
A. ALBERT, A. HAMPTON, F. R. SELBIE and ROSEMARY D. SIMON.

From the Department of Medical Chemistry* of the Australian National University, and the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W.1.

Received for publication November 12, 1953.

It has been shown that 8-hydroxyquinoline is not toxic for Staphylococcus aureus unless traces of iron (or copper) are present in the medium (Rubbo, Albert and Gibson, 1950). It was next shown that a large excess of either iron or oxine prevented a rapid bactericidal action. This led to the hypothesis that oxine gained access to the cells as a complex having one atom of the metal bound between two molecules of oxine (to be referred to briefly as the 1 : 2 complex (XIII)); and that it exerted its toxic effect inside the cells in the form of the unsaturated complex which consists of one molecule of oxine bound to one atom of the metal (i.e., the 1 : 1 complex (XII)) (Albert, Gibson and Rubbo, 1953).

It will be noted that this hypothesis postulates that oxine acts within the cell, and not on the outside as the aminoacridines do (Albert, 1951). The present work was carried out to test that assumption by varying the lipophilic nature of oxine by means of suitable substituents in the molecule. It is generally agreed that in a series of related substances the ease of penetration through a cell membrane is proportional to the lipophilic nature of the substance (Davson and Danielli, 1943; Höber, 1945). This lipophilic nature can be conveniently measured in terms of oil/water partition coefficients.

In the present work the coefficient of oxine has been lowered by the insertion of one or more ring-nitrogen atoms, and the coefficients of some of the resulting substances have been raised again by the insertion of alkyl groups. It will be shown that the antibacterial properties disappear as the coefficient is lowered, and re-appear when it is raised again. These facts provide support for the hypothesis that oxine acts inside the cell.

**EXPERIMENTAL.**

**Bacteriology.**

The antibacterial activities of the substances described below were assessed by finding the greatest dilution that would prevent visible growth. In order to preserve continuity with previous studies in this series, these tests were performed with the six species of bacteria used previously (Albert, Rubbo, Goldacre and Balfour, 1947). The strains of Gram-positive bacteria used (Clostridium welchii, Streptococcus pyogenes and the Oxford strain of Staphylococcus aureus) were not the same as in the earlier series and under the conditions of the tests were about 4 times less sensitive to oxine. Hence oxine was included as a control substance in all experiments so that a relative assessment of the antibacterial action of the new substances could be made in spite of differences in the strains used and in the conditions of the experiments. In the final experiment (Table II), another strain of Str. pyogenes, equal in sensitivity to the original strain, was introduced. The Gram-negative strains used

* Present address: 183, Euston Road, London, N.W.1.
(Bacterium coli, Proteus, Pseudomonas pyocyanea) were similar in sensitivity to the original strains.

The bacteriostatic tests consisted of inoculating a series of twofold dilutions of the substance in broth (pH 7.3) and inspecting the solutions for evidence of growth after 48 hr. incubation at 37°C. Each substance was tested in duplicate. The solutions of the test substances (m/800 in broth) were autoclaved at 15 lb. for 15 min. (120°C) in an air-tight screw-capped container. From this stock solution, serial dilutions were prepared in autoclaved broth.

The metal-depleted broth used for the experiment shown in Table III was prepared by repeated extraction of broth with oxine and chloroform as described by Rubbo et al. (1950). In this experiment fourfold serial dilutions of oxine (or its analogue) were made in oxine-treated broth and in untreated broth, and then distributed in 2 ml. amounts. Solutions of FeSO₄·7H₂O (Analar) and CoSO₄·7H₂O (Analar) were made in treated and untreated broth at a concentration of m/12,500. These metal solutions were added in 1 ml. amounts to sets of serial dilutions of oxine as required, and the volume of each tube was made up to 4 ml. with the appropriate broth where necessary. Control tubes containing the metals without oxine were also set up. The tubes were then inoculated with 3 drops of a 24-hr. culture of the Oxford staphylococcus in oxine-treated broth and the results were read after incubation for 18 hr.

Chemistry.

The ionization constants, stability constants and partition coefficients are taken from Albert and Hampton (1954). The substances were prepared as described by Albert and Hampton (1952, 1954). These substances are described uniformly as aza-oxines, using the same numbering as for oxine (I). Alternative names also exist for them and have been placed under each formula to assist indexers.

FORMULAE.
It will be seen that the substances first investigated are analogues of oxine carrying one extra ring-nitrogen in every possible position (II to VII). Others carry two or three extra ring-nitrogens (VIII to XI). These form No. 2–11 of Table I. The insertion of even one ring-nitrogen greatly decreases the lipophilic properties of (I), as Table I shows.

Later, homologues of (II) and (III) were prepared in which a methyl (–CH₃), propyl (–CH₂CH₃CH₃) or allyl (–CH₂CH : CH₃) side-chain was included for the purpose of restoring lipophilic properties. These syntheses produced 4-methyl-2-aza-oxine (4-methyl-8-hydroxyquinoline), 4-methyl-3-aza-oxine (4-methyl-8-hydroxyquinazoline), 4-propyl-3-aza-oxine and 7-allyl-3-aza-oxine (No. 17–20). Their formulae can be easily visualized with the help of numbering included in formulae (II) and (III).

All of the above substances form tightly bound complexes with the ions of metals by the process of chelation, i.e., by building the metal ions into a new heterocyclic ring, cf. (XII). Some non-chelating isomerides of the above substances were prepared as controls. These (No. 12–16) are not capable of chelation because their hydroxyl-group is not peri to a ring-nitrogen.

RESULTS.

Table I gives the results of the bacteriostatic testing of oxine, its aza-derivatives and some non-chelating isomerides of the latter. It is evident that the mono-aza-oxines fall into two sub-classes, (a) those having a partition-coefficient about one-tenth of that of oxine (No. 2, 3, 4); and (b), those having a still lower coefficient (No. 5, 6, 7). The antibacterial action of (a) is less than that of oxine, but quite appreciable, the antibacterial action of (b) is much less. The poly-aza-oxines (No. 8–11) resemble group (b), having low partition coefficients and lacking antibacterial activity.

The substances No. 12–16 were synthesized to provide a series of isomerides of No. 2–4. These differ only in that they do not chelate with metallic ions. Thus No. 12 is isomeric with No. 2, No. 13–14 with No. 3, and No. 15–16 with No. 4. It is evident that none of these non-chelating isomerides have the antibacterial powers of their chelating prototypes.

### Table I.—Bacteriostatic Action of Oxine and Related Substances.

Highest dilutions (expressed as 1/m) completely preventing visible growth in 48 hr. at 37° (medium: broth, pH 7.3).

<table>
<thead>
<tr>
<th>No.</th>
<th>Substance</th>
<th>Gram-positive organisms</th>
<th>Gram-negative organisms</th>
<th>Partition coefficient (oleyl alcohol/water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oxine</td>
<td>25,600</td>
<td>12,800</td>
<td>25,600</td>
</tr>
<tr>
<td></td>
<td>Mono-aza-oxines :</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2-Aza-oxine</td>
<td>1,600</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>3</td>
<td>3-Aza-oxine</td>
<td>3,200</td>
<td>1,000</td>
<td>1,600</td>
</tr>
<tr>
<td>4</td>
<td>4-Aza-oxine</td>
<td>3,200</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>5</td>
<td>6-Aza-oxine</td>
<td>1,600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7-Aza-oxine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5-Aza-oxine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poly-aza-oxines :</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4:5-Diaza-oxine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4:7-Diaza-oxine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5:7-Diaza-oxine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>4:5:7-Triaza-oxine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-chelating isomerides of No. 2–4:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>4-Hydroxyquinoline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>4-Hydroxyquinazoline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>6-Hydroxyquinazoline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2-Hydroxyquinoline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>6-Hydroxyquinoline</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

— Signifies growth at M/800. It is not convenient to use solutions stronger than M/800 because of poor solubility.
In order to provide a series of mono-aza-oxines which would be more lipophilic than No. 2-4, substances were synthesized similar to these but having, in addition, a short aliphatic side-chain in order to increase the partition coefficients. As it was evident from preliminary tests that these new substances (No. 17-20) were giving results of considerable theoretical interest, special care was used in designing the biological test, the results of which are reported in Table II. All the substances mentioned in this table were tested at the same time, whereas those in Table I were done in batches of five or six (always including oxine as a control). As has been mentioned, the strain of Str. pyogenes used in this experiment was more sensitive than that used in the earlier tests; in the meantime, the Oxford staphylococcus had apparently become more resistant in that there was a greater tendency for subsequent growth to appear in tubes showing bacteriostasis at 24 hr.

Besides the new alkyl-substituted aza-oxines (No. 17-20), it was decided to include in this test not only oxine, but all the aza-oxines which had shown any antibacterial activity in Table I. It will be seen that for substances common to Tables I and II, the results for Cl. welchii agree to within one tube, which is what is to be expected for this type of test. The results with Str. pyogenes are naturally higher in Table II, but even the use of the more sensitive strain has not elicited any activity from No. 7. The results obtained with Staph. aureus lie so near the bottom of the scale, that comparison with Table I is difficult.

It is evident from Table II that the alkylated aza-oxines (No. 17-20) have higher partition coefficients than their lower homologues, No. 2 and 3. In fact in two cases the coefficients surpass that of oxine. It is also evident that the antibacterial activity of these substances surpasses that of the unsubstituted aza-oxines (No. 2-7), but even in No. 20 it remains a little short of that of oxine (i.e., by one tube for each organism).

Apart from these three Gram-positive organisms, all the substances in Table II were tested simultaneously against the three Gram-negative organisms listed in Table I. However, little or no activity was found, a feature typical of the 8-hydroxyquinolines as a class (Albert et al., 1947). It was thought, however, that more specific tests were desirable to show that the mode of action of the alkylated compounds was of the same nature as that of oxine. The results of such tests (Table III) show that the action of representative alkylated aza-oxines is biologically similar to that of oxine.

In order to discover the relative metal-binding powers of the various chelating substances in Tables I and II, potentiometric titrations were carried out in the

### Table III.—Comparison of the Bacteriostatic Properties of Oxine and Representative Alkylated Aza-oxines.

Highest dilutions (as 1/10) completely preventing visible growth of Staph. aureus at 37°C (pH 7.3) after 18 hr.

<table>
<thead>
<tr>
<th>No.</th>
<th>Substance</th>
<th>Nutrient broth (as used in Table II)</th>
<th>Without additions</th>
<th>Plus—m/50,000 FeSO₄</th>
<th>Plus—m/50,000 CoSO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oxine</td>
<td>&gt;100,000</td>
<td>1,600</td>
<td>&gt;100,000</td>
<td>25,000</td>
</tr>
<tr>
<td>18</td>
<td>4-Methyl-3-aza-oxine</td>
<td>6,400</td>
<td>&lt;1,600</td>
<td>&gt;100,000</td>
<td>25,000</td>
</tr>
<tr>
<td>20</td>
<td>4-Allyl-3-aza-oxine</td>
<td>6,400</td>
<td>1,600</td>
<td>&gt;100,000</td>
<td>25,000</td>
</tr>
</tbody>
</table>
### Table II.—Bacteriostatic Action of Oxine and Related Substances (continued).

Highest dilutions (expressed as 1/μl) completely preventing visible growth in 48 hr. at 37°C (medium: broth, pH 7·3). Partition coefficients, stability constants and \( \bar{n} \) values.

<table>
<thead>
<tr>
<th>No.</th>
<th>Substance</th>
<th>Organisms</th>
<th>Average bacteriostatic titre</th>
<th>Partition coefficient (oleyl alcohol/water)</th>
<th>Stability of 1:2 complexes (log ( K_a ))</th>
<th>Average no. of mols. bound by one metallic cation (pH 7·3 and M/5000).</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oxine</td>
<td>Cl. welchii, Str. pyogenes, Staph. aureus</td>
<td>25,600, 200,000, 6,400</td>
<td>77,000</td>
<td>67</td>
<td>33, 23, 13, 15</td>
</tr>
<tr>
<td>2</td>
<td>2-Aza-oxine</td>
<td>3,200, 12,800</td>
<td>800</td>
<td>5,600</td>
<td>6</td>
<td>26, 17, 15</td>
</tr>
<tr>
<td>3</td>
<td>3-Aza-oxine</td>
<td>1,600, 12,800</td>
<td>—</td>
<td>4,800</td>
<td>5</td>
<td>28, 17.5, 14.5</td>
</tr>
<tr>
<td>4</td>
<td>4-Aza-oxine</td>
<td>3,200, 6,400</td>
<td>—</td>
<td>3,200</td>
<td>8</td>
<td>26, b, 14.5</td>
</tr>
<tr>
<td>5</td>
<td>6-Aza-oxine</td>
<td>3,200, 1,600</td>
<td>800</td>
<td>1,000</td>
<td>1</td>
<td>26, 14, b</td>
</tr>
<tr>
<td>6</td>
<td>5-Aza-oxine</td>
<td>800</td>
<td>—</td>
<td>—</td>
<td>270</td>
<td>&lt;0·02</td>
</tr>
</tbody>
</table>

**Mono-aza-oxines:**

**Alkylated Mono-aza-oxines:**

17. 4-Methyl-2-aza-oxine | 6,500, 25,600, 6,500 | 13,000 | 16 | b, b | 15 | b | b | 1·8, b |
18. 4-Methyl-3-aza-oxine | 6,500, 51,200, 3,200 | 20,000 | 17 | b, b | 14·5 | b | b | 1·9, b |
19. 4-Propyl-3-aza-oxine | 12,800, 100,000, 1,600 | 38,000 | 135 | b, b | 14·5, b | b | b | 1·8, b |
20. 7-Allyl-3-aza-oxine | 12,800, 100,000, 3,200 | 39,000 | 310 | b, b | 14·5, a, b | b | b | 1·8, b |

---

Signifies growth at M/800.  
* a. From analogy with No. 3, 18 and 19.  
* b. Complex is too insoluble to measure.  
* c. Referred to in text as \( \bar{n} \) (the maximum possible value of \( \bar{n} \) is 2·0).
presence of the following cations: Fe\textsuperscript{+++}, Cu\textsuperscript{++}, Ni\textsuperscript{++} and Fe\textsuperscript{++} (Albert and Hampton, 1953). From these titrations, the overall stability constant \(K_s\) was calculated for each complex. This constant \(K_s\) is a measure of the mass action equilibrium between the metallic cations and the anions of the chelating substances (on the one hand) and the 1 : 2 complexes (on the other). The greater the affinity between the ions, the more stable is the complex, and the higher the \(K_s\). This overall stability constant is the product of two partial stability constants, of which \(K'\) governs the formation of the 1 : 1 complex, whereas \(K''\) governs the formation of the 1 : 2 complex from the 1 : 1 complex (in the presence of a further supply of chelating substance). Thus \(K_s = K' . K''\); hence log \(K_s = \log K' + \log K''\). (In the case of trivalent iron, \(K_s = K' . K''. \ K'''\) because a 1 : 3 complex is formed).

The reason for including nickel in these studies is as follows: In carrying out this work, precipitates were often encountered when titrating the iron and copper complexes, and this often prevented values being obtained directly for \(K_s\) (when \(K'\) is known, the overall stability constant can be derived indirectly, from the approximation: \(\log K_s = 2 \log K' - 1\)). The generally greater solubility of the nickel complexes almost always allowed complete titration curves to be obtained, so that the oxine-like character of the chelation process could be inspected. As the figures in Table II show, the constants for nickel tend to lie between those for Fe\textsuperscript{++} and Cu\textsuperscript{+}. In some cases the insolubility of the iron and copper complexes was so great that no figures for \(K'\) could be obtained: in such cases, the availability of constants for nickel provided useful information on the relative metal-binding powers of the various substances.

It was thought desirable to know not only the stability constants, but also the \(\bar{n}\) values, i.e., the average number of molecules of substance bound by one atom of metal under the experimental conditions (\(\bar{n}\) varies with \(K_s\), dilution and pH, whereas \(K_s\) is independent of \(\bar{n}\), dilution and pH). To calculate \(\bar{n}\) from \(K_s\), two new equations have now been evolved by reversing the usual derivation of \(K_s\) from experimentally determined values of \(\bar{n}\) (Albert, 1950, 1952). These equations are simplified to suit the case where, as here, a 1 : 2 ratio exists between the concentration of the metallic salt and that of the metal-binding substance. Equation (i) is for values of \(\bar{n}\) between 0 and 1; equation (ii) gives values between 1 and 2. As these are quadratic equations, two answers are obtained, one rational (lying between the above limits) and one absurd (e.g., 297). Unless (i) gives a value that is approx. 1, there is no need to solve equation (ii).

\[
\begin{align*}
\bar{n} = & \frac{3[M^0]K' + \alpha \pm \sqrt{[M^0]^2(K')^2 + 6\alpha[M^0]K' + \alpha^2}}{2[M^0]K'} \\
\bar{n} = & \frac{4[M^0]K'' + \alpha \pm \sqrt{4[M^0]K'' + \alpha}}{2[M^0]K''}
\end{align*}
\] (i) (ii)

where \([M^0]\) is the concentration of metal, free and combined, \(K'\), \(K''\) and \(\bar{n}\) have the meanings given above,

\[
\alpha = \frac{[H^+]^2}{K_aK_a'} + \frac{[H^+]}{K_a} + 1
\]

(where \(K_a\) is the higher (and \(K_a'\) the lower) acidic ionization constant).
Several \( \bar{n} \) values are included in Table II. The dilution of M/5000 has been chosen as an intermediate value within the range of limiting bacteriostatic dilutions (\( \bar{n} \) tends to decrease on dilution). It can be found, from equations (i) and (ii), that two substances may have different stability constants and still give the same value of \( \bar{n} \) under experimental conditions, provided that the substance with the smaller \( K_s \) is compensated by having a larger \( K_a \) (a larger \( K_a \) implies a smaller \( pK_s \)). Because ring-nitrogens promote the ionization of acids, it is not surprising that several of the aza-oxines are compensated in this way (Albert and Hampton, 1953).

It is evident that Table II contains seven substances which combine with metals as effectively as oxine but which have partition coefficients covering a wide range (from 5 to 310, oxine being 67). Thus it is clear that a series of substances has been synthesized having the physical properties requisite to test the hypothesis.

DISCUSSION.

The present work was undertaken to obtain evidence as to whether the action of oxine could take place inside the bacterial cell. Oxine itself is not toxic to staphylococci unless traces of iron (or copper) ions are present. The fact that the concentration of these ions relative to that of the oxine is critical, led to a postulate that the mode of action was inside the cell (Albert et al., 1953). It is desirable now to review the evidence for this postulate. The hypothesis, of which it forms an essential part, is that the 1:1 complex (XII) is the actual toxic substance (likely, because it is unsaturated), but that it cannot penetrate the cell (likely, because (XII) is ionized and hence not lipophilic); oxine enters the cell as such and as the 1:2 complex (XIII) (likely, because they are highly lipophilic); this 1:2 complex is non-toxic (likely, because it is saturated); in the absence of excess oxine inside the cell, the 1:2 complex is broken down to the toxic 1:1 complex and death results; in the presence of excess oxine, rapid death does not occur because the 1:2 complex persists in the cell and is harmless; in the presence of excess iron, rapid death does not occur because no 1:2 complex can be formed, according to the law of mass action.

FORMULAE.

\[
\begin{align*}
\text{(XII)} & \quad \begin{array}{c}
\text{O} \\
\text{Fe}\overset{2+}{}
\end{array} \\
\text{(XIII)} & \quad \begin{array}{c}
\text{O} \\
\text{Fe} \\
\text{N} \\
\text{N}
\end{array}
\end{align*}
\]

One piece of evidence was offered (by Albert et al., 1953) in support of action being confined to the inside of the cell, namely that 8-hydroxyquinoline-5-sulphonic acid, although its complexes have the same stability constants as oxine, is not antibacterial (neither this acid nor any of its complexes is liposoluble). A possible objection to this evidence is that this acid and all of its complexes
must carry a strong negative charge (on the sulphonylic acid group), and hence they do not belong to the same charge-type as oxine and its complexes. Thus they may conceivably be repelled from the surface of bacteria by a coulombic effect.

To meet this possible objection, a series of analogues of oxine has been synthesized in which lipophilic properties are progressively lowered by the insertion of extra heterocyclic nitrogen atoms; later, other substances with increased lipophilic properties have been synthesized by the insertion of small alkyl groups into these aza-oxines. Some of these new substances are even more lipophilic than oxine. These operations do not change the charge-type from that of oxine. If action takes place inside the cells, it would be expected to fall off with decreasing oil/water partition coefficients, which are believed to be a measure of the ease with which a substance penetrates the cell membrane (Davson and Danielli, 1943; Höber, 1945). Conversely, antibacterial action should be restored when the substances with low coefficients are chemically modified so that their coefficients approach that of oxine. To what extent these expectations have been realized will now be discussed.

As can be seen from Table I, the mono-aza-oxines (i.e., oxines with one extra ring-nitrogen) have various partition coefficients, all much lower than that of oxine. These substances are all much less antibacterial than oxine, and the loss of antibacterial properties is, on the whole, greatest in those with the lowest coefficients. Further, the di- and tri-aza-oxines (No. 8–11) have partition coefficients so low that they could not be measured, and they all lack antibacterial activity.

An important step in the discovery that oxine owes its antibacterial activity to chelation was the examination of the six hydroxyquinolines isomeric with oxine (Albert et al., 1947). None of these isomers has a ring-nitrogen atom in the peri position relative to the hydroxyl group: hence it cannot bind metals by chelation. It was recognized as significant that none of these isomers was antibacterial. Hence it was thought important in the present studies to examine isomers of the antibacterial mono-aza-oxines. Hence isomers (No. 12–16) of 2-, 3- and 4-aza-oxine were prepared. For the structural reasons just discussed none of these substances can chelate with metallic ions (this lack of affinity for metallic ions was verified experimentally). It is evident from Table I that none of these substances is antibacterial. Hence it seems certain that what activity the mono-aza-oxines possess is due to their chelating ability, just as with oxine.

At this stage it should be explained why oleyl alcohol was used in determining the oil/water partition coefficients. It has been shown that the distribution of a series of substances between water and various poorly miscible liquids always follows the same order, although the less water-soluble liquids give the greater spread of values (Collander, 1947). Oleyl alcohol was used in preference to vegetable oils in the present studies because it is less viscous and more readily purified. It is a representative of the fatty alcohols found in cytoplasmic membranes.

To see if raising the partition coefficient would restore antibacterial activity to the aza-oxines, some homologues were synthesized of two aza-oxines (No. 2 and 3) which had shown moderate antibacterial activity. These homologues (No. 17–20) differed only in the possession of an alkyl group containing from one to three carbon atoms. The results of this investigation are given in Table II, and the opportunity was taken to test, at the same time, all substances from Table I which had shown activity (see under “Results” for comments on the strains of organisms used).
It is evident from Table II that the device of inserting small alkyl groups has been successful in creating a range of mono-aza-oxines having increased partition coefficients (the two highest exceed that of oxine). Simultaneously, the antibacterial action has increased until it is almost as great as that of oxine (these tests are conducted with two-fold dilutions; thus No. 20 is consistently one tube less active than oxine). It is evident that the antibacterial action of oxine has been decreased, and even extinguished, by altering the molecule so as to lower the oil/water partition coefficient, and then almost completely restored by further alterations which increase the coefficient. In this connection it is interesting to recall that the antibacterial activity of oxine itself is not improved by increasing the partition coefficient which seems to be optimal (5-methyl- and 5-propyl-oxine have about the same antibacterial activity as oxine (Albert et al., 1947)).

The question now arises whether in altering the chemical structure of oxine so as to vary the partition coefficients, the metal-binding powers of oxine were also being decreased, thus introducing a second variable. Investigation of the data provided in Table II shows that this has indeed taken place in the case of No. 5 and 7, but that there are seven other substances which have metal-binding powers of the same order as those of oxine, and yet vary widely in partition coefficient and antibacterial action. These substances are No. 2, 3, 4, 17, 18, 19 and 20. The basis of comparison is the $\bar{\pi}$ value (given in the last columns of Table II), i.e., the average number of molecules of the substance bound by one atom of metal. (The special suitability of nickel as a reference metal has been discussed under "Results," where it is also shown how two substances having different values of $K_x$ can have the same value of $\bar{\pi}$).

It was important to discover whether the mode of action of the highly effective alkylated aza-oxines was of the same nature as that of oxine. Oxine has the following characteristic biological properties: it shows (i) reduced antibacterial activity in metal-depleted broth, (ii) full antibacterial activity in metal-depleted broth to which a trace of a ferrous salt has been added, and (iii) substantial antagonism of the increase described under (ii) when a cobalt salt is added at the same concentration as the iron (Albert et al., 1953). It is evident from Table III that the alkylated aza-oxines show all these effects and hence are exerting their toxic action through a mechanism similar to that of oxine.

The hypothesis outlined at the beginning of this discussion demands free access to the cell of both oxine and its 1:2 complexes. Hence not only should oxine and its analogues be lipophilic to be effective (as has been demonstrated above), but the 1:2 complexes should be lipophilic also. That they are even more lipophilic than the metal-free substances follows from theoretical grounds because the 1:2 complexes have fewer centres of hydration (the 1:1 complexes, being ions, are highly hydrated). It would be most satisfactory if partition coefficients for the 1:2 complexes could be determined experimentally, but this is difficult because the metals can only be held entirely as 1:2 complexes when an excess of the metal-free substance is present (law of mass action), and its presence would vitiate the calculations. A satisfactory compromise is to find whether the aza-oxines are more completely extracted by oil from water when metallic ions are present and if the most lipophilic aza-oxines are those most completely removed from water in this way: both of these expectations have been realized experimentally (Albert and Hampton, 1954).

The most biologically active substance to emerge from this work is 7-allyl-
3-aza-oxine (No. 20), and it was thought of interest to see whether it had chemotherapeutic powers in the mouse. The L.D$_{50}$ (subcutaneous) was found to be $>0.2$ g./kg., and hence it is considerably less toxic than oxine (0.03 g./kg., on the same strain of mice). Hence it has a higher in vitro chemotherapeutic index than oxine. However, as with oxine, no chemotherapeutic effect was demonstrable in mice (infected with Str. pyogenes, Richards strain) when 50 mg./kg. was injected with or without 5 mg./kg. of copper sulphate. Moreover its in vitro bacteriostatic effect was strongly lowered in the presence of intact red blood cells. Oxine, also, has little antibacterial action in the presence of red blood cells (Rubbo et al., 1950). It is known that the partition coefficient of oxine between red blood cells and normal saline is 7, which does not seem unduly high (Shaw, 1928). Whether these cells merely withdraw oxine (and its analogues) from circulation, or whether they chemically alter it, e.g., by conversion to glucuronate or sulphate (which would be non-chelating and hence inactive), would be information worth acquiring.

**SUMMARY.**

To obtain further evidence for the site of action of 8-hydroxyquinoline (oxine), analogues were examined having from one to three extra ring-nitrogen atoms. These substances covered a range of oil/water partition coefficients, all of which were lower than that of oxine. It was found that, as the coefficients fell, the antibacterial activity also fell, and tended to disappear.

Representative examples of these aza-oxines were then structurally modified by inserting small alkyl groups so that the partition-coefficients increased and eventually exceeded that of oxine. It was found that concomitant with this restoration of high coefficients, an antibacterial activity almost equal to that of oxine was realized.

These new substances (of which 7-allyl-3-aza-oxine is the most active) exert their antibacterial activity by the same mechanism as oxine. Like oxine, they are less active in metal-depleted broth, their activity is restored on the addition of iron, and it can then be antagonized by cobalt.

It is concluded that this new evidence provides support for the hypothesis that the site of action of oxine is inside the bacterial cell.

One of us (A. H.) expresses his thanks to the Australian National University for a Scholarship.

**REFERENCES.**


Davson, H., and Danielli, J.—(1943) 'The Permeability of Natural Membranes.' Cambridge (University Press), Table XXII, p. 108.

Höber, R.—(1945) 'Physical Chemistry of Cells and Tissues.' Philadelphia (Blakiston Co.), Chapter X.
