IRON-CONTAINING ANTIBIOTICS AND MICROBIC GROWTH FACTORS

V. PRELOG

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In the course of our systematic studies of the metabolic products of actinomycetes carried out in collaboration with the Institute of Special Botany of the ETH and with the Research Laboratories of the Pharmaceutical Department of Ciba Ltd. in Basel[1], we have encountered a group of highly active, iron-containing antibiotics which were named ferrimycins[2]. Upon testing the activity of the culture filtrates and of the extracts containing these antibiotics erratic results were often obtained. This led eventually to the discovery that actinomycetes also produce iron-containing factors antagonistic to the ferrimycins[3]. With the help of a simple and specific antagonism test, the detection and determination and, ultimately, the isolation of the antagonists was made possible. Moreover, the isolated iron-containing factors could in turn be used in the same test for the detection and determination of the ferrimycins and related antibiotics[1, 3].

The antagonism test is carried out in the following way: a filter paper strip impregnated with the antibiotic is placed at right angles on a second filter paper strip impregnated with the antagonist and both are then disposed on an agar dish. After inoculation with a suitable micro-organism and incubation, the action of the antagonist causes a wedge of microbic growth to appear in the germ-free zone produced by the antibiotic. Under standardized conditions, the size of the wedge can be used for the quantitative determination of the antagonistic factors (*Figure 1*).

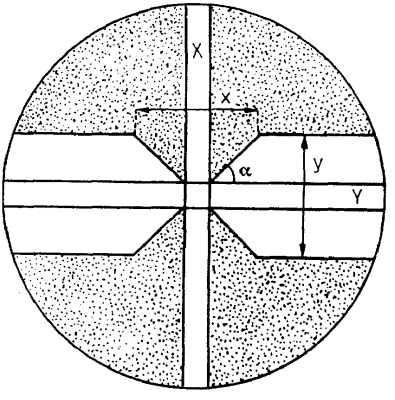

Figure 1. Antagonism test

At the time when the antagonism between iron-containing antibiotics and antagonistic factors was discovered, a number of iron-containing microbic growth-factors such as ferrichrome[4], coprogen[5] and terregens factor[6], had already been described, and it was easily shown that these factors also are active against the ferrimycins in the antagonism test. It was also found that the antagonists of the ferrimycins isolated by us acted as growth-factors for several heterotrophic micro-organisms used for testing substances for growth-promoting activity. The result of such a growth-promoting test with *Microbacterium lacticum* ATCC 8181 is shown in *Figure 2*. Other iron-containing antibiotics were also known for some time, *e.g.* grisein[7] and the albomycins[8]. These can replace the ferrimycins in the antagonism test.

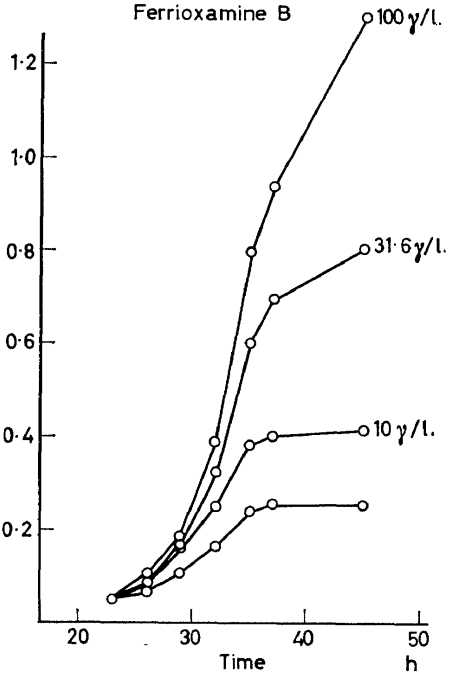

Figure 2. Growth promotion test with *Microbacterium lacticum*

As could be shown by numerous experiments, the observed antagonism is a case of biological competition of the iron-containing factors essential for the growth of the micro-organism by the iron-containing antibiotics. The antagonistic action is not due, however, to a chemical reaction between the two groups of compounds, as the chemically and biologically unchanged antibiotic and antagonist can be separated easily by electrophoresis from a biologically inactive mixture. With the help of the antagonism test it was soon found that iron-containing growth factors and antibiotics are widely distributed in micro-organisms. Several of these compounds were isolated in the pure state and in many cases their structure could be completely, or at least partially, established. Some have recently also been prepared by synthesis. The red-brown iron-containing metabolites with a characteristic

absorption band at 420–440 mμ have been given the general name siderochromes. Siderochromes with growth-promoting properties are called sideramines, those with antibiotic properties sideromycins[2]. The siderochromes isolated and characterized up to now are listed in *Figure 3.*

	Sideramines	*Sideromycins*
Ferrichrome	Ferrioxamine A	Grisein
Coprogen	Ferrioxamine B	Albomycin
Terregens factor	Ferrioxamine C	
	Ferrioxamine D_1	Ferrimycin A_1
	Ferrioxamine D_2	Ferrimycin A_2
	Ferrioxamine E	Ferrimycin B
	Ferrioxamine F	ETH 22765
	Ferrioxamine G	
		LA 5352
	Ferrichrysin	LA 5937
	Ferriocrocin	
	Ferrirhodin	
	Ferrirubin	

Figure 3. Siderochromes

The fact that iron-free organic compounds easily convertible into siderochromes by addition of iron(III) ions can be obtained in high yield in cultures containing only small amounts of iron greatly facilitated the task of preparing them in larger quantities by microbiological methods. The isolation of siderochromes was largely based on the methods used in the isolation of the cobalt complexes of the vitamin B_{12} group.

The greater part of my paper will be devoted to the sideramines, the group which has been best studied so far.

The cultures of actinomycetes yield several closely related sideramines, which we have called ferrioxamines[2]. At present, eight representatives of the ferrioxamines are known. They are the ferrioxamines A, B, C, D_1, D_2, E, F and G, which are mostly produced as mixtures by the same micro-organism. Ferrioxamine B or E are the main components of such mixtures[9].

The structures of the ferrioxamines B, D_1, E and G have been established fully, the one of ferrioxamine A is partially known. In all five cases the compounds are iron(III) complexes of organic trihydroxamates. The iron can be removed easily as iron(III) chloride by extraction with ether from a solution of the ferrioxamine in concentrated hydrochloric acid, or as iron(III) hydroxide by precipitation with alkali or, finally, as insoluble oxinate upon addition of 8-hydroxyquinoline. The organic desferri compounds can then be isolated as colourless substances.

It was observed at an early date that the hydroxylamine derivatives formed upon hydrolysis of the ferrioxamines and related compounds react with the iron(III) ions, yielding complex mixtures of oxidation and reduction products as shown in *Figure 4* for 1-amino-5-hydroxylaminopentane[10]. The mixture obtained upon hydrolysis thus contains many artefacts and the interpretation of the results is difficult. It is, therefore, preferable to use as starting materials for the hydrolytic degradations desferri compounds carefully freed of iron(III) ions.

As an example, the structure elucidation[10] of ferrioxamine B, the biologically most active sideramine of this series, will be discussed.

Ferrioxamine B and its desferri derivative are monoacidic nitrogen bases. The energetic hydrolysis of desferri-ferrioxamine B yields approximately three moles of 1-amino-5-hydroxylaminopentane[11], two moles of succinic acid and one mole of acetic acid. As shown in *Figure 5*, the desferri compounds can be derived formally from the hydrolysis products by elimination of five

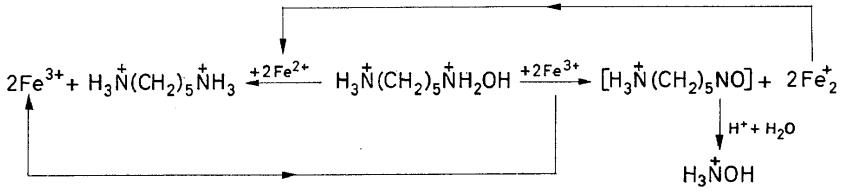

Figure 4. Reactions of 1-amino-5-hydroxylaminopentane with Fe^{3+} and Fe^{2+}

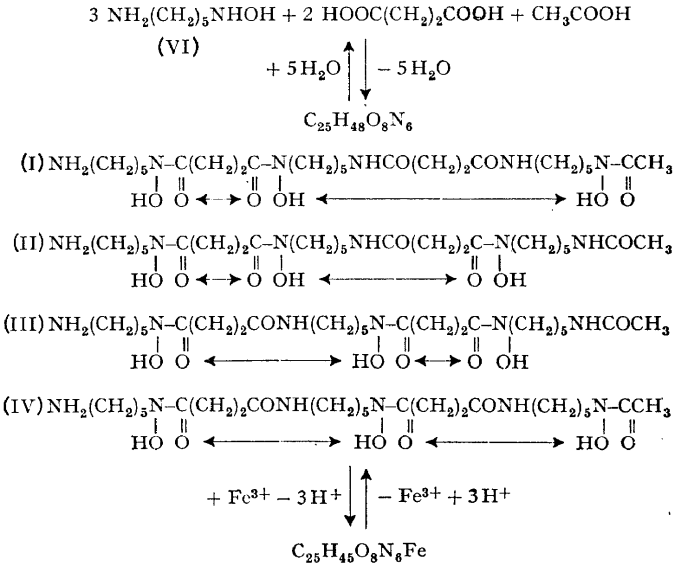

Figure 5. Determination of the structure of ferrioxamine B

moles of water and consequent disappearance of five basic and five acid functions. The remaining basic group must be an amino and not a hydroxylamino group as shown by its pK^{*}_{MCS} of 9·7 (ref. 28). Therefore, in the condensation process three hydroxamic acid and two amide groups must be formed. Formally, this can take place in four different ways. The magnetic moment of ferrioxamine B, and the very large stability constant ($>10^{30}$), prove that ferrioxamine B is a very stable octahedral ionic iron (III) complex. Hence it must be assumed that the steric conditions are particularly favourable to the formation of such a complex. A study of molecular models shows that this is the case for only one of the four possible combinations, namely for the one in which there are no short chains between the hydroxamic acid groups. All other physical and chemical properties of ferrioxamine B are in

best agreement with the structural formula deduced in this way and shown in *Figure 6*. It was confirmed, as will be shown later, by an unambiguous synthesis.

The structure of four further ferrioxamines D_1[12], E[13] and G[14] was established in a similar way by determination of their genuine hydrolysis products and their neutral, basic or amphoteric character. Thus, neutral ferrioxamine D_1 yields three moles of 1-amino-5-hydroxylaminopentane, two moles of

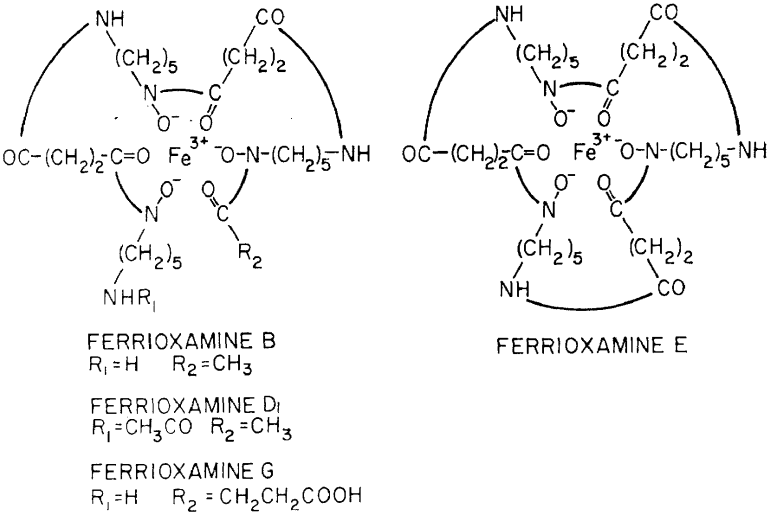

Figure 6. Structures of ferrioxamines

succinic acid and two moles of acetic acid upon hydrolysis. Ferrioxamine D_1 obviously must be the *N*-acetyl derivative of ferrioxamine B, a deduction which is confirmed by its formation upon mild acetylation of the latter compound. From ferrioxamine G, an amphoteric compound, there were obtained upon hydrolysis three moles of 1-amino-5-hydroxylaminopentane and three moles of succinic acid, but no acetic acid. From these results one may conclude that ferrioxamine G has the structure shown in *Figure 6*. Desferri-ferrioxamine E, finally, a neutral compound, yields the same hydrolysis products as desferri-ferrioxamine G, but contains one mole of water less. Consequently, it must be a cyclic compound with a thirty-three-membered ring. This conclusion was proved by its partial synthesis upon treatment of ferrioxamine G with dicyclohexylcarbodiimide[14] (*Figure 7*).

It should be mentioned that desferri-ferrioxamine E is identical with nocardamine, isolated by other authors[15] from cultures of a Nocardia species. Its correct structure was derived in our laboratory on the basis of the results mentioned above.

The structure of ferrioxamine A has not yet been fully established. The compound is a monoacidic base, which yields two moles of 1-amino-5-hydroxylaminopentane, one mole of 1-amino-4-hydroxylaminobutane, two moles of succinic acid and one mole of acetic acid upon hydrolysis. It may, therefore, be assumed to be analogous to ferrioxamine B and to contain

one mole of 1-amino-5-hydroxylaminobutane in place of one mole of the next homologue[16]. We plan to establish the sequence of the components by synthesis.

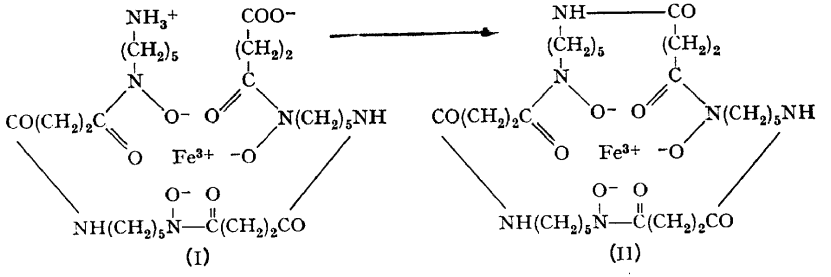

Figure 7. Partial synthesis of ferrioxamine E

For the synthesis of the ferrioxamines the following reactions represented schematically in *Figure 8* are of fundamental importance.

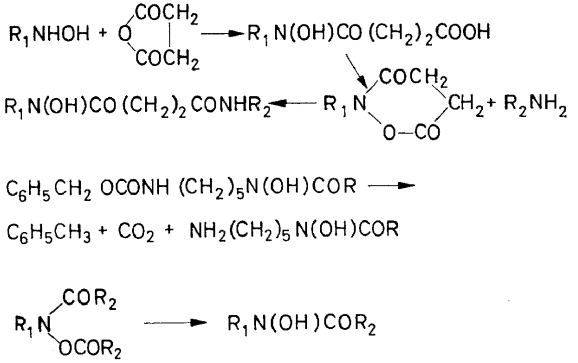

Figure 8. Reactions used for syntheses of ferrioxamines

(*i*) The *N*-substituted hydroxylamines, upon treatment with succinic anhydride and pyridine and subsequent cyclization with dicyclohexylcarbodiimide or thionylchloride yield 3,6-dioxotetrahydro-oxazine derivatives, which react with primary amines under mild conditions to form succinic monoamide monohydroxamic acids.

(*ii*) The carbobenzoxy derivatives, containing hydroxamic acid groups, can be de-carbobenzoxylated under mild conditions by catalytic hydrogenation without reduction of the hydroxamic acid groups.

(*iii*) The *O*-acyl-hydroxamic acids can be saponified by mild alkaline hydrolysis or ammonolysis, without suffering attack on the hydroxamic acid groups.

The multi-step syntheses of ferrioxamines B[17] and G[18] based on these reactions are summarized in *Figures 9* and *10*, respectively.

After the structure of ferrioxamine B had been established, Emery and Neilands[19] published their work on ferrichrome and ferrichrome A, the latter an inactive siderochrome, and advanced proposals for their structure. The results of the American authors show that the two compounds are, as

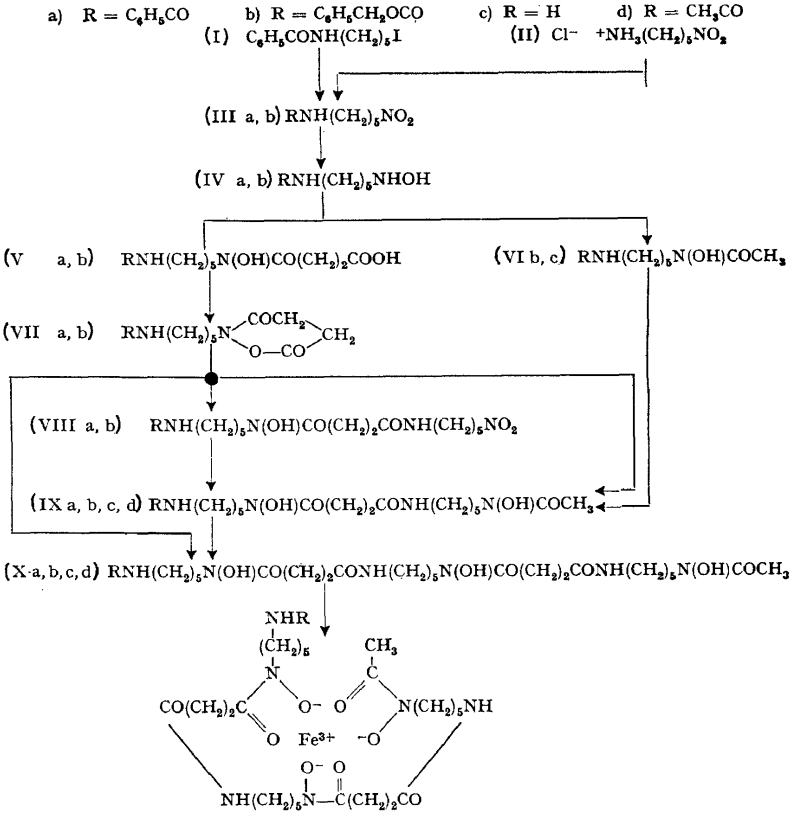

Figure 9. Syntheses of ferrioxamines B and D_1

a) R = $C_6H_5CH_2OCO$ b) R = H c) R = CH_3CO

(I) $RNH(CH_2)_5N$ (COCH$_2$, CH$_2$, O—CO)

(II a, b) $RHN(CH_2)_5N(OH)CO(CH_2)_2COOCH_3$

(III a, b) $RNH(CH_2)_5N(OH)CO(CH_2)_2CONH(CH_2)_5N(OH)CO(CH_2)_2COOCH_3$

(IV a, b, c)
$RNH(CH_2)_5N(OH)CO(CH_2)_2CONH(CH_2)_5N(OH)CO(CH_2)_2CONH(CH_2)_5N(OH)CO\,(CH_2)_2COOCH_3$

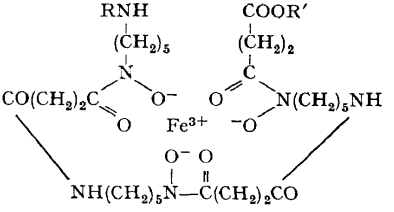

Figure 10. Synthesis of ferrioxamine G

ours, iron(III) trihydroxamate complexes. The iron-free trihydroxamic acid, however, differs considerably from those found in the ferrioxamines. The desferri-ferrichromes are cyclohexapeptides built up of three L-δ-*N*-acyloxy-ornithines and three other amino-acids. In ferrichrome the acyl group is acetyl and the amino acid glycine (three moles); in ferrichrome A the acyl group is β-methyl-glutaconyl—an interesting discovery—and the amino-acids are L-serine (two moles) and glycine (one mole). Obviously, despite the similarity in biological activity, the components and their combination in the ferrichromes are quite different from the ones of the ferrioxamines.

In a systematic search by the antagonism test our research group has succeeded in isolating from various Penicillium, Aspergillus and Paecilomyces species several other compounds related to the ferrichromes. Four of these, namely ferrichrysin, ferricrocin, ferrirhodin and ferrirubin, have been studied in closer detail[20]. All of these sideramines are iron(III) trihydroxamate complexes of cyclohexapeptides consisting of three moles of L- δ-*N*-acyloxy-ornithine and three other amino-acids. As in the case of the ferrioxamines, the iron-free trihydroxamic acids can be prepared easily and these in turn by mild acid hydrolysis can be de-acylated. Such a reaction sequence is shown in *Figure 11* for ferrichrysin. Treatment of desferri-ferrichrysin with

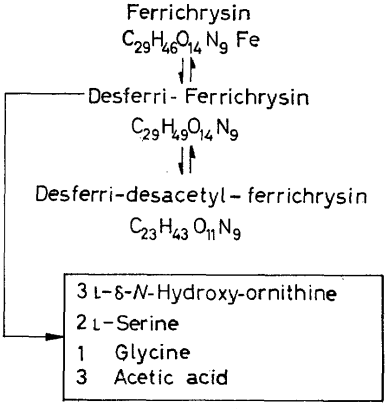

Figure 11. Determination of the structure of ferrichrysin

iron(III) salts regenerates the original complex, and desferri-desacetyl-ferrichrysin upon mild acetylation is reconverted into desferri-ferrichrysin. The components of the cyclohexapeptide can best be obtained by hydrolysis of desferri-ferrichrysin followed by reduction of the unstable L-δ-*N*-hydroxy-ornithine to ornithine. The routine methods of peptide chemistry were then employed in the isolation and identification of the amino-acids. Their sequence in the cyclohexapeptide has not been determined so far. We assume—in contrast with Emery and Neilands[19]—that the L-δ-*N*-acyloxy-ornithine units are distributed regularly in the cyclohexapeptide. The structures of the sideramines of the ferrichrome group derived on the basis of this assumption are shown in *Figures 12* and *13* which clearly depict the common general structure and the differences of the single components. The

acyl group of ferrirhodin deserves special mention. It is a 5-hydroxy-3-methylpent-2-enoic acid residue formally derivable from mevalonic acid by dehydration or from β-methylglutaconic acid (which occurs in ferrichrome A), by reduction of a carboxyl group. The biochemical significance of the presence of these compounds, whose importance for the biosynthesis of terpenoids is well known, is not clear, but their occurrence is certainly most remarkable.

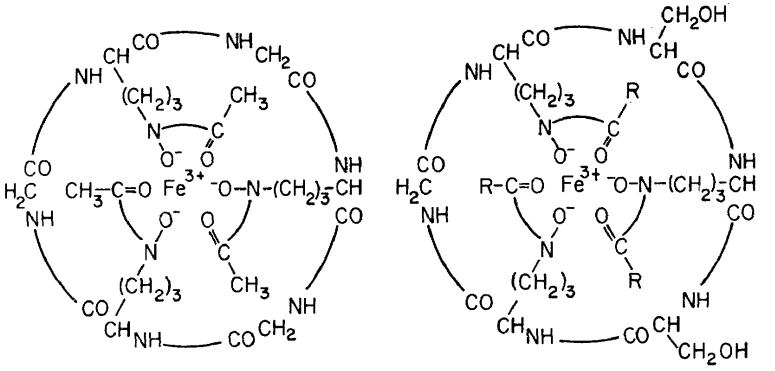

Figure 12. Structures of ferrichromes

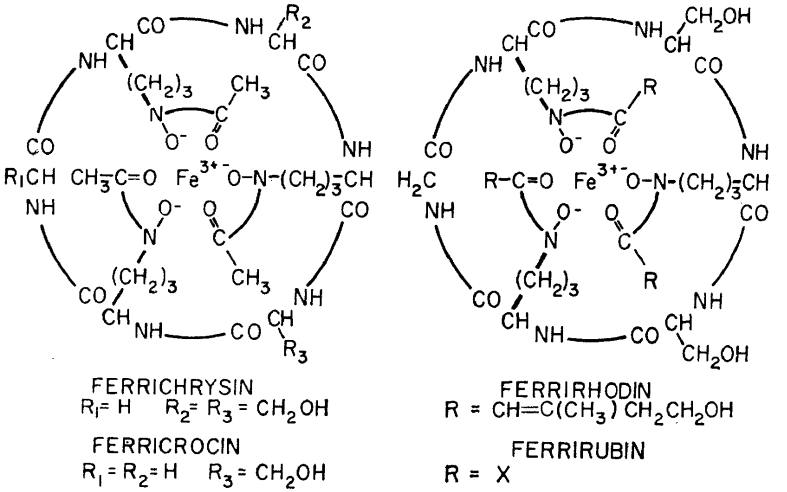

Figure 13. Structures of some sideramines from fungi

The high growth-promoting properties of the sideramines lead one to expect that they play an important biological rôle[21] the nature of which is not yet known with certainty. Most of the experimental results available so far have been achieved in a study of the biochemical function of ferrichrome, the longest known sideramine. Several laboratories are engaged at present in the study of the biochemistry of the ferrioxamines.

For some time it had been believed that the sideramines are iron transport factors having the task of conveying the iron(III) ions through certain cell membranes. More recent work appears to indicate that they are coenzymes. Experiments with intact and homogenized Arthrobacter JG-9—a micro-organism which requires haemin to synthesize its porphyrin enzymes (*e.g.* catalase)—have shown that less than 0·01 mole of ferrichrome can replace one mole of haemin and may be considered as an indication that ferrichrome is the coenzyme of an enzyme which transfers iron to proto-haemin. Ferrimycin blocks this activity of ferrichrome but is without effect on haemin. It is interesting to mention that not only in Arthrobacter JG-9 but also in all other sideramines demanding heterotrophic micro-organisms large amounts of haemin can replace sideramines.

Of the chemical properties of the desferri-ferrioxamines the most remarkable is their ability to bind iron(III) specifically, giving rise to very stable complexes. A comparison of the complex stability constants of desferri-ferrioxamine B and of EDTA for various biochemically important ions is shown in *Figure 14* as a striking illustration of this property[22].

	Desferri-ferrioxamine	EDTA
	log *K*	log *K*
Fe^{3+}	30·6	25·1
Ca^{2+}	2·5	10·6
Co^{2+}	10·3	16·1
Zn^{2+}	11·1	16·1
Cu^{2+}	14·1	18·3

Figure 14. Complex stability constants

The high and specific capacity to bind iron in a stable complex and the low toxicity of desferri-ferrioxamine B has led to its use in the treatment of pathological iron accumulations in the body, with a view to the removal of deposits of iron in the form of water-soluble stable complexes which can be eliminated through the kidneys. Such pathological iron deposits in organs occur, for instance, in primary and secondary haemochromatoses and sideroses. The clinical results in pathological cases of these types, which usually take a lethal course, appear to be promising.

The chemistry of the sideromycins is far less advanced than the one of the sideramines, because these compounds are unstable substances of difficult purification. The following results, which have been achieved with ferrimycin A, the main component of the ferrimycin mixture, appear, however, significant enough to justify their mention.

Ferrimycin A is a diacidic base with pK^{*}_{MCS} values of 4·1 and 7·9 differing considerably from the one of the basic ferrioxamines[23]. The iron-free component of ferrimycin A can be obtained by treatment of the antibiotic with 8-hydroxyquinoline. Addition of iron(III) salts reconverts the desferri compound into the original, fully active complex (*Figure 15*)[24]. This regeneration is of particular interest, because treatment of ferrimycin A with 2M sodium acetate at room temperature for 24 hours leads to complete loss of antibiotic activity and formation of a sideramine, which we have named

sideramine Z. Acid hydrolysis of desferri-ferrimycin A yields, among other compounds, three moles of 1-amino-5-hydroxylaminopentane, two (or possibly three) moles of succinic acid and one mole of acetic acid. In other words, the same components found in ferrioxamine B occur in ferrimycin A[25]. The rest of the constituents, which are responsible for the antibiotic activity, is under active investigation in our laboratory.

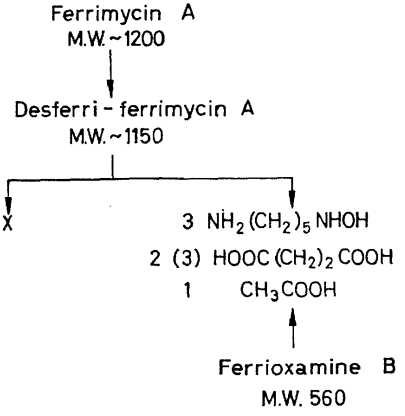

Figure 15. Partial structure of ferrimycin A

The partial similarity of structure of ferrimycin A and ferrioxamine B, as well as the ease with which the former is converted in a sideramine, accounts for the rapid development of resistance to the antibiotic by microorganisms. It should be pointed out in this connection that several microorganisms transform ferrimycin A in a sideramine which is not identical with sideramine Z.

The fast rise of resistant strains hinders the practical use of the ferrimycins, despite their high activity against Gram-positive pathogenic microorganisms, which sometimes exceeds considerably the one of penicillin. An analogous property of producing rapid resistance is also characteristic of the two oldest representatives of the sideromycin group, grisein and albomycin, which might be identical, or at least very closely related[26]. The latest results indicate that albomycin is closer in chemical structure to the sideramines of the ferrichrome group than to those of the ferrioxamine type[27].

I should like to express my gratitude to my colleagues Dr W. Keller-Schierlein and Dr H. Zähner in Zürich, Dr H. Bickel and Dr E. Vischer in Basle as well as to the many co-workers mentioned in the references.

Finally, I should like to thank Dr B. G. Engel for translating the German manuscript.

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