75. *The Chemistry of Colour Reactions: The Tyrosine Test of Gerngross, Voss, and Herfeld.*

By T. J. King and C. E. Newall.

The reaction of *p*-alkylphenols with 1-nitroso-2-naphthol under acid conditions in the presence of an oxidising agent has been shown to give a mixture of 10-alkylbenzo[*a*]phenoxazin-9-ones [*e.g.*, (I)] and -5-ones [*e.g.*, (VII)], the latter predominating. The colour produced in the tyrosine test under the standard conditions is not due to the formation of these phenoxazinones. A suggestion is made as to the possible nature of the species responsible for the test colour.

Amongst the various methods for the detection and estimation of tyrosine in protein hydrolysates one of the most unambiguous and reproducible is the colour reaction devised by Gerngross, Voss, and Herfeld.[1] This uses a solution of 1-nitroso-2-naphthol in dilute nitric acid, the presence of tyrosine being shown by the formation of a cherry-red colour. Gerngross, Voss, and Herfeld also observed that the nitric acid could be replaced by any strong mineral acid plus an oxidising agent, *e.g.*, manganese dioxide. They also suggested that the role of the oxidising agent was to convert the nitrosonaphthol into the nitronaphthol. This cannot be so as no red colour is formed when the nitro-compound is substituted for the nitroso-compound in the test. Other attempts [2,3] have been made to explain the chemistry of the test, and Udenfriend and Cooper [4] isolated a coloured salt from the reaction of tyrosine with 1-nitroso-2-naphthol and nitric acid for which analysis led to a formula $C_{18}H_{14}N_2O_2,HNO_3$, but they did not propose a structure for it.

p-Cresol was chosen as a suitable model for tyrosine and readily gave a positive test under the standard conditions. In experiments to isolate the coloured products, sulphuric acid and manganese dioxide were substituted for nitric acid to obviate possible nitration of the products or intermediates. When the red solution so obtained from *p*-cresol was diluted with a large volume of water a brown precipitate was formed which was purified by chromatography to give a yellow crystalline material soluble in acid to a red solution with a very similar absorption spectrum to that of the original test solution.

The reaction closely resembles that of Fischer and Hepp [5] who condensed *p*-nitrosophenol with 2-naphthol in acetic acid with zinc chloride to give the benzophenoxazinone (I; R = R′ = H). This reaction presumably goes through the intermediate (II; R = R′ = H) which cyclises either by direct oxidative coupling or by addition of the hydroxyl group to the quinonoid system followed by oxidation of the hydroxyphenoxazine so produced, perhaps by excess of *p*-nitrosophenol. The intermediate (II; R = R′ = H) would also be produced by the condensation of 1-nitroso-2-naphthol with phenol itself, but with a *para*-substituted phenol the initial condensation product would have to be of the type (III) and its tautomer (IIIa). An intermediate of this kind, if it cyclised by the same route as must operate in the Fischer–Hepp synthesis, would give a benzo[*a*]phenoxazin-11-one, which is not a known system. Under the standard test conditions phenol does not give a positive result but, under the preparative conditions used, two phenoxazinones are formed. That produced in smaller amount was red and proved identical with the benzophenoxazinone (I; R = R′ = H) prepared by the method of Fischer and Hepp. The major product, however, was a yellow compound, isomeric with the red one, and which,

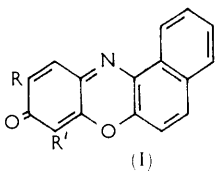
[1] Gerngross, Voss, and Herfeld, *Ber.*, 1933, **66**, 435; Cobbett, Kenchington, and Ward, *Biochem. J.*, 1962, **84**, 468; Ceriotti and Spandrio, *ibid.*, 1957, **66**, 607; Thomas, *Arch. Biochem.*, 1944, **5**, 175.
[2] Ramachandran and Sarma, *J. Sci. Ind. Res., India*, 1951, **101**, 246.
[3] Giral, *Anales Inst. Invest. cient. (Univ. Nuevo Leon, Monterrey, Mex.)*, 1944, **1**, 115 (*Chem. Abs.*, 1947, **41**, 2107e).
[4] Udenfriend and Cooper, *J. Biol. Chem.*, 1952, **196**, 227.
[5] Fischer and Hepp, *Ber.*, 1903, **36**, 1807.

from its ultraviolet absorption, was clearly analogous in structure to the product formed under the same conditions from *p*-cresol.

A reaction under our preparative conditions with 2,6-xylenol which can couple only in the *para*-position to give the intermediate (II; R = R′ = Me), afforded, in very small yield, only one benzophenoxazinone, which was red and must have the structure (I; R = R′ = Me). Confirmation of this structure was obtained by synthesis of the compound from 2-naphthol and 2,6-dimethyl-4-nitrosophenol by the Fischer–Hepp route.

The yellow benzophenoxazinones formed in the reaction with phenol and *p*-cresol presumably come from intermediates of type (III) and it is not improbable that cyclisation occurs between the two oxygen groups in this system.

Direct cyclisation of a compound of type (III) to give a phenoxazonium salt (IV) cannot be the cause of the colour formation because an oxidising agent is necessary for the production of colour. At one time it seemed probable to us that the intermediates (III) were oxidised to other uncyclised intermediates, *e.g.*, (V) or (VI), which then cyclised by loss of water to give phenoxazin-9-ones (I) and -5-ones (VII), which, in acid solution as the corresponding phenoxazonium salts, could be responsible for the colour of the test solutions. According to this idea an *ortho*-coupled intermediate (III) could give both benzophenoxazinones (I) and (VII), but it would be expected that the latter would be formed in greater amount because of the greater stability of the tautomer (IIIa).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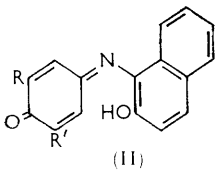
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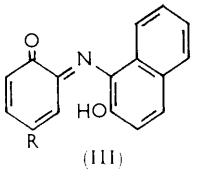
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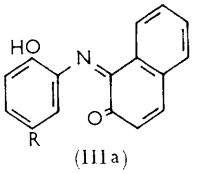
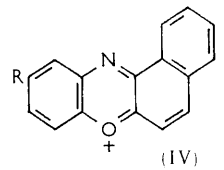
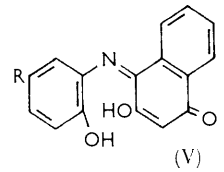

(IIIa)


(IV)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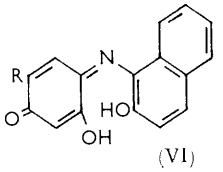
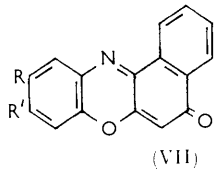

(VI)


(VII)

According to this scheme the yellow product from *p*-cresol would have structure (VII; R = Me, R′ = H), and this was established by a synthesis from 2-hydroxy-1,4-naphthoquinone and 2-amino-4-methylphenol. Further examination of the tail fractions from the chromatographic purification of this compound from the test solution by thin-layer chromatography showed the presence of a red benzophenoxazinone which we believed to be compound (I; R = Me, R′ = H) produced by the less favoured route. The structure of the yellow compound from phenol was established as (VII; R = R′ = H) by synthesis from 2-hydroxy-1,4-naphthoquinone and *o*-aminophenol. A red benzophenoxazinone prepared by the Fischer–Hepp method from 2-methyl-4-nitrosophenol and 2-naphthol, which could have had structure (I; R = Me, R′ = H or *vice versa*) was shown by thin-layer chromatography to be different from the *p*-cresol product and must therefore have been the 8-methyl isomer. 3,4-Xylenol was also treated with 1-nitroso-2-naphthol under preparative conditions and gave a single yellow benzophenoxazinone, identical with that produced from 2-hydroxy-1,4-naphthoquinone and 2-amino-4,5-dimethylphenol; it must therefore have been the expected 9,10-dimethylbenzo[*a*]phenoxazin-5-one (VII; R = R′ = Me).

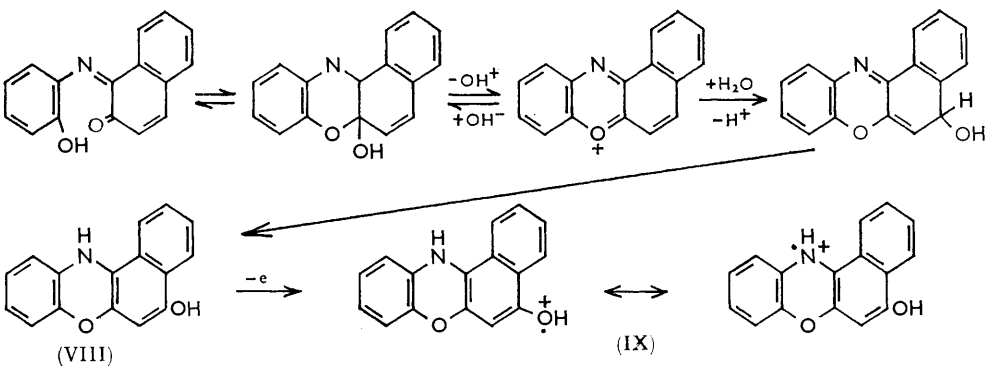
In addition to the phenols already mentioned the test was carried out with *o*- and *m*-cresol, 2,4- and 3,5-xylenol, 2-isopropyl-4,5-dimethylphenol, and 1- and 2-naphthol. All these phenols could be made to give a positive result with the sulphuric acid–manganese dioxide reagent, but under the milder conditions specified for the tyrosine test, with the

understandable exception of the reactive 2-naphthol, only those phenols with a *para*-alkyl substituent and at least one *ortho*-position free gave a positive reaction. A large-scale reaction between *p*-cresol and 1-nitroso-2-naphthol in the presence of dilute nitric acid gave the same yellow benzophenoxazinone (IV; R = Me, R′ = H) as was obtained under the more vigorous conditions generally employed in the preparative experiments.

Two observations of interest were made during the thin-layer chromatography of these products. All the yellow compounds, on alumina, when spotted with concentrated sulphuric acid, gave a blue colour which faded to lilac, whilst all the red compounds gave a green spot fading to lilac with a blue edge. Further, in the red series the R_F value increased with increasing alkyl substitution whilst in the other (benzophenoxazin-5-one) series the R_F decreased with increasing alkyl substitution.

The above experiments amply confirmed the nature of the end-products of the reaction. However, further studies showed conclusively that these end-products themselves could not be the cause of the colour produced in the test. Thus, although the benzophenoxazin-5-one from *p*-cresol would dissolve in strong acid to give a solution whose absorption spectrum was very similar to that of the test solution there were slight differences in these spectra and, more important, at the acid concentration employed in the test (<1N), the benzophenoxazinones were insoluble and did not give red solutions. It was further noticed that, when manganese dioxide was used as the oxidising agent in the test and removed by filtration after the colour had developed, then the colour of the filtrate disappeared slowly in the dark but much more quickly in daylight. This observation suggested that the colour might be due to a free radical and this view was reinforced when it was seen that the colour was discharged rapidly by the action of nitric oxide on the solution.

The formation of phenoxazinones by one-electron oxidation of phenoxazine derivatives is well known. The work of Kehrmann and Boubis [6] first suggested this and it was confirmed by Michaelis and his co-workers.[7] Phenoxazines are known to be readily oxidised in the position *para* to the bridging nitrogen atom by many oxidising agents, and if this takes place by a two-step process it is quite reasonable in the light of Michaelis's work to suggest that a radical-ion intermediate is sufficiently stable to be responsible for the colour of



the " tyrosine test." There are many possible ways in which such a scheme could be formulated and one which we believe to be the most reasonable can be represented as shown. The phenoxazonium salt (IV), which would only be present in very small concentration, could suffer nucleophilic attack by solvent at position 5 to give, after the appropriate tautomeric shift, the hydroxyphenoxazine (VIII). Removal of one electron from this would give the resonance-stabilised radical-ion (IX), and subsequent hydrogen abstraction would yield a phenoxazonium salt and, hence, the phenoxazinones which have been shown

[6] Kehrmann and Boubis, *Ber.*, 1917, **50**, 1662.
[7] Michaelis, Granick, and Schubert, *J. Amer. Chem. Soc.*, 1940, **62**, 1802; 1941, **63**, 351.

to be the final products. A very similar mechanism has been suggested [7] for the oxidation of 3-hydroxyphenoxazine itself.

Experimental

Light petroleum was the fraction of b. p. 60—80°. Alumina for column chromatography was Spence's grade H. Thin-layer chromatography was performed on a 275 μ layer of Merck " Aluminiumoxid G " with benzene as the eluant. Ultraviolet absorptions were measured for chloroform solutions with a Unicam S.P. 700 instrument. M. p.s were determined on a Kofler hot-stage apparatus.

10-*Methylbenzo*[a]*phenoxazin*-5-*one* (VII; R = Me, R′ = H).—(*a*) Manganese dioxide (10 g.) was added to a solution of 1-nitroso-2-naphthol (4 g.) and *p*-cresol (5 g.) in methanol (50 c.c.) at 0°. Concentrated sulphuric acid (10 c.c.) was added dropwise with shaking and after 5 min. the red solution was poured into water (1 l.). The suspension was heated for 2 hr. on a steam-bath and the brown precipitate was collected, washed with dilute alkali and water, and dried. The solid was extracted continuously with light petroleum until no more coloured material was extracted. The extract was evaporated and the residue purified by chromatography in chloroform on alumina. The first coloured band was blue and was discarded and the second band (yellow with a red tail) was crystallised from chloroform–light petroleum giving 10-*methylbenzo*[a]*phenoxazin*-5-*one* as golden-brown needles (2 g.), m. p. 216—217° (Found: C, 77·8; H, 4·2; N, 5·3. $C_{17}H_{11}NO_2$ requires C, 78·15; H, 4·2; N, 5·4%), $\lambda_{max.}$ 240 (ε 20,400), 248, (ε 22,600), 262 (ε 14,900), 286 (ε 10,000), 361 (ε 12,900), and 456 mμ (ε 14,400).

(*b*) A solution of 1-nitroso-2-naphthol (1·7 g.) and *p*-cresol (2 g.) in ethanol (200 c.c.) was added to 2N-aqueous nitric acid (100 c.c.), and the mixture was heated at 60—70° for 2 hr. The resulting red solution was neutralised with 2N-aqueous sodium hydroxide and extracted with ether. The washed and dried extract was evaporated and the residue was purified as in (*a*) to give the same benzophenoxazinone as yellow needles, m. p. and mixed m. p. 216—218°, identical in its spectral properties with the product obtained in (*a*).

(*c*) 4-Methyl-2-nitrophenol [8] (10 g.) in acetic acid (80 c.c.) was shaken with hydrogen and palladised charcoal at room temperature and pressure until reduction was complete. The resulting solution, freed from catalyst, was treated with 2-hydroxy-1,4-naphthoquinone (5 g.) and water (20 c.c.) and heated for 2 hr. on a steam-bath. Next day, the resulting precipitate was collected and crystallised from chloroform–benzene (1 : 1) (charcoal) to give the benzophenoxazinone (1·6 g.) as yellow needles, m. p. 215—217°.

Light Absorption Properties of the Test Solutions.—The maximal absorption in the visible range of test solution was at *ca.* 500 mμ, broad peak, and that of solutions of the pure benzophenoxazin-5-one in 20% sulphuric acid was *ca.* 503 mμ, broad peak, the shapes of the absorption envelopes were slightly different. After 30 min., in the absence of oxidising agent, the absorption of a test solution showed a maximum at *ca.* 450 mμ similar to that shown by the benzophenoxazin-5-one in neutral or weakly acid solutions.

A test solution freed from oxidising agent (MnO_2) faded to yellow in 10 min. on the presence of diffuse daylight, kept in the dark the solution was still red after 30 min. A red test solution, free of oxidising agent, was decolourised in 30 sec. by treatment, under nitrogen, with a stream of nitric oxide.

Benzo[a]*phenoxazin*-5-*one* (VII; R = R′ = H).—A solution of phenol (2 g.) and 1-nitroso-2-naphthol (1·7 g.) was treated as in (*a*) above. Chromatography on alumina in benzene separated the product into a yellow compound, which was eluted first, and a red compound. Rechromatography and sublimation of the yellow product gave the benzophenoxazinone (50 mg.) as needles, m. p. 195—197° undepressed by an authentic specimen [9] (m. p. 197—198°), $\lambda_{max.}$ 238 (ε 21,300), 246 (ε 21,800), 260 (ε 14,900), 283 (ε 8600), 358 (ε 12,200), 373 (ε 11,800), and 439 mμ (ε 13,600).

Benzo[a]*phenoxazin*-9-*one* (I; R = R′ = H).—The red fraction from the preceding experiment, on sublimation, gave benzo[*a*]phenoxazin-9-one (10 mg.) as needles, m. p. 225—226° identical with an authentic specimen,[5] $\lambda_{max.}$ 255 (ε 26,800), 261 (ε 26,800), 291 (ε 16,600), 312 (ε 9300), 407 (ε 6100), and 496 mμ (ε 17,900).

8,10-*Dimethylbenzo*[a]*phenoxazin*-9-*one* (I; R = R′ = Me).—(*a*) A solution of 2,6-xylenol

[8] Schultz, *Ber.*, 1907, **40**, 4324.
[9] Kehrmann, *Ber.*, 1895, **28**, 353.

(5 g.) and 1-nitroso-2-naphthol (4 g.) in methanol (50 c.c.) was treated with manganese dioxide and sulphuric acid as above. Chromatography of the product on alumina in chloroform, followed by crystallisation of the red eluate from chloroform–light petroleum and sublimation at 140°/0·2 mm., gave the benzophenoxazin-9-one (35 mg.) as red needles, m. p. 187—188°, identical with the material prepared by method (*b*).

(*b*) A solution of 2-naphthol (4 g.) and 2,6-dimethyl-4-nitrosophenol [10] (6 g.) in acetic acid (50 c.c.) was shaken with anhydrous zinc chloride (11 g.) for 20 hr. The precipitated zinc salt was collected and decomposed by boiling it for 1 hr. with 25% sulphuric acid. The free *benzophenoxazin-9-one* was collected and crystallised from chloroform–ethanol to give red needles (0·8 g.), m. p. 188° (Found: C, 78·4; H, 4·8. $C_{18}H_{13}NO_2$ requires C, 78·5; H, 4·8%), $\lambda_{max.}$ 264 (ε 24,000), 271 (ε 23,300), 293 (ε 17,100), 314 (ε 8800), 389 (ε 9000), 405 (ε 9000), and 495 mμ (ε 14,700).

8-*Methylbenzo*[a]*phenoxazin-9-one.*—2-Methyl-4-nitrosophenol [11] (6 g.) and 2-naphthol (4 g.) were condensed as in the preparation of the 8,10-dimethyl analogue above. The *benzophenoxazinone* (0·2 g.), red needles, m. p. 215—217° (from chloroform–light petroleum) (Found: C, 78·1; H, 4·25; N, 5·45. $C_{17}H_{11}NO_2$ required C, 78·15; H, 4·25; N, 5·35%), $\lambda_{max.}$ 291 (ε 14,600), 313 (ε 9000), 402 (ε 7300), and 494 mμ (ε 15,700), was distinct from the 10-methyl isomer on thin-layer chromatography (see below).

9,10-*Dimethylbenzo*[a]*phenoxazin-5-one* (VII; R = R′ = Me).—(*a*) 3,4-Xylenol (5 g.) and 1-nitroso-2-naphthol (4 g.) were condensed under the usual conditions. The *benzophenoxazinone* was obtained, after chromatography, as yellow needles, m. p. 220—222° (from chloroform–light petroleum) (Found: C, 78·2; H, 4·7; N, 5·1. $C_{18}H_{13}NO_2$ requires C, 78·5; H, 4·8; N, 5·1%).

(*b*) 4,5-Dimethyl-2-nitrophenol [12] (3·8 g.) was reduced catalytically in acetic acid over palladium–charcoal and the amine produced was condensed, without isolation, with 2-hydroxy-1,4-naphthoquinone (2 g.) to give the dimethylbenzo[*a*]phenoxazin-5-one as yellow needles (1 g.), m. p. 226—228°, undepressed by the product from method (*a*).

Detection of 10-*Methylbenzo*[a]*phenoxazin-9-one from the Test with* p-*Cresol.*—The red tailings from the chromatographic purification of 10-methylbenzo[*a*]phenoxazin-5-one were concentrated by further chromatography on alumina in benzene and were then examined by thin-layer chromatography. Five coloured spots were observed, two of which showed strong fluorescence in ultraviolet light. The yellow spot was shown to be due to 10-methylbenzo[*a*]phenoxazin-5-one by direct comparison with a pure sample, the red spot (red fluorescence) showed the characteristics (see below) of the authentic benzo[*a*]phenoxazin-9-ones and is presumably produced by 10-methylbenzo[*a*]phenoxazin-9-one.

Thin-layer Chromatography of the Above Benzophenoxazinones.—All benzo[*a*]phenoxazin-5-ones gave yellow spots with a yellow fluorescence and a characteristic colour in concentrated sulphuric acid; the benzo[*a*]phenoxazin-9-ones gave red spots with red fluorescence. The R_F values were reproducible within a factor of 10% for different plates and were determined by the degree of alkylation present. Isomeric benzophenoxazinones showed small but real differences in R_F within their appropriate group. The values obtained were: benzo[*a*]phenoxazin-5-one, no methyl group R_F 0·44, 1 methyl group R_F 0·35, 2 methyl groups R_F 0·30; benzo[*a*]-phenoxazin-9-ones, no methyl group R_F 0·13, 1 methyl group R_F 0·25, 2 methyl groups R_F 0·48.

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[10] Auwers and Markovits, *Ber.*, 1908, **41**, 2335.
[11] Bridge and Morgan, *Amer. Chem. J.*, 1898, **20**, 766.
[12] Diepolder, *Ber.*, 1909, **42**, 2917.