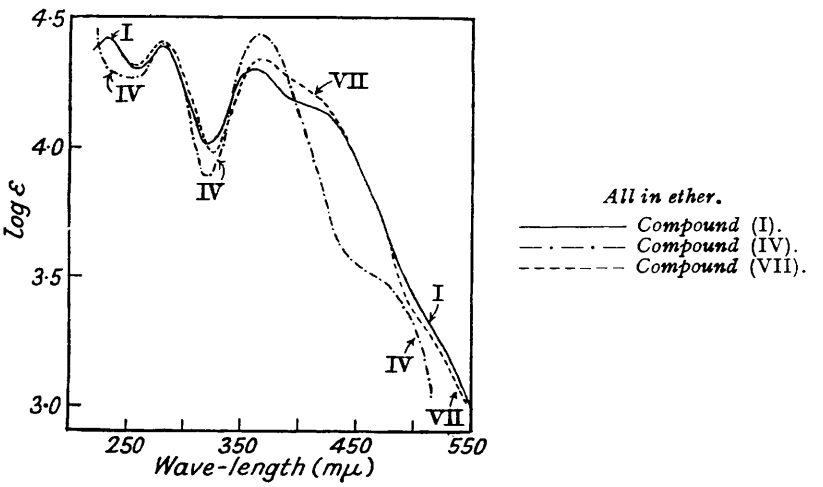
## 169. *Studies in Peroxidase Action. Part VIII.* The Oxidation of* p-*Chloroaniline. A Reaction involving Dechlorination.*

By D. G. H. Daniels and B. C. Saunders.

The peroxidase oxidation of *p*-chloroaniline at room temperature gave as the main product 2-amino-5-*p*-chloroanilinobenzoquinone di-*p*-chloroanil (I), the constitution of which has been established. Chloride ion was produced in amount sufficient to account for the production of (I). When (I) was boiled with acetone, or the crude oxidation product extracted with this solvent, 2-*p*-chloroanilino-5-*iso*propylideneaminobenzoquinone di-*p*-chloroanil (IV) was formed, and its structure established by catalytic hydrogenation to the corresponding *iso*propylaminobenzoquinone (VII).

It has already been shown that the system consisting of hydrogen peroxide and the enzyme peroxidase is capable (among other reactions) of eliminating the *p*-methyl group from mesidine (Chapman and Saunders, *J.*, 1941, 496), and the *p*-methoxyl group from 4-methoxy-2 : 6-dimethylaniline (Saunders and Watson, *Biochem. J.*, 1950, **46**, 629) and from *p*-anisidine (Daniels and Saunders, *J.*, 1951, 2112). In all these reactions, hydrogen peroxide was added intermittently (see below) and coloured crystalline compounds were obtained and their constitution determined.




In view of the stability of the chlorine atom in *p*-chloroaniline, it seemed desirable to investigate the action of the enzyme system on this substrate, particularly as *p*-toluidine is readily oxidised by the enzyme at pH 4·5 to coloured products without the elimination of the *p*-methyl group (Saunders and P.J.G. Mann, *J.*, 1940, 769). Throughout the oxidation of *p*-toluidine in dilute acetic acid, the pH remained constant at pH 4·5 in the absence of added buffer solution.

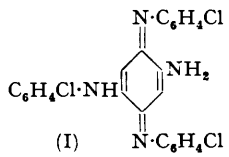
In exploratory experiments, hydrogen peroxide was added to a dilute solution of *p*-chloroaniline in aqueous acetic acid (pH 4·5), and the oxidation started by addition of the

* Part VII, *J.*, 1951, 3433.

enzyme whereupon a deep red colour was produced. Hydrogen peroxide was added intermittently (30 hours) : only a small yield of red-brown solid oxidation product was obtained and the pH had fallen to 3·5. This fall was due to accumulation of hydrochloric acid (cf. below), and the low yield of solid could be attributed in part to the inhibition of the reaction by the change in pH and in part to retardation of the enzymatic oxidation by the consequent accumulation of hydrogen peroxide relative to the enzyme, since it is known that a large excess of hydrogen peroxide also inactivates the enzyme.

Therefore, in later experiments, the oxidation of *p*-chloroaniline was carried out in acetate buffer at pH 4·6 which remained constant. In this way high yields of a red-brown solid were obtained and the method finally adopted for separating this product into its components involved (*a*) extraction with *cyclo*hexane and crystallisation of the residue, giving a dark red compound (II), (*b*) concentration of the extract and vacuum-sublimation of the crystals obtained (giving orange 4 : 4′-dichloroazobenzene), and (*c*) crystallisation of the involatile material from benzene–methanol, giving a red compound (I).

This is the first time that more than a trace of an azo-compound has been produced in peroxidase oxidation of an amine : this point will be discussed in a later paper. Elementary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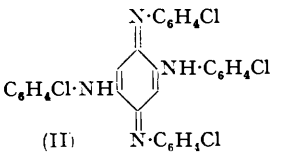
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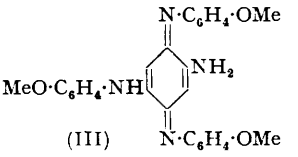
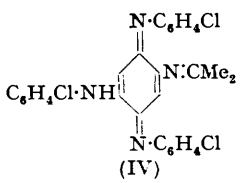

(III)

analysis and acetylation showed that the compound (I) had the partial formula $C_{24}H_{15}N_3Cl_3(NH_2)$. Its general properties clearly indicated that it was 2-amino-5-*p*-chloroanilinobenzoquinone di-*p*-chloroanil (I), analogous to a compound which we had recently obtained by the oxidation of *p*-anisidine (Daniels and Saunders, *loc. cit.*) and shown unambiguously to be (III). Further evidence in favour of structure (I) is given below.

The properties of the dark red crystals (II) agreed with those of tetra-*p*-chloroazophenine (Ikuta, *Annalen*, 1888, **243**, 288). Only a small quantity was, however, produced in the oxidation.

The aminochloroanil (I) could be produced from four mols. of *p*-chloroanil provided that dechlorination of one mol. of the latter takes place. We therefore examined the filtrate after removal of the solid product, and found that it contained chloride ion in amount approximately equal to that theoretically required for the formation of (I).

During preliminary investigations of the crude solid product, the isolation of a stable orange crystalline compound, $C_{27}H_{21}N_4Cl_3$, from acetone extracts caused some confusion until it was recognised as an artefact. It could not be acetylated, but reacted with *p*-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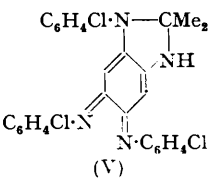
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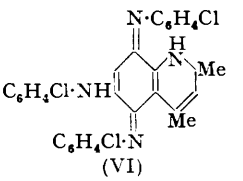
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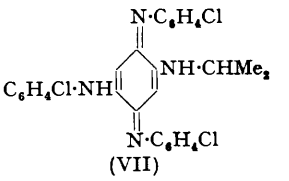
(VII)

chloroaniline, in the presence of hydrochloric acid, with the elimination of ammonia, giving tetra-*p*-chloroazophenine (II). The subsequent isolation of the pure aminochloroanil (I) from the oxidation product by *cyclo*hexane extraction rendered it probable that the orange compound was the Schiff's base (IV), or possibly (V). The cyclic structure (V) was at first thought to be more likely because of the well-known instability of the anils of acetone. Examination of a model of (V) showed that it could not be ruled out on steric grounds. Reddelien and Thurm (*Ber.*, 1932, **65**, 1511) have, furthermore, shown that the supposed Schiff's base of acetone and aniline is a dihydroquinoline derivative formed by ring closure of a dimeric form of the true anil (with elimination of aniline). The corresponding product from our amino-anil (I) would be compound (VI), $C_{30}H_{25}N_4Cl_3$, which is not in agreement with our analytical figures.

Three reactions showed that the Schiff's base formula (IV) was correct. (i) Acetone was liberated when the compound was hydrolysed with dilute sulphuric acid. (ii) When a mixture of (IV), *p*-chloroaniline, and concentrated hydrochloric acid was heated, tetra-*p*-chloroazophenine (II) was produced. Incidentally this reaction provides further support for the structure of (I) since (II) is of known constitution. (iii) Reduction over a platinum catalyst gave a leuco-base from which air yielded a new quinonoid compound, $C_{27}H_{23}N_4Cl_3$, the absorption spectrum of which was almost identical with that of the amino-compound (I), and the new compound was accordingly allotted the constitution 2-*p*-chloroanilino-5-*iso*propylaminobenzoquinone di-*p*-chloroanil (VII). (The light absorption curves of these compounds are appended.) It follows that the orange compound was 2-*p*-chloroanilino-5-*iso*propylideneaminobenzoquinone di-*p*-chloroanil (IV). Its stability, compared with the Schiff's base of acetone and aniline, may be connected with the more extended conjugated system (including the chloroanil groups in the 1 and the 4 position) of which the *iso*propylidene double bond is a part. The corresponding double bonds in the stable anils of aromatic aldehydes are similarly part of extended conjugated systems.

The conditions under which (IV) was formed from (I) and acetone were investigated. Attempted catalysis of the reaction by acetic acid, sodium acetate, hydrochloric acid, and sodium hydroxide separately was ineffective. Quantitative conversion was achieved by prolonged heating of (I) under reflux with acetone, sodium acetate, and acetic acid, preferably in the presence of a drying agent such as anhydrous sodium sulphate. Even so, the reaction was much slower than that occurring when the crude oxidation product was extracted with acetone. The nature of the catalyst in the latter case is unknown.

## Experimental

*Oxidation of* p-*Chloroaniline.*—The *p*-chloroaniline was distilled at 116°/17 mm., and was then colourless : m. p. 69—70°. The enzyme had a P.N. of 25—30, and, for use, 0·25 g. was dissolved in water (20 ml.) giving a solution containing 0·3 unit per ml. *p*-Chloroaniline (10 g.) was dissolved, with warming, in 1500 ml. of a 1·0M-buffer solution of pH 4·6, containing acetic acid (90 g.) and sodium acetate (204 g. of hydrated salt). After 16 hours, 3·5 g. of *p*-chloroaniline had crystallised. This was filtered off, leaving 6·5 g. still in solution. To this solution was added hydrogen peroxide (1 ml.; 20-vol.), no change being observed. The enzyme solution (1 ml.) was then added and a deep red colour was produced. After a few minutes the solution became turbid and an orange-brown precipitate separated, leaving the supernatant liquid colourless. Hydrogen peroxide (1 ml.) and enzyme solution (1 ml.) were added every 30 minutes until 40 ml. of each reagent had been added. At each addition of the reagents the solution became deep red again. The mixture was then filtered and the precipitate washed and dried (5·9 g.). Further addition of the reagents to the almost colourless filtrate produced, on standing, a negligible amount of solid. It was therefore concluded that the oxidation was at an end.

*The filtrate.* In a typical experiment, 6·80 g. of *p*-chloroaniline were oxidised and 5·86 g. of solid were filtered off. Aliquots of the filtrate and washings were estimated by Volhard's method (Found : Cl′ in whole filtrate, 0·31 g.). As shown later, 20% of the solid product of the reaction is 4 : 4′-dichloroazobenzene. Hence in 5·86 g. of solid, 1·17 g. is the azo-compound and 4·69 g. is non-azo-compound. On the assumption that all this is the aminobenzoquinone anil (I), and is formed by the elimination of one chlorine atom from four chloroaniline residues, then chloride ion released should be 0·355 g. As the non-azo-compound is not all compound (I) (see below), the agreement can be considered good.

*Treatment of the solid oxidation product.* The washed solid was dried *in vacuo* ($P_2O_5$–NaOH). The solid (5·9 g.) was extracted (Soxhlet) with ether until the extract was colourless. Evaporation of the brown solution to dryness gave red-brown crystals. These gave a deep violet colour with concentrated sulphuric acid. A single recrystallisation from ethanol gave red needles, m. p. 192·5°. Crystallisation of the red-brown solid from dioxan gave first red crystals, m. p. 159—161°, then on concentration of the mother-liquor yellow-brown plates, m. p. 179—180°, which on recrystallisation from methanol gave orange prisms, m. p. 182—183°. The last gave with concentrated sulphuric acid an orange colour identical with that obtained from 4 : 4′-dichloroazobenzene, An alternative method of separating the red crystals (vacuum-sublimation) is described below. By neither method was it possible to obtain as high an m. p. of the azo-compound as recorded (187·5—188·5°) by Bamberger and Baudisch (*Ber.*, 1909, **42**, 3578).

The red-brown solid residue after the ether-extraction was then extracted exhaustively with methanol which removed a small quantity of dark tar. The small amount of residual red solid dissolved completely in benzene and was examined chromatographically on alumina. The lowest, main band was eluted with more benzene, and evaporation of the red eluate gave a small quantity of dark red crystals, m. p. 240—242°. The susbtance gave with concentrated sulphuric acid a violet colour characteristic of tetra-*p*-chloroazophenine. After several recrystallisations from toluene or xylene, the m. p. was 261—262° (decomp.) (Found : C, 62·4; H, 3·8; Cl, 24·5. Calc. for $C_{30}H_{20}N_4Cl_4$ : C, 62·3; H, 3·5; Cl, 24·5%). Fischer and Hepp (*Ber.*, 1888, **21**, 678) gave m. p. 265°.

*Alternative treatment of solid oxidation product.* The original crude solid (5·75 g.) was extracted (Soxhlet) with *cyclo*hexane (150 ml.). When the extract was cooled, brown crystals separated and were filtered off (*A* ; 1·60 g.). The mother-liquor was concentrated to small bulk, and, on its cooling, a further crop (*B* ; 3·02 g.) of brown crystals separated. The dark solid residue after the *cyclo*hexane extraction was extracted with methanol to remove tar and then with benzene. From the latter solvent, crystalline tetra-*p*-chloroazophenine was obtained by the method described above.

The brown crystals obtained from *A* and *B* were heated, in portions of *ca.* 0·25 g., in a small flask provided with a "cold thimble." At 140°/0·5 mm. orange crystals rapidly sublimed, with negligible blackening of the residue. The temperature had to be carefully controlled, as at 150°/0·5 mm. decomposition of the residue was rapid, whereas below 135°/0·5 mm. sublimation was so slow that the residue had to be exposed to the elevated temperature for an unduly long period. At the optimum temperature, each portion was heated for 15 minutes only. The yields of sublimates from the crops *A* and *B* were 0·60 and 0·35 g. respectively.

The sublimates, m. p. 176—178°, were mixed and after recrystallisation from methanol had m. p. 182—183°. This substance was further purified by sublimation in a tube (60 × 0·5 cm.), of which the bottom three-quarters was surrounded by a copper jacket. On insertion into an electrically-heated block, traces of two colourless impurities (m. p. 109—111° and 162—164° severally) sublimed. At 130°/0·1 mm., the 4 : 4′-dichloroazobenzene moved only 10 cm., forming magnificent orange plates, m. p. 183—184° (Found : C, 57·5; H, 3·65; N, 11·4. Calc. for $C_{12}H_8N_2Cl_2$ : C, 57·4; H, 3·2; N, 11·15%).

The dark red non-sublimable residue was washed free from tar with a small volume of cold benzene, and recrystallised from benzene–methanol (1 : 2) as scarlet needles of 2-*amino*-5-p-*chloroanilinobenzoquinone di*-p-*chloroanil*, m. p. 196—197° (Found : C, 62·1; H, 3·95. $C_{24}H_{17}N_4Cl_3$ requires C, 61·6; H, 3·7%), giving a violet colour with cold sulphuric acid. Recrystallisation from ether gave dark red needles containing solvent of crystallisation retained on drying for 3 hours at 50°/0·1 mm. [Found : N, 11·1. $C_{24}H_{17}N_4Cl_3,\frac{1}{2}(C_2H_5)_2O$ requires N, 11·1%], but lost in 20 hours at 100°/0·1 mm. (Found : C, 61·5; H, 4·0; N, 11·95. $C_{24}H_{17}N_4Cl_3$ requires C, 61·6; H, 3·7; N, 12·0%). The solvate slowly became lighter in colour because of loss of solvent.

2-*Acetamido*-5-p-*chloroanilinobenzoquinone Di*-p-*chloroanil*.—The amino-compound (I) (60 mg.) was boiled under reflux with acetic anhydride for 10 minutes. On cooling, the mixture became almost solid, and fine silky red needles were filtered off. After recrystallisation from ethanol, the *acetyl* derivative had m. p. 205—206° (Found : C, 61·1; H, 4·1; N, 10·8. $C_{26}H_{19}ON_4Cl_3$ requires C, 61·2; H, 3·77; N, 11·0%).

2-p-*Chloroanilino*-5-iso*propylideneaminobenzoquinone Di*-p-*chloroanil*.—The crude oxidation product (3·8 g.) was extracted with acetone (Soxhlet), and the dark extract concentrated to small bulk and cooled, whereupon orange crystals (2·2 g.) separated. After recrystallisation from acetone or ethanol the iso*propylidene* compound had m. p. 197—198°, and mixed m. p. with (I) 175—180° [Found : C, 63·5; H, 4·2; N, 11·0; Cl, 21·3% ; *M* (cryoscopic in dioxan), 450. $C_{27}H_{21}N_4Cl_3$ requires C, 63·8; H, 4·2; N, 11·0; Cl, 21·0% ; *M*, 507·5. $C_{30}H_{25}N_4Cl_3$ (VI) requires C, 65·7; H, 5·1; N, 10·25; Cl, 19·5%]. The compound gave a brown colour with concentrated sulphuric acid in the cold, changed to blue-violet on heating. This sulphuric acid solution when diluted with water showed a red fluorescence in ultra-violet light.

*Attempted acetylation.* (i) The substance (50 mg.) was warmed with acetic anhydride for 10 minutes and was recovered unchanged. (ii) A mixture of the substance and acetic anhydride was heated on the steam-bath for 1½ hours. The solution, which had darkened considerably, gave ill-defined material on dilution with sodium hydroxide solution. (iii) Similar results were obtained when equal parts of acetic anhydride and pyridine were used, but a small amount of starting material was recovered.

*Reaction of* 2-p-*Chloroanilino*-5-iso*propylideneaminobenzoquinone Di*-p-*chloroanil* (IV) *with*

p-*Chloroaniline*.—A mixture of the *iso*propylidene compound (0·1 g.), *p*-chloroaniline (1·15 g., *ca.* 5 mols.), and concentrated hydrochloric acid (0·1 ml.) was heated on a steam-bath for 8 hours. The resultant mass was washed with cold methanol and then recrystallised from toluene, giving dark red crystals, m. p. and mixed m. p. with tetra-*p*-chloroazophenine, 258°. The compound gave the violet colour with concentrated sulphuric acid characteristic of the azophenine.

*Hydrolysis of* (IV).—The *iso*propylidene compound was dried at 60°/0·1 mm. for 8 hours to remove absorbed solvents. The compound (42·7 mg.) was boiled in dilute sulphuric acid (15 ml. of 20% acid diluted with 15 ml. of ethanol) for 20 minutes under reflux. The solution was then distilled and the first 2 ml. collected; 0·5 ml. of this distillate was treated with alkaline sodium nitroprusside (Feigl, " Qualitative Analysis by Spot-Tests," Cleaver Hume Press, 1947, p. 349). A pink colour was produced indicating the presence of acetone. A " blank " test omitting the *iso*propylidene compound was negative.

*Catalytic Hydrogenation of* (IV).—(a) *Over palladium oxide.* The substance (32·0 mg.) was added after the catalyst (8·0 mg.) had been reduced in dioxan (10 ml.). The uptake of hydrogen was 1·40 ml. (at N.T.P.) (1 mol. = 1·4 ml.). On exposure to air, the colourless solution soon regained its colour, and compound (IV) was recovered. After recrystallisation from alcohol it had m. p. and mixed m. p. 196—197°.

(b) *Over platinum oxide.* Hydrogenation of (IV) (29·2 mg.) in dioxan (10 ml.) over Adams' platinum oxide (10·0 mg.) was carried out similarly. The reaction did not reach a definite end-point, but was stopped after 30 hours when 7·90 ml. of hydrogen (at N.T.P.) had been absorbed (6 mols. = 7·74 ml.). When 87·7 mg. of (IV) and 49·1 mg. of catalyst (49·1 mg.) in dioxan or pure ethanol (25 ml.) were used, uptake was 32·0 ml. (at N.T.P.) (*ca.* 8 mols.) in 30 hours. The colourless solution became red in air. The catalyst was filtered off and the solvent distilled, leaving fine red needles, m. p. 212—213°. After recrystallisation from acetone–methanol (1 : 1) 2-p-*chloroanilino*-5-iso*propylaminobenzoquinone di*-p-*chloroanil* had m. p. 213—214° (Found: C, 63·7; H, 4·5; N, 10·9. $C_{27}H_{23}N_4Cl_3$ requires C, 63·6; H, 4·55; N, 11·0%); it gave a violet colour with concentrated sulphuric acid.

*Synthesis of* 2-p-*Chloroanilino*-5-iso*propylideneaminobenzoquinone Di*-p-*chloroanil under Controlled Conditions*.—The formation of this compound during the extraction of the crude oxidation product appeared to be fairly rapid. Thus in one experiment *ca.* 0·8 g. was extracted with acetone (Soxhlet) completely in about 2 hours. On concentration of the extract and cooling, orange crystals, m. p. 197—198°, were obtained.

The most likely catalyst present in the crude solid was considered to be acetic acid, although other possibilities are sodium acetate and hydrochloric acid. Acetone solutions of the pure amino-quinone (I) (0·1 g.) were boiled for 3 hours with these reagents: the starting material was recovered unchanged in each case. The possibility that air was essential was ruled out by extraction of a mixture of the pure aminochloroanil and these reagents in a small Soxhlet apparatus. In each case there was no apparent change after 3 hours.

An extraction of a mixture of the aminochloroanil (0·17 g.), anhydrous sodium acetate (0·1 g.), and glacial acetic acid (0·1 ml.) for 12 hours yielded a very small crop of orange crystals giving the characteristic brown colour with concentrated sulphuric acid.

Finally, the amino-compound (35 mg.) was mixed with anhydrous sodium acetate (13 mg.) and moistened with glacial acetic acid (0·5 ml.). Anhydrous sodium sulphate (0·5 g.) and acetone (25 ml.; dried over $K_2CO_3$) were added and the mixture boiled under reflux at 60°. After 4 hours a few orange crystals began to separate, although the solution was still dark red, and a drop of it gave a violet colour with concentrated sulphuric acid. After 24 hours' heating, however, the solution was orange. The metallic salts were filtered off, the filtrate was concentrated, and orange crystals separated on cooling (yield, 24·6 mg., 65%). After recrystallisation from acetone and methanol (1 : 1), these had m. p. and mixed m. p. with authentic 2-*p*-chloroanilino-5-*iso*propylideneaminobenzoquinone di-*p*-chloroanil, 196—198°.

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