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Superoxide-dependent formation of hydroxyl radicals and lipid peroxidation in the presence of iron salts

Detection of 'catalytic' iron and anti-oxidant activity in extracellular fluids

John M. C. GUTTERIDGE,* David A. ROWLEY† and Barry HALLIWELL†
**National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, U.K., and †Department of Biochemistry, University of London King's College, Strand, London WC2R 2LS, U.K.*

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Synovial fluid from rheumatoid patients and normal cerebrospinal fluid contains micromolar concentrations of non-protein-bound iron salts that can promote lipid peroxidation and also the superoxide-dependent formation of hydroxyl radicals from hydrogen peroxide. These iron catalysts of oxygen radical reactions cannot be detected by conventional assays unless interfering high-molecular-weight substances, probably proteins, are removed by ultrafiltration or inactivated by exposure to low pH values. The bleomycin assay for 'catalytic' iron [Gutteridge, Rowley & Halliwell (1981) *Biochem. J.* **199**, 263–265] does not suffer from these artifacts.

The biochemical mechanisms responsible for oxygen toxicity include lipid peroxidation and the reduction of O_2 to the superoxide radical, $O_2^{-\cdot}$, by several cellular systems (for reviews see Fridovich, 1975, 1978; Chance *et al.*, 1979; Halliwell, 1978*a*, 1981*a*). The rate of lipid peroxidation *in vitro*, however, is extremely low unless transition-metal ions are present, iron being especially effective in the presence of ascorbate (Wills, 1965, 1969; Gutteridge *et al.*, 1979). Superoxide in aqueous solution is not highly reactive, and it has been suggested that many of its damaging effects are due to the $O_2^{-\cdot}$-dependent formation of hydroxyl radicals, $OH^\cdot$, from H_2O_2. This process also requires traces of iron salts and it may be represented by the overall equation (McCord & Day, 1978; Halliwell, 1978*b*,*c*, 1981*a*,*b*):

$$O_2^{-\cdot} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe salt}} OH^\cdot + OH^- + O_2 \quad (1)$$

If the above processes are physiologically relevant, then traces of non-protein-bound iron salts must be present *in vivo*. That they are present can be argued from the results of much previous research work (Halliwell, 1981*b*, 1982; Blake *et al.*, 1981*a*), and direct experimental evidence for their existence both intracellularly in liver (Fong *et al.*, 1976) and in bacteria (Repine *et al.*, 1981) and extracellularly in animal tissue fluids (Gutteridge *et al.*, 1981; Gutteridge, 1982) has been reported. Gutteridge *et al.* (1981) found micromolar concentrations of non-protein-bound iron in animal pleural-exudate fluid and in human synovial or cerebrospinal fluid. No free iron was found in human serum, presumably because of its high content of iron-binding proteins that are normally only partially iron-loaded, but it was detected in the serum from patients suffering from iron-overload diseases (Hershko *et al.*, 1978).

By contrast, Winterbourn (1981) exposed several human body fluids, including synovial fluid, to an $O_2^{-\cdot}$-generating system and attempted to detect $OH^\cdot$ radicals. No physiological catalyst of reaction (1) was found. Unfortunately, the methional–ethene assay that she used is subject to many artifacts (Bors *et al.*, 1976; Pryor & Tang, 1978) and detects oxidizing species in addition to $OH^\cdot$ (Elstner *et al.*, 1980; Youngman *et al.*, 1982). Further, it may be that the extracellular fluids contained anti-oxidants that interfered with this assay but not with the method used by Gutteridge *et al.* (1981). Although such fluids contain little superoxide dismutase or catalase activities (McCord, 1974; Blake *et al.*, 1981*b*), the protein caeruloplasmin, which inhibits several oxygen-radical reactions and scavenges $OH^\cdot$ (Stocks *et al.*, 1974; Gutteridge *et al.*, 1980; Bannister *et al.*, 1980), is present, and there is evidence for other anti-oxidant proteins as well (Stocks *et al.*, 1974).

In an attempt to explain the results obtained by Winterbourn (1981), we have tested to see whether or not the non-protein-bound iron detected in human body fluids by the bleomycin assay (Gutteridge *et al.*, 1981) can catalyse radical reactions. Two

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such reactions were studied: ascorbate-induced peroxidation of bovine brain phospholipid liposomes, which requires traces of iron salts (Gutteridge *et al.*, 1979), and the O_2^{-}·-dependent formation of OH· radicals by a hypoxanthine/xanthine oxidase system, which again requires iron salts (Halliwell, 1978*b*,*c*; McCord & Day, 1978; Richmond *et al.*, 1981).

Materials and methods

Reagents

Catalase (bovine liver; thymol-free; specific activity 27000 μmol of H_2O_2 decomposed/min per mg under the assay conditions given in the Sigma catalogue), superoxide dismutase [bovine erythrocyte; specific activity 2900 units/mg under the assay conditions given by McCord & Fridovich (1969)], hypoxanthine, calf thymus DNA (type 1), 2-deoxy-D-ribose, diethylenetriaminepenta-acetic acid and albumin (human; fatty acid-free) were obtained from Sigma Chemical Co. Xanthine oxidase was from Boehringer, desferrioxamine (as Desferal) from CIBA–GEIGY, Chelex-100 from Bio-Rad Laboratories and bleomycin from Lundbeck (Luton, Beds., U.K.). All other reagents were of the highest quality available from BDH Chemicals. Pathologically normal cerebrospinal fluids were obtained from Mr. P. Lamport at the Whittington Hospital, London, U.K. Synovial fluids were obtained from Dr. D. Blake at the University of Birmingham Medical School, Birmingham, U.K. The water used to make up all solutions had been Chelex-treated before use. Samples were received either in glass tubes previously acid-cleaned for serum iron determination or in new plastic tubes which are known not to introduce contaminating iron (Gutteridge *et al.*, 1981).

Ultrafiltration

This was performed with an Amicon Diaflow PM10 (cut-off mol.wt. 10000) membrane with N_2 at 0.2 MPa (30lbf/in^2) above atmospheric pressure.

Bleomycin assay for non-protein-bound iron

This was performed as described by Gutteridge *et al.* (1981).

Superoxide-dependent formation of hydroxyl radicals

This was assayed by their ability to attack deoxyribose and form thiobarbituric acid-reactive products. Assays were performed essentially as described by Halliwell & Gutteridge (1981) except that incubations were for 2h and $FeNH_4(SO_4)_2$ was omitted from the reaction mixture. Where indicated 0.03ml of body fluid was included in the reaction mixture. In studies at pH4.9 KH_2PO_4 adjusted to pH4.9 with KOH was added.

Lipid peroxidation

This was studied with bovine brain phospholipids obtained as described by Gutteridge (1977) and Gutteridge *et al.* (1979). Reaction mixtures contained, in a final volume of 0.49ml, 2.04mg of phospholipid as liposomes at pH7.4, 0.49mM-ascorbate and, where indicated, 0.03ml of body fluid. They were incubated at 37°C for 2h. Then 0.5ml of a 1% (w/v) solution of thiobarbituric acid in 0.05M-NaOH was added, plus 0.5ml of 25% (v/v) HCl. After being heated at 100°C for 15min the tube contents were mixed with 1.5ml of butan-1-ol to extract the chromogen. After centrifugation the A_{532} of the butanol phase was measured. In studies at pH4.0 the reaction mixtures also contained 12mM-sodium acetate buffer at this pH and ascorbate at a concentration of 1.2mM.

Results

Experiments were performed on samples of cerebrospinal fluid and synovial fluid that had been drawn from patients for authentic medical purposes. The cerebrospinal fluids had been classified as 'pathologically normal' in that they contained less than 0.6mg of protein/ml and less than 6 cells/mm^3 and in that no bacteria were grown on culture. Synovial fluid was drawn from the knee-joints of patients who fulfilled the criteria of the American Rheumatism Association for classical or definite rheumatoid arthritis. Blood-stained fluids were rejected because of the possibility of contamination by catalase and superoxide dismutase from lysed eyrthrocytes (Blake *et al.*, 1981*b*). Plasma was obtained from healthy volunteers.

Table 1 (column A) shows the iron content of the body fluids as determined by the bleomycin assay: results were fully comparable with those obtained previously on different patients. The bleomycin assay is specific for non-protein-bound iron and is not interfered with by normal constituents of body fluids (Gutteridge *et al.*, 1981).

Catalytic activity of iron in ascorbate-dependent lipid peroxidation

The ability of ascorbate to induce lipid peroxidation in bovine brain phospholipid liposomes is absolutely dependent on traces of non-protein-bound iron salts and can be inhibited by the specific iron chelator desferrioxamine (Gutteridge *et al.*, 1979). Pure bovine brain phospholipids were mixed with ascorbate in Chelex-treated solutions (see the Materials and methods section). Addition of micromolar concentrations of inorganic iron salts induced rapid peroxidation, but despite their iron content

Table 1. *Presence and catalytic activity of non-protein-bound iron in human extracellular fluids*
For details see the text. Abbreviation: CSF, cerebrospinal fluid. In columns B and C the results have been corrected for the low rate of peroxidation seen in the presence of ascorbate only. The fluids themselves contained no interfering amounts of thiobarbituric acid-reactive material when assayed under the conditions described. In columns D and E the results have been corrected for the low rates of $OH^{\bullet}$ formation in the absence of added fluid. The values presented in each column are the mean values for at least three determinations, the variation between assays being 5% or less.

Column ...		A	B	C	D	E
Patient code	Fluid collected	Iron content by bleomycin assay (μmol/l)	Rate of ascorbate-dependent lipid peroxidation (as ΔA_{532}) in the presence of 30μl of ultrafiltered fluid	As B, but at pH 4.0 and with 30μl of native fluid	Rate of $O_2^{-\bullet}$-dependent formation of $OH^{\bullet}$ in the presence of 30μl of ultrafiltered fluid	As D, but at pH 4.9 and with 30μl of native fluid
R	CSF	1.2	0.03	0.39	0.04	0.08
M	CSF	0.5	0.07	0.20	0.02	0.12
W	CSF	4.0	0.11	0.29	0.06	0.15
C	CSF	1.0	0.13	0.76	0.15	0.13
B	Synovial	3.3	0.04	0.00	0.04	0.01
L	Synovial	2.5	0.03	0.00	0.04	0.00
D	Synovial	3.1	0.03	0.00	0.05	0.03
G	Plasma	0.0	0.00	0.00	0.00	0.00
A	Plasma	0.0	0.00	0.00	0.00	0.00
S	Plasma	0.0	0.01	0.00	0.02	0.01

none of the body fluids listed had this effect (results not shown), results essentially consistent with those of Winterbourn (1981). It follows either that the iron salts detected by the bleomycin assay were not in a form that could induce peroxidation, or else that anti-oxidants in the fluid were inhibiting lipid peroxidation and preventing an effect from being seen.

Most of the known anti-oxidants are proteins (Stocks *et al.*, 1974), and so as a test of this second hypothesis the effect of removing them by ultrafiltration was examined. Column B of Table 1 shows that stimulatory activity could then easily be detected in synovial and cerebrospinal fluids. The ultrafiltration process did not cause release of iron from iron proteins, since no significant catalytic activity was detected in the ultrafiltrate of plasma samples, which contain very high concentrations of partially-iron-loaded proteins. (It could be argued that the iron-binding proteins of cerebrospinal fluid are more labile than those of serum. Although this seems unlikely, since iron-binding proteins in other body fluids are similar to each other, proper testing of it will require a detailed analysis of the proteins present.) That the catalytic activity was due to iron was shown by its inhibition by the chelating agents desferrioxamine and diethylenetriaminepenta-acetic acid (Table 2), which inhibit iron-dependent radical reactions (Buettner *et al.*, 1978; Halliwell, 1978*b*,*c*; Gutteridge *et al.*, 1979; Richmond *et al.*, 1981).

The anti-oxidant activity of some extracellular fluids is greatly diminished, although not eliminated, at pH values below 5 (Stocks *et al.*, 1974), and the effect of performing assays of lipid peroxidation at pH 4.0 with the native fluids is shown in column C of Table 1. A stimulatory effect could be detected with cerebrospinal fluid, which was prevented by desferrioxamine (Table 2), but not with synovial fluid or plasma.

Attempts were made to destroy the anti-oxidant activity by heating the fluids, but the results were complicated by the apparent release of iron from iron proteins, since a large stimulatory effect was observed with plasma samples heated at 100°C for 8 min although these do not contain non-protein-bound iron (Table 1, columns A and B).

Catalytic activity of iron in superoxide-dependent formation of hydroxyl radicals

A mixture of hypoxanthine and xanthine oxidase generates $O_2^{-\bullet}$ and H_2O_2, which, in the presence of micromolar concentrations of free iron salts, can interact to form $OH^{\bullet}$. The $OH^{\bullet}$ radical may be detected by its ability to degrade deoxyribose, and degradation is inhibited by catalase, superoxide dismutase and desferrioxamine (Halliwell & Gutteridge, 1981). Omission of iron salts from the assay mixture gives a very low rate of degradation of deoxyribose. Addition of the native body fluids listed in Table 1 did not increase the rate (results not shown), again in agreement with the results obtained by Winterbourn (1981).

As with lipid peroxidation, however, ultrafiltrates of synovial or cerebrospinal fluids were effective in increasing formation of $OH^{\bullet}$, and this was prevented by desferrioxamine (Table 1, column D, and

Table 2. *Catalytic activity of non-protein-bound iron in human extracellular fluids: inhibition by chelating agents and radical scavengers*

Concentrations of added reagents are the final concentrations obtained in the reaction mixtures. For further details see Table 1 and the Materials and methods section. Abbreviations: CSF, cerebrospinal fluid; DETAPAC, diethylenetriaminepenta-acetic acid. Cerebrospinal fluid from patient M was chosen for study simply because the volume of it in our possession allowed sufficient experiments to be done. The results of a typical experiment are presented, but similar results were obtained in several experiments with fluids both from patient M and from other patients.

System studied	Fluid used	Reagent added	Rate of process
Ascorbate-induced lipid peroxidation (rates as $\Delta A_{532}/2h$)	CSF (patient M)	None	0.05
		Desferrioxamine (1 mM)	0.00
		DETAPAC (1 mM)	0.00
Ascorbate-induced lipid peroxidation, pH 4.0 (rates as $\Delta A_{532}/2h$)	CSF (patient M)	None	0.20
		Desferrioxamine (1 mM)	0.00
Superoxide-dependent formation of $OH^{\bullet}$ (rates as $\Delta A_{532}/2h$)	CSF (patient M)	None	0.02
		Desferrioxamine (1 mM)	0.00
		Catalase (0.5 mg/ml)	0.00
		Superoxide dismutase (0.5 mg/ml)	0.00
		Bovine serum albumin (1.0 mg/ml)	0.04

Table 2). As expected, superoxide dismutase and catalase inhibited $OH^{\bullet}$ formation, but bovine serum albumin, used as a control for non-specific protein effects, did not (Table 2).

If the pH of the assay was changed to 4.9, the stimulatory effect could be demonstrated directly on the native fluid (Table 1, column E), although it was much more obvious with cerebrospinal fluid than with synovial fluid. $OH^{\bullet}$ formation at pH 4.9 was inhibited by catalase, superoxide dismutase or desferrioxamine (results not shown).

Discussion

The results given in the present paper show that the non-protein-bound iron detected in cerebrospinal fluid and rheumatoid synovial fluid by the bleomycin assay method of Gutteridge *et al.* (1981) can indeed catalyse oxygen-radical reactions if interfering substances are removed by ultrafiltration. A similar effect can be achieved in cerebrospinal fluid by conducting assays at acid pH values, but this is much less marked with synovial fluid. A detailed study of the nature of these inhibitors is required to explain the differences, but presumably they are proteins and include caeruloplasmin (Stocks *et al.*, 1974) as well as others as yet unknown.

The results reported by Winterbourn (1981) thus cannot be taken as evidence for the absence of physiological iron catalysts in body fluids other than plasma, and their presence is supported by overwhelming circumstantial evidence (McCord, 1974; Fridovich, 1975, 1978; Halliwell, 1978*a*,*b*,*c*, 1981*a*,*b*; Blake *et al.*, 1981*a*) as well as by the direct demonstration reported in the present paper. The $HO^{\bullet}$ radical rapidly reacts with lipoproteins, caeruloplasmin, other proteins and other body-fluid constituents such as hyaluronic acid (Halliwell, 1981*a*). In conventional assays these body-fluid constituents compete with added detector molecules such as methional for any $OH^{\bullet}$ formed, and as they react with it they are damaged or destroyed. Until they are removed by ultrafiltration it is difficult to demonstrate the presence of iron catalysts of $OH^{\bullet}$ formation.

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References

Bannister, J. V., Bannister, W. H., Hill, H. A. O., Mahood, J. F., Willson, R. L. & Wolfenden, B. S. (1980) *FEBS Lett.* **118**, 127–129

Blake, D. R., Hall, N. D., Bacon, P. A., Dieppe, P. A., Halliwell, B. & Gutteridge, J. M. C. (1981*a*) *Lancet* **ii**, 1142–1144

Blake, D. R., Hall, N. D., Treby, D. A., Halliwell, B. & Gutteridge, J. M. C. (1981*b*) *Clin. Sci.* **61**, 483–486

Bors, W., Lengfelder, E., Saran, M., Fuchs, C. & Michel, C. (1976) *Biochem. Biophys. Res. Commun.* **70**, 81–87

Buettner, G. R., Oberley, L. W. & Leuthauser, S. W. H. C. (1978) *Photochem. Photobiol.* **28**, 693–695

Chance, B., Sies, H. & Boveris, A. (1979) *Physiol. Rev.* **59**, 527–605

Elstner, E. F., Osswald, W. & Konze, J. R. (1980) *FEBS Lett.* **121**, 219–221

Fong, K. L., McCay, P. B., Poyer, J. L., Misra, H. P. & Keele, B. B. (1976) *Chem.-Biol. Interact* **15**, 77–89
Fridovich, I. (1975) *Annu. Rev. Biochem.* **44**, 147–159
Fridovich, I. (1978) *Science* **201**, 875–880
Gutteridge, J. M. C. (1977) *Anal. Biochem.* **82**, 76–82
Gutteridge, J. M. C. (1982) *Biochem. Soc. Trans.* **10**, 72–73
Gutteridge, J. M. C., Richmond, R. & Halliwell, B. (1979) *Biochem. J.* **184**, 469–472
Gutteridge, J. M. C., Richmond, R. & Halliwell, B. (1980) *FEBS Lett.* **112**, 269–272
Gutteridge, J. M. C., Rowley, D. A. & Halliwell, B. (1981) *Biochem. J.* **199**, 263–265
Halliwell, B. (1978*a*) *Cell Biol. Int. Rep.* **2**, 113–128
Halliwell, B. (1978*b*) *FEBS Lett.* **92**, 321–326
Halliwell, B. (1978*c*) *FEBS Lett.* **96**, 238–242
Halliwell, B. (1981*a*) in *Age Pigments* (Sohal, R. S., ed.), pp. 1–62, Elsevier/North-Holland Biomedical Press, Amsterdam
Halliwell, B. (1981*b*) *Bull. Eur. Physiopathol. Respir.* **17**, 21–28
Halliwell, B. (1982) *Biochem. J.* **205**, 461–462
Halliwell, B. & Gutteridge, J. M. C. (1981) *FEBS Lett.* **128**, 347–352
Hershko, C., Graham, G., Bates, G. W. & Rachmilewitz, E. A. (1978) *Br. J. Haematol.* **40**, 255–263
McCord, J. M. (1974) *Science* **185**, 529–531
McCord, J. M. & Day, E. D. (1978) *FEBS Lett.* **86**, 139–142
McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **224**, 6049–6055
Pryor, W. A. & Tang, R. H. (1978) *Biochem. Biophys. Res. Commun.* **81**, 498–503
Repine, J. E., Fox, R. B. & Berger, E. M. (1981) *J. Biol. Chem.* **256**, 7094–7096
Richmond, R., Halliwell, B., Chauhan, J. & Darbre, A. (1981) *Anal. Biochem.* **118**, 328–335
Stocks, J., Gutteridge, J. M. C., Sharp, R. J. & Dormandy, T. L. (1974) *Clin. Sci.* **47**, 215–222
Wills, E. D. (1965) *Biochim. Biophys. Acta* **98**, 238–251
Wills, E. D. (1969) *Biochem. J.* **113**, 325–332
Winterbourn, C. C. (1981) *Biochem. J.* **198**, 125–131
Youngman, R. J., Osswald, W. F. & Elstner, E. F. (1982) *Biochem. Pharmacol.* **31**, 603–606