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THE ACTION OF NINE CHELATORS ON IRON-DEPENDENT RADICAL DAMAGE

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Nine iron chelators were tested in five systems for their effects on radical-generation and conversion at chelator: iron molar ratios from 0.1 to 10. Stimulatory actions might distinguish toxic from safer chelators. Radical-generating reactions which represent different aspects of iron (ferrous and ferric) availability were studied: a) the reaction with hydrogen peroxide to hydroxylate benzoate; b) the oxidation of ascorbate; c) the reaction with hydrogen peroxide to fragment proteins; d) the reaction with hydrogen peroxide to permit amplified chemiluminescence; and e) the induction of peroxidation of mitochondrial membrane lipids. The compounds used were HBED, CP130, Desferal, EDTA, pyridine-hydrazone (CGP 43'902B), Ferrozine, CP 94 (CGP 46'700), L1 (CGP 37 391) and rhodotorulic acid (CGP 45 274). Only the hexadentate compounds HBED, CP130 and Desferal were uniformly inhibitory ("protective"). The protective compounds were also apparently more stable during radical fluxes than the other chelators.

KEY WORDS: Free Radicals; iron; chelators; protein oxidation; lipid oxidation; metal reduction.

INTRODUCTION

Iron chelators are widely used in clinical practice, in dealing with thalassaemia, iron overload, and other conditions[1,2]. Desferal is amongst the most important of these chelators, and has been studied intensively for more than 30 years[3]. It is also commonly used in free radical research, being one of the most potent inhibitors of iron-dependent radical reactions[4] by virtue of its retention of ferric ions, and its facilitation of the oxidation of ferrous to ferric ions[5].

While desferal has proved to be quite safe even at high dose and during chronic use[1,6], it must be given by daily subcutaneous infusions (8–12 hours each) because of its low oral bioavailability and short systemic halflife it. Therefore, substantial efforts have been made to develop an orally active iron chelator, which would have a much improved patient acceptance[7]. Unfortunately, until now all orally active iron chelators have proved substantially more toxic than Desferal when compared at equi-effective doses.

The present study was undertaken in order to better understand the reasons for such toxicity. For example, an iron chelator which facilitates the redox reactions of iron (such as Fenton chemistry) which generate damaging hydroxyl radicals[8], might be toxic *in vivo*. Therefore, we have studied a range of iron chelators, representing each of the structural types currently under development or in use, focussing on the capacity of the chelators to restrict the participation of iron in several radical generating reactions[9]. We present information relevant to the reducibility of the metal, and its redox availability for reactions which lead to damage to aromatic compounds, proteins, and lipids.

Compounds which permit such radical reactions may thereby be toxic, whereas those which do not are less likely to be toxic. Although the toxicity of xenobiotics is influenced by many other factors, such as bioavailability[1], for iron chelators, radical reactions must be considered.

MATERIALS AND METHODS

Iron Chelators

The following iron chelators were obtained from Ciba-Geigy, Basel: CGP-53'650 or **HBED** (n,n'-Di-(2-hydroxybenzyl)-ethylenediamine-N,N'-diacetic acid)[10]; Desferrioxamine B methanesulphonate (**Desferal**); **CP-130** the hexadentate pyridinone of Dr R. Hider and colleagues[11]; **CGP-37'391** (1,2-Dimethyl-3-hydroxy-pyrid-4-on; "L1'), and CGP-46'700 (1,2-Diethyl-3-hydroxy-pyrid-4-on; also known as **CP94**)[12]; **CGP-45'274** (Rhodotorulic acid); **CGP-43'902B** (called pyridine-hydrazone or more precisely, 1-(N-Ethoxycarbonylmethyl-pyrodoxylidenium)-2-(2'-pyrimidyl) hydrazone, methanesulfonate, being an analogue of pyridoxal isonicotinoylhydrazone, PIH). Other chelators used were **Ferrozine** (3-(2-Pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, Sigma) and **EDTA** (BDH AnalaR). Ferrous sulphate (only for the benzoate assay, a) below), ferrous chloride and ferric chloride were used as iron sources (from Sigma).

Methods

All nanopure water and solutions (other than metal stocks) were pretreated with chelex resin to remove contaminating multivalent metals (with the exception that hydrogen peroxide cannot be mixed with chelex). When supplied, chelators were always added before iron, so as to restrict iron oxidation or precipitation. Iron stock solutions were made daily. Ferrous stocks were made at 6 mM and 600 μM in water, and kept tightly capped in volumes of 20 ml for not more than 5 hours. All assays were performed with 1 μM and 30 μM iron, but in several cases, the reaction was only detectable at the higher concentration. The concentrations are chosen to reflect respectively, possible physiological and pathological concentrations of iron available in low molecular weight form[4,9,13,14,15]. With the exception of amplified chemiluminescence, reactions were progressive with time and monophasic. When possible a linear range was used, but many of the reactions of ferrous are non-linear and extremely rapid, because of autoxidation and precipitation. All assays were done with chelator: metal molar ratios from 0.1:10.

The benzoate assay was done with duplicates (always within 5%), and averages are shown. The ascorbate oxidation, protein fragmentation and lipid peroxidation assays were normally done in triplicate, and averages and sds are shown on the graphs. When no error bar is visible it is obscured by the corresponding data point.

a) Benzoate Hydroxylation

This primarily measures hydroxyl radicals (though higher oxidation states of iron may play a role[16]). These hydroxylate benzoate to fluorescent products (308 nm excitation and 410 nm emission)[4,17]. 1 mM benzoic acid (Sigma) was incubated at room temperature for 1 hour in 10 mM sodium phosphate (pH 7.4) with 5 mM

hydrogen peroxide, an iron chelator and either ferrous sulfate or ferric chloride (1 μM or 30 μM). The reaction was started by the addition of iron and kept in the dark before measuring the fluorescence. Salicylate was used as a standard with 10 μM giving a fluorescence of about 530, varying slightly from day to day. A limited series of hydroxylation experiments were conducted with ferrous chloride instead of sulphate, and these gave indistinguishable results (not shown); thus in all the assays below, the chloride was used for both ferrous and ferric salts. Salicylate was used to determine quenching by the chelators and complexes.

b) Ascorbate Oxidation

Fresh ascorbic acid (0.1 mM) was incubated in sodium phosphate buffer (10 mM, pH 7.4) in the presence of ferric ions (30 μM) and an iron chelator at room temperature for 40 minutes[18,19]. Absorbance at 265 nm was measured after 10 minutes and after 40 minutes and the decrease was calculated. The reaction was started by the addition of ascorbic acid. A change in absorbance of 0.1 cm^{-1} represents a change of 70 μM in the ascorbate concentration. Many of the chelators gave high absorbances at 265 nm, but the rate of ascorbate consumption was sufficient to be detected.

c) Protein Fragmentation

BSA was labelled with ^{14}C formaldehyde[20] to a specific radioactivity of about 100,000 cpm/mg. 1 mg/ml ^{14}C-BSA was incubated (final volume: 1 ml) in 10 mM sodium phosphate (pH 7.4) with 5 mM hydrogen peroxide, iron ions (30 μM only, since 1 μM was insufficient) and a chelator at 37°C for 24 hours. A 200 μl sample was taken to measure the total radioactivity. Then 100 μl 100 mg/ml BSA and 200 μl 30% TCA were added to the remaining sample. After cooling to 4°C and spinning, an aliquot was taken to measure the TCA soluble fragments. Fragmentation was expressed as TCA soluble as percentage of total radioactivity.

d) Amplified Chemiluminescence

Radicals (and other excited species), were detected in a β-scintillation counter in out-of-coincidence mode[17,21]. Lucigenin (1.54 mM) was incubated in sodium phosphate buffer (pH 7.4) with 5 mM hydrogen peroxide, 300 μM iron and chelators, in very low light. Before the addition of iron the samples were followed until a stable baseline was obtained. After iron was added the samples were allowed to cycle in the counter overnight at room temperature. The data show the behaviour of individual samples, representative of two.

Possible quenching of the chemiluminescence by the chelators was tested in a metal-independent system. The thermolabile radical generator AAPH (50 mM)[22,23] was added to the standard system but without peroxide at 37 degrees Centigrade, and readings taken at regular intervals by transferring the vials from the incubator (light-protected) to the counter. The chelators all quenched the chemiluminescence from 60–80% when using 300 μM of both ferrous ion and chelator. This effect does not obscure qualitative and kinetic differences, and is slighter at lower chelator and metal concentrations. However, the data should not be interpreted quantitatively.

e) Lipid Oxidation

Rat liver sub-mitochondrial particles (SMPs)[24,25] (50 μg protein/ml) were incubated in 10 mM-phosphate buffer with iron and chelators. After 90 minutes at room temperature TCA was added to a final concentration of 5% then the solution was mixed with an equal volume of 0.67% thiobarbituric acid (TBA). This mixture was heated for 15 minutes at 90–100°C, cooled, spun, and the fluorescence of the supernatant measured at 537 nm excitation and 558 nm emission. This measures sensitively the TBA-reactive malondialdehyde. As a standard, 1,1,3,3-tetramethoxy propane in 1% sulfuric acid was also heated with TBA. 1 μM 1,1,3,3-tetramethoxy propane converted into malondialdehyde (2 μM) gave a fluorescence of about 170.

f) Destruction of Chelators during Radical Fluxes

Simple experiments on this were undertaken using the protein fragmentation system, because it involved prolonged reaction. Visual observations were made of the loss of the coloured chelator-metal complexes, and whether these could be regenerated by the addition of fresh metal.

RESULTS

The results from all 5 assay systems are summarised in Table 1, and presented in more detail in the sections which follow. In most cases systems supplied with ferrous ions are more active than those receiving ferric. The data extend the limited published information on EDTA, and desferal[26,27] and reveal the behaviour of the newer chelators.

a) Benzoate Hydroxylation

Figures 1a,b show the expected[26,27,28] dramatic stimulation of ferrous-initiated benzoate hydroxylation by EDTA, and the inhibitory actions of most of the other chelators (in accord with other studies of superoxide conversion to hydroxyl radicals by various iron chelates[29]). Pyridine hydrazone (CGP 43902B) shows a biphasic effect, with stimulation at low C:Fe. These data are presented after correction for fluorescence quenching by the chelator (similarly in Figure 2). Quenching was sufficient to cast doubt on the qualitative interpretation only for C:Fe > 5 (where the quenching was approx. 70% when C was 150 μM); however, with CP130 the formation of highly coloured complexes precluded clear interpretation of the hydroxylation data. However it is clear that with all chelators but EDTA and CP130 inhibition is incomplete even at C:Fe = 10. This is probably because of binding of metal by benzoate. An alternative hypothesis is that benzoate can be hydroxylated by an "outer sphere" electron tunnelling reaction of the C:Fe complexes, which would not be expected to be inhibitable by adding more chelator.

Benzoate hydroxylation (unlike most of the reactions studied) was detectable with 1 μM ferrous sulphate. EDTA again stimulated considerably, and was still becoming more effective at C:Fe = 10. Desferal and L1 had hardly any effect, but this is probably due to the fact that in their presence or in the absence of chelator, the ferrous

TABLE 1
Influences of chelators on five iron-dependent radical reactions

Chelator	Benzoate hydroxylation		Ascorbate consumption	Protein fragmentation	Chemiluminescence		Mitochondrial lipid peroxidation	
	Fe2+	Fe3+	Fe3+	Fe2+ or Fe3+	Fe2+	Fe3+	Fe2+	Fe3+
HBED	I partial	I almost complete	I	I	I	I slight	I	I
EDTA	S	S max. at low C:Fe	S	S huge	S early and late	S gradual	I	I
CGP 43'902B	S at low C:Fe I at high C:Fe	As for Fe2	S at low C:Fe; nil at high	S	I (S slight and late)	S slight and late	I at low C:Fe; S at high	S
Desferal	I partial	I near complete	I	I	I	I slight	I	I
CP130	Not determined		I	I	I	I slight	I	I
Ferrozine	not done	not done	I	S slight	I	I slight	S at low C:Fe I at high	S at low C:Fe I at high
CGP 46'700	I partial	I almost complete	I (S slight at low C:Fe)	S at low C:Fe; I at high	I slight	I slight	I	I
CGP 37 391	I partial	I almost complete	I (S slight at low C:Fe)	S at low C:Fe; I at high	I	I slight	I	I
CGP 45 274	I partial	I almost complete	I	S huge	I slight	I slight	I	I

I: Inhibition
S: Stimulation
C: Chelator

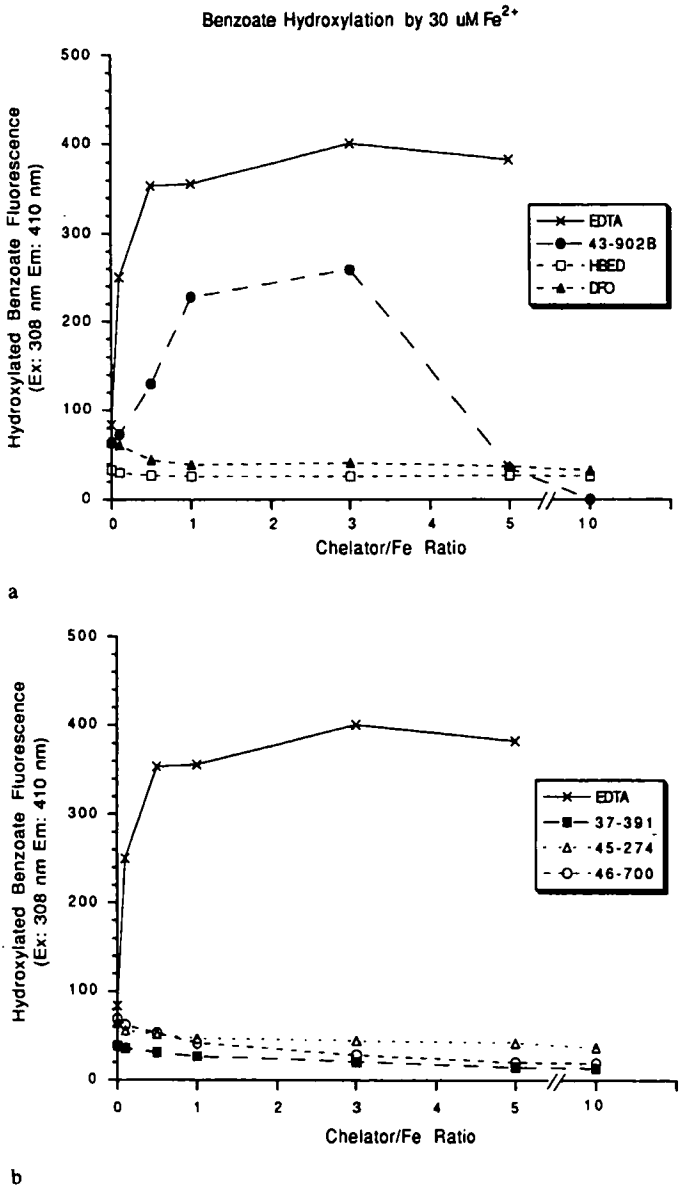

FIGURE 1a,b Benzoate hydroxylation by hydrogen peroxide upon addition of 30 μM-ferrous iron and chelators as specified was determined as described in Methods. Data from two different experiments (each concerning different chelators) are presented, with zero chelator fluorescence values of about 35 and about 60.

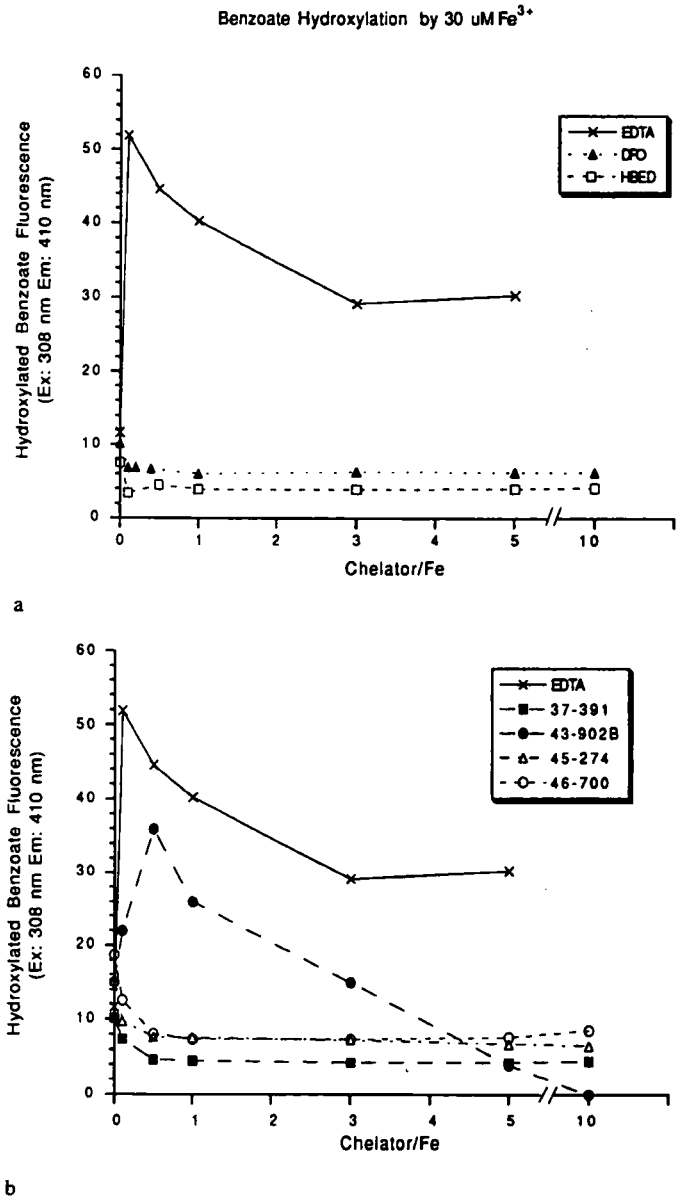

FIGURE 2a,b Benzoate hydroxylation by hydrogen peroxide upon addition of 30 μM-ferric iron and chelators as specified was determined as described in Methods. Data are from several different experiments, and hence zero chelator values vary.

ions are oxidised so rapidly by oxygen that the radical reactions are very brief. EDTA probably prolongs them.

Figures 2a and b show the benzoate hydroxylation by ferric iron (30 μM) with the several chelators. The extent of inhibition is greater with most chelators than for ferrous iron, and EDTA and pyridine-hydrazone again stimulate. In both Figures 1 and 2, inhibition is maximal at C:Fe = 1.

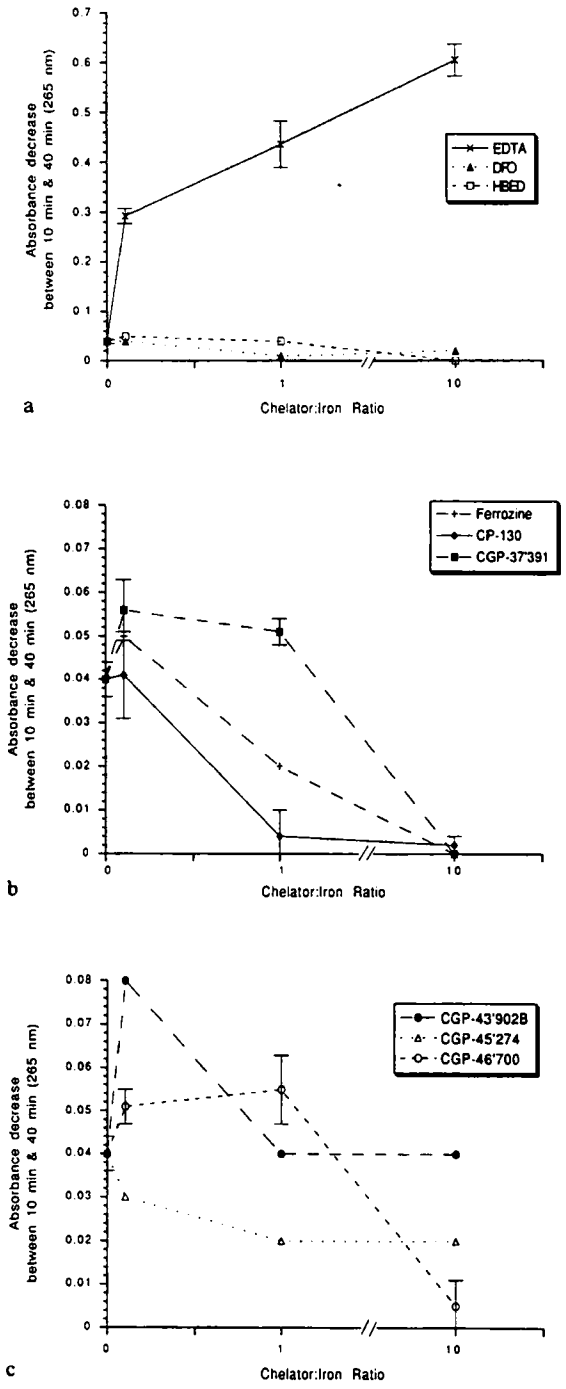

FIGURE 3a,b,c Ascorbate Oxidation as an index of metal reducibility. Ascorbate consumption by ferric iron in the presence of various chelator concentrations was determined as detailed in Methods.

b) Ascorbate Oxidation

Figure 3a-c shows that the effects of the various chelators on reducibility of ferric iron (judged by the oxidation of ascorbate) parallel those in the benzoate system. However, the pyridinones stimulate at low C:Fe (around 1), and in most cases higher C:Fe ratios are needed for maximal inhibition than with benzoate. This is probably because of greater access of ascorbate to the bound metal than of the combination of peroxide and benzoate; in addition ascorbate may bind the metal more avidly than does benzoate.

c) Protein Fragmentation

Figures 4 (ferrous) and 5 (ferric) show the results for protein fragmentation. The stimulation by EDTA contrasts dramatically with the complete inhibition by HBED. Desferal inhibits ferric more than ferrous; while the slight stimulation by ferrozine may be due to its capacity to hold ferric iron in solution, and redox active. The pyridinones again stimulate at low C:Fe, and inhibit at high.

The similarity between these results and those of assays a) and b) suggests that rather similar factors influence them all. The literature has emphasised roles of protein-bound metal in dictating sites of protein damage[30,31]. The displacement of metal from protein to chelator in these experiments may be partly responsible for inhibitory actions. Such displacement from target to chelator may be important in each system.

d) Amplified Chemiluminescence

This reaction was conveniently detectable only with 30 μM iron. The kinetics are complex and multiphasic (Figures 6a-c). The main reaction is very rapid with ferrous ion (Figure 6a) and much delayed with ferric (data not shown). The variations in the rapid peak with ferrous and the various chelators parallels the observations in other systems, so that EDTA stimulates, while the other compounds (except CP 94) inhibit to varying degrees. The initiation of a delayed second phase reaction in the ferrous system with both EDTA (Figure 6a) and pyridine hydrazone (Figure 6b) is a feature not shared by the other chelators.

With ferric ion (not shown) there is virtually no initial peak, but a small "delayed" peak (around 200 minutes) with 43 902B. The reaction with EDTA gradually accelerates. In the presence of the other chelators there is even less chemiluminescence than in the absence of ferric iron.

We investigated the reaction of pyridine-hydrazone further by adding reagents at the time the "delayed" peak normally commences. When the system is complete, the addition of further iron, peroxide or both (in each case a fresh quantity contributing the same concentration as initially present), enhances in an additive way. When the system lacks iron until 135 minutes, a subsequent delayed peak can occur after about a further 135 min, if ferrous or ferric ions and peroxide are added. Again the effects are approximately additive. The iron- and peroxide-dependence of the "delayed" reaction in the presence of pyridine-hydrazone are thus confirmed.

e) Lipid Peroxidation

Figures 7 (ferrous) and 8 (ferric) illustrate the influence of the chelators on peroxidation induced by 30 μM iron. The fluorescence contribution of the chelates

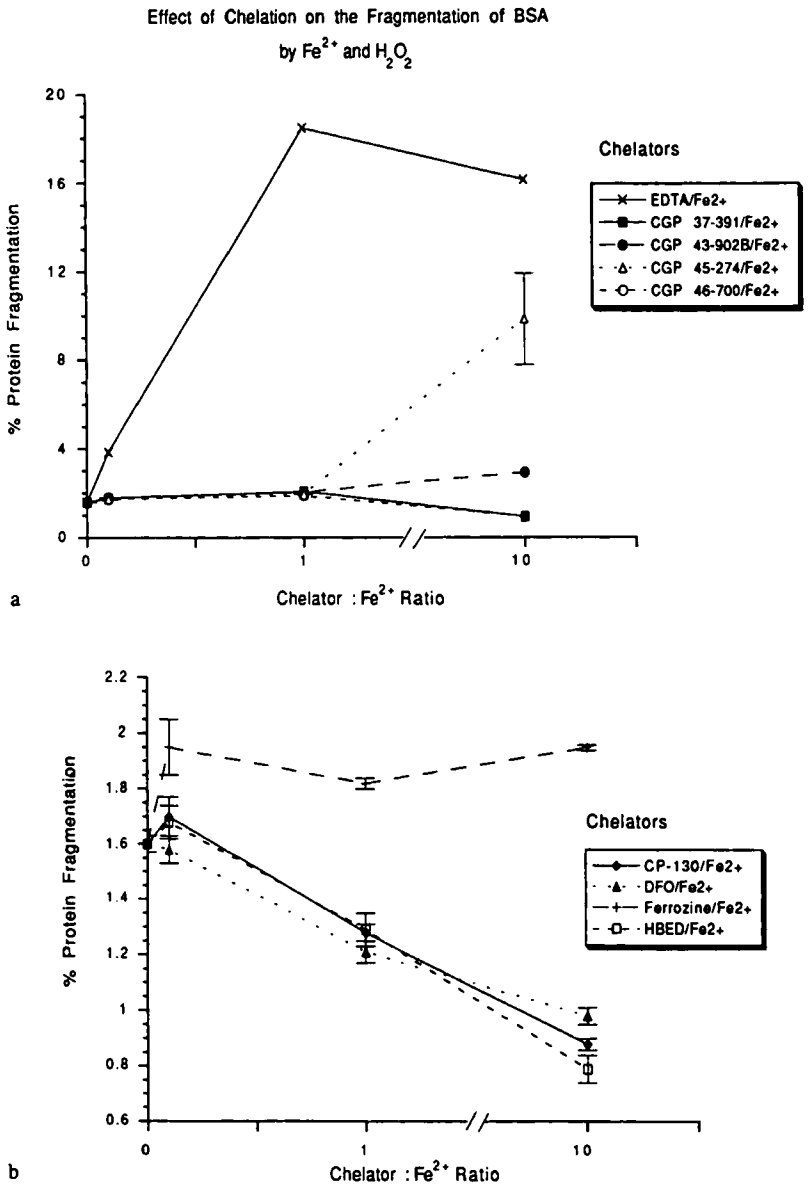

FIGURE 4a,b Protein fragmentation by 30 μM-ferrous iron and peroxide in the presence of a range of chelators. The graphs are presented with widely different scales on the y axis, for clarity and derive from a single experiment. The determinations were made as described in Methods.

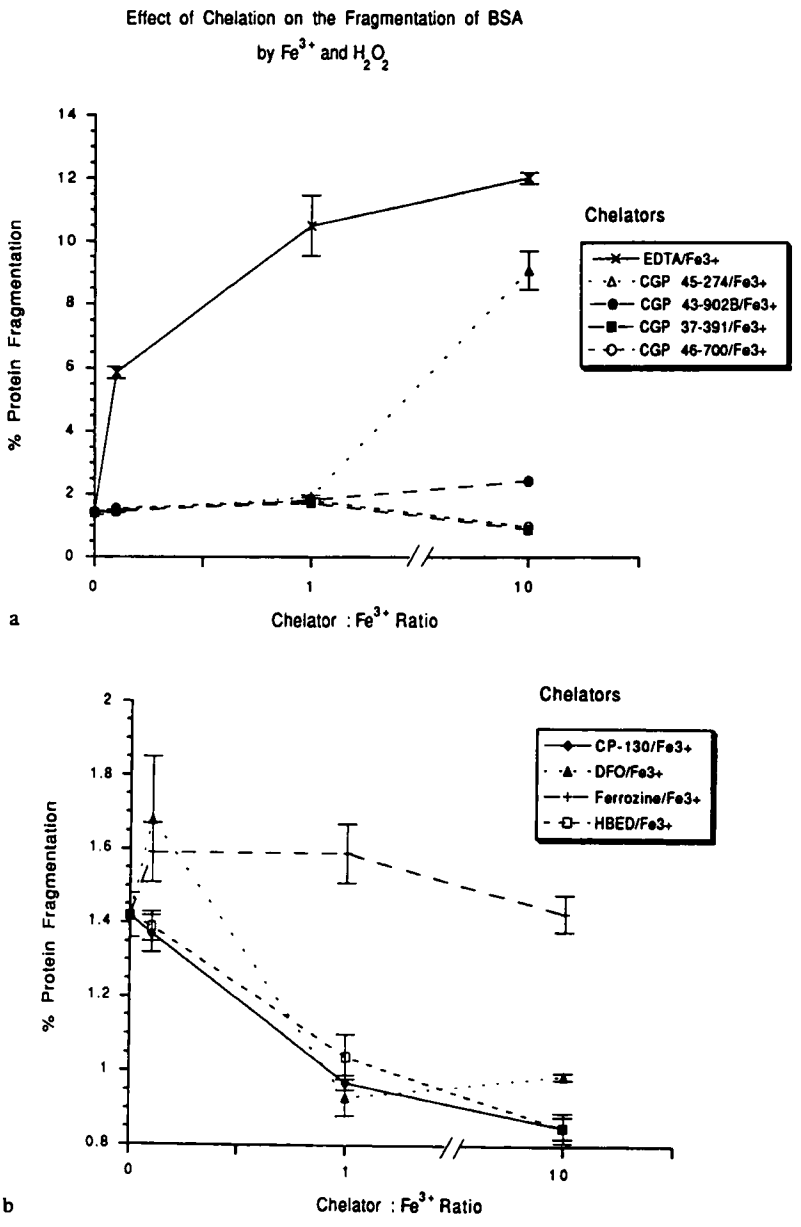

FIGURE 5a,b Protein fragmentation by 30 μM-ferric iron and peroxide in the presence of a range of chelators. The graphs (from a single experiment) have widely different y axis scales, for clarity, and the determinations were made as described in Methods.

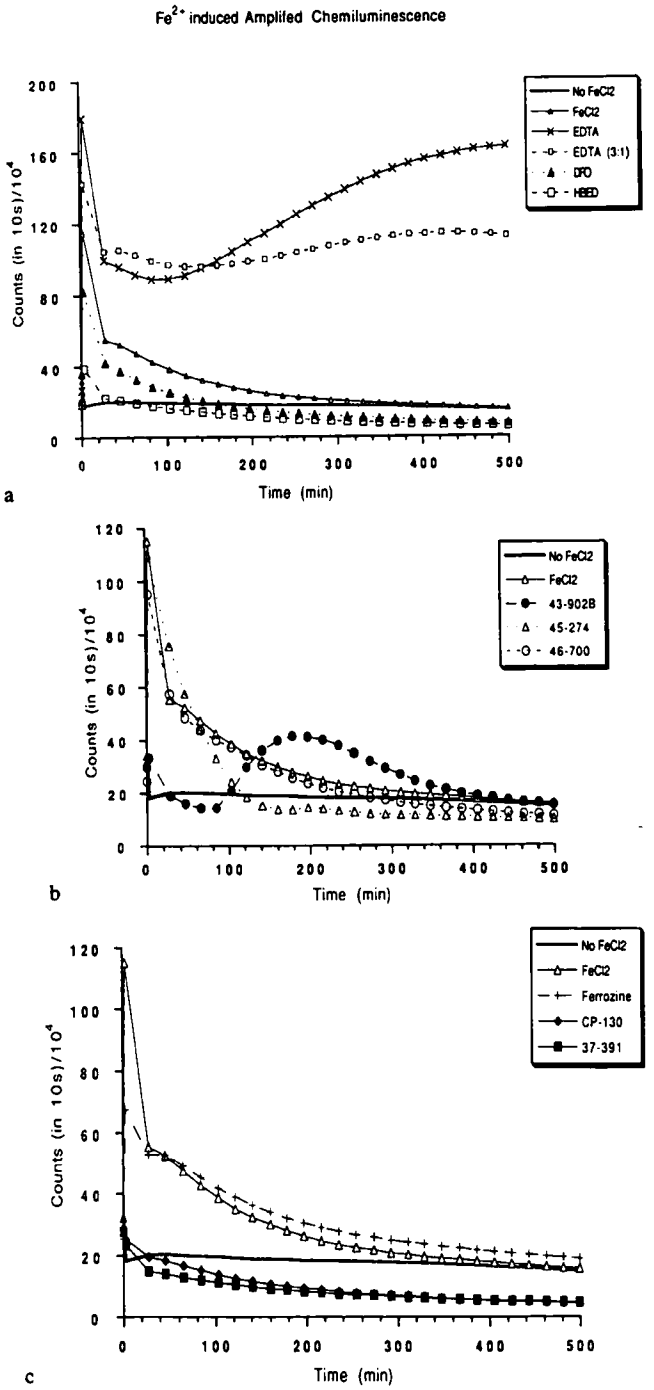

FIGURE 6a,b,c Kinetics of amplified chemiluminescence induced by the reaction of 300 μM-ferrous iron and hydrogen peroxide. Chelators were also at 300 μM, though EDTA was studied additionally at a 3:1 molar ratio with metal. The chemiluminescence was measured by scintillation counting in the out-of-coincidence mode, as detailed in Methods.

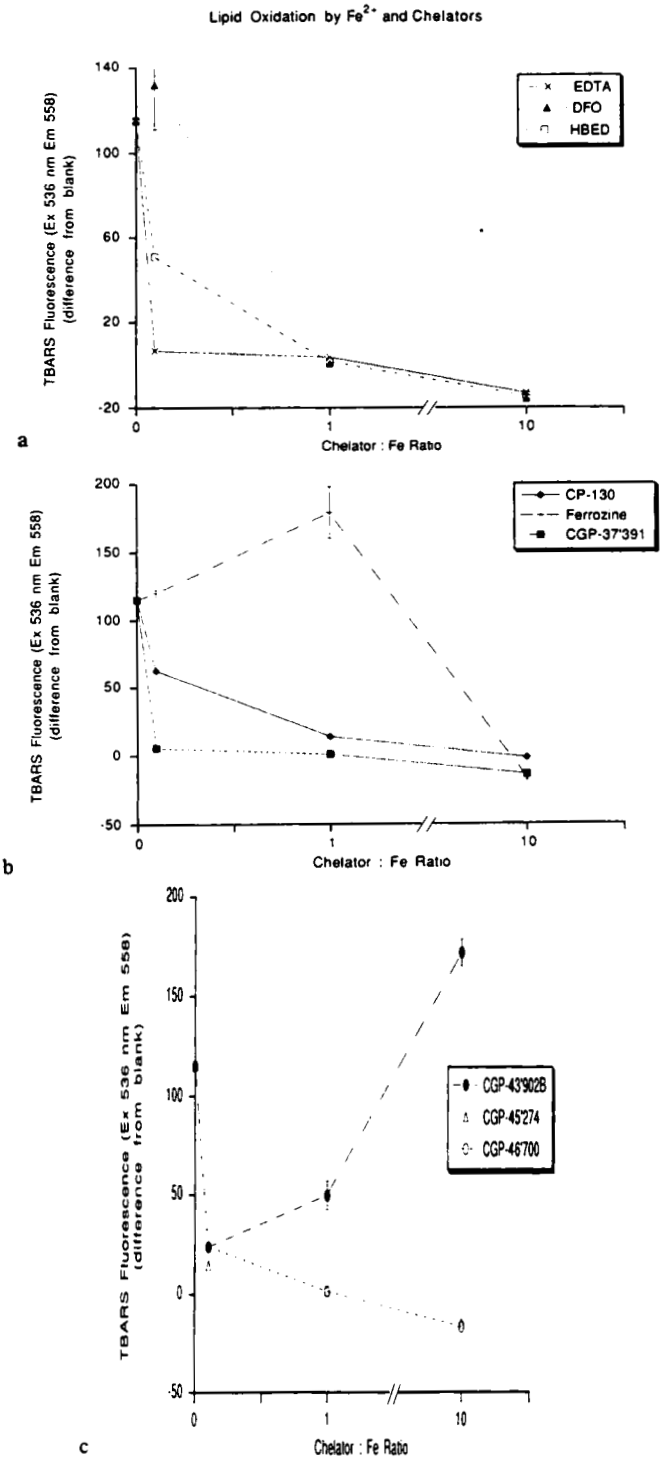

FIGURE 7a,b,c Lipid oxidation induced by 30 μM-ferrous iron, in the presence of various chelators. Results are expressed as the fluorescence difference between the experimental value and the blank incubation (without chelator). Details are defined in Methods.

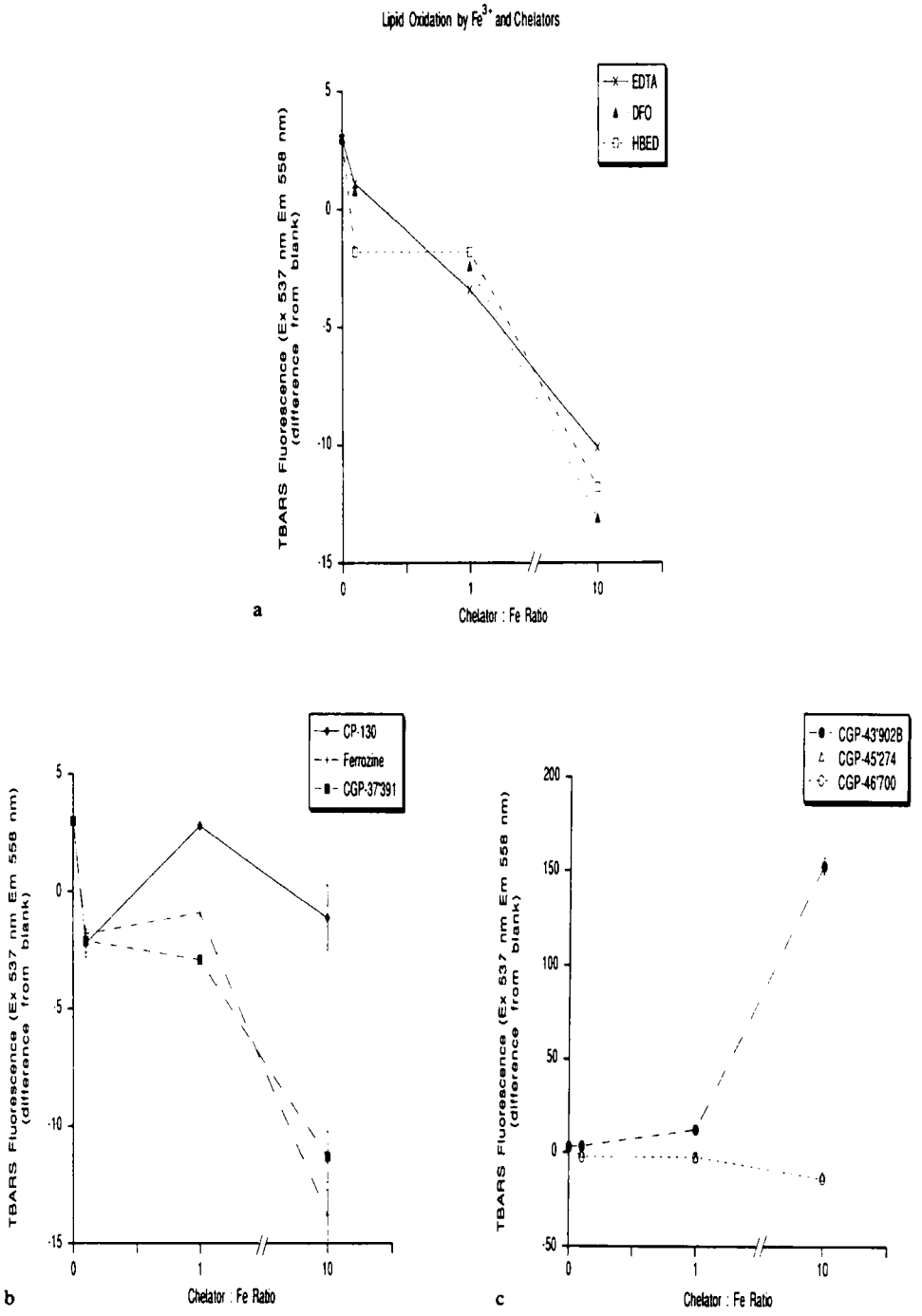

FIGURE 8a,b,c Lipid oxidation induced by 30 μM-ferric iron, in the presence of various chelators. Results are expressed as the fluorescence difference between the experimental value and the blank incubation (without chelator). Details are defined in Methods.

themselves was negligible, with the exception of CP 130 for which it was only slight. No corrections have been made. The data were obtained in several experiments in each of which the several chelators were studied at one concentration. The blank peroxidation fluorescence value (measured after incubations with no added iron) varied significantly between experiments, increasing with the storage of the membranes over a 2 week period; thus the values are presented with the relevant blank value subtracted, and so they can be positive or negative. Zero time values (unincubated) were virtually unaffected by the presence of iron and the various chelator concentrations, indicating that there is no significant variation in the completeness of generation of TBA-adducts.

More chelators (notably, EDTA) are inhibitory in this than the other systems, probably because metals are kept away from the weak binding sites available on the membrane. Ferrozine is again stimulatory at low C:Fe, though inhibitory at high. Surprisingly, with ferrous ions, pyridine-hydrazone inhibits at low C:Fe, but then progressively stimulates as C:Fe rises. With ferric ions, where the reaction is in any case very slight, pyridine-hydrazone stimulates progressively as C:Fe is raised. This effect is not a fluorescence artefact and may reflect some specialised binding between C:Fe complexes and membrane which is not shared by free metal. It is established that inhibition by lipophilic chelators (e.g. steroid conjugates) is greater than by their non-lipophilic congeners, and also depends on the tightness of their ferrous complexes[32]. Simulation could also result from the same factors.

f) Qualitative Observations on Chelator Destruction during Radical Generation

After prolonged incubation in the protein fragmentation system the initial visible colour of the chelator-metal complexes in many cases disappeared. A simple (visual) investigation established whether this reflected damage to the chelator by the radical fluxes or sequestration of the metal by the protein. EDTA was not studied since its complexes are not coloured. With the standard conditions of the fragmentation assay, but with 150 μM ferrous ion and 300 μM chelator, 3 incubations of each chelator were set up: one set was complete, one without BSA, and one without peroxide. CP130, desferal, HBED and L1 were not grossly decolourised after incubation, and thus were presumably not grossly degraded. However, pyridine-hydrazone, rhodotorulic acid and CP94 were decolourised in a peroxide dependent manner, but not by BSA alone: these were presumably radical damaged. Ferrozine was decolourised by peroxide alone, but more so by BSA alone, presumably indicating some loss of metal to the protein.

When fresh metal (at the original concentration) was added back to the peroxide-decolourised compounds ferrozine, pyridine-hydrazone, rhodotorulic acid or CP94, colour was not regenerated, confirming that these chelators had been chemically damaged. Colour could be restored in ferrozine which had been incubated with BSA and without peroxide, confirming that it was chemically intact in these circumstances.

These results again suggest desferal, CP130, HBED and to some extent L1 are stable protective compounds.

DISCUSSION

a) Mechanisms of Chelator Interference in Iron-Dependent Radical Reactions

There are many influences on these apparently simple *in vitro* systems. At physiological pH ferrous ion rapidly oxidises in oxygenated buffer, and much precipitates unless one of a limited range of ions (e.g. citrate) or chelators are present. In the chelator-free physiological buffer system (phosphate) used here, both oxidation and precipitation are pronounced 9. The modes of action of iron under these circumstances are still not entirely clear, and whether reactions of higher oxidation states participate, and roles of superoxide as well as peroxide, are still a matter of debate[16].

Most importantly for the present work, the actions of the chelators are themselves diverse and include:

i) maintaining the metal in solution (particularly in the case of ferrous ion)
ii) competing with the "target" molecules in the system for binding non-precipitated metal
iii) changing the redox potential and reactivity of the metal. These factors include its redox potential in the chelate, and also the ability of the metal in the chelate to undergo redox-cycling reactions with reducing radicals formed on other components of the system[33,34]. When complex molecules such as the "Good" buffers (Hepes, Mes etc) are present, these perturb the latter factor considerably[17,33] and hence they were avoided in this work, but inevitably the target "indicator" molecule can have such an effect.
iv) influencing the accessibility of the metal to water and other reactants. This is partly a matter of the number of ligand centres offered by the chelator (e.g. six with HBED, desferal and CP130, which can thus satisfy all the iron coordination sites; and smaller numbers with the other chelators).
v) undergoing reactions with the generated radical fluxes.

The literature tends to emphasise ii) particularly when considering reactions with proteins[30], and it is becoming increasingly obvious that it is an important factor in most systems. For example, we now know of complexes of iron with sugars, peptides, ascorbate and aromatic molecules[27].

Hexadentate ligands might be most effective in inhibiting metal dependent radical reactions because the equilibrium with metal would only comprise free metal and completely coordinated metal[27]. In contrast, the equilibria with bidentate ligands would include CM complexes in which 4 or 2 of the available iron coordination sites might be unoccupied by the chelator, and hence reactive. Such reactive "incomplete complexes" appear to be formed between EDTA and iron. Although EDTA is nominally hexadentate, it is too small to reach completely around the iron atom and leaves one or more coordination sites unoccupied. Hence the EDTA-iron chelates were reactive (i.e. damaging) in all systems even when the chelator was in large excess. All other hexadentate chelators which are of adequate size to properly chelate iron, namely HBED, desferal and CP130, are uniformly protective.

The systems give remarkably consistent results, generally with both ferrous and ferric (in spite of significant differences in association constants for the two valency states for some of the chelators). Only desferal, CP 130 and HBED inhibit all the damaging reactions. This may indicate the uniform importance of factor ii), and

that these three chelators are able to sequester metal away from most kinds of target molecule while other chelators are only able to do so from lipids.

b) Implications of the Results for Toxicology of the Chelators

It needs to be borne in mind that the toxicology of these chelators will depend on a range of factors. An incomplete list includes the following factors:

i) The reactivity and availability of the metal in the chelate in any given solution.
ii) The bioavailability and pharmacokinetics of the chelators, and their possible tissue-selective distribution[35]
iii) The efficiency with which the chelators gain metal from its pre-existent *in vivo* binding sites[31,37]. The degree of metal binding of the chelator may have a significant influence as may the concentration of competing metals such as copper and zinc[38].
iv) The degree of influence the chelators have on the distribution in space and time of the endogenous metal, being a cumulation of factors ii and iii.

The experiments conducted here only addressed issue i. Their predictive power must therefore be limited. But clearly one can expect some damaging reactions to be permitted by most of the chelators, and in some circumstances to be enhanced. On the other hand CP 130, desferal, and HBED might be expected to be hardly damaging *in vivo*, since no stimulatory actions were found *in vitro*. Their stability was also favourable in comparison with the other chelators. However, even with these chelators inhibition was incomplete in several systems.

A more complete understanding of the toxicology of the present molecules will probably depend particularly on the study of issues iii and iv above.

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