Free Rad. Res. Comms., Vol. 8, No. 4-6, pp. 241-249
Reprints available directly from the publisher
Photocopying permitted by license only

THE REDUCTIVE DEGLYCOSYLATION OF ADRIAMYCIN IN AQUEOUS MEDIUM: A PULSE RADIOLYSIS STUDY

J.S. LEA, F.A.P. RUSHTON, E.J. LAND and A.J. SWALLOW*

Paterson Institute for Cancer Research, Christie Hospital & Holt Radium Institute, Manchester M20 9BX, UK

(Received June 20, 1989; accepted July 31, 1989)

The free radical (II) produced by one-electron reduction of adriamycin (I) exists in aqueous solution at pH 7.0 in equilibrium with the parent and the two-electron reduced form (III). Over some hundreds of milliseconds deglycosylation takes place yielding an aglycone (IV) which subsequently rearranges to form a more stable aglycone, 7-deoxyadriamycinone (V). The changes in the optical absorption spectrum accompanying these processes are reported. The rate constant for III → IV is $1.1\,s^{-1}$ and for IV → V is $1.5 \times 10^{-2}\,s^{-1}$. At pH 4.0 the two electron reduced form of adriamycin exists predominantly in a different tautomeric form (VII). It is suggested that this deglycosylates via a free radical mechanism involving the acidic form of the semiquinone free radical (VI).

KEY WORDS: Adriamycin, semiquinone, reductive deglycosylation, aglycone tautomer, 7-deoxyadriamycinone, pulse radiolysis.

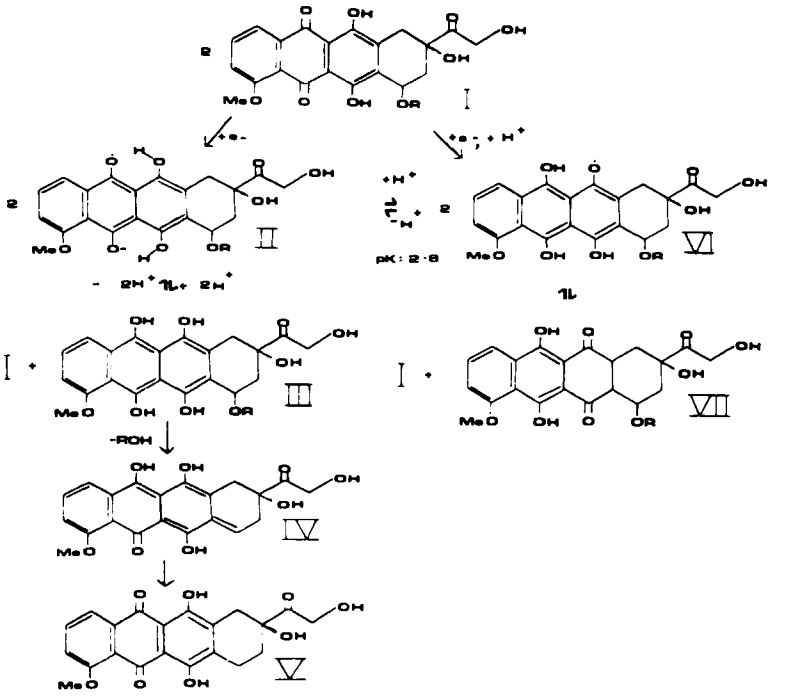
INTRODUCTION

The anthracycline antibiotics continue as one of the major classes of chemotherapeutic agents.[1] Of these, adriamycin (I, see Scheme 1) (doxorubicin) possesses the widest range of activity as an anti-tumour agent.[2] However, its utility is marred by dose-related cytotoxic and cardiotoxic effects.[3] The metabolism and mode of action of this drug and the chemically similar daunomycin have been extensively studied and much of the current studies revolve around the redox chemistry.[4]

Reduction of the parent quinone drug leads to the formation of the semiquinone free radical species (II)[5] which, as shown with daunomycin, in turn gives rise to the production of 7-deoxyaglycone (V)[6] probably via a deglycosylation mechanism involving successively the hydroquinone (III) and a tautomer of the aglycone (IV). These intermediates have been the subject of chemical studies employing reduction by the radical dimer, bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) in methanolic systems[6] and pulse radiolytic reduction in aqueous solutions using very high doses of radiation[7] with the drug daunomycin.

Whilst the mode of action of the anthracyclines does not necessarily involve free radicals, the drugs, like many other quinones, augment the flow of electrons from NADPH to molecular oxygen.[8] This process leads to the production of reactive oxygen species which may indeed form an aspect of the anti-tumour activity but are more likely to be of importance in connection with the cardiotoxicity of the drug.

*Correspondence to: A.J. Swallow.



Building upon previous experiments in this laboratory[5,9,10] and the mechanism earlier outlined for daunomycin,[6] we have now studied the formation and decay of the tautomeric (reductive) deglycosylation product (IV) at pH = 7.0. Changes in the mechanism in acidic solution have also been observed with evidence for the presence under these conditions of a different tautomer (VII) of the hydroquinone, which has already been reported for daunomycin.[11] The studies of tautomer (IV) decay employed the 'single-shot' long timescale pulse radiolysis system recently developed,[12] which incorporated a diode array U.V./vis spectrophotometer. This facilitated the collection of complete spectra over the wavelength range studied, at 5–45 second intervals, up to 300 seconds after the pulse.

MATERIALS AND METHODS

Adriamycin (doxorubicin) hydrochloride was obtained from Farmitalia and used as received. Comparison with a sample obtained from Sigma revealed no difference in behaviour. Solutions were freshly made up and kept at room temperature for the minimum amount of time (< 1 hour) before pulse radiolysis. Water was redistilled from alkaline permanganate. All other chemicals were AnalaR grade from BDH or Hopkin and Williams. Solutions were buffered as necessary by borate/OH^-, $H_2PO_4^-$/HPO_4^{2-} and $HCOOH/HCOO^-$. All solutions were purged for at least 30 min using argon (Air Products Ltd). The purged solutions were passed through a capillary flow system into a cylindrical quartz capillary optical cell of 3 mm diameter, and optical path length 2.5 cm.

Single pulses of electrons from the Paterson Institute linear accelerator[12] were used to irradiate the solution. The changes produced were analyzed (maximum time scale of 300 seconds) using a tungsten light beam. Doses ranged from 4–450 Gy (1 Gy = 1 JKg^{-1}). For most of the pulse radiolysis studies the light transmitted through the solution was passed through a Kratos monochromator into an EMI 9558Q photomultiplier (using bandwidths of 10 nm). Changes in optical transmission with time were then recorded using a HP 9836S computer fitted with a Tektronix 7612 AD digitiser. Absorbed doses were measured from the transient $(SCN)_2^-$ formation from oxygen saturated aqueous 10^{-2} mol dm^{-3} potassium thiocyanate, using $G = 2.9$ per 100 eV and $\varepsilon_{500} = 7.1 \times 10^3$ dm^3 mol^{-1} cm^{-1}.[13]

Long-timescale measurements were made by passing the light transmitted through the solution through an optical fibre and directly into an HP8451A Diode Array Spectrophotometer. Standard conditions when employing the 2.5 cm capillary cell necessitated maintaining the solution under pressure to enable the solution under test to be exchanged after exposure to radiation. For the longer timescale experiments, in order to establish that leakage of solution did not distort the results, certain critical experiments were repeated with a stoppered standard 1 cm spectrophotometric cuvette. The results were indistinguishable from those employing the 2.5 cm capillary cell.

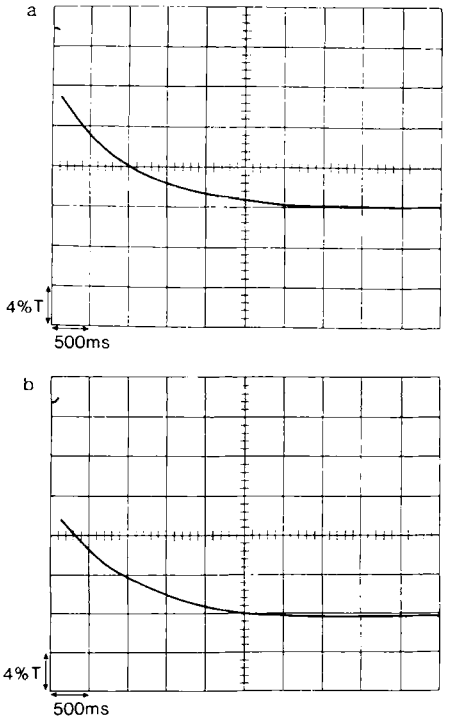


FIGURE 1 Computer traces showing the changes in optical transmission (a) at 620 nm and (b) at 380 nm as a function of time after delivery of a pulse (10 Gy) to argon bubbled aqueous solutions containing 10^{-1} mol dm^{-3} sodium formate and 3.9×10^{-5} mol dm^{-3} adriamycin in 10^{-3} mol dm^{-3} phosphate buffer.

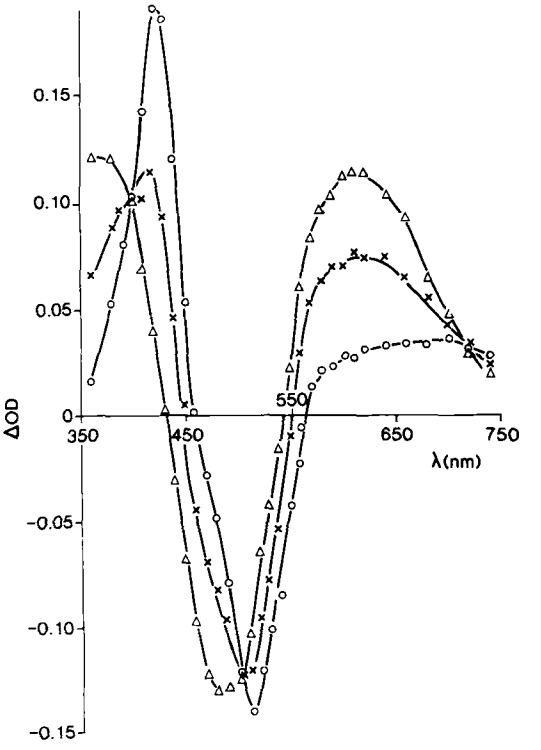


FIGURE 2 Difference between the spectrum of the parent adriamycin and the products of reduction at pH = 7.0 at increasing times after the pulse (16 Gy), ○ = 24 ms, × = 600 ms and △ = 3.91 s. Argon flushed solution contained 10^{-1} mol dm^{-3} sodium formate, 4×10^{-5} mol dm^{-3} adriamycin and 10^{-3} mol dm^{-3} phosphate buffer.

RESULTS AND DISCUSSION

It has been shown by Mukherjee *et al.*[10] that on pulse radiolytic reduction of adriamycin, an equilibrium of the type;

$$2 \text{ Semiquinone} \rightleftharpoons \text{Hydroquinone} + \text{Parent} \qquad (1)$$

is established within a few milliseconds of the pulse. Experiments at longer timescales,[9] based on absorption measurements at 380 and 608 nm, showed changes consistent with deglycosylation of the hydroquinone, as observed for daunomycin in methanol by Kleyer and Koch.[6]

Figure 1 displays typical traces at pH = 7.0, with a total timescale of 5 seconds at the comparable wavelengths 380 and 620 nm. The initial decrease in transmission is attributed to the establishment of the equilibrium as in reaction (1), with the subsequent slow reaction being consistent with the formation of the longer lived product, an aglycone tautomer. This appears to be supported by the changes in the difference spectrum (350 → 750 nm) as shown in Figure 2. At increasing times after the pulse (24 ms → 3.91 s) peaks attributed to tautomer absorption increase (380 and 620 nm) whilst there is a reduction in the absorption assigned to the hydroquinone (420 nm).

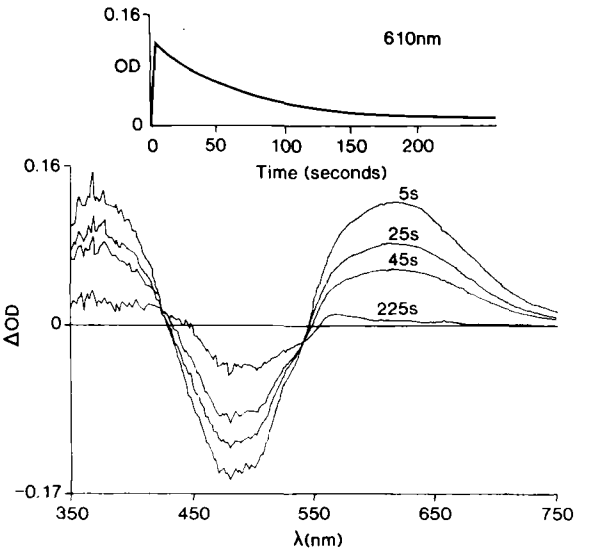


FIGURE 3 Upper: Loss of absorbance of the tautomer (IV) peak at 610 nm attributed to rearrangement to 7-deoxyadriamycinone (V). Lower: Full difference spectra at increasing times demonstrating the rearrangement of the tautomer (IV) into 7-deoxyadriamycinone (V). Solution and dose were as for Figure 2.

When studies are extended to even longer times (up to 300 seconds) subsequent reaction causes the absorption due to this tautomer to decrease. Figure 3 summarises these very slow changes. The upper trace displays the loss of absorption close to the peak of the tautomer (610 nm) at this longer timescale. This decrease is attributed to the rearrangement of the tautomer of the aglycone into the final product, 7-deoxyadriamycinone, consistent with the corresponding rearrangement of the tautomer of the aglycone of daunomycin observed in methanol by Kleyer and Koch.[6] The final product possesses a similar absorption spectrum to the parent quinone,[14] therefore the lower trace (225 s) of Figure 3 supports this rearrangement. The difference spectra recorded in the lower section of this figure show the loss of absorption at wavelengths assigned to the tautomer and a recovery in absorption at the peak of both parent and 7-deoxyadriamycinone absorption. Very similar results were obtained with aqueous solutions of daunomycin.

The mechanism can be interpreted in terms of the left hand side of Scheme 1. To test the fit of Scheme 1 to experimental data obtained with the solution for Figures 2 and 3, the expected concentration of the various species was calculated as a function of time by numerical integration of the corresponding rate equations. The initial concentration of radicals produced by the pulse was calculated from the dose assuming a G value of 6.5 radicals per 100 eV and was found to be to 11×10^{-6} mol dm^{-3}. The equilibrium constant was obtained from measurements of the semiquinone radical at 24 ms after the pulse using the absorption at 720 nm, and was found to be 8, consistent with the value reported by Mukherjee *et al.*[10] at this pH (7.0). Values for the forward and back reactions of the equilibrium were set at 1×10^{9} and 1.3×10^{8} mol dm^{-3} s^{-1}, respectively, although the conclusion was unaffected when both rate constants were taken to be one tenth of these values. Optimal correspon-

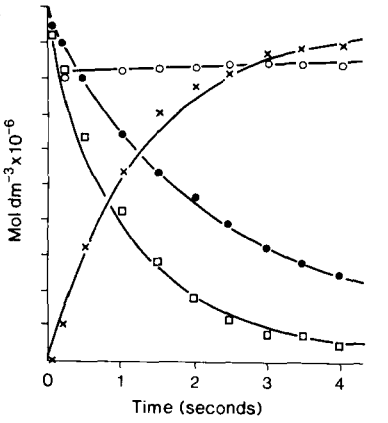


FIGURE 4 Calculated changes (full lines) of the concentration of various species at times up to 4 seconds based on Scheme 1, together with experimentally derived concentrations (points) at selected times after the pulse (○ = parent (I), ● = semiquinone (II), □ = hydroquinone (III) and × = tautomer (IV)). The vertical axis differs in each case. Each division for the parent = 4×10^{-6} mol dm^{-3}, the semiquinone = 4×10^{-7} mol dm^{-3}, the hydroquinone = 4×10^{-7} mol dm^{-3} and the tautomer = 5×10^{-7} mol dm^{-3}. Solution and dose were as for Figure 2.

dence (Figure 4 and 5) between the changes in concentrations of the relevant species calculated in this way and the experimental results (see below) could be obtained if the rate constant for the deglycosylation (III → IV) is 1.1 s^{-1} and the rate constant for the rearrangement (IV → V) is 1.5×10^{-2} s^{-1}. These rate constants may be compared with the corresponding rate constant for daunomycin at pH = 8.0 of Fisher *et al.*[15] (2 s^{-1} for the deglycosylation (III → IV) and 4.6×10^{-2} s^{-1} for the rearrangement (IV → V)), and for daunomycin at pH = 7.4 of Bird *et al.*[11] (3.3×10^{-2} s^{-1} for the rearrangement (IV → V)). The slow changes in transmission shown in Figure 1 are dominated by the first order deglycosylation process with a rate

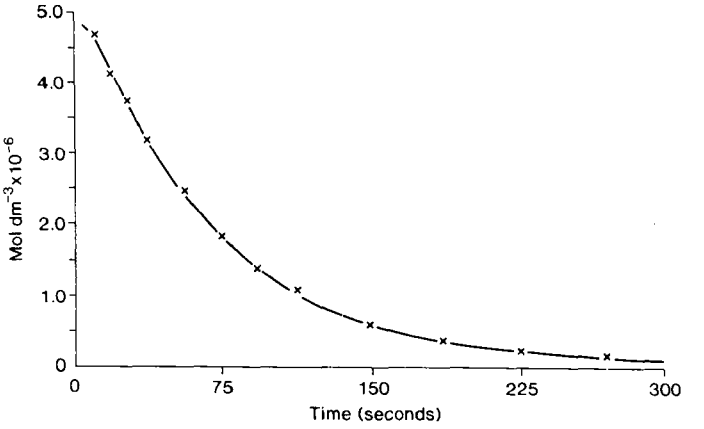


FIGURE 5 Calculated change (full line) of concentration of tautomer (IV) at times up to 300 seconds based on Scheme 1, together with experimentally derived points at selected times after the pulse. Solutions and dose were as for Figure 2.

TABLE I
Effect of varying conditions on the rate of tautomer formation

Dose (Gy)	Wavelength (nm)	k (s^{-1})	[ADR] (μmol dm^{-3})
24	620	1.12	50
34	620	1.33	50
24	620	1.01	150
34	620	1.15	150
11	380	1.03	150
23	380	1.07	50

constant of 1.1 s^{-1}. The rate constant was independent of the parent adriamycin concentration within the range 50–150 $\times$ 10^{-6} mol dm^{-3} and the dose per pulse range of 11–34 Gy (Table I).

The concentrations of the parent, semiquinone, hydroquinone and aglycone tautomer for Figures 4 and 5 were calculated from optical densities at 420, 510, 620 and 720 nm in the solution for Figures 2 and 3 and the extinction coefficients of each species at these wavelengths. The absolute absorption spectra of the parent and the semiquinone are already known.[6] The extinction coefficients of the hydroquinone

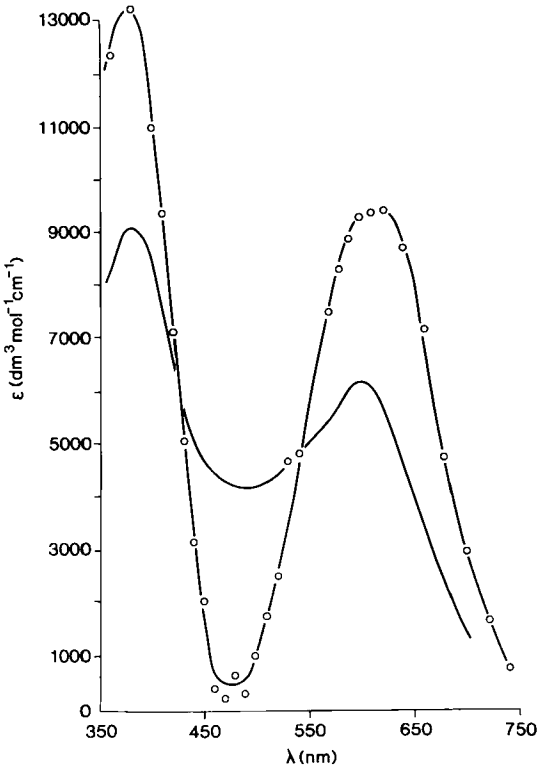


FIGURE 6 Absolute absorption spectrum of the aglycone tautomer (IV) in aqueous solution at pH = 7.0 calculated as described in the text (○), compared to that obtained for the corresponding derivative of daunomycin in methanol by Kleyer and Koch.[6]

were obtained from spectroscopic measurements at the position of equilibrium (24 ms) where only the parent, semiquinone and hydroquinone are present. Provisional values of the extinction coefficient for the aglycone tautomer were first obtained from optical densities at 3.5 seconds where the concentration was at its highest. They were then adjusted to allow for decay setting in before formation was complete, using the rate constants for III → IV and IV → V as given above. The full absorption spectrum of the tautomer calculated in this way is shown in Figure 6, compared with that obtained for daunomycin in methanol by Kleyer and Koch.[6]

No evidence was obtained for any other intermediates involved in the process III → IV, unlike Houee-Levin *et al.*,[7] even when mimicking the high irradiation dose experimental conditions of these workers.

At an early stage (12 ms) after reduction of adriamycin in slightly acid solutions, Mukherjee *et al.*[10] observed the presence of unidentified species which were not seen in neutral solution. Correspondingly, in acid solutions of daunomycin, Bird *et al.*[11] demonstrated the presence of isomers of a hydroquinone differing from the equivalent of III. Unlike the daunomycin equivalent of III, the hydroquinone was stable to deglycosylation. The isomers of the hydroquinone were characterised as the equivalent of VII by optical, n.m.r., and mass spectroscopy. All forms of the hydroquinone display strong absorptions near 420 nm. We now find that several seconds after reduction of adriamycin in acid solutions there is a strong absorption at 420 nm whereas in neutral solutions, the absorption at 420 nm had largely disappeared by this time. This absorption is attributed to a tautomer of the hydroquinone, VII, analogous to that observed with daunomycin. We attribute the formation of this species to a disproportionation of the acidic form of the semiquinone.

$$2\ \mathrm{VI} \rightarrow \mathrm{I} + \mathrm{VII} \tag{2}$$

Bird *et al.*[11] observed that although stable at pH = 3.0 the tautomer of the hydroquinone produced in acid solution deglycosylates at pH 7.4. This can, tentatively, be explained in terms of the corresponding reactions of adriamycin shown in Scheme 1. The leuco form of the hydroquinone (VII) is oxidised (by I in Scheme 1) followed by deprotonation of the semiquinone (VI) to give the anionic semiquinone (II), which then disproportionates[10] and deglycosylates as demonstrated above:

$$\mathrm{I} + \mathrm{VII} \rightarrow 2\ \mathrm{VI} \tag{3}$$

$$\mathrm{VI} \rightarrow \mathrm{II} + \mathrm{H}^+ \tag{4}$$

$$2\ \mathrm{II} + 2\ \mathrm{H}^+ \rightarrow \mathrm{I} + \mathrm{III} \tag{5}$$

$$\mathrm{III} \rightarrow \mathrm{IV} \tag{6}$$

It may be noted that this scheme incorporates a free radical chain reaction for the conversion of VII into III, with I acting as a catalyst. Further work would be required to test this scheme and put it on a quantitative basis.

The importance of the above reactions is demonstrated by recent studies[16,17] which identify aglycones as detectable metabolites within the plasma of cancer patients treated with anthracyclines.

Acknowledgements

This work was supported by grants from the Cancer Research Campaign.

References

1. Arcamone, F. Doxorubicin anticancer antibiotics: Academic Press, New York, (1981).
2. Arcamone, F. Properties of antitumour anthracyclines and new developments in their application. *Cancer Research*, **45**, 5995–5999, (1985).
3. Green, M.D., Speyer, J.L. and Muggia, F. Cardiotoxicity of anthracyclines. *Eur. J. Cancer Clin. Oncol.*, **20**, 293–296, (1984).
4. Abdella, B.R.J. and Fisher, J. A chemical perspective on the anthracycline antibiotics. *Environ. Health Perspect.*, **64**, 3–18, (1985).
5. Land, E.J., Mukherjee, T., Swallow, A.J. and Bruce, J.M. One-electron reduction of adriamycin: Properties of the semiquinone. *Arch. Biochem. Biophys.*, **225**, 116–121, (1983).
6. Kleyer, D.L. and Koch, T.H. Spectroscopic observation of the tautomer of 7-deoxydaunomycinone from elimination of daunosamine from daunomycin hydroquinone. *J. Am. Chem. Soc.*, **105**, 2504–2505, (1983).
7. Houée-Levin, C., Gardès-Albert, M. and Ferradini, C. Pulse radiolysis study of daunorubicin redox reactions: Redox cycles or glycosidic cleavage. *J. Free Rad. Biol. Med.*, **2**, 89–97, (1986).
8. Bachur, N.R., Gordon, S.L., Gee, M.V. and Kon, H. NADPH cytochrome P450 reductase activation of quinone anticancer agents to free radicals. *Proc. Nat. Acad. Sci. U.S.A.*, **76**, 954–957, (1979).
9. Land, E.J., Mukherjee, T., Swallow, A.J. and Bruce, J.M. Possible intermediates in the action of adriamycin – A pulse radiolysis study. *Br. J. Cancer*, **51**, 515–523, (1985).
10. Mukherjee, T., Land, E.J., Swallow, A.J. and Bruce, J.M. One-electron reduction of adriamycin and daunomycin: Short-term stability of the semiquinones. *Arch. Biochem. Biophys.*, **272**, 450–458, (1989).
11. Bird, D.M., Boldt, M. and Koch, T.H. Leucodaunomycin, a tautomer of daunomycin hydroquinone. *J. Am. Chem. Soc.*, **111**, 1148–1150, (1989).
12. Butler, J., Hodgson, B.W., Hoey, B.M., Land, E.J., Lea, J.S., Lindley, E.J., Rushton, F.A.P. and Swallow, A.J. A study of some moderately fast processes initiated by radiation. *Rad. Phys. Chem.*, **34**, 633–646, (1989).
13. Adams, G.E., Boag, J.W., Currant, J. and Michael, B.D. The pulse radiolysis of aqueous solutions of thiocyanate ions. In Pulse Radiolysis (Eds. M. Ebert, J.P. Keene, A.J. Swallow and J.H. Baxendale). Academic Press, London, 117–129, (1965).
14. Kleyer, D.L. and Koch, T.H. 7-Deoxydaunomycinone, a catalyst for achieving a two-electron reduction with a one-electron reducing agent. *J. Am. Chem. Soc.*, **105**, 5911–5912, (1983).
15. Fisher, J., Abdella, B.R.J. and McLane, K.E. Anthracycline antibiotic reduction by spinach ferredoxin-$NADP^+$ reductase and ferredoxin. *Biochemistry*, **24**, 3562–3571, (1985).
16. Cummings, J., Milroy, R., Banham, S.W. and Kaye, S.B. Method for the determination of 4-demethoxydaunorubicin, its quinone and hydroquinone metabolites in human plasma and urine by high performance liquid chromatography. *Cancer Chemotherapy and Pharmacol.*, **19**, 296–300, (1987).
17. Cummings, J., Morrison, J.G. and Willmot, N. Determination of anthracycline purity in patient samples and identification of in-vitro chemical reduction products by application of a multi-diode array spectrophotometric detector. *J. Chromatography*, **381**, 373–384, (1986).

Accepted by Prof. H. Sies/Prof. E. Cadenas