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IRON–EDTA STIMULATED REDUCTION OF INDICINE *N*-OXIDE BY THE HEPATIC MICROSOMAL FRACTION, ISOLATED HEPATOCYTES, AND THE INTACT RAT

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Abstract—Fe(III) complexes of EDTA and diethylenetriamine pentaacetic acid (DETAPAC) at low concentrations (between 1 and 100 μM) produced up to a 20-fold increase in anaerobic microsomal NADPH- and NADH-dependent reduction of indicine *N*-oxide. Under aerobic conditions microsomal indicine *N*-oxide reduction was stimulated to half the levels seen under anaerobic conditions. EDTA alone was much less effective at stimulating indicine *N*-oxide reduction, while $FeCl_3$ alone had no effect on reduction. Other complexes of Fe(III) had little or no effect in stimulating microsomal indicine *N*-oxide reduction. Fe(III)–EDTA stimulated indicine *N*-oxide reduction by purified NADPH–cytochrome P-450 reductase and NADPH. It is probable that iron serves to transfer electrons between microsomal flavoprotein reductases and indicine *N*-oxide. The redox potential and the presence of an exchangeable ligand, such as water, in the inner ligand sphere of the iron complex are suggested to be important factors in determining which iron complexes will stimulate indicine *N*-oxide reduction. EDTA complexes of other transition metal ions do not stimulate indicine *N*-oxide reduction. Hydroxyl radicals, detected as the spin adduct of 5,5-dimethyl-1-pyroline-*N*-oxide, appear to be formed during Fe(II)–EDTA-dependent reduction of indicine *N*-oxide under anaerobic conditions. Fe(III)–EDTA at concentrations between 50 and 250 μM stimulated indicine *N*-oxide reduction by rat isolated hepatocytes up to 5-fold under anaerobic conditions and to half these values under aerobic conditions. By themselves, EDTA and $FeCl_3$ at similar concentrations produced a small stimulation of indicine *N*-oxide reduction by hepatocytes under anaerobic conditions. Fe(III)–EDTA stimulated indicine *N*-oxide reduction by murine leukemia P-388 cells under aerobic conditions and by rat caecal flora under anaerobic but not aerobic conditions. Fe(III)–EDTA, EDTA or $FeCl_3$ administered to rats produced a 3-fold increase in the 24-hr urinary excretion of indicine following an i.p. dose of indicine *N*-oxide.

Indicine *N*-oxide is a pyrrolizidine alkaloid *N*-oxide with antitumor activity in animals and man [1, 2]. Hepatic microsomal reduction of indicine *N*-oxide to indicine is mediated by cytochrome P-450 [3]. Preliminary experiments showed, however, that low (μM) concentrations of Fe(III)–EDTA produced a large increase in the microsomal reduction of indicine *N*-oxide to indicine. An increased reduction of *N*-oxides to their tertiary amines could have important consequences for the biological activity of these compounds. Pyrrolizidine alkaloid bases exhibit a greater biological reactivity and toxicity than their *N*-oxide derivatives [4]. In this paper, we report some characteristics of the stimulation by Fe(III)–EDTA and other chelates of indicine *N*-oxide reduction by microsomal enzymes, by isolated hepatocytes, and by the whole rat. A mechanism for Fe(III)–EDTA stimulated reduction of indicine *N*-oxide with formation of hydroxyl radicals is proposed.

MATERIALS AND METHODS

Enzyme preparations. Male rats of the Sprague–Dawley strain (Sprague–Dawley, Madison, WI), weighing between 150 and 200 g, were used. Animals were allowed free access to food and water at all times. Livers were removed and flushed retrogradely with 50 ml of cold 0.15 M KCl, and the hepatic microsomal fraction was prepared by ultracentrifugation following homogenization in 0.25 M sucrose as described by Ernster *et al.* [5]. The microsomes were washed by resuspension in 20 vol. of 0.15 M KCl and then collected by ultracentrifugation before being suspended in 0.15 M KCl at a concentration of 10 mg protein/ml. Protein was determined by the method of Lowry *et al.* [6], using crystalline bovine serum albumin as a standard. NADPH–cytochrome P-450 reductase (EC 1.6.2.4) was prepared from hepatic microsomes of phenobarbital-induced rats (80 mg/kg, i.p., for 3 days) by the method of Yasukochi and Masters [7].

Cell preparations. Hepatocytes were prepared from rat liver by the method of Stewart and Inaba [8]. Cell viability was assessed by exclusion of trypan blue and was typically 80–90%. Gut microflora were collected by suspending the caecal contents from a rat in 20 ml of 0.9% NaCl, filtering through 100 μm nylon mesh and adjusting the filtrate to a protein concentration of 10 mg/ml. Murine leukemia P-388 cells were grown in the ascitic form in male BDF_1 mice for 7 days following the i.p. injection of 10^6 P-338 cells. The ascitic fluid was aspirated and P-388 cells were washed twice in Dulbecco's phosphate-buffered saline.

Metabolism studies. Microsomal incubations contained 5 mg microsomal protein, 0.3 mmole Tris–

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HCl buffer (pH 7.4), 15 μmoles $MgCl_2$ and 3 μmoles indicine *N*-oxide in a final volume of 3 ml. The incubation mixture was preincubated with shaking for 10 min at 37° and the vessel was alternately evacuated and filled with the appropriate gas phase. High purity N_2 or CO was passed through a deoxygenating solution as described by Meites and Meites [9] or through an Oxy-Trap (Regis Chemical Co., Morton Grove, IL). The reaction was initiated by addition of 9 μM reduced pyridine nucleotide in 10 μl of degassed buffer and the reaction was terminated after 10 min by the addition of 5 ml chloroform. Incubations with whole cells contained hepatocytes (0.2×10^7 cells/ml), or P-388 cells (2×10^7 cells/ml), or caecal microflora (2 mg protein/ml) in 10 ml of Dulbecco's phosphate-buffered saline (pH 7.4) containing 10 mM glucose. The mixture was preincubated for 10 min at 37° under the appropriate gas phase and the reaction was started by addition of 1 mM indicine *N*-oxide. At 15-min intervals for 1 hr, 1 ml of incubation mixture was withdrawn and added to 5 ml of chloroform. The rate of indicine formation by hepatocytes was linear over 1 hr. Acid labile conjugates were not detected, indicating that indicine was liberated by hepatocytes as the free base. The rate of indicine formation was measured over the 1-hr incubation.

Electron spin resonance (e.s.r.) spectroscopy spin trap studies. Incubation mixtures contained 88 mM 5,5-dimethyl-1-pyroline-*N*-oxide (DMPO) and 3 mM indicine *N*-oxide in 50 mM potassium phosphate buffer (pH 7.4). The mixture was deoxygenated by bubbling with O_2-free N_2 for 20 min and the reaction was initiated by addition of a deoxygenated solution of $FeSO_4$–EDTA to give a final concentration of 0.1 mM. After a 10-min incubation at 37°, a sample of the reaction mixture was transferred under anaerobic conditions to an e.s.r. sample tube and e.s.r. measurements were made on a Bruker 410 ESR spectrometer. In studies employing NADPH–cytochrome P-450 reductase, the incubation mixture contained 1 unit/ml NADPH–cytochrome P-450 reductase in addition to DMPO and indicine *N*-oxide. The reaction was initiated by adding an anaerobic solution of $FeCl_3$–EDTA to give 0.1 mM, followed closely, where appropriate, by an anaerobic solution of NADPH to give 0.1 mM.

Whole animal studies. Rats received indicine *N*-oxide (400 mg/kg body weight, i.p.) and were placed in glass and stainless steel metabolism cages. Urine was collected for 24 hr.

Assays. Indicine *N*-oxide and indicine formed from indicine *N*-oxide were assayed by a gas chromatographic method previously described [10].

Drug and chemicals. Indicine *N*-oxide and razoxane were obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. NADPH and NADH were purchased from Boehringer Mannheim, Indianapolis, IN. Ethylenedinitrilotetraacetic acid, disodium salt (EDTA) was obtained from Mallinckrodt, St. Louis, MO; cyclohexanediamine tetraacetic acid, disodium salt (CDTA) from Hach Chemical, Ames, IA: and diethylenetriamine pentaacetic acid (DETAPAC), ADP and DMPO from the Sigma Chemical Co., St. Louis, MO. DMPO was purified by distillation under reduced pressure. Desferoxamine mesylate (DFO) was obtained from Ciba-Geigy, Summit, NJ; 2,2'-dipyridyl from the Aldrich Chemical Co., Milwaukee, WI; and 1,10-phenanthroline from the Fisher Scientific Co., Fair Lawn, NJ. All other reagents were the purest grade available.

RESULTS

Microsomal reduction. Fe(III)–EDTA at concentrations as low as 10 μM produced up to a 20-fold increase in the rate of NADPH-dependent reduction of indicine *N*-oxide by the hepatic microsomal fraction under anaerobic conditions (Fig. 1). One micromolar Fe(III)–EDTA produced a 3-fold increase in indicine *N*-oxide reduction. EDTA by itself was much less effective at promoting reduction and gave a maximum 3-fold stimulation at 500 μM EDTA. $FeCl_3$ did not stimulate microsomal indicine *N*-oxide reduction. Hepatic microsomal indicine *N*-oxide reduction is inhibited by oxygen and is normally undetectable under aerobic conditions [3]. However, under aerobic conditions in the presence of Fe(III)–EDTA, indicine *N*-oxide reduction was stimulated to almost 50 per cent of the increased values seen with Fe(III)–EDTA under anaerobic conditions. EDTA by itself failed to stimulate aerobic indicine *N*-oxide reduction. NADH is almost equally effective as NADPH in supporting hepatic microsomal indicine *N*-oxide reduction [3]. Fe(III)–EDTA and EDTA produced similar effects on the NADH-dependent microsomal reduction of indicine *N*-oxide, as were seen with NADPH-dependent reduction.

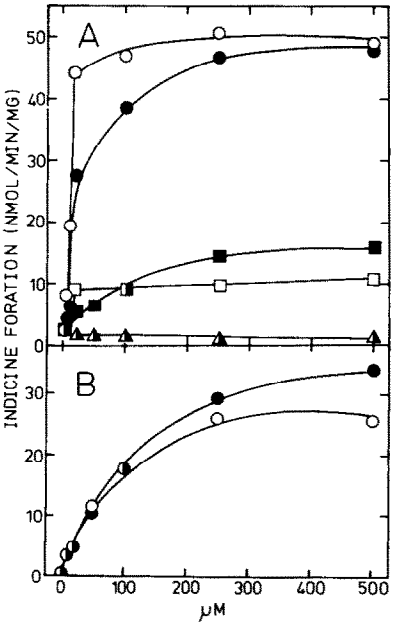

Fig. 1. Effects of Fe(III), EDTA and Fe(III)–EDTA at various concentrations on hepatic microsomal reduction of indicine *N*-oxide under anaerobic conditions (A) and under aerobic conditions (B). Open symbols: with NADPH as cofactor; closed symbols: with NADH as cofactor. Key: (◑) Fe(III)–EDTA; (◨) EDTA; and (▲) $FeCl_3$.

Table 1. Effect of phenobarbital pretreatment and CO on stimulation of indicine *N*-oxide reduction by Fe(III)–EDTA*

	NADPH (nmoles·min^{-1}·mg^{-1})	NADPH + Fe(III)–EDTA (nmoles·min^{-1}·mg^{-1})
Microsomes		
Control N_2	3.9 ± 0.1	44.8 ± 4.8†
CO	0.8 ± 0.0‡	35.3 ± 6.0†
Phenobarbital N_2	12.9 ± 4.1	172.6 ± 8.2†
CO	1.8 ± 0.2‡	140.9 ± 9.2†
NADPH–cytochrome P-450 reductase N_2	0.0 ± 2.7	259.5 ± 22.0†
CO	0.0 ± 0.5	162.4 ± 31.0†,‡

* Each value is the mean ± S.E.M. of four determinations. Fe(III)–EDTA was present at 10^{-4} M, and NADPH–cytochrome P-450 reductase at 0.15 units.
† $P < 0.05$ compared to the appropriate control without Fe(III)–EDTA.
‡ $P < 0.05$ compared to the appropriate control under N_2.

In the absence of iron chelates the hepatic microsomal reduction of indicine *N*-oxide is mediated almost exclusively by cytochrome P-450 and is inhibited by carbon monoxide [3]. Carbon monoxide had no significant effect on Fe(III)–EDTA stimulated reduction of cytochrome P-450 (Table 1). Phenobarbital pretreatment, which increases microsomal cytochrome P-450 and NADPH–cytochrome P-450 reductase, increased the extent of stimulation of indicine *N*-oxide reduction by Fe(III)–EDTA. The increased rate of reduction was not inhibited significantly by carbon monoxide. Indicine *N*-oxide was not reduced by purified NADPH–cytochrome P-450 reductase and NADPH unless Fe(III)–EDTA was present. Carbon monoxide produced a small but significant inhibition of Fe(III)–EDTA-dependent indicine *N*-oxide reduction by NADPH–cytochrome P-450 reductase.

The ability of some other iron chelates to stimulate indicine *N*-oxide reduction is shown in Table 2. Only Fe(III)–EDTA, Fe(III)–DETAPAC, and to a lesser extent Fe(III)–CDTA stimulated reduction. The effect of EDTA complexes of some first series transition metals on microsomal indicine *N*-oxide reduction is shown in Table 3. Only Fe(III)–EDTA stimulated indicine *N*-oxide reduction.

* G. Powis and C. DeGraw, unpublished observations.

Hepatocytes and other cells. The reduction of indicine *N*-oxide by isolated hepatocytes is linear for at least 1 hr under anaerobic conditions and is completely inhibited by low levels of oxygen.* Fe(III)–EDTA stimulated the reduction of indicine *N*-oxide by hepatocytes up to 5-fold at 250 μM Fe(III)–EDTA under anaerobic conditions and to almost half this value under aerobic conditions (Fig. 2). EDTA by itself produced only a small stimulation of indicine *N*-oxide reduction by hepatocytes under anaerobic conditions and had no effect under aerobic conditions. Low concentrations of $FeCl_3$ (50 μM) produced a small increase in indicine *N*-oxide reduction under aerobic and anaerobic conditions but this effect disappeared at higher concentrations of $FeCl_3$.

Fe(III)–EDTA stimulated indicine *N*-oxide reduction by murine leukemia P-388 cells *in vitro* under both anaerobic and aerobic conditions (Fig. 3). Gastrointestinal microflora also reduce indicine *N*-oxide to indicine under anaerobic conditions [11]. Fe(III)–EDTA stimulated indicine *N*-oxide reduction by gastrointestinal flora over 5-fold under anaerobic conditions but had relatively little effect in promoting reduction under aerobic conditions (Fig. 4).

Whole animal studies. The effect of Fe(III) and EDTA upon the ability of the whole animal to reduce indicine *N*-oxide to indicine was measured as the

Table 2. Stimulation of microsomal indicine *N*-oxide by Fe(III) complexes*

	Cofactor	
	NADPH (nmoles·min^{-1}·mg^{-1})	NADH (nmoles·min^{-1}·mg^{-1})
Control	2.5 ± 0.2	2.2 ± 0.2
Fe(III)–EDTA	69.5 ± 1.5	65.6 ± 2.5
Fe(III)–DETAPAC	59.8 ± 2.3	62.3 ± 4.0
Fe(III)–CDTA	11.9 ± 1.5	9.5 ± 0.9
Fe(III)–pyrophosphate	2.1 ± 0.2	1.2 ± 0.1
Fe(III)–razoxane	0.3 ± 0.1	2.6 ± 0.1
Fe(III)–ADP	1.9 ± 0.7	1.7 ± 0.1
Fe(III)–DFO	1.5 ± 0.1	1.6 ± 0.1
Fe(III)–bipyridyl	2.5 ± 0.1	2.0 ± 0.2
Fe(III)–phenanthroline	3.7 ± 0.4	1.7 ± 0.2

* Each value is the mean ± S.E.M. of six determinations. All compounds were at 10^{-4} M.

Table 3. Effect of metal ions on EDTA stimulated microsomal indicine *N*-oxide reduction*

	NADPH (nmoles·min^{-1}·mg^{-1})	NADH (nmoles·min^{-1}·mg^{-1})
Control	2.7 ± 0.2	2.0 ± 0.2
Fe(III)	60.4 ± 2.0	58.4 ± 2.7
Cu(II)	2.2 ± 0.1	2.5 ± 0.1
Co(II)	2.2 ± 0.2	2.8 ± 0.2
Ni(II)	2.6 ± 0.3	3.2 ± 0.1
Mn(II)	2.1 ± 0.4	2.1 ± 0.2

* Each value is the mean ± S.E.M. of four determinations. EDTA and metal ions were present at 10^{-4} M.

24-hr urinary excretion of indicine following a dose of 400 mg indicine *N*-oxide/kg body weight. The doses of Fe(III)–EDTA, Ca–EDTA and $FeCl_3$ chosen were half the reported LD_{50} for these agents in mice [12–14]. Fe(III)–EDTA produced over a 3-fold increase in the 24-hr urinary excretion of free indicine (Table 4). Ca–EDTA and $FeCl_3$ produced slightly less than a 3-fold increase in free indicine excretion. None of the agents had a significant effect on the 24-hr urinary excretion of unchanged indicine *N*-oxide.

Electron spin resonance spin trap studies. The spin trap DMPO was used to study the formation of free radicals during the reduction of indicine *N*-oxide by Fe(II)–EDTA under anaerobic conditions. An e.s.r. signal consisting of a 1:2:2:1 quartet was seen which was similar to that formed from DMPO in the presence of an anaerobic hydroxyl radical generating system of Fe(II) and H_2O_2 (Fig. 5). This suggests that the signal seen during the Fe(II)–EDTA catalyzed reduction of indicine *N*-oxide was due to the DMPO–OH radical adduct. No spin adduct signal was seen using $FeSO_4$ or $FeCl_3$ and indicine *N*-oxide and DMPO, or using indicine, Fe(II)–EDTA and DMPO. A similar spin adduct signal was seen when NADPH–cytochrome P-450 reductase was incubated with Fe(III)–EDTA, indicine *N*-oxide and DMPO in the presence, but not in the absence, of NADPH. No spin adduct signal was seen with any of the reaction mixtures in the absence of either indicine *N*-oxide or DMPO.

DISCUSSION

The greatly enhanced microsomal reduction of indicine *N*-oxide in the presence of Fe(III)–EDTA is probably due to the iron complex acting as an intermediate in the electron transfer from microsomal flavoprotein reductases to indicine *N*-oxide. This is suggested by the ability of Fe(III)–EDTA to stimulate the reduction of indicine *N*-oxide by purified NADPH–cytochrome P-450 reductase. Free Fe(III) is only slowly reduced by flavoenzymes such as NADPH–cytochrome P-450 reductase, possibly

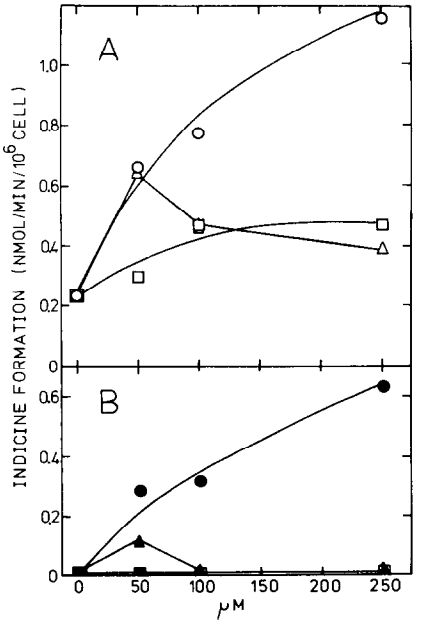

Fig. 2. Effects of Fe(III)–EDTA and EDTA at various concentrations on indicine *N*-oxide reduction by isolated hepatocytes under anaerobic conditions (A) and under aerobic conditions (B). (◑) Fe(III)–EDTA; (◨) EDTA; and (▲) $FeCl_3$.

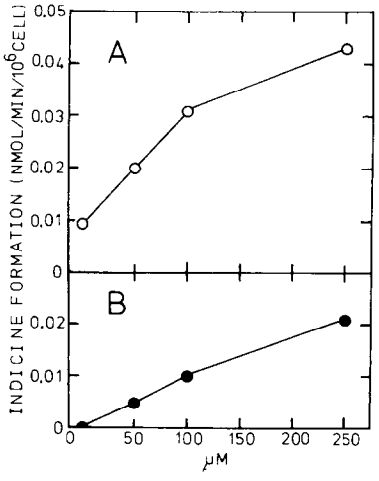

Fig. 3. Effect of Fe(III)–EDTA concentration on indicine *N*-oxide reduction by murine leukemia P-388 cells. Key: (○) anaerobic conditions, and (●) aerobic conditions.

Table 4. *In vivo* metabolism of indicine *N*-oxide*

Treatment	N	24-hr Urinary excretion (% dose) Indicine *N*-oxide	Indicine
Control	6	18.2 ± 5.5	5.3 ± 1.2
Fe(III)–EDTA (10 mg/kg)	3	15.9 ± 6.9	17.4 ± 5.4†
Ca–EDTA (250 mg/kg)	3	21.3 ± 1.1	12.8 ± 1.3†
$FeCl_3$ (34 mg/kg)	3	15.2 ± 4.7	15.2 ± 2.0†

* Rats were injected i.p. with indicine *N*-oxide (400 mg/kg), and urine was collected for 24 hr. Each value is the mean ± S.E.M. of N animals, expressed as a per cent of the dose administered.
† $P < 0.05$.

due to the low solubility of Fe(III) at neutral pH. Complexation of iron leads to increased rates of reduction due to increased solubility of the iron [15]. Indicine *N*-oxide is known to be reduced to indicine by Fe(II) [16]. It is well known that the reducing power of Fe(II) is enhanced by complexation by EDTA [17]. EDTA chelates Fe(III) more strongly than Fe(II), which results in a decrease in the redox potential of the Fe(III)/Fe(II) couple from +0.75 to +0.12 V. Complexation by DETAPAC and CDTA results in a lowering of the Fe(III)/Fe(II) redox potential to +0.09 and +0.08 V respectively [17]. Although lowering of the redox potential probably plays an important role in the Fe(II) complex promoted reduction of indicine *N*-oxide, it is unlikely to be the only factor involved. Differences in the stereochemistry of the iron complexes may also be important. Fe(III)–CDTA is much less effective in stimulating the microsomal reduction of indicine *N*-oxide than either Fe(III)–EDTA or Fe(III)–DETAPAC. Aqueous EDTA [18] and DETAPAC [19] iron complexes contain a water molecule in the inner ligand sphere, while the CDTA complex does not [19, 20]. Electron transfer between *N*-oxides and iron complexes is likely to involve an inner ligand sphere reaction. The *N*-oxide must occupy an inner ligand sphere position and probably does so by exchanging with the weaker water ligand [21]. With CDTA there is no water ligand and indicine *N*-oxide can enter the inner ligand sphere only by displacing one of the ligand groups of the sexadentate CDTA moiety, a thermodynamically unfavorable reaction. Fe(III)–ADP has one or more weak inner sphere ligands which could probably be displaced by indicine *N*-oxide, but the reduction potential, theoretically calculated to be +0.72 V from the data of Taqui-Khan and Martell [22] and the Irving–Williams

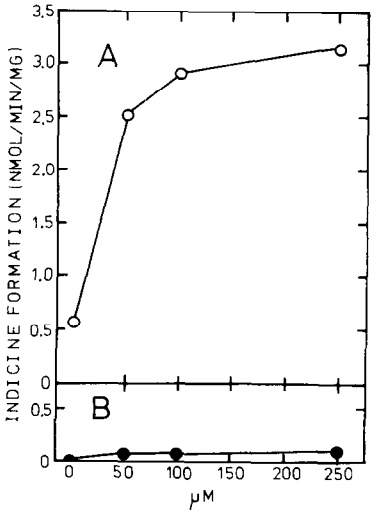

Fig. 4. Effect of Fe(III)–EDTA concentration on indicine *N*-oxide reduction by rat caecal microflora under anaerobic conditions (○) and aerobic conditions (●).

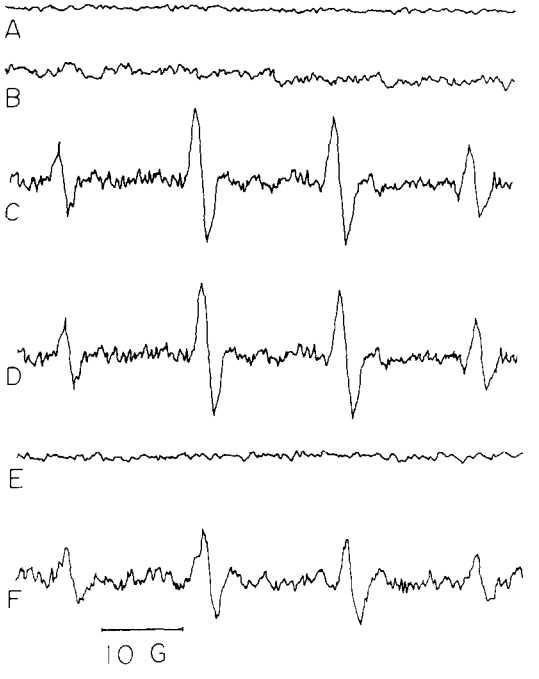

Fig. 5. DMPO spin adducts formed during iron promoted reduction of indicine *N*-oxide. Electron spin resonance spectrometer settings: field 3351G, microwave frequency 9.38 GHz, and field modulation 1.0 Gpp. Signals are the average of sixteen individual scans. Key (A) 0.1 mM Fe(II)–EDTA; (B) 3 mM indicine *N*-oxide; (C) 0.1 mM Fe(II)–EDTA and 3 mM indicine *N*-oxide; (D) 0.1 mM $FeSO_4$ and 30 μM H_2O_2; (E) 0.1 mM Fe(III)–EDTA, 1 Unit/ml NADPH cytochrome P-450 reductase and 3 mM indicine *N*-oxide; and (F) 0.1 mM Fe(III)–EDTA, 1 Unit/ml NADPH–cytochrome P-450 reductase, 3 mM indicine *N*-oxide and 0.1 mM NADPH.

order, is evidently too high to allow the reduction of indicine *N*-oxide. Complexes of Cu(II), Co(II), Ni(II) and Mn(II) with EDTA do not stimulate indicine *N*-oxide reduction because indicine *N*-oxide does not bind to the central cation [21].

It was a striking finding that reduction of indicine *N*-oxide was observed under aerobic conditions when Fe(III)–EDTA was added to microsomal or hepatocyte preparations at rates almost half those seen under anaerobic conditions. The reduction of indicine *N*-oxide by microsomal cytochrome P-450 and by hepatocytes in the absence of Fe(III)–EDTA is completely inhibited under aerobic conditions. Fe(II)–EDTA is readily oxidized by air to Fe(III)–EDTA [23], but clearly competition by O_2 with indicine *N*-oxide for reduction by EDTA–Fe(II) is much less effective than with cytochrome P-450. Carbon monoxide, which inhibits microsomal cytochrome P-450-dependent reduction of indicine *N*-oxide, did not significantly inhibit Fe(III)–EDTA stimulated microsomal reduction of indicine *N*-oxide. It should be noted, however, that carbon monoxide produced a small but significant inhibition of Fe(III)–EDTA-dependent reduction of indicine *N*-oxide by purified NADPH–cytochrome P-450 reductase. Carbon monoxide is known to bind weakly to reduced iron complexes [24].

Possible mechanisms of reduction of *N*-oxides by iron have been reviewed [25–29]. Ferris *et al.* [30] have presented evidence for the formation of an amminium cation free-radical intermediate during Fe(II) catalyzed decomposition of tertiary amine oxides to produce tertiary and secondary amines and aldehyde. Mattocks [27, 28] has suggested that Fe(II) catalyzed conversion of pyrrolizidine *N*-oxides to pyrrolizidine base and pyrrole might also be explained by a free-radical mechanism involving an amminium cation radical. We have so far been unable to detect the formation of such a radical by e.s.r. spectroscopy during Fe(II)–EDTA-dependent reduction of indicine *N*-oxide. Pyrrolizidine *N*-oxide alkaloids are weakly basic in aqueous solution but can undergo protonation of the oxygen atom [31]. A protonated *N*-oxide is isoelectronic with H_2O_2. We reasoned, therefore, that the reaction between indicine *N*-oxide and Fe(II)–EDTA might be similar to the well-known Fenton reaction which leads to the production of hydroxyl radical (OH·) [32]. In the presence of DMPO, an e.s.r. signal not inconsistent with that of DMPO–OH, the radical adduct, was observed during Fe(II)–EDTA catalyzed reduction of indicine *N*-oxide under anaerobic conditions. A proposed mechanism for the reduction of indicine *N*-oxide, and perhaps other pyrrolizidine alkaloid *N*-oxides, by Fe(II)–EDTA is shown in Fig. 6.

Fe(III)–EDTA stimulated indicine *N*-oxide reduction by the microsomal fraction, by isolated hepatocytes, and by the whole animal. EDTA by itself was much less effective at stimulating indicine *N*-oxide reduction by the microsomal fraction and presumably must complex with free iron in the incubation media or within the cells to produce an effect. EDTA administered to the whole animal was almost as effective as Fe(III)–EDTA in increasing the urinary excretion of indicine. $FeCl_3$ did not stimulate microsomal indicine *N*-oxide reduction but, at low

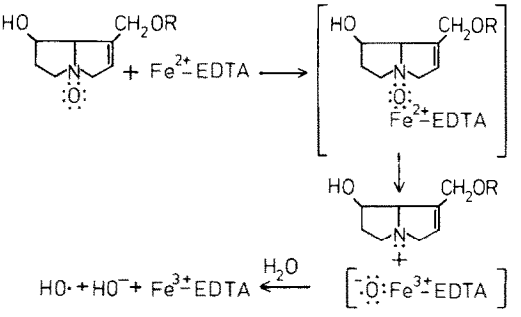

Fig. 6. Mechanism for reduction of indicine *N*-oxide by Fe(II)–EDTA. R = $C_7H_{12}O_3$. Brackets represent proposed intermediates.

concentrations, stimulated indicine *N*-oxide reduction by hepatocytes. $FeCl_3$ administered to the whole animal stimulated urinary indicine excretion. It is probable that Fe(III) was complexed by endogenous chelators that could catalyze the transfer of electrons from intracellular reductases to indicine *N*-oxide. Such endogenous chelators would probably not include ADP or pyrophosphate since Fe(III) chelates of both of these compounds failed to stimulate microsomal indicine *N*-oxide reduction. Hepatocytes were not the only cells in which Fe(III)–EDTA stimulated the reduction of indicine *N*-oxide. The effect was seen with leukemia P-388 cells under aerobic and anaerobic conditions and with gut microflora, also a site of indicine *N*-oxide reduction [11], under anaerobic but not aerobic conditions.

The biological consequences of an increase in indicine *N*-oxide reduction by Fe(III)–EDTA and $FeCl_3$ are not known. Conversion to indicine does not appear to be essential for the antitumor activity of indicine *N*-oxide [1] but may be the cause of hepatotoxicity seen in patients receiving high doses of indicine *N*-oxide [2]. Possible changes in the therapeutic index of indicine *N*-oxide accompanying changes in the metabolic profile of the drug with Fe(III)–EDTA and $FeCl_3$, and the role hydroxyl radicals might play in the biological activity of indicine *N*-oxide, are under investigation in our laboratory.

In summary, iron chelates of EDTA and DETAPAC at concentrations around 10^{-4} M produced up to a 20-fold increase in microsomal NADPH- and NADH-dependent reduction of indicine *N*-oxide under anaerobic and aerobic conditions. Other iron complexes had little or no effect. Fe(III)–EDTA and Fe(III)–DETAPAC stimulated indicine *N*-oxide reduction by isolated hepatocytes, leukemia P-388 cells, and rat caecal microflora. Fe(III)–EDTA, EDTA and $FeCl_3$ stimulated indicine formation by intact rats given indicine *N*-oxide. A mechanism is proposed to account for the apparent formation of hydroxyl radicals during Fe(II)–EDTA catalyzed reduction of indicine *N*-oxide to indicine under anaerobic condtions.

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