, 79·75; H, 6·3; N, 8·7%).

4 : 4′-*Dimethyldiphenylamine and* 4 : 4′-*Dimethylazobenzene*.—The cold alcoholic filtrate (*A*) was evaporated to dryness, extracted with boiling light petroleum (b. p. 60—80°), and filtered from a small quantity of ill-defined black material. The extract was shaken with dilute hydrochloric acid (1 : 6); a bluish-black precipitate (*B*) was produced which was filtered off from the aqueous layer (*C*) and the petrol layer (*D*). The last was dried and concentrated, long, nearly colourless, needles of 4 : 4′-dimethyldiphenylamine (di-*p*-tolylamine) separating, m. p. 79°. Yield, about 2 g. (Found : C, 85·2; H, 7·6; N, 7·1. Calc. for $C_{14}H_{15}N$: C, 85·3; H, 7·6; N, 7·1%).

The mother-liquor from the ditolylamine was evaporated to dryness, and the residue dissolved in glacial acetic acid and allowed to cool; a very small amount of 4 : 4′-dimethylazobenzene and of ditolylamine crystallised. The deep yellow needles of the former were separated mechanically and had m. p. 145° (mixed m. p. with an authentic specimen 144°). The dimethylazobenzene produced by the enzyme reaction also gave the characteristic deep yellow coloration with concentrated sulphuric acid.

4-p-*Toluidino*-2 : 5-*toluquinone*-2-p-*tolylimine*.—The precipitate (*B*) was ground with 10% sodium hydroxide solution and extracted with light petroleum. The extract was concentrated, and on standing produced crystals of ditolylamine; the mother-liquor on long standing sometimes gave crystals of 4-*p*-toluidino-2 : 5-toluquinone-2-*p*-tolylimine, m. p. 178°. The analysis of the light petroleum extract was, however, carried out much more satisfactorily by chromatographic methods using aluminium oxide; a violet band due to the toluidinotoluquinone-*p*-tolylimine was clearly visible, and after elution with more petrol and evaporation, the characteristic colour reaction with concentrated sulphuric acid (see above) was obtained. (A band due to the corresponding bis-*p*-tolylimine was also obtained.)

Treatment of the Aqueous Layer (C).—This was neutralised with sodium hydroxide solution, and the resulting reddish-brown precipitate (*D*) was filtered off, dissolved in light petroleum, and examined chromatographically with aluminium oxide. A lower violet band on elution with petrol and subsequent concentration gave, on standing, a very small quantity of yellowish needles, m. p. 167°. The amount of pure substance obtained was so small as to render identification uncertain (a rather impure specimen gave N, 8·6%). With concentrated sulphuric acid the substance gave a red coloration, becoming pale yellow-green on dilution. The substance could be sublimed and also decolorised by sulphur dioxide, the colour being restored by ferric chloride.

Chromatographic Analysis.—When it is necessary to elute a many-banded chromatogram continuously with a given solvent, it is convenient to fix the chromatographic tube into a filter of the type described by Irvine (*Biochem. J.*, 1915, **9**, 321). In this way, it is possible to wash out successive bands and to separate the different coloured solutions so obtained without dismantling the apparatus. When the Buchner flask is used (Zechmeister and Cholnoky, " Die Chromatographische Adsorptions-methode "), the elution has to be stopped and the flask removed every time a band is washed through.

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