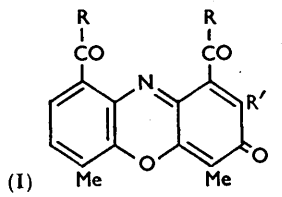
306. *Actinomycin. Part III.* The Reaction of Actinomycin with Alkali.*

By S. J. Angyal, E. Bullock, W. G. Hanger, W. C. Howell, and A. W. Johnson.

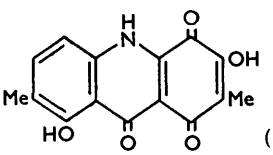
The production and purification of actinomycin B are described. Addition of dilute alkali to an alcoholic solution of actinomycin causes a marked hypsochromic effect which is reversible. The reaction obeys second-order kinetics and is interpreted as a fission of the oxygen bridge of the phenoxazin-3-one nucleus, giving rise to a highly strained and non-planar quinone anil. The reverse reaction involves two stages; ring closure and elimination of water. Isatin, another molecule which shows a hypsochromic shift in the spectrum after addition of alkali, is shown to possess spectral characteristics which differ from those of actinomycin in several respects.

Actinomycin B [1] has been prepared from strain "X-45" [2] and has been shown by chromatography [3] to consist of at least four components. Brockmann and Vohwinkel [4] have produced evidence to suggest that the chromophore is common to all members of the actinomycin group and consequently crystalline actinomycin B has been used for most of our degradative work aimed at the elucidation of the nature of the chromophore.

In a paper [5] describing its isolation and chemistry it was stated that actinomycin was probably a hydroxyquinone, on the basis of its reversible reduction and formation of acetyl and *leuco*-acetyl derivatives. This view was unchallenged until 1955 when, in a preliminary communication,[6] we pointed out that the absorption spectrum of actinomycin (max. at 234 and 444 mμ; log ε 4·50 and 4·33) was not of the usual hydroxyquinone type. The recent derivation of the 2-aminophenoxazin-3-one peptide structure (I; R′ = NH_2; R = L-threonyl-D-*alloiso*leucyl-L-prolylsarcosyl-L-*N*-methylvaline with the terminal carboxyl group esterified by the hydroxyl of the threonyl fragment) for actinomycin C_3 [7] has



(I)



(II)

justified our earlier misgivings, although before this publication other chemical properties were discovered which were not in accord with the hydroxyquinonoid formulation. Thus,

* Part II, *J.*, 1952, 2672.

[1] Dalgliesh, Johnson, Todd, and Vining, *J.*, 1950, 2946.
[2] Lehr and Berger, *Arch. Biochem.*, 1949, **23**, 503.
[3] Brockmann and Gröne, *Naturwiss.*, 1953, **40**, 222, 224; 1954, **41**, 65; *Chem. Ber.*, 1954, **87**, 1036; Vining *et al.*, *Science*, 1954, **120**, 389; *J.*, 1956, 2469.
[4] Brockmann and Vohwinkel, *Naturwiss.*, 1954, **41**, 257.
[5] Waksman and Tishler, *J. Biol. Chem.*, 1942, **142**, 519.
[6] Angyal, Bullock, Hanger, and Johnson, *Chem. and Ind.*, 1955, 1295.
[7] Brockmann, Bohnsack, Franck, Gröne, Muxfeldt, and Süling, *Angew. Chem.*, 1956, **68**, 70.

the conversion of a hydroxyquinone into its anion usually involves a pronounced bathochromic shift in the absorption spectrum, whereas the initial yellow colour of a dilute solution of actinomycin in aqueous sodium hydroxide rapidly fades until an almost colourless solution (max. at 282—285, 343 and a low-intensity band at 458 mμ) is formed. Actinomycin can be recovered from the alkaline solution provided that it is not heated or even kept at room temperature for more than about one hour. After that, evolution of ammonia becomes pronounced and a neutral degradation product is produced, which on further reaction with alkali forms the salt of an acid. The product derived from actinomycin B with cold alkali described in our earlier paper [8] has now been shown to be a mixture of these compounds which can be separated by preliminary extraction of solutions in organic solvents with aqueous sodium hydrogen carbonate, followed by counter-current distribution in benzene–ethanol–water. In the actinomycin C series, the parallel substances have been investigated in detail by Brockmann and Franck.[9] The neutral

FIG. 1. *Rate of reaction of actinomycin with alkali calculated from* (a) *the rate of appearance of the* 343 mμ *peak* (×) *and* (b) *the rate of disappearance of the* 444 mμ *peak* (⊙).

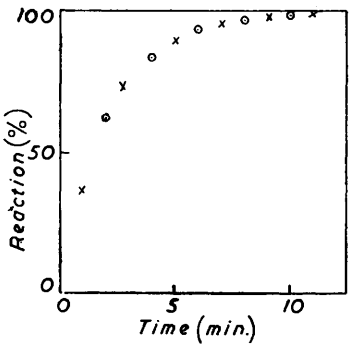
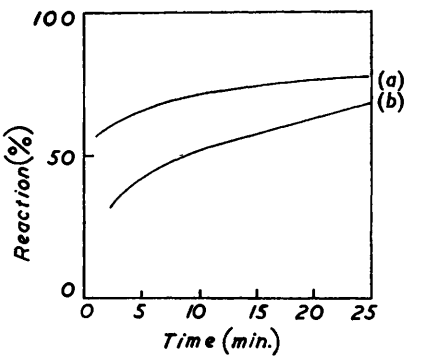


FIG. 2. *Rate of reaction of dilute acid with an alkaline solution of actinomycin calculated from* (a) *the rate of disappearance of the* 343 mμ *peak and* (b) *the rate of appearance of the* 444 mμ *peak.*



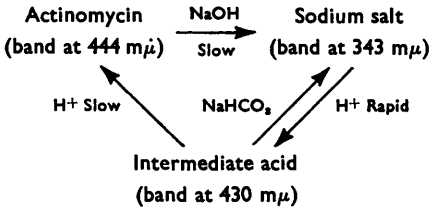
product, " deaminoactinomycin," was formed by acid hydrolysis of the 2-aminophenoxazin-3-one fragment (I; R = peptide; R′ = NH_2) to the corresponding 2-hydroxyphenoxazin-3-one (I; R = peptide; R′ = OH) and the acid degradation products, actinomycinic acid and the corresponding " deaminoactinomycinic acid " (NH_2 ⟶ OH), were formed by the opening of the peptide lactone groups with methanolic sodium hydroxide. Vigorous treatment of actinomycin with aqueous barium hydroxide causes extensive rearrangement, to give actinomycinol [8] or depeptidoactinomycin [10] (II).

The alkaline decolorisation of actinomycin is not rapid, *e.g.*, under the experimental conditions used, decolorisation was complete only after about 30 minutes. The comparatively slow reaction enabled the kinetics to be determined by spectroscopic methods. It is of the second order, the rate being proportional to the concentration both of actinomycin and of alkali, and hence the decolorisation is not merely an ionisation but probably involves the addition of hydroxyl ion. The rate of disappearance of the band at 444 mμ is equal to the rate of appearance of the 343 mμ band (Figs. 1 and 2) and it is reasonable to conclude that no intermediate of appreciable stability is involved in the decolorisation process.

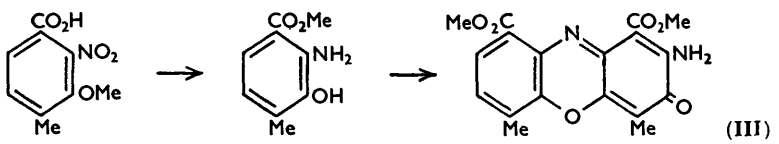
Reversal of the above reaction by acidification of the alkaline solution is not so straightforward; the 343 mμ band disappears much faster than the 444 mμ band appears and it

[8] Johnson, Todd, and Vining, *J.*, 1952, 2672.
[9] Brockmann and Franck, *Chem. Ber.*, 1954, **87**, 1767; *Angew. Chem.*, 1956, **68**, 68.
[10] Brockmann *et al.*, *Chem. Ber.*, 1956, **89**, 1373, 1379, 1393; see also Part V of this series, *J.*, in the press.

was not until this reaction was studied with a Cary self-recording spectrophotometer that the true state of affairs was established. It transpired that the 343 mμ band disappears immediately (within the limitations of the intrument) after acidification and is replaced by a band at 430 mμ with a shoulder at 445 mμ. This phase of the reaction is reversed when excess of aqueous sodium hydrogen carbonate is added to the freshly acidified solution, and in this respect the new product differs from actinomycin, solutions of which are unchanged by addition of sodium hydrogen carbonate. However, the solution of the product obtained by acidifying an alkaline solution of actinomycin is unstable and the spectrum slowly changes until that of the original actinomycin is obtained. The situation can therefore be represented :

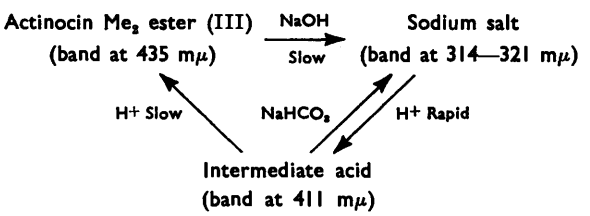


The same spectral changes are shown by deaminoactinomycin B and deaminoactinomycin acid (formed by prolonged reaction of actinomycin B with alkali) and also by actinocin dimethyl ester [11] (III), now synthesised by a modified procedure from 3-methoxy-2-nitro-*p*-toluic acid ($CO_2H = 1$) :



(III)

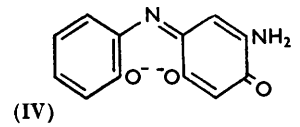
The ester (III) reacts more slowly with acid and alkali than does actinomycin, and the absorption maxima of the products are more widely spaced, so that it is easier to follow the various reactions by the spectral changes :

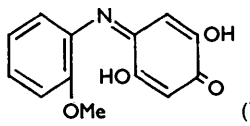


A similar cycle of reactions has been observed with dimethyl 2-amino-3-oxophenoxazine-1 : 9-dicarboxylate and it therefore appears that these reactions are characteristic of the phenoxazin-3-one nucleus. By contrast, acidification of solutions of sodium isatinate showed no immediate change in the spectrum; the strong peak at 370 mμ slowly faded, giving rise to the isatin peak at 415 mμ. The oxygen bridge in the phenoxazin-3-one system is part of a vinylogous ester system and it is probable that alkali causes a slow fission of the heterocyclic ring,[12] giving rise to an anion of a 2 : 2′-dihydroxyquinone anil. Quinone anils containing substituents in the 2- and/or 2′-position are known to be appreciably sterically hindered [13] and the consequent loss of planarity and inhibition of resonance has a marked effect on the absorption spectrum. In an attempt to prepare

[11] Brockmann and Muxfeldt, *Angew. Chem.*, 1956, **68**, 69.
[12] Cf. Butenandt, Biekert, and Neubert, *ibid.*, p. 379.
[13] McOmie and White, *J.*, 1955, 2619; Vittum and Brown, *J. Amer. Chem. Soc.*, 1947, **69**, 152; Horner and Sturm, *Chem. Ber.*, 1955, **88**, 329.

a crystalline derivative from the dianion (IV) an alkaline solution of 2-aminophenoxazin-3-one was treated with dimethyl sulphate and gave a red solid which was not obtained completely pure but appeared nevertheless to correspond to a molecular formula $C_{13}H_{11}O_4N$. The assigned structure (V) was derived from the dianion (IV) by replacement of the amino-group by hydroxyl and by methylation of the 2′-phenoxide group.


(IV)

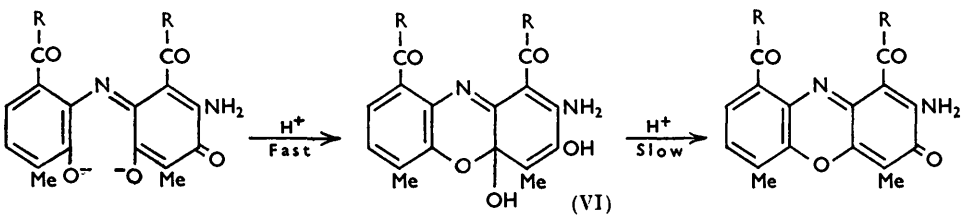

(V)

The spectrum of the ether (V) showed a marked qualitative resemblance to that of quinone anil [13] and the molecule must therefore have assumed a planar configuration, presumably by rotation about the nitrogen atom. By contrast, in the anion derived from actinomycin, the presence of bulky groups in all four positions *ortho* to the nitrogen bridge atom prevents rotation and thus contributes to the strong hypsochromic shift in the spectrum after the addition of alkali to actinomycin.

Acidification of the alkaline solution derived from actinomycin causes an immediate bathochromic shift of *ca.* 90 mμ and hence the product cannot be formulated as the acid corresponding to the anion. The intense band at 430 mμ in the spectrum of the acidification product suggests that the whole molecule is once more planar and examination of models suggests that this can be achieved only if the oxygen bridge is re-formed. This intermediate is tentatively formulated as (VI; R = peptide), a molecule which slowly reverts to the original phenoxazin-3-one structure by elimination of water.

The bathochromic shift of *ca.* 20 mμ observed when actinomycin is dissolved in acids [14] is attributed to salt formation on the heterocyclic nitrogen atom. The same phenomenon has been observed with xanthommatin, another substituted phenoxazin-3-one.[15]

Before it was appreciated that actinomycin was a derivative of phenoxazin-3-one, a number of other chromophoric systems reversibly decolorised by alkali, were considered. For example, this behaviour recalled certain hydroxy-derivatives of methylenequinones (*e.g.*, phenolphthalein with excess of alkali) but such compounds, unlike actinomycin, are not reversibly reduced unless other structural features are also present as in fuscin.[16] Another molecule which reproduces many of the properties of actinomycin including the reversible reduction is isatin which forms colourless salts of isatic acid on addition of alkali. When it was found [17] that 7-hydroxy-6-methylisatin, like actinomycin, could be

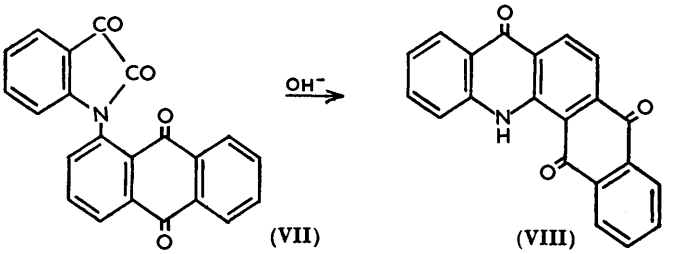

(VI)

converted into 7-methylbenzoxazolone-4-carboxylic acid (or the corresponding peptide) by oxidation with alkaline hydrogen peroxide, it seemed possible that isatin or a derived structure might be part of the actinomycin chromophore. The related structures indane-2 : 3-dione and coumaran-2 : 3-dione were eliminated because of the reported instability [18] and bathochromic spectral shift [19] with alkali respectively.

[14] See also Brockmann and Franck, *Naturwiss.*, 1954, **41**, 451.
[15] Butenandt, Scheidt, and Biekert, *Annalen*, 1954, **588**, 106.
[16] Barton and Hendrickson, *J.*, 1956, 1028.
[17] Bullock and Johnson, following paper.
[18] Perkin, Roberts, and Robinson, *J.*, 1912, **101**, 234.
[19] Cram, *J. Amer. Chem. Soc.*, 1950, **72**, 1028.

Little to distinguish actinomycin from the isatins could be derived from infrared spectra; thus chloroform solutions of actinomycin and isatin showed bands at 1754 cm.$^{-1}$ with inflections at 1770 cm.$^{-1}$ in the carbonyl region. In a polarographic examination of actinomycin B, kindly determined for us by Dr. N. S. Hush at the University of Manchester, a marked feature was the semiquinone formation in acid solution at 0·15 v. Isatin likewise showed this phenomenon at 0·28 v.[20] The formation of a purple anion from isatin [21] was also simulated when actinomycin was treated with alcoholic sodium ethoxide.

However, the ultraviolet and visible absorption spectrum of actinomycin showed appreciable differences from those of typical isatins. Thus, the intensity (log ε) of the



(VII) (VIII)

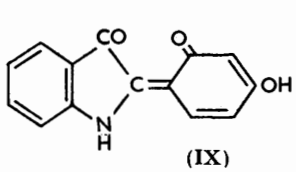
absorption band in the visible region of the spectrum rarely exceeded 3·0 for simple isatins whereas for actinomycin at 443—445 mμ it was 4·33. Treatment with dilute aqueous sodium hydroxide caused a hypsochromic effect (Δmμ) generally less (30—80 mμ) for the isatins than for actinomycin (98 mμ), and the intensity of the new band in the spectrum of the alkaline solution was greatly increased in the case of the isatins (Δ log ε 0·4—1·3) but unchanged for actinomycin. In some ways, this behaviour of actinomycin resembled more closely that of the *N*-acylisatins but these compounds, once converted into the isatic acids by treatment with alkali, did not re-form the parent acylisatins on acidification, possibly because of the competing quinoline formation.[22] Acidification of alkaline solutions of hydroxyisatins, unlike the case of actinomycin, caused an immediate *hypsochromic* effect, because of the neutralisation of the phenoxide ion, and this was followed by a slow bathochromic shift marking the appearance of the original isatin.

Nevertheless in view of the established acridone-quinone structure (II) for actinomycinol we were impressed by the alkaline rearrangement of *N*-(1-anthraquinonyl)isatin (VII) to 1 : 2-phthaloylacridone (VIII) [23] and it seemed that the carbon atoms of the quinonoid ring of actinomycinol must have been already present as a quinonoid ring.[24] Had this potential quinone ring been attached to the isatin group through the nitrogen atom as in (VII), the actinomycin chromophore on catalytic hydrogenation should have absorbed more hydrogen than the one mol. observed.[8] For this reason we attempted to prepare certain 3-oxindolylidene derivatives where the precursor of the quinone ring of actinomycinol was attached to the isatin through $C_{(3)}$.

Friedlaender and his co-workers [25] have described several members of the corresponding indoxylylidene series (*e.g.*, IX or a tautomeric form) which they prepared by condensation of indoxyl, isatin α-chloride or α-anil with a variety of phenols. The products were reversibly reduced and addition of alkali caused a marked hypsochromic shift of the absorption spectrum. As with actinomycin, the colour of the original compound was restored immediately after acidification of the alkaline solution, suggesting that the reaction with alkali involved addition of a hydroxyl group rather than ring opening. We

[20] Sumpter, Williams, Wilkin, and Willoughby, *J. Org. Chem.*, 1949, **14**, 713.
[21] Sumpter, Wilkin, Williams, Wedemeyer, Boyer, and Hunt, *ibid.*, 1951, **16**, 1777.
[22] Jacobs, Winstein, Linden, Robson, Levy, and Seymour, *Org. Synth.*, Coll. Vol. III, 1955, p. 456.
[23] Bayer and Co., G.P. 286,095; *Chem. Zentr.*, 1915, II, 568.
[24] Cf. the formation of 2 : 5-dihydroxybenzoquinone from *apo*terramycin, Hochstein, Stevens, Conover, Regna, Pasternak, Gordon, Pilgrim, Brunings, and Woodward, *J. Amer. Chem. Soc.*, 1953, **75**, 5455.
[25] Friedlaender *et al.*, *Monatsh.*, 1908, **29**, 375, 387, and later papers.

were unable, however, to obtain corresponding compounds of the oxindolylidene series from similar condensations using *N*-methyloxindole, isatin β-dichloride, β-anil, β-*p*-



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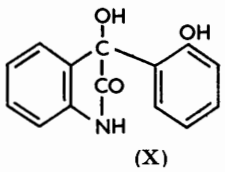

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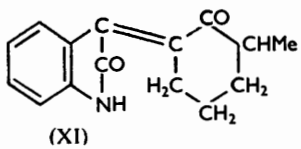

(XI)

methylanil,[26] or β-*p*-nitroanil. In another approach isatins were treated with the Grignard derivatives of *o*-methoxyaryl halides [27] and the products demethylated to yield 3-*o*-hydroxyaryldioxindoles, *e.g.*, (X), but in spite of numerous attempts these compounds could not be dehydrated. The 3-*o*-methoxyaryldioxindoles were also reduced to the corresponding oxindoles with stannous chloride,[28] and the methoxy-groups were hydrolysed, but in all the cases investigated the colourless phenolic products resisted purification and, as they clearly did not possess the required oxindolylidene structures, this approach was abandoned. In other experiments, 2-3′-dioxindolyl-6-methyl*cyclo*hexanone was dehydrated to 2-methyl-6-3′-oxindolylidene*cyclo*hexanone [29] (XI), but attempted dehydrogenation with selenium dioxide failed to give a coloured product.

All this evidence, coupled with the fact that actinomycin, unlike isatin, failed to react with *o*-phenylenediamine or thioindoxyl,[30] led us to abandon structures for actinomycin based on the isatin chromophore.

Experimental

95% Ethanol was used as the solvent in determinations of absorption spectra, and infrared spectra were determined on mulls in Nujol except where otherwise stated.

Production of Actinomycin B (with Messrs. R. Elsworth, J. W. S. Ford, R. Harris-Smith, and V. Williams, of the Ministry of Supply Microbiological Research Station, Porton).—Sub-cultures of strain X-45 [2] were prepared on tomato-agar slopes. After inoculation these were incubated at 25° for 4 days in 1 oz. screw-capped bottles with the caps loosened. They were stored in a refrigerator with the caps tightened, and the sub-cultures were renewed at monthly intervals. The medium was prepared from glucose (10 g.), peptone (1 g.), homogenised canned tomato (20 g.), dipotassium hydrogen phosphate (1 g.), calcium carbonate (2 g.), and agar (15 g.) in tap-water (to 1 l.), and if necessary the pH was adjusted to 7·0 with 5N-potassium hydroxide.

Primary seeds were prepared by inoculation from a slope into the medium (80 c.c.) in a 40 oz. narrow-necked screw-capped bottle. It was shaken (98 strokes/min.; 2″ amplitude) for 48 hr. at 25° after which there was a nodular deposit on the walls of the bottle and a diffuse growth in the medium. The primary seed medium was prepared from dextrin (10 g.), maize-steep liquor (5 c.c.), and dipotassium hydrogen phosphate (1 g.) in distilled water (to 1 l.). The pH was adjusted to 6·5 with 5N-potassium hydroxide as before, and calcium carbonate (5 g.) was added.

Secondary seeds were prepared by transferring the primary seed growth described above into a 20-l. aspirator containing the main culture medium. The contents were incubated at 25° for 48 hr. with thorough aeration (30 l./min.) to produce an evenly dispersed nodular growth. The main culture was grown at 25° in 80-l. batches after inoculation with the secondary seed (8 l.) until the yield of actinomycin reached a maximum (*ca.* 7 days). The main culture solution was prepared from peptone (Evans; 1% w/v), maize-steep liquor (0·5% w/v), brown sugar (2% w/v), lard oil (edible grade; 0·3% w/v), and dipotassium hydrogen phosphate (0·1% w/v) at a pH of 6·0—7·0 adjusted before sterilisation.

Most of the actinomycin was in the mycelium and consequently the liquid phase was rejected. Hyflo Supercel (0·625%) was added to the broth, and the mycelium separated in the centrifuge.

[26] Pummerer and Goettler, *Ber.*, 1910, **43**, 1381.
[27] Inagaki, *J. Pharm. Soc. Japan*, 1939, **59**, 5.
[28] Marschalk, *Bull. Soc. chim. France*, 1952, 949.
[29] Cf. Braude and Lindwall, *J. Amer. Chem. Soc.*, 1933, **55**, 325.
[30] Harley-Mason, *J.*, 1942, 404.

The wet mycelium was extracted in ½-gallon bottles with 95% ethanol (3·3 l. per kg.). After mixing, the bottle was kept overnight, shaken for 2 hr., heated at 60° for 1 hr. on the water-bath, and again shaken for 2 hr. The solids were separated and the alcoholic solution (containing *ca.* 33% of the total amount of actinomycin produced) used in the next stage of the purification. By a modified cylinder-plate method of microbiological assay,[31] the production was subjected to analytical control at all stages.

Isolation of Actinomycin B.—Removal of the solvent from the aqueous-alcoholic extract (60 l.) as prepared above, at 30° *in vacuo*, yielded a thick orange emulsion (5 l.) which was repeatedly extracted with benzene (6 × 500 c.c.). Removal of the solvent from the combined benzene extracts gave a deep red semi-solid gel (300 g.). Addition of ether (2 l.) precipitated the actinomycin as a sticky orange solid which, after repeated washing with ether (10 × 100 c.c.) and drying, gave an orange solid (25 g.). Evaporation of the ethereal filtrates gave a deep red residue which was treated with light petroleum (b. p. 60—80°; 2 l.), a further quantity of crude actinomycin being precipitated. After another treatment with light petroleum, nearly all of the actinomycin was obtained free from lard oil and other fatty substances present in the mycelium. Crystalline actinomycin B was obtained as bright red prisms, m. p. 250—253°, from the combined solids (40 g.) by crystallisation from ethanol. As actinomycin was sparingly soluble in ethanol, it was preferable to dissolve the solid in chloroform, then to add excess of ethanol and remove most of the chloroform by evaporation.

The product was examined by paper chromatography on Whatman No. 1 paper which had been soaked in 4% aqueous sodium naphthalene-2-sulphonate [3] and dried between other sheets of filter paper. The actinomycin was applied to the paper as a benzene solution, and the chromatogram developed with pentyl acetate which had been equilibrated with the sodium naphthalene-2-sulphonate solution. By this means the product was shown to contain four coloured substances, of R_F 0·74, 0·38, 0·10, and 0·03, which were estimated to occur in the following approximate proportions : 80—90, 10, 5, and <1% respectively.

Chromatographic Separation of the Actinomycin B Complex.—Activated alumina (100 g.) was warmed with aqueous 10% acetic acid (100 c.c.) at 70° on the water-bath for 2 hr. with constant stirring. The alumina was separated and washed with hot distilled water until the pH of the filtrate was 4—5. It was then suspended in methanol for 20 min., separated, washed with methanol, and dried at room temperature for 24 hr. This alumina has a low capacity and only small quantities of actinomycin could be separated at a time.

Actinomycin B complex (20 mg.) was applied to a column of alumina (15 × 1 cm.), and the sharp, uppermost, orange-red band developed with gradually increasing concentrations of chloroform in benzene. The process of the separation was followed by chromatography on paper as above but the early fractions contained only the actinomycin with R_F 0·74 which was followed by the component of R_F 0·38 and finally that of R_F 0·10. The pure actinomycin, R_F 0·74, was recrystallised from ethanol. Light absorption : max. at 232—236 and 443—445 mμ (log ε 4·50 and 4·33). Infrared absorption : max. at 3340, 3250, 1750, 1652, 1588, 1515, 1315, 1300, 1270, 1190, 1100, 1075, 1055, 1010, 980, 950, 875, and 760 cm.$^{-1}$. Determinations (i) in hexachlorobutadiene showed additional max. at 2940, 1407, and 1360 cm.$^{-1}$ and (ii) in chloroform at 1635 cm.$^{-1}$.

Reaction of Actinomycin with Alkali.—Aqueous 2·5N-sodium hydroxide (0·1 c.c.) was added to a 50% aqueous-ethanolic solution (5 c.c.) of actinomycin, the concentration of which (3 mg. actinomycin in 50 c.c.) was suitable for spectroscopic examination. Light absorption : max. at 285, 344, and 458 mμ (log ε 4·13, 4·38, and 3·05 respectively). The changes in intensity at 344 and 444 mμ were recorded and the results plotted (Fig. 1).

Further runs were made by adding varying amounts of aqueous sodium hydroxide to the actinomycin solution (2·5 c.c. as above) : (i) 0·1N-NaOH (0·3 c.c.) + H_2O (0·4 c.c.); (ii) 0·1N-NaOH (0·5 c.c.) + H_2O (0·2 c.c.); and (iii) 0·1N-NaOH (0·7 c.c.) : the calculated half-lives in the three cases were 8·3, 5, and 3·75 min. respectively, *i.e.*, proportional to the concentration of sodium hydroxide.

Acidification of an Alkaline Solution of Actinomycin.—A solution (2·5 c.c.) of actinomycin in 50% ethanol (as in the previous experiment) was mixed with 0·1N-aqueous sodium hydroxide (0·3 c.c.) and kept for 4·5 hr. It was then diluted to twice its original volume, 0·3N-hydrochloric acid (0·1 c.c.) was added, and spectral readings were taken as before. The results are plotted in Fig. 2.

[31] Brownlee, Delves, Dorman, Green, Grenfell, Johnson, and Smith, *J. Gen. Microbiol.*, 1938, **2**, 40.

*3-Hydroxy-2-nitro-*p*-toluic Acid* (CO_2H = 1).—3-Methoxy-2-nitro-*p*-toluic acid (7·6 g.; prepared by methylation [32] and subsequent nitration [33] of 3-hydroxy-*p*-toluic acid [34]) and freshly prepared pyridine hydrochloride (22 g.) were heated in an oil-bath at 190—200° for 45 min. The cooled mixture was poured into water (200 c.c.) and extracted with ether (3 × 100 c.c.). The combined ethereal extracts were washed and dried, the solvent was removed, and the residue crystallised from water, to give the *product* as lemon-yellow needles, m. p. 185·5—186·5° (6·7 g., 94%) (Found, on a sample sublimed at 140°/0·05 mm.: C, 48·9; H, 3·5; N, 7·5. $C_8H_7O_5N$ requires C, 48·7; H, 3·6; N, 7·1%).

*Methyl 3-Hydroxy-2-nitro-*p*-toluate.*—A mixture of 3-hydroxy-2-nitro-*p*-toluic acid (6·6 g.), methanol (4·1 c.c.), ethylene dichloride (10 c.c.), and concentrated sulphuric acid (0·5 c.c.) was heated under reflux for 24 hr. After cooling, the mixture was diluted with water (100 c.c.) and extracted with ether (3 × 100 c.c.). The combined ethereal extracts were washed with water and dried and the solvent was removed to give the crude *ester* (7·1 g.) which crystallised from aqueous methanol in needles (5·35 g., 76%), m. p. 116—116·5°. The mixture of ester and unchanged acid from the mother-liquors can be re-esterified. For analysis a sample was sublimed at 90°/0·05 mm. (Found: C, 51·4; H, 4·1; N, 6·9. $C_9H_9O_5N$ requires C, 51·2; H, 4·3; N, 6·6%).

Methyl 3-Hydroxy-4-methylanthranilate Hydrochloride.—The foregoing nitro-ester (2·76 g.) was hydrogenated in absolute ethanol (35 c.c.) and at room temperature and pressure over Raney nickel (*ca.* 1 g.). The catalyst was separated and the product isolated as its hydrochloride by dilution of the filtrate with ether (200 c.c.) and saturation of the filtrate with dry hydrogen chloride. The *product* (2·65 g., 93%) was obtained as colourless needles, m. p. 168—170° (decomp.) after two recrystallisations from ethanol–ether (Found: C, 49·7; H, 5·3; Cl, 15·9. $C_9H_{11}O_3N$,HCl requires C, 49·7; H, 5·55; Cl, 16·3%).

Dimethyl 2-Amino-4:6-dimethyl-3-oxophenoxazine-1:9-dicarboxylate (*Actinocin Dimethyl Ester*).—A solution of methyl 3-hydroxy-4-methylanthranilate hydrochloride (2·5 g.) in phosphate buffer (3 l.; pH 7·1) was kept at 40—45° while a solution of potassium ferricyanide (7·65 g.) in water (300 c.c.) was added dropwise with stirring. After cooling, the product (1·52 g., 74%) which had formed as a bright orange flocculent precipitate, was separated, washed, and dried. Crystallisation from ethyl acetate or benzene gave *actinocin dimethyl ester* as fine orange needles, decomp. 195—198° (Found: C, 60·9; H, 4·4; N, 7·9. $C_{18}H_{16}O_6N_2$ requires C, 60·7; H, 4·5; N, 7·9%). Light absorption: (i) max. at 240 and 435 mμ (log ε 4·57 and 4·49 respectively); (ii) in 1:1-aqueous-ethanolic 0·05N-NaOH: max. at 316 and 445 mμ (log ε 4·25 and 3·62).

3-Hydroxy-2-nitrobenzoic Acid.—3-Methoxy-2-nitrobenzoic acid [35] (1 g.) was fused with freshly prepared pyridine hydrochloride (2·5 g.) at 220° (oil-bath) for 40 min. The cooled melt was treated with water (20 c.c.) and cooled in ice. The brown precipitate was separated (0·9 g.) and crystallised from water, then having m. p. 172—174° (Hegedus [36] gives m. p. 176—178°); it was used directly for the next stage.

Methyl 3-Hydroxy-2-nitrobenzoate.—The foregoing acid (0·9 g.) was heated under reflux with dry methanol (2 c.c.), ethylene dichloride (20 c.c.), and concentrated sulphuric acid (3 drops) for 24 hr. The resulting solution was washed with water, and the solvent removed *in vacuo*. The methyl ester was thus obtained as white needles, m. p. 108—110° (lit.,[37] 115°) after crystallisation from water.

Dimethyl 2-Amino-3-oxophenoxazine-1:9-dicarboxylate.—Methyl 3-hydroxy-2-nitrobenzoate (1·5 g.) was hydrogenated in ethanol (40 c.c.) over Raney nickel (1 g.) at room temperature and pressure. Three mols. of hydrogen were absorbed in 4 hr., then the catalyst was separated and the filtrate diluted with ether (100 c.c.). The solution was treated with dry hydrogen chloride for 1½ hr. and the white hydrochloride (1·3 g.), m. p. 197° (decomp.), of methyl 3-hydroxyanthranilate which was formed was separated. The hydrochloride (1·2 g.) was heated in a phosphate buffer (3 l.; pH 7·4) to 40°. A solution of potassium ferricyanide (4·5 g.) in water (300 c.c.) was added slowly with stirring, then the product was cooled and the pale orange

[32] Perkin and Weizmann, *J.*, 1906, **89**, 1658.
[33] Simonsen and Rau, *J.*, 1921, **119**, 1339.
[34] Meldrum and Perkin, *J.*, 1908, **93**, 1416.
[35] Nyc and Mitchell, *J. Amer. Chem. Soc.*, 1948, **70**, 1847.
[36] Hegedus, *Helv. Chim. Acta*, 1951, **34**, 611.
[37] Senoh, Seki, and Kikkawa, *J. Chem. Soc. Japan*, 1953, **74**, 251.

crystalline *product* (0·8 g.) separated. After crystallisation from chloroform–ethyl acetate it formed orange needles, m. p. 225—226° (decomp.) (Found: C, 58·64; H, 3·7; N, 8·5. $C_{16}H_{12}O_6N_2$ requires C, 58·55; H, 3·7; N, 8·5%), readily soluble in polar organic solvents but only slightly soluble in water. Light absorption: (i) max. at 237 and 433 mμ (log ε 4·51 and 4·43); (ii) in 95% EtOH–10% aqueous NaOH (1 : 1): max. at 318 and 468 mμ (log ε 4·26 and 3·45); (iii) in 95% ethanol–10N-hydrochloric acid (1 : 4): max. at 231, 468, and 500 mμ (log ε 4·63, 4·45, and 4·38 respectively).

Methylation of an Alkaline Solution of 2-Aminophenoxazin-3-one.—2-Aminophenoxazin-3-one was prepared according to Zincke and Hebebrand,[38] forming very dark needles, m. p. 245° (decomp.) (from benzene). Light absorption: max. at 236 and 432 mμ (log ε 4·34 and 4·21).

The amino-compound (500 mg.) was dissolved in dioxan (50 c.c.) and diluted with water (50 c.c.). Aqueous 2·5N-sodium hydroxide (30 c.c.) was added, the solution kept for 30 min., and the precipitated 2-aminophenoxazin-3-one (280 mg.) separated. The alkaline solution was treated at room temperature with a large excess of dimethyl sulphate (7·8 g.) in six portions, during 2 hr. with constant shaking. Aqueous ammonia (3 c.c.; *d* 0·88) was added to decompose the excess of dimethyl sulphate, and the solution then acidified with hydrochloric acid to pH 3. After dilution with water (100 c.c.), the product was extracted with chloroform (3 × 100 c.c.), and the combined extracts were washed and dried. Removal of the solvent under reduced pressure gave a red gum which was treated with aqueous 2·5N-sodium hydroxide (10 c.c.) and any insoluble material separated. The alkaline solution was carefully acidified and the dark red precipitate removed, washed with water, and dried *in vacuo*. The product (70 mg.), m. p. 77° (decomp.), could not be crystallised satisfactorily (Found: C, 63·0; H, 5·1; N, 5·5; OMe, 9·0. Calc. for $C_{13}H_{11}O_4N$: C, 63·6; H, 4·5; N, 5·7; 1OMe, 12·6%). It was very soluble in polar organic solvents and only slightly soluble in water. It dissolved in aqueous sodium hydrogen carbonate solution with liberation of carbon dioxide. Light absorption: max. at 502, 306, 283, and 219 mμ (log ε 3·36, 3·93, 3·97, and 4·14 respectively).

5-Hydroxy-2-indoxylylidenecyclohexa-3 : 5-dienone [25] (*from Ethanol*).—The product obtained from the condensation of isatin-α-chloride and resorcinol formed thick, almost black needles with a green reflex. Light absorption: (i) max. at 229, 288, and 518 mμ (log ε 3·24, 3·39, and 3·90 respectively); (ii) in EtOH–0·1N-aqueous sodium hydroxide (1 : 1), max. at 225 and 327 mμ (log ε 3·44 and 4·18 respectively).

Reaction of Isatin with Arylmagnesium Bromides.—Magnesium (0·9 g., 1·5 mol.; previously etched with 1% nitric acid for 2 min., then washed and dried), ethyl bromide (2 drops), and dry ether (10 c.c.) were heated under reflux until the reaction had ceased. The aryl bromide (0·5 g.) was added, the mixture again heated, and a solution of the remainder of the aryl bromide (4·7—5·8 g., 1 mol.) in ether (50 c.c.) added slowly. The solution was heated under reflux for 3 hr., then isatin (1·5 g., 1 mol.) was gradually introduced by means of a Soxhlet extractor (6—8 hr.). The product was poured into cold 10% sulphuric acid (300 c.c.), unchanged isatin being precipitated and removed. The ethereal layer was separated and the aqueous solution again extracted with ether. The combined ethereal extract was washed and dried, the solvent removed, and the colourless crystalline residue recrystallised. Yields were of the order of 30%. Thus were prepared:

3-o-*Methoxyphenyldioxindole* (1·0 g.), colourless needles, m. p. 238°, from aqueous ethanol (Found: C, 70·6; H, 5·1; N, 5·5. $C_{15}H_{13}O_3N$ requires C, 70·6; H, 5·1; N, 5·5%). The product could be readily purified by sublimation *in vacuo*.

3-p-*Methoxyphenyldioxindole* (1·2 g.), colourless needles, m. p. 180° (from ethanol) (Found: N, 5·55%).

3-(2 : 4-*Dimethoxy*-m-*tolyl*)*dioxindole* (0·6 g.) [from 3-bromo-2 : 6-dimethoxytoluene (see below)], needles, m. p. 227° (decomp.), from aqueous methanol (Found: C, 68·1; H, 5·7; N, 4·7. $C_{17}H_{17}O_4N$ requires C, 68·2; H, 5·7; N, 4·7%). The product was also purified by sublimation at 170°/0·1 mm.

3-Bromo-2 : 6-dimethoxytoluene.—2 : 6-Dimethoxytoluene (20 g.) in chloroform (75 c.c.) was treated with bromine (23 g.) in chloroform (75 c.c.), and the chloroform then removed by distillation. The residual brown oil (34 g.) was distilled through a short Vigreux column to give the *bromo-derivative*, b. p. 147—149°/15 mm. (Found: Br, 33·1. $C_9H_{11}O_2Br$ requires Br, 33·4%).

[38] Zincke and Hebebrand, *Annalen*, 1882, **226**, 61.

3-o-*Methoxyphenyloxindole*.—3-(*o*-Methoxyphenyl)dioxindole (0·5 g.) was dissolved in glacial acetic acid (10 c.c.), a solution of stannous chloride (1 g.) in acetic acid (20 c.c.) and concentrated hydrochloric acid (1 c.c.) was added, and the mixture heated on the water-bath at 80—90° for 10 min. The product was poured into water (150 c.c.), giving white needles of 3-(o-*methoxyphenyl*)*oxindole* (300 mg.), m. p. 148° (from aqueous ethanol) (Found : C, 75·4; H, 5·55; N, 5·85. $C_{15}H_{13}O_2N$ requires C, 75·3; H, 5·5; N, 5·85%).

3-p-*Methoxyphenyloxindole*.—Prepared from 3-*p*-methoxyphenyldioxindole (500 mg.) by a method similar to the foregoing, this *product* (400 mg.) formed colourless crystals, m. p. 163—165° (decomp.), from aqueous ethanol (Found, in a sample sublimed under reduced pressure : C, 75·4; H, 5·7; N, 5·8%). Light absorption (in *NN*-dimethylformamide) : log $\varepsilon_{max.}$ 4·42 at 259 mμ.

3-(2 : 4-*Dimethoxy*-m-*tolyl*)*oxindole*.—Prepared by reduction of the corresponding dioxindole derivative as above, this *product* (240 mg.) was obtained as colourless crystals, m. p. 162°, which after crystallisation from aqueous ethanol could be sublimed under reduced pressure (Found : N, 4·85. $C_{17}H_{17}O_3N$ requires N, 4·95%). Light absorption (in *NN*-dimethylformamide) : log $\varepsilon_{max.}$ 4·19 at 268 mμ.

Isatin β-p-*Nitroanil*.—Isatin (5 g.) in ethanol (150 c.c.) was heated with an alcoholic solution of *p*-nitraniline (5 g.) under reflux for 7 hr. and, on cooling, the *product* (8 g.) crystallised as red needles, m. p. 159° (Found, on a sample sublimed at 130°/0·1 mm. : C, 63·0; H, 3·3; N, 15·9. $C_{14}H_9O_3N_3$ requires C, 62·9; H, 3·4; N, 15·7%).

2-(3-*Dioxindolyl*)-6-*methyl*cyclo*hexanone*.—Isatin (5 g.), 2-methyl*cyclo*hexanone (3·9 g.), and piperidine (2 g.) were dissolved in dry dioxan and kept at room temperature for 24 hr. A small quantity of 3 : 3-dipiperidino-oxindole which separated was removed; when the filtrate was diluted with water (150 c.c.) a further quantity of the dipiperidino-oxindole was obtained, and again separated. After being kept a week, the *product* (2·3 g.) separated from the solution as colourless prisms, m. p. 168° (decomp.) after crystallisation from aqueous ethanol (Found : C, 69·1; H, 6·4; N, 5·4. $C_{15}H_{17}O_3N$ requires C, 69·5; H, 6·6; N, 5·4%).

2-*Methyl*-6-3′-*oxindolylidene*cyclo*hexanone*.—2-(3-Dioxindolyl)-6-methyl*cyclo*hexanone (1 g.) was suspended in ethanol (2 c.c.) and concentrated hydrochloric acid (0·5 c.c.) was added. The mixture was warmed, a clear orange solution being formed which slowly deposited the dehydration *product* as orange prisms (0·8 g.), m. p. 142° (from aqueous ethanol) (Found : C, 74·7; H, 6·1; N, 6·0. $C_{15}H_{15}O_2N$ requires C, 74·7; H, 6·2; N, 5·8%).

Absorption Spectra of Substituted Isatins.—Spectra of the following isatin derivatives were determined in (i) 95% ethanol and (ii) 95% ethanol–0·1N-aqueous sodium hydroxide (1 : 1).

5-*Hydroxy*- :[39] (i) max. at 482—485, 302, 256, and 214 mμ (log ε 2·95, 3·34, 4·27, and 3·99 respectively); (ii) max. at 422—424 and 248 mμ (log ε 3·52 and 4·16 respectively).

5-*Methoxy*- :[40] (i) max. at 472—475, 300, 254, and 214 mμ (log ε 2·84, 3·29, 4·32, and 4·09 respectively); (ii) max. at 391—394 and 233—224 mμ (log ε 3·61 and 4·17 respectively).

1-*Acetyl*-5-*methoxy*- : (i) max. at 374—375 and 238—240 mμ (log ε 3·06 and 4·08); (ii) max. at 325, 258—260, and 228—230 mμ (log ε 3·30, 3·85, and 4·18 respectively).

7-*Methoxy*-1-*methyl*- :[41] (i) max. at 462—467, 322, and 228 mμ (log ε 2·94, 3·48, and 4·26 respectively); (ii) max. at 393, 278, and 238 mμ (log ε 3·56, 3·44, and 4·28 respectively).

1-*Benzyl*- : (i) max. at 421—425, 298, 251, and 245 mμ (log ε 2·67, 3·37, 4·23, and 4·30 respectively); (ii) max. at 390, 268, and 230 mμ (log ε 3·78, 3·87, and 4·27 respectively).

1-*Phenyl*- : (i) max. at 419—422 and 244 mμ (log ε 2·56 and 4·36 respectively); (ii) max. at 398, 282, 253, and 228 mμ (log ε 3·87, 3·95, 4·16, and 4·17 respectively).

1-*Acetyl-isatin* :[22] (i) max. at 341—343, 272, 265, and 236 mμ (log ε 3·56, 3·91, 3·91, and 4·36 respectively); (ii) max. at 326, 264, and 231 mμ (log *z* 3·47, 3·99, and 4·29 respectively).

Isatin and 1-methylisatin : see Ault, Hirst, and Morton.[42]

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[39] Hartmann and Panizzon, *Helv. Chim. Acta*, 1936, **19**, 1327.
[40] Bachmann and Picha, *J. Amer. Chem. Soc.*, 1946, **68**, 1601.
[41] Cook, Loudon, and McCloskey, *J.*, 1952, 3904. We are very grateful to Dr. Loudon for the gift of a sample of this compound.
[42] Ault, Hirst, and Morton, *J.*, 1935, 1653.

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