88. *Bacterial Inhibition by Metabolite Analogues. Part V. Reactions and Antibacterial Properties of* p-*Diazine Di*-N-*oxides.*

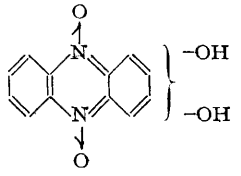
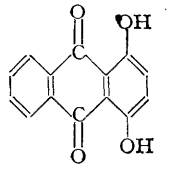
By Henry McIlwain.

The bacterial pigment iodinin has been regenerated from the product of its reduction, and its structure as a dihydroxyphenazine di-*N*-oxide confirmed. A number of quinoxaline di-*N*-oxides have been prepared for examination as antibacterial agents, for reasons outlined below, and they exhibited the following properties. The oxides retained the basic characters of the diazines, but those with saturated 2-substituents had also acidic properties which could be explained as due to tautomeric oxime forms. 2-*Methylquinoxaline di*-N-*oxide* changed in alkali to a rich blue product, and the conversion was greatly accelerated by light. Two quinoxaline di-*N*-oxides characterised by unsubstituted 2-positions reacted with keto-methylene compounds in dilute alkaline solutions. All the di-*N*-oxides were readily reduced to the diazines, and quinoxalines, but not phenazines, underwent further fission by zinc in acid solution to *o*-phenylenediamine and monoketones.

All the di-*N*-oxides were inhibitory to bacterial growth in concentrations in which their parent diazines were inactive.

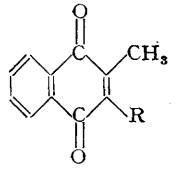
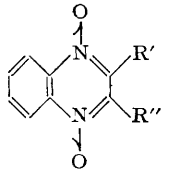
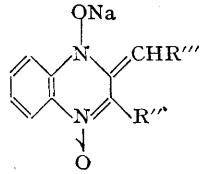
The pigment of *Chromobacterium iodinum*, here termed iodinin, was considered by analysis, degradation, and comparison with related compounds to be the di-*N*-oxide of a dihydroxyphenazine (I) (Clemo and McIlwain,

J., 1938, 479). In concentrations of less than 1 μg. per ml. it inhibited growth of some pathogenic bacteria (McIlwain, *Nature*, **1941**, **148**, 628); the inhibition was relatively little influenced by extracts of many natural materials, but was antagonised by some containing naphthaquinone and anthraquinone derivatives, and by certain pure hydroxyanthraquinones, *e.g.*, by quinizarin (II) (McIlwain, *Biochem. J.*, in press). The mutual interaction of (I) and (II) may be by competition for common receptor sites of the affected organism, as there

(I.)  (II.) 

is a structural and polar similarity between the two compounds analogous to that existing between other molecules which interact as bacterial inhibitors and their antagonists, for example, between sulphanilamide and *p*-aminobenzoic acid. The latter relationship has been successfully used in designing further inhibitory compounds corresponding to carboxylic acids other than *p*-aminobenzoic acid (McIlwain, *Lancet*, **1942**, **1**, 412). This subject has been pursued in the present investigation, which is of compounds related to antihæmorrhagic substances in the manner in which iodinin is related to the antagonistic anthraquinones.

2-Methyl-1 : 4-naphthaquinone (III, R = H) promotes growth of Johne's bacillus (Woolley and McCarter, *Proc. Soc. exp. Biol., N.Y.*, **1940**, **45**, 357); unknown compounds of antihæmorrhagic activity occur in other bacteria (Almquist, Pentler, and Mecchi, *ibid.*, **1938**, **38**, 336) and phthiocol (III, R = OH) in *M. tuberculosis*. If these materials are of functional value to the organisms producing them, related quinoxaline di-*N*-oxides

(III.)  (IV.)  (V.) 

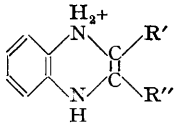
may be inhibitory. Previous descriptions of such oxides have not been found, and the following representatives have now been prepared. 2-Methylquinoxaline was obtained from *iso*nitrosoacetone and *o*-phenylenediamine (Bennett and Willis, J., 1928, 1966), and 2-methyl-3-*n*-amylquinoxaline by a similar reaction with *iso*nitrosomethyl *n*-hexyl ketone. These bases, and also quinoxaline and 1 : 2 : 3 : 4-tetrahydrophenazine (2 : 3-*cyclo*tetramethylenequinoxaline), were converted into their di-*N*-oxides by hydrogen peroxide in acetic acid solution. Iodinin was regenerated from the dihydroxyphenazine produced on reduction, by perbenzoic acid in benzene. 2-*Methylquinoxaline di*-N-*oxide* (IV; R′ = CH_3, R″ = H) is isosteric with the antihæmorrhagic 2-methylnaphthaquinone, and the *oxide* of 2-methyl-3-*n*-amylquinoxaline (IV; R′ = CH_3, R″ = $[CH_2]_4{\cdot}CH_3$) approximates to similar relationship with the K vitamins.

The diazine di-*N*-oxides were basic, forming slightly soluble phosphotungstates in 2N-sulphuric acid, but no precipitate with lead, silver, or mercuric salts. The substituted quinoxaline oxides showed also acidic properties which were probably due to their tautomeric forms (V); thus, while sodium hydroxide facilitated extraction of phenazine di-*N*-oxide from aqueous solution by ether or chloroform, it hindered such extraction of 2-methylquinoxaline, 2-methyl-3-*n*-amylquinoxaline, and of 1 : 2 : 3 : 4-tetrahydrophenazine oxides. Examples of tautomerism which, as (IV, R′ = CH_2R''') $\rightleftharpoons$ (V), involve saturated 2-substituents of *N*-substituted quinoxalines have previously been observed in quaternary quinoxaline salts (McIlwain, J. 1937, 1701). These cases also were associated with a valency change of the 1-N atom, and again analogous reactions did not occur with phenazine derivatives. Secondary changes took place subsequently in alkaline solutions of 2-methylquinoxaline and 1 : 2 : 3 : 4-tetrahydrophenazine di-*N*-oxides. A reaction peculiar to the former oxide was its conversion in alkaline solution into a rich blue product. The change was accelerated by illumination, and could be seen proceeding in sunlight in a few seconds. A much slower photochemical change in 2-methylquinoxaline itself was observed by Böttcher (*Ber.*, 1913, **46**, 3085). Rapid photochemical reactions have previously been encountered in phenazonium salts (McIlwain, J., 1937, 1704); the isosteric 2-methyl-1 : 4-naphthaquinone is also photosensitive and the K-vitamins are also sensitive to alkali.

The nature of the product of the photochemical change has not been established, nor that of the secondary change in 1 : 2 : 3 : 4-tetrahydrophenazine di-*N*-oxide, which was not noticeably accelerated by light. Further indication of the reactivity of the *N*-oxides was obtained through reactions with ketomethylene compounds. The di-*N*-oxides of quinoxaline and its 2-methyl derivative reacted in very dilute alkaline solutions with acetone, producing pronounced brown products. As the oxides of 2-methyl-3-*n*-amylquinoxaline, phenazine, and its 1 : 2 : 3 : 4-tetrahydro-derivative did not so react, the change probably involves a hydrogen atom of the 2- or 3-position.

All the diazine di-*N*-oxides investigated liberated iodine from acid solutions of potassium iodide and were reduced by neutral or slightly acid sodium hydrosulphite to the parent diazines. The quinoxaline oxides were peculiar in undergoing more extensive degradation with zinc in acid solutions, yielding, in addition to the

1 : 2 : 3 : 4-tetrahydro-compound, *o*-phenylenediamine and a monoketone or aldehyde (IV ⟶ IX). This was not primarily a reaction of the *N*-oxides [and is thus not analogous to rearrangements of certain *N*-oxides to ketones in acids observed by Haworth and Perkin (J., 1926, 445, 1769)], but was given also by the diazines themselves. It was a reaction of quinoxalines as distinct from phenazines, which under similar conditions yielded their *NN'*-dihydro-compounds. 1 : 2 : 3 : 4-Tetrahydrophenazine gave a mixture of 1 : 2 : 3 : 4 : 9 : 10 : 11 : 12-octahydrophenazines, *o*-phenylenediamine, and *cyclo*hexanone. The reaction is not a hydrolysis of 1 : 2 : 3 : 4-tetrahydroquinoxalines (the products of alkaline reduction), which were found stable to acid; nor is it analogous to Emde's reaction or to the fission of benziminazole salts to alkylated *o*-phenylenediamines, which occur in alkaline solution. In dihydrophenazines, as distinct from dihydroquinoxalines, the 2 : 3-double bond does not exist as such, forming part of an aromatic system; also, alkylated *o*-phenylenediamines can form dihydrochlorides, while dihydrophenazine forms only a monohydrochloride. Both these points increase the mesomeric forms possible to the diazine ring in dihydroquinoxaline cations. One such form is (VII), in which the tendency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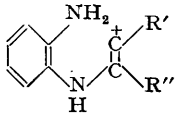
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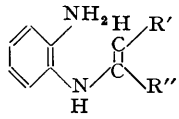
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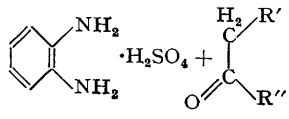
(IX.)

of N^+ to acquire electrons is satisfied by rupture of the 1 : 2-C–N bond; the energy for this breakage may be supplied by resonance, further salt formation at the amino-groups formed, and supply of electrons and a proton to the 2-C atom to give the Schiff's base (VIII), capable in the acid reaction solution of further fission to a monoketone and amine salt (IX). Electrons supplied immediately to the form (VI) would constitute the normal reduction to a 1 : 2 : 3 : 4-tetrahydroquinoxaline, occurring simultaneously in acid solution. In alkali, forms such as (VII) would not exist and some of the associated processes, such as salt formation with the product, would not occur; reduction to the 1 : 2 : 3 : 4-tetrahydro-compound only can then be understood.

All the di-*N*-oxides were found to inhibit growth of *Streptococcus hæmolyticus*; the parent bases in comparable or higher concentrations were usually indifferent. *C. diphtheriæ* also was inhibited by the compounds investigated. The concentrations required for bacteriostasis were in all cases greater than those required of iodinin; the compounds have, however, not yet been tested with organisms requiring pre-formed K-vitamins for growth.

Experimental.

p-Diazine Di-N-oxides.—The method of Clemo and McIlwain (J., 1938, 479) can be advantageously carried out with less reagents, the procedure here employed being: the base (1/200 mol.) in glacial acetic acid (20 ml.) with hydrogen peroxide (5 ml. of 100 vol.) was heated at 50° for 16 hours. Separation of the products is described individually below.

The quinoxaline oxides were yellow or brown, and the phenazine oxides orange or red, crystalline solids, melting (with decomposition and evolution of gas) higher than the bases from which they were derived. Heating just below 100°, alone or in solution, also usually caused slow decomposition. The oxides liberated iodine from iodides; satisfactory conditions for discriminating between this reaction and the liberation of iodine which is caused by certain diazines themselves (especially phenazines, by their being reduced to their *NN'*-dihydro-compounds) were: the oxide (1—2 mg.) in 50% aqueous acetic acid (0·5 ml.) with potassium iodide (0·25 ml. of 10% aqueous solution) was heated at 100° for 5 minutes, diluted with water, and starch solution added. Degradative reduction was carried out as follows: the oxide or quinoxaline (0·5 g.) in 5N-sulphuric acid (20 ml.) with excess of zinc, after a few minutes at room temperature smelt of the ketone or aldehyde, and the reaction was completed by warming. The colour changed through yellow-green (of the semi-quinone) to paler yellow; the aldehyde or ketone was separated by aspiration or ether-extraction of the acid solution, and *o*-phenylenediamine obtained by ether extraction of the basified aqueous layer. In this way acetaldehyde- and *cyclo*hexanone-2 : 4-dinitrophenylhydrazones and *o*-phenylenediamine were isolated from quinoxaline and 1 : 2 : 3 : 4-tetrahydrophenazine and from their di-*N*-oxides, and keto-compounds and *o*-phenylenediamines detected from 2-methyl- and 2-methyl-3-*n*-amyl-quinoxaline di-*N*-oxides. No phenolic or ketonic material was detected in similar reductions of phenazine or phenazine di-*N*-oxide, nor from reductions of them in more or in less acid solutions.

Quinoxaline Di-N-oxide.—Quinoxaline (b. p. 106—107°/20 mm., m. p. 33°; from *o*-phenylenediamine and glyoxal sodium bisulphite) was oxidised, ice and excess of 10N-sodium hydroxide added, and the oxide extracted by shaking four times with equal volumes of chloroform. The dried (sodium sulphate) extract was evaporated, and the *product* which crystallised collected in two batches and recrystallised from water (16 ml.), forming bright yellow needles, which were dried at 60°. Yield 55%, m. p. 238—239° to a yellow liquid which almost immediately darkened and evolved gas (Found: N, 16·9. $C_8H_6O_2N_2$ requires N, 17·3%).

Colourless dilute solutions of the oxide (*e.g.*, of 4 mg./ml.) in N-sodium hydroxide produced with acetone (20 mg./ml.) a yellow colour deepening in a few minutes to brown and forming also a light-coloured flocculent precipitate. The reaction was given to varying degrees by acetophenone, ethyl acetoacetate and oxaloacetate, by sodium pyruvate and α-ketoglutarate but not by benzaldehyde, acetate, butyrate, glycine or glucose. Alkaline hyrogen peroxide caused no comparable reaction with the ketomethylene compounds; the oxide itself became brown only in more concentrated (5N) alkali.

2-Methylquinoxaline Di-N-oxide.—The quinoxaline (b. p. 124—125°/20 mm., from *o*-phenylenediamine and *iso*nitrosoacetone in 40—50% yield; see below) was oxidised, ice added, the mixture neutralised with 10N-sodium hydroxide (excess of alkali caused decomposition and hindered extraction) and extracted six times with equal volumes of chloroform. The dried extracts were evaporated to 5 ml., light petroleum (30 ml.) added, the precipitation repeated with the evaporated mother-liquors, and the combined precipitates crystallised from benzene; yield 65%; m. p. 180—181° (Found: C, 61·3; H, 4·5; N, 16·0. $C_9H_8O_2N_2$ requires C, 61·4; H, 4·5; N, 15·9%).

Dilute alkaline solutions of the oxide, which were almost colourless (*e.g.*, of 2 mg./ml.) gradually became strongly green or blue, and a Prussian-blue-like amorphous material separated from them. The change was more rapid in N- than in N/10-sodium hydroxide, and was much accelerated by sunlight. The blue product was apparently complex:

it was insoluble in chloroform, ether, and in 2N-acid or alkali, and on reduction in neutral solution by hydrosulphite did not yield a volatile base. The oxide resembled that of quinoxaline in its reaction with acetone.

2-Methyl-3-n-amylquinoxaline.—*iso*Nitrosomethyl *n*-hexyl ketone was prepared according to Behr-Bregowski (*Ber.*, 1897, **30**, 1515) and recrystallised from light petroleum by ice-cooling. To a mixture of water (20 ml.), glacial acetic acid (6 ml.), and concentrated hydrochloric acid (3 ml.), *o*-phenylenediamine (7·2 g.) and the *iso*nitroso-compound (10·9 g.) were added and shaken; the mixture became warm and by further heating was refluxed for 3 minutes. An oily layer formed and was separated by extracting the cooled mixture with light petroleum, leaving the aqueous layer (a). The extract was filtered from a solid (b) which separated, washed successively with a little 0·5N-sulphuric acid (c) and 2N-sodium hydroxide (d), dried, and evaporated; the product distilled in a vacuum (175°/12 mm.) as a very pale yellow liquid (6·0 g.) solidifying on cooling. It was recrystallised from light petroleum, forming rosettes of stout colourless prisms, m. p. 46°, which were very slightly soluble in water.

Various modifications in procedure failed to afford a higher yield. Solid (b) separated also from the reaction mixture (a) on neutralisation and in all weighed 3·2 g.; the crude substance, m. p. 160°, on heating in acid liberated methyl hexyl ketone and on reduction in acid yielded *o*-phenylenediamine. It was considered a product of condensation of 2 mols. of the *iso*nitrosoketone with one of *o*-phenylenediamine, as a corresponding excess (2·9 g.) of the diamine was found in (a) and (c) but little or no *iso*nitroso-ketone or other compound could be recovered from (d). Qualitative evidence was obtained of a similar side reaction in the preparation of 2-methylquinoxaline, of which the yield was similarly low.

2-Methyl-3-n-amylquinoxaline Di-N-oxide.—The quinoxaline was oxidised for 40 hours, ice added, and the mixture neutralised with 10N-sodium hydroxide; the product, which separated as an oil that solidified on scratching, was filtered off, washed with water, and dried in a vacuum. It crystallised from methanol or aqueous methanol, with ice-cooling, in yellow needles (58%) of the *oxide*, m. p. 107° (Found: C, 67·9; H, 7·4; N, 11·65. $C_{14}H_{18}O_2N_2$ requires C, 68·3; H, 7·3; N, 11·4%). The oxide was more soluble in water than was the parent base, and with strong alkali gave a yellow solution from which it was not extracted by ether.

1 : 2 : 3 : 4-Tetrahydrophenazine Di-N-oxide.—The base (Clemo and McIlwain, J., 1934, 1991) was oxidised to a brown solution, which was neutralised with ice-cooling. A buff-coloured granular precipitate separated and was filtered off, washed with water, and dried in a vacuum. It partly decomposed on crystallising from water, but was obtained in brown-yellow needles from alcohol or benzene; m. p. on rapid heating, 188° (Found: N, 13·2. $C_{12}H_{12}O_2N_2$ requires N, 12·95%). Alkaline solutions of the *oxide*, initially brown, changed slowly through orange to red-purple; the change was accelerated by heating but little if at all by light.

Phenazine Di-N-oxide.—Prepared by the general method, most of the oxide (cf. Clemo and McIlwain, J., 1938, 479) crystallised from the mixture and was separated in 90—95% yield by dilution with water, cooling and filtration. Oxidation by perbenzoic acid was also attempted to act as model in the preparation of iodinin; the same compound, m. p. 204°, was obtained in 60% yield.

Iodinin.—The general method of *N*-oxide formation was not found applicable to iodinin. Following Meisenheimer (*Ber.*, 1926, **59**, 1848) and Puschkareva (*J. Gen. Chem. Russia*, 1938, **8**, 151, 158), perbenzoic acid was found successful. The acid was prepared in chloroform solution, and assayed according to Tiffeneau (*Org. Synth., Coll. Vol.*, 1932, **1**, 422) and was transferred to benzene by repeated addition of the solvent and partial evaporation under reduced pressure.

The dihydroxyphenazine (15 mg.) derived (Clemo and McIlwain, J., 1938, 479) from the pigment, in benzene (10 ml.), was kept at room temperature with a benzene solution of perbenzoic acid calculated to contain 4 equivs. of available oxygen. The solution gradually became red (but similar ones in chloroform did not); aliquots diluted 20 to 50 times in benzene were compared colorimetrically with a standard solution of iodinin and showed a 40% conversion in 1 day, 55% after 3 days, and no further increase after 5 days. The bulk was then added to a column of activated alumina prepared with benzene; brown-yellow material was strongly adsorbed and the red product formed a wide band below it. The chromatogram was developed with benzene; the yellow material remained at the top of the column while the red band moved rapidly down and changed to blue. A benzene solution (red) of iodinin was adsorbed on alumina as a blue band, and the transitory red appearance of the adsorbate of the reaction mixture was probably due to the initial presence of much benzoic acid, which was partly removed by the solvent first passing through the column. The blue band from the reaction mixture was also washed through the column, the resulting red solution evaporated to dryness, benzoic acid extracted with a little ether, and the pigment crystallised from benzene, yielding purple needles (3 mg.) with a bronze metallic glint; m. p. alone or with iodinin, 236°.

Bacteriological Testing.—Aqueous solutions of the *N*-oxides were sterilised by filtration, and different quantities added to media, which were then inoculated and examined for growth during the following 7 days. General methods are described elsewhere (McIlwain, *Biochem. J.*, in press); the present tests used three strains of *Streptococcus hæmolyticus* (the Richards, another Group A, and a Group G organism) grown in the peptone medium *b* (*loc. cit.*) and three of *C. diphtheriæ* (one *gravis* and two *mitis* strains) grown in a casein hydrolysate medium (McIlwain, *Brit. J. exp. Path.*, 1942, **23**, 95). The concentrations needed for different degrees of bacteriostasis are shown in the Table. When strains reacted differently, a range of values is given.

Antibacterial Effects of N-Oxides.

Organism.	Base or *N*-oxide.	Concentration (*M*) of base or *N*-oxide causing— no inhibition.	prevention of visible growth for (days): 1.	2—4.	5 or more.
Strep. hæm.	Iodinin	10^{-7}	5×10^{-7}	10^{-6}	$1·5—2 \times 10^{-6}$
C. diphtheriæ	,,	10^{-7}	$10^{-7}—10^{-6}$	$10^{-6}—10^{-5}$	10^{-5}
Strep. hæm.	Phenazine di-*N*-oxide	4×10^{-6}	$2—4 \times 10^{-5}$	$4—8 \times 10^{-5}$	$6 \times 10^{-5}—10^{-4}$
,, ,,	1 : 2 : 3 : 4-Tetrahydrophenazine	10^{-3}	—	—	—
,, ,,	,, ,, ,, ,, di-*N*-oxide	4×10^{-5}	2×10^{-4}	$5 \times 10^{-4}—10^{-3}$	10^{-3}
C. diphtheriæ	1 : 2 : 3 : 4-Tetrahydrophenazine	10^{-3}	—	—	—
,, ,,	,, ,, ,, ,, di-*N*-oxide	4×10^{-5}	$4 \times 10^{-5}—2 \times 10^{-4}$	$2 \times 10^{-4}—10^{-3}$	10^{-3}
Strep. hæm.	2-Methyl-3-*n*-amylquinoxaline	5×10^{-4}	—	—	—
,, ,,	,, ,, ,, ,, di-*N*-oxide	2×10^{-4}	5×10^{-4}	—	—
C. diphtheriæ	2-Methyl-3-*n*-amylquinoxaline	5×10^{-4}	—	—	—
,, ,,	,, ,, ,, ,, di-*N*-oxide	10^{-4}	2×10^{-4}	10^{-3}	—
Strep. hæm.	2-Methylquinoxaline	$5 \times 10^{-4}—2 \times 10^{-3}$	2×10^{-3}	—	—
,, ,,	,, ,, ,, di-*N*-oxide	$2 \times 10^{-5}—2 \times 10^{-4}$	$2 \times 10^{-4}—10^{-3}$	—	$5 \times 10^{-4}—10^{-3}$

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