Biochemical Pharmacology, Vol. 37, No. 3, pp. 409–413, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

DIALURIC ACID AUTOXIDATION

EFFECTS OF TRANSITION METALS ON THE REACTION RATE AND ON THE GENERATION OF "ACTIVE OXYGEN" SPECIES

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(*Received* 10 *June* 1987; *accepted* 27 *July* 1987)

Abstract—The autoxidation of dialuric acid, a process which is believed to be of crucial importance in the diabetogenic action of alloxan, was found to be strongly catalysed by copper, iron and manganese. Superoxide radical and hydrogen peroxide were generated in both the uncatalysed and the metal-catalysed reactions. In contrast, hydroxyl radical was formed during dialuric acid autoxidation only in the presence of added iron salts. Production of the latter radical was strongly inhibited by catalase but only weakly by superoxide dismutase, implying that the metal-catalysed Haber–Weiss reaction is of comparatively little importance in hydroxyl radical generation from dialuric acid.

Alloxan (2,4,5,6[1H, 3H]-pyrimidinetetrone) induces diabetes in experimental animals through destruction of the insulin-producing β-cells of the pancreas [1]. Although the mode of action of this substance is not fully understood, there is evidence [2–5] that the tissue damage is initiated by intracellular generation of "active oxygen" species, formed in a cyclic reaction involving alloxan and its reduction product, dialuric acid. The autoxidation of the latter has been shown to generate superoxide radical [9, 10] and hydrogen peroxide [9–12]; it is proposed [4–8] that hydroxyl radical, formed from these species via the so-called Haber–Weiss reaction, is the agent ultimately responsible for the observed pancreatic damage.

The importance of metal catalysis in autoxidation reactions has been recognised for many years; the oxidation of numerous compounds, including thiols [13], quinols [14, 15] and hydrazine derivatives [16, 17] is known to be partly or wholly mediated by transition metal ions. Little is known, however, about the possible involvement of metal salts in dialuric acid autoxidation. In view of the suggested importance of this reaction in the diabetogenic action of alloxan and because of the obligatory metal catalysis of the Haber–Weiss reaction [18], the effects of various transition metals on the rate of autoxidation of dialuric acid, and on the formation of "active oxygen" species in this process, have been investigated. The results of this study are described in the present report.

MATERIALS AND METHODS

Dialuric acid was synthesised by the method of Biltz and Damm [19]. Catalase (from bovine liver) was from Boehringer. Superoxide dismutase (from bovine erythrocytes) and diethylenetriaminepentaacetic acid (DTPA) were products of Sigma. Desferrioxamine methanesulphonate (Desferal) was from Ciba-Geigy. Chelex-100 was purchased from BioRad Laboratories and equilibrated to pH 7.0 before use. Metal salts—cupric sulphate (BDH), ferric ammonium sulphate (Riedel de Hahn), ferrous ammonium sulphate (May & Baker), cobaltous chloride (BDH), manganese chloride (BDH) and nickel chloride (BDH)—were of analytical grade.

The rate of autoxidation of dialuric acid was followed by rate of absorbance decrease at 273 nm ($\varepsilon = 16{,}000\ \mathrm{l\ mol^{-1}\ cm^{-1}}$ [6]) on an Aminco DW 2a spectrophotometer. The buffer employed was 0.05 M potassium phosphate, pH 7.0, which, except in a few preliminary experiments, was freed from trace metal contamination by treatment with Chelex resin. The temperature was maintained at 25°; recording of A_{273} was started immediately after initiation of the reaction by addition of dialuric acid to a final concentration of 50 μM. In experiments in which the effects of free-radical scavengers, metals and metal-chelating agents were investigated, these were added to the assay mix before the dialuric acid. The effect of transition metal salts was quantified by rate measurements at various metal concentrations. In each case, the rate of autoxidation proved to be directly proportional to the concentration of metal; the concentration of each metal required to double the rate of autoxidation was calculated by linear regression analysis.

Formation of hydroxyl radical during the autoxidation of dialuric acid was detected using the aromatic hydroxylation technique of Richmond *et al.* [20]. Dialuric acid (500 μM) was incubated at 25° in pH 7.0 buffer in the presence of potassium salicylate (2.5 mM) for 2 hr. After acidification, the hydroxylated products formed from salicylate by reaction with $OH^{\cdot}$ were extracted and assayed colorimetrically [20], being quantified by reference to a standard curve prepared using 2,3-dihydroxybenzoic acid.

Table 1. Effect of chelating agents, superoxide dismutase and catalase on dialuric acid autoxidation

Addition	Initial rate of autoxidation (μmoles l^{-1} min^{-1})	
None	13.8 ± 0.4	
EDTA	9.6 ± 0.4	(−30%)
DTPA	10.1 ± 0.4	(−27%)
Desferal	10.1 ± 0.3	(−27%)
SOD	4.4 ± 0.5	(−68%)
CAT	10.4 ± 0.3	(−25%)
SOD + CAT	3.1 ± 0.3	(−78%)
DTPA + SOD	3.2 ± 0.3	(−77%)
DTPA + CAT	9.4 ± 0.6	(−32%)
DTPA + SOD + CAT	2.0 ± 0.1	(−86%)

Buffer was treated with Chelex resin. The concentration of the chelating agents was 100 μM, that of superoxide dismutase (SOD) 25 μg ml^{-1} and of catalase (CAT), 50 μg ml^{-1}. Results shown are the means and SEM of 4 determinations.

RESULTS

Effect of metal-chelating agents, superoxide dismutase and catalase on the rate of autoxidation of dialuric acid

In preliminary experiments, dialuric acid autoxidation was studied in unpurified buffers. Under these conditions, however, problems were encountered with reproducibility, with marked variations in reaction rate being observed with different batches of buffer. All buffer solutions were subsequently treated with the metal-complexing resin, Chelex-100, which lowered the autoxidation rate by a factor of 2–3 and eliminated the inter-batch variation.

Even using Chelex-treated buffers, further decreases in reaction rate were induced by addition of the metal-chelating agents EDTA, DTPA and Desferal (Table 1).

The rate of autoxidation of dialuric acid was markedly decreased in the presence of superoxide dismutase and, to a lesser degree, in the presence of catalase. These effects were observed both in the presence and absence of metal-chelating agents (Table 1).

No inhibition of dialuric acid autoxidation was observed in the presence of superoxide dismutase and catalase which had been inactivated by boiling. Indeed, the rate of the reaction was considerably *increased* in the presence of boiled superoxide dismutase (Table 2). This effect was abolished by addition of EDTA.

Table 3. Metal catalysis of dialuric acid autoxidation

Metal	Concentration required to double the rate of autoxidation (μmoles l^{-1})	
Cu$^{\mathrm{II}}$	0.10	(0.08–0.12)
Mn$^{\mathrm{II}}$	1.05	(0.93–1.16)
Fe$^{\mathrm{II}}$	1.29	(1.18–1.40)
Fe$^{\mathrm{III}}$	6.78	(6.20–7.36)
Co$^{\mathrm{II}}$	>100*	
Ni$^{\mathrm{II}}$	>100*	

* No catalysis was observed with these metals at a concentration of 100 μmoles l^{-1}.

Assays were conducted as described in Materials and Methods. Results shown are the means and 95% confidence intervals.

Effect of transition metals on the rate of autoxidation of dialuric acid

Copper was a potent catalyst of dialuric acid autoxidation, doubling the reaction rate at a concentration of 0.1 μmoles l^{-1} (Table 3). Manganese and ferrous iron were also very effective catalysts, while ferric iron was somewhat less active. No catalysis was observed with nickel and cobalt at a concentration of 100 μmoles l^{-1} (Table 3).

In the presence of metals, as in their absence, the rate of autoxidation was decreased in the presence of superoxide dismutase and catalase (Table 4).

The catalytic action of copper and manganese was eliminated in the presence of excess EDTA or DTPA; even at 100 μmoles l^{-1}, no catalysis by the complexed forms of these metals was observed. Similarly, DTPA and Desferal abolished the catalytic activity of iron salts. In contrast, the EDTA complexes of iron were effective catalysts of dialuric acid autoxidation, Fe$^{\mathrm{II}}$–EDTA and Fe$^{\mathrm{III}}$-EDTA doubling the reaction rate when present at concentrations of 22.2 and 23.0 μmoles l^{-1} respectively.

Table 2. Effect of inactivated superoxide dismutase and catalase on dialuric acid autoxidation

Addition	Initial rate of autoxidation (μmoles l^{-1} min^{-1})
None	14.2 ± 0.4
EDTA	10.6 ± 1.3
Inactivated SOD	25.4 ± 1.4
Inactivated SOD + EDTA	10.2 ± 0.9
Inactivated CAT	14.9 ± 0.5
Inactivated CAT + EDTA	10.6 ± 0.7

Superoxide dismutase (SOD) and catalase (CAT), inactivated by boiling, were added at concentrations equivalent to 25 μg ml^{-1} and 50 μg ml^{-1} respectively. Where indicated, EDTA was added at a concentration of 100 μM. Results shown are the means and SEM of 4 determinations.

Table 4. Inhibition of metal-catalysed autoxidation of dialuric acid by superoxide dismutase and catalase

Catalyst	Percent decrease in initial rate of autoxidation in the presence of: SOD	CAT	SOD + CAT
None*	68 ± 4	25 ± 2	78 ± 3
Cu$^{\mathrm{II}}$ (0.1 μM)	84 ± 4	67 ± 3	91 ± 4
Mn$^{\mathrm{II}}$ (1 μM)	80 ± 3	52 ± 3	88 ± 3
Fe$^{\mathrm{II}}$ (1 μM)	54 ± 3	47 ± 8	89 ± 4
Fe$^{\mathrm{III}}$ (5 μM)	78 ± 5	35 ± 4	89 ± 5

* Data from Table 1.

The concentrations of superoxide dismutase (SOD) and catalase (CAT) were 25 μg ml^{-1} and 50 μg ml^{-1} respectively. Results shown are the means and SEM of 3 determinations.

Table 5. Hydroxyl radical production during dialuric acid autoxidation

Addition	2,3-Dihydroxybenzoate formed (μmoles l^{-1})
None	4.8 ± 0.6
Cu^{II}	6.0 ± 0.7
Mn^{II}	5.4 ± 0.6
Fe^{II}	19.1 ± 0.9
Fe^{III}	12.4 ± 1.1
Fe^{II}-EDTA	111.5 ± 4.3
Fe^{III}-EDTA	115.2 ± 3.7
Fe^{II}-DTPA	41.5 ± 1.0
Fe^{III}-DTPA	34.4 ± 2.2
Fe^{II}-Desferal	0.9 ± 0.5
Fe^{III}-Desferal	2.2 ± 0.4

The concentrations of the unchelated metals were the same as those shown in Table 4; with the iron complexes, the iron concentration in all cases was 20 μM and that of the chelating agents 100 μM. Results shown are the means and SEM of 4 determinations.

Formation of hydroxyl radical during the autoxidation of dialuric acid

Using the aromatic hydroxylation technique [20], which detects OH$^{\bullet}$ by virtue of its ability to form 2,3-dihydroxybenzoic acid from salicylate, no significant hydroxyl radical production was observed during the uncatalysed autoxidation of dialuric acid or that occurring in the presence of copper, manganese or iron–Desferal. Hydroxyl radical formation was recorded, however, in the presence of uncomplexed iron, iron–DTPA and, particularly, iron–EDTA (Table 5).

The formation of hydroxyl radical from dialuric acid mediated by iron salts was greatly decreased in the presence of catalase, although superoxide dismutase had little effect (Table 6).

DISCUSSION

Buffers and reagents are invariably contaminated with trace amounts of metal, particularly copper and iron [13, 21, 22]. The potent catalysis of dialuric acid autoxidation by salts of these metals would account for the marked decrease in rate observed after treatment of buffers with Chelex resin. Such treatment was not, however, sufficient totally to remove catalytic metal from the system since a further decrease in rate was induced by addition of EDTA, DTPA or Desferal to the reaction mixture. The autoxidation rate in the presence of the three chelating agents was closely similar; the effectiveness of the iron-specific chelator, Desferal, implies that iron was the contaminating metal.

Superoxide dismutase and catalase inhibited the uncatalysed autoxidation of dialuric acid. This observation is consistent with previous reports [9–12] of generation of superoxide radical and hydrogen peroxide during this reaction and indicates that these "active oxygen" species participate in the oxidation. This is in accord with the mechanism of dialuric acid autoxidation proposed by Houée-Levin *et al.* [23, 24] in which the reaction is initiated by oxidation of dialuric acid (AH_2) by molecular oxygen

$$AH_2 + O_2 \longrightarrow AH^{\bullet} + O_2^{-} + H^{+} \tag{1}$$

The alloxan radical ($AH^{\bullet}$) may form alloxan (A) and dialuric acid through dismutation (Reaction 2) or react with oxygen or superoxide radical (Reactions 3 and 4)

$$2AH^{\bullet} \longrightarrow A + AH_2 \tag{2}$$

$$AH^{\bullet} + O_2 \longrightarrow A + O_2^{-} + H^{+} \tag{3}$$

$$AH^{\bullet} + O_2^{-} + H^{+} \longrightarrow A + H_2O_2 \tag{4}$$

Dialuric acid is oxidised [23, 24] by both superoxide and hydrogen peroxide (Reactions 5 and 6):

$$AH_2 + O_2^{-} + H^{+} \longrightarrow AH^{\bullet} + H_2O_2 \tag{5}$$

$$AH_2 + H_2O_2 \longrightarrow A + 2H_2O \tag{6}$$

The decreased rate of dialuric acid autoxidation in the presence of superoxide dismutase reflects inhibition of Reactions 4 and 5 while abolition of Reaction 6 would account for the inhibitory effect of catalase.

In agreement with early reports [25, 26], iron was found to be an effective catalyst for dialuric acid autoxidation; copper and manganese were also identified as catalysts. The potent effect of copper may account for the observation that a sample of superoxide dismutase which had been inactivated by boil-

Table 6. Effect of superoxide dismutase and catalase on iron-mediated hydroxyl radical production during dialuric acid autoxidation

	Percent decrease in 2,3-dihydroxybenzoate formation in the presence of:	
Iron compound	SOD	CAT
Fe^{II}	19 ± 1	78 ± 5
Fe^{III}	25 ± 4	81 ± 8
Fe^{II}-EDTA	5 ± 2	88 ± 3
Fe^{III}-EDTA	4 ± 4	89 ± 3
Fe^{II}-DTPA	21 ± 1	85 ± 2
Fe^{III}-DTPA	14 ± 3	94 ± 7

The concentrations of the iron compounds were as detailed in the legend to Table 5. The concentration of both superoxide dismutase (SOD) and catalase (CAT) was 200 μg ml^{-1}. Results shown are the means and SEM of 3 determinations.

ing increased the rate of dialuric acid autoxidation; copper released from this enzyme following denaturation has been shown to stimulate other autoxidation reactions which are catalysed by this metal [27, 28].

It is likely that the catalytic effect of iron, copper and manganese is brought about by electron transfer from dialuric acid to metal, followed by re-oxidation of the latter by molecular oxygen (Reactions 7 and 8):

$$AH_2 + M^{n+} \longrightarrow AH^{\cdot} + M^{(n-1)+} + H^+ \quad (7)$$

$$M^{(n-1)+} + O_2 \longrightarrow M^{n+} + O_2^{-} \quad (8)$$

The sum of these reactions is equivalent to Reaction 1, the rate-limiting step of the autoxidation [24] and if these reactions are comparatively fast, an increase in overall rate is to be expected. As in the uncatalysed reaction, superoxide radical and hydrogen peroxide were formed during the metal-catalysed autoxidation of dialuric acid, as indicated by decreases in reaction rate in the presence of superoxide dismutase and catalase. The effect of the latter enzyme was greater in the catalysed reaction than in the uncatalysed, suggesting facilitation of Reaction 6 by metal salts.

The catalytic activity of copper and manganese was abolished by EDTA and DTPA. The latter chelating agent also eliminated iron catalysis, as did Desferal. In contrast, EDTA, while reducing the effectiveness of iron, did not completely abolish the catalytic effect of this metal, possibly reflecting reduction of Fe^{III}-EDTA by dialuric acid, as discussed below.

The formation of hydroxyl radical during dialuric acid autoxidation was investigated using the sensitive and specific aromatic hydroxylation technique [20]. Little hydroxyl radical production occurred during the uncatalysed reaction, in accord with the observation [24] that the alloxan radical is not readily oxidised by hydrogen peroxide (Reaction 9).

$$AH^{\cdot} + H_2O_2 \longrightarrow A + OH^{\cdot} + H_2O \quad (9)$$

Neither copper nor manganese promoted hydroxyl radical formation, but a trace was formed in the presence of unchelated iron and more with iron–DTPA. By far the best catalyst, however, was iron–EDTA, which is known to be a particularly effective promoter of hydroxyl radical formation in other systems generating "active oxygen" species [29].

The necessity for iron catalysis of hydroxyl radical formation from dialuric acid identified in the present study is in contrast to a previous report [10] in which no requirement for metal ions was documented. In the latter study, however, the buffer (which contained EDTA) was noted to be contaminated with significant quantities of iron and it is therefore likely that hydroxyl radical formation was promoted by chelated iron.

The formation of hydroxyl radicals mediated by iron salts involves oxidation of ferrous iron by hydrogen peroxide (Reaction 10), with the catalytic cycle maintained by reduction of the metal by superoxide (Reaction 11) [18, 30, 31] or by other reducing agents [21, 32–34], which, in the present instance, may be dialuric acid or the alloxan radical (Reactions 12 and 13).

$$Fe^{II} + H_2O_2 \longrightarrow Fe^{III} + OH^{\cdot} + OH^- \quad (10)$$

$$Fe^{III} + O_2^{-} \longrightarrow Fe^{II} + O_2 \quad (11)$$

$$Fe^{III} + AH_2 \longrightarrow Fe^{II} + AH^{\cdot} + H^+ \quad (12)$$

$$Fe^{III} + AH^{\cdot} \longrightarrow Fe^{II} + A + H^+ \quad (13)$$

Reactions 10 and 11 constitute the iron-catalysed Haber–Weiss reaction, which has been shown to be responsible for hydroxyl radical formation in many systems generating superoxide radical and hydrogen peroxide [18, 30, 31]. It cannot, however, contribute significantly to hydroxyl radical formation from dialuric acid, since this process, while strongly inhibited by catalase (reflecting inhibition of Reaction 10) was only marginally influenced by superoxide dismutase; the Haber–Weiss reaction is strongly inhibited by both catalase and superoxide dismutase [18, 22, 35]. Furthermore, significant hydroxyl radical production was observed in the presence of iron–DTPA, which is not a catalyst for the Haber–Weiss reaction [31, 35]. In the case of dialuric acid, therefore, it would appear that metal reduction is mediated via Reactions 12 and/or 13. The former reaction is equivalent to Reaction 7, and an increase in the overall rate of autoxidation is therefore to be expected; this process may thus be important in the reduction of iron–EDTA. In contrast, the reduction of iron–DTPA, which was not associated with augmentation of the reaction rate, may be mediated solely by Reaction 13 in a manner analogous to that described for the paraquat [33] and adriamycin [34] radicals.

Hydroxyl radical is believed to be the proximate toxic agent in alloxan diabetes, with the metal-catalysed Haber–Weiss reaction being held responsible for the formation of this species [4–8]. This concept is based upon observations that DTPA and hydroxyl radical scavengers ameliorate alloxan toxicity both in isolated cells [6–8, 36, 37] and in the whole animal [2, 3, 38, 39]. The results of the present investigation, however, indicate that the Haber–Weiss reaction is unlikely to be a major source of hydroxyl radicals in the alloxan/dialuric acid cycle. Furthermore, the potent catalytic effect of transition metals on dialuric acid autoxidation suggests that results of experiments with this substance or with dialuric acid involving metal-chelating agents need to be interpreted with care.

Acknowledgements—The skilled technical assistance of Mrs E. A. Fowke is gratefully acknowledged. Thanks also to Dr C. C. Winterbourn for valuable discussion.

REFERENCES

1. C. C. Rerup, *Pharmac. Rev.* **22**, 485 (1970).
2. R. E. Heikkila, B. Winston, G. Cohen and H. Bardon, *Biochem. Pharmac.* **25**, 1085 (1976).
3. J. Tibaldi, J. Benjamin, F. S. Cabbat and R. E. Heikkila, *J. Pharmac. exp. Ther.* **211**, 415 (1979).
4. W. J. Malaisse, *Biochem. Pharmac.* **31**, 3527 (1982).
5. L. J. Fischer and A. W. Harman, in *Pathology of Oxygen* (Ed. A. P. Autor), p. 261. Academic Press, New York (1982).
6. J. J. van Hemmen and W. J. A. Meuling, *Archs Biochem. Biophys.* **182**, 743 (1977).
7. K. Grankvist, S. Marklund, J. Sehlin and I. Täljedal, *Biochem. J.* **182**, 17 (1979).

8. F. Ishibashi and B. V. Howard, *J. biol. Chem.* **256**, 12134 (1981).
9. L. R. DeChatelet, P. S. Shirley, P. R. Goodson and C. E. McCall, *Antimicrob. Agents Chemother.* **8**, 146 (1975).
10. G. Cohen and R. E. Heikkila, *J. biol. Chem.* **249**, 2447 (1974).
11. R. E. Heikkila and G. Cohen, *Molec. Pharmac.* **8**, 241 (1972).
12. D. W. Deamer, R. E. Heikkila, R. V. Panganamala, G. Cohen and D. G. Cornwell, *Physiol. Chem. Phys.* **3**, 426 (1971).
13. R. Munday, *J. appl. Toxicol.* **5**, 69 (1985).
14. T. H. James and A. Weissberger, *J. Am. chem. Soc.* **60**, 98 (1938).
15. Y. Kono, *J. Biochem. (Tokyo)* **91**, 381 (1982).
16. H. P. Misra and I. Fridovich, *Biochemistry N.Y.* **15**, 681 (1976).
17. A. L. Green, *Biochem. Pharmac.* **13**, 249 (1964).
18. J. M. McCord and E. D. Day, *FEBS Lett.* **86**, 139 (1978).
19. H. Biltz and P. Damm, *Ber. dt. chem. Ges.* **46**, 3662 (1913).
20. R. Richmond, B. Halliwell, J. Chauhan and A. Darbre, *Analyt. Biochem.* **118**, 328 (1981).
21. S. F. Wong, B. Halliwell, R. Richmond and W. R. Skowroneck, *J. inorg. Biochem.* **14**, 127 (1981).
22. J. DiGuiseppi and I. Fridovich, *Archs Biochem. Biophys.* **205**, 323 (1980).
23. C. Houée-Levin, M. Gardes-Albert, C. Ferradini and J. Pucheault, *Radiat. Res.* **83**, 270 (1980).
24. C. Houée-Levin, M. Gardes-Albert, C. Ferradini and J. Pucheault, *Radiat. Res.* **88**, 20 (1981).
25. G. M. Richardson, *Biochem. J.* **26**, 1959 (1932).
26. E. S. Hill, *J. biol. Chem.* **92**, 471 (1931).
27. N. S. Brownlee, J. J. Huttner, R. V. Panganamala and D. G. Cornwell, *J. Lipid Res.* **18**, 635 (1977).
28. R. Munday, *Chem. Biol. Interact.* **41**, 361 (1982).
29. E. Graf, J. R. Mahoney, R. G. Bryant and J. W. Eaton, *J. biol. Chem.* **259**, 3620 (1984).
30. M. S. Baker and J. M. Gebicki, *Archs Biochem. Biophys.* **234**, 258 (1984).
31. J. Butler and B. Halliwell, *Archs Biochem. Biophys.* **218**, 174 (1982).
32. C. C. Winterbourn, *Biochem. J.* **182**, 625 (1979).
33. C. C. Winterbourn, J. M. C. Gutteridge and B. Halliwell, *J. Free Radicals Biol. Med.* **1**, 43 (1985).
34. C. C. Winterbourn and H. C. Sutton, *Archs Biochem. Biophys.* **235**, 116 (1984).
35. B. Halliwell, *FEBS Lett.* **96**, 238 (1978).
36. K. Grankvist, S. Marklund and I. Täljedal, *FEBS Lett.* **105**, 15 (1979).
37. L. J. Fischer and S. A. Hamburger, *Diabetes* **29**, 213 (1980).
38. R. E. Heikkila and F. S. Cabbat, *Experientia* **38**, 378 (1982).
39. R. E. Heikkila, *Eur. J. Pharmac.* **44**, 191 (1977).