

**100.** *The Phenazine Series. Part VII. The Pigment of Chromobacterium Iodinum; the Phenazine Di-N-oxides.*

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The pigment of *Chromobacterium iodinum* (nov. sp.) has been isolated and shown to be the NN'-dioxide of a dihydroxyphenazine (probably the 1:2-dihydroxy-derivative). Other derivatives of the hitherto unknown phenazine di-N-oxide (IV) have been prepared, and their reactions studied.

THIS new species of *Chromobacterium* was isolated at the National Institute for Research in Dairying, Reading, during milk examination. The name *Chromobacterium iodinum* was proposed on account of a violet, copper-glinting pigment occurring outside the organism at the later stages of its growth. Cultures, and an account of the examination of the organism carried out at Reading, were sent by Mr. J. G. Davies, to whom our thanks are due; we are also indebted to Prof. Dunlop of the Department of Bacteriology, King's College, for growing several batches of the organism, from which was extracted the pigment used in the present investigation.

The bacteria were grown on a solid agar medium, and after about 15 days had attained maximal pigmentation; the colouring matter was removed by washing with water and extraction with chloroform, and, on concentration of the extract, separated as uniform crystals, deep purple in colour and with coppery lustre, in a yield of about 1 g. per sq. m. of medium. Analytical data indicate for the pigment an empirical formula  $C_{12}H_8O_4N_2$ ; it is not sufficiently soluble in camphor to permit the determination of molecular weight by Rast's method. Its homogeneity is indicated by the fact that, though not adsorbed by sugar or chalk, the pigment gives a single sharp narrow band on alumina. It is not basic, being insoluble in acids except concentrated sulphuric acid, but is soluble in sodium hydroxide, giving a brilliant blue solution which deposits green crystals of the sodium salt. This deposit can be seen under the microscope to dissolve transitorily in water to a rich blue solution, which almost immediately deposits purple crystals of the original pigment; such salt formation and hydrolysis indicate how the pigment, insoluble in water, could be deposited outside the bacterial cell. Alkaline solutions of the pigment are reduced by hyposulphite, the colour changing through red to yellow; the last stage only is reversible by air or by persulphate oxidation. The pigment and its reduction product are precipitated from their alkaline solutions by excess of bicarbonate or sulphur dioxide.

The pigment was shown to be a phenazine derivative by zinc dust distillation of its reduction product, which yielded phenazine itself. The formula  $C_{12}H_8O_4N_2$  is that of a tetrahydroxyphenazine; of these compounds, the 1:2:3:4-derivative and a methyl

homologue are known (Kehrmann and coll., *Ber.*, 1887, 20, 324, 3150; 1888, 21, 1227; 1890, 23, 2448) and are consistently described as yellow-brown needles. As various tri-, di-, and mono-hydroxyphenazine derivatives are also yellow, the pigment is not likely to be a simple hydroxyphenazine, though it is phenolic in character.

The action of acetic anhydride is not simple acetylation; it leads to a product whose analytical figures indicate loss of oxygen during the process.

The reduction of the pigment (best in alcoholic solution with platinum and hydrogen) leads to the replacement of two oxygen atoms by two of hydrogen—such as, for example,  $-\text{NO}_2 \rightarrow -\text{NH}_2$ , but no product of biological origin has yet been obtained with a nitro-group—and the compound produced,  $\text{C}_{12}\text{H}_{10}\text{O}_2\text{N}_2$ , is readily converted on recrystallisation into  $\text{C}_{12}\text{H}_8\text{O}_2\text{N}_2$ . Such facile dehydrogenation would be expected to occur with an *NN'*-dihydrophenazine and the somewhat high molecular weight recorded is understandable on account of association as in the case of pyocyanine. The reduction product is still phenolic; it gives a green coloration with ferric chloride in alcoholic solution, and forms a diacetyl derivative. Its alcoholic solution gives with lead acetate an intense violet lake which closely resembles that given by alizarin; quinizarin under similar conditions gives no lake. Catechol gives a colourless precipitate and quinol none with alcoholic lead acetate.

In the search for phenomena comparable with these results, the observations of Wohl on phenazine *N*-oxide (*Ber.*, 1901, 34, 2442; 1903, 36, 4140) were encountered. This compound yields *NN'*-dihydrophenazine on reduction and phenazine itself on refluxing with acetic anhydride; it is not, however, markedly coloured. The above results of degradation, and also the observed decomposition of the pigment with vigorous gas evolution on melting, would be explained were it a di-*N*-oxide. Such compounds have not been described in the phenazine series; indeed *N*-oxides have never been made directly from the corresponding phenazines, Wohl's product being obtained by heating aniline and nitrobenzene in the presence of sodium hydroxide, and substituted compounds being prepared by Bamberger and Ham (*Annalen*, 1911, 382, 82) by condensation of *p*-substituted nitrosobenzenes.

In attempts to prepare a di-*N*-oxide, phenazine was recovered unchanged from the action of hydrogen peroxide or benzoyl peroxide in neutral solution (compare Bamberger and Tschirner, *Ber.*, 1899, 32, 342, 1882; Meisenheimer, *Ber.*, 1926, 59, 1848), but with Caro's acid (Bamberger and Rudolf, *Ber.*, 1902, 35, 1082) or, better, with hydrogen peroxide in glacial acetic acid, a product different from the Wohl *N*-oxide, crystallising in orange-red needles, m. p. (with gas evolution) 204°, was obtained. The analytical data corresponded to a di-*N*-oxide (IV) and from the products of its thermal decomposition phenazine mono-*N*-oxide and phenazine were obtained.

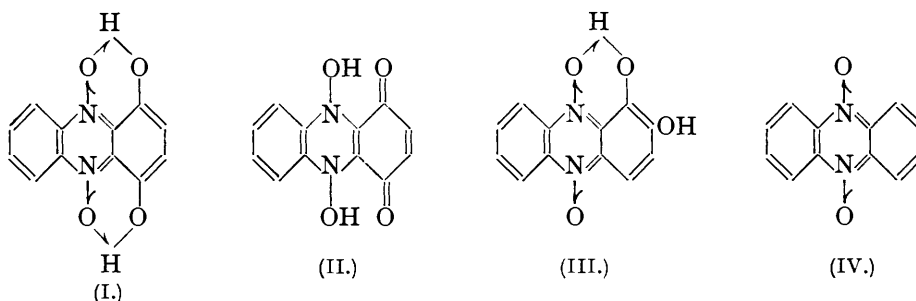
The di-*N*-oxide produced similarly from 1-hydroxyphenazine prepared by the method of Wrede (*Z. physiol. Chem.*, 1929, 177, 177) shows great similarity to the natural pigment. It is deep red, and forms in alkalis blue solutions which are reduced by hyposulphite irreversibly to red (the colour of the salt of 1-hydroxyphenazine) and further, reversibly, to yellow (the colour of the alkaline salt of 1-hydroxydihydrophenazine). The phenazine *N*-oxides have, in common with other *N*-oxides and with the pigment, the property of liberating iodine from potassium iodide in acid solution. This occurs to only a very small and readily distinguishable extent with phenazine itself, which is partly reduced in the process. It has not yet been found possible to reconvert into the pigment the dihydroxyphenazine formed on its reduction; this may be compared with the greater difficulty of oxidation of 1-hydroxyphenazine in comparison with phenazine, and the lower yield of dioxide obtained.

The configuration of the dihydroxyphenazine obtained on reduction has not yet been definitely established. Two acetoxyphenazines, the 2 : 3- and 2 : 6-, have been described, but are different from that isolated from the pigment.

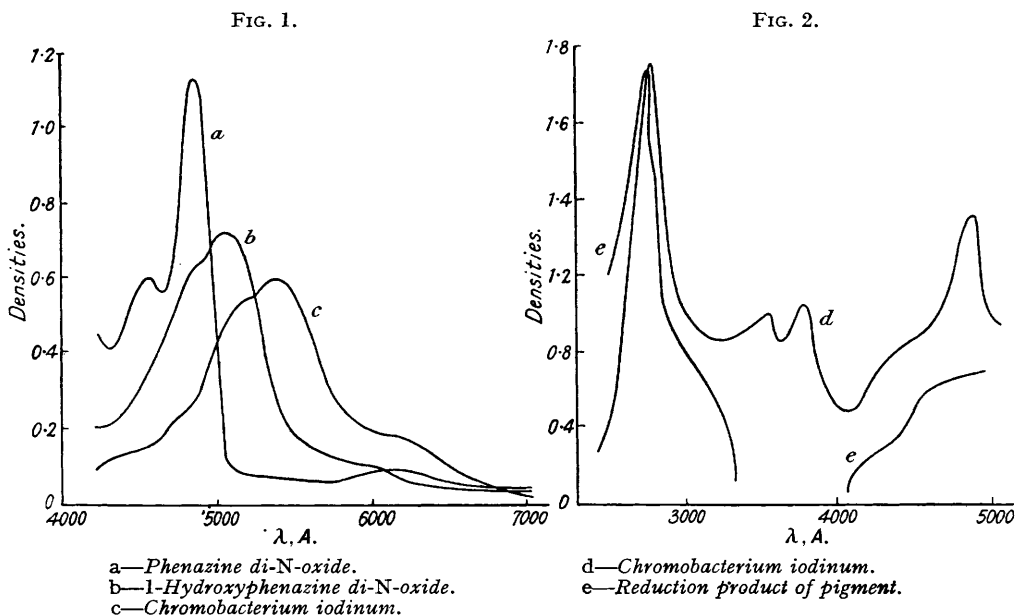
Fischer and Hepp (*Ber.*, 1890, 23, 8141) and Ullmann and Mauthner (*Ber.*, 1902, 35, 4305) claim to have prepared 2 : 3-dihydroxyphenazine and its diacetoxy-derivative. We find their dihydroxy-derivative, which gives no lake with alcoholic lead acetate, gives a bright yellow solution in sodium hydroxide, and although their diacetoxyphenazine melts

at 230°, its m. p. is depressed to 210—215° by the diacetoxyphenazine from the pigment. Eight other dihydroxyphenazines are possible. The following evidence is brought forward in this connection. Oxidation of the dihydroxydihydrophenazine is complete after the absorption of 10 atoms of oxygen; as oxidation with the conversion of one homocyclic ring into two carboxyl groups requires 8 atoms of oxygen, whereas similar oxidation of the other ring in addition would require 17 atoms, this result is taken to indicate the presence of both hydroxyl groups in the same benzene nucleus of the phenazine skeleton, but so far quinoxaline-2 : 3-dicarboxylic acid has not been isolated from the reaction.

The pigment is insoluble in boiling water, whereas a dihydroxyphenazine is somewhat soluble and phenazine di-*N*-oxide is more soluble in water than phenazine. Similarly 1-hydroxyphenazine di-*N*-oxide is less water-soluble than 1-hydroxyphenazine itself. These results would appear to indicate some interaction between the hydroxyl and the *N*-oxidic oxygen atom, such as by co-ordination in (I), or possibly by tautomerism as in (II), both forms being possible in a 1 : 4-derivative.



The absorption spectra of 1/100,000 w/v chloroform solutions of phenazine di-*N*-oxide, 1-hydroxyphenazine di-*N*-oxide, and the pigment in the visible region are shown



in Fig. 1, where it is seen that the action of the 1-hydroxy-group in lowering the extinction coefficient, shifting the absorption maximum to the red, and broadening the band is carried still further by the two hydroxyl groups of the pigment. The 2-hydroxy-compound is unfortunately not at present available for comparison, but the absorption spectrum of the

pigment resembles that of alizarin and not that given by quinizarin. Ultra-violet absorption spectra of the pigment and its reduction product are given in Fig. 2.

Further, chloroform solutions of the pigment and of alizarin scarcely fluoresce in ultra-violet light, whereas that of quinizarin gives an intense fluorescence.

The conclusion is reached, therefore, that the pigment is probably 1 : 2-dihydroxy-phenazine di-N-oxide (III) and this is being put to the test of synthesis.

The phenazine compounds which have been isolated from natural sources are now three in number (pyocyanine, chlororaphine, and the present pigment), and, though all are bacterial pigments, phenazine compounds have been shown to exhibit biological activity in a variety of organisms (Friedheim, *J. Exper. Med.*, 1931, **54**, 207), tissues (Friedheim, *Biochem. J.*, 1934, **28**, 173; Young, *J. Biol. Chem.*, 1937, **120**, 659; Dickens, *Biochem. J.*, 1936, **30**, 1064, 1233), and enzyme systems (Green, Stickland, and Tarr, *Biochem. J.*, 1934, **28**, 1812; Weil-Malherbe, *ibid.*, 1937, **31**, 2080). It is anticipated that phenazine derivatives, possibly *N*-alkyl compounds (cf. Dickens, Weil-Malherbe), will be found much more widely distributed in nature. The present pigment is not itself readily reversibly oxidised and reduced, and is regarded as related to some compound of functional value to the bacterium.

#### EXPERIMENTAL.

*Extraction of the Pigment.*—The bacteria had been grown in medicine bottles lying on their broad sides, each thus affording a surface of beer-wort-agar of 110–120 sq. cm. The cultures were incubated at 30° for 5 days and then allowed to lie at room temperature for 7–9 days, during which a deep purple layer of coppery lustre developed. Water (20 c.c.) was added to each, the bottle shaken, and the resultant suspension removed by decantation. Two, or occasionally three, such operations were sufficient to remove the pigment. The washings were allowed to stand, and the suspension of bacteria decanted from the precipitated pigment, which was further washed in the same manner and extracted with chloroform. On concentration, the pigment separated as purple crystals retaining the coppery glint; it was recrystallised from chloroform; the m. p., 236° (decomp.), was unchanged by further crystallisation. The chloroform mother-liquors contained a relatively small amount of non-pigmented material. The average yield was 10 mg. per bottle, or about 1 g./sq. m. of medium.

Qualitative tests showed the pigment to contain nitrogen; sulphur, phosphorus, and metals were absent (Found : C, 58.9, 59.1; H, 3.3, 3.5; N, 11.6, 11.6.  $C_{12}H_8O_4N_2$  requires C, 59.0; H, 3.3; N, 11.5%).

The pigment (0.5 mg.) dissolved in glacial acetic acid to a red solution, which on warming with the addition of 10% potassium iodide solution (0.1 c.c.) liberated iodine, readily detected by its coloration with starch. The compound, after being refluxed for 3 hours in methylalcoholic potassium hydroxide, was precipitated unchanged by sulphur dioxide.

*Reduction.*—The pigment (0.1 g.), platinum oxide (0.2 g.), and absolute alcohol (10 c.c.) were shaken in hydrogen (100 lb./sq. in.) for 24 hours. Filtration removed the catalyst, but no organic matter: this, considering the small solubility of the pigment in alcohol, indicated completion of the reaction. The filtrate, concentrated to crystallisation, yielded fine old-gold plates (0.06 g.), m. p. on rapid heating in a sealed tube, 260° (Found : C, 67.5, 67.7; H, 4.8, 4.7; N, 13.4; *M*, 245.  $C_{12}H_{10}O_2N_2$  requires C, 67.3; H, 4.7; N, 13.1%; *M*, 214). When the substance was recrystallised from alcohol or ethyl acetate, yellow prisms were obtained, m. p. 273–274° (Found : C, 67.9, 67.3; H, 3.8, 3.8.  $C_{12}H_8O_2N_2$  requires C, 67.9; H, 3.8%). The same compound was obtained when the pigment was refluxed for 15 hours with an alcoholic solution of *o*-phenylenediamine in a test for the presence of an ortho-quinonoid grouping.

*Acetylation of the Reduction Product.*—The compound (10 mg.) was refluxed for 1 hour in pyridine (1 c.c.) and acetic anhydride (4 drops), the solvent removed under reduced pressure, and the residue crystallised from alcohol; light yellow needles separated, m. p. 234° [Found : C, 64.5; H, 4.6; N 9.7.  $C_{12}H_8O_2N_2(CO\cdot CH_3)_2$  requires C, 64.8; H, 4.1; N, 9.5%]. The compound is not soluble in cold dilute acids, but with cold dilute sodium hydroxide hydrolysis occurs slowly, giving a pink solution.

*Conversion into Phenazine.*—The reduction product (10 mg.) was ground with zinc dust (2 g.) and placed in a hard-glass tube with a layer of zinc dust on top; this was strongly heated, and the heating then extended to the mixture. On the cold part of the tube there collected an almost colourless, crystalline sublimate (2 mg. approx.), which crystallised from light petroleum in very pale yellow prisms, m.p. 171°, not depressed by authentic phenazine.

*Phenazine Di-N-oxide.*—Phenazine (0.9 g.) in glacial acetic acid (50 c.c.) and hydrogen peroxide (5 c.c. of 100 vol.) were heated at 50° for 16 hours; the solution, which had then become a richer yellow, was diluted with an equal volume of hot water. On cooling, the *dioxide* separated in fine orange-red needles, which were washed with water and dried at 50° (1.0 g.). Chloroform extraction of the basified (sodium carbonate) mother-liquor yielded a little more (0.1 g.). The whole was recrystallised from alcohol (600 c.c.), yielding orange needles, m. p. 204° (gas evolution) after browning at 150° or lower according to the rate of heating (Found: C, 67.8, 68.2; H, 3.9, 3.6.  $C_{12}H_8O_2N_2$  requires C, 67.9; H, 3.8%).

*1-Hydroxyphenazine Di-N-oxide.*—1-Hydroxyphenazine (0.5 g.) in glacial acetic acid (20 c.c.) and hydrogen peroxide (2 c.c. of 100 vol.) were heated at 50° for 24 hours. The solution had become rich red, and no further apparent change took place in the course of 24 hours; addition of excess of sodium bicarbonate produced a red sludge. This was collected and extracted with aqueous sodium hydroxide, the resulting blue solution treated with sulphur dioxide, and the red precipitate collected, dried, and crystallised from chloroform–methyl alcohol (1 : 2), *1-hydroxyphenazine di-N-oxide* separating in deep red prisms, with a metallic sheen, m. p. (gas evolution) 189–190° (Found: C, 62.5; H, 3.5.  $C_{12}H_8O_3N_2$  requires C, 63.1; H, 3.5%).

*Decomposition of Phenazine Di-N-oxide.*—(1). The dioxide (0.1 g.) was refluxed with acetic anhydride (2 c.c.); it gradually dissolved and the solution had become yellow-brown in 10 minutes. It was concentrated to crystallisation; phenazine mono-*N*-oxide, which separated in yellow prisms, was recrystallised from alcohol (0.07 g.; m. p. 223°, alone or mixed with material prepared by the method of Wohl). On longer refluxing, the decomposition to phenazine described by Wohl took place.

(2). When the dioxide (0.1 g.) was heated at 180°, it became brown, phenazine (0.04 g.) sublimed, and an alkali-soluble material was also produced.

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