

*Studies in Peroxidase Action. Part IX.\* Reactions involving the Rupture of the C-F, C-Br, and C-I Links in Aromatic Amines.*

By G. M. K. HUGHES and B. C. SAUNDERS.

[Reprint Order No. 5664.]

Peroxidase oxidation of *p*-fluoroaniline gave chiefly 2-amino-5-*p*-fluoroanilino-*p*-benzoquinone di-*p*-fluoroanil, involving rupture of a C-F bond, with elimination of fluorine as F<sup>-</sup>. Oxidations of *p*-bromo- and *p*-iodoaniline follow analogous courses. The eliminated I<sup>-</sup> was oxidised during the reaction to I<sub>2</sub>, and this iodinated some unchanged amine. Unlike *p*-fluoroaniline, *p*-bromo- and *p*-iodoaniline gave appreciable quantities of the corresponding azo-compounds.

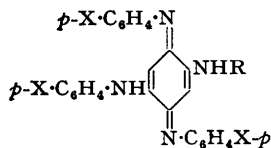
The mechanisms of the reactions are discussed, and the main products can be accounted for by unsymmetrical pairing of initially formed anilino-radicals, followed by elimination of hydrogen halide.

Thyroxine was not among the products of the oxidation of 3 : 5-di-iodo-tyrosine.

THE peroxidase-catalysed oxidation of *p*-chloroaniline (Daniels and Saunders, *J.*, 1953, 822) demonstrated the ease with which the oxidising system can eliminate *para*-substituents from certain amines. This oxidation was unique in that appreciable quantities of 4 : 4'-dichloroazobenzene were also formed, whereas azo-compounds were produced in negligible quantities from other amines. We have now extended the work to the other *para*-halogenated amines, it being of especial interest to ascertain whether the strong C-F bond in *p*-fluoroaniline could be broken, and whether azo-compound formation was general for these amines.

Routine oxidations were carried out in acetate buffer, as preliminary experiments had shown that there was a fall of pH. With *p*-fluoroaniline the change was from pH 4.5 to pH 3.9, and the yield of oxidation product was only 25%. However, even in a buffered medium the yields were never greater than 35%. This was attributed to the accumulation of fluoride ion.

The red-brown solid oxidation product was split into fractions by solvent extraction, and each extract was chromatographed on alumina. Petroleum-soluble material (62.5%)



- |   |                     |
|---|---------------------|
| (I) R = C <sub>6</sub> H <sub>4</sub> F, X = F.               | (IV) R = H, X = Br. |
| (II) R = H, X = F.  | (V) R = H, X = I.   |
| (III) R = <i>p</i> -C <sub>6</sub> H <sub>4</sub> Br, X = Br. | (VI) R = H, X = Cl. |

gave two red compounds (I) and (II). The latter was identified by elementary analysis, acetylation, and reaction with *p*-fluoroaniline, as 2-amino-5-*p*-fluoroanilino-*p*-benzoquinone di-*p*-fluoroanil. Infra-red, visible, and ultra-violet absorption spectra were compared with

\* Part VIII, *J.*, 1953, 822.

those of the corresponding chloro-compound (VI). A major feature of the infra-red spectrum is three bands in the 3200—3500-cm.<sup>-1</sup> region, which are assigned to three N—H bonds. These three bands are present in all compounds having R = H, while only one in the 3250—3340-cm.<sup>-1</sup> region appears in compounds having R = C<sub>6</sub>H<sub>4</sub>X, where X is H or Me. The ultra-violet and visible maxima are recorded in the Experimental section for compounds (II), (IV), and (V), and provide important corroborative evidence. More detailed analysis of these spectra will be published later.

The product (I) was identified by synthesis from benzofurazan oxide and *p*-fluoroaniline (cf. Ruggli and Buchmeier, *Helv. Chim. Acta*, 1945, **28**, 850). The structure of the simpler anil (II) being known, that of its homologue (I) follows from formation of the latter from the former by the reaction with *p*-fluoroaniline.

Chromatography of the ether-soluble part of the oxidation product gave further quantities of (I) and (II), with traces of an orange compound, probably 4 : 4'-difluoroazobenzene.

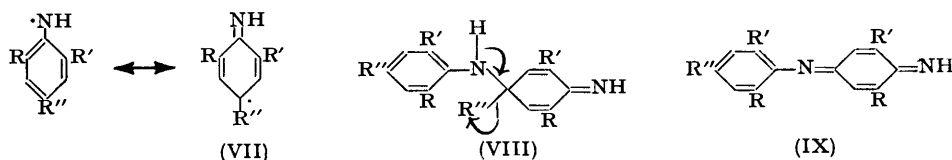
The formation of the anils (I) and (II) requires that one fluorine atom should be eliminated between five and four molecules of *p*-fluoroaniline respectively. Calculation shows that in a typical experiment, proceeding to 30% completion, the concentration of F<sup>-</sup> would be ca.  $7 \times 10^{-3}$ M. Actually no fluoride ion could be detected by the addition of calcium chloride to the red filtrate of the reaction mixture, but its presence on the walls of the reaction vessel was demonstrated easily by the potassium dichromate-sulphuric acid reaction (cf. Saunders and Stacey, *J.*, 1948, 1773). Since peroxidase action is inhibited (though not totally in our experience) by concentrations of fluoride greater than about  $1 \times 10^{-3}$ M (Sumner and Myrback, "The Enzymes," Academic Press, New York, 1951, Vol. II, p. 426), it is clear that this is the cause of the low yields in this oxidation. (Large amounts of enzyme added to small test portions of the reaction mixture did, in fact, give greater yields, confirming this view.)

Unlike *p*-chloroaniline, *p*-fluoroaniline gave only traces of azo-compound and, to investigate whether this was in accord with the mechanism of the reactions, we oxidised *p*-bromoaniline and *p*-iodoaniline in aqueous buffer. *p*-Bromoaniline afforded the analogous anils (III) and (IV), and in addition 4 : 4'-dibromoazobenzene was isolated (17.5% of the product). The anils were identified as in the previous case. The colourless filtrate from this oxidation gave a positive test for bromide ion.

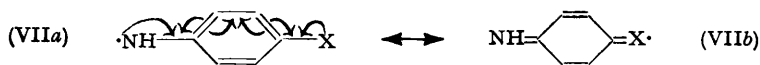
From the solid oxidation product of *p*-iodoaniline were isolated, initially, 4 : 4'-di-iodoazobenzene and an anil (V). Infra-red and visible absorption spectra indicated that this anil was the analogue of (II), (IV), and (VI). Analysis and acetylation confirmed that it was 2-amino-5-*p*-iodoanilino-*p*-benzoquinone di-*p*-iodoanil. Like its analogues, it must be formed with elimination of iodine at some stage, yet the filtrate contained neither iodine nor any common iodine-containing anion. It is known that peroxidase catalyses the oxidation of iodide ion to molecular iodine (Bansi and Ucko, *Z. physiol. Chem.*, 1926, **159**, 235) and that quinones also effect this oxidation. Thus iodine was most likely to be present as I<sub>2</sub>. However, *p*-iodoaniline reacted immediately with aqueous iodine. That this had been the fate of the iodine was shown by the isolation, from the mother-liquors from an initial recrystallisation of the anil (V), of 2 : 4-di-iodoaniline, which was synthesised by the action of iodine monochloride (generated *in situ*) on aniline (cf. Bradfield, Orton, and Roberts, *J.*, 1928, 782).

It has been postulated that peroxidase-catalysed oxidations involve the free radical (VII) (cf. Saunders and Mann, *J.*, 1940, 769), formed either by direct loss of a hydrogen atom or by electron-removal followed by loss of a proton. Symmetrical pairing of two such radicals would give hydrazo-compounds which would be oxidised to the corresponding azo-compounds. The problem remaining is the mechanism by which compounds (I)—(VI) are formed from these radicals. An initial unsymmetrical dimerisation of the radical (VII) would give a substituted quinone di-imine (IX) by elimination of R'', when R'' = F, Cl, Br, or I and R' = R'' = H; the imine (IX), by a series of established addition and oxidation reactions, would give the products (I)—(VI). In Part VI (*J.*, 1951, 2112) it was postulated that the formation of the imine (IX) involved the coupling of a radical with a molecule of unchanged amine. In the light of the present work it is regarded as unlikely that a radical (VII) reacts with an amine, as there are no products involving

substitution *meta* to the amino-group. Hey and his co-workers (*J.*, 1951, 2892; 1952, 2094) have shown that radical substitution leads to a mixture of all isomers, though the *meta*-isomer is formed to the least extent. Thus we postulate unsymmetrical pairing of the radical (VII) to give (VIII) ( $R'' = \text{halogen}$ ), which, by a one- or a two-stage process, gives the imine (IX). This simple explanation accounts for both the fall in pH and the production of the ion  $R''^-$ .



When the side reactions of iodination are taken into account, the yields of the azo-compounds from the *p*-chloro-, *p*-bromo-, and *p*-iodo-aniline represent 15% of the total solid products, in contrast to a virtual absence in the case of *p*-fluoroaniline. The formation of an azo-compound will depend on the degree of localisation of the lone electron on the nitrogen atom in the radical (VII). The relative order of this localisation will be the reverse of that in the parent amines, where it is manifested by their basic strengths. These strengths,  $10^{12}K_b$  in 30% ethanol, are X = F 120, Cl 28.8, Br 21.9, I 15.1, and H 126 (Ingold "Structure and Mechanism in Organic Chemistry," Bell, London, 1953, p. 741). The same  $+I$  and  $-E$  effects will operate in the radical and will determine the relative contributions of the forms (VIIa and b). Since *p*-fluoroaniline is a considerably stronger base than the other halogenated anilines, the electron release is much greater and the radical (VIIb) will play a proportionately greater part. This decreases the relative amount of azo-compound formed, as we have shown experimentally.



The elimination of iodine from *p*-iodoaniline suggested the study of another iodine-containing substrate, namely, 3:5-di-iodotyrosine. Oxidation of this resulted in the liberation of iodine. This indicated that thyroxine could not be among the oxidation products, and that the reaction had proceeded by the dimerisation of two radicals (in which the electron may be considered as localised on the carbon atom *ortho* to the hydroxyl group) with elimination of iodine. This is in agreement with the mode of oxidation of phenols by peroxidase investigated in this laboratory (unpublished work). Examination of the products as described by Reincke and Turner (*J. Biol. Chem.*, 1946, **162**, 369) failed to reveal any thyroxine. We believe these results are in agreement with the findings (unpublished) of Harington and Pitt-Rivers.

#### EXPERIMENTAL

*Oxidation of p-Fluoroaniline.*—*p*-Nitrofluorobenzene, b. p. 85.5°/14 mm., was prepared on the 0.33-molar scale from fluorobenzene in 60–70% yield (Bradlow and VanderWerf, *J. Amer. Chem. Soc.*, 1948, **70**, 654). Synthesis by a Schiemann reaction on *p*-nitroaniline gave a yield of only 38%. Reduction to the amine was effected both catalytically (nickel in alcohol at 40°/50 atm.), and by tin and hydrochloric acid, in 80–85% yield on the 0.14-molar scale.

Since preliminary experiments showed a fall in pH during oxidation in an unbuffered medium, subsequent oxidations of this amine and *p*-bromo- and *p*-iodo-aniline were conducted in 0.5M-acetate buffer, pH 4.73 (NaOAc, 3H<sub>2</sub>O 68 g.; AcOH 57 ml.; H<sub>2</sub>O to 1 l.).

To *p*-fluoroaniline (10 g.), dissolved in the buffer solution (500 ml.), were added hydrogen peroxide (2 ml.; 20-vol.), and a diluted solution of highly purified horse-radish peroxidase (1 ml.; P.N. 15) (kindly supplied by Prof. Keilin), and the additions were repeated intermittently to a total volume of 40 ml. of hydrogen peroxide. (No immediate reaction was observed in the absence of enzyme, though 2% of oxidation occurred in 3 weeks.) The introduction of enzyme immediately produced a red colour, which deepened during the oxidation to a dark permanganate hue, and a red-brown solid was slowly precipitated. The mixture was filtered, and the residue washed, dried (2.5–3.5 g.), and examined as below. The filtrate [pH unaltered (4.73)]

gave no precipitate with calcium chloride, but the walls of the reaction vessel, after thorough washing to remove all organic material, gave a positive reaction for fluoride ion with hot potassium dichromate and concentrated sulphuric acid, exhibiting the characteristic non-wettability.

The red-brown product (5.4 g.) was extracted (Soxhlet) successively with light petroleum (4 × 150 ml., b. p. 40–60°; 2 × 150 ml., b. p. 80–100°) and ether (200 ml.). The combined red petroleum extracts on evaporation gave a red-brown semi-crystalline mass (3.37 g.). The ether extract similarly gave an orange crystalline mass (1.26 g.).

The petroleum-soluble material (2.15 g.) was chromatographed on alumina in benzene. The lowest band gave a red crystal (35 mg.), m. p. 260–261°, identified by synthesis (below) as 2 : 5-di-*p*-fluoroanilino-*p*-benzoquinone di-*p*-fluoroanil (I). The next band, the major constituent, gave, on concentration of its eluate, 2-amino-5-*p*-fluoroanilino-*p*-benzoquinone di-*p*-fluoroanil (II), as red crystals (1.30 g.), m. p. 191–192°, unaltered on recrystallisation from methanol-benzene (Found : C, 69.1; H, 4.4; N, 12.9. C<sub>24</sub>H<sub>17</sub>N<sub>4</sub>F<sub>8</sub> requires C, 68.8; H, 4.1; N, 13.4%). Its infra-red spectrum showed maxima at, *inter alia*, 3311, 3362, and 3484 cm.<sup>-1</sup>, in ether there were max. at 274 and 354 μ (ε 19,000 and 20,300 respectively) with an inflexion at 405–407 μ (ε 12,700).

The ether-soluble material (855 mg.) was chromatographed on alumina in benzene, giving at least six bands. The first yielded only 1 mg. of orange material which gave a deep yellow colour with concentrated sulphuric acid, indicating the presence of 4 : 4'-difluoroazobenzene. The second band on concentration and addition of methanol gave red crystals (10 mg.), m. p. and mixed m. p. with the anil (I), 261–262°. The third band similarly gave further quantities of the anil (II) (306 mg.), m. p. and mixed m. p. 191–192°.

*Reactions of the Amino-anil (II).*—(a) The product (II) (79 mg.) was heated under reflux for 20 min. with acetic anhydride (5 ml.), and from the cooled solution 2-acetamido-5-*p*-fluoroanilino-*p*-benzoquinone di-*p*-fluoroanil crystallised as dark red needles; recrystallised from ethanol it had m. p. 211° (Found : C, 68.0; H, 4.6; N, 12.0. C<sub>26</sub>H<sub>19</sub>ON<sub>4</sub>F<sub>8</sub> requires C, 67.9; H, 4.1; N, 12.2%).

(b) The product (II) (98 mg.) was heated in acetic acid (10 ml.) and water (5 ml.) with *p*-fluoroaniline (1 ml.) at 100° for 2 hr. After cooling, the mixture was filtered, and the residue washed with hot methanol and dried (75 mg.; m. p. 245–246°). After two recrystallisations from benzene-light petroleum (b. p. 100–120°) the product had m. p. 260° and the mixed m. p. with 2 : 5-di-*p*-fluoroanilino-*p*-benzoquinone di-*p*-fluoroanil was 261–262°.

*Synthesis of 2 : 5-Di-*p*-fluoroanilino-*p*-benzoquinone Di-*p*-fluoroanil (I)* (cf. Ruggli and Buchmeier, *loc. cit.*).—Benzofurazan oxide (Green and Rowe, *J.*, 1912, 101, 2455) (405 mg.) was added during 5 min. to a stirred mixture of *p*-fluoroaniline (4 ml.) and *p*-fluoroaniline hydrochloride (450 mg.) at 150–155°. After a further 15 minutes' heating, the mixture was set aside overnight. The blue mixture was then suspended in ethanol and filtered, and the solid was washed with hot ethanol until the washings were almost colourless. The residue of 2 : 5-di-*p*-fluoroanilino-*p*-benzoquinone di-*p*-fluoroanil (red needles from toluene) had m. p. 261–262° alone or mixed with the material from the peroxidase reaction (Found : C, 70.6; H, 4.5; N, 10.8. C<sub>30</sub>H<sub>20</sub>N<sub>4</sub>F<sub>4</sub> requires C, 70.3; H, 3.9; N, 10.9%).

*Oxidation of *p*-Bromoaniline.*—*p*-Bromoaniline was distilled (b. p. 102–104°/3–4 mm.), and the distillate recrystallised from ethanol (m. p. 68°). The amine (17.60 g.) was dissolved in warm acetate buffer (1 l.), and the solution filtered after being set aside at room temperature for 24 hr. The residue weighed 13.06 g., leaving 4.54 g. in solution. On oxidation by the established procedure, addition of hydrogen peroxide gave no immediate reaction, but the first drops of enzyme solution gave a brown colour, which deepened as the oxidation proceeded. A total of 20 ml. of hydrogen peroxide was added, and a precipitate (3.40 g.) separated. The colourless filtrate contained bromide ion [Found : Br<sup>-</sup> (Volhard), 0.280 g.; formation of the anils (III) and (IV) requires the elimination of 0.221 g. of Br<sup>-</sup>].

The brown solid (3.38 g.) was extracted (Soxhlet) successively with light petroleum (3 × 300 ml.; b. p. 60–80°) and ether (300 ml.). The red extracts were evaporated to dryness and gave crystalline residues (2.83 g. and 0.50 g. respectively).

Petroleum-soluble material (1.53 g.) was chromatographed on alumina in 1 : 1 benzene-light petroleum. The lowest band gave, on evaporation, orange leaflets, m. p. 202–204° (0.27 g.). A portion on sublimation had m. p. 204–206° (4 : 4'-dibromoazobenzene has m. p. 205°) (Found : C, 42.7; H, 2.8; N, 8.5. Calc. for C<sub>12</sub>H<sub>8</sub>N<sub>2</sub>Br<sub>2</sub> : C, 42.4; H, 2.4; N, 8.2%). The next band gave, on evaporation, orange needles, m. p. 256° (30 mg.), identified by synthesis as 2 : 5-di-*p*-bromoanilino-*p*-benzoquinone di-*p*-bromoanil (III) (see below). The third band, the major constituent, gave, on evaporation, red crystals, m. p. 200–203° (740 mg.);

recrystallisation from benzene-light petroleum (b. p. 100—120°) gave 2-amino-5-*p*-bromoanilino-*p*-benzoquinone di-*p*-bromoanil (IV), m. p. 214—215° (Found: C, 48.15; H, 3.0; N, 9.5.  $C_{24}H_{17}N_4Br_3$  requires C, 47.96; H, 2.9; N, 9.3%). The infra-red spectrum (bands at, *inter alia*, 3257, 3338, and 3460  $cm^{-1}$ ) was consistent with there being three N-H bonds in the molecule. Light absorption in  $Et_2O$ : max. at 232, 284, and 362  $m\mu$  ( $\epsilon$  28,900, 26,900, and 21,500, respectively); inflexion at 400—402  $m\mu$  ( $\epsilon$  15,700).

Chromatography on alumina of the ether-soluble material gave a further quantity of the anil (IV) (250 mg.), m. p. 212—213° (from benzene-light petroleum), together with numerous minor bands.

*Reactions of the Dianil (IV).*—Acetylation gave 2-acetamido-5-*p*-bromoanilino-*p*-benzoquinone di-*p*-bromoanil, needles (from ethanol), m. p. 217° (Found: N, 8.85.  $C_{26}H_{19}ON_4Br_3$  requires N, 8.7%).

The product (IV) (98 mg.) was heated in acetic acid (25 ml.) and water (25 ml.) with *p*-bromoaniline (0.5 g.) at 100° for 2.5 hr., cooled, and filtered; the residue, thrice recrystallised from benzene-light petroleum (b. p. 100—120°), had m. p. 253° alone or mixed with (III).

*Synthesis of 2:5-Di-*p*-bromoanilino-*p*-benzoquinone Di-*p*-bromoanil (III).*—Benzofurazan oxide (1.36 g.) was added portionwise during 20 min. to a stirred mixture of *p*-bromoaniline (10 g.) and *p*-bromoaniline hydrochloride (2.08 g.) at 150°. The dark blue mass was then suspended in ethanol and filtered, and the residue washed with hot ethanol. Since washing did not dissolve the blue impurity (presumably a thermal decomposition product of *p*-bromoaniline), the residue was extracted with benzene (Soxhlet), giving a dark brown solution containing very little blue material. The extract was evaporated and the residue of 2:5-di-*p*-bromoanilino-*p*-benzoquinone di-*p*-bromoanil (III) was recrystallised from 1:9 benzene-light petroleum (b. p. 100—120°) as orange-red needles (60 mg.), m. p. 253°, alone or mixed with (III) obtained by peroxidase action (Found: N, 7.6.  $C_{30}H_{20}N_4Br_4$  requires N, 7.4%).

*Oxidation of *p*-Iodoaniline.*—*p*-Iodoaniline, b. p. 116—117°/4 mm., recrystallised from light petroleum (b. p. 60—80°), had m. p. 62—63°. The amine (1.59 g.) was dissolved in acetate buffer (1 l.). Addition of hydrogen peroxide alone caused no immediate reaction, though a control experiment showed oxidation to be 10% complete in 8 weeks. One drop of enzyme solution gave an immediate red colour, which deepened to red-brown as the oxidation proceeded. A total of 15 ml. of hydrogen peroxide was added, and solid (1.41 g.) was obtained. The brown solid (6.40 g.) was extracted (Soxhlet) successively with light petroleum (2 × 300 ml.; b. p. 80—100°) and ether (300 ml.). The red extracts were evaporated to dryness, giving amorphous solids (5.0 g. and 0.64 g. respectively).

The petroleum-soluble material (5.0 g.) was chromatographed on alumina in benzene. The lowest band gave, on evaporation to dryness, 4:4'-di-iodoazobenzene as orange leaflets (0.46 g.) which after sublimation had m. p. 237—238° (Found: C, 32.9; H, 2.3; N, 6.3. Calc. for  $C_{12}H_8N_2I_2$ : C, 33.2; H, 1.9; N, 6.5%). The next band gave on evaporation red crystals (3.45 g.). Two recrystallisations from chloroform-ethanol gave 2-amino-5-*p*-iodoanilino-*p*-benzoquinone di-*p*-iodoanil (V), m. p. 214—215°, having infra-red bands at, *inter alia*, 3247, 3344, and 3484  $cm^{-1}$ , and max. (in  $Et_2O$ ) at 240, 288, and 364  $m\mu$  ( $\epsilon$  36,600, 29,400, and 21,700 respectively) and an inflexion at 408—410  $m\mu$  ( $\epsilon$  16,600) (Found: C, 38.97; H, 2.2; N, 7.54.  $C_{24}H_{17}N_4I_3$  requires C, 38.85; H, 2.3; N, 7.55%). Acetylation gave the 2-acetamido-derivative, needles (from ethanol), m. p. 218° (Found: N, 7.2.  $C_{26}H_{19}ON_4I_3$  requires N, 7.14%).

Evaporation of the mother-liquors from the first recrystallisation of the anil (V) gave a red mass, which under a microscope showed colourless needles contaminated by red material. The mixture (0.54 g.) was fractionally recrystallised on paper, by the technique of Vasisth and Muthana (*Nature*, 1953, 172, 862), and pure colourless 2:4-di-iodoaniline (0.20 g.) was isolated having m. p. and mixed m. p. 93°.

2:4-Di-iodoaniline (cf. Bradfield *et al.*, *loc. cit.*).—Dichloramine-T (0.02 mol.) was dissolved in acetic acid (100 ml.), and sodium iodide (0.02 mol.) added slowly. The solution was added dropwise to a stirred solution of aniline (0.02 mol.) in acetic acid (50 ml.). Water (600 ml.) was added, and brown crystals of 2:4-di-iodoaniline separated (m. p. 90—91°; 4.85 g., 70%). Recrystallised from ethanol, this had m. p. 94—96° (Found: C, 20.6; H, 1.4. Calc. for  $C_8H_6NI_2$ : C, 20.8; H, 1.5%). Chromatography of the ether-soluble material gave numerous bands, none of which warranted further investigation.

We thank Dr. G. W. Kenner and Dr. R. N. Haszeldine for valuable discussions, and are grateful to the D.S.I.R. for a maintenance grant (to G. M. K. H.).