EVIDENCE FOR THE PRODUCTION OF HYDROXYL RADICALS FROM THE ADRIAMYCIN SEMIQUINONE AND H_2O_2

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1. Introduction

The antitumour activity of adriamycin (doxorubicin–HCl) and other quinone-like antibiotics is thought to involve intercalation of the drug molecules with DNA and free radical-dependent scission of the DNA strands [1–4]. Their cardiotoxicity is also thought to be a result of redox cycling of the drug and free radical reactions [5–8]. It has been postulated that these processes depend on the production of O_2^- and H_2O_2 from the reaction of the adriamycin free radical ($Adr^\cdot$) with O_2, with subsequent formation of $OH^\cdot$ radicals [2,3,5,6]. However, it is not yet clear how O_2^- produced in biological systems can give rise to $OH^\cdot$ radicals [9–11].

This paper describes a reaction between $Adr^\cdot$ and H_2O_2 that produces the $OH^\cdot$ radical, or a related species with very similar reactivity. Neither a metal catalyst nor O_2^- are required for this reaction, although in air it is inhibited by superoxide dismutase. The characteristics of this reaction are such that it could be of major significance in the mechanism of action of adriamycin.

2. Methods

Adriamycin radicals and O_2^- were generated, at 25°C, by the reaction of xanthine oxidase with xanthine, with and without adriamycin, in 0.05 M phosphate buffer (pH 7.4) in N_2 and air/N_2 mixtures. Rates of radical generation were determined by measuring the rate of reduction of 15 μM cytochrome *c* (ΔA_{550}, $\epsilon = 21.1$) in the presence of catalase (30 μg/ml). For the measurement of ethylene production, reactions were carried out in 2.5 ml total vol. in rubber-stoppered 12 ml tubes. Further experimental details are given under each figure or table. At intervals, 0.4 ml gas samples were removed and the ethylene concentration measured as in [12]. This was quantitated by comparing the GLC peak height with heights of peaks obtained by oxidizing known amounts of methional with excess $OH^\cdot$ produced from H_2O_2 and $FeSO_4$.

All biochemicals were obtained from Sigma (St Louis MO) except superoxide dismutase (Diagnostic Reagents, Oxon) and adriamycin (Pharmitalia, Barnet).

Abbreviations: $Adr^\cdot$, adriamycin semiquinone radical; O_2^-, superoxide; $OH^\cdot$, hydroxyl radical; DTPA, diethylenetriamine penta-acetic acid

3. Results

A wide range of quinones can replace O_2 as an electron acceptor from xanthine oxidase, and transfer electrons via their semiquinone radical to cytochrome *c* [13–15]. Adriamycin has been shown to behave in this way [16], and its reduction by xanthine oxidase under N_2 therefore provides a convenient method for continuous production of the semiquinone. In air, O_2^- is the main radical product of the reaction [16], but subsequent reactions could involve either O_2^- or $Adr^\cdot$ because of the equilibrium:

$$Adr^\cdot + O_2 \rightleftharpoons O_2^- + \text{adriamycin} \quad (1)$$

The reaction of adriamycin with xanthine oxidase and xanthine in N_2 in the presence of H_2O_2 resulted in the production of ethylene from methional (fig.1).

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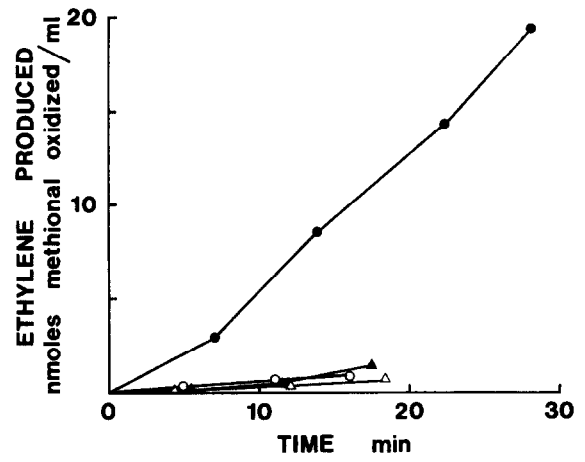

Fig.1. Rate of ethylene production from Adr˙ and H_2O_2. Reactions were carried out in N_2-bubbled solutions containing xanthine (0.2 mM), DTPA (0.1 mM), methional (2 mM), H_2O_2 (300 μM), adriamycin (120 μM) and xanthine oxidase (5×10^{-3} U/ml). Radical generation rate 15 nmol . ml^{-1} . 10 min^{-1}. (●) All reactants present; (○) no adriamycin; (▲) no H_2O_2; (△) no xanthine oxidase.

After an initial lag of ~5 min, ethylene accumulated in the gas phase at a linear rate. There was very little reaction unless xanthine oxidase, adriamycin and H_2O_2 were all present (fig.1). The reaction rate (measured after 5 min over the linear portion of the curve) depended on the concentrations of xanthine oxidase and adriamycin (fig.2) and there was an absolute requirement for H_2O_2, as shown by the virtually complete inhibition by catalase (table 1). However the rate was maximal and independent of H_2O_2 over 100–750 μM. Ethylene was a major product of the overall reaction. The rate of production was the same with 1–4 mM methional, indicating efficient trapping of the precursor radical, and under the conditions of fig.1, the amount of methional oxidized corresponded to approximately half of the radicals produced by the xanthine oxidase.

The reaction rate was the same in the presence of either diethylenetriamine–penta-acetic acid (DTPA) or EDTA (0.1 mM) as with no chelator present. Addition of 10 μM Fe^{2+} (EDTA), in the absence of DTPA,

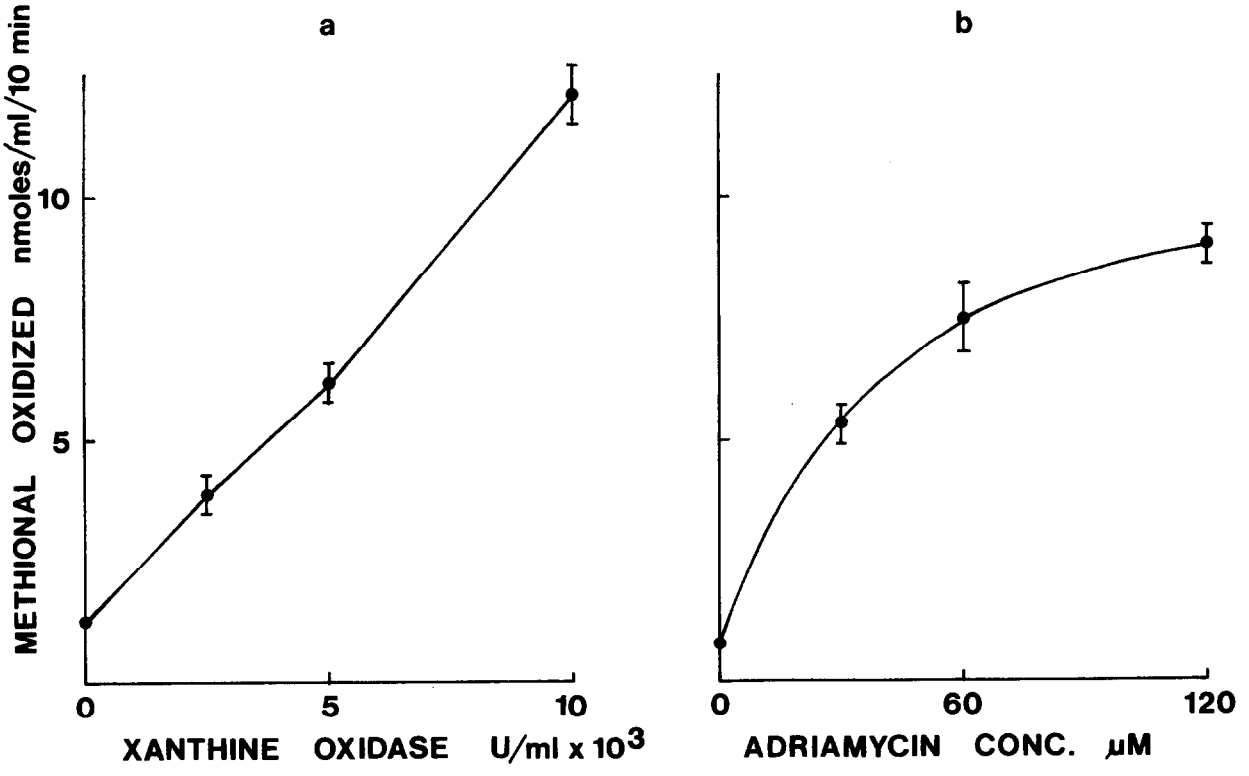

Fig.2. Ethylene production from Adr˙ and H_2O_2. Dependence on: (a) xanthine oxidase concentration; (b) adriamycin concentration. Apart from the variable reactant, concentrations were as in fig.1.

Table 1
Effects of inhibitors on ethylene production

Inhibitor	Rate of ethylene production from $Adr^{\cdot} + H_2O_2$ (Δ peak height/10 min)		Inhibition (%)	Inhibition (%) of ethylene production from O_2^-, H_2O_2 and Fe^{2+} [b]
	No inhibitor	With inhibitor		
Catalase[a] 30 μg/ml	19	1	96	
Superoxide dismutase (μg/ml) 10	122	112	8 ± 6	
2 (15% air)	65	18	72	
10 (15% air)		13	80	
2 (30% air)	45	12	73	
10 (30% air)		7	84	
2 (air)	21.5	5	77	
10 (air)		1	94	
Boiled 10 (30% air)	27	32	0	
Na-benzoate (mM) 10	74	30	60	75
20		14	81	75
Mannitol (mM) 10	74	60	40	57
20		42	58	79
Formate (mM) 10	54	39	28	44
20		39	28	54
Ethanol (mM) 10	54	36	35	23
20		27	50	39

[a] Reactions were carried out in the absence of added H_2O_2
[b] The xanthine oxidase reaction was carried out in air, with no adriamycin added, in the presence of 10 μM $FeSO_4$ and 100 μM EDTA

Reaction conditions were as in fig.1, except that adriamycin was 60 μM and methional 1 mM. Reactions were carried out in N_2-bubbled solution unless otherwise indicated. Figures quoted are the means of duplicates, that differed by <10%

decreased the initial lag slightly, but did not alter the rate of ethylene production.

The $OH^{\cdot}$ scavengers, benzoate, mannitol, formate and ethanol all inhibited ethylene production from $Adr^{\cdot}$ and H_2O_2 (table 1). The effects of these scavengers were also examined on ethylene production from a known $OH^{\cdot}$-generating system [17], xanthine plus xanthine oxidase in air in the presence of Fe^{2+} (EDTA) (table 1). There was no difference between the 2 systems in the extent of inhibition by benzoate, mannitol and ethanol, although formate gave less inhibition with the adriamycin-dependent reaction.

Increasing the O_2 concentration resulted in a gradual decrease in the rate of ethylene production for $Adr^{\cdot}$ and H_2O_2 (fig.3a), until in air it was only slightly higher than the basal rate in the absence of adriamycin. This basal ethylene production was due to O_2^--dependent $OH^{\cdot}$ production by the Haber–Weiss reaction, catalysed by metal contaminants, and was very low because of the presence of DTPA [18]. The rate of radical generation by the xanthine oxidase reaction was higher in air than N_2, and slightly higher in air when adriamycin was present. Thus the difference in radical production rate could only account for the small difference in ethylene production in air, but not at lower O_2 concentrations. Ethylene production in the absence of O_2 was decreased to ~15% if no H_2O_2 was added. Addition of O_2, which provides a source of H_2O_2 by reacting with either xanthine oxidase or $Adr^{\cdot}$, resulted in a sharp increase in rate of

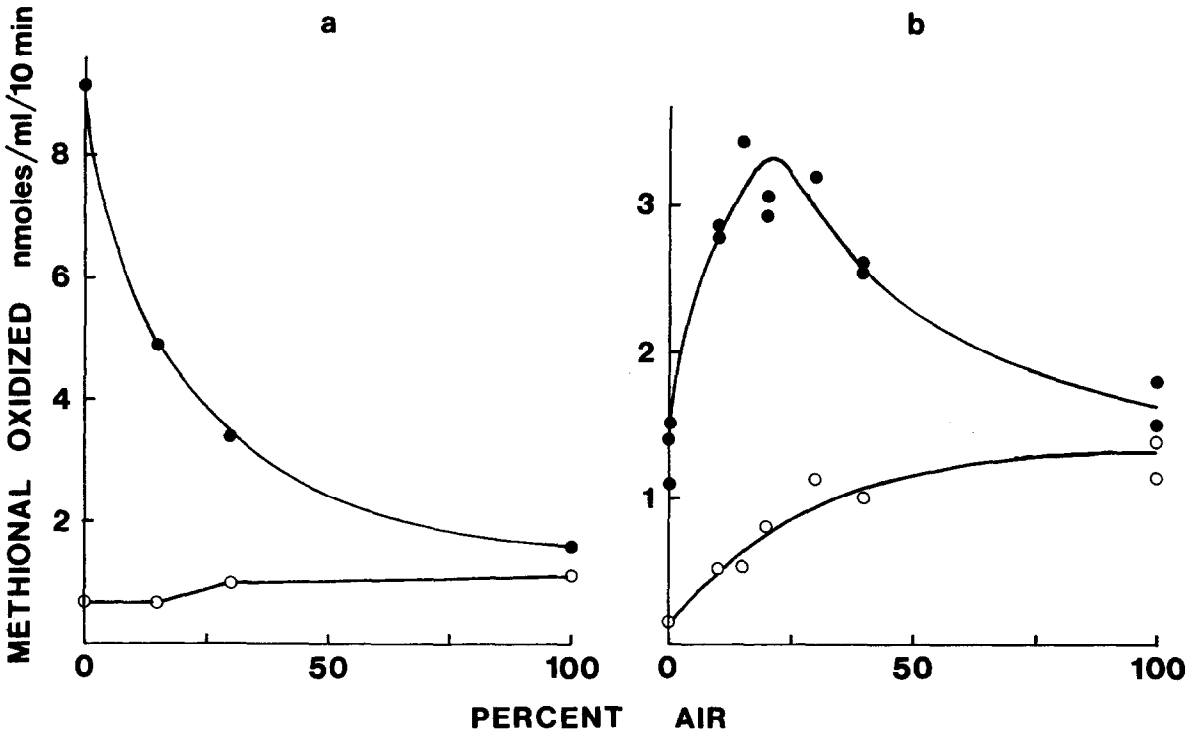

Fig.3. Effect of O_2 concentration on the rate of ethylene production: (a) in the presence of 300 μM H_2O_2; (b) with no added H_2O_2. Reaction conditions are otherwise as in fig.1. Air/N_2 mixtures were prepared by flushing the reaction vessel with N_2 and using a syringe, replacing the required volume of N_2 with air. Each point is the mean of duplicates which differed by <10%; (•) 120 μM adriamycin present. The radical generation rate (measured as cytochrome *c* reduction) increased gradually from 15 nmol . ml^{-1} . min^{-1} in N_2 to 41 nmol . ml^{-1} . 10 min^{-1} in air; (○) no adriamycin present. The radical generation rate increased gradually from <2.5 nmol . ml^{-1} . 10 min^{-1} in N_2 to 36 nmol . ml^{-1} . 10 min^{-1} in air.

ethylene production (fig.3b), which then declined gradually as normal air concentration was approached.

Ethylene production from Adr˙ and H_2O_2 was not inhibited by superoxide dismutase in N_2. (The very slight inhibition measured on some occasions was most likely due to the presence of traces of O_2.) However, in air and air/N_2 mixtures, native, but not denatured, superoxide dismutase inhibited the reaction almost completely. The data in table 1 suggest that more enzyme was required to inhibit the reaction at lower O_2 concentrations than in air.

Essentially the same results were obtained when α-keto-γ-methiol–butyric acid was substituted for methional. There was H_2O_2 and Adr˙-dependent production of ethylene in N_2, and the rate of production declined as the O_2 concentration increased. Superoxide dismutase inhibited the reaction in the presence but not the absence of O_2. With 1 mM α-keto-γ-methiol–butyric acid in N_2, the reaction was inhibited by 5 mM sodium benzoate (81% inhibition), 10 mM sodium formate (84% inhibition), 10 mM mannitol (69% inhibition) and 20 mM ethanol (75% inhibition).

4. Discussion

The adriamycin semiquinone, generated from adriamycin by xanthine oxidase, in combination with H_2O_2, has been shown to produce ethylene from methional and α-keto-γ-methiol–butyric acid. Whether this reaction indicates OH˙ production is generally assessed by examining the effects of scavengers [19,20]. In this case, 4 OH˙ scavengers inhibited the reaction, which is strong support for OH˙ involvement. The only equivocal finding is that inhibition by formate of ethylene production from methional was less than with another known OH˙-producing system. However, using ESR with spin traps, OH˙ was pro-

duced when adriamycin is reduced in aqueous solution [6] and although in [20] electron donor–H_2O_2 complexes gave ethylene production from methionine, their systems contrasted with ours in that there was no inhibition by mannitol or formate. Overall, therefore, the evidence strongly favours $OH^{\cdot}$ as the product of the reaction of $Adr^{\cdot}$ with H_2O_2. If not, it must be a species with very similar properties to $OH^{\cdot}$. Regardless, the product would be highly reactive and potentially damaging to cell constituents, and the biological significance of the reaction is not therefore dependent on which of these interpretations is correct.

That the reaction occurred in N_2 indicates that $Adr^{\cdot}$ rather than O_2^- was directly responsible for $OH^{\cdot}$ production. The reaction was not stimulated by Fe^{2+} (EDTA) nor inhibited by DTPA. It does not therefore require an iron catalyst, in contrast with the equivalent reaction of O_2^- (the Haber–Weiss reaction). Another mechanism of $OH^{\cdot}$ production from adriamycin has been proposed [5] involving O_2^- production from $Adr^{\cdot}$, and the Haber–Weiss reaction. Although this reaction may be significant if the necessary metal catalyst is present, the reaction we have described can occur whether or not this condition is met, and could also explain some of the observations in [5].

The reaction of the adriamycin semiquinone with H_2O_2 appears to be fast, with $OH^{\cdot}$ as a major product, even in the presence of low concentrations of H_2O_2. In the presence of O_2, however, it has to compete with the very fast reaction between the semiquinone and O_2 to give O_2^-, and this explains why the yield of $OH^{\cdot}$ was decreased. In air, no reaction between $Adr^{\cdot}$ and H_2O_2 was detectable, but in partially deoxygenated solution, H_2O_2 was able to compete with O_2 and production of $OH^{\cdot}$ was observed. In the absence of added H_2O_2, where O_2 was required as a source of H_2O_2, $OH^{\cdot}$ production was maximum in 2–6% O_2. This is significant because biological systems would also derive their H_2O_2 from O_2, and tissue O_2 concentrations would commonly be in this range.

Although O_2^- was clearly not responsible for adriamycin-dependent $OH^{\cdot}$ production, the reaction in air (but not N_2) was inhibited by superoxide dismutase. This phenomenon has been observed in a number of reactions [16,21,22], including a comparable one between the paraquat radical and H_2O_2 [23] and the reaction of $Adr^{\cdot}$ with methaemoglobin [16]. The explanation is that superoxide dismutase inhibits reactions of free radical precursors of O_2^- by affecting equilibrium (1) [15].

The cytotoxicity of adriamycin is dependent on a number of factors. Other investigators have shown that reductive cycling to give the semiquinone, and the presence of O_2 and H_2O_2 are important [3–5,7], and there is some evidence for protection by superoxide dismutase [1,2]. Decreased cytotoxicity in high pressure O_2 has also been observed [24]. One explanation for the cell damage is that it is due to O_2^- production and subsequent formation of $OH^{\cdot}$, possibly via a metal-catalysed Haber–Weiss reaction [1–6]. Although this can occur with metal chelates of antitumour antibiotics [1,2,6], it is known whether suitable metal catalysts are available at the sites were the drugs act in vivo. In their absence, O_2^- is unlikely to be sufficiently reactive to induce the cell damage associated with adriamycin [10,11]. $OH^{\cdot}$ production directly from $Adr^{\cdot}$, however, also has requirements for H_2O_2 and O_2 (as a source of H_2O_2), it is inhibited by superoxide dismutase, and by high O_2 pressure, yet it does not require a metal catalyst. The reaction, therefore, meets the criteria for it to be responsible for the cytotoxicity of adriamycin.

Acknowledgement

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References

[1] Cone, R., Hasan, S. K., Lown, J. W. and Morgan, A. R. (1976) Can. J. Biochem. 54, 219–223.
[2] Lown, J. W., Sim, S. K., Majumdar, K. C. and Chang, R. Y. (1977) Biochem. Biophys. Res. Commun. 76, 705–710.
[3] Lown, J. W. and Chen, H. H. (1981) Can. J. Chem. 59, 390–395.
[4] Berlin, V. and Haseltine, W. A. (1981) J. Biol. Chem. 256, 4747–4756.
[5] Thayer, W. S. (1977) Chem. Biol. Interact. 19, 265–278.
[6] Goodman, J. and Hochstein, P. (1977) Biochem. Biophys. Res. Commun. 77, 797–803.
[7] Bozzi, A., Mavelli, I., Mondovi, B., Strom, R. and Rolilio, G. (1981) Biochem. J. 194, 369–372.
[8] Myers, C. E., McGuire, W. P., Liss, R. H., Ifrim, I., Grotzinger, K. and Young, R. C. (1977) Science 197, 165–167.
[9] Halliwell, B. (1978) Cell Biol. Int. Rep. 2, 113–128.

[10] Fee, J. A. (1980) in: Metal Ion Activation of Dioxygen (Spiro, T. G. ed) pp. 209–237, Wiley, New York.
[11] Winterbourn, C. C. (1981) Biochem. J. 198, 125–131.
[12] Winterbourn, C. C. (1979) Biochem. J. 182, 625–628.
[13] Muraoka, S., Enomoto, H., Sugiyama, M. and Yamazaki, H. (1967) Biochim. Biophys. Acta 143, 408–415.
[14] Nakamura, M. and Yamazaki, I. (1969) Biochim. Biophys. Acta 189, 29–37.
[15] Winterbourn, C. C. (1981) Arch. Biochem. Biophys. 209, 159–167.
[16] Bates, D. and Winterbourn, C. C. (1981) Biochem. J. in press.
[17] Diguiseppi, J. and Fridovich, I. (1980) Arch. Biochem. Biophys. 205, 323–329.
[18] Halliwell, B. (1978) FEBS Lett. 92, 321–326.
[19] Cohen, G. and Sinet, P. M. (1980) in: Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase (Bannister, J. V. and Hill, H. A. O. eds) pp. 24–37, Elsevier/North-Holland, Amsterdam, New York.
[20] Elstner, E. F., Osswald, W. and Konze, J. R. (1980) FEBS Lett. 121, 219–221.
[21] Winterbourn, C. C., French, J. K. and Claridge, R. F. C. (1978) FEBS Lett. 94, 269–272.
[22] Sutton, H. C. and Sangster, M. F. (1981) J. Chem. Soc. Faraday I in press.
[23] Winterbourn, C. C. (1981) FEBS Lett. 128, 339–342.
[24] Wheeler, R. H., Dirks, J. W., Lunardi, I. and Nemiroff, M. J. (1979) Cancer Res. 39, 370–375.