Biochem. J. (1979) **184**, 469–472
Printed in Great Britain

Inhibition of the Iron-Catalysed Formation of Hydroxyl Radicals from Superoxide and of Lipid Peroxidation by Desferrioxamine

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(*Received* 9 *August* 1979)

The peroxidation of membrane phospholipids induced *in vitro* by ascorbic acid or by dialuric acid (hydroxybarbituric acid) does not occur in the absence of traces of metal ions. Peroxidation induced by adding iron salts to phospholipids can either be promoted or inhibited by the chelators EDTA, diethylenetriaminepenta-acetic acid and bathophenanthrolinesulphonate, depending on the ratio [chelator]/[iron salt]. The iron chelator desferrioxamine inhibits peroxidation at all concentrations tested, and it also inhibits the iron-catalysed formation of hydroxyl radicals ($OH^{\bullet}$) from superoxide ($O_2^{-\bullet}$). Since desferrioxamine is approved for clinical use, it might prove a valuable tool in the treatment of inflammation, poisoning by autoxidizable molecules and radiation damage.

O_2 is essential for the life of aerobes, but it has long been known to be toxic to them when supplied at concentrations only slightly greater than those in normal air (Haugaard, 1968). The biochemical mechanisms responsible for O_2 toxicity include lipid peroxidation and the generation of H_2O_2 together with the superoxide free radical, $O_2^{-\bullet}$. In biochemical systems, $O_2^{-\bullet}$ and H_2O_2 react together to form the highly reactive hydroxyl radical, $OH^{\bullet}$, which can attack and destroy almost all known biomolecules (Fridovich, 1975; Halliwell, 1978*a*).

Lipid peroxidation *in vitro* has been much studied, and several workers have found that it does not occur unless chelated iron or haem compounds are present (e.g. Wills, 1965; Tyler, 1975; Kaschnitz & Hatefi, 1975; Svingen *et al.*, 1978). Induction of lipid peroxidation by easily oxidizable molecules, such as ascorbate, dihydroxyfumaric acid or dialuric acid, has also been reported (Wills, 1966; Fee *et al.*, 1975).

Similarly, the reaction between $O_2^{-\bullet}$ and H_2O_2 to give $OH^{\bullet}$ does not occur unless traces of iron salts are present in the system (McCord & Day, 1978; Buettner *et al.*, 1978; Halliwell, 1978*b*). This iron-dependent generation of $OH^{\bullet}$, often called the 'iron-catalysed Haber–Weiss reaction', can be inhibited by the metal-ion chelators diethylenetriaminepenta-acetic acid or bathophenanthrolinesuphonate (Buettner *et al.*, 1978; Halliwell, 1978*b*,*c*). Halliwell (1978*c*) suggested that these chelators might be clinically useful in the treatment of inflammatory joint diseases, provided that they are not toxic. For example, in rheumatoid arthritis the hyaluronic acid present in synovial fluid has an abnormally low intrinsic viscosity (Kofoed & Barcelo, 1978). During the acute inflammatory stage of the disease, there is extensive infiltration of polymorphonuclear leucocytes into the joint space, and it is known that these cells release $O_2^{-\bullet}$ (Babior, 1978). Exposure of hyaluronic acid to a superoxide-generating system *in vivo* causes depolymerization due to formation of $OH^{\bullet}$ by an iron-catalysed Haber–Weiss reaction (McCord, 1974; Halliwell, 1978*c*).

Paraquat poisoning, for which present treatments are grossly inadequate, also involves generation of oxygen free radicals and lipid peroxidation *in vivo* (Autor, 1974). We have therefore examined the effect of the above chelators on lipid peroxidation *in vitro* and we have extended our previous work to include desferrioxamine, a powerful iron chelator whose clinical use is already approved (Editorial, 1978).

Materials and Methods

Reagents

Superoxide dismutase (EC 1.15.1.1) from bovine blood, hyaluronic acid type III-P, bathophenanthrolinesulphonate, diethylenetriaminepenta-acetic acid and penicillamine were obtained from Sigma Chemical Co. Xanthine oxidase was obtained from Boehringer, and Desferal (desferrioxamine B methanesulphonate) from CIBA Laboratories, Horsham, Sussex, U.K. All other reagents were of the highest quality available from BDH Chemicals, and double-distilled water was used throughout. Dialuric acid (hydroxybarbituric acid) was purchased from Koch–Light.

Measurement of lipid peroxidation

Phospholipid was extracted from fresh bovine brains as described by Gutteridge (1977*a*). Liposomes were prepared by vortex-mixing phospholipid (5mg/ml) in 0.15M-NaCl adjusted to pH7.4 with $NaHCO_3$. Solutions were purged with N_2 before being vortex-mixed, and the liposomes swollen at

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4°C before use. Incubations were carried out at 37°C for 2h. The fluorescent lipid-peroxidation products (Bidlack & Tappel, 1973) were extracted into chloroform and determined by a fluorimetric method as described by Gutteridge (1977*a*). A Perkin–Elmer MPF-4 spectrofluorimeter was employed (excitation at 360nm, emission scanned in the range 425–435nm). The fluorescence standard was quinine sulphate (1μg/ml in 0.05M-H_2SO_4). Chelex-treated water was used to make up all solutions. Solutions of AnalaR ammonium ferrous sulphate were made up in Chelex-treated water purged with N_2 and used immediately.

Measurement of hydroxylation and hyaluronic acid degradation

Hydroxylation of salicylate by the xanthine/xanthine oxidase system was measured as described by Halliwell (1978*b*) in the presence of 100μM-EDTA. Hyaluronate degradation was determined by the decrease in viscosity of a reaction mixture containing 0.24ml of hyaluronic acid (8.5mg/ml in 67.5mM-Na_2HPO_4, pH7.4), 0.01ml of $FeSO_4$ (10mM), 0.658ml of a 0.38mM solution of the sodium salt of xanthine in 0.0675M-Na_2HPO_4 buffer adjusted to pH7.4 with KOH and 0.082ml of water. Reactions were initiated by adding 10μl of a solution containing 20μg of xanthine oxidase and 0.1μmol of EDTA. The reaction mixture was contained in the cup of a Brookfield LVT cone-plate viscometer (cone-plate angle 1.565°) at 37±0.2°C. Viscosity was measured at appropriate intervals at a shear rate of 230s; water flow through the cup jacket was 100ml/min (Wells *et al.*, 1961). Formation of uric acid in the reaction mixture was measured by the increase in A_{290}.

Results

Lipid peroxidation

Pure ox brain phospholipid liposomes containing no detectable preformed lipid peroxide were used

Table 1. *Effect of metal ion chelators on Fe^{2+}-induced lipid peroxidation*

Incubations were carried out as described in the Materials and Methods section. The final concentration of Fe^{2+} in the system was 0.143mM and the chelators were present at the final concentrations stated. The chelators themselves did not induce any peroxidation. Measurement of lipid peroxidation by the thiobarbituric acid method (Gutteridge 1977*a*,*b*) gave similar results to those reported here obtained by using the fluorescence method. Abbreviations: DETAPAC, diethylenetriaminepanta-acetic acid; BPS, bathophenanthrolinesulphonate.

Chelator added	Concn. (mM)	Rate of peroxidation (fluorescence units/2h)	Stimulation or inhibition of peroxidation (%)
None	—	61	—
EDTA	0.014	115	89 stim.
	0.072	149	144 stim.
	0.143	162	166 stim.
	0.286	117	92 stim.
	0.572	21	66 inhib.
DETAPAC	0.014	125	105 stim.
	0.072	200	227 stim.
	0.143	103	69 stim.
	0.286	51	16 inhib.
	0.572	18	70 inhib.
BPS	0.014	64	— —
	0.072	73	20 stim.
	0.143	72	18 stim.
	0.286	59	— —
	0.572	16	74 inhib.
Penicillamine	0.014	82	34 stim.
	0.072	133	118 stim.
	0.143	152	149 stim.
	0.286	99	62 stim.
	0.572	82	34 stim.
	1.144	74	21 stim.
Desferrioxamine	0.007	56	8 inhib.
	0.014	50	18 inhib.
	0.072	46	25 inhib.
	0.143	15	75 inhib.
	0.286	10	84 inhib.
	0.572	7	89 inhib.

in the experiments listed below. All reagents were made up in water freed of endogenous metal ions by Chelex treatment.

If care was taken to remove endogenous metal ions by Chelex treatment of solutions, neither ascorbic acid (final concentration 1 mM) nor dialuric acid (1 mM) induced any lipid peroxidation in our system. However, peroxidation in the presence of these compounds was detected at once if micromolar concentrations of Fe^{2+} or Fe^{3+} were added. Similarly, dihydroxyfumarate-induced lipid peroxidation is prevented by removal of trace metal ions from the system (A. Bindoli, personal communication).

In agreement with previous results (Gutteridge, 1977*b*), addition of Fe^{2+} to the liposomes, at a final concentration of 0.143 mM, induced rapid peroxidation. The effect of metal ion chelators on this process was examined (Table 1). When equimolar amounts of iron salt and EDTA were present, peroxidation was greater than in the presence of iron alone, but at higher [EDTA]/[Fe^{2+}] ratios peroxidation was decreased. Diethylenetriaminepenta-acetic acid and bathophenanthrolinesulphonate gave similar results: at some critical ratio of [chelator]/[Fe^{2+}] there is a change from pro-oxidant to anti-oxidant effects. Results of this type have been obtained in at least 30 different experiments, although sometimes there is a slight variation in the concentration of chelator at which the change from stimulation to inhibition occurs. Similar results were obtained whether lipid peroxidation was measured by the fluorescence method or by the thiobarbituric acid method (Table 1). In all our experiments, however, penicillamine stimulated Fe^{2+}-dependent peroxidation, whereas desferrioxamine inhibited it at every concentration tested (Table 1). The well-known anti-oxidant propyl gallate also inhibited peroxidation at all concentrations tested (results not shown).

Iron-catalysed Haber–Weiss reaction

Generation of OH• radicals from O_2^- and H_2O_2 can be detected by the ability of OH• radicals to hydroxylate aromatic compounds (Halliwell, 1978*b*). Hydroxyl-radical generation can be demonstrated in a model system containing xanthine plus xanthine oxidase (to generate O_2^- and H_2O_2), salicylate and traces of Fe^{2+} or Fe^{3+}. Formation of diphenolic products from salicylate in this system is inhibited by superoxide dismutase, by catalase, or by scavengers of OH• radicals (Halliwell, 1978*b*). Millimolar concentrations of EDTA increase the rate of OH• generation, whereas similar concentrations of bathophenanthrolinesulphonate and diethylenetriaminepenta-acetic acid inhibit (Halliwell, 1978*c*). Table 2 shows that desferrioxamine is also a powerful inhibitor of OH• radical generation, being effective at lower concentrations than is bathophenan-

Table 2. *Effect of desferrioxamine on OH• generation by the xanthine/xanthine oxidase/Fe^{2+} system*

Assays of hydroxylation and xanthine oxidase activity were carried out as described by Halliwell (1978*b*). Desferrioxamine had no effect on the assay used to measure hydroxylated product formation, nor on xanthine oxidase activity.

Final concn. of desferrioxamine present in reaction mixture (mM)	Diphenol produced (nmol/h)	Inhibition of hydroxylation (%)
0	42	0
0.005	38	9
0.05	18	57
0.2	9	79

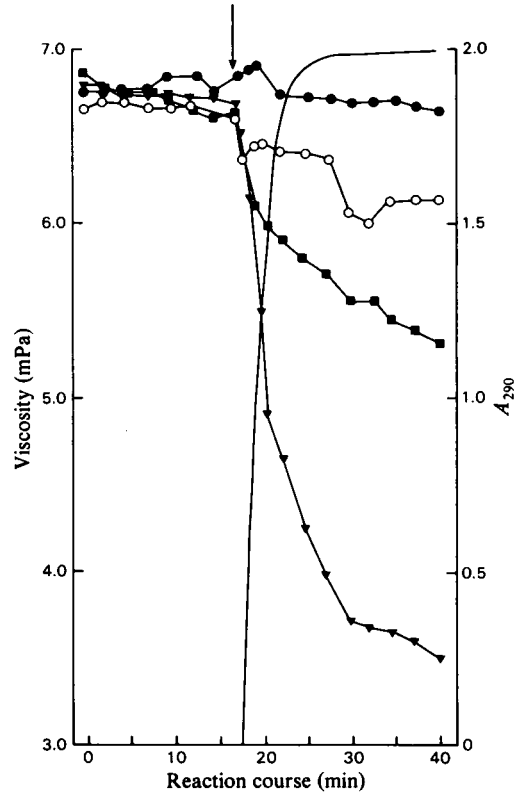

Fig. 1. *Effect of desferrioxamine on degradation of hyaluronic acid by the xanthine/xanthine oxidase system*

Degradation was measured by the decrease in viscosity (mPa) as described in the Materials and Methods section. Xanthine oxidase was added to start the reaction at the point indicated by the arrow. ▼, Complete reaction mixture; ■, +0.3 mM-desferrioxamine; ○, +1.5 mM-desferrioxamine; ●, xanthine oxidase replaced by an equivalent amount of bovine serum albumin. ——, Urate production followed as ΔA_{290}. Desferrioxamine had no effect on xanthine oxidase activity.

throlinesulphonate or diethylenetriaminepenta-acetic acid.

Degradation of hyaluronic acid on exposure to a xanthine/xanthine oxidase system *in vitro*, which is a model for inflammatory joint diseases such as rheumatoid arthritis (see above), is also due to a metal-ion-catalysed Haber–Weiss reaction (Halliwell, 1978*c*). Fig. 1 shows that this degradation is powerfully inhibited by desferrioxamine.

Discussion

The damaging effect of oxygen free radicals and lipid peroxidation *in vivo* plays an important role in mediating pathological processes (McCord, 1974; Oyanagui, 1976) and radiation damage (Fridovich, 1975; Petkau, 1978). Since $OH^{\cdot}$ generated from O_2^- and H_2O_2 plays an important role in these processes (Symposium on Singlet Oxygen and Related Species in Chemistry and Biology, 1978), Halliwell (1978*c*) suggested that inhibitors of the iron-catalysed Haber–Weiss reaction, such as bathophenanthrolinesulphonate and diethylenetriaminepenta-acetic acid, might have a clinical use.

The results in the present paper lend strength to the idea that lipid peroxidation *in vitro*, and presumably *in vivo* as well, only occurs if traces of metal ions are present. Iron salts, which are found in all biological systems, are the most likely catalysts *in vivo* (Kon, 1978). Our observation that bathophenanthrolinesulphonate and diethylenetriaminepenta-acetic acid can sometimes stimulate iron-catalysed lipid peroxidation casts some doubt on their proposed clinical utility, although protective effects have been demonstrated with the use of isolated lung cells (Autor & Fox, 1979). Why the effect of these chelators on peroxidation varies with the [chelator]/[iron salt] ratio is not at present clear. Attempts to relate this to liposome size or batch of phospholipid used have proved negative, and our experimental conditions are rigorously controlled. Possibly two metal ions complexed to one chelator molecule, as at low [chelator]/[iron] ratios, act as promoters of peroxidation, whereas different complexes inhibit peroxidation.

Desferrioxamine is an iron chelator isolated from *Streptomyces pilosus*. It is available as Desferal (desferrioxamine B methanesulphonate) and it is approved for clinical use in the treatment of iron poisoning. We have shown that it inhibits lipid peroxidation, the iron-catalysed Haber–Weiss reaction and O_2^--induced degradation of hyaluronic acid. Since it is approved for clinical use, it would seem an ideal tool for investigating the role of the above processes in human pathology.

We are grateful to the Wellcome Trust for financial support and to Dr. Richard Naftalin for the use of his viscometric equipment. R. R. thanks the Science Research Council for a research studentship.

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