THE INTERACTION OF CHELATING AGENTS WITH BACTERIA

PART I. 8-HYDROXYQUINOLINE (OXINE) AND *Staphylococcus aureus*

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The interaction of solutions containing oxine or iron or both (in a 1 : 1 molar ratio) with *Staph. aureus* is examined with respect to the chelating agent and metal ion binding by the bacteria. Iron enhances the amount of oxine uptake by the organism although the presence of the chelating agent does not affect the amount of iron bound. Iron is shown to be present in the ferric state in dilute solutions containing oxine. Two types of iron receptor sites in the bacterial surface are postulated and iron is shown to mediate oxine binding by the bacteria.

THE antibacterial activity of certain compounds for example 8-hydroxyquinoline, the tetracycline antibiotics, and *iso*nicotinic acid hydrazide, has been attributed at least in part, to their ability to chelate trace metals[1]. The importance of the latter in enzyme systems, and particularly those involving the metalloflavoproteins, is being increasingly realised.

Because various hypotheses have been advanced for the mechanism of antibacterial action of chelating agents, it is of interest to provide further information concerning the interaction of such substances with bacteria in the presence and absence of trace metals.

EXPERIMENTAL

Oxine. Analar 8-hydroxyquinoline (oxine) was recrystallised from ethanol, m.p. 76° (uncorr.), Heilbron and Bunbury[2] gave 75 to 76°; log ϵ 4·50 at λ max 240 mμ in water. *Ferrous ammonium sulphate.* Solutions of the Analar reagent in water were prepared immediately before use. *Metal depletion of media.* Distilled water, obtained from an all-glass still, was used throughout this work. Normal saline: 9 g. sodium chloride (Analar) was dissolved in 250 ml. of water and 1 ml. chloroform containing 25 μg. of oxine was added. The solution was shaken vigorously and set aside one hour before extraction with chloroform (3 $\times$ 25 ml. portions). This treatment with oxine was repeated thrice. The solution was finally extracted with 6 $\times$ 25 ml. portions of chloroform to remove all traces of oxine, boiled to expel dissolved solvent, cooled and diluted to 1000 ml. with water. *Spectrophotometer.* A Hilger H 700 spectrophotometer was used in conjunction with matched fused silica cuvettes. *Organism. Staphylococcus aureus* (originally N.C.T.C. 6571) was maintained on nutrient agar slopes. Cultures were incubated for 18 to 24 hours at 37°.

Preparation of bacterial suspensions. The bacteria were harvested from slope cultures using metal-depleted normal saline; the suspension was centrifuged at 8500 *g* for 10 minutes and the cells resuspended and again washed with the same medium. The bacteria were finally suspended in

metal-depleted normal saline at a concentration of 165×10^8 organisms/ml. Routine standardisation was carried out nephelometrically.

Preparation of suspensions of heat-killed bacteria. Washed suspensions of *Staph. aureus* (approx. 165×10^8 organisms/ml.) in metal-depleted normal saline were maintained at 70° for 30 minutes. The bacteria were centrifuged, washed twice and resuspended in metal-depleted saline (165×10^8 organisms/ml.).

Preparation of suspensions of isolated cell walls. Suspensions of isolated cell walls of *Staph. aureus* were prepared following the method of Salton and Horne[3].

Preparation of iron-oxine solutions. Solutions were prepared to contain 1 μg./ml. oxine and 2·7 μg./ml. ferrous ammonium sulphate, that is, oxine and iron in a 1 : 1 molar ratio. Spectrophotometric examination of the solution (4 cm. cuvettes) revealed the presence of a single absorption peak at 241 mμ which underwent a gradual bathochromic shift to 248 mμ on standing. The intensity of the latter peak reached a steady figure after 48 hours; all oxine-iron solutions were, therefore, stored for 48 hours before use.

General Method for Drug-bacteria Contact

Solutions containing oxine or iron, or both, were introduced into glass centrifuge tubes which were immersed in a water bath maintained at 25° (±1°). The total volume was adjusted to 42 ml. with water. 3 ml. volumes of bacterial suspension were added to each solution after allowing sufficient time for temperature equilibration. The final concentration of bacteria in the test suspension was 11×10^6 organisms/ml. unless otherwise stated. After 60 minutes, unless otherwise specified, the bacteria were removed from the suspension by centrifuging twice at 8500 *g* for 10 minutes before spectrophotometric examination of the supernatant solution between 220 to 300 mμ (1 cm. cuvettes).

Determination of Unchanged Oxine in Solutions after Contact with Bacteria

The intensity of the ultra-violet absorption spectra of the reaction solutions was frequently greater than that attributable to the initial concentration of oxine. This was due to the liberation from the bacteria of cellular constituents having λ max 260 mμ; these substances will be referred to as cell exudate[4]. Solutions containing cell exudate are unaffected by extraction of the solutions with chloroform, whereas oxine is completely removed from an aqueous solution by this means. This separation may be effected quantitatively and the oxine concentration determined by subtraction of the ultra-violet absorption spectrum of the chloroform-extracted solution from that of the initial reaction solution. 20 ml. portions of the reaction solutions were extracted with chloroform (reagent grade, 5×20 ml.) and a total volume of 20 ml. of water was used for washing. The solvent was removed from the aqueous layer by boiling and the final volume was re-adjusted to 20·0 ml. after cooling.

Colorimetric Determination of Iron

Iron was determined by formation of the 3 : 1 ferrous-*o*-phenanthroline complex (log $K_s 21{\cdot}3^5$) in the presence of a reducing agent.

The concentration of iron remaining in solution after contact with suspensions of *Staph. aureus* was relatively low but the following procedure was found to give satisfactory results under these conditions. To a suitable volume of the solution was added 4·0 ml. of a 4 per cent w/v solution of hydroxylamine hydrochloride in water followed by 2·0 ml. of a 0·4 per cent w/v solution of *o*-phenanthroline in 50 per cent v/v aqueous methanol. The final volume was adjusted to 100 ml. and the optical density of the solution measured at λ max 510 mμ in 4 cm. cuvettes. Colour development was complete within a few minutes and the optical density at 510 mμ was unchanged after 24 hours at room temperature.

This method also proved satisfactory for the determination of iron *in the presence of oxine*, using an appropriate calibration curve.

Recovery of Iron from Bacterial Suspensions

Staph. aureus suspensions were exposed to solutions of ferrous ammonium sulphate (18·66 μg./ml.) for known periods of time (between 2 to 60 minutes); solutions of hydroxylamine hydrochloride and *o*-phenanthroline were then added, the final concentrations of these reagents being identical with those used for the colorimetric determination of iron. After a further 15 minutes, the bacteria were removed by centrifuging and the optical density of the supernatant solution measured at 510 mμ.

Reaction of Staph. aureus *with Solutions containing Iron-phenanthroline Complex*

The bacteria were added to solutions containing iron (2·5 to 20 μg./ml.), hydroxylamine hydrochloride and *o*-phenanthroline in the same concentrations as those used for the colorimetric determination of iron. After 15 minutes the bacteria were removed by centrifuging and the supernatant solutions were examined spectrophotometrically at 510 mμ.

RESULTS

The Uptake of Oxine by Staph. aureus

Rate of uptake. The amount of oxine bound by *Staph. aureus* from a solution containing 14·9 μg./ml. was independent of the contact time between 10 and 60 minutes. The supernatant solution contained 14·05 μg./ml.

Relationship between the uptake of oxine and its initial concentration in solution. Figure 1 shows results obtained using solutions initially containing up to 15 μg./ml. of oxine. More concentrated drug solutions were not examined because the accuracy of the results obtained would have been adversely affected. At an initial concentration of 9·33 μg./ml., 6·3 per cent of the available oxine was bound by the bacteria. The amount of oxine bound by isolated cell walls from *Staph. aureus* is also shown in Figure 1.

The Uptake of Iron by Staph. aureus

The results obtained for ferrous ammonium sulphate solutions (up to 20 μg./ml. initially)* and whole cell and isolated cell wall preparations are shown in Table I.

A very slight increase in the amount of iron bound by whole cells (maximum 3·5 per cent) from solutions initially containing 12 to 19 μg./ml. ferrous iron was observed in the presence of 1 μg./ml. of oxine. These solutions were stored before use.

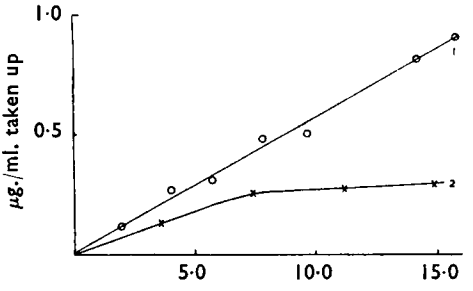


FIG. 1. Uptake of oxine by whole cells (curve 1) and isolated cell walls (curve 2) of *Staph. aureus*.

The results using a solution of ferrous ammonium sulphate (18·66 μg./ml.) and contact times of between 2 to 60 minutes (see Figure 2) indicate a rapid initial uptake followed by a relatively slow uptake of iron. No saturation point was reached within 60 minutes contact time.

TABLE I

COMPARISON OF THE AMOUNT OF IRON BOUND BY WHOLE CELLS AND ISOLATED CELL WALLS OF *Staph. aureus*

μg./ml. available	μg./ml. taken up by			Percentage uptake (compared with whole, viable cells) by	
	Whole viable cells	Heat-killed cells	Isolated cell walls	Heat-killed cells	Isolated cell walls
4·66	4·49	3·39	2·58	75·5	57·6
9·33	8·80	6·41	4·33	73·0	49·2
14·0	12·66	9·20	5·60	72·6	44·3
18·66	16·41	11·83	6·39	72·1	39·0

Recovery of Iron from Staph. aureus *Suspensions*

The concentrations recoverable after 2 to 60 minutes contact time, under conditions comparable to those used in the previous section (see Fig. 2) decreased with increasing time of contact and also decreased at a much faster rate than that at which iron was taken up by the organisms. Table II presents the results for a fixed contact time (60 minutes) and ferrous ammonium sulphate solutions initially containing 2 to 19 μg./ml.

The Reaction of Staph. aureus *with Solutions containing Iron and Oxine (1 : 1 Molar Ratio)*

After removal of the bacteria from the contact suspension (λ max of the initial solution 248 mμ), the supernatant solution (curve 2 of Fig. 3) contained a component having an absorption spectrum (λ max 240 mμ) resembling that of oxine. This solution, after addition of iron and 24

* The iron concentrations are expressed as ferrous ammonium sulphate throughout this paper.

hours storage again showed an absorption maximum at 248 mμ. The amount of oxine bound (0·933 μg./ml. available) in the presence of iron was 0·373 μg./ml. or 40 per cent of that available. The time taken for the bacteria to effect the hypsochromic shift of the iron-oxine solution (248 to 240 mμ) was 15 minutes; the absorption peak shifted to 240 to 241 mμ within 5 minutes.

TABLE II

IRON BINDING BY *Staph. aureus* IN THE ABSENCE OF OXINE

Iron available μg./ml.	Iron recovered μg./ml. (*a*)	Iron* bound as complex μg./ml. (*b*)	Sum of (*a*) and (*b*)	Iron non-recoverable μg./ml.
2·33	0·8	0·33	1·13	1·20
4·66	1·4	0·66	2·06	2·60
7·0	2·15	0·96	3·11	3·89
9·33	3·2	1·33	4·53	4·80
11·66	5·23	1·55	6·78	4·88
14·0	6·78	1·90	8·68	5·32
16·33	8·28	2·23	10·51	5·82
18·66	10·0	2·46	12·46	6·20

* Ferrous-*o*-phenanthroline complex.

Reaction of Staph. aureus *with Solutions containing Iron and* o-*Phenanthroline*

The results are presented in Table II. This Table shows the corresponding amounts of iron which could be recovered with *o*-phenanthroline at the same initial concentration levels of iron.

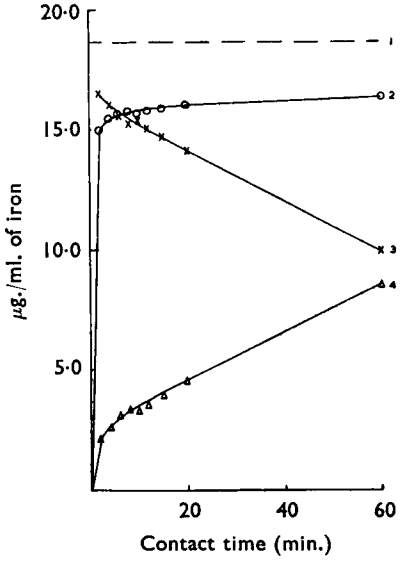


FIG. 2. Interaction of iron with *Staph. aureus*.
Curve 1, the amount of iron available.
Curve 2, the uptake of iron.
Curve 3, the recovery of iron using *o*-phenanthroline.
Curve 4, the amount of iron non-recoverable from the bacteria.

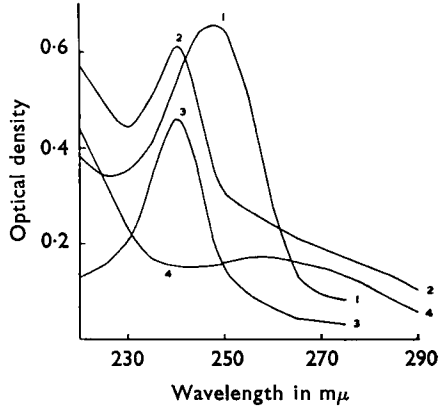


FIG. 3. Ultra-violet absorption curves of solutions containing iron and oxine (1:1 molar ratio) before and after contact with *Staph. aureus*.
Curve 1, reference solution.
Curve 2, the supernatant solution after removal of the bacteria from the contact suspension.
Curve 3, curve 2 corrected for the presence of cell exudate (curve 4).

Experiments with Heat-killed Bacteria

Reaction with iron-oxine solution. The absorption spectrum of the supernatant solution, corrected for the presence of exudate, showed a peak at 248 mμ. The amount of oxine bound accounted for 54 per cent of the initial concentration.

Rate of uptake of iron. In contrast with the results obtained with viable organisms, iron uptake by heat-killed cells was complete after 3 minutes contact time.

Uptake of iron in presence and absence of oxine. There was very little difference in the amount of iron bound by heat-killed cells in the presence and absence of oxine, as was also the case using viable cells. However, more iron was bound by living organisms, as may be seen in Table II.

Recovery of iron with o-phenanthroline. After 2 minutes contact time, 9·9 per cent of the iron available (18·66 μg./ml.) could not be recovered from the bacteria with *o*-phenanthroline. This figure increased to only 11·6 per cent after 60 minutes contact with heat-killed bacteria.

Discussion

Analytical Methods

In related unpublished studies with several antibacterial drugs and cell exudate from different bacteria, it has been shown that the method used in the present work effects the quantitative separation of the drug from the exudate. The method has the advantage that the spectrophotometric identity of the components may be verified.

State of Oxidation of Iron in Iron-Oxine Solutions

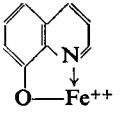
The iron-oxine solutions were prepared from fresh solutions of ferrous ammonium sulphate in water. The ultra-violet absorption spectrum of the iron-oxine solution (1 : 1 molar ratio) underwent a gradual bathochromic shift on standing which could be attributed to (*a*) slow chelation, (*b*) slow oxidation of the ferrous iron and subsequent chelation with oxine or (*c*) formation of a ferrous-oxine chelate and subsequent oxidation of the metal ion. The location of the absorption peak observed on addition of iron, in the ferric state, to a solution of oxine was 248 mμ; thus, *the iron-oxine solutions examined contained ferric iron.* Colorimetric checks using *o*-phenanthroline in the absence of a reducing agent demonstrated the disappearance of ferrous iron in the presence of oxine. (A ferrous salt was used to prepare the solutions because it is easier to handle in neutral aqueous solutions than a ferric salt.)

The valency state of the iron offered to the bacteria appears to be unimportant, as the extent of iron binding was identical whether ferrous (ferrous ammonium sulphate) or ferric (ferrous ammonium sulphate and oxine) iron solutions were used. Further, the actual valency state of the iron bound at the bacterial surface is not known and cannot be inferred from a knowledge of the valency state of iron in the bulk phase.

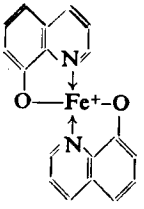
Ferric Iron-Oxine Complexes

The relative proportion of the complex(es) present in the iron-oxine solutions (1 : 1 molar ratio) used in the present work is not yet clarified.

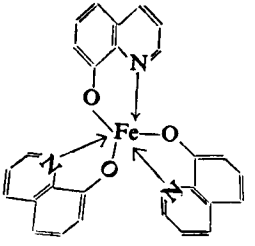
Three are possible, namely, the charged 1:1 (I) and 1:2 (II) and the electrochemically neutral 1:3 (III) complexes.



(I)



(II)



(III)

Oxine Binding by Staph. aureus

Relatively little oxine is bound by the bacteria in the absence of added heavy metal ions. Possibly, the binding observed is due to residual traces of heavy metals in the system. The uptake of the chelating agent from solution is rapid and is complete within 10 minutes of addition of the bacteria. Secondly (see Fig. 1), less than half the amount of oxine bound by whole cells is taken up by isolated cell walls. Standardisation of cell wall preparations, in terms of the number of organisms from which they were derived, is difficult and losses inevitably occur during isolation. Further, almost double the surface area may be made available for binding on separation from the intact bacteria. Therefore, the percentage of oxine bound by isolated cell walls may bear little relation to the amount bound by the same structure in viable bacteria. Probably, the drug is bound preferentially by the cytoplasmic membrane, at least in the living organism.

Iron Binding by Staph. aureus

The relationship between the uptake of iron and the time of contact with *Staph. aureus* indicates at least two mechanisms of binding. Most of the iron was bound within 10 minutes of contact with the bacteria but no saturation was observed after a further 50 minutes. The sum of the amount of iron recovered from the cells and the amount of iron bound as the ferrous-*o*-phenanthroline complex (see Table II) does not correspond with the total iron bound by *Staph. aureus*. Possibly iron gradually penetrates the bacterial cell, or alternatively, some molecular rearrangement occurs at the surface of the cell which results in an increase in the iron binding forces. It is significant that oxine does not affect the final extent of iron binding (see later under mechanism of antibacterial action).

The percentages of iron bound by isolated cell walls of *Staph. aureus* in relation to those observed for the intact organisms (cf. Table II) are subject to the same comments as those made in the case of oxine.

Oxine Binding in the Presence of Iron

The ultra-violet absorption curves presented in Figure 3, indicate that the bacteria remove iron from its complex with oxine in aqueous solution. This reaction was rapid, being completed within 15 minutes. Thus some component of the bacterial surface (cytoplasmic membrane) was capable of binding iron *more firmly* than oxine under the conditions of these experiments. *However, the presence of iron considerably increased the amount of oxine bound by* Staph. aureus from 6 to 40 per cent of the total available oxine; in the presence of a ten molar excess of iron, the increase was only to 15 per cent of the total available oxine.

When iron was presented to *Staph. aureus* as the ferrous-*o*-phenanthroline complex, the bacteria did not bind the metal preferentially. Either the stability constant of the complex is too great or the bacteria actually bind iron in the ferric state only; alternatively steric factors may prevent any interaction of the complexed metal ion with the receptor sites of the bacterial surface.

Results Using Heat-killed Bacteria

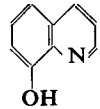
Heat-killed bacteria appear to be incapable of removing iron from its complex with oxine and in fact the complex itself is bound by the cells. The amount of iron bound by heat-killed cells is also reduced compared with viable cells and uptake is complete within 10 minutes. Further, the amount of iron recoverable from the cells with *o*-phenanthroline was virtually the same after initial contact times of 2 to 60 minutes. Only about 10 per cent of the iron available to the bacteria could not be recovered by this method.

General Discussion

Two types of iron binding sites are postulated at the cytoplasmic membrane of *Staph. aureus* to account for the results described, the first capable of loosely binding iron by ionic forces ("anionic receptor sites") and the second capable of firmer binding, possibly involving chelation ("iron chelating sites"). Similar sites may also be present in isolated cell walls but as the site of toxic action of oxine-metal complexes is probably the cytoplasmic membrane, the discussion will be restricted to this structure.

The "anionic receptor site" is capable of binding hydrated positively charged ferrous ions and charged ferric iron-oxine complexes (I and II), because the amount of iron bound remains unchanged in the presence of oxine and yet the oxine uptake increases in the presence of iron.

The limited extent of oxine binding in the absence of added heavy metal ions may be due to either the lack of a formal charge on the oxine molecule



(IV)

(cf. the difference between IV and I) reducing the possibility of binding to anionic (or cationic) sites in the cell surface, or alternatively to the lack

of a heavy metal ion to mediate binding. If the latter were true, no oxine would be bound in the complete absence of heavy metal ions but there is no known method for removing every trace of such metals from a medium.

As the charged iron-oxine complex is capable of binding to the "anionic receptor site," it is reasonable to assume iron mediation of oxine binding by formation of a ternary oxine-metal-receptor complex. Such a concept accounts for the observed increase in oxine binding on addition of metal ions. The work of Klotz and his associates[6] on the facilitated binding of dye molecules to proteins in the presence of metal ions may be cited in support of this hypothesis.

The decreased uptake of oxine on addition of increasing molar proportions of iron indicates competition between the hydrated metal ions and the charged metal-oxine complexes (I and II) for the "anionic receptor sites." Possibly, van der Waals' forces between the aromatic nucleus and complementary receptor surface may reinforce the ionic attractive forces, since not all the oxine is displaced by a large molar excess of iron.

The presence of a second type of iron binding receptor, probably of chelating character, is postulated for three reasons.

Firstly, the bacteria can compete successfully for oxine chelated iron, stability constants log K_1 12·3, log K_2 11·3 and log K_3 10·3[7], under the conditions employed.

The alternative explanation, that free iron penetrates the surface, thus being removed from the equilibrium with oxine, is precluded since only slow penetration of the bacterial surface occurs with free iron and bacteria rapidly remove iron from its complex with oxine. Probably the iron-oxine complex is attracted to the surface where iron is bound at the "iron chelating sites" and oxine liberated into solution.

Secondly, even after short periods of iron-bacteria contact, a proportion of the iron cannot be recovered from the bacteria with *o*-phenanthroline (log K_s 21·3[5]).

Thirdly, heat-killed bacteria bind less iron than viable cells and nearly all may be recovered with *o*-phenanthroline. These bacteria are no longer capable of removing iron from its complex with oxine, probably due to modification of the "iron chelating sites." If these "iron chelating sites" are anionic in character, heating apparently only destroys their chelating ability, since binding of 5-aminoacridine, as a cation, to the same organism is unaffected by similar treatment[8].

That chelation of trace heavy metals, iron and copper, is implicated in the antibacterial action of oxine was demonstrated when the seven other position isomers of oxine were found to be inactive and oxine itself was devoid of activity in heavy metal depleted media[9]. Since the actual antibacterial activity of iron and oxine apparently varies with their molar ratios, it has been suggested that an equilibrium exists between the lipophilic 1:2 (*ferrous*-oxine) and the hydrophilic 1:1 (*ferrous*-oxine) complexes and that, whereas the former facilitates membrane penetration, the latter is the toxic entity. Recently[1], the charged 1:1 (I) and 1:2 (II) and the electrochemically neutral 1:3 (III) *ferric* iron-oxine complexes

have been considered in this respect. The ability to form a lipophilic complex is considered a necessary property if an oxine derivative is to show significant antibacterial activity (cf. work with azaoxines[7,10,11]); an intracellular site of action was postulated.

If a 1 : 3 iron-oxine complex, because of its lipid solubility, penetrated the bacterial cell from a 1 : 1 molar iron-oxine solution, one would expect liberation of *free iron* into the biophase unless the iron were also held at a site in the cell wall or cytoplasmic membrane. The fact that *free oxine* is speedily liberated into the biophase upon presentation of the above solution to the bacteria indicates that at least a gross migration of the 1 : 3 complex across a lipoid boundary has not occurred. The possibility of the chelate acting at the cytoplasmic membrane must, therefore, be considered. Addition of iron does not reduce the bactericidal activity of an iron-oxine solution (1 : 1 molar ratio) under conditions identical to those described for the uptake measurements (unpublished work). Because we have shown that oxine is not implicated in the uptake of iron, whereas iron is involved in the uptake of oxine, it seems possible, contrary to current views, that the antibacterial activity of oxine in the presence but not the absence of iron is due to the latter constituting a bridge to bind the drug to an important site in the cytoplasmic membrane. The iron-oxine complex may be regarded as interacting in an enzyme system in a manner analogous to the metal of a metalloflavoprotein and its substrate reacting with the rest of the flavoprotein; such an interaction could inhibit the normal processes of electron transfer with consequent impairment of enzyme function. The oxine inhibitor derived from erythrocytes (erythrochelatin[12,13]) probably protects bacteria against this inhibition of enzyme function since it has been demonstrated not to interfere with iron-oxine chelation.

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DISCUSSION

The Paper was presented by DR. A. E. ROBINSON.

THE CHAIRMAN. Were the bacteriostatic or bactericidal concentrations of oxine diminished in the presence of iron? What was the appropriate

calibration curve used for the determination of iron in the presence of oxine and how was it prepared? In Fig. 2 what does 'available' iron mean?

MR. G. SYKES (Nottingham). Excessive concentrations of certain metals may turn the metallic compound from a nutrient to an antangonist. Was an extra oxidation-reduction system being introduced? It seemed unnecessary at present to consider the idea of "anionic receptor sites" and "iron chelating sites". He thought that the latter was possibly an enzyme or a unit of an enzyme. Care must be exercised in contrasting the activities of live and heat-killed organisms.

DR. L. SAUNDERS (London). Was the assumption that the exudate gave no chloroform-soluble extractive justified? Had the use of chelating ion exchange resins which could effectively remove traces of copper from solution been considered?

MR. H. D. C. RAPSON (Betchworth). Was the uptake of oxine fast or slow?

DR. A. M. COOK (London). Did the excess iron have any effect on the bactericidal activity of the oxine-iron complex? Had the use of *Strep. faecalis* been considered where the iron-containing enzymes were probably not so important as in the very aerobic *Staph. aureus*?

DR. ROBINSON replied. The use of bacteriostatic tests had been deliberately avoided since metal ions were present and the results would not be significant in relation to the uptake work. Preliminary results showed that iron had a very marked effect on the bactericidal activity of iron-oxine solutions. The appropriate curve for the determination of iron in the presence of oxine meant that allowance had been made for the slight deviation from the calibration curve for iron and *o*-phenanthroline observed in the presence of oxine. The "available" iron in Fig. 2 was the amount added initially. The "iron chelating sites" might form an integral part of an enzyme system, but the Authors preferred to use a general, rather than a specific, term at this stage. Structural changes undoubtedly occurred in the cytoplasmic membrane on heat-killing bacteria but work with 5-aminoacridine had indicated that an anionic centre survived the treatment. She was satisfied that cell exudate could be separated from the unchanged drug by the method used. Other methods for removal of trace metals had not been tried. The uptake of oxine was complete within 10 minutes. Bactericidal evaluation of the system was not yet complete but the addition of further iron to an iron-oxine solution could alter the death time of the bacteria in that solution from 130 to 45 minutes. Complete data was not yet available for any other organism.