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SHORT COMMUNICATION

Comparative Radical Scavenging Ability of Bidentate Iron(III) Chelators

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ABSTRACT. Iron chelators can reduce radical damage inflicted on cells by two mechanisms, either direct scavenging of the radicals or by scavenging loosely bound iron which under aerobic conditions can generate radicals. Frequently it is not possible to distinguish between these two modes of action. 3-Hydroxypyridin-4-ones, in contrast to many iron(III) chelators are poor radical scavengers and therefore have potential in analysing mechanisms involved in biochemical and physiological processes which are centered on radical-induced cell injury. BIOCHEM PHARMACOL **55**;8:1327–1332, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. iron chelators; radical scavengers; hydroxyurea; hydroxypyridinones

Iron has been reported to induce radical damage to a range of tissues, particularly when subjected to ischaemic conditions [1, 2]. Thus as a class, iron chelators may have potential for the treatment of stroke [3], heart attack [4] and neurodegenerative diseases [5]. DFO† has been reported to reduce radical damage in a range of tissues [6] but there is some uncertainty as to the precise mode of action. There is no doubt that DFO is a potent chelator of iron(III); however, it is also a powerful scavenger of radicals [7, 8]. Similarly, 3-hydroxypyridin-4-ones are recorded as providing protection to the heart [9], hepatocytes [10, 11] and inflammed tissue [12]. Again the mode of action is not clear. In principle these chelators could act *via* radical scavenging or iron(III) scavenging. This paper sets out to compare the ability of a range of iron(III) chelators to quench ROS and RNS under well defined conditions. DPPH was selected as a convenient source of a nitrogen centred radical. It was hoped that a class of iron chelator could be selected with excellent iron(III) chelating ability, but with poor radical scavenging ability. Such a compound could greatly facilitate the present understanding of the mode of action of iron chelators under radical generating conditions.

The range of bidentate ligands indicated in Fig. 1 were selected because most have previously been used in biological experiments. DFO has been included as a well characterised standard. AHA and DHBA are the bidentate chelating elements of many synthetic and natural hexadentate ligands, including DFO. The pyridinones CP20 and CP08 have been selected as representatives of the 3-hydroxypyridin-4-one and 3-hydroxypyridin-2-one classes respectively. Maltol and 8-HQ are well characterised monobasic bidentate ligands. Although HU is generally considered as a radical scavenger, it contains the hydroxamate function and, therefore, is also an iron chelator. Phenol is included as a noniron chelating control.

MATERIALS AND METHODS

Materials

DFO was supplied from Ciba Geigy. CP20 and CP08 were synthesised as previously reported [13, 14]. Maltol was purchased from Pfizer. Ascorbic acid, deoxyribose, *N*-hydroxy-2-pyridinethione sodium salt, DPPH, HU, AHA, phenol, 8HQ, DHBA, trichloroacetic acid and thiobarbituric acid were purchased from Sigma Chemical Company.

DPPH Assay

The chelator to be tested, dissolved in ethanol, was added at various concentrations (10–200 μM) to an ethanolic solution of DPPH (final concentration 200 μM). The mixture (2 mL) was incubated at room temperature for 20 min and the absorbance recorded at 517 nm [15, 16]. Experiments without a chelator were used as controls. The radical quenching ability of a chelator was recorded as a percentage of the radical remaining after 20 min.

Interaction with ROS

For this study it was essential to select a source of ROS which was independent of iron or copper, as most of the

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† *Abbreviations:* AHA, acetohydroxamic acid; CP20, 1,2-dimethyl-3-hydroxypyridin-4-one; CP08, 1-carboxymethyl-3-hydroxypyridin-2-one; DFO, desferrioxamine; DHBA, 2,3-dihydroxybenzoic acid; DPPH, 1,1-diphenyl-2-picryhydrazine; 8HQ, 8-hydroxyquinoline; HU, hydroxyurea; NPG, *N*,*N*′-(5-nitro-1,3-phenylene) bisglutarimide; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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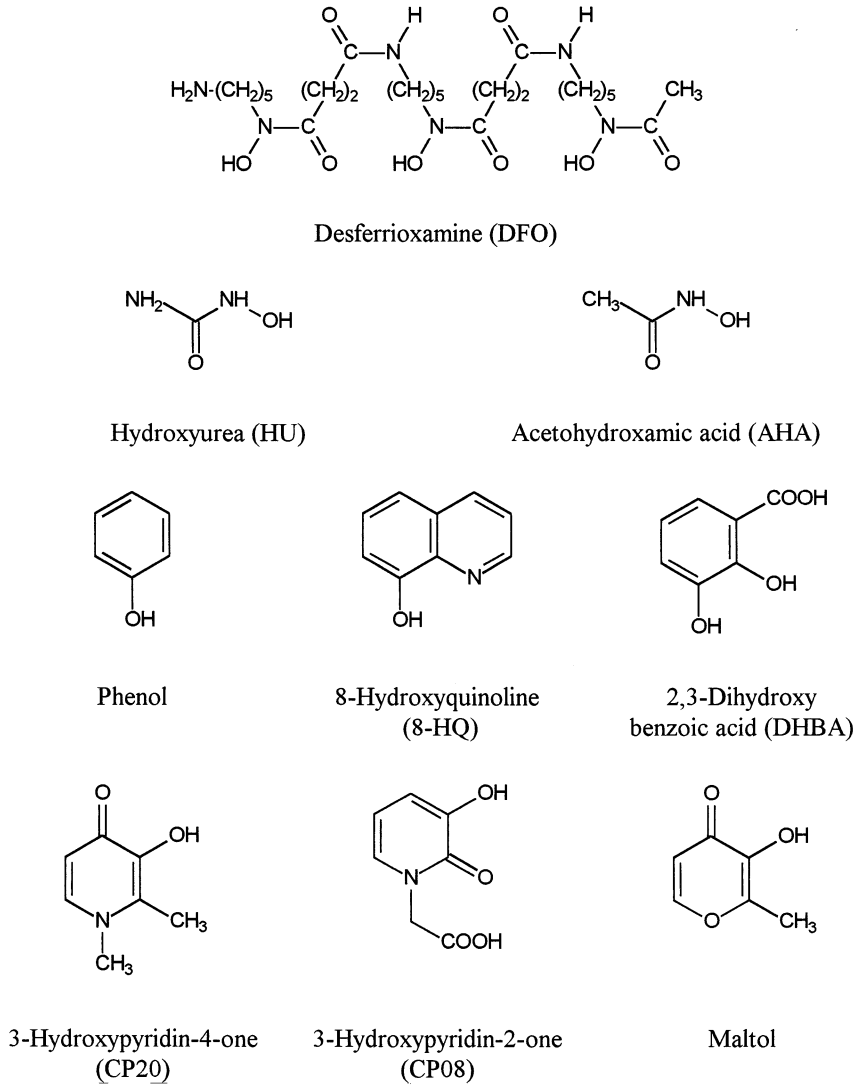


FIG. 1. Iron chelators used in this study.

compounds to be tested are metal binding agents. Initially, peroxynitrite was used a source for ROS and NPG was used as the radical trap [17]. However the hydroxyl radical was found to be only a minor breakdown product of peroxynitrite [18, 19], thus a more efficient source of ROS was sought. As the irradiation of *N*-hydroxy-2-pyridinethione by visible light has been shown to be a good source of hydroxyl radicals [20], we decided to utilise this system as a ROS source together with the deoxyribose detection assay. *N*-Hydroxy-2-pyridinethione (5 mM) was reacted with deoxyribose (10 mM) in the presence and absence of chelator (10 mM) in 50 mM of borate buffer at pH 8. The total reaction volume was 1 mL. The reaction tube was placed 13 cm in front of a 300-W light bulb in a steady stream of air for 4 hr during which time the temperature of the solution was maintained between 38 and 42°. The blank samples contained the same solution components, but were not exposed to radiation. After the reactions were complete, 740 μL of 2.8% (w/v) trichloroacetic acid and 740 μL of 1% (w/v) thiobarbituric acid in 50 mM of NaOH were added and the solutions vortexed and incubated at 100° for 10 min. Samples were cooled to room temperature before recording the absorbance of the solutions at 532 nm for the thiobarbituric acid-malonodialdehyde product chromophore. Ascorbic acid was not included in this assay as it decomposed when exposed to radiation, generating a coloured product. All other chelators included in this study were found to be stable at the level of radiation used.

Hydroxyurea pKa Determination

Equilibrium potentiometric analysis of HU was undertaken using an automated system [21]. A blank titration of 0.1 M of KCl 25 mL was carried out to determine the electrode zero using Gran's method [22]. A standard hydrogen electrode and a silver chloride electrode (Metrohm), were used to calibrate the electrode zero. The solution, 0.1 M of KCl 25 mL, which is contained in a jacketed titration cell, was acidified by 0.15 mL of 0.2 M of HCl. The titration was then carried out against 0.2 M of KOH using increments of 0.01 mL, dispensed from a Metrohm 665 dosimat. The solution was maintained at 25° under an argon atmosphere.

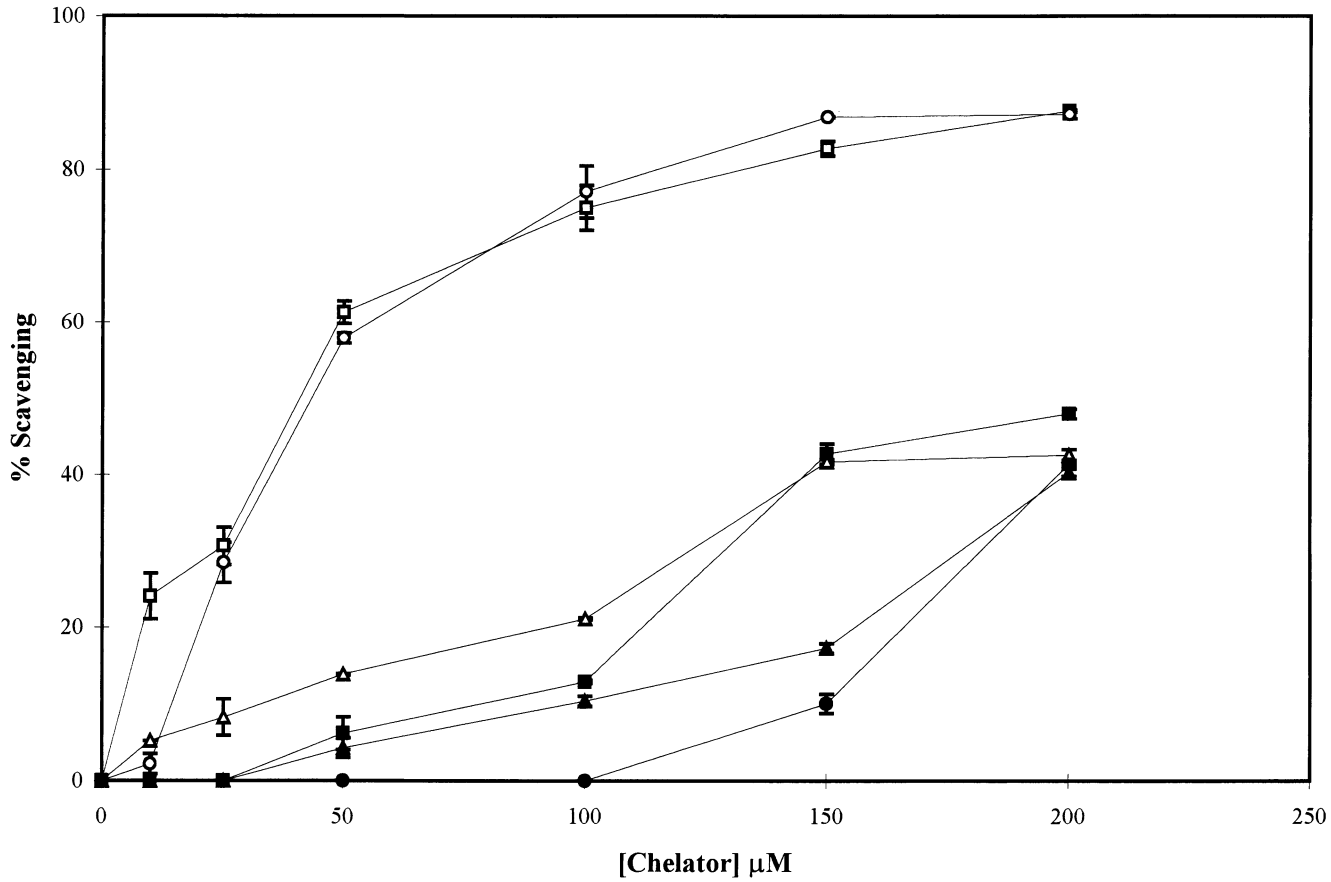


FIG. 2. DPPH radical scavenging by chelators. DPPH was incubated with chelators (10–200 μM). Radical quenching was assessed by quantifying the percentage of radical remaining after 20 min incubation. The concentration of DPPH radical was estimated spectrophotometrically at 517 nm. (□) DFO; (○) CP08; (△) AHA; (■) HU; (●) CP20; (▲) 8HQ. Values are mean ± SD (*N* = 4).

The above titration was repeated in the presence of HU (3.433×10^{-3} M). The data obtained from this titration were inserted into TITRFIT program [23].

HU-Iron(III) Affinity Constant Determination

The affinity constants of iron(III)-HU were determined by spectrophotometric titration using the automated system [21]. The iron(III) complex of HU was prepared in the presence of ligand (10^{-4} M) and iron(III) (10^{-5} M) in 0.1 M of KCl. The resulting spectrophotometric data were processed using STABOPT [23].

RESULTS

Characterisation of HU as a Iron(III) Chelator

The pKa value of HU was determined as 10.32 which is markedly higher than AHA (9.4). This relatively high value predicts that the chelation of iron(III) by HU will be subjected to a greater competition from protons than the analogous hydroxamate or pyridinone moieties (pKa ≈ 9.0). The K_1, K_2 and K_3 values for iron(III) were determined as 14.66, 12.67 and 7.56 respectively leading to a relatively high β_3 value of 34.9. This value is higher than the corresponding values for 3-hydroxypyridin-2-ones, 32 and AHA, 28 [24]. The high value of the β_3 constant associated with HU is somewhat misleading as the high pKa value effectively weakens the interaction with iron(III) at physiological pH values. Thus the pFe^{3+} value for HU is 18, the identical value to that of the hydroxyl anion [25]. As a result, HU is not capable of competing effectively with the hydroxyl anion in the pH range 5–8 and, consequently, the attempted formation of HU iron(III) complexes is accompanied with the simultaneous formation of iron hydroxide precipitates over this pH range.

Interaction with the DPPH Radical

A dose response investigation with the selected chelators (Fig. 1) was undertaken over the chelator concentration range 10–200 μM (Fig. 2). The most effective radical scavenger was found to be DFO, which was more effective than ascorbic acid in the concentration range 10–50 μM (for instance at 25 μM; DFO, 30.6 ± 2.45%; ascorbic acid, 14.6 ± 2.00%). The next most potent scavenger was found to be the 3-hydroxypyridin-2-one CP08, the inhibitory properties of which were virtually indistinguishable from DFO apart from the lowest concentration (Fig. 2). AHA and HU were the next most potent quenchers, but both were markedly less efficient than DFO and CP08 over the concentration range 25–200 μM. The remaining com-

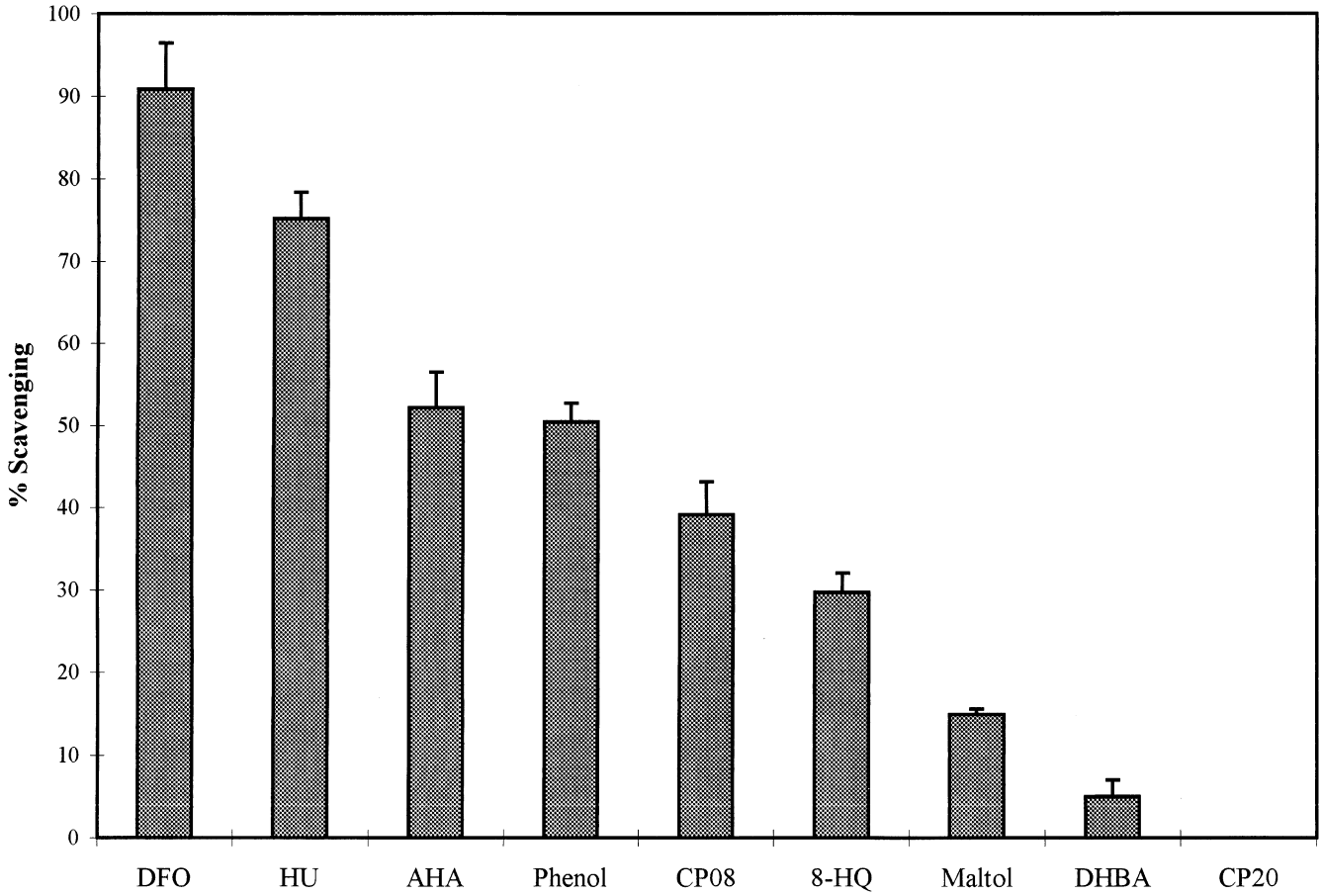


FIG. 3. Hydroxyl radical scavenging by chelators. Hydroxyl radicals were generated by the photolysis of N-hydroxy-2-pyridinethione and detected by the thiobarbituric acid assay. The chelators (10 mM) were exposed to hydroxyl radicals in 50 mM of sodium borate buffer (pH 8.0) at 40°. Values are mean ± SD (N = 4).

pounds including phenol, were found to be ineffective at concentrations ≤100 μM and only quenched appreciably at 200 μM. These are typified by CP20 response curve (Fig. 2).

Interaction with ROS

The most effective scavenger in this assay at the equi-molar ratio of deoxyribose to the inhibitor (iron chelator) tested, was again found to be DFO (Fig. 3). The 3-hydroxypyridin-2-one, CP08, possessed good activity but less than HU, AHA and phenol. Maltol and DHBA were very poor inhibitors and, surprisingly, the 3-hydroxypyridin-4-one CP20 was found to totally lack activity in the presence of deoxyribose. High concentrations of ligands are required to compete effectively for ROS.

DISCUSSION

As outlined in the introduction the aim of this investigation is to differentiate between the iron binding ability and radical scavenging ability of a range of well characterised chelators. We have used two classes of radical interaction, ROS and RNS. As expected DFO emerged as an outstanding quencher of both radical types (Fig. 2 and 3) possibly due to the stability of the hydroxamate radical **(1)** (see Fig. 4) [8, 26]. Significantly, AHA and HU are also potent DPPH radical scavengers, both are capable of forming a hydroxamate-type radical **(1)** and **(2)** respectively. The 3-hydroxypyridin-2-one (CP08) is equipotent with DFO in its ability to quench DPPH radicals (Fig. 2), presumably the corresponding radical **(3)** possesses similar properties to that of the hydroxamate radical **(1)**. Again with the ROS the most potent quenching molecules were the hydroxamate-containing chelators, DFO, AHA and HU. The 3-hydroxypyridin-2-one possessed good scavenging activity but was less efficient than the hydroxamates. The other chelators (8HQ, Maltol, DHBA and CP20) proved to be weak scavengers.

Although HU is well known as a radical scavenger [27] it is not generally considered to be an iron chelator, although substituted HUs form ternary complexes with metalloenzymes, for instance 5-lipoxygenase [28]. Surprisingly, the pKa value and affinity constant for iron(III) have not been previously reported. The observation that the affinity constant for iron(III) is greater for that of HU than for AHA is related to increased electron density on the carbonyl oxygen atom Fig. 1. The affinity for iron(III) is directly related to the electron density on the coordinating ligands

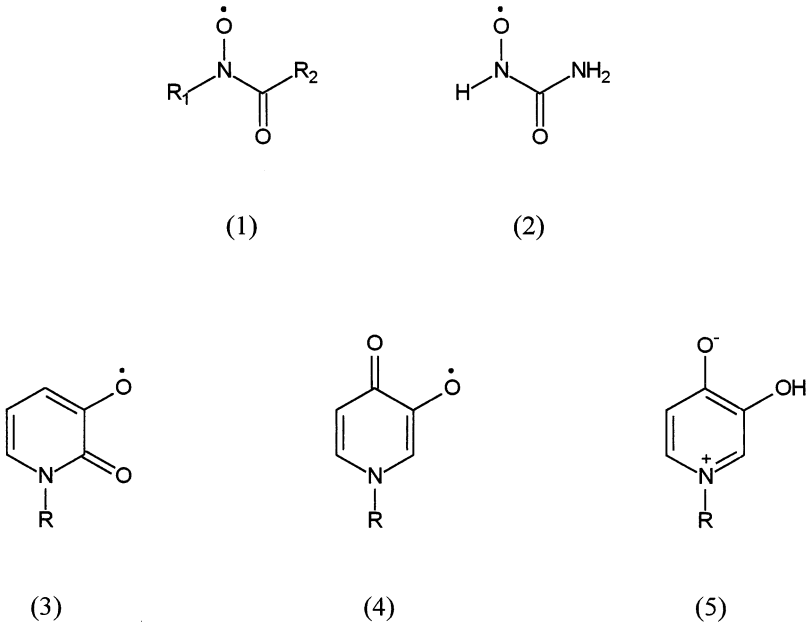
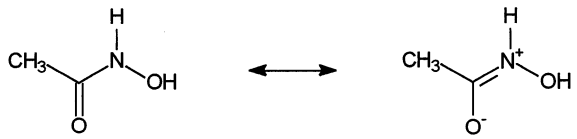


FIG. 4. Hydroxamate and pyridinone radicals.

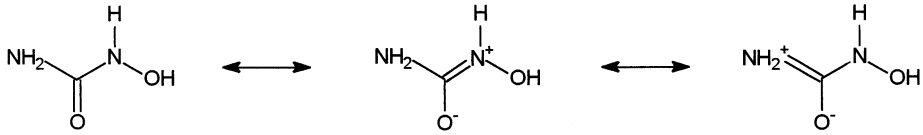
[24]. However by virtue of the relatively high pKa value (10.32), HU is not a good iron chelator in aqueous solution. Competition with protons at the active site of an enzyme is less critical than in free solution, which accounts for the ability of HU to form ternary complexes with certain iron-containing enzymes while not being able to scavange iron in aqueous solution in the pH range 6–8.

In contrast to hydroxamate-based chelators and 3-hydroxypyridin-2-ones (CP08), 3-hydroxypyridin-4-ones as typified by CP20, were found to be exceedingly weak radical scavengers. CP20 apparently failed to interact with ROS in the presence of deoxyribose even at concentrations of 10^{-2} M and it also only interacted with DPPH weakly at concentrations above 10^{-4} M. Clearly formation of the radical-type **(4)** must be unfavourable. A likely reason for the marked difference between the behaviour of the pyridin-2-one and pyridin-4-one structures, is unlike the former molecule, the dominant mesomeric form of the pyridin-4-one [13, 29] adopts a zwitterionic nature **(5)**. It is for this very same reason that 3-hydroxypyridin-4-ones are outstandingly good metal chelators, possessing a particularly high affinity for iron(III). This fortunate contrasting character of pyridin-4-ones, namely weak radical scavenging combined with avid iron(III) binding ability, endows this class of molecule with properties suitable for mechanistic investigation.

Recently it has been reported that 3-hydroxypyridin-4-



Acetohydroxamic acid



Hydroxyurea

SCHEME 1. Acetohydroxamic acid (AHA) possesses two mesomeric forms, only one of which involves delocalisation of electrons on the carbonyl oxygen atom, whereas hydroxyurea (HU) possesses three mesomeric forms, two of which involve delocalisation of electrons on the carbonyl oxygen atom.

ones are potent inhibitors of both 5-lipoxygenase [30] and ribonucleotide reductase [31]. In view of the findings reported herein it seems most probable that both inhibitory actions result from iron chelation and not from the direct quenching of free radicals generated at the active site of the respective enzymes.

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