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Reactions of Adriamycin with haemoglobin

Superoxide dismutase indirectly inhibits reactions of the Adriamycin semiquinone

Diana A. BATES and Christine C. WINTERBOURN
Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand

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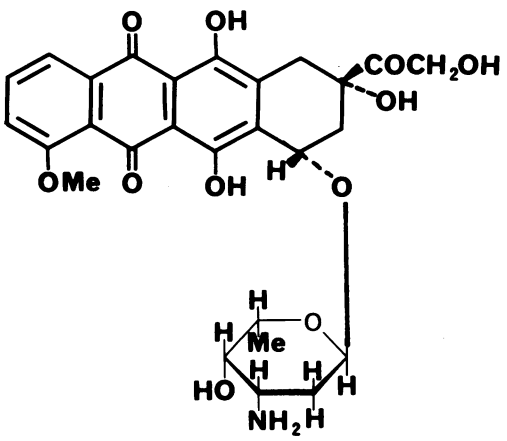
The Adriamycin semiquinone produced by the reaction of xanthine oxidase and xanthine with Adriamycin has been shown to reduce both methaemoglobin and cytochrome *c*. In air, but not N_2, both reactions were inhibited by superoxide dismutase. With cytochrome *c*, superoxide formed by the rapid reaction of the semiquinone with O_2, was responsible for the reduction. However, even in air, methaemoglobin was reduced directly by the Adriamycin semiquinone. Superoxide dismutase inhibited this reaction by removing superoxide and hence the semiquinone by displacing the equilibrium:

$$\text{Semiquinone} + O_2 \rightleftharpoons \text{quinone} + O_2^{-\bullet}$$

to the right. This ability to inhibit indirectly reactions of the semiquinone could have wider implications for the protection given by superoxide dismutase against the cytotoxicity of Adriamycin. Oxidation of haemoglobin by Adriamycin has been shown to be initiated by a reversible reaction between the drug and oxyhaemoglobin, producing methaemoglobin and the Adriamycin semiquinone. Reaction of the semiquinone with O_2 gives superoxide and H_2O_2, which can also react with haemoglobin. Catalase, by preventing this reaction of H_2O_2, inhibits oxidation of oxyhaemoglobin. Superoxide dismutase, however, accelerates oxidation, by inhibiting the reaction of the semiquinone with methaemoglobin by the mechanism described above. Although superoxide dismutase has a detrimental effect on haemoglobin oxidation, it may protect the red cell against more damaging reactions of the Adriamycin semiquinone.

Anthracycline antibiotics such as Adriamycin [doxorubicin hydrochloride (I)] are widely used in cancer chemotherapy, but their clinical use is limited by dose-related cardiotoxic side effects. These anthracyclines contain quinone structures that can undergo redox cycling, resulting in the production of semiquinone radicals and subsequently superoxide ($O_2^{-\bullet}$) and H_2O_2 (Handa & Sato, 1975; Bachur *et al.*, 1977, 1979; Thayer, 1977; Berlin & Haseltine, 1981). Redox cycling of Adriamycin has been shown to initiate free-radical reactions with cell constituents. Free-radical-induced scission of DNA appears to be important for the antitumour activity of the drug (Cone *et al.*, 1976; Lown *et al.*, 1977), and lipid peroxidation could be the cause of its cardiotoxicity (Goodman & Hochstein, 1977; Myers *et al.*, 1977).



(I)

Abbreviations used: $Adr^{-\bullet}$, Adriamycin semiquinone radical; oxyHb, oxyhaemoglobin; metHb, methaemoglobin; Q, quinone; Q^{-}, semiquinone.

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Some reactions of quinone antitumour drugs are inhibited by superoxide dismutase (Cone *et al.*, 1976; Lown *et al.*, 1977), suggesting that they are caused by either superoxide, or the product of a subsequent reaction of superoxide. However, it has been shown that superoxide dismutase can indirectly inhibit reactions of other semiquinones with cytochrome *c* and methaemoglobin (Winterbourn *et al.*, 1978; Winterbourn, 1980, 1981). If the same were true with Adriamycin, effects generally attributed to superoxide could be due to the Adriamycin semiquinone radical ($Adr^{-\bullet}$). To test this proposal we have examined the effect of superoxide dismutase on reactions of $Adr^{-\bullet}$ with cytochrome *c* and methaemoglobin.

Adriamycin has been shown to oxidize haemoglobin (Shinohara & Tanaka, 1980), and to produce superoxide in red cells (Henderson *et al.*, 1978). This may result in haemolysis, particularly in glucose 6-phosphate dehydrogenase-deficient red cells (Sagone & Burton, 1979). Although it has been suggested that this reaction is initiated by NADPH-dependent reduction of Adriamycin (Henderson *et al.*, 1978), menadione, which is structurally related to Adriamycin, reacts directly with oxyhaemoglobin to produce the semiquinone (Winterbourn *et al.*, 1979). The results of the present study, in which we have examined the effects of catalase, superoxide dismutase and radical scavengers on the reaction, lead us to conclude that this is also the case with Adriamycin.

Materials and methods

Superoxide was generated from the xanthine oxidase-catalysed reaction between xanthine and O_2. $Adr^{-\bullet}$ was also produced by this system, but with Adriamycin present. Reactions of the radicals with cytochrome *c* and methaemoglobin were carried out at 22°C in 0.05M-phosphate buffer, pH 7.4, containing catalase. The rate of cytochrome *c* reduction was determined by measuring ΔA_{550} ($\Delta\varepsilon$ reduced − oxidized $= 2.1 \times 10^4$) (Fridovich, 1970). Rates of reaction with methaemoglobin or oxyhaemoglobin were determined by measuring ΔA_{577} ($\Delta\varepsilon$ methaemoglobin − oxyhaemoglobin $= 1.188 \times 10^4$) (Benesch *et al.*, 1973). Reactions in the absence of air were carried out either in evacuated tonometers or in solutions that had been bubbled with N_2 for 5min, and the change in A_{560} was monitored ($\Delta\varepsilon$ methaemoglobin − deoxyhaemoglobin $= 0.883 \times 10^4$) (Benesch *et al.*, 1973). Haemoglobin concentrations are expressed in terms of haem groups.

Reactions of oxyhaemoglobin with Adriamycin were carried out at 37°C in 0.05M-phosphate buffer, pH 7.4, containing 1mM-EDTA. At intervals, concentrations of oxyhaemoglobin, methaemoglobin and haemichrome were calculated from the absorbance of the solution at 700, 630, 577 and 560nm (French *et al.*, 1978), relative to an appropriate Adriamycin blank. In some experiments, Adriamycin was removed by passing the solution through a column of Sephadex G-25. If necessary, the solution was first centrifuged for 10min at 12000*g*. It was realized from initial experiments analysed in this way that little if any soluble haemichrome was present. In subsequent experiments, therefore, concentrations of oxyhaemoglobin and methaemoglobin were determined from the absorbance of the solution at 577 and 630nm. Close agreement between the two methods of analysis was found. The amount of precipitation was determined as the difference in A_{700} before and after centrifugation. Reactions between deoxyhaemoglobin and Adriamycin were carried out at 37°C under vacuum, with continuous monitoring of either A_{550} or A_{630}.

Haemoglobin oxidation and Heinz-body formation were measured in twice-washed human red cells [5% suspension in phosphate-buffered saline (0.01M-sodium phosphate/0.14M-NaCl), pH 7.4], incubated at 37°C with Adriamycin (0.4mg/ml). Haemoglobin oxidation was determined by diluting portions of the cell suspension in 5mM-phosphate buffer, pH 7.4, centrifuging, and analysing spectrophotometrically as described above. Heinz bodies were stained with Methyl Violet.

The preparation of purified human oxyhaemoglobin and methaemoglobin was as described by French *et al.* (1978). Adriamycin (as Adriamycin/lactose, 1:5) was obtained from Pharmitalia (U.K.) Ltd., Barnet, Herts, U.K. Lactose-free Adriamycin was obtained from the Sigma Chemical Corp., St Louis, MO, U.S.A. Both batches of Adriamycin produced the same rate of oxidation of oxyhaemoglobin. Xanthine, xanthine oxidase, catalase and cytochrome *c* were also obtained from Sigma, and superoxide dismutase was obtained from Diagnostic Reagents Ltd., Thame, Oxon, U.K.

Results

Reactions of the Adriamycin semiquinone with cytochrome c and methaemoglobin

In N_2-bubbled solution, cytochrome *c* was reduced by xanthine and xanthine oxidase in the presence of Adriamycin (Table 1). In the absence of Adriamycin, there was very little reduction, and that which occurred was presumably due to the incomplete removal of O_2. Thus xanthine oxidase reacted directly with Adriamycin to produce a species capable of reducing cytochrome *c*. By analogy with other quinones that react similarly (Nakamura & Yamazaki, 1969; Winterbourn, 1981), this product was almost certainly $Adr^{-\bullet}$. Cytochrome *c* reduction in the presence of 60μM-Adriamycin was about

Table 1. *Cytochrome c and methaemoglobin reduction by superoxide and the Adriamycin semiquinone*
Reaction mixtures contained xanthine oxidase (4.5×10^{-3} units/ml), xanthine (0.2mM) and catalase (30μg/ml) in 0.05M-phosphate buffer, pH 7.4, and either 14μM-cytochrome *c* (in air or N_2-bubbled solution), or 8μM-methaemoglobin (in air or under vacuum). The results presented here are from a single set of experiments, but are representative of those obtained on several occasions.

		In air		In absence of air	
	[Adriamycin] (μm)	Rate ($\Delta\mu$M/100s)	Inhibition by superoxide dismutase (8μg/ml) (%)	Rate ($\Delta\mu$M/100s)	Inhibition by superoxide dismutase (8μg/ml) (%)
Cytochrome *c*	0	4.17	97	0.38	
	60	4.67		1.55	
	120	4.70	97	1.71	20
	250	4.87			
Methaemoglobin	0	0.42	100	0	
	47	1.54			
	95	2.25	93	1.35	24
	190	2.67			

three times faster in air than in N_2 (Table 1), and in air it was slightly faster than reduction by superoxide alone. Increasing the Adriamycin concentration did not alter the rate.

Superoxide dismutase completely inhibited cytochrome *c* reduction in air, both in the presence and absence of Adriamycin, but gave virtually no inhibition in N_2 (Table 1). The amount of superoxide dismutase required for half-maximum inhibition was the same in the presence or absence of Adriamycin (Table 2).

In accordance with previous observations (Sutton *et al.*, 1976), methaemoglobin was slowly reduced to oxyhaemoglobin by superoxide generated by the xanthine oxidase-catalysed oxidation of xanthine. Addition of Adriamycin gave a concentration-dependent increase in the rate of reduction, with 190μM-Adriamycin giving an approx. 6-fold increase in rate (Table 1). No reaction occurred in the absence of xanthine oxidase. In the absence of air, $Adr^{-\bullet}$ reduced methaemoglobin (to deoxyhaemoglobin) at approx. 60% of the rate found in air. No reaction occurred if either Adriamycin or xanthine oxidase was not present. Allowing for the 3-fold decrease in rate of radical generation (measured by cytochrome *c* reduction) the reaction was slightly more efficient in N_2 than in air.

In air, superoxide dismutase completely inhibited the reduction of methaemoglobin (Table 1). Superoxide dismutase caused very little inhibition in evacuated solutions, however, and this slight effect was most likely due to the presence of traces of air.

Although superoxide can both oxidize oxyhaemoglobin and reduce methaemoglobin (Sutton *et al.*, 1976), no reaction was detected between $Adr^{-\bullet}$ and oxyhaemoglobin. Adriamycin itself caused the direct oxidation of oxyhaemoglobin (see below) and, provided catalase was present to remove the H_2O_2 generated, addition of xanthine oxidase caused a slight decrease in this rate.

Table 2. *Inhibition by superoxide dismutase of cytochrome c reduction by xanthine oxidase and Adriamycin*
Reactions were carried out in air, and conditions were the same as for Table 1. $I_{max.}$ and K_i were obtained from plots of 1/percentage inhibition against 1/[superoxide dismutase].

	Inhibition (%)	
[Superoxide dismutase] (ng/ml)	No Adriamycin	120μM-Adriamycin
1.67	49.0	50.2
4.17	70.4	69.0
8.33	80.4	81.4
$I_{max.}$	97.0	97.0
K_i	1.60	1.60

Reaction of Adriamycin with oxyhaemoglobin

Incubation with Adriamycin resulted in the oxidation of oxyhaemoglobin. Methaemoglobin was the main product, with less than 5% haemichrome being detected in the solution. However, continued incubation resulted in some haemoglobin precipitation. No reaction was detected between Adriamycin and deoxyhaemoglobin by monitoring either A_{550} or A_{630} for up to 2h. Inositol hexaphosphate (2mol/mol of haemoglobin tetramer), which stabilizes the deoxyhaemoglobin configuration, had no effect on the rate of oxidation of oxyhaemoglobin by Adriamycin.

The rate of oxidation of oxyhaemoglobin increased with increasing Adriamycin concentration (Fig. 1). The reaction rate was approximately halved by the addition of catalase, but superoxide dismutase

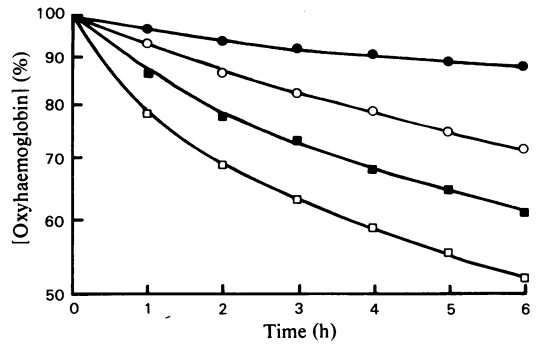


Fig. 1. *Reaction between oxyhaemoglobin and Adriamycin*

The dependence on Adriamycin concentration (semilogarithmic plot) is shown: ●, 0 μM; ○, 60 μM; ■, 120 μM; □, 240 μM. The oxyhaemoglobin concentration was 32 μM. Other conditions are given in the Materials and methods section.

caused a marked increase in rate (Fig. 2*a*). In the presence of superoxide dismutase the reaction followed first-order kinetics, which meant that the stimulatory effect of superoxide dismutase became more marked as the methaemoglobin/oxyhaemoglobin ratio built up. Superoxide dismutase increased the reaction rate both in the presence and absence of catalase. Its effect was slight in the presence of catalase when the initial solution contained only oxyhaemoglobin and the methaemoglobin/oxyhaemoglobin ratio remained low. However, with 30% methaemoglobin, superoxide dismutase caused a 3-fold increase in the initial rate, both in the presence and absence of catalase (Fig. 2*b*).

The rate of reaction between Adriamycin and oxyhaemoglobin was unaffected by either ascorbate (50 or 100 μM, in the presence of catalase) or reduced glutathione (20 μM). Haemoglobin oxidation also occurred in red cells incubated with Adriamycin. After 20 h, 40% of the haemoglobin was oxidized and 20% of the cells were lysed, but very few Heinz bodies were detected.

Discussion

One aim of the present study was to determine whether superoxide dismutase could indirectly inhibit reactions of the Adriamycin semiquinone. Adriamycin in the presence of xanthine and xanthine oxidase and in the absence of O_2 reduced both cytochrome *c* and methaemoglobin. This indicates that, in common with many other quinones (Nakamura & Yamazaki, 1969; McCord & Fridovich, 1970; Winterbourn, 1981; Sutton & Sangster, 1982), Adriamycin can accept an electron from xanthine oxidase to form the semiquinone,

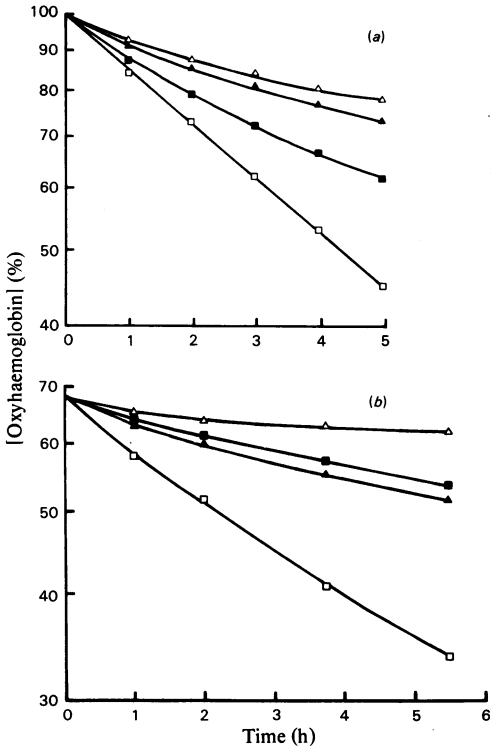
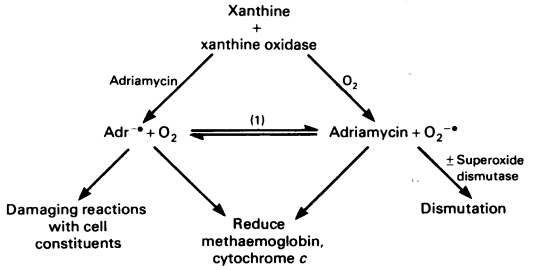


Fig. 2. *Effects of catalase and superoxide dismutase on the rate of reaction between oxyhaemoglobin and Adriamycin*

Semilogarithmic plots are shown: (*a*) initial solution 100% oxyHb; (*b*) original solution 70% oxyHb, 30% metHb. ■, Total haemoglobin concentration 32 μM, Adriamycin concentration 120 μM; △, plus catalase (30 μg/ml); □, plus superoxide dismutase (8 μg/ml); ▲, plus catalase and superoxide dismutase. Other conditions are given in the Materials and methods section. The results shown are from one experiment but are representative of those obtained on several occasions.

which can reduce cytochrome *c* and methaemoglobin. The rate of cytochrome *c* reduction by $Adr^{-\bullet}$ in N_2 was slower than the rate of reduction in air. The rate in air was only slightly affected by the presence of Adriamycin (it gave a slight increase in rate), suggesting that superoxide was the main radical produced from the enzyme. However, because of the equilibrium between superoxide and the semiquinone, either radical could have reduced cytochrome *c* (as in the reaction sequence shown in Scheme 1). The contribution by the semiquinone has been found to depend on its redox potential, and can be determined by comparing the concentrations of superoxide dismutase required to inhibit the reaction in the presence and absence of quinone (Winterbourn, 1981; Sutton & Sangster, 1982). In the



Scheme 1. *Sequence of reactions involving superoxide, Adriamycin, cytochrome c and methaemoglobin*

presence of quinones such as menadione (E_7^0 $Q/Q^- = -0.20$ V; Ilan *et al.*, 1976) the semiquinone makes a major contribution to the reaction and considerably more superoxide dismutase is required. Anthraquinone-2-sulphonate (E_7^0 $Q/Q^- = -0.38$ V; Meisel & Neta, 1975), however, does not alter the concentration of superoxide dismutase required for inhibition. Our finding that K_i was the same in the presence and in the absence of Adriamycin (E_7^0 $Q/Q^- = -0.29$ V; Svingen & Powis, 1981) is in agreement with the results obtained with anthraquinone-2-sulphonate. For these low-redox-potential quinones, equilibrium (1) (Scheme 1) is displaced far to the right, apparently keeping the semiquinone concentration too low for it to contribute significantly to the reaction with cytochrome *c*. Cytochrome *c* reduction by Adriamycin and xanthine oxidase in air was therefore due to superoxide, and the inhibition by superoxide dismutase can be explained on this basis.

The contribution of superoxide to the reduction of methaemoglobin was much less, however. Superoxide produced from xanthine and xanthine oxidase in air reduced methaemoglobin very slowly, but in the presence of Adriamycin, reduction was much more rapid. This was not due to an increased rate of radical production by the enzyme, since Adriamycin did not give a similar increase in cytochrome *c* reduction, and therefore cannot be due to the reaction of superoxide. It must therefore represent direct reduction of methaemoglobin by the semiquinone, produced predominantly by equilibrium (1) (Scheme 1). Although this equilibrium favours superoxide, the reaction of superoxide with methaemoglobin has a low rate constant and does not compete well with spontaneous dismutation of superoxide (Sutton *et al.*, 1976). It appears that the rate constant for the reaction of $Adr^{-\bullet}$ with methaemoglobin is sufficiently high that even with very low $Adr^{-\bullet}$ compared with superoxide concentrations, most of the methaemoglobin reduction is due to $Adr^{-\bullet}$. This has been found to be the case with anthraquinone-2-sulphonate (Winterbourn, 1980; Sutton & Sangster, 1982). Methaemoglobin reduction occurred more rapidly in N_2 than in air. This was presumably because in air there was some competition with the fast reaction of $Adr^{-\bullet}$ with O_2 and subsequent dismutation of the superoxide formed.

Although $Adr^{-\bullet}$ was responsible for methaemoglobin reduction, the reaction was inhibited by superoxide dismutase. This was not due to a direct reaction of superoxide dismutase with the semiquinone, as superoxide dismutase gave no inhibition of either cytochrome *c* or methaemoglobin reduction in N_2. These findings are analogous to those reported for a number of other semiquinones with methaemoglobin (Winterbourn *et al.*, 1979; Winterbourn, 1980). They can be explained by superoxide dismutase reacting with superoxide and displacing equilibrium (1) to the right, thereby lowering the $Adr^{-\bullet}$ concentration and preventing its reaction with methaemoglobin.

It can be concluded, therefore, that superoxide dismutase indirectly inhibits the reaction of $Adr^{-\bullet}$ with methaemoglobin. This mechanism could equally well apply for other reactions of $Adr^{-\bullet}$, and indeed one of us has recently found that superoxide dismutase inhibits its reaction with H_2O_2 to produce a highly reactive hydroxyl-radical-like species (Winterbourn, 1982). These findings have implications when considering reactions such as lipid peroxidation and DNA breakage that are initiated by Adriamycin and inhibited by superoxide dismutase (Handa & Sato, 1975; Cone *et al.*, 1976; Lown *et al.*, 1977; Goodman & Hochstein, 1977; Thayer, 1977). It could be that, in these situations also, superoxide dismutase is inhibiting a reaction of the semiquinone radical rather than superoxide.

Adriamycin has previously been shown to cause oxidation of haemoglobin (Shinohara & Tanaka, 1980). We have found that the reaction requires oxyhaemoglobin and not deoxyhaemoglobin, is inhibited by catalase, but is accelerated by superoxide dismutase in both the presence and absence of catalase. The reaction of oxyhaemoglobin with menadione has these same features, although it is several times faster (Winterbourn *et al.*, 1979), and both reactions are compatible with the following mechanism (where SOD is superoxide dismutase):

$$\text{oxyHb} + \text{Adriamycin} \rightleftharpoons \text{metHb} + Adr^{-\bullet} + O_2 \quad (2)$$

$$Adr^{-\bullet} + O_2 \rightleftharpoons O_2^{-\bullet} + \text{Adriamycin} \quad (3)$$

$$2O_2^{-\bullet} + 2H^+ \xrightarrow{\pm SOD} O_2 + H_2O_2 \quad (4)$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \quad (5)$$

$$1/2H_2O_2 + \text{oxyHb} \dashrightarrow \text{metHb} + OH^- + O_2 \quad (6)$$

$$O_2^{-\bullet} + \text{metHb} \dashrightarrow \text{oxyHb} \quad (7)$$

$$2H^+ + O_2^{-\bullet} + \text{oxyHb} \dashrightarrow \text{metHb} + H_2O_2 \quad (8)$$

The first step is the direct reaction of Adriamycin with oxyhaemoglobin, to give the semiquinone (reaction 2). As we have shown, however, the

semiquinone readily reacts with methaemoglobin, so reaction (2) is reversible and the back reaction is able to compete with the reaction of $Adr^{-\bullet}$ with O_2 to give superoxide (reaction 3). As described above, superoxide dismutase can inhibit methaemoglobin reduction by the semiquinone via reaction (3). This would displace equilibrium (3) to the right and explains why superoxide dismutase accelerated haemoglobin oxidation. This would also prevent product inhibition, explaining why the reaction followed first-order kinetics in the presence of superoxide dismutase. The effect of catalase would be to prevent haemoglobin oxidation by H_2O_2 (reaction 6) formed by dismutation of superoxide. Since superoxide would react with oxyhaemoglobin (reaction 8) rather than methaemoglobin (reaction 7) in solutions containing predominantly oxyhaemoglobin (Sutton *et al.*, 1976), inhibition of a direct reaction between superoxide and haemoglobin cannot explain the effect of superoxide dismutase. However, the finding of only slight acceleration by superoxide dismutase when the oxyhaemoglobin/methaemoglobin ratio was very high suggests that, under these conditions, reaction (8) makes some contribution to the overall reaction.

Although the reaction of oxyhaemoglobin with Adriamycin is a free-radical one, glutathione and ascorbate, the main free-radical scavengers present in the red cell, had no effect on the reaction. This is probably because both these species are only good scavengers of oxidizing radicals, whereas $Adr^{-\bullet}$ is reducing. Henderson *et al.* (1978) have proposed that anthracyclines undergo cyclic oxidation–reduction in the red cell, acting as mediators of electron transfer from NADPH via the semiquinone to oxyhaemoglobin. However, our findings that Adriamycin itself reacts with oxyhaemoglobin, and that the semiquinone reacts with methaemoglobin rather than oxyhaemoglobin, do not support this interpretation. In the red cell, however, Adriamycin does stimulate the hexose monophosphate shunt and cause glutathione oxidation, and this could pose a haemolytic threat in glucose 6-phosphate dehydrogenase deficiency (Sagone & Burton, 1979). Oxyhaemoglobin reacts more slowly with Adriamycin than with most oxidant drugs, and very few Heinz bodies were found in normal red cells incubated for several hours with Adriamycin. However, this produced substantial red-cell lysis.

Adriamycin has also been shown to inhibit ATPase activity and K^+ transport, and to produce membrane changes in red cells (Shinohara & Tanaka, 1980). These changes may be more damaging to the cell and a more important cause of haemolysis than haemoglobin denaturation. In this respect, although superoxide dismutase had a detrimental effect on haemoglobin oxidation, it may be more important in preventing other more damaging reactions of $Adr^{-\bullet}$.

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