# OXYGEN FREE-RADICALS AND LIPID PEROXIDATION: INHIBITION BY THE PROTEIN CAERULOPLASMIN

John M. C. GUTTERIDGE+, Ramsay RICHMOND and Barry HALLIWELL
+*National Institute for Biological Standards and Control, Holly Hill, Hampstead, London, NW3 6RB* and *Department of Biochemistry, University of London King's College, Strand, London, WC2R 2LS, England*

Received 12 February 1980

## 1. Introduction

Oxygen is essential for the survival of aerobes, but it has long been known to be toxic to them when supplied at concentrations greater than those in normal air [1]. The biochemical mechanisms responsible for $O_2$ toxicity include lipid peroxidation and the generation of $H_2O_2$ plus the superoxide free radical, $O_2^{\cdot-}$ [2]. In biochemical systems $O_2^{\cdot-}$ and $H_2O_2$ react together to form the hydroxyl radical, which can attack and destroy almost all known biomolecules [2–4]. Neither lipid peroxidation nor generation of hydroxyl radicals ('OH) occur in vitro, however, unless traces of metal ions, especially iron or copper, are present in the system [4,5]. Traces of such metal ions are present in all biological systems and any increase in the normal concentration of free iron or copper salts, e.g., after decompartmentalisation due to tissue damage, will potentiate the toxic effects of $O_2$ [5].

Lipid peroxidation and 'OH generation can often be prevented by the enzymes superoxide dismutase, catalase and glutathione peroxidase, which are important protective mechanisms in vivo [1–5]. Unfortunately, extracellular fluids contain little of these enzymes, although they contain transition metals in abundance [5] and can be exposed to $O_2^{\cdot-}$ generated by white blood cells or by enzymes released from damaged cells [2–5]. However tissue fluids do contain caeruloplasmin, a copper protein which is capable of catalysing the oxidation of $Fe^{2+}$ to $Fe^{3+}$, the electrons being transferred to $O_2$ with the production of water [6]. It seemed logical to us that the ferroxidase activity of caeruloplasmin could be a mechanism by which lipid peroxidation and the generation of 'OH promoted by iron salts could be prevented, since caeruloplasmin should keep the iron in the $Fe^{3+}$ state and so prevent it from undergoing the redox cycles necessary to initiate toxic effects [4–6]. Indeed, human blood serum and purified caeruloplasmin have already been reported to inhibit the lipid peroxidation occurring in a crude ox-brain homogenate, although this is a complex system to study [7].

Here, we report the effects of caeruloplasmin, catalase and superoxide dismutase on lipid peroxidation in well-defined systems and on the degradation of DNA induced by exposure to iron salts.

## 2. Materials and methods

### 2.1. *Reagents*

Superoxide dismutase (spec. act. 2900 units/mg protein), catalase (bovine liver, thymol free), albumin (bovine, fatty acid free) and caeruloplasmin (human, type III) were obtained from Sigma Chemical Corp. The catalase was further purified on Sephadex G25. Bacteriophage PM2 DNA was obtained from Boehringer Ltd. All other reagents were of the highest quality available from BDH Chemicals Ltd. Bovine brain phospholipids were extracted as in [8].

### 2.2. *Incubation of liposomes*

Liposomes (5 mg/ml) were prepared in 0.15 M NaCl buffered to pH 7.4 with $NaHCO_3$. Water used for the preparation of all reagents was treated with chelex to remove contaminating metal ions. After vortexing and swelling for 1 h under nitrogen at 4°C the liposomes were sonicated for 30 s (Rapidis 180). Liposomes (0.5 ml) together with 0.1 ml appropriate inhibitor were adjusted to 0.8 ml final reaction vol. by adding buffered NaCl and were pre-incubated for 5 min at 37°C. The reaction was started by the addi-

tion of a solution of freshly prepared $Fe^{2+}$ (purged with nitrogen) and the incubation continued for 30 min at 37°C. On completion of incubation 1 ml buffered saline was added to each tube. A final iron concentration in the reaction mixture of 0.125 mmol/l was used for the thiobarbituric acid (TBA) method, but it was necessary to raise this to 0.25 mmol/l for the fluorescence method.

2.3. *Incubation of PM2-DNA*

Closed covalent circular PM2-DNA was dissolved in 0.15 M NaCl (pH 7.4) to give 1 unit/ml final conc. Fluorescence studies were based on the method in [9] using the intercalation of ethidium bromide. DNA (0.1 ml) together with 0.1 ml inhibitor, finally adjusted to give 0.4 ml reaction vol. in each tube, were pre-incubated at 37°C for 5 min. The reaction was initiated by the addition of 0.1 ml $Fe^{2+}$ prepared as described and incubation was continued for 60 min at 37°C.

2.4. *Measurement of metal-ion-catalysed free-radical damage*

2.4.1. Lipid peroxidation

For TBA reactivity, 1 ml 25% (v/v) HCl followed by 1 ml 1% TBA reagent were added and the tubes heated at 100°C for 15 min. The tubes were centrifuged and the resulting chromogen in the supernatant was scanned from 500–600 nm. The $A_{532}$ was calculated by subtraction of the $A_{600}$. For measurement of native fluorescence 3 ml chloroform:methanol (2:1) was added to each tube followed by vigorous vortex mixing for 2 min. The phases were separated by centrifugation and the upper methanol–water layer discarded. Methanol (0.5 ml) cleared the chloroform phase prior to measurement in the spectrofluorimeter. Native fluorescence of peroxidised phospholipids was measured as in [8]. Units were expressed as relative fluorescence intensity compared to a standard reference block containing $10^{-7}$ M tetraphenylbutadiene. This was set to 100 units using the following instrument setting on a Perkin-Elmer MPF-4: excitation 350 nm; emission 440 nm; filter 430 nm; slits 10 nm; sensitivity × 1. Peroxidation fluorescence was measured at 360 nm excitation, 430 nm emission, filter 390 nm, slits 10 nm and sensitivity × 1.

2.4.2. DNA nicking

Nicking of closed covalent circular DNA converts it to open circular DNA. Intercalation of ethidium bromide followed by a heating and cooling cycle results in loss of fluorescence due to separation of the strands. After sample incubation 4.0 ml ethidium bromide was added and the tubes heated for 4 min at 96°C, then rapidly cooled to 20°C for 5 min. Fluorescence was measured with reference to a block standard containing $3 \times 10^{-6}$ M rhodamine B set to 100 units under the following instrument settings: excitation 480 nm; emission 580 nm; slits 10 nm; no filter; sensitivity × 1. Test fluorescence was measured at 525 nm excitation, 600 nm emission, no filter, slits 10 nm and sensitivity × 10.

## 3. Results

3.1. *Lipid peroxidation*

Purified ox-brain-phospholipid liposomes do not undergo peroxidation in vitro unless metal ions are present [4]. However, addition of $FeSO_4$ at a 0.125 mmol/l final conc. induced rapid peroxidation, in agreement with [4,10]. Lipid peroxidation was partially inhibited by superoxide dismutase or by catalase, but even large quantities of these enzymes could not prevent peroxidation completely (table 1). In contrast, caeruloplasmin was a much more powerful inhibitor. Heat-denatured caeruloplasmin or bovine serum albumin had much less effect. These results were obtained whether peroxidation was measured by a fluorescence technique for aldehydes or by the TBA method for lipid peroxides. Although caeruloplasmin, like most copper complexes and proteins [5], can scavenge $O_2^{\cdot-}$, its rate constant for reaction with this radical is only $7 \times 10^5$ as compared to $2 \times 10^9$ for copper-zinc superoxide dismutase [11]. Hence inhibition of lipid peroxidation by caeruloplasmin cannot be attributed to its low dismutase activity since it is a far more effective inhibitor than pure superoxide dismutase (table 1).

If the ability of caeruloplasmin to inhibit $Fe^{2+}$-induced lipid peroxidation were due to its ferroxidase activity, as suggested in section 1, it should have no effect on peroxidation induced by other metal ions such as $Cu^{2+}$. However, table 2 shows that caeruloplasmin was a powerful inhibitor of $Cu^{2+}$-catalysed peroxidation.

3.2. *DNA degradation*

Experiments using closed circular viral DNA have shown that iron salts at low concentrations are capable

Table 1
Effect of inhibitors on lipid peroxidation induced by $Fe^{2+}$

| Inhibitor added | Extent of peroxidation | | % Inhibition of peroxidation | |
|---|---|---|---|---|
| | As $A_{532}$ | As fluorescence units at 430 nm | As $A_{532}$ | As fluorescence units at 430 nm |
| None | 0.21 | 28 | 0 | 0 |
| Superoxide dismutase (0.04 mg/ml) | 0.15 | 21 | 29 | 25 |
| Catalase (0.04 mg/ml) | 0.17 | 23 | 19 | 18 |
| Superoxide dismutase + catalase (both at 0.04 mg/ml) | 0.14 | 20 | 33 | 29 |
| Caeruloplasmin (0.06 mg/ml) | 0.08 | 11 | 62 | 61 |
| Bovine serum albumin (0.08 mg/ml) | 0.20 | 25 | 5 | 11 |
| Denatured caeruloplasmin (0.06 mg/ml) | 0.14 | 25 | 33 | 11 |

Peroxidation was measured either by the TBA method (results expressed as $A_{532}$) or by the fluorescence method (results expressed as fluorescence units) A typical set of results is shown, but they were highly reproducible. Where indicated, caeruloplasmin was denatured by heating at 100°C for 5 min

of activating oxygen with resulting damage to the DNA molecule. Damage can be decreased by adding superoxide dismutase and catalase [12]. We have been able to confirm these results, and have also found in several experiments that caeruloplasmin at 0.12 mg/ml in the reaction mixture inhibits DNA degradation by 25–46%, whereas bovine serum albumin does not.

## 4. Discussion

Caeruloplasmin at the concentrations used here, which are well below the mean value of normal human serum (0.3 mg/ml) [6], is an effective inhibitor of iron-dependent lipid peroxidation and DNA degradation. Since serum caeruloplasmin levels rise in response to tissue damage or inflammation [6] it seems logical

Table 2
Effect of caeruloplasmin on lipid peroxidation induced by $Cu^{2+}$

| Inhibitor added | Extent of peroxidation | | % Inhibition of peroxidation | |
|---|---|---|---|---|
| | As $A_{532}$ | As fluorescence units at 430 nm | As $A_{532}$ | As fluorescence units at 430 nm |
| None | 0.17 | 39 | 0 | 0 |
| Caeruloplasmin (0.06 mg/ml) | 0.05 | 13.5 | 71 | 65 |
| Denatured caeruloplasmin | 0.12 | 26 | 29 | 33 |

Peroxidation was measured either by the TBA method (results expressed as $A_{532}$) or by the fluorescence method (results expressed as fluorescence units). A typical set of results is shown, but they were highly reproducible. Where indicated, caeruloplasmin was denatured by heating at 100°C for 5 min

to suppose that it functions in vivo to protect the extracellular fluids against the damaging effects of decompartmentalised iron salts arising from pathological processes. Although caeruloplasmin, like most copper proteins and complexes, has a weak superoxide dismutase activity [11] its anti-oxidant action is far greater than can be attributed to this.

It is tempting to explain the inhibitory effects of caeruloplasmin on iron-catalysed processes by its ferroxidase activity, since heat-denaturation greatly reduces the effect. This cannot be the whole story, however, since caeruloplasmin is an equally powerful inhibitor of $Cu^{2+}$-catalysed peroxidation. Further studies of these inhibitory effects are clearly required.

## Acknowledgements

We are grateful to the Wellcome Trust and to the Central Research Fund of the University of London for financial support. R.R. thanks the Science Research Council for a research studentship.

## References

[1] Haugaard, N. (1968) Physiol. Rev. 48, 311–345.
[2] Halliwell, B. (1978) Cell Biol. Int. Rep. 2, 113–128.
[3] Fridovich, I. (1975) Ann. Rev. Biochem 44, 147–159.
[4] Gutteridge, J. M. C., Richmond, R. and Halliwell, B. (1979) Biochem. J. 184, 469–472.
[5] Halliwell, B. (1980) in: Age Pigments (Sohal, R. S. ed) Academic Press, in press.
[6] Gutteridge, J. M. C. (1978) Ann. Clin. Biochem. 15, 293–296.
[7] Stocks, J., Gutteridge, J. M. C., Sharp, R. J. and Dormandy, T. L. (1974) Clin. Sci. Mol. Med. 47, 223–233.
[8] Gutteridge, J. M. C. (1977) Anal. Biochem. 82, 76–82.
[9] Morgan, A. R. and Pulleyblank, D. E. (1974) Biochem. Biophys. Res. Commun. 61, 396–403.
[10] Gutteridge, J. M. C. (1977) Biochem. Biophys. Res. Commun. 77, 379–386.
[11] Goldstein, I. M., Kaplan, H. B., Edelson, H. S. and Weissman, G. R. (1979) J. Biol. Chem. 254, 4040–4045.
[12] Lown, J. W. and Sim, S.-K. (1977) Biochem. Biophys. Res. Commun. 77, 1150–1157.