# Development of Iron Chelators for Cooley's Anemia

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(Received May 21, 1987)

## Abstract

The rationale employed in the design of new chelating ligands for strong binding of Fe(III) at physiological pH for the treatment of iron overload disease such as Cooley's anemia is explained and the preparation and evaluation of forty new iron(III) chelators are described. The new ligands investigated include the following classifications: five analogs of ethylenediaminetetraacetic acid (EDTA) with amino and carboxylate donor groups; ten ligands with phenolic (or potential phenolic) donor groups in addition to amino and carboxylate donors: five ligands containing phenolic groups substituted on pyridine rings in addition to amino and carboxylate donors; six aminophosphonic acid or ester groups with additional phenolate and amino donors; eight macrocyclic polyamines containing auxiliary carboxylate and/or phenolate donor groups; three trishydroxamic acids; two triscatechols; and one multidentate ligand with coordinating amide groups. These chelating agents were administered to male $BDF_1$ hybrid mice overloaded with iron by blood transfusion. Fecal and urinary elimination of iron were measured, as well as the retention of iron in the spleen and liver. The results are compared with the action of desferriferrioxamine B (DFB). Potencies relative to a Desferal® dose of 250 mg/kg tested simultaneously and assigned a potency of 1.0 are reported, and relative toxicities (*LD*50s and toxic signs) are also described. Nine of the ligands tested showed sufficient potency to warrant further development as iron chelating drugs, while six of them have potencies comparable to or greater than that of DFB.

## Introduction

### *Cooley's Anemia*

The iron-loading anemias, in particular β-thalassemia (Cooley's anemia) constitute a major health problem throughout the Mediterranean region, the Middle East, India and Southeast Asia. This disease is characterized by progressive iron accumulation resulting from increased iron absorption, or from repeated blood transfusion therapy, or from both. Treatment by venesection generally is not feasible because of the associated anemia, and the only method of preventing iron-loading, or removal of iron once it has accumulated in the body, is specific iron chelation therapy.

The iron chelating drug that has provided the best treatment thus far is desferriferrioxamine B (Desferal®)†, DFB, a naturally-occurring sideramine, and is now the reference drug to which new potential iron chelating drugs are compared. Its selectivity for $Fe^{3+}$ is excellent, and when available compounds of Fe(III) are present, forms the Fe(III) chelate to the virtual exclusion of biologically important trace metal ions. The iron(III)–DFB chelate clears rapidly from the plasma with a half time of 1–2 h.

In spite of its apparent success, DFB must be given peritoneally because it is ineffective when administered by the oral route. It is metabolized in the plasma and other tissues so that to be effective it must be administered slowly, over a period of time, which results in severe problems in administration to children, who constitute nearly all of the patients with this disease. For further information on iron overload resulting from Cooley's anemia and its treatment with chelating agents the reader is referred to recent publications on this subject [1, 2].

This paper is concerned with the synthesis and testing with experimental animals of a series of new iron chelators as candidates for the treatment of Cooley's anemia. The chelating agents described contain a wide variety of functional groups, and therefore probably have considerable differences in solubility, membrane permeability, biodistribution, and bioavailability. Also of considerable interest is the approach described in this paper to the molecular design and synthesis of chelating agents that are

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†Desferal® is the copyrighted name employed by the supplier (Ciba) for the methanesulfonate salt of DFB.

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selective for iron(III), and form chelates with sufficient thermodynamic stability to prevent transfer of the iron to apotransferrin or apoferritin or other metal ion receptors present in the body.

*General Principles of Ligand Design*

The principles involved in designing new chelating agents for strong complexing of iron(III) in aqueous solution at neutral and high pH were described in considerable detail in review papers by Pitt and Martell [3] and Martell [4]. Briefly the plan was to design molecules containing six or more 'hard' donor groups of the type that have high iron(III) affinity, arranged so that they may bind a single metal ion simultaneously with a minimum of steric restraints and coulombic repulsions. Larger assemblies of donor groups that may bind more than one ferric ion in the same molecule are not precluded. Because $Fe^{3+}$ is a 'hard' metal ion, it is most strongly complexed by small negative ions such as alkoxide or aryloxide groups. It is of course very strongly bound by hydroxide ion, a tendency that must be overcome by the donor groups of the ligand to prevent precipitation of ferric hydroxide. The following are the most effective donor groups for coordination of the ferric ion.

*Unidentate Groups*

*Negative donors*

$RO^-$ > $RCH{=}CH{-}O^-$ > $C_6H_5{-}O^-$ > $R{-}P(O)(O^-)_2$ > $ROP(O)(O^-)_2$ >

$RC(O){-}O^-$ > $R_2C{=}N{-}O$ > $RCO\bar{N}{-}R'$ > $R{-}S^-$

*Neutral donors*

$R_3N$ > imidazole > pyridine > $R_2O$ > $R_2S$

*Bidentate combinations*

catecholate ($C_6H_4(O^-)_2$) > $R{-}CH(O^-){-}COO^-$ > $R{-}C(=O){-}N(O^-){-}R'$

$R{-}C(=O){-}CH{=}C(O^-){-}R$ > $R{-}C(=O){-}N(R){-}\bar{N}{-}R''$

There are many other combinations of donor groups, including mixtures of aromatic and aliphatic derivatives, that form highly stable iron(III) chelates.

The molecular design necessary to arrange these donor groups in favorable positions is a complex process which must take into account the chelate, macrocyclic, and cryptate effects. Neutralization of ionic charge is a favorable factor. Thus some negative charge on the ligand stabilizes the complex, but the accumulation of excess negative charge, greater than −3, around the central metal ion decreases the stability of the resulting chelate. In aqueous solution competition between the ferric ion and hydrogen ion for the ligand donor groups is an important and ever present consideration. Thus ligands with very high metal ion affinities (e.g., catecholate$^{2-}$) may lose much of their effectiveness because of very high protonation tendencies as measured by their p*K* values. For the polycatecholate ligands developed by Raymond *et al.* [5] part of the adverse effect of high basicity was mitigated by the substitution of electron-withdrawing carbonyl functional groups adjacent to the benzene rings.

The individual coordinating tendencies of monodentate and bidentate functional groups are greatly amplified by developing structures that take the fullest possible advantage of the chelate effect. This requires that the ligands be sexadentate, as compact as possible, with minimization of any steric effects that would prevent the donor atoms from occupying the preferred octahedral coordination sites around the Fe(III) ion. The design of chelating agents satisfying these requirements is a complicated process, given the large number of donor groups available, and the restrictions imposed by the practical requirement that convenient synthetic methods must be available for tying the donor groups together in a molecular framework. Frequently synthetic requirements and molecular design require the use of donor groups that are not particularly effective for Fe(III). Thus aliphatic nitrogen donors have low affinity for Fe(III), but are incorporated into chelating agents such as ethylenediaminetetraacetic acid (EDTA) and *N,N'*-bis-(*o*-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED) in order to provide the chain branching needed for the attachment of other more effective donor groups.

In many cases the donors have steric requirements that impose restrictions on molecular design of the ligand. Thus the bidentate pairs of oxygen donors in hydroxamic acids and catechols must be symmetrically bound to the iron(III) in order to be fully effective.

[Structures: hydroxamate chelate R–C(=O)–N(R)–O⁻ bound to $Fe^{3+}$; tetra-R-substituted catecholate bound to $Fe^{3+}$]

Because of the trigonal nature of the bridging atoms, they must be built into the chelate structure through remote positions indicated by R.

The highly-touted macrocyclic and cryptate effects may be useful in further amplifying the stabilities of the iron(III) complexes beyond what may be achieved by the chelate effect, but the use of such structures involves difficulties in synthesis, especially because of the exacting steric requirements of obtaining a close fit between the donor groups of the macrocycle (or macrobicycle) and the metal ion.

The so-called macrocyclic ligands synthesized by Raymond *et al.* [5], and those reported in this research (Table IE, below) have donor groups exocyclic to the macrocyclic ring, and hence do not employ the ring structures for enhancement of stability and selectivity. In these ligands the macrocyclic rings merely provide a framework on which to hang the donor groups.

With endocyclic donor groups such as those containing iron(III)–specific hydroxamate and catecholate functions, as suggested some time ago [4], full advantage may be taken of the additional stabilization provided by the macrocyclic rings, if upon complex formation the size of the rings and spacing of the donor groups are adjusted so as to closely fit six donor oxygens into the coordination sphere of the Fe(III) ion. In other words the cavity must be adjusted to the correct size and shape. Very little work has been completed in this area, but some new synthetic attempts are in progress. Recent publications include the synthesis of endocyclic triscatechols [6], endocyclic bishydroxamates [7], and biscatechols [8]. These interesting ligands have yet to be submitted for animal tests for iron overload.

## Experimental

### *Synthetic Methods*

#### *EDTA analogs, Table IA*

EDTA and its analogs listed in Table IA are available from commercial sources. The higher molecular weight ligands, though not commercial materials, are known compounds, for which synthetic methods have been described in the literature. Complete bibliographies for these compounds may be found in a single series of general references [9].

Conversions of these ligands to the salts and calcium chelates illustrated in Table IA were accomplished by standard laboratory procedures for conversion of organic ligands to their metal complexes.

#### *HBED analogs, Table IB*

HBED and analogous ligands (HDTT) were prepared by methods described in the literature [9]. HBEDDA and its conversion to HBED have been described elsewhere [10]. PGHC and TGTC were obtained commercially as a mixture (W. R. Grace and Co., Hampshire Division) and were separated and purified by the method described by Yoshida *et al.* [11]. The syntheses of the remaining HBED analogs are described below. Esters of these compounds were prepared by standard laboratory procedures. The synthesis of PLED was improved following the work described in this paper, and will be published later [12].

#### *HBED pyridoxyl analogs, Table IC*

These ligands were prepared by Schiff base formation between pyridoxal and the appropriate amine, followed by reduction and alkylation with a haloacetic acid. Improvements in the synthesis are being developed and will be published later.

#### *HBED phosphonic acid analogs, Table ID*

The syntheses of the phosphonic acid esters and half esters in Table ID are described below. The free phosphonic acid $Ca_2Na_2$SHEMP was synthesized by the same method as that employed for the tetraester HBP4E, except that phosphorous acid was employed in place of triethylphosphite.

#### *Macrocyclic ligands, Table IE and catechol derivatives, Table IG*

These ligands were prepared by alkylation of the corresponding polyamines. The work was carried out by Sala in this laboratory, and will be described in a separate publication [13].

#### *Hydroxamic acids, Table IF*

The hydroxamic acids investigated, BAMTPH, BTAMGH, BZCO3MHA, were synthesized by Dr. I. Murase of Kyushu University in collaboration with this research group. The synthesis of BAMTPH has been reported [14]. Synthesis of BTAMGH is described below. BZCO3MHA was synthesized by the same procedure as that employed for BAMTPH except for the following two modifications: ethyl 4-aminobutyrate instead of 3-aminopropionate; and *N*-methylhydroxylamine instead of hydroxylamine. The colorless hygroscopic oil could not be entirely freed from solvent water, however its identity was checked by NMR, by its reaction with $Fe^{3+}$, and by potentiometric titration.

#### *Ligand with coordinating amide groups, Table IH*

The synthesis and purification of this ligand are described in a paper reporting the stabilities of its metal chelates [15].

TABLE I. Summary of Chelating Agents Submitted for Testing

| Name, abbreviation | Graphic and empirical formulas | $-\log[Fe^{3+}]$, (pM) at p[H] 7.4 |
|---|---|---|
| **IA EDTA Analogs** | | |
| Calcium trisodium diethylenetriaminepenta-acetate, $CaNa_3DTPA$ | $C_{14}H_{18}O_{10}N_3Na_3Ca$ | 23.8 |
| Dicalcium disodium triethylenetetraamine-hexaacetate, $Ca_2Na_2TTHA$ | $C_{18}H_{24}O_{12}Na_2Ca_2$ | 22.0 |
| Dicalcium trisodium tetraethylenepentamineheptaacetate, $Ca_2Na_3TPHA$ | $C_{22}H_{30}N_5O_{14}Na_3Ca_2$ | 22[a] |
| Dicalcium tetrasodiumpentaethylenehexamineoctaacetate, $Ca_2Na_4PHOA$ | $C_{26}H_{36}N_6O_{16}$ | 21[a] |
| 1,4-Bis(2',5',5'-tricarboxymethyl-2',5'-diazapentyl)benzene, PXED3A | $C_{24}H_{32}N_4O_{12}\cdot 4HCl$ | 21.6 |
| **IB HBED Analogs** | | |
| *N,N''*-bis-*o*-hydroxybenzyldiethylenetriamine-*N,N',N''*-triacetic acid dihydrochloride dihydrate, HDTT | $C_{24}H_{31}N_3O_8\cdot 2HCl\cdot 2H_2O$ | 24[a] |
| *N,N'*-bis(*o*-hydroxybenzyl)ethylenediamine-*N,N'*-diacetamide, HBEDDA | $C_{20}H_{26}N_4O_4$ | 30.0[b] |

*(continued)*

TABLE I. *(continued)*

| Name, abbreviation | Graphic and empirical formulas | $-\log[Fe^{3+}]$, (pM) at p[H] 7.4 |
|---|---|---|
| Bis[3-(2-hydroxy-5-methyl-benzylaminomethyl)-2-hydroxy-5-methylbenzyl]amine-*N*,*N*′,*N*″-triacetic acid, TGTC | $C_{40}H_{47}N_3O_{10} \cdot 3NaCl \cdot 3H_2O$ | 26.2 |
| Bis(3-[3′-(2″-hydroxy-5″-methylbenzylamino-methyl)-2′-hydroxy-5′-methylbenzylamino-methyl]-2-hydroxy-5-methylbenzyl)amine-*N*,*N*′,-*N*″,*N*‴,*N*″″-pentaacetic acid, PGHC | $C_{62}H_{73}N_5O_{16} \cdot HCl \cdot 4H_2O$ | 26.5 |
| *N*-(2-hydroxy-5-methylbenzyl)iminodiacetic acid, CMIDA | $C_{12}H_{15}O_5N \cdot 2H_2O$ | 17[a] |
| 2,6-Bis(aminomethyl)-4-methylphenol-*N*,*N*,*N*′,*N*″-tetraacetic acid, PC2IDA | $C_{17}H_{22}N_2O_9$ | 18[a] |
| *N*-(2-hydroxybenzyl)ethylenediamine-*N*,*N*′,*N*″-triacetic acid, HBED3A | $C_{15}H_{20}N_2O_7$ | 25[a] |
| *N*-(2-methoxybenzyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid, MBED3A | $C_{16}H_{22}N_2O_7$ | 25.1[b] |
| Triethyl *N*-(2-hydroxybenzyl)ethylenediamine-*N*,*N*′,*N*′-triacetate, HBED3A3Et | $C_{21}H_{32}N_2O_7$ | 25.1 |
| *N*,*N*′,*N*″-tris-(hydroxybenzyl)-1,5,9-triazanonane trihydrochloride, 3HBDP | $C_{27}H_{35}N_3O_3 \cdot 3HCl$ | 18.1[a] |

*(continued)*

TABLE I. *(continued)*

| Name, abbreviation | Graphic and empirical formulas | $-\log[Fe^{3+}]$, (pM) at p[H] 7.4 |
|---|---|---|
| IC HBED Pyridoxyl analogs | | |
| *N,N'*-bispyridoxylethylenediamine-*N,N'*-diacetic acid trihydrochloride, PLED | $C_{22}H_{30}N_4O_8 \cdot 3.6HCl \cdot 2NH_4X \cdot 4H_2O$ | 28.0 |
| *N,N'*-bispyridoxyldiethylenetriamine-*N,N'*(*N"*)-diacetic acid dihydrochloride dihydrobromide hydrate, BPDIENDA | $\cdot 2HCl \cdot 2HBr \cdot H_2O$<br>$C_{24}H_{35}N_5O_8 \cdot 2HCl \cdot 2HBr \cdot H_2O$ | 32.6[a] |
| *N,N'*-bipyridyoxylethylenediamine-*N,N'*-diacetic acid trihydrochloride, $PLEDNH_4$ | $C_{22}H_{30}N_4O_8 \cdot 3.6HCl \cdot 2NH_4X \cdot 4H_2O$ | 28.0 |
| *N,N'*-bipyridoxylethylenediamine-*N,N'*-diacetic acid dihydrochloride, PLEDHCl | $\cdot 2HCl$<br>$C_{22}H_{32}N_4O_8Cl_2$ | 28.0 |
| *N,N*-bis(pyridoxyl)glycine, BPG | $C_{18}H_{23}N_3O_6 \cdot 3HCl$ | 22[a] |
| ID Phosphonic acid HBED analogs | | |
| Calcium disodium chelate of bis-(*p*-sulfo-*o*-hydroxybenzyl)ethylenediamine-bis-(methylenephosphonate), $Ca_2Na_2SHEMP$ | $\cdot 2Na^+$<br>$C_{18}H_{22}N_2S_2P_2O_{14}Na_2Ca$ | >27[a] |
| Tetraethyl *N,N'*-bis(2-hydroxybenzyl)-ethylenediamine-*N,N'*-bis(methylene-phosphonate), HBP4E | $C_{26}H_{42}O_8N_2P_2$ | >27[a, b] |

*(continued)*

TABLE I. *(continued)*

| Name, abbreviation | Graphic and empirical formulas | $-\log[Fe^{3+}]$, (pM) at p[H] 7.4 |
|---|---|---|
| Bis-2-hydroxybenzylethylenediamine-dimethylenephosphonic acid trisodium salt, HBEDPO | $C_{18}H_{23}O_8N_2P_2Na_3$ | >27[a] |
| Diethylene $N,N'$-bis(2-hydroxybenzyl)-ethylenediamine-$N,N'$-bis(methylene-phosphonate), HBP2E | $C_{22}H_{34}O_8N_2P_2$ | 191[a] >27[b] |
| Tetramethyl $N,N'$-bis(2-hydroxybenzyl)-ethylenediamine-$N,N'$-bis(methylene-phosphonate), HBP4Me | $C_{23}H_{40}N_2O_{10}P_2$ | >27[a, b] |
| Dimethyl $N,N'$-bis(2-hydroxybenzyl)-ethylenediamine-$N,N'$-bis(methylene-phosphonate), HBP2Me | $C_{20}H_{30}N_2P_2O_8$ | 19.0 >27[a, b] |
| IE Macrocyclic ligands (exocyclic donors) | | |
| 1,4,8,11-tetraazacyclotetradecane-$N,N',N'',N'''$-tetraacetic acid tetrakis hydrochloride CYTA | $C_{18}H_{36}N_4O_8Cl_4$ | 23[a] |
| $N,N',N'',N'''$-tetra-(2-hydroxybenzyl)-1,4,8,11-tetraazacyclotetradecane, 4HBCY | $C_{30}H_{48}N_4O_4$ | 23[a] |
| $N,N',N''$-tri-(2-hydroxybenzyl)-1,4,8,11-tetraazacyclotetradecane, 3HBCY | $C_{31}H_{42}N_4O_3 \cdot 4HBr \cdot NaBr \cdot 3NaCl \cdot 4H_2O$ | 22[a] |

*(continued)*

TABLE I. *(continued)*

| Name, abbreviation | Graphic and empirical formulas | $-\log[Fe^{3+}]$, (pM) at p[H] 7.4 |
|---|---|---|
| *N,N',N'',N'''*-bis(2-hydroxybenzyl)-bis(acetic acid(1,4,8,11-tetraazacyclotetradecane, CY2BZ2AC | $R_1 = -CH_2-C_6H_4OH$; $R_2 = -CH_2COOH$; $C_{28}H_{40}N_4O_6 \cdot 4HBr \cdot 10H_2O$ | 23[a] |
| *N,N',N'',N'''*-tetrabis(2-hydroxy-5-sulphonobenzyl)-1,4,8,11-tetraazacyclotetradecane tetra sodium salt, 4SHBCY | $R = -CH_2-C_6H_3(OH)SO_3^-Na^+$; $C_{38}H_{44}N_4S_4O_{16}Na_4$ | 19[a] |
| 1,4,7,10,13,16-hexa(2-hydroxybenzyl)-1,4,7,10,13,16-hexaazacyclooctadecane, 6HBN6C18 | $R = -CH_2-C_6H_4OH$; $C_{54}H_{66}N_6O_6$ | 23[a] |
| 1,4,7,10,13,16-hexa-(2-hydroxy-5-sulphobenzyl)-1,4,7,10,13,16-hexaazacyclo-octadecane hexasodium salt, 6SHBN6C18 | $R = -CH_2-C_6H_3(OH)SO_3^-Na^+$; $C_{54}H_{60}N_6O_{24}Na_6$ | 25[a] |
| 1,4,7,10,13,16-hexaazacyclooctadecane-*N,N',N'',N''',N'''',N'''''*-hexaacetic acid hexahydrochloride, N6C18AC6 | $C_{24}H_{48}Cl_6N_6O_{12}$ | 21[a] |
| IF Hydroxamic acids | | |
| 1,3,5-benzenetricarboxamide-*N,N',N''*-tris(3-propriohydroxamic acid), BAMTPH | CONHCH$_2$CH$_2$CONHOH (×3 on benzene); $C_{18}H_{24}N_6O_9$ | 20.6 |
| 1,3,5-benzenetri(acetamidoglycylhydroxamic acid), BTAMGH | CH$_2$CONHCH$_2$CONHOH (×3 on benzene) · 1/2 H$_2$O; $C_{18}H_{24}N_6O_9 \cdot 1/2H_2O$ | 25[a] |

*(continued)*

TABLE I. *(continued)*

| Name, abbreviation | Graphic and empirical formulas | $-\log[Fe^{3+}]$, (pM) at p[H] 7.4 |
|---|---|---|
| 1,3,5-benzenetricarboxamide-*N*,*N*′,*N*″-tris(4-butyro-*N*‴-methylhydroxamic acid), BZCO3MHA | $C_{24}H_{36}N_6O_9$ | 25[a] |
| DesferriferrioxamineB (Desferal®), DFB | $C_{25}H_{48}N_6O_8$ | 25.6 |
| IG Catechol derivatives | | |
| *N*,*N*′,*N*″-tris-(2,3-dihydroxybenzoyl)-1,5,9-triazadecane, DP3DHBO | $C_{27}H_{29}N_3O_9$ | 28[a] |
| Trisodium-*N*,*N*′,*N*″-tris(2,3-dihydroxy-5-sulphobenzoyl)-1,5,9-triazanonane, DP3SDHBO | $C_{27}H_{26}N_3O_{18}S_3Na_3$ | 28[a] |
| IH Ligand with coordinating amide groups | | |
| *N*,*N*′-bis(2-aminoethyl)oxalamide-*N*″,*N*″,*N*‴,*N*‴-tetraacetic acid, BAOTA | $C_{14}H_{22}O_{10}N_4$ | 20[a] |

[a]Calculated from estimated log *K* and p*K*s based on molecular structure, number and arrangement of functional groups, and analogy with ligands for which stability constants have been measured. [b]The pM value is based on the assumption that amide and ester groups would be hydrolyzed *in vivo*. Error estimates for pM values are ±0.05 where stability constants are known; for pM values indicated by [a], errors are estimated as ± 1 log unit.

## *Detailed Synthetic Procedures*

### *N,N′,N″-Tris(ethoxycarbonylmethyl)-1,3,5-benzenetriacetamide*

1,3,5-Benzenetriacetic acid chloride (6.2 g, *ca.* 0.02 mol) in dry ether (100 ml) was added dropwise to an ether solution (100 ml) of ethyl glycinate (14.4 g, 0.14 mol) with vigorous stirring at 2–4 °C. The mixture was further stirred at room temperature for 1 h. The resulting precipitates were collected by filtration, washed with water to remove ethyl glycinate hydrochloride, and recrystallized from meth-

anol. Yield 8.7 g (87%), melting point (m.p.) 154–155 °C. The sample was further recrystallized from methanol, m.p. 155–157 °C. IR (KBr) 3280 (s, NH), 1750 (s, ester C=O), 1650 (s, amide I), 1540 $cm^{-1}$ (s, amide II). *Anal.* Calc. for $C_{24}H_{33}N_3O_9$: C, 56.78; H, 6.57; N, 8.28. Found: C, 56.30; H, 6.60; N, 8.34%.

*N,N',N"-Tris(N-hydroxycarbamoylmethyl)-1,3,5-benzenetriacetamide, BTAMGH*

An alkaline solution of hydroxylamine was prepared by mixing hydroxylamine hydrochloride (2 g, 0.03 mol) in methanol (20 ml) with potassium hydroxide (3.3 g, 0.05 mol) in methanol (10 ml) at the temperature below 30 °C followed by filtering off the resulting potassium chloride. To the filtrate was added a methanol solution (50 ml) of the ester described above (2.5 g, 0.005 mol) and the mixture was stirred for 3 days at room temperature. The solvent was distilled off by means of a rotary evaporator and the oily residue was dissolved in water (70 ml). The aqueous solution was passed through a column of a cation exchange resin (Amberlite 120, H form, 50 ml) and eluted with water. The combined eluate was concentrated to near dryness under reduced pressure and a large amount of ethanol was added to the residue. The resulting sticky mass solidified on standing 2 days at room temperature. It was dried under vacuum over $P_2O_5$. Yield 1.9 g (81%). It did not show a sharp melting point and started to decompose around 127–130 °C. IR (KBr) 1640 (s, broad, amide I), 1535 $cm^{-1}$ (s, broad, amide II). *Anal.* Calc. for $C_{18}H_{24}N_6O_9 \cdot \frac{1}{2}H_2O$: C, 45.27; H, 5.29; N, 17.60. Found: C, 45.24; H, 5.23; N, 17.11%.

*2,6-Bis(aminomethyl)p-cresol-N,N,N',N'-tetraacetic acid, PC2IDA*

This ligand was prepared by a modification of the method of Schwarzenbach *et al.* [16]. *p*-Cresol (10.87 g, 0.1 mol) was dissolved in 100 ml methanol in a 3-neck flask, and 42 g (0.21 mol) of the disodium salt of iminodiacetic acid was added. To the resulting turbid reaction mixture 2/3 of a sample of formaldehyde solution (25 g, 36%, 0.3 mol) was added through a dropping funnel and the solution was refluxed under nitrogen for two hours. The remaining formaldehyde was then added, and refluxing was continued for two more hours. Solvent was then stripped off and 400 ml of ethanol was added to the residue and the reaction mixture was stirred overnight. About 60 g of the crude sodium salt was collected.

The product (15 g) was dissolved in hot water, concentrated HCl was added to make the solution strongly acidic, and it was then passed through the acid form of a cation exchange column. The eluate was concentrated and the desired product was dissolved in alcohol and precipitated with ether. About 7.0 g of the product was recovered (yield 72%).

*2-Aminomethyl-p-cresol-N,N-diacetic acid, CMIDA*

The procedure employed for the synthesis of CMIDA is identical to the one employed for the synthesis of PC2IDA, with the exception that the Mannich reaction was allowed to reflux half as long. The poor yield (*ca.* 45%) may be improved by repeating the reaction with a smaller amount of iminodiacetic acid. The product was identified and distinguished from PC2IDA by NMR: m.p. 212–215 °C (dec.); potentiometric p*K*s 2.28, 8.02, >11; $M_r$ calc. 289; found 292.

*N-2-Hydroxybenzylethylenediamine, HBEN*

Salicylaldehyde (13.2 g) and 10.2 g *N*-acetylethylenediamine were mixed in 100 ml methanol and heated for 15 min. The solution was hydrogenated with a Pd/C catalyst, the catalyst was filtered off, the solution was concentrated under reduced pressure and dissolved in 150 ml 6 M HCl. After the reaction mixture was refluxed for 2 days solvent was removed and a colorless solid appeared when 200 ml of ethanol was added to the solution. The crude product was isolated as the dihydrochloride and was used for the next step directly.

*N-2-Hydroxybenzylethylenediamine-N,N',N'-triacetic acid, HBED3A*

A 24 g sample of HBEN and 42 g bromoacetic acid were dissolved in 200 ml of water, and the solution was made strongly alkaline with approximately 50 ml of 10 M NaOH which was added dropwise. After 3 h heating at 50 °C, the solvent was stripped off and the sodium bromide was filtered off. The filtrate was acidified and then passed through a cation exchange column ($H^+$ form). The strong acid fraction was discarded. The eluates were concentrated and evaporated to almost dryness (yield 70%). The HBED3A was crystallized from isopropyl alcohol and was identified by NMR; potentiometric p*K*s 2.97, 5.38, 9.46, 11.04; $M_r$ calc. (HBED3A·HCl·$5H_2O$) 404; found 406.

*Tetramethyl-N,N'-bis(2-hydroxybenzyl)ethylenediamine-N,N'-bis(methylenephosphonate), HBP4ME*

The Schiff base 51.7 g (0.193 mol) from salicylaldehyde and ethylenediamine was hydrogenated with 7.3 g (0.192 mol) of sodium borohydride in a vessel containing 400 ml isopropyl alcohol. The solution was heated for 1 h at 55 °C. The cooled solution was diluted with 80 ml of water and was poured into 800 ml of $H_2O$. The product, *N,N'*-bis(2-hydroxybenzyl)ethylenediamine (DHBEN) was then filtered off; yield 41.8 g (80%). A solution of 5.1 g (0.0188 mol) of 1.10 g (0.0375 mol) of paraformaldehyde in 30 ml of toluene was heated to reflux and 4.10 g (0.0375 mol) of dimethyl phosphite

was then added. After 45 min reflux, the reaction was terminated and the solvent removed under vacuum. The product HBP4ME, a pale yellow oil, was then redissolved in methanol and passed through an alumina column. The final yield of tetraester obtained after removal of the solvent was 5.80 g (60%). *Anal.* Calc. for $C_{22}H_{34}O_8N_2P_2 \cdot CH_3OH \cdot H_2O$: C, 49.02; H, 6.75; N, 4.97. Found: C, 48.76; H, 6.67; N, 4.84%.

*Tetraethyl-N, N'-bis(2-hydroxybenzyl)ethylene-diamine-N, N'-bis(methylenephosphonate), HBP4E*

To 30 ml of toluene, 5.10 g (0.0188 mol) of DHBEN and 1.10 g (0.0375 mol) of paraformaldehyde were added under nitrogen, with some heating required, to form a clear and colorless solution. The solution was then heated to reflux and 4.10 g (0.0375 mol) of diethyl phosphite was slowly added. After $2\frac{1}{2}$ h of reflux, the solvent was removed and the pale yellow product was redissolved in ethanol and passed through a column containing alumina; yield 6.20 g (57%). *Anal.* Calc. for $C_{26}H_{42}N_2O_8P_2$: C, 54.55; H, 7.34; N, 4.90. Found: C, 54.52; H, 7.07; N, 5.22%.

*Diethyl N, N'-bis(2-hydroxybenzyl)ethylene-diamine-N, N'-bis(methylenephosphonate), HBP2E*

A solution of 16.9 g HBP4E (0.0295 mol) and 4.7 g (0.118 mol) NaOH in 30 mol absolute ethanol was refluxed for 4.5 h under nitrogen, and 12.0 ml concentrated HCl was added. The solution was filtered and the solvent was removed under vacuum. The residue was dissolved in methanol and the ester was precipitated with ether. Isopropyl alcohol was then added to the diester and the mixture was stirred until the ester solidified. The product was filtered off and dried under vacuum; yield 9.60 g (63.2%). *Anal.* Calc. for $C_{22}H_{36}N_2O_8P_2 \cdot H_2O$: C, 49.4; H, 7.1; N, 5.2; P, 11.6. Found: C, 50.11; H, 6.91; N, 5.29; P, 11.47%.

*Dimethyl N,N'-bis(2-hydroxybenzyl)ethylene-diamine-N, N'-bis(methylenephosphonate), HBP2ME*

A solution consisting of 13.8 g HBP4ME (0.0267 mol) and 4.3 g sodium hydroxide (0.1068 mol) in isopropyl alcohol was heated and refluxed for $2\frac{1}{2}$ h under nitrogen. The isopropyl alcohol was removed under vacuum and the solid residue was dissolved in methanol. To the methanol solution, 9.0 ml of concentrated hydrochloric acid (0.108 mol) were added. The insoluble sodium chloride was filtered off, and the methanol was partially removed under vacuum. The product, HBP2ME, crystallized out of the methanol solution; yield 4.20 g (32%). *Anal.* Calc. for $C_{20}H_{30}N_2P_2O_8 \cdot CH_3OH$: C, 50.00; H, 6.75; N, 5.56; P, 12.3. Found: C, 49.63; H, 6.49; N, 5.82; P, 11.54%.

*Other Synthetic Procedures*

Detailed procedures for any of the ligands described in this paper that are not given above and are otherwise not previously published may be obtained by writing to the senior author (AEM). An improved procedure for PLED is also available.

*Bioassay Protocol*

The basic concepts and parameters of the *in vivo* mouse screen utilized were previously reported [4]. In the present assay fecal iron measurements and more extensive attention to gross toxic signs were incorporated [17, 18]. Male $BDF_1$ hybrid mice, tested to be free of common murine viruses, were 6 to 7 weeks old and weighed 16–20 g (Simonsen Laboratories, Inc., Gilroy, Calif. or Charles River Breeding Laboratories, Wilmington, Mass.). The mice were randomized by the card method into groups of 10 housed in 26 X 19 X 22 cm stainless-steel wire mesh metabolism cages. They were quarantined 7 days in their treatment room with a controlled environment: 12-hour light/dark cycle, 23 ± 2 °C, 50–70% relative humidity and 8 changes of fresh air each hour. Pellet feed (Wayne Lab-Blox, Chicago, Ill.) and water were freely available.

Two purebred adult beagles (Marshall Beagles, Inc., North Rose, N.Y.) were the constant source of transfusion blood. Erythrocytes from both dogs were pooled, washed twice, heated at 50 °C for 30 min (to minimize immunological response), and resuspended in isotonic saline. Mice received 0.6 ml transfusate i.p. every other day for 3 days and treatment with a test compound commenced on the third day after the last transfusion. A transfused control group received vehicle and a non-transfused control group was included to monitor disposition of transfused iron and development of immune responses (the latter did not occur). Analysis of aliquots of the transfusate quantified the amount of iron each mouse was given. Administration of test compound was continued for 7 days irrespective of route and the injection volume was usually 0.5 ml/mouse/day.

Daily observations of mortality, behavorial derangements, appetite and measurements of excreta were made. The day after the last treatment, mice were sacrificed by inhalation of ether, organs were examined for gross pathology, the aorta severed to remove blood from the spleen and liver which were weighed and homogenized in 0.073 M saline. Iron levels in tissue homogenates of 1–5%, fecal specimens (7-day pools) hydrolyzed in 1 N HCl at 90 °C for 30 min, and sediment-free 7-day urine pools were determined by atomic absorption spectroscopy.

One group in each bioassay (10 groups) was treated with DFB thus permitting direct comparison of the effects of the clinical drug with those of test chelates. In 44 separate assays, a 250 mg/kg i.p. dose of DFB induced an approximate 26 ± 5% decrease in liver iron and 250 ± 10% increase in urinary iron with little or no effect on splenic and fecal iron levels. A dose-response curve for Desferal was obtained and there was generally a linear response of the sum of percent iron reduction in liver plus percent increase in urinary iron excretion *versus* dosage between 50–400 mg /kg (curve constructed three times). Precision of dose response was monitored by repeated measurement of the 250 mg/kg point. To adjust dose response of Desferal to a specific test compound dose and response, the following simple proportion was used:

$$\frac{250 \text{ mg/kg DFB}}{\text{liver iron reduction + urinary iron excretion}} = \frac{\text{DFB dose equal to test compound}}{X}$$

thus permitting calculation of the relative potency ratio:

$$\frac{\text{sum of response of test compound}}{\text{sum of response of DFB (on transfused test animal)}}$$

Essentially no assumptions were made in calculating relative potency but percent change (tissue or excreta) of less than 8% (error limit) was considered zero in calculating sum of responses. Dosages of test compounds generally were between 100–500 mg/kg and when necessary, an acute toxicity study was performed to aid in selection of dose and to become familiar with gross toxic signs. LD50 values were estimated by the method of Weil [19].

## Results and Discussion

The chelating agents synthesized and submitted to the mouse screen are presented in Table I, together with the chemical affinity for Fe(III), as measured by pM (−log aquo Fe(III) ion at p[H] 7.4 in the presence of equimolar excess ligand). For information on the calculation of pM and the related equilibrium constants, the reader is referred to papers dealing with stabilities of metal chelates of these ligands (e.g. refs. 4, 5, 11, 14, 15, 20, 21). The chelating agents in Table I are grouped on the basis of the nature of the donor functions and resemblance to other, well-known ligands. All test results on samples subjected to the seven-day mouse screen are presented in Table II. It should be emphasized that all the samples listed in Table II were subjected to the same test routine and on that basis the test results indicated in Table II as potency *versus* that of DFB are considered reasonably comparable. The abbreviations and acronyms employed to identify the iron chelators in Tables I and II are in most cases self-explanatory on the basis of the graphic formulas also presented. All terms employed are defined precisely in Table I.

### *Chelating Agents Prepared for Animal Tests, Tables I and II*

*Table IA, EDTA analogs*

The chelating ligands $Ca_2Na_3$TPHA and $Ca_2Na_4$-PHOA represent an extension of the EDTA-type polyaminopolycarboxylic acids to tetraethylenepentamine and pentaethylenehexamine derivatives with seven and eight carboxylic acid groups, respectively. The results show moderate activity, but represent no significant advantage over diethylenetriamine-pentaacetic acid (DTPA), one of the parent compounds.

It should be noted that several of these ligands are supplied in the form of the Ca(II) chelates. These ligands bind calcium(II) ion very strongly and would produce hypocalcemic shock when administered i.v. or i.p. This could not take place, however, if the calcium(II) chelates themselves were employed as the iron chelators. Since the calcium(II) chelates are labile, and are always much less stable than the corresponding iron(III) chelates, rapid exchange of Ca(II) for Fe(III) takes place, with complete conversion to the iron(III) chelate *in vivo* as well as *in vitro*. This use of calcium(II) chelates is a device that has been employed with success previously (e.g., the use of CaEDTA for lead poisoning) and is highly recommended when the chelator employed binds calcium(II) strongly.

The decadentate ligand PXED3A represented a new concept in the design of Fe(III) chelators: the formation of a binuclear chelate and stabilization of the complex through formation of an intramolecular $\mu$-oxo dimer through partial hydrolysis. The development of additional ligands that take advantage of $\mu$-oxo dimer formation would seem worthwhile, because of the considerable stability enhancement resulting from intramolecular oxo bridge formation.

*Table IB, HBED analogs*

The success of HBED, EHPG and their esters suggest the synthesis and study of other ligands with *o*-hydroxylbenzyl groups substituted into the polyaminopolyacetate framework. Some of the new ligands of this type that have been synthesized and submitted are listed in Table IB (HDTT, HBEDDA, PGHC, TGTC, CMIDA, PC2IDA, HBED-3A, MBED3A, HBED3A3ET, 3HBDP). The triamine HDTT has an obvious relationship to DTPA.

The diamide of HBED, HBEDDA, was made available by Gulf Research and Development Company as an inexpensive source of HBED, but efficient

TABLE II. Changes in Splenic, Hepatic, Fecal and Urinary Iron in Transfused $BDF_1$ Male Mice Treated with Potential Iron Chelators for 7 Days

| Test compound | Dose[a] (mg/kg) | Number survivors | Toxic signs | *LD*50[b] | Percent iron changes *vs.* control | | | | Potency *vs.* DFB[c] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Spleen | Liver | Feces | Urine | |
| $CaNa_3DTPA$ | 200 | 10/10 | none | >800 | −8 | +16 | −1 | +89 | 0.4[d] |
| $Ca_2Na_2TTHA$ | 200 | 10/10 | none | >800 | −12 | +20 | ±0 | +69 | 0.3[d] |
| $Ca_2Na_3TPHA$ | 100 | 10/10 | none | >2400 | +5 | +9 | −7 | +38 | 0.4[d] |
| $Ca_2Na_4PHOA$ | 100 | 10/10 | none | >2400 | −1 | +1 | +24 | +33 | 0.3[d] |
| PXED3A | 300 | 5/10 | CNS↓, body wt.↓ | >300 | +14 | +20 | −62 | −30 | [e] |
| HDTT | 375 | 10/10 | body wt. sl.↓ | 1286 | −9 | −26 | −23 | +53 | 0.2[d, e] |
| HBEDDA | 400 | 10/10 | none | >2400 | −4 | +2 | +22 | +39 | 0.1 |
| TGTC | 300 | 10/10 | none | >500 | +2 | −28 | −8 | +140 | 0.4 |
| PGHC | 300 | 10/10 | sl. body wt.↓ | >500 | −6 | −27 | −10 | +72 | 0.3 |
| CMIDA | 300 | 10/10 | sl. body wt.↓ | >400 | −10 | −5 | −38 | +31 | 0.1[d, e] |
| PC2IDA | 100 | 10/10 | intestinal bloat | 200 | −1 | −3 | −27 | +5 | 0.1 |
| HBED3A | 300 | 10/10 | none | >500 | −20 | −19 | +15 | +188 | 0.8 |
| MBED3A | 100 | 10/10 | body wt.↓ | >100 | +2 | −13 | −35 | +100 | 0.9 |
| HBED3AEt | 300 | 8/10 | CNS↓, body wt.↓ | 350 | −12 | +2 | −18 | −19 | [e] |
| 3HBDP | 50 | 10/10 | none | 350 | −1 | −6 | −12 | +7 | 0.3 |
| PLED | 400 | 10/10 | none | >1120 | −15 | −17 | +9 | +376 | 0.8 |
| BPDIEDNA | 300 | 10/10 | CNS↑, body wt.↓ | >400 | −3 | 0 | −4 | +28 | 0.1[d, e] |
| $PLEDNH_4$ | 214 | 4/10 | CNS↓, excreta↓ | 300 | −27 | +12 | −70 | −4 | 0.1[e] |
| PLEDHCl | 300 | 8/8 | none | >500 | +5 | +4 | +9 | +110 | 0.5 |
| BPG | 193 | 4/10 | CNS↓, excreta↓ | >200 | −28 | +30 | −86 | −75 | 0.2[e] |
| $Ca_2Na_2SHEMP$ | 200 | 10/10 | sl. CNS↑ | >600 | −22 | −9 | −2 | +349 | 1.3[d] |
| HBP4E | 50 | 10/10 | none | 212 | +6 | +5 | +7 | −15 | 0 |
| HBEDPO | 150 | 10/10 | CNS↓, enteritis | 369 | −13 | −10 | −16 | +9 | 0.2[d, e] |
| HBP2E | 400 | 10/10 | none | 1697 | −7 | +7 | +25 | +178 | 0.4[d] |
| HBP4Me | 300 | 10/10 | none | >500 | +3 | +6 | −7 | ±0 | 0 |
| HBP2Me | 300 | 10/10 | none | >400 | +23 | +8 | −7 | +33 | 0.1[d] |
| CYTA | 300 | 9/10 | CNS↓, body wt.↓ | >400 | −2 | +13 | −54 | −54 | [e] |
| 4HBCY | 250 | 10/10 | spleen↑ | >400 | +9 | +3 | −3 | −16 | 0 |
| 3HBCY | 250/100 | 0/10; 3/3 | CNS↑↓, excreta↓ | 200 | +32 | +24 | −18 | −57 | [e] |
| CY2B2Z2AC | 193 | 6/10 | CNS↑, excreta↓ | >200 | −13 | +32 | −79 | −78 | 0.1[e] |
| 4SHBCY | 300 | 10/10 | none | >400 | +2 | +30 | −5 | +8 | 0.1 |
| 6HBN6C18 | 250 | 10/10 | CNS↓, excreta↓ | >400 | +13 | +20 | −64 | −32 | 0[e] |
| 6SHBN6C18 | 300 | 6/10 | body wt.↓, excreta↓ | >500 | +1 | +1 | −48 | +42 | 0.1[e] |
| N6C18AC6 | 250 | 10/10 | body wt.↓, liver↓ | >400 | −13 | −9 | −52 | −41 | 0.1 |
| BAMTPH | 300 | 10/10 | none | >500 | +7 | +5 | +2 | ±0 | 0 |
| BAMTGH | 400 | 10/10 | none | >600 | +3 | −8 | +2 | +28 | 0.1 |
| BZCO3MHA | 500 | 10/10 | none | >800 | −6 | −9 | +12 | +65 | 0.2 |
| DFB | 250 | 260/260 | none | >2000 | −2 | −26 | +7 | +250 | 1.0 |
| DP3DHBO | 300 | 10/10 | liver↓ | >500 | −16 | −4 | −10 | +27 | 0.1 |
| DP3SDHBO | 300 | 4/10 | CNS↓, organs↓, feces↓ | 300 | −48 | +1 | −69 | +136 | 0.5[e] |
| BAOTA | 300 | 10/10 | none | >400 | +11 | +10 | −21 | −6 | 0 |

[a]Route i.p. [b]*LD*50 values were derived from acute toxicity studies, or from screen data, and the symbol > indicates the largest dose tested for lethality. [c]Relative potency is a ratio of the sum of the decreases in splenic and liver iron plus increased in fecal and urinary iron for test compound to the corresponding sum for DFB corrected for dosage. [d]Source of excreted iron is not established. [e]Toxicity affected iron changes.

hydrolysis or esterification of the amide groups had not previously been successful. In our hands, it was completely hydrolyzed as the Fe(III) chelate at pH 5.0 to give a 100% yield of the Fe(III)–HBED chelate and ammonia. Treatment with 8-hydroxyquinoline and solvent extraction removed the iron(III), and gave a quantitative yield of metal-free HBED. This process, however, may not be suitable for large-scale synthesis, because of the necessity of repeating the solvent extraction step. This synthetic method has been reported [10].

Ligands TGTC and PGHC were fractionated from a crude mixture supplied by W. R. Grace and Company (Hampshire Division). The mixture was apparently synthesized by a Mannich reaction carried out on *p*-cresol, formaldehyde, and glycine. The triamine

TGTC has been subjected to detailed equilibrium studies in this laboratory, and has been reported separately [11]. Although samples PGHC and TGTC seem to have only moderate effectiveness in removal of iron overload, these chelators, as well as their lower analogs CMIDA and PC2IDA, should be given more attention. They are all readily synthesized in an essentially one-step Mannich reaction from inexpensive starting materials. Because they are inexpensive and easily synthesized, minor variations in molecular structure, such as esterification, should be carried out to improve their bioavailability, as was done successfully for HBED.

A surprising and interesting development in this research program is the unexpectedly encouraging effectiveness of HBED3A, the monophenolic analog of HBED and EDTA, and the slightly improved potency of its methoxy ether MBED3A and its triethyl ester HBED3A3ET. These chelators are about as potent as DFB, as measured by the tests carried out thus far, and may be further improved by minor changes in structure and method of administration.

*Table IC, HBED-pyridoxyl analogs*

The ligand PLED is very similar to HBED, except for the pyridine ring and the ring substituents derived from vitamin $B_6$. While its stability constants for metal ions are not as high as those of HBED, it has the advantage of lower basicity of the phenolate groups, thus reducing hydrogen ion competition in solution, resulting in Fe(III) complexation at about the same level as the parent compound (HBED). There is also the possibility that the natural pyridine ring in this ligand would be less toxic than the benzene rings of HBED and EHPG. The initial test shows that the compound is effective, seemingly close in potency to DFB itself. Also there is the possibility that esterification will further increase its effectiveness in the removal of iron overload.

The variations in potency between samples PLED, $PLEDNH_4$, and PLEDHCl shows that the presence of extraneous, non-chelating, salts of crystallization can have considerable influence on effectiveness in iron(III) removal. Further improvements in the potency of PLED may be expected by refinement in the purity of the compound to eliminate salts of crystallization, and perhaps by conversion of the hydrophilic hydroxymethyl groups to more lipophilic derivatives.

The potential of PLED as a chelator for trivalent metal ions, with comparisons to EHPG and HBED, has been investigated separately, and has been published recently [20, 21]. It has been noted [22] that the presence of pyridine rings in PLED make possible protonation of the Fe(III), Ga(III), and In(III) chelates without extensive loss in stability, thus reducing charge on the complex and increasing the possibility of membrane permeability [22].

*Table ID, HBED, phosphonic acid analogs*

The methylenephosphonate-containing ligands (HBEDPO, $Ca_2Na_2SHEMP$, HBP4E, HBP2E, HBP-4ME, HBP2ME) also contain phenolic groups to further increase affinity for Fe(III). Continuing interest in phosphonate-containing ligands is based on the high affinity of this group for the ferric ion, and its occurrence in biological systems in the form of aminophosphonic acids.

Those ligands in which the phosphonate groups are fully esterified (HBP4E, HBP4ME) seem to be completely inactive. This may be due to lack of bioavailability, or to the well known stability of phosphonate esters toward hydrolysis. The use of the esters of chelating agents is based on the assumption that hydrolysis will occur *in vivo* before or at the time of combination with the metal ion of interest.

The half-esters HBP2ME and HBP2E are of interest because phosphate half esters, such as diethylpyrophosphate, are known to be effective chelating agents. On this basis it was thought that these ligands might also show high affinity for Fe(III) ions. This has been verified by studies reported elsewhere [23]. It should be pointed out that HBP2E and HBP2ME are unique, and that they are the first ever to be reported with half phosphonate ester donor groups.

Although moderate to low activity has been reported for the bisphosphonic acid (HBEDPO) and for the half ester HBP2E, the very soluble sulfonic acid derivative $Ca_2Na_2SHEMP$ seems to be very effective for Fe(III) removal. Thus it appears that low activity in these ligands may be due to low solubility (partially protonated iron(III) phosphonate chelates are known to be very insoluble), and that further studies on phosphonate ligands could be based on the incorporation of solubilizing groups in ligand design.

*Table IE, macrocyclic ligands*

A number of tetraaza and hexaaza macrocyclic compounds with acetate and phenolic groups, as well as various combinations of the two, were screened. Compounds CYTA, 4HBCY, 3HBCY, CY2BZ2AC and 4SHBCY are based on the well known cyclam backbone and are of interest for their ability to be alkylated at the secondary nitrogens with appropriate exocyclic substituents having high affinity for Fe(III). Animal tests showed that these compounds are uniformily toxic to the central nervous system, depress excretion, and some adversely affect body weight. The only exception is the sulfonated derivative 4SHBCY, which was non-toxic and showed some activity.

The larger macrocycles behave in a similar fashion. The hexaphenol (6HBN6Cl8), the sulfonated hexaphenol (6SHBN6Cl8) and the hexaacetate (N6Cl8-

AC6) were shown to be toxic, but are slightly more active for iron(III) removal than the smaller macrocyclic derivatives.

*Table IF, hydroxamic acids*

Compounds BAMTPH and BTAMGH are structural isomers of tripodal trishydroxamic acids, somewhat mimicking the natural tris hydroxamic acids. While BAMTPH showed no activity, BTAMGH did show a potency of 0.1 relative to DFB. This can be rationalized on the basis that BAMTPH has restricted rotational flexibility of the conjugated amide carbonyls, whereas BTAMGH does not have this steric problem. The metal ion affinities of BAMTPH have been studied in depth and are reported elsewhere [14].

Addition of a methylene group at each bridge of BAMTPH results in BZCO3MHA, which shows quite improved potency for iron(III) removal. The *N*-methyl group on the hydroxamic acid moiety probably helps prevent degradation of the ligand, while increasing its donor properties. In the future, secondary hydroxamic acids should be chosen over their primary counterparts for the design of iron(III) chelators.

*Table IG, catechol derivatives*

Compound DP3DHBO differs from the well-publicized spermidine derivatives in that the backbone to which the three catecholate groups are attached consists of symmetrical (1,3-trimethylene) bridges between the three nitrogens. Although DP3DHBO shows some liver toxicity, it nevertheless possesses some activity in Fe(III) mobilization.

The sulfonated compound DP3SDHBO shows much improved potency (0.5) but it produces side effects, including CNS depression.

*Table IH, ligand with coordinating amide groups*

The ligand BAOTA is unique in that it contains two amide groups that become strong donor groups by proton dissociation in the presence of metal ions having sufficient polarizing power to displace the amide protons. This type of ligand appears to have little or no potency for iron(III) removal. The affinity of this ligand for various transition metal ions is reported separately [15].

*Toxicity versus Potency*

The predominant application of the mouse screen was to provide evaluation of the usefulness of new synthetic iron chelators in two directions: (1) to establish the most efficacious structural moieties *in vivo*; and (2) to focus efforts on producing active oral derivatives. In order to attain these objectives, the simultaneous accumulation of toxicity data on likely candidates could narrow endeavors toward minimally toxic drugs. For example, some potential chelators were quite active but were toxic (MBED3A and $Ca_2Na_2$SHEMP) whereas others exhibited good activity in the absence of gross toxicity (HBED3A and PLED). There were instances in which toxicity precluded estimates of relative potency or may have caused lower estimates of potency *versus* Desferal (Table II, footnote).

Interpretation of efficacy should also take into consideration solubility in aqueous drug formulations and the fact that i.p. administration of test compounds avoids the initial liver detoxification processes. Indeed, in general, among very active chelators relative potency declined when the oral route was used (*i.e.*, MBED3A; the i.p. relative potency was 0.9 and the oral relative potency was 0.3). As to poor water solubility of chelators, bioavailability is diminished and local irritability of tissues could be increased. These latter two factors as well as route of administration can play a role in determination of the *LD*50 (Table II). As to effects on the CNS, severe debilitation induced by a chelator must be eliminated as a cause of depression before concluding that a purely central effect on motor activity has been evoked by the test compound.

*Stability of Fe(III) Chelates versus Potency*

The pM values (negative logarithm of concentration of free (aquo) ferric ion under specified conditions, 100% excess free ligand, $-\log[H^+]$ 7.4) listed in Table I provide the best relative measure of the chemical effectiveness of the chelating agents in combining with iron(III). Those with pM values near 25 would be expected to sequester iron(III) as effectively as DFB. Those with higher values are chemically more effective than DFB, to the extent indicated by the incremental pM value. Inspection of Tables I and II indicates that while many chelating agents have the requisite Fe(III) affinity, only a few of them are effective *in vivo*. Therefore it is apparent that lack of bioavailability, for whatever reason, is an important factor in limiting potency. Conversely, however, it is seen that all of the chelating agents showing effectiveness have the requisite stabilities of their Fe(III) chelates.

*Macrocyclic Ligands with Endocyclic Hydroxamic Acid and Catechol Groups*

The most recent phase of this research program involved the synthesis of macrocycles containing endocyclic hydroxamic acid or catechol groups (formulas A, B, and C). Such compounds had long been envisaged as having the greatest potential for high affinity and selectivity for iron(III). An example of this is the projected synthesis of a biscatechol macrocycle described by Martell [4]. Several macrocyclic ligands of this type, with endocyclic catecholate or hydroxamate groups, have now been

Endocyclic bishydroxamic acid

Endocyclic biscatechol (30-membered ring)

Endocyclic biscatechol (26-membered ring)

synthesized. The endocyclic bishydroxamate A has been described [7]. Metal ion affinities of the macrocyclic biscatechols are currently being investigated, and a paper describing initial results has recently appeared [8].

## Acknowledgement

This work was supported by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, U.S. Public Health Service under contracts No. NO1AM-O-2228 with Texas A and M Research Foundation, and No. NO1AM-O-2206 with EG and G Mason Research Institute.

## References

1 A. E. Martell, W. F. Anderson and D. G. Badman (eds.), 'Development of Iron Chelators for Clinical Use', Elsevier North-Holland, New York, 1981.
2 A. Bank, W. F. Anderson and E. C. Zaino (eds.), 'Fifth Cooley's Anemia Symposium', *Ann. N.Y. Acad. Sci., 445*, 1–466 (1985).
3 C. G. Pitt and A. E. Martell, 'Inorganic Chemistry in Biology and Medicine', American Chemical Society, Washington, D.C., 1980, pp. 297–312.
4 A. E. Martell, 'Development of Iron Chelators for Clinical Use', Elsevier North-Holland, New York, 1981, pp. 67–104.
5 K. N. Raymond, G. Pecoraro and F. L. Weitl, 'Development of Iron Chelators for Clinical Use', Elsevier North-Holland, New York, 1981, pp. 165–187.
6 S. J. Rodgers, C. Y. Ng and R. N. Raymond, *J. Am. Chem. Soc., 107*, 4094 (1985).
7 Y. Sun, A. E. Martell and R. J. Motekaitis, *Inorg. Chem., 24*, 4343 (1985).
8 Y. Sun, A. E. Martell and R. J. Motekaitis, *Inorg. Chem., 25*, 4780 (1986).
9 R. M. Smith and A. E. Martell, 'Critical Stability Constants', Vols. 1–5, Plenum, New York, 1974, 1975, 1976, 1977, 1982.
10 A. E. Martell, R. J. Motekaitis, E. T. Clarke and J. J. Harrison, *Can. J. Chem., 64*, 449 (1986).
11 I. Yoshida, R. J. Motekaitis and A. E. Martell, *Inorg. Chem., 22*, 2795 (1983).
12 Y. Sun, A. E. Martell, M. J. Welch, R. J. Motekaitis and C. J. Mathias, to be published.
13 L. Sala and A. E. Martell, *Inorg. Chim. Acta*, to be submitted.
14 I. Yoshida, I. Murase, R. J. Motekaitis and A. E. Martell, *Can. J. Chem., 61*, 2740 (1983).
15 R. M. Smith, R. J. Motekaitis and A. E. Martell, *Inorg. Chem., 24*, 1132 (1985).
16 G. Schwarzenbach, G. Anderegg and R. Sallman, *Helv. Chim. Acta, 35*, 1785 (1952).
17 C. G. Pitt, G. Gupta, W. E. Estes, H. Rosenkrantz, J. J. Metterville, A. L. Crumbliss, R. A. Palmer, K. W. Nordquest, K. A. Sprinkle Hardy, D. R. Whitcomb, B. R. Byers, J. E. L. Arceneau, C. G. Gaines and C. V. Sciortono, *J. Pharmacol. Exp. Ther., 208*, 12 (1979).
18 A. Winston, C. V. P. R. Varaprasad, J. J. Metterville and H. Rosenkrantz, *J. Pharmacol. Exp. Ther., 232*, 644 (1985).
19 C. S. Weil, *Biometrics, 8*, 249 (1959).
20 C. H. Taliaferro, R. J. Motekaitis and A. E. Martell, *Inorg. Chem., 23*, 1188 (1984).
21 C. H. Taliaferro and A. E. Martell, *Inorg. Chem., 24*, 2408 (1985).
22 M. A. Green, M. J. Welch, C. J. Mathias, P. Taylor and A. E. Martell, *Int. J. Nucl. Med. Biol., 12*, 38 (1985).
23 C. H. Taliaferro and A. E. Martell, *Inorg. Chim. Acta, 85*, 9 (1984).