Biochemical Pharmacology, Vol. 30, No. 18, pp. 2626–2629, 1981.
Printed in Great Britain.

0006-2952/81/182626-04 $02.00/0
Pergamon Press Ltd.

Role of one-electron and two-electron reduction products of adriamycin and daunomycin in deoxyribonucleic acid binding

(*Received* 24 *October* 1980; *accepted* 11 *March* 1981)

A number of chemotherapeutic agents, including adriamycin and daunomycin, interact with nucleic acids. Intercalative binding of adriamycin and daunomycin to DNA and consequent inhibition of important cellular functions are believed to be responsible for the anti-cancer properties of these agents [1–4]. Adriamycin and daunomycin, however, are also mutagenic and carcinogenic in humans [5]. Studies from our laboratory have shown recently that adriamycin and daunomycin, in the presence of reducing agents, bind covalently to nucleic acids and proteins [6–8]. Furthermore, the covalent binding to DNA correlates with their ability to induce sister chromatid exchanges [6].

The exact species derived from adriamycin and daunomycin that alkylates cellular marcomolecules is not known at this time. Moore [9], however, has proposed that C_7-quinone methide, formed from two-electron reduction of the parent drug, may act as an alkylating species. Recently we proposed that, in addition to the C_7-quinone methide, a C_7-free radical metabolite (Fig. 1), formed from one-electron reduction, may also alkylate cellular macromolecules [7]. In this paper we describe the role of these species in binding to DNA.

Materials. Adriamycin·HCl (NSC 123127) and daunomycin (NSC 82151) were obtained from the Natural Products Division, National Cancer Institute, (Bethesda, MD). Calf thymus DNA (type I, highly polymerized), NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, α-tocopherol and reduced glutathione were purchased from the Sigma Chemical Co. (St. Louis, MO). Ethyl xanthate was prepared according to a method published previously [10].

Methods. Male C–D rats (Charles River Breeding Laboratories, Wilmington, MA), maintained on Purina Rat Chow, were used to prepare hepatic microsomes and the 105,000 *g* supernatant fraction. Food and water were available *ad lib*. Livers were removed and homogenized in 3 vol. of 150 mM KCl–50 mM Tris buffer (pH 7.4). Microsomes and the 105,000 *g* supernatant fractions were isolated according to methods published previously [11, 12]. Protein was determined by the method of Sutherland *et al.* [13], using bovine serum albumin as a standard.

Microsomal activation of the drugs was carried out under anaerobic conditions as described previously [7]. Studies with inhibitors (10-fold molar excess over drugs) were carried out by adding the inhibitor to the incubation mixtures and then activating the drugs with NADPH under anaerobic conditions. Activation of drugs with the 105,000 *g* supernatant fraction with or without dicumarol was carried out similarly.

The drug–DNA complexes were isolated by extraction with equal volumes of phenol–chloroform (1:1) for 15 min and centrifguation at 6000 *g* for 10 min. The clear supernatant fraction was removed and re-extracted with phenol–chloroform. The supernatant fraction was then extracted with equal volumes of 1-butanol, and the drug–DNA complexes were precipitated in 20-fold volumes of ethanol. The ethanol precipitates were cooled to −20° for 4 hr, and the complexes were isolated by centrifugation, washed well with ethanol, and dried with nitrogen. The complexes were dissolved in 5 ml of 10 mM phosphate buffer containing 50 mM NaCl (pH 7.4) and were dialyzed extensively against the phosphate buffer. The binding ratios, defined here as the molar ratio of the mononucleotide unit to the drug, were obtained spectrophotometrically as described previously [7].

The samples for e.s.r. studies were prepared under nitrogen and transferred (under N_2) into quartz flat cells. The samples were introduced into the E-238 TM_{110} cavity and the e.s.r. spectrum was recorded with a Varian E-109 spectrometer operating at 100 kHz.

Results and discussion. Sato *et al.* [14] and Bachur *et al.* [15] have shown that adriamycin and daunomycin, in the presence of microsomes and NADPH, generate semiquinone free radicals. The e.s.r. spectrum (Fig. 2A) obtained

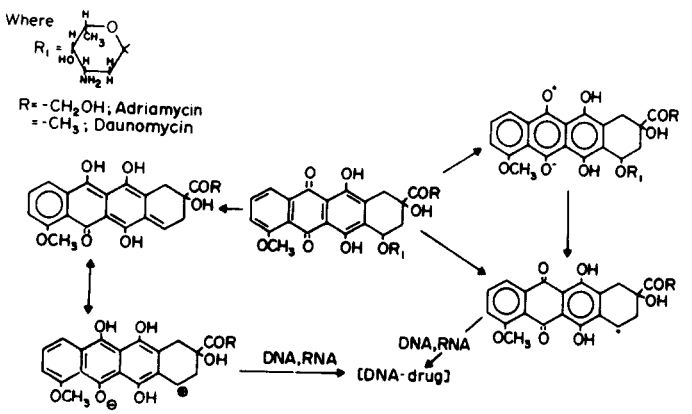

Fig. 1. Proposed formation of reactive species from adriamycin and daunomycin by one-electron and two-electron reduction.

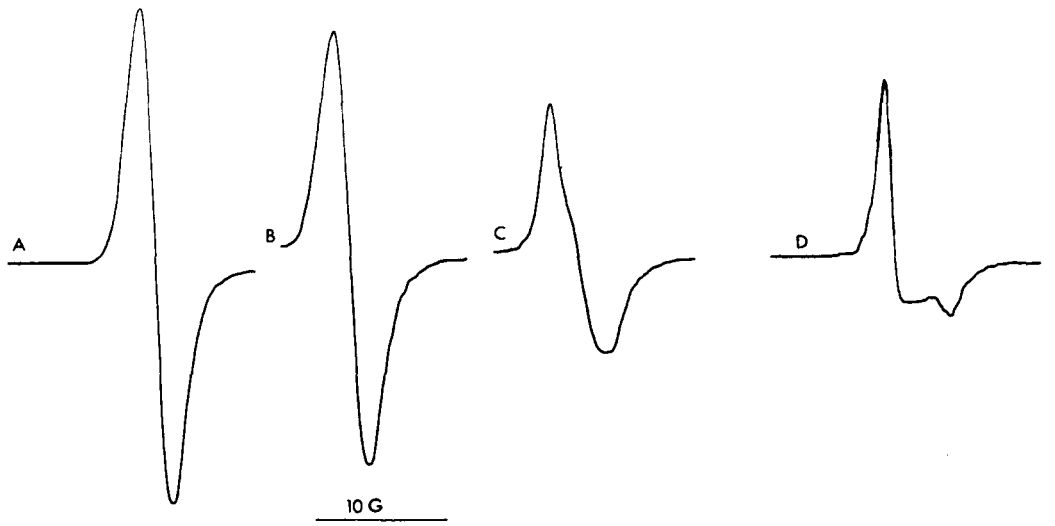

Fig. 2. Electron spin resonance time-profile of daunomycin (1 mg/ml) in the presence of GSH (10-fold excess), microsomes (1.5 mg/ml protein), and NADPH regenerating system under anaerobic conditions. The nominal microwave power was 20 mW and the modulation amplitude was 0.6 G. A: 2.0 min (receiver gain 4×10^3); B: 10 min (receiver gain 8×10^3); C: 3 min (receiver gain 8×10^3); and D: 2.0 hr (receiver gain 1.6×10^4).

with daunomycin in the presence of microsomes and NADPH under anaerobic conditions is similar to that obtained by others [14, 15]. Recently, Kalyanaraman *et al.* [16] have shown that this e.s.r. spectrum is time dependent and progressively changes from a symmetric motionally narrowed spectrum into a completely immobilized spectrum. Although the reason is not clear, binding of the semiquinone or its decomposition products to microsomes and/or polymerization of the anthracyclines have been proposed [14, 16]. The presence of either GSH or α-tocopherol (10-fold molar excess over drugs) had no effect on the formation of the initial semiquinone radical(s) (Fig. 2). Furthermore, neither of these agents prevented the time-dependent progressive immobilization of the e.s.r. spectra (Fig. 2, B, C and D).

Iyanagi and Yamazaki [12] and Kato *et al.* [17] have shown that DT-diaphorase (NADPH-reductase) catalyzes the reduction of quinones. In contrast to the microsomal NADPH reduction, this DT-diaphorase-mediated reduction is believed to proceed through a two-electron transfer. Furthermore, diaphorase-catalyzed reductions are inhibited by dicumarol. The 105,000 *g* supernatant fraction has been shown to contain DT-diaphorase [12]. Incubation of daunomycin with the 105,000 *g* supernatant fraction and NADPH under anaerobic conditions did not result in the formation of the semiquinone radical(s). With time, however, a weak e.s.r. signal was detected, which became progressively more pronounced until it reached its maximum amplitude at 20 min (Fig. 3). No progressive immobilization of this spectrum was observed. The addition of ethyl xanthate, which has been shown to react with positively charged species and was recently used to trap active species derived from mitomycin C [18], initially lengthened the time of the appearance of the radical(s) (Fig. 4) and simultaneously decreased the radical concentration about 50 per cent. This time-dependent appearance of semiquinone free radical must be due to the initial fully reduced hydroquinone which undergoes the well known disproportionation–comproportionation reaction [19, 20]

$$Q + QH_2 \rightleftharpoons 2\,Q^- + 2H^+$$

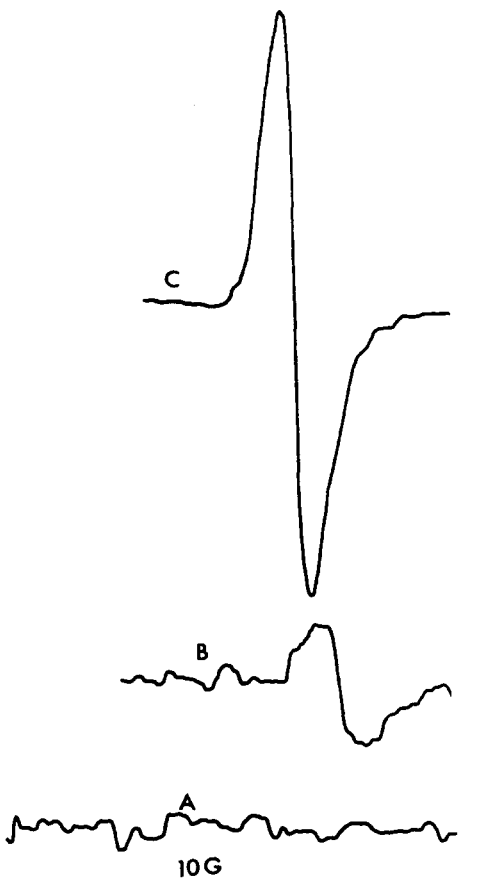

Fig. 3. Electron spin resonance time-profile of daunomycin (1 mg/ml) in the presence of 1.5 mg/ml of 105,000 *g* protein and NADPH regenerating system under anaerobic conditions. The spectrometer settings were identical to those for Fig. 2. A: 2 min (receiver gain 5×10^4); B: 8 min (receiver gain 5×10^4); and C: 20 min (receiver gain 2.5×10^4).

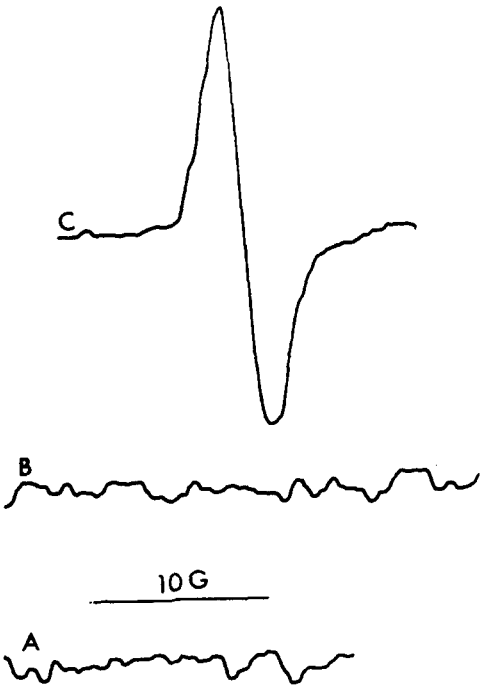

Fig. 4. electron spin resonance time-profile of daunomycin (1 mg/ml) in the presence of 1.5 mg/ml of 105,000 *g* protein, NADPH regenerating system, and ethyl xanthate (10-fold excess). The spectrometer settings were identical to those for Fig. 3. A: 2 min; B: 8 min; and C: 20 min.

These observations also suggest that the fully reduced product may decompose to form the quinone methide which then either produces aglycone or reacts with xanthate.

To evaluate the role of the free radical and/or the quinone methide in the alkylation of the cellular macromolecules, binding studies with DNA were carried out under various conditions. Incubation of drugs with DNA and heat-treated (denatured) microsomes or native microsomes in the absence of NADPH-generating system resulted in a small amount of drug bound to DNA which could not be extracted. However, when the drugs, activated by native (non-heated) microsomes and NADPH, were incubated with DNA, a 4- to 5-fold increase in binding was observed (Table 1). The presence of α-tocopherol, a presumed free radical scavenger, had no effect on this binding. In contrast, GSH reduced considerably the binding of adriamycin and daunomycin to DNA. In addition, ethyl xanthate also reduced this binding by 50 per cent (Table 1).

Since α-tocopherol did not inhibit the binding of these agents to DNA, it is likely that α-tocopherol neither prevents the formation nor reacts with the active species generated. Reduced glutathione, which neither prevents the formation of the semiquinone nor inhibits its immobilization, considerably diminished the binding. This then suggests that the active species that alkylates DNA is not the semiquinone but is derived from the semiquinone. Reduced binding in the presence of GSH further suggests that GSH either reacts with or inhibits the formation of these active species. The observation that adriamycin and daunomycin form glutathione-adducts (B. K. Sinha, unpublished results) supports the idea that GSH reacts with the active species generated and thereby effectively reduces the concentration available for DNA alkylation.

To explore further the role of two-electron reduced quinone methide in DNA binding, studies were carried out with the 105,000 *g* supernatant fraction and NADPH systems. The binding ratios obtained under these conditions (Table 1) were somewhat higher than those obtained from microsomal systems. Nevertheless, these results indicate that the 105,000 *g* supernatant fraction did catalyze the activation of these drugs and that the species derived from two-electron reduction was bound to DNA. Furthermore, DNA was added at 3 min, when the concentration of this active species was maximum before any disproportionation–comproportionation reaction formed the free radical-derived species. The presence of dicumarol inhibited this binding only slightly, possibly due to the solubility problems encountered with this inhibitor.

In summary, our e.s.r. studies show that incubation of adriamycin and daunomycin with microsomes and NADPH resulted in the formation of semiquinone free radicals. In

Table 1. Binding of adriamycin and daunomycin to DNA in the presence of microsome and DT-diaphorase and effects of various inhibitors on binding*

Enzymes	Inhibitors	Molar binding ratios	
		Adriamycin	Daunomycin
Heat-treated microsomes†		650 ± 20	~1000 ± 40
Native microsome‡		625 ± 15	~1050 ± 35
Native microsomes		125 ± 12	280 ± 5
Native microsomes	α-Tocopherol	120 ± 10	325 ± 4
Native microsomes	GSH	265 ± 15	690 ± 20
Native microsomes	Xanthate	222 ± 14	580 ± 30
105,000 *g* Supernatant fraction		184 ± 12	260 ± 18
105,000 *g* Supernatant fraction	Dicumarol	220 ± 20	300 ± 20

* Reaction conditions: drugs (1.0 mg/ml) in KCl–Tris buffer (pH 7.4) were activated with 3.0 mg protein/ml-NADPH regenerating systems under N_2 and after 3 min DNA (1.0 mg/ml) was added. The reaction mixtures were incubated for 18 hr and the complexes were isolated as described in Methods. Binding ratio is defined here as molar ration of mononucleotide unit to the drug; for details see Methods.

† Microsomes were heated to 70° for 30 min.

‡ Binding studies were carried out with native microsomes (twice washed) in the absence of NADPH generating system and isolating the complexes as described in Methods.

addition, studies using α-tocopherol and reduced glutathione show that these agents did not inhibit the formation of the initial semiquinone radicals nor did they prevent their progressive immobilization. Binding studies with DNA indicate that the active species (C_7-free radical) derived from one-electron reduction product were bound to DNA. Furthermore, identical binding ratios obtained with and without α-tocopherol suggest that α-tocopherol neither inhibited the formation nor was bound to the active species formed. A significantly reduced binding in the presence of GSH, on the other hand, indicates that GSH was bound to this active alkylating species. A reduced binding with ethyl xanthate, which is known to react with positively charged species, suggests that microsomal NADPH activation produced both one and two-electron reduction products of adriamycin and daunomycin. Our e.s.r. studies suggest that the 105,000 *g* supernatant fraction also catalyzed the formation of two-electron reduction products and that ethyl xanthate reduced the concentration of this active species (C_7-quinone methide) available for alkylation. Binding studies with DNA and the 105,000 *g* supernatant fraction show that the active species derived from the two-electron reduction products also bind to DNA.

These observations confirm, then, our earlier suggestions that species derived from both one-electron and two-electron reduction products (C_7-free radical and C_7-quinone methide) of adriamycin and daunomycin alkylate nucleic acids and proteins.

Laboratory of Environmental Biophysics
National Institute of Environmental Health Sciences
Research Triangle Park NC 27709, U.S.A.

BIRANDRA K. SINHA*
J. LEWIS GREGORY

* Author to whom all correspondence should be addressed.

REFERENCES

1. A. DiMarco, F. Arcomone and F. Zunino, in *Mechanism of Action of Antimicrobial and Antitumor Agents* (Eds. J. Cockran and F. H. Hahn), p. 101. Springer, Berlin (1975).
2. A. DiMarco, *Cancer Chemother. Rep.* **59**, (Part 3), 91 (1975).
3. A. Theologides, J. Yarbro and B. J. Kennedy, *Cancer, N.Y.* **21**, 16 (1968).
4. F. Zunino, A. DiMarco, A. Zaccara and G. Juone, *Chem. Biol. Interact.* **9**, 25 (1974).
5. S. M. Sieber and R. H. Adamson, *Adv. Cancer Res.* **22**, 57 (1975).
6. B. K. Sinha and C. F. Chignell, *Chem.-Biol. Interact.* **28**, 301 (1979).
7. B. K. Sinha, *Chem.-Biol. Interact.* **30**, 67 (1980).
8. B. K. Sinha and R. H. Sik, *Biochem. Pharmac.* **29**, 1867 (1980).
9. H. W. Moore, *Science* **197**, 527 (1977).
10. A. I. Vogel, in *A Textbook of Practical Organic Chemistry*, Third Edition, p.499. John Wiley, New York (1956).
11. R. P. Mason and J. L. Holtzman, *Biochem. biophys. Res. Commun.* **67**, 1267 (1975).
12. T. Iyanagi and I. Yamazaki, *Biochim. biophys. Acta* **216**, 282 (1970).
13. E. W. Sutherland, C. F. Cori, R. Hayes and N. S. Olsen, *J. biol. Chem.* **180**, 825 (1949).
14. S. Sato, M. Iwaizumi, K. Handa and Y. Tamura, *Gann* **68**, 603 (1977).
15. N. R. Bachur, S. L. Gordon and M. V. Gee, *Molec. Pharmac.* **13**, 901 (1977).
16. B. Kalyanaraman, E. Perez-Reyes and R. P. Mason, *Biochim. biophys. Acta* **630**, 119 (1980).
17. R. Kato, A. Takahashi and T. Osmima, *Biochem. Pharmac.* **19**, 45 (1970).
18. V. Hornemann, P. J. Keller and J. F. Kozlowski, *J. Am. chem. Soc.* **101**, 7121 (1979).
19. M. Tomasz, C. M. Mercado, J. Olson and N. Chatterjie, *Biochemistry* **13**, 4878 (1974).
20. R. P. Mason, in *Reviews in Biochemical Toxicology* (Eds. L. Hodson, J. R. Bend and R. M. Philpott), Vol. 1, p. 151. Elsevier/North Holland, New York (1979).