THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTIBACTERIAL ACTIVITY. PART VIII. 2-MERCAPTOPYRIDINE-N-OXIDE, AND SOME GENERAL OBSERVATIONS ON METAL-BINDING AGENTS

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8-HYDROXYQUINOLINE (oxine) (I) and its derivatives are powerful bactericides, and the antibacterial action has been traced to their ability to chelate, *i.e.*, to form cyclic complexes with the ions of heavy metals (Albert, Rubbo, Goldacre and Balfour, 1947). Further, these substances are innocuous if traces of iron are removed from the testing medium (Rubbo, Albert and Gibson, 1950). Evidence points to the 1 : 1 oxine : iron complex as the true toxic agent, and to the interior of the bacterial cell as its site of action (Albert, Gibson and Rubbo, 1953). This work led to the examination of hydroxy-derivatives of quinoxaline, quinazoline and similar substances all derived from the 8-hydroxyquinoline series by the insertion of further ring-nitrogen atoms. It was concluded that a high oil/water partition coefficient (*i.e.*, in favour of lipids) is necessary (Albert, Hampton, Selbie and Simon, 1954). As high coefficients are known to assist penetration into the cell, this was taken as supporting the hypothesis that action takes place within the cell.

It has often been postulated, but not upheld, that some chelating agents act against bacteria by sequestering essential heavy metals such as iron. For example, it has been suggested that the 2-substituted pyridine-N-oxides act by this mechanism (Lott and Shaw, 1949). As such a starvation mechanism is entirely different from the one which has been established for oxine in this series of papers, an investigation of these pyridine-N-oxides, *e.g.*, (III), was undertaken and is reported here. It is found that the mode of action is apparently identical with that of oxine although the substances are chemically so different. Further work is reported on the broader issue of what properties a metal-binding agent must possess to be antibacterial.

EXPERIMENTAL

Microbiology

The greatest dilution preventing visible growth was used as the criterion in the bacteriostatic tests. The strains of bacteria used were intermediate (in sensitivity to oxine) between various strains used in earlier papers in this series. Hence oxine was included in the tests, as a control, so that the antibacterial action of the new substances could be compared with it as a standard.

The bacteriostatic tests consisted of inoculating two series of twofold dilutions (beginning at M/200 and M/2000) of the substance in broth (pH 7·3) and inspecting the solutions for growth after 48 hours' incubation at 37°. Each test was made in duplicate. The bactericidal tests reported in Table IV were carried out by the technique of Miles and Misra (1938).

The mould *Penicillium spinulosum* was grown in 2 per cent malt extract (Allen and Hanbury). To 100 ml. of the solution 20 ml. of McIlvaine's citric acid disodium phosphate buffer of the appropriate pH was added. The pH was adjusted to the desired figure (by adding additional citric acid or disodium phosphate) using a glass electrode, the medium bottled in appropriate volumes and sterilised by steaming. The inoculum in all cases was one drop of a spore suspension obtained from growth of the mould on solid medium.

Chemistry

The preparation, ionisation constants, stability constants and partition coefficients of the various substances used are given in Table I.

Ionisation constants were determined by titration of 0·0005 M of the dried specimen in air-free water under nitrogen, using a Cambridge pH set with glass and calomel electrodes (standardised to pH 4·00 with 0·05 M potassium hydrogen phthalate, and to 9·20 with 0·05 M borax, at 20°). Results were acceptable only when agreement on re-standardisation with these buffers after titration lay within ± 0·01 unit. Nine-tenths of an equivalent of 0·1 N hydrochloric acid, or potassium hydroxide (carbon-dioxide free), was added in nine equal portions. The nine pK_a values for each pH reading were calculated, when acid was the titrant, from the formula :

$$pK_a = pH - \log([B] + [H^+] / [BH^+] - [H^+])$$

where $[BH^+]$ and [B] are the concentration of the molecule, protonated and non-protonated respectively, neglecting hydrolysis corrections which are taken care of by the remainder of the formula. When alkali was the titrant, the following formula was used :

$$pK_a = pH + \log([AH] + [OH^-]/[A^-] - [OH^-])$$

The nine pK values were converted into antilogarithms before averaging, then reconverted to logarithms. The values were rejected if the spread exceeded ± 0·06.

Stability constants were determined by adding an equivalent of 0·1 N potassium hydroxide (carbonate-free) in ten equal portions to aqueous solutions (0·001–0·0025 M) which contained the substance and one equivalent of the metallic ion (*e.g.*, cobalt nitrate). When titrating substances No. 8 and 9 in the presence of copper, prior addition of hydrochloric acid was necessary to reveal the lower values of $\bar{n}$ (defined as the average number of molecules of chelating substance combined with one atom of metal). The titrations were carried out in boiled-out water, under nitrogen. Calculations of the stability constants were made as described by Albert (1950, 1952). The symbol K′ refers to the equilibrium betwen the substance and the 1 : 1-complex, *e.g.*, (IV) ; whereas K″ refers to the equilibrium between the 1 : 1- and the 2 : 1- complex, *e.g.*, (V).

Partition coefficients, between purified oleyl alcohol and 0·05 M buffer, were carried out (under nitrogen for No. 2) as described by Albert and Hampton (1954).

The substances were prepared as in Table I, or as follows. 2-(2′-Hydroxyphenyl) pyridine (IX), prepared according to Geissman (1946), proved to be not an oil, as reported, but a solid of m.p. 53°. (Found : C, 76·7 ; H, 5·3 ; N, 7·9. Calculated for $C_{11}H_9ON$, C, 77·1; H, 5·3 ; N, 8·2 per cent). 3-Butyryl-2-hydroxy-6-propyl-γ-pyrone, prepared according to Deshapande (1932), and fractionated melted 9° higher (*viz.* 25°) than in the literature. (Found : C, 64·0 ; H, 7·0. Calculated for $C_{12}H_{16}O_4$, C, 64·3 ; H, 7·2 per cent.) *o*-Hydroxyacetophenone (XIV) was a gift from Professor A. Birch. 1-Hydroxy-2-acetonaphthone was prepared according to Witt and Braun (1914), m.p. 98°. Hydrazine-N : N-diacetic acid (XI) was made according to Bailey and Read (1914). (Found : C, 32·3 ; H, 4·9 ; N, 18·9. Calculated for $C_4H_8O_4N_2$, C, 32·4 ; H, 5·4 ; N, 18·9 per cent). 4-Butyl-2-aminophenol (XIII) was prepared from phenyl butyrate by rearrangement, reduction (Rice and Harden, 1936), nitration (Close, Tiffany and Spielman, 1949), and final reduction (Baranger, 1931).

RESULTS

The results of the bacteriostatic testing of five heterocyclic N-oxides (No. 2–6) bearing mercapto- or hydroxy- groups in the 2-position are given in Table II. Oxine is included as a standard, for comparison. Logarithmic stability constants

TABLE I.—*Preparation and Physical Properties of Test-substances*

No.	Substance.	Preparation.	Ionisation (pKa, 20°). Basic.	Ionisation (pKa, 20°). Acidic.	Stability constants (logs ; 20°). Copper. K′.	Copper. K″.	Nickel. K′.	Nickel. K″.	Zinc. K′.	Zinc. K″.	Cobalt. K′.	Cobalt. K″.	Ferrous. K′.	Ferrous. K″.	Partition coefficients (oleyl alcohol/water) 20°. pH 7·3.	pH 3·0.
1	8-Hydroxyquinoline (oxine) for comparison	a	5·13	9·89	12·2[l]	n	9·9	n	8·6	p	9·1	p	8·0	p	67	—
2	2 - Mercaptopyridine - N - oxide (III)	b	<0·5	4·57	m	m	m	m	5·9	5·4	5·5	4·5	4·7	m	1·07	—
3	2 - Mercapto - 4 - *n* - propyl - pyridine-N-oxide	c	j	j	m	m	m	m	m	m	m	m	m	m	q	—
4	2 - Hydroxypyridine - N - oxide	d	−0·62[k]	5·97	7·0	6·2	5·7	4·4	5·5	4·3	5·3	4·3	m	m	0·03	—
5	2 - Hydroxyquinoline - N - oxide (VI)	e	<1	6·88	m	m	5·5	m	5·4	m	5·1	m	m	m	3·18	—
6	2-Hydroxy-4-methyl-5 : 6-benzoquinoline - N - oxide (VIII)	c	j	j	m	m	m	m	m	m	m	m	m	m	r	—
7	2-(2′-Hydroxyphenyl) pyridine (IX)	f	4·20	10·63	m	m	6·1	m	m	m	—	—	—	—	—	—
8	2-(2′-Hydroxyphenyl) imidazoline	g	6·63 / <1	12·58	11·7	m	8·1	m	7·9	m	7·5	6·5	m	m	0·36	—
9	2 - (2′ - Hydroxynaphthyl - 3′-) imidazoline (X)	c	7·01	10·85	11·3	10·6	—	—	—	—	—	—	—	—	7·47	—
10	Hydrazine - N : N - diacetic acid (XI)	f	7·32	2·40 / 3·12	m	m	7·8	5·2	6·7	m	—	—	—	—	0·05[s]	—
11	Dehydracetic acid (3 - acetyl-2-hydroxy-6-methyl-γ-pyrone) (XII)	a	<1	5·53	5·6	4·3	4·1	m	m	m	m	m	m	m	0·3	8·9
12	3-Propionyl-2-hydroxy-6-ethyl-γ-pyrone	h	<1	5·38	5·3	m	3·9	m	m	m	m	m	m	m	2·3	156
13	3 - Butyryl - 2 - hydroxy - 6 - propyl-γ-pyrone	f	<1	5·47	m	m	m	m	m	m	m	m	m	m	10·5	18,800

— = Not attempted.
a = Commercial, recrystallised to constant m. pt.
b = Shaw *et al.* (1950).
c = Rees (1956).
d = Lott and Shaw (1949).
e = Newbold and Spring (1948).
f = See text, under " Experimental."
g = Johnston and Freiser (1954) [but higher m.p., *viz.* 203–4°].
h = Deshapande (1932).
j = Not determinable because of poor solubility.
k = Determined spectrometrically.

l = All oxine figures from Albert (1953), except zinc which is from Näsänen (1952).
m = Sparing solubility interfered with attempted determination.
n = Not exactly determinable because of sparing solubility, but estimated to be *two* units less than log K′.
p = As n, but *one* unit less.
q = as m, but estimated to be > 30.
r = As m, but estimated to be > 10.
s = The oleyl alcohol layer was diluted with ethanol and titrated with alkali.

for the binding of zinc are included as a sample of the metal-combining powers of each substance (other stability constants are available from Table I). It is evident that all the N-oxides have similar stability constants, which are about a thousand times less than that of oxine [*e.g.*, or No. 1 and 4, antilog (8·6–5·5) = 1300]. One substance (2-mercaptopyridine-N-oxide) has an antibacterial action much greater than that of oxine. The antibacterial action of the 2-hydroxy-derivatives is far inferior to that of the 2-mercapto-derivatives. In the 2-hydroxy- series, the antibacterial action is seen to increase as the partition coefficient rises (*i.e.*, as the molecule is modified so as to favour dissolution in lipids at the expense of water).

Substances 2–6 differ from oxine in that the metal is located between two oxygen atoms, or between an oxygen and a sulphur atom. Substances 7–10, 14 and 15 were synthesised to provide the same type of chelation as in oxine, the metal being held in a five or six-membered ring between an oxygen and a nitrogen atom. None of these substances approaches oxine in antibacterial power, but in the pairs 8–9 and 14–15 an increase in activity with increasing lipophilic nature can be seen.

TABLE II.—*Bacteriostatic Action of Test Substances*

Highest M dilutions preventing visible growth in 48 hr. at 37° (medium : broth, pH 7·3.

No.	Substance.	*Str. pyogenes.*	*Staph. aureus.*	*Bact. coli.*	Stability constant (log K′) zinc.	Partition coefficient pH 7·3 (see Table I).
1	Oxine (for comparison)	40,000	40,000	<5,000	8·6	67
2	2-Mercaptopyridine-N-oxide (III)	160,000	160,000	10,000	5·9	1·07
3	2-Mercapto-4-*n*-propyl-pyridine-N-oxide	64,000	32,000	3,000	5·9†	>30†
4	2-Hydroxpyridine-N-oxide	100	200	100	5·5	0·03
5	2-Hydroxyquinoline-N-oxide (VI)	1,000	1,000	1,000	5·4	3·18
6	2-Hydroxy-4-methyl-5 : 6-benzoquinoline-N-oxide (VIII)	4,000	4,000	2,000	5·5†	>10†
7	2-(2′-Hydroxyphenyl)-pyridine (IX)	—	200	—	6†	—
8	2-(2′-Hydroxyphenyl)-imidazoline	800	800	<200	7·9	0·36
9	2-(2′-Hydroxynaphthyl-3′)-imidazoline (X)	2,000	2,000	<1,000	7·9†	7·47
10	Hydrazine-N : N′-diacetic acid (XI)	100	200	100	6·7	0·05
14	2-Aminophenol	3,200	1,600	200	4‡	0·40
15	4-Butyl-2-aminophenol (XIII)	8,000	4,000	<4,000*	4‡	7·06

— = Not attempted.
* = M/4000 is strongest solution obtainable in broth, and this was inactive.
† = Estimated from values of nearest analogues.
‡ = Estimated from Charles and Freiser (1952) who worked in dioxane.

Table III relates the antifungal action of dehydracetic acid, and its homologues, to the pH at which the test is carried out, and hence to the percentage of non-ionised substance present. At a pH suitable for testing bacteria, these

substances are completely ionised and it can be seen from Table III that they are not antibacterial.

TABLE III.—*Fungistatic and Bacteriostatic Action in the Dehydracetic Acid Series*

No.	Substance.	*Penicillium spinulosum.** Highest dilution preventing visible growth. pH 4.	pH 8 (buffered).	Percentage not ionised. pH 4.	pH 8.	*Str. pyogenes*,† *Staph. aureus* and *Bact. coli.* Highest concentrations obtainable. These did not prevent growth.	Percentage not ionised at pH 7·5.
11	Dehydracetic acid (3-acetyl-2-hydroxy-6-methyl-γ-pyrone (XII)	4,000	200	97	0·32	200	0·99
12	3-Propionyl-2-hydroxy-6-ethyl-γ-pyrone	16,000	<1,000	96	0·25	1,000	0·79
13	3-Butyryl-2-hydroxy-6-propyl-γ-pyrone	32,000	2,000	97	0·32	2,000	0·99

* Heavy inoculum of spores, incubated for 7 days in 2 per cent malt extract at 30°. † Each in broth at pH 7·5 and 30°.

As 2-mercaptopyridine-N-oxide (No. 2) has high antibacterial activity, it was subjected to tests which had previously done much to reveal the mode of action of oxine (Albert *et al.*, 1953). Table IV shows that, like oxine, this substance is bactericidal only in the presence of iron, this activity being prevented by traces of cobalt, but not of nickel. Increasing the ratio of substance to iron abolishes the activity, as with oxine.

TABLE IV.—*The Effect of Traces of Metallic Cations on Bactericidal Action in Glass-distilled Water (pH 6·0–6·8) at 20°*

Organism : *Staph. aureus.*

Tube. No.	2-Mercaptopyridine-N-oxide 1/M.	Ferric sulphate 1/M.	Growth after exposure (hr.). 0.	2.
1	20,000	*Nil*	+++*	+++
2	20,000	20,000	−†	−
3	40,000	*Nil*	+++	+++
4	40,000	40,000	+++	−
5	80,000	*Nil*	+++	+++
6	80,000	80,000	+++	−
7	160,000	*Nil*	+++	+++
8	160,000	160,000	+++	+
9	80,000	80,000	+++	−
10	8,000	80,000	+++	+++
11	800	80,000	+++	+++
12	40,000	40,000 / +40,000 Co^{++}	+++	+++
13	40,000	40,000 / +40,000 Ni^{++}	+++	−

* +++ signifies discrete colonies, but uncountable ; + signifies 10 to 50 colonies ; −no growth.
† Rapid bactericidal action before count could be made.

Finally, the partition coefficients of oxine and some of its derivatives were determined to provide necessary data for comparison. These are reported in Table V, together with the bacteriostatic figures for these substances.

TABLE V.—*Correlation of Bacteriostatic Activity and Partition Coefficient in the Oxine Series*

Highest dilution (expressed as 1/M) preventing visible growth in 48 hr. at 37° (medium : broth, pH 7·3).

No.	8-Hydroxyquinoline.	*Str. pyogenes.**	*Staph. aureus.**	Partition coefficient oleyl alcohol/water 20° : pH 7·3.
1	(Unsubstituted, *i.e.*, oxine)	100,000	100,000	67
17	5 : 7-Dichloro-	100,000	100,000	294
18	5 : 6-Benzo-	200,000	26,000	234
19	-5-Carboxylic acid	3,200	<800	3·90

* Bacteriostatic values from Albert *et al.* (1947), the strains being slightly more sensitive than in the present work.

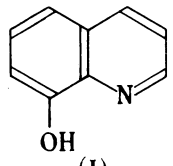
DISCUSSION

2-*Mercaptopyridine-N-oxide* (III)

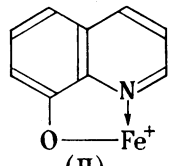
High antibacterial powers were claimed for this substance at the time of its discovery (Shaw, Bernstein, Losee and Lott, 1950; Pansy, Stander, Koerber and Donovick, 1953). However, it has never been subjected to comparative testing alongside oxine (8-hydroxyquinoline) or other potent antibacterials. This has now been done and it is evident from Table II that this substance is four times as active as oxine against the Gram-positive species. It also shows significant activity against the Gram-negative organism, but of a lower order.

Because 2-mercaptopyridine-N-oxide (also known as N-hydroxy-2-pyridine-thione) is so potent, its mode of action was investigated. As recorded in Table I, it forms complexes with the cations of heavy metals. Yet it binds these metals differently from oxine, because it holds the metal between oxygen and sulphur, see (IV), whereas oxine holds the metal between oxygen and nitrogen, see (II). Nevertheless a common mode of action is not excluded, and hence the mercapto-compound was submitted to four tests which are characteristic of oxine, and which have shed much light on the mode of action of oxine. These tests are reported in Table IV.

It is characteristic of oxine that (*a*) it is rapidly bactericidal, (*b*) it is bactericidal only if traces of iron are present, (*c*) such traces of iron do not activate oxine if traces of cobalt (but not nickel) are added, and (*d*) traces of iron do not activate in the presence of an excess of oxine (Albert *et al.*, 1953). It is evident from Table IV that 2-mercaptopyridine-N-oxide follows the same pattern. Tube 2 reveals its rapid bactericidal action, and a comparison of tubes 1 and 2, 3 and 4, 5 and 6 shows that it is inactive in the absence of small amounts of iron. The de-activating effect of cobalt on an iron-activated mixture is seen by comparing tubes 12 and 4 (tube 13 shows that, as with oxine, nickel has no comparable effect). Finally the quenching effect of an excess of 2-mercaptopyridine-N-oxide is seen in tubes 9–11.



(I)



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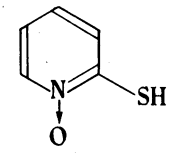

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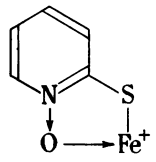

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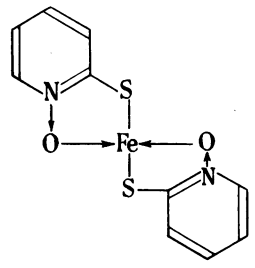

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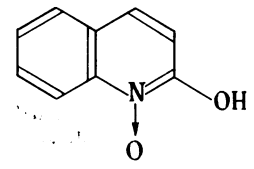

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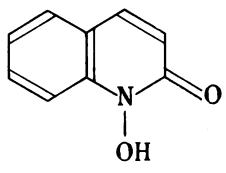

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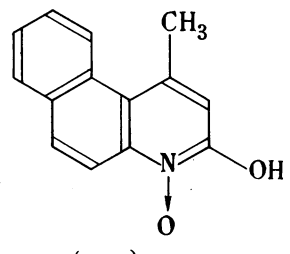

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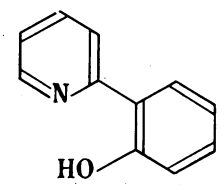

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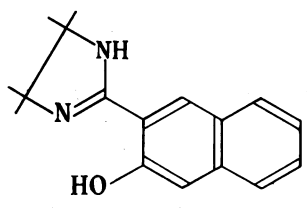
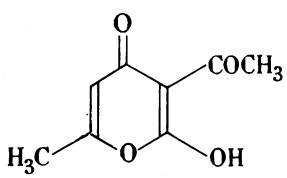


(X)

$H_2N{\cdot}N(CH_2CO_2H)_2$

(XI)



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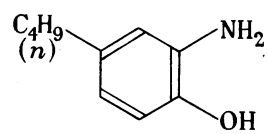

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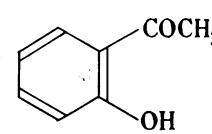

(XIV)

[The de-activating effect of cobalt has been demonstrated in two other types of metal-binding antibacterials : 5-bromosalicylal-thiosemicarbazone and 1-nitroso-2-naphthol (Schraufstätter, 1950).]

Thus it must be concluded that, like oxine, 2-mercaptopyridine-N-oxide is not of itself toxic, but that it forms a toxic 1 : 1-complex (IV). From tubes 9–11 of Table IV, it is evident that the 2 : 1-complex (V) is not toxic, just as in the case of oxine.

The hypothesis has been put forward that the oxine-metal complex acts inside the cell (Albert *et al.*, 1953). This concept received strong support from further work in which a series of analogues of oxine were tested. In these substances the high oil-water partition coefficient of oxine was lowered by inserting an extra ring-nitrogen atom in all possible positions (Albert *et al.*, 1954). Because of the low coefficients, such substances are less likely to enter the cell. As the partition-coefficients fell, the antibacterial activity also fell ; and when (still retaining the extra nitrogen atom) the partition coefficient was raised by inserting a small alkyl-group, the antibacterial action also increased, exactly as the hypothesis demands. At the time, no investigation was made to see if the partition coefficient of oxine was optimal, or if raising the coefficient would improve the action. This has now been investigated, and it would seem from Table V that a fourfold increase in the coefficient confers no extra benefit (compare No. 1, 17 and 18). Likewise, the carboxylic acid derivative of oxine (No. 19) which has only feeble antibacterial properties, is now seen to have a low coefficient, as was predicted earlier (Albert *et al.*, 1947). It must be pointed out that these correlations of partition coefficients with antibacterial activity of the substances in Table V are valid only if the stability constants do not vary appreciably. This may safely be assumed for substances 1, 18 and 19 at least, because annelation has little effect (compare No. 4 and 5 in Table I), and oxine-5-sulphonic acid has almost the same stability constants as oxine (Albert, 1953).

This work on the derivatives of oxine (particularly that reported in 1954) suggests that other series of antibacterials which act in conjunction with metals would also be benefited by increasing the partition coefficient, at least until an optimal value was reached. It is not simple to subject 2-mercaptopyridine-N-oxide to this hypothesis as it is somewhat prone to destruction by oxidation, and this tendency is greatly magnified by the introduction of alkyl-groups necessary to raise the partition coefficient. Indeed, another analogue intended for the present series (2-mercapto-4-methyl-5 : 6-benzoquinoline-N-oxide) was so readily oxidised by air (Rees, 1956) that difficulty was found in determining the physical constants. Thus, it is not necessarily contrary to the hypothesis that No. 3 (Table II) seems to be less antibacterial than No. 2.

Fortunately the corresponding hydroxy-compounds (No. 4, 5 and 6) have no tendency to oxidise. These three substances demonstrate the postulated increase in antibacterial action with rising partition coefficient, a satisfactory comparison because the affinity for metals (as shown by the stability constants) is of the same order. Further small series showing this effect are No. 8 and 9, also No. 11, 12 and 13 in Table III and apparently No. 14 and 15 (Table II).

However, it is evident that such a rule can be applied only within a series of related substances. For example, 8-hydroxyquinoline-5-carboxylic acid is almost inactive because its partition-coefficient is 3·9, a figure that is too low for activity in the oxine series (Table V ; also see Albert *et al.*, 1954 for much other relevant

data). Yet, in another series, the lower figure of 1·07 suffices for an outstandingly active substance, 2-mercaptopyridine-N-oxide (No. 2).

The stability constants of this mercapto-compound also fall below those of oxine. Obviously, for a substance to be able to act through forming metal complexes the stability constants must exceed certain critical values. It is now suggested that these critical values are the stability constants of the common amino-acids, in so far as the antibacterial will be in competition with these (free and combined) in the medium, and inside the cell. The figures for glycine (these are typical of those of amino-acids in general, excepting histidine and cysteine, which have higher avidity) are :

Logarithmic stability constants for glycine

Copper.		Nickel.		Zinc.		Cobalt.		Iron (ferrous).	
K′	K″	K′	K″	K′	K″	K′	K″	K′	K″
8·5	6·9	6·1	5·0	5·3	4·1	5·1	3·9	4·3	3·7

2-Mercaptopyridine-N-oxide has K′ values not much above this supposed critical level. For example its affinity for zinc is :

$$\text{antilog } (5{\cdot}9–5{\cdot}3) = 4$$

that is to say only four times greater than glycine, comparing the 1 : 1 complexes, *e.g.*, (IV). But an 80-fold difference in favour of the mercapto-compound is seen if the second stability constant (K″) is also used, and thus the 2 : 1-complex, *e.g.*, (V), is taken into consideration. Actually, the 2 : 1 complex of oxine, being liposoluble like oxine itself, has been suggested to be the form in which oxine penetrates the cell, because the 1 : 1-complex is non-liposoluble, and presumably non-penetrating. In the presence of excess oxine the 2 : 1-complex is held as such in the cell and so the cell is unharmed, but in the absence of excess oxine, the 2 : 1-complex is broken down to the toxic 1 : 1-complex or further, according to this hypothesis (Albert *et al.*, 1953). It was shown above that the mercapto-compound has the same mode of action as oxine, and hence the constants of the 2 : 1-complex should be significant for this substance also. Analogues of oxine with reduced stability constants seem to be less active than oxine (Albert *et al.*, 1954), so no virtues can be claimed for the mercapto-compound's nearness to the supposed critical figures:

To sum up : 2-mercaptopyridine-N-oxide has lower stability constants, and lower partition coefficients than oxine, but is nevertheless more active biologically. This points to the likelihood of a third factor playing a part. This factor can hinge on a very small change in the molecular structure as is seen by comparing No. 2 with No. 4 and 5 (Table II). These substances have stability constants and partition coefficients of the same order, yet No. 4 and 5 are much poorer as antibacterials. The significant difference between these substances is whether they have sulphur or oxygen in the 2-position, the substances with oxygen being chemically stable, whereas those with sulphur are prone to oxidative destruction. As was mentioned above, the metal-binding antibacterials are thought to enter the bacterial cell as the 2 : 1-complex with iron, but to act only after this has been broken down. Such a breakdown would be particularly easy in the case of mercapto-compounds

which readily form disulphides, a class of substances which cannot bind metals. It is therefore suggested that the third factor is this ease of chemical change. Thus a limited proneness to chemical decomposition may be an advantage.

General observations on metal-binding agents

Given that these three factors are significant in imparting antibacterial properties to a metal-binding substance, the question arises : How can knowledge of these factors lead to the discovery of new types of metal-binding antibacterials?

The partition-coefficient is the factor most easily brought under control. It can be raised considerably by inserting a small side-chain of perhaps not more than three carbon atoms, as in the aza-oxines (Albert *et al.*, 1954). But this rule does not apply if the substance is ionised at the pH of the biological tests (information which is readily obtained from the ionisation constant).

By their nature, ions have low partition coefficients. Substances 11, 12 and 13 are of special interest in this connection. As Table I shows, the insertion of four carbon atoms into the molecule of dehydracetic acid increases the partition coefficient 2000-fold. However, this is true only of the neutral molecule, which is the form present at pH 3·0. On the other hand, the anion of dehydracetic acid (*i.e.*, the form present at pH 7·3) has an intrinsically lower coefficient, and the insertion of four carbon atoms raises it only 35-fold. Table III shows some further correlations for these substances. At pH 4, a condition under which substances are commonly tested for fungistatic activity, these substances are not appreciably ionised and are highly fungistatic ; at pH 8, these substances are entirely ionised and are only feebly fungistatic. At pH 7·5, where bacteria are commonly tested, they are only feebly antibacterial, presumably for the same reason. These results may shed light on yet other substances believed to be fungistatic but not antibacterial. (Substances 11–13 are such weak metal-binders that we do not wish to claim that metal-binding is responsible for their antifungal action.)

Of all the numerous metal-binding substances evolved by analysis, the majority are ionised at pH 7, and hence unlikely to be antibacterial. Ethylenediamine tetra-acetic acid (versene), which has very high stability constants but is ionised, is an outstanding example of this effect. Substance 10 (Table II) is a further example. Several other ionised reagents, of the most diverse chemical types, have been found to have little or no action on bacteria (Albert *et al.*, 1947). Further development of such substances requires modification of the molecule to repress ionisation.

Thus the rules for improving an unfavourable partition coefficient are known. But it is otherwise with stability constants. In spite of all that has been written on the subject, it is still impossible to assess the magnitude of a stability constant in advance of measurement. Hence many substances must be synthesised and much time spent on titrating them in the presence of metals (solubility permitting) for an occasional substance with high constants to be discovered. Some substances with reputedly high avidities have been found, when measured, to have only modest values. A useful list of stability constants has been made by Irving and Williams (1953), but it must be borne in mind that values obtained in solvents other than water are not relevant to biological problems.

Two other classes of drugs will now be mentioned which bind metals with avidity equalling, or somewhat exceeding that shown by the common amino-

acids. First, isoniazid, and other anti-tubercular hydrazides, which become inactive if modified so that metals can no longer be bound (Cymerman-Craig, Rubbo, Willis and Edgar, 1955), but in which another factor is also involved (Albert, 1956). Again, the tetracyclines (*e.g.*, aureomycin) have considerable affinity for metals (Albert, 1956), particularly for ferric iron (for which the common amino-acids have little affinity). But we find that the tetracyclines kill bacteria so much more slowly than oxine and 2-mercaptopyridine-N-oxide that they cannot act by exactly the same mechanism. *o*-Hydroxyacetophenone (XIV) and 1-hydroxy-2-acetonaphthone (partition coefficient, 32) were examined, because of their structural resemblance to the metal-binding part of the tetracyclines. Both showed a high avidity for ferric ion (*e.g.*, log K′ and K″ of *o*-hydroxyacetophenone were found to be 10·4 and 9·6). However, both lacked antibacterial properties.

Clinical possibilities.

Although little affected by serum, oxine is inactivated by red blood cells (Albert *et al.*, 1947). We have now examined 5-methyl-, 5-chloro-, 7-chloro- and 5 : 7-dichloro-oxines, and 2-mercaptopyridine-N-oxide in the presence of red cells : all were inactivated. The cause of the inactivation of oxine is believed to be a diffusible antagonist liberated by the red cell (Professor G. A. H. Buttle and Dr. W. G. Smith, personal communication).

Professor Buttle has kindly determined the average lethal doses (LD_{50}) in mg./kg. (for mice) of 2-mercaptopyridine-N-oxide (the corresponding values for oxine, obtained at the same time, are given in brackets) : oral, 650 (190) ; subcutaneous, 280 (17) ; intravenous, 255 (22). Although the mercapto-compound is more active against bacteria than oxine, and has less mammalian toxicity, the inactivation by red cells would limit it to the uses commonly made of oxine, which are principally on the intact mucous membrane.

A more interesting substance for laboratory development may be 2-(2′-hydroxynaphthyl-3′)imidazoline (No. 9, Table II) which is almost unaffected by blood under conditions where the activity of oxine drops to one-twentyfifth.

SUMMARY

The antibacterial action of 2-mercaptopyridine-N-oxide was seen to follow the pattern shown to be typical for oxine, *viz.*, it was rapidly bactericidal, but inactive in the absence of traces of iron, inactive in the presence of iron if traces of cobalt were added, and inactive in concentrated solution.

Stability constants and partition coefficients in conjunction with the above evidence indicate that there is no reason to assume that the 2-substituted pyridine-N-oxides act by sequestering essential heavy metals such as iron. It is suggested that 2-mercaptopyridine-N-oxide enters the cells as a 2 : 1 complex with iron, but that it acts only after this has been broken down.

As with oxine, 2-mercaptopyridine-N-oxide is inactivated by red cells.

A general discussion on the factors which make a chelating substance antibacterial is presented.

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