Iron-induced DNA damage and synthesis in isolated rat liver nuclei

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Incubation of iron with isolated rat liver nuclei stimulated fragmentation of single-stranded DNA, incorporation of [^3H]thymidine into DNA and the binding of ^{59}Fe to DNA. FeCl_2 was about twice as active as FeCl_3 . Lipid peroxidation took place in nuclei incubated with FeCl_2 , but not with FeCl_3 . Generation of reactive forms of oxygen was required for iron-mediated DNA damage, but evidence for direct interaction of reactive oxygen with DNA was not found. Apparent adducts of iron bound to DNA seemed to be formed by an enzymic mechanism.

Lipoperoxidation in biological membranes releases products capable of diffusing over a significant range to damage distant biological structures (Benedetti *et al.*, 1979). Products of lipoperoxidation such as malondialdehyde have been reported to react with nucleic acids (Brooks & Klammer, 1968) and to be mutagenic (Mukai & Goldstein, 1976; Shamberger *et al.*, 1979). In the eukaryotic cell nucleus, the nuclear envelope is a reported site for lipoperoxidation (Baird *et al.*, 1980). Given the juxtaposition of nuclear chromatin to the envelope, the question arises whether the subjacent DNA is vulnerable to lipoperoxidative epoxides in the envelope.

Lipoperoxidation *in vitro* requires iron in hepatic microsomal fractions stimulated by NADPH (May & McCay, 1968; Wills, 1969; Svingen *et al.*, 1979; Kornbrust & Mavis, 1980). The metal is active as inorganic salt or when chelated with ADP or pyrophosphate. Iron seems to have a role in all stages of the complex lipoperoxidative sequence (initiation, propagation and degradation of peroxide products), but initial activity appears to require maintenance of iron as Fe(II) by the membrane's NADPH:cytochrome *c* reductase (see Svingen *et al.*, 1979; Kornbrust & Mavis, 1980).

Iron also has been shown to produce single-strand DNA breaks when incubated *in vitro* with purified DNA, acting either in an inorganic form or when complexed with any of several antitumour antibiotics such as bleomycin (Takeshita *et al.*, 1978; Sausville *et al.*, 1978; Burger *et al.*, 1979; Povirk, 1979). Complexes of iron and bleomycin were markedly more active for DNA cleavage than was either iron or bleomycin alone. Cleavage of bacteriophage- ϕX1 DNA incubated with the iron–bleomycin

complex was promoted by various reducing agents such as superoxide anion, H_2O_2 , ascorbate or mercaptoethanol and was inhibited by the chelators deferoxamine or EDTA (Sausville *et al.*, 1978). The iron–bleomycin complex in the presence of a reducing agent interacts with DNA, and small non-enzymic strand cleavage results from the oxidation of the Fe(II)–bleomycin–DNA complex (Sausville *et al.*, 1978; Povirk, 1979). Iron-mediated formation of $\text{OH}^\bullet$ or $\text{O}_2^{\bullet-}$ arising from the O_2 –bleomycin–Fe(II) complex may be involved in DNA cleavage (Sausville *et al.*, 1978; Lesko *et al.*, 1980). Iron acting in concert with antibiotic preferentially cleaves DNA at guanidyl 3'-phosphate residues compared with the apparently random cleavage found after incubation with inorganic iron salts (D'Andrea & Haseltine, 1978; Takeshita *et al.*, 1978).

Given the actions *in vitro* of iron against membrane unsaturated fatty acid and against purified DNA, the metal might be suspected of acting on both targets in the eukaryotic-cell nucleus. Alternatively, the reactive degradation products of iron-stimulated lipoperoxidation occurring in the envelope of the nucleus might alter interior DNA themselves. In the study reported here, the DNA of isolated nuclei was studied after incubation *in vitro* with iron chloride.

Materials and methods

Materials

Superoxide dismutase (EC 1.15.1.1) was obtained from Truett Laboratories (Dallas, TX, U.S.A.), and crystalline catalase (EC 1.11.1.6) from Sigma (St. Louis, MO, U.S.A.). Diethylenetriaminepenta-acetic

acid (DETAPAC) and mannitol were from Fisher Scientific Co. (Fairlawn, NJ, U.S.A.), and all iron salts from J. T. Baker Chemical Corp. (Phillipsburg, NJ, U.S.A.). Sucrose (density-gradient grade) was from Schwartz-Mann (New York, NY, U.S.A.), and light petroleum (b.p. 35–60°C) from Mallinckrodt (St. Louis, MO, U.S.A.). All other compounds, unless otherwise specified, were obtained from Sigma.

Methods

Isolation of nuclei. Nuclei were isolated from the livers of 250–300g male rats (Holtzman Co., Madison, WI, U.S.A.). They were kept in wire cages and fed on Rodent Laboratory Chow (no. 5001; Ralston Purina Co., Minneapolis, MN, U.S.A.) *ad libitum* in quarters darkened between 18:00 and 06:00h daily.

Isolation followed the procedure of Burgoyne *et al.* (1974). The excised liver was homogenized in 0.34M-sucrose containing 60mM-KCl, 15mM- β -mercaptoethanol, 0.15mM-spermidine, 0.5mM-spermine, 15mM-NaCl, 15mM-Tris/HCl (pH 7.4 at 25°C), 2mM-disodium EDTA and 0.5mM-sodium EGTA. Homogenate (10–15ml) was layered over 10ml of 1.37M-sucrose in homogenizing buffer and centrifuged in a Sorvall HB-4 rotor (Dupont Instruments, Newtown, CT, U.S.A.) for 20 min at 9500rev./min at 2°C in an RC-5 Sorvall centrifuge.

[³H]dTTP incorporation into DNA

For incubations, isolated nuclei were suspended to a concentration of 20 A_{260} units/ml (1 A_{260} unit = approx. 60 μ g of DNA) in buffer containing 15mM-Tris/HCl (pH 7.6 at 37°C), 0.25M-sucrose, 60mM-KCl, 15mM-NaCl, 0.15mM-spermine and 0.5mM-spermidine. All incubations, unless otherwise noted, were in a total volume of 0.415ml containing 42.5 μ M each of dGTP, dATP, dCTP and dTTP, plus 0.6 μ Ci of [³H]dTTP ([methyl-³H]-dTTP, ammonium salt; 47Ci/mmol; Amersham Corp., Arlington Heights, IL, U.S.A.).

Experiments were performed with paired incubations, one with and one without iron. Incorporation was measured as the difference between radioactivity (d.p.m.) incorporated/ μ g of DNA in the iron-containing incubations minus radioactivity in the paired control. The optimum pH for iron-stimulated incorporation was 7.3–7.4; incorporation was linearly related to temperature between 4 and 37°C; the incubation time that gave maximum incorporation was 15 min at 25°C; and a reliance on the simultaneous presence of the four deoxynucleotide triphosphates was demonstrated by the nearly complete inhibition of incorporation if any one of the four was omitted. No requirement for ATP was demonstrable, nor did its inclusion at up to 120 μ M stimulate incorporation. Cytosine arabinoside at

0.5–3.8mM also has no effect on iron-stimulated incorporation. However, incubations in 100% N₂ were about half as active as those performed in O₂/CO₂ (19:1).

Two methods for measurement of [³H]dTTP incorporation into DNA were used: caesium salt gradients and an acid-precipitation/alkaline-hydrolysis technique. The caesium-salt-gradient method was modified from Shaw *et al.* (1975) as follows: in a Beckman SW 56 centrifuge tube, 0.7ml of a CsCl (20.21%, w/v) and Cs₂SO₄ (11.95%, w/v) solution ($d = 1.82$) was layered on the bottom. Next, in order, were 0.7ml layers of 19.96% CsCl ($d = 1.72$) and 13.76% CsCl ($d = 1.50$). A nuclear pellet was suspended in a solution containing 6.88% CsCl, 2.99M-NaSCN and 0.1% *N*-lauroylsarcosine (Sarkosyl), and 1.5ml was overlaid on the gradient, followed by 0.7ml of Sarkosyl (0.1%)/NaSCN (2.99M). The rotor was spun at 28000rev./min for 17h at 22°C. Then ten-drop fractions were collected by using an ISCO model 183 density-gradient fractionator (Instrumentation Specialities Co., Lincoln, NE, U.S.A.). The density of fractions was determined with a Bausch and Lomb refractometer on samples collected under mineral oil.

For the acid-precipitation/alkaline-hydrolysis method, the nuclear pellet was suspended in an excess volume of aq. 95% (v/v) ethanol, sedimented at 1600rev./min for 2min, resuspended and sedimented once in diethyl ether, and once in 95% ethanol. The nuclear pellets were then suspended in 1.0ml of a 1% solution of bovine albumin and 1ml of cold 10% (v/v) trichloroacetic acid was added. The precipitate was collected and washed three times in cold 5% trichloroacetic acid and twice in 0.2M-HClO₄. At that point, if radioactivity was detected in the supernatant, additional HClO₄ precipitations were performed until no more radioactivity was released. Pellets were then taken up in 1ml of 0.3M-NaOH and incubated for 1h at 37°C. After hydrolysis, 0.5ml of 1.2M-HClO₄ was added, and the pellets were dissolved in 0.2ml of 99% formic acid at 37°C for 2h and transferred to 10ml of Aquasol (New England Nuclear, Boston, MA U.S.A.) for radioactivity counting.

Determination of single-strand DNA fragmentation

The alkaline elution technique of Kohn *et al.* (1976) and Fornace *et al.* (1976) was followed. All filtration and elution solutions contained 40mM-EDTA. Filters were eluted in pairs, one filter carrying prelabelled purified rat liver DNA as a control for the other filter loaded with incubated nuclei. Prelabelled DNA was prepared from partially hepatectomized rats injected with 50 μ Ci of [methyl-³H]thymidine (18Ci/mmol; Amersham) nine times, every 4h, starting 16h after surgery. No sooner than

2 weeks later, nuclei were isolated and DNA was extracted as described by Kirby & Cook (1967).

Analytical procedures

For phospholipid fatty acid analysis, incubated nuclei were extracted as described by May & McCay (1968). Fatty acids were analysed with a Hewlett-Packard gas chromatograph (model 5830A) fitted with a glass column packed with 10% SP2340 and 100–200 Chromosorb W AW (Supelco, Bellefonte, PA, U.S.A.). Individual fatty acid methyl esters were identified by comparative retention times of authentic standard esters obtained from Supelco. Phospholipid P was measured by the method of Fiske & Subbarow (1925). Iron was measured with a Perkin-Elmer atomic-absorption spectrophotometer (model 360) by using a graphite furnace. Samples of nuclei for analysis were hydrolysed together with an exogenous iron standard (1000 p.p.m.; ArRo Labs, Joliet, IL, U.S.A.) in sealed vessels with conc. HNO_3 for 5–7 h at 95°C. All assays were performed in triple-distilled and deionized water whose endogenous iron content was assessed before use. DNA was assayed by the Burton (1956) procedure. Malondialdehyde was prepared from malonaldehyde bis(dimethylacetal) (obtained from Pfaltz and Bauer, Flushing, NY, U.S.A.) with 1M-HCl and measured by using a molar absorption coefficient of 23 000 at 270 nm at pH 7.5 (Shires, 1975). Scintillation counting was done with a Beckman LS-250 scintillation spectrometer, and gamma radioactivity of ^{59}Fe (Amersham-Searle, Oakbrook, IL, U.S.A.) was determined with a Beckman gamma counter, model 310.

Results

Incubation of isolated nuclei with FeCl_3 resulted in increased fragmentation of DNA (Fig. 1). The relative extent of fragmentation was related to iron concentration. The possibility that free iron or active iron complexes remained with the nuclei past the post-incubation washes was tested by adding ^3H -labelled DNA to washed nuclei (previously incubated with 0.75 mM- FeCl_3) before they were lysed on the filters. No elution of this marker DNA from the filter was found, indicating the improbability of DNA breakdown as an artifact during the elution analysis (results not shown).

Precursor incorporation into the DNA of isolated nuclei was stimulated by FeCl_2 and FeCl_3 as well as by several agents known to promote lipoperoxidation. In Fig. 2, the ability of the lipoperoxidative NADPH and ascorbate systems to stimulate precursor incorporation was compared on the basis of concentration-dependent effect with other systems. Activity was found with both systems that was

Fig. 1. Analysis for single-strand fragmentation by alkaline elution of DNA from isolated nuclei incubated with iron

All experiments were carried out with nuclei isolated from hepatectomized rats labelled with ^3H -thymidine, except that control pattern (■) which shows the elution of purified ^3H -labelled liver DNA. The other elution patterns shown are from nuclei incubated with (□) 0.75 mM- or (▲) 1.5 mM- FeCl_3 . FeCl_3 was absent from nuclear incubations represented by ●. All nuclear incubations contained 4 A_{260} units of nuclei, in 0.415 ml, and lasted 15 min at 25°C before sedimentation of the nuclei.

roughly one-tenth that with FeCl_2 . The weak activity of the lipoperoxidative by-product malondialdehyde would seem to disqualify it from consideration as a potentially active intermediate in the stimulation of incorporation. The established alkylating agent, methyl methanesulphonate, was surprisingly weak compared with iron and the lipoperoxidizing systems. Iron was more active as Fe(II) than as Fe(III) whether incubated as chloride (Fig. 2) or sulphate salts (results not shown). Iron-dextran (Imferon; Merrell-National, Cincinnati, OH, U.S.A.) or $\text{FeNH}_4(\text{SO}_4)_2$ was virtually inactive (results not shown), as were MnCl_2 and CoCl_2 (up to 1.45 mM). CuCl_2 was about one-sixth as active as FeCl_3 at 0.75 mM.

Relation to lipid peroxidation

Lipoperoxidation was demonstrated by measuring the proportionate decline in phospholipid polyunsaturated fatty acids after incubation of nuclei with iron (May & McCay, 1968; Pfeifer & McCay, 1972). The relative content of fatty acids from nuclei incubated with iron was compared with their content in incubations from which the metal was omitted

(Table 1). Fe(II) as FeCl₂ at 1.5 mM decreased the unsaturation index by 33%, but Fe(III) as FeCl₃ produced no convincing changes in unsaturation at concentrations of 0.75 and 1.5 mM. FeCl₃-stimulated incorporation, however, was demonstrable at both 0.75 and 1.5 mM (cf. Fig. 2), as was increased single-strand DNA fragmentation (cf. Fig. 1). These data argue that iron-stimulated precursor incorporation does not require lipoperoxidation. However, NADPH, which was able to stimulate incorporation somewhat weakly (cf. Fig. 2), decreased the unsaturation index by 12%.

Since precursor incorporation when stimulated by

Fig. 2. Comparison of the activities of lipoperoxidative and alkylating agents for stimulation of incorporation of [³H]dTTP into the DNA of isolated nuclei

All incubations were at 25°C for 15 min with 4 *A*₂₆₀ units of nuclei. In the NADPH system, ADP and FeCl₃ were added at constant concentration ratios (mM) of 4:3:0.012 (ADP:NADPH:iron). Total iron, including that contaminating reagents and buffer (measured by atomic absorption), was 13 μM. Generally incorporation was not detectable in nuclei at <0.1 mM-FeCl₃.

FeCl₂ or NADPH (but not by FeCl₃) appeared to be concomitant with lipoperoxidation, the effects of various inhibitors of peroxidation on incorporation promoted by these three systems was examined (Table 2). The hydroxyl-radical scavenger mannitol inhibited the incorporative activity stimulated by both FeCl₂ and FeCl₃. Antioxidant inhibitors of the propagation phase of lipoperoxidation, hydroxybutyltoluene and α-tocopherol, caused a decrease in the incorporative activity stimulated by NADPH and both forms of iron, as did the chelator EDTA. Enzymic destruction of peroxide by catalase inhibited the activities of both iron forms, whereas superoxide dismutase had no effect on NADPH activity but decreased the incorporation stimulated by both FeCl₂ and FeCl₃.

Each of these inhibitors also decreased the iron-stimulated fragmentation of DNA (Fig. 3). The rank order of the relative effect of individual agents in decreasing fragmentation was about the same as that observed for inhibition of FeCl₃-stimulated incorporation shown in Table 2. Elution profiles of DNA (not shown) from nuclei incubated with FeCl₂ in the presence of mannitol and of α-tocopherol indicated that both agents effectively curtailed the extent of fragmentation stimulated by this form of iron. DNA-fragmentation analysis for NADPH (also not shown) was difficult to interpret, because the extent of fragmentation by the NADPH system itself was much lower and more variable than was seen with iron. On balance, these inhibitor studies suggest an involvement of active forms of oxygen in single-strand DNA fragmentation and in precursor incorporation.

Disulphide and thiol groups were also involved in iron-stimulated incorporation (Table 2), as indicated by a marked enhancement of incorporation by 5,5'-dithiobis-(2-nitrobenzoic acid). *N*-Ethylmaleimide had virtually no effect, whereas mild reductive cleavers of disulphide bonds, β-mercaptoethanol and dithiothreitol, markedly decreased incorporation.

Table 1. Alteration in the proportions of phospholipid fatty acids in isolated nuclei incubated with iron or NADPH. Nuclei (10 *A*₂₆₀ units) were incubated in a final volume of 1 ml for 30 min at 25°C (unless otherwise indicated) omitting deoxynucleoside triphosphates. The unsaturation index is the sum of each fatty acid's mole fraction multiplied by number of its unsaturated double bonds.

	Content (mole fraction)							Unsaturated index
	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:4}	C _{22:6}	
Without iron	19.8	0.6	21.1	8.0	10.2	32.4	7.9	205.9
Without iron at 37°C	17.6	—	21.0	7.6	13.8	32.5	7.5	210.1
0.75 mM-FeCl ₃	17.4	1.7	18.0	10.7	14.9	30.0	7.3	204.3
1.5 mM-FeCl ₃	21.5	—	22.6	7.7	10.7	30.6	7.7	197.7
0.75 mM-FeCl ₂	21.7	—	23.9	7.2	11.3	28.9	7.0	187.4
1.5 mM-FeCl ₂	19.7	0.5	28.6	15.6	13.2	17.1	5.3	142.2
1 mM-NADPH + 1.3 mM-ADP at 37°C	19.1	—	23.5	11.4	12.0	27.3	6.7	184.6

Table 2. *Effects of thiol reagents and peroxidation inhibitors on iron-stimulated [^3H]dTTP incorporation into DNA* Incubations were performed as described in Table 1. Controls were incubated in the absence of inhibitors. Results are means $\pm$ S.E.M. for 3–11 experiments. Each experiment was performed with its own control incubation. Tocopherol and hydroxybutyltoluene were dissolved in dimethyl sulphoxide, whose final concentration was 3.3 mM. Ancillary studies showed that this concentration of the solvent had no independent effect on iron-stimulated incorporation. All other agents were dissolved in buffer. Dashes indicate experiments that were not done. Not indicated are additional control experiments for the two enzymes in which either dismutase or catalase was heat-inactivated by boiling for 1 h before use. No inhibition of incorporation was observed with these inactivated preparations.

Inhibitor and its concentration	Incorporation (% of control)		
	FeCl_3 (0.75 mM)	FeCl_2 (0.75 mM)	NADPH/ADP (1 mM- NADPH, 1.3 mM-ADP)
α -Tocopherol (2 mM)	20 $\pm$ 3	18 $\pm$ 5	17 $\pm$ 7
Hydroxybutyltoluene (2 mM)	60 $\pm$ 4	41 $\pm$ 6	28 $\pm$ 10
Mannitol (10 mM)	42 $\pm$ 5	28 $\pm$ 10	—
EDTA (2.4 mM)	16 $\pm$ 4	10 $\pm$ 6	11 $\pm$ 3
Catalase (20 units/0.4 ml)	32 $\pm$ 10	43 $\pm$ 12	—
Superoxide dismutase (270 units/0.4 ml)	46 $\pm$ 20	58 $\pm$ 18	100 $\pm$ 0
Dithiothreitol (10 mM)	9 $\pm$ 1.8	7 $\pm$ 0.2	—
Mercaptoethanol (70 mM)	12 $\pm$ 2.3	16 $\pm$ 1.4	100 $\pm$ 0
<i>N</i> -Ethylmaleimide (60 mM)	100 $\pm$ 2.0	100 $\pm$ 1.9	100 $\pm$ 0
5,5'-Dithiobis-(2-nitrobenzoic acid) (10 mM)	198 $\pm$ 10.2	201 $\pm$ 8.3	—

Table 3. *Effect of chelators on [^3H]dTTP incorporation into DNA*

Results are expressed as the percentage of the incorporation compared with control incubations performed with the omission of chelator. All incubations contained 4 A_{260} units of nuclei, 42.5 μM each of four deoxynucleoside triphosphates, 0.6 μCi of [^3H]dTTP, and 0.75 mM-iron chloride, and were performed at 25°C for 30 min. When free chelator was involved, it was added to the incubation mixture before addition of iron. Addition of free iron or iron chelate marked the start of incubation. Chelates were prepared as follows: for FeCl_3 , the iron salt in water was mixed with ADP at a ratio (mM) of 1:17, EDTA at 1:1.1, DETAPAC (diethylenetriaminepenta-acetic acid) at 1:1.1, and sodium citrate at 1:5.75. The pH of the mixtures was adjusted to 6.9 with bicarbonate, and oxygen was bubbled through the solution. For FeCl_2 , the iron salt in water was mixed in the presence of 0.22 mM-ascorbate with each chelator in the same mM ratios as those used for Fe(III) and at pH 7.4. The final concentration of a particular chelator and of iron was the same in all incubations whether added as chelation complexes or independently. The data are the mean percentages $\pm$ S.D. from three to five experiments. Dashes indicate experiments not done.

Chelator (final concn. free or complexed)	Incorporation (%)			
	FeCl_3 (0.75 mM)		FeCl_2 (0.75 mM)	
	Added as chelate	Free chelator mixed with nuclei + iron	Added as chelate	Free chelator mixed with nuclei + iron
Citrate (4.3 mM)	6.7 $\pm$ 2.9	1.5 $\pm$ 0.9	2.6 $\pm$ 1.3	10.3 $\pm$ 9.5
ADP (12.75 mM)	5.1 $\pm$ 3.8	8.9 $\pm$ 2.1	24.1 $\pm$ 9.8	19.1 $\pm$ 11.6
EDTA (0.825 mM)	11.6 $\pm$ 5.2	33.7 $\pm$ 10.3	4.6 $\pm$ 4.0	9.0 $\pm$ 3.7
DETAPAC (0.825 mM)	—	100.0 $\pm$ 0.0	0.8 $\pm$ 0.7	18.0 $\pm$ 10.8

Reaction of iron with nuclear DNA

To find out if iron itself was interacting with DNA in isolated nuclei, and under what conditions binding might occur, trace amounts of $^{59}\text{FeCl}_3$ were incubated with nuclei in the presence of unlabelled 0.75 mM- FeCl_3 , and the radioactive labelling of

DNA by ^{59}Fe was examined in $\text{CsCl/Cs}_2\text{SO}_4$ gradients (Fig. 4a). About 0.12% of the applied ^{59}Fe radioactivity was recovered from the DNA band of the gradients ($d = 1.64$). If the unlabelled FeCl_3 was omitted, virtually none of the trace ^{59}Fe appeared with the DNA band. In all experiments, the

Fig. 3. Effects of antioxidants on FeCl_3 -stimulated alterations of the alkaline elution pattern of DNA

Nuclei were incubated in buffer with 0.75 mM- FeCl_3 and 2 mM- α -tocopherol (□), 20 units of catalase (■), 10 mM-mannitol (△), 270 units of superoxide dismutase (▲), or 2 mM-hydroxybutyltoluene (○). Controls were incubated in the presence of iron but in the absence of antioxidant and are shown by ●. All experiments were performed as indicated in Fig. 1.

concentration of radioactive tracer was 1.72 nM, a value too low to support detectable incorporation by itself.

Inclusion of the agents mannitol or superoxide dismutase suppressed the appearance of ^{59}Fe in the DNA band (Fig. 4a). The thiol reagent 5,5'-dithiobis-(2-nitrobenzoic acid), which markedly enhanced FeCl_3 -stimulated incorporative activity (see Table 2), also enhanced the extent of ^{59}Fe binding (cf. Figs. 4b and 4a), and dithiothreitol, which inhibited iron-stimulated incorporation, also inhibited ^{59}Fe binding (Fig. 4b). Thus, in general, iron binding to DNA followed the same response pattern observed with iron-stimulated precursor incorporation.

Metal chelators were found to depress markedly iron-stimulated precursor incorporation (Table 3). For citrate, ADP or EDTA, both FeCl_3 and FeCl_2 effects were depressed either when the chelators were directly added to nuclear incubation mixtures containing free iron salt or when equivalent amounts of iron were chelated with the individual reagents before

Fig. 4. $\text{CsCl}/\text{Cs}_2\text{SO}_4$ gradient analysis of ^{59}Fe binding to DNA of isolated nuclei

Isolated nuclei ($4 A_{260}$ units) were incubated with 0.75 mM- FeCl_3 (unlabelled) (a, □) in the presence of 1.0 μCi of ^{59}Fe (2.4 μM) and unlabelled deoxy-nucleoside triphosphates for 15 min at 25°C. In (a) the additional presence of superoxide dismutase (270 units) is indicated by ■, and that of 10 mM-mannitol by ○. Incubations from which unlabelled iron was omitted are indicated by ●. In all cases the entire incubation mixture was layered on the gradients. The density 1.64 g/ml occurred at fraction 12. In (b), all conditions were the same as in panel (a) except for the presence of 10 mM-5,5'-dithiobis-(2-nitrobenzoic acid) (▲) or 5 mM-dithiothreitol (△).

their addition to the nuclear mixture. The chelator diethylenetriaminepenta-acetic acid (DETAPAC) had no effect on FeCl_3 -stimulated incorporation, but was inhibitory against FeCl_2 .

Iron effects on nuclei stripped of the nuclear envelope

If the process of lipoperoxidation was important to FeCl_2 -stimulated incorporation, removal of the nuclear envelope before incubation with iron might be expected to decrease incorporation caused by

Fig. 5. Alkaline-elution patterns of detergent-treated nuclei

Nuclei were suspended in 0.5% Triton X-100, and washed in buffer. Control nuclei were not treated with detergent. Triton-treated nuclei were incubated with 0.75 mM-FeCl₃ (●) or without iron (□). Control nuclei were also incubated with (▲) or without iron (■). Alkaline elution was then performed as indicated in Fig. 1.

Fig. 6. Binding of ⁵⁹Fe to DNA in nuclei treated with Triton X-100 as analysed on caesium salt gradients. Nuclei were isolated and treated with Triton as indicated in Fig. 5. Incubations of 4 A₂₆₀ units of Triton-treated nuclei were carried out with 0.75 mM-FeCl₃ (unlabelled) in the presence of 1.0 μCi of ⁵⁹Fe and unlabelled deoxynucleosides for 15 min at 25°C (□) or with the addition of 2 mM-hydroxybutyltoluene (■) or 20 units of catalase (●). Nuclei not treated with detergent but incubated with unlabelled iron and ⁵⁹Fe are indicated by ○.

Table 4. Effect of detergent treatment on iron-stimulated incorporation of [³H]dTTP into DNA and production of malondialdehyde-like substance

Nuclei were suspended at 10 A₂₆₀ units/ml in buffer with 0.5% Triton X-100 and incubated at 2°C for 5 min. After centrifugation, the pellet was incubated in buffer with 0.5% Triton a second time under the same conditions as the first. The Triton-treated pellets were then washed three times in buffer and finally suspended in buffer at a concentration of 10 A₂₆₀ units/ml. Control nuclei were incubated twice at 2°C for 5 min in buffer from which Triton was omitted and then washed three times in that buffer. Triton-treated or control nuclear preparations were then incubated with or without 0.75 mM- or 1.5 mM-iron for 15 min at 25°C and then incorporation or phospholipid P was determined. The data shown represent the mean values of three to six experiments.

	Incorporation (c.p.m./μg of DNA)		Lipid (mmol/mg of DNA)	
	Control	+ Triton	Control	+ Triton
No iron	0.58	0.11	0.033	0.013
0.75 mM-FeCl ₃	228	109	0.035	0.010
1.5 mM-FeCl ₃	503	261	0.038	0.011
0.75 mM-FeCl ₂	283	132	—	—
1.75 mM-FeCl ₂	617	293	0.033	0.009

FeCl₂, but not that caused by FeCl₃. Nuclei were treated with 0.5% Triton X-100 (Whittle *et al.*, 1968) to disperse elements of the nuclear envelope. As shown in Table 4, about one-third of the original phospholipid phosphorus remained with the Triton-treated nuclei. The unsaturation index for the preparation's fatty acids was 74.7 (compared with 208 for nuclei not exposed to the detergent) with no detectable C_{22:6} or C_{20:4} fatty acid. When the detergent-treated nuclei were incubated with either FeCl₂ or FeCl₃, incorporative activity for both was 47–48% of that observed for preparations not treated with detergent (Table 4). This result argues that incorporation caused by either form of iron did not significantly require concurrent lipoperoxidation.

The single-strand DNA fragmentation in detergent-treated nuclei after incubation with 0.75 mM-FeCl₃ was much decreased compared with preparations not treated with detergent (Fig. 5). However, ⁵⁹Fe binding to DNA in detergent-treated nuclei in CsCl/Cs₂SO₄ gradients (Fig. 6), compared with control nuclei, was markedly increased. This increase could be partially reversed by hydroxybutyltoluene or by catalase (Fig. 6), but not by superoxide dismutase. Thus Triton X-100 treatment probably inhibited the removal of DNA-iron ad-

ducts as well as DNA cleavage. This result contrasts with reports of the metal's spontaneous non-catalytic cleavage of pure DNA (Takeshita *et al.*, 1978; Povirk, 1979), and perhaps implies a different mechanism in whole nuclei.

Discussion

Reactive oxygen intermediates play an important role in iron-stimulated incorporation, single-strand fragmentation, and the metal's binding to the DNA of isolated nuclei. Iron in aqueous aerobic solutions at neutrality is known to promote formation of reactive oxygen intermediates such as $\cdot\text{OH}$, $-\text{OOH}$ and $\text{O}_2^{\cdot-}$ (Martell & Khan, 1975; Halliwell, 1978), and these intermediates can cleave purified DNA under conditions *in vitro* (Massie *et al.*, 1972; Lesko *et al.*, 1980; Floyd, 1981). Thus, in addition to iron, these intermediates could be primary cleavage agents in iron-stimulated incorporation. However, the inhibitory effect of iron chelators argues against a major involvement of oxygen intermediates in DNA cleavage, since it is well known that co-ordinate linkage of iron does not curtail the metal's interaction with molecular oxygen nor the appearance of reactive intermediates (Halliwell, 1978; Svingen *et al.*, 1979). If DNA alteration had been dependent on direct reaction with oxygen intermediates, complexation of iron by chelators should have decreased iron-stimulated DNA alterations. Further, since lipoperoxidation represents an alternative source of reactive oxygen, its absence in incubations with ferric iron also favours iron and not reactive oxygen intermediates as being responsible for iron-stimulated DNA synthesis in these isolated nuclei.

The role of reactive oxygen intermediates may be alternatively viewed as supporting iron as a DNA interactant through their reducing capabilities. The inhibitor studies are consistent with this view. However, there is reason to be guarded in relying on inhibitors as unequivocal means for detecting and identifying any one particular intermediate. For instance, the polyol mannitol, an $\cdot\text{OH}$ scavenger, can form co-ordinate linkage with iron (Martell & Khan, 1975). Its effect on isolated nuclei thus may not result completely from its scavenging activity. Superoxide dismutase has been reported to interact directly with DNA (Galvan *et al.*, 1980), although the consequences of this are unknown. Despite the shortcomings of any one particular inhibitor, there was a common pattern of inhibitor effect shared by all agents (mannitol, catalase, α -tocopherol, hydroxybutyltoluene and superoxide dismutase), thus supporting a role for the intermediates in fostering iron interaction with DNA.

The NADPH and ascorbate lipoperoxidative systems were found to stimulate incorporation, albeit

weakly compared with iron. The premise that reactive lipoperoxidative products might destructively interact with DNA is thus not ruled out, even though ferric-iron-stimulated incorporation was clearly independent of lipoperoxidation. The promotion of lipoperoxidation by ferrous but not by ferric iron in isolated nuclei follows the pattern reported for microsomal membrane preparations (Poyer & McCay, 1971; Kornbrust & Mavis, 1980). Hydrated ferric iron complexes probably penetrate poorly into the lipid phase of the membranes, unlike ferrous iron.

Extrapolation of these nuclear studies to cells and tissues is currently prevented by the absence of any definitive information on the intracellular concentrations of DNA-reactable iron. Given the large number of organic groups and low-molecular-weight compounds in the cell that could co-ordinate with free iron, the chance for the intracellular occurrence of such reactable iron seems very low. Indeed, the fact that most known life forms have successfully based much of their redox and respiratory function on iron-mediated catalysis is an argument against the significant routine intracellular presence of reactable iron. It is possible this apparent avoidance of DNA-reactable iron by intracellular iron metabolism may be less successful in cells chronically loaded with excess iron. In the absence of any relevant information about DNA in the many forms of haemosiderosis (benign iron overload) or haemochromatosis (pathological iron overload), it remains to be seen whether the potential for iron-induced damage to nuclear DNA, demonstrated here, is realized in whole cells *in vivo*.

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