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# Semiquinone Free Radical Formation by Daunorubicin Aglycone Incorporated into the Cellular Membranes of Intact Chinese Hamster Ovary Cells

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The production of semiquinone free radicals has been measured by electron paramagnetic resonance spectroscopy (EPR) in Chinese hamster ovary cells in which 7-hydroxy daunorubicin aglycone had been incorporated. The highly lipophilic daunorubicin aglycone was incorporated into the cellular membrane by swirling a cell suspension over a thin layer of daunorubicin aglycone. Thus, the observed semiquinone free radical was likely formed directly in the lipophilic environment of the cellular membrane. The linewidth of the observed EPR signal suggested that a neutral protonated semiquinone species was formed. In the presence of the cell-impermeant paramagnetic line broadening agent chromium(III) oxalate, no detectable signal was observed. This result indicates that even though the semiquinone is embedded in the membrane, it is still partly accessible to the external chromium(III) oxalate. Analysis of chloroform extracts of the cells after EPR experiments indicated that daunorubicin aglycone was extensively metabolized. The results of a growth inhibition assay carried out on cells into which daunorubicin aglycone had been incorporated showed almost no effect on cell growth. This result indicates that in spite of significant daunorubicin aglycone-induced radical formation taking place directly in the cell membrane, little cell damage results.

*Key words:* daunorubicin, aglycone, membrane, cell, semiquinone, radical

## INTRODUCTION

The clinical use of the antitumor anthracyclines including daunorubicin and doxorubicin is limited by a unique cumulative dose-limiting cardiotoxicity.[1,2] There is now a considerable body of evidence to suggest that the cardiotoxicity may be due to iron-based oxygen free radical-induced oxidative stress[1,3,4] on the relatively unprotected heart muscle.[5] The anthracyclines can be reductively activated[6,7] to their semiquinone free radical form. The semiquinone can react rapidly with oxygen[8] to produce superoxide, which then dismutates to hydrogen peroxide. The extremely damaging hydroxyl radical is produced by the reduction of hydrogen peroxide by iron(II)-anthracycline complexes.[9] Both daunorubicin and doxorubicin are extensively metabolized *in*

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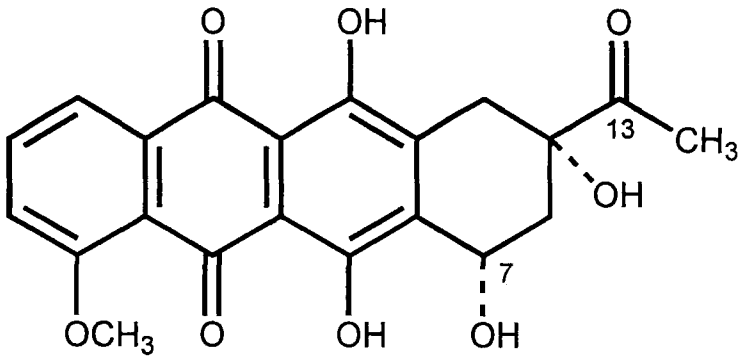


FIGURE 1 Structure of daunorubicin aglycone.

*vivo*.[10–12] The 7-hydroxy (Figure 1) and 7-deoxy daunorubicin aglycone metabolites are formed either from the hydrolytic or reductive loss, respectively, of the daunosamine sugar from daunorubicin. These aglycones are much more lipophilic than the parent compound and, thus, might be expected to partition into the plasma membrane. A semiquinone free radical produced from an anthracycline aglycone should also be able to transfer an electron to oxygen dissolved in the plasma membrane. This would result in superoxide (or in the acidic environment of the membrane, its protonated form, hydroperoxyl radical) being formed directly in the membrane. The hydroperoxyl radical is a more powerful reductant, oxidizing agent and nucleophile than is superoxide itself.[5] In addition, the hydroperoxyl radical dismutates to hydrogen peroxide much faster than does superoxide.[5] Thus, the formation of a semiquinone radical directly in the membrane could lead to site-specific damage to the cell membrane. This study was carried out to see if daunorubicin aglycone semiquinone could be formed in cells which had daunorubicin aglycone incorporated in the membrane. The doxorubicin aglycone has been shown to have dramatic effects on isolated heart mitochondria, resulting in increased oxidation of pyridine nucleotides, sulfhydryl group modification, and an increase in inner membrane permeability.[13] While daunorubicin aglycone has been shown to form in microsomal suspensions,[14] daunorubicin aglycone has not been identified as a major metabolite in humans, though its 7-deoxy derivative has.[10]

## MATERIALS AND METHODS

### Materials

Daunorubicin hydrochloride, HEPES, Dulbecco's phosphate buffered saline (PBS, pH 7.4), xanthine oxidase (from buttermilk), and MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma (St. Louis, MO). Minimum Essential Media ($\alpha$-MEM, nucleotide free), calf serum, penicillin and streptomycin were obtained from Gibco Laboratories (Burlington, Canada). Potassium chromium(III) oxalate ($K_3Cr(C_2O_4)_3 \bullet 3H_2O$), was from Aldrich (Milwaukee, WI). Hypoxanthine was from Eastman (Rochester, NY). Strontium oxide (SrO) was from Johnson Matthey (Seabrook, NH). The gas permeable ultra thin wall capillary Teflon tubing (1 mm i.d., 0.04 mm wall thickness) used in the electron paramagnetic resonance (EPR) experiments was from Zeus Industrial Products (Raritan, NJ). Daunorubicin aglycone was prepared by mild acid hydrolysis of daunorubicin, essentially as described.[15] The product gave only a single spot by

TLC, and the $^1$H-NMR spectra was consistent with 7-hydroxy daunorubicin aglycone. An extinction coefficient of 9060 $M^{-1}cm^{-1}$ at 480 nm in chloroform was measured and was used to determine daunorubicin aglycone concentrations.

### Cell culture and cytotoxicity assay

CHO cells (type AA8) obtained from the American Type Culture Collection (Rockville, MD) were grown in $\alpha$-MEM containing 20 mM HEPES, 100 units/ml penicillin G, 100 $\mu$g/ml streptomycin, 10% calf serum (Gibco, iron supplemented and enriched) in an atmosphere of 5% $CO_2$ and 95% air at 37°C (pH of 7.4). Cells in exponential growth were harvested, and after counting were suspended in $\alpha$-MEM containing 20 mM HEPES and placed in an erlenmeyer flask in a shaker bath at 37°C at a cell density of $6 \times 10^6$ cells/ml. Lysed cells for EPR control experiments were prepared by subjecting packed cells to seven freeze-thaw cycles between liquid nitrogen and a 37°C bath. Cells into which different amounts of daunorubicin aglycone had been incorporated were seeded at 5000 cells/well in 96-well microtiter plates (200 $\mu$l/well) and allowed to grow for a further 48 hr in order to determine if any growth inhibition had occurred. The cell growth was determined by MTT assay, basically as described.[16] Cellular cytosolic reductases act on MTT to produce a dye, the absorbance of which at 490 nm is proportional to cell number. Typically six replicates were measured at each drug concentration. For the daunorubicin cytotoxicity assay, the drug was added to cells that had been plated at 2000 cells/well 24 hr before and allowed to grow in the presence of drug for a further 48 hr.

Daunorubicin aglycone was incorporated into the PBS-washed CHO cells by gently swirling the cells ($6 \times 10^6$ cells/ml in 1 ml) in a test tube containing a thin coat of daunorubicin aglycone. The thin film was deposited on the inside of the test tube by using a stream of nitrogen to completely evaporate the solvent from a solution of daunorubicin aglycone dissolved in chloroform (0.5 ml, 1 mM). After the daunorubicin aglycone had been incorporated into the cells, the cell suspension was concentrated by centrifugation at 20 $g$ for 5 min, and the pellet was resuspended in PBS to give a final cell density of $10^8$ cells/ml.

### EPR experiments

A 50 $\mu$l aliquot of the cell suspension was injected into the gas permeable Teflon tubing, folded at both ends and inserted into a quartz EPR tube open at both ends and placed in the EPR cavity. Pre-purified grade nitrogen, thermostatted at 37°C was flowed continuously over the sample with a flow rate of approximately 200 l/h. Recording of the first-derivative EPR spectra was started after one minute of being placed in the EPR cavity. A total of 10 spectra were recorded over 11 minutes and their signals were averaged. The EPR spectra were recorded with a Varian E-12 spectrometer with a Nicolet 1180 data system and a Bruker variable temperature controller. The instrument settings were as follows: microwave power 20 mW, microwave frequency 9.02 GHz, modulation amplitude 1.0 G, time constant 0.1 s, and magnetic field centred at 3215 G with a 40 G scan range. The radical detected was quantitated by measuring the double integrals of the baseline-corrected spectra, referred to a 5 $\mu$M 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) standard EPR spectrum that was obtained under identical conditions. Strontium oxide (SrO) containing a trace amount of paramagnetic manganese ion was used as an accurate $g$-standard[17] to obtain accurate peak positions and spectral ranges. The daunorubicin semiquinone was produced in PBS under hypoxic conditions by a xanthine oxidase (0.1 milliunits/ml)/ hypoxanthine (400 $\mu$M) system in the presence of 3 mM daunorubicin, essentially as described.[18]

### Spectrofluorometric studies

The optimum excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) wavelengths of daunorubicin aglycone in

chloroform were determined to be $\lambda_{ex}$ 490 nm, and $\lambda_{em}$ approximately 554 and 588 nm. The daunorubicin aglycone was extracted from the cell suspensions by shaking the cell suspensions with 3 ml of chloroform. The aglycones were analyzed by TLC (silica gel) using a solvent system consisting of chloroform/methanol/acetic acid in the ratio 100 : 2 : 5 (v : v : v).[10] The daunorubicin aglycone octanol-aqueous buffer partition coefficient $P_{oct}$ was measured to be 180 ± 30 (n = 2) by shaking 1-octanol containing a known concentration of daunorubicin aglycone with PBS, and measuring the fluorescence in the aqueous layer, assuming the same fluorescence intensity for aqueous solutions as in chloroform. Unless indicated, all errors quoted are ± SEM.

## RESULTS

### EPR measurements of daunorubicin aglycone semiquinone formation in CHO cells

When a suspension of CHO cells was swirled in a test tube which contained a thin layer of daunorubicin aglycone, the cells took on a progressively pink color over a period of several minutes. As shown in Figure 2 the amount incorporated into the cells depended upon the length of time the cells were in contact with the daunorubicin aglycone layer. Under a bright field microscope the cells displayed heavily stained red membranes, indicating that the daunorubicin aglycone had partitioned into the cell membrane.

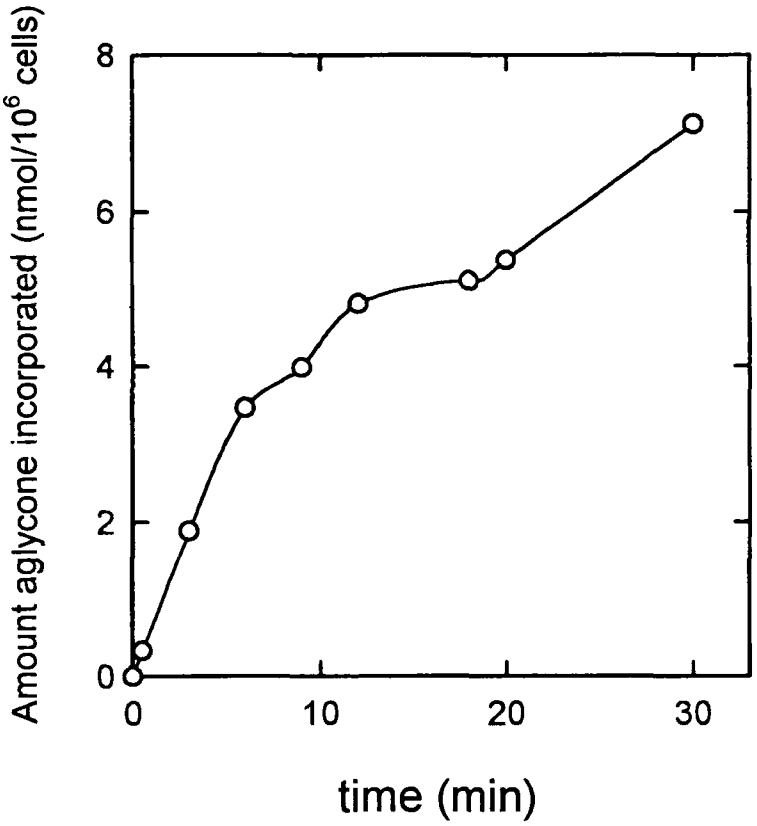


FIGURE 2 Amount of daunorubicin aglycone incorporated into CHO cells suspended in PBS by swirling the cells at room temperature over a thin layer of daunorubicin aglycone deposited on the inner surface of a test tube. The daunorubicin aglycone was quantitated by extracting the cells with chloroform and measuring the fluorescence.

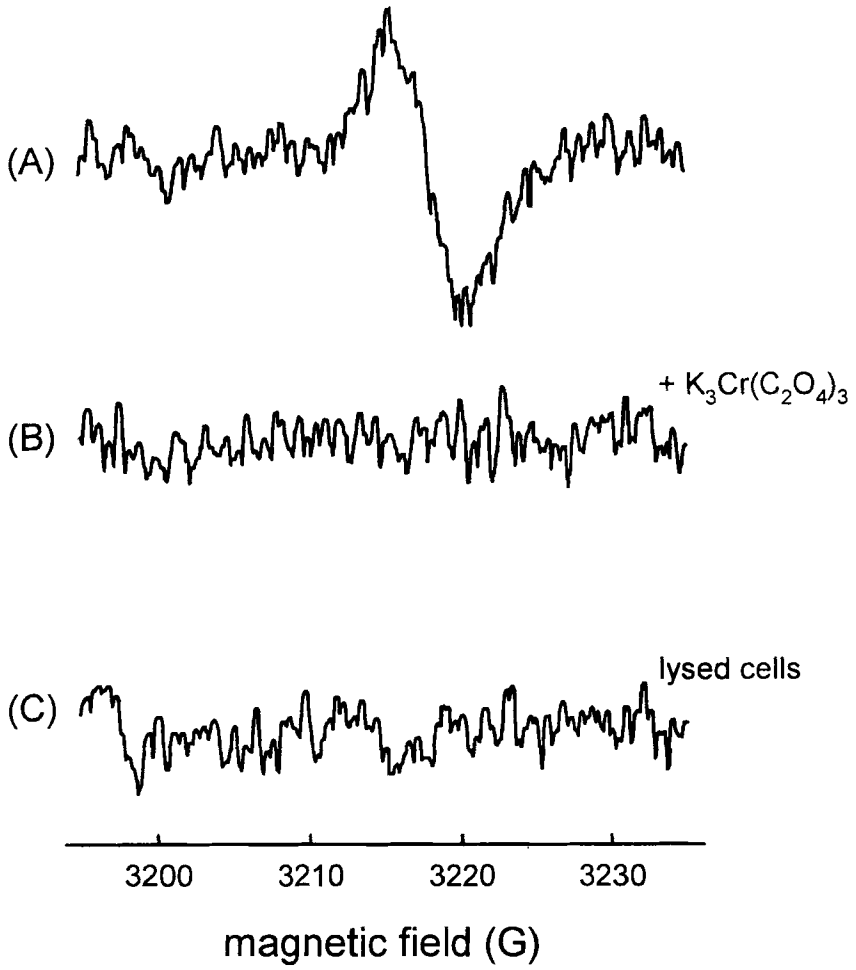


FIGURE 3 (A): The EPR spectrum produced under hypoxic conditions by CHO cells ($1 \times 10^8$ cells/ml in PBS at 37°C, pH 7.4) into which daunorubicin aglycone had been incorporated. The scan range was 40 G and the modulation amplitude was 1.0 G. The spectrum shown is the average of 10 scans recorded over a period of 11 min. The concentration of the radical formed was 0.9 $\mu$M. (B): As in (A) above, but in the presence of 50 mM chromium(III) oxalate. (C): As in (A) above, but with cells that were lysed through multiple freeze-thaw cycles.

Hypoxic cell suspensions into which daunorubicin aglycone had been incorporated displayed an EPR signal (Figure 3A) with a $g$ of 2.005 and a peak-to-peak linewidth $\Delta H_{pp}$ of 5.1 G. After about 15 min the EPR signal disappeared. The daunorubicin aglycone-derived EPR spectra showed significant asymmetry suggesting the semiquinone formed is partly immobilized in the cell membrane. The introduction of air into a hypoxic cell suspension displaying an EPR signal, resulted in a complete and rapid loss of the radical signal. Cells into which no daunorubicin aglycone had been incorporated, did not display a detectable EPR signal (data not shown). The xanthine oxidase/hypoxanthine-induced daunorubicin semiquinone free in solution was measured under identical conditions to have a $\Delta H_{pp}$ of 2.7 G and a $g$ of 2.006 (data not shown). The observed $g$ of 2.005 for daunorubicin aglycone in the cell suspension is close to this value and is also close to a $g$ value of 2.0056 ($\Delta H_{pp}$ of 1.0 G) found for daunorubicin aglycone semiquinone formed in a

DMSO-potassium superoxide system.[19] Double integration of the radical signal gave a semiquinone radical concentration in the cell suspension of 0.66 ± 0.19 $\mu$M (n = 3). From this value it can be estimated that there are approximately four million semiquinone radicals present in each cell. It can also be estimated, that of the total daunorubicin aglycone incorporated, 0.13 % was detected as semiquinone radical. Cells in which daunorubicin aglycone had been incorporated and were subsequently lysed, displayed very little or no radical signal (Figure 3C), indicating that only intact cells can produce the daunorubicin aglycone semiquinone radical.

As shown in Figure 3B, the addition of the cellular impermeant line broadening agent chromium(III) oxalate (50 mM) to the cell suspension completely eliminated the daunorubicin aglycone-induced EPR signal. This paramagnetic metal ion complex, which is completely excluded from the intracellular space, has been previously used to identify whether the doxorubicin semiquinone is located intracellularly or extracelluarly.[20] The lack of any EPR signal is consistent with the semiquinone being accessible to chromium(III) oxalate.

### Metabolism of daunorubicin aglycone in CHO cell suspensions

The fluorescence spectrum of daunorubicin aglycone extracted from cells on which EPR studies were carried out is shown in Figure 4 (lower curve), and is significantly different than

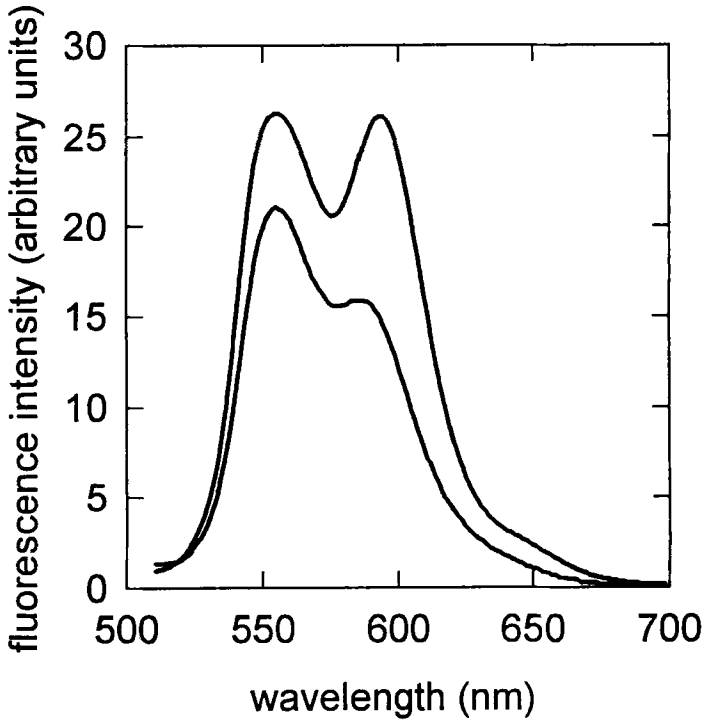


FIGURE 4 Normalized fluorescence spectra ($\lambda_{ex}$ 490 nm) in chloroform of daunorubicin aglycone (upper curve), and daunorubicin aglycone (lower curve) extracted from CHO cells after the hypoxic EPR experiments described in the caption to Figure 3. The spectral changes indicate that daunorubicin aglycone was metabolized by the cells.

daunorubicin aglycone alone (upper curve). Spectral changes similar to these have been previously observed for daunorubicin aglycone metabolites.[10] Since these results suggested that daunorubicin aglycone was undergoing metabolism in the anoxic cell suspensions, the chloroform extracts were subjected to TLC using a previously described solvent system.[10] Extensive metabolism had occurred under both aerobic and hypoxic conditions. For the hypoxic experiments only a small amount of parent daunorubicin aglycone could be detected, while for the aerobic experiments no parent was seen. For the hypoxic experiments two polar compounds with $R_f$'s of 0.59 and 0.43 were seen, compared to a $R_f$ of 0.74 for daunorubicin aglycone itself. Under aerobic conditions at least two polar compounds were seen. In either case no spot was seen that could have corresponded to the less polar 7-deoxy daunorubicin aglycone. These more polar compounds could not be positively identified, due to the limited quantities of sample available. Metabolites more polar than daunorubicin aglycone that have been identified[10] include daunorubicinol aglycone, deoxydaunorubicinol aglycone and demethyldeoxy daunorubicinol aglycone.

### Inhibition of the growth of CHO cells by daunorubicin aglycone and daunorubicin

The ability of daunorubicin aglycone to inhibit the growth of CHO cells over 48 hr (approximately eight cell doublings) was measured by MTT assay. As shown in Figure 5, in spite of the fact that significant amounts of daunorubicin aglycone were incorporated into the cell membrane, no significant growth inhibition occurred (slope = $-20 \pm 40$ million cells/nmol). For comparison, the

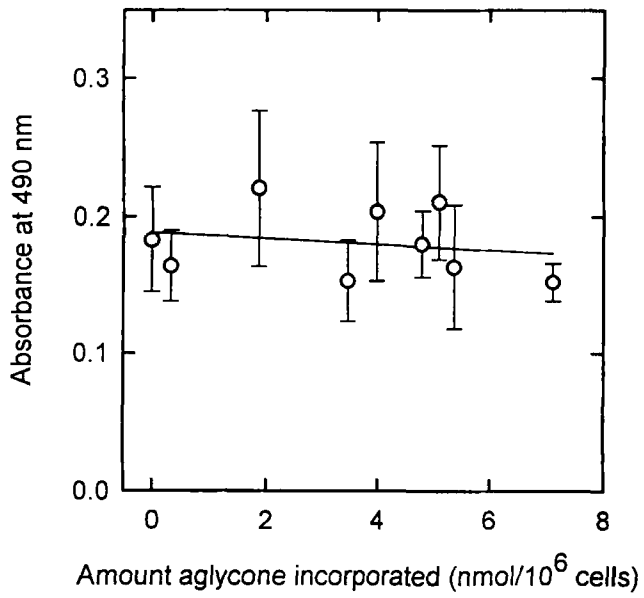


FIGURE 5 Growth inhibition assay for CHO cells into which varying amounts of daunorubicin aglycone have been incorporated. The plot has a slope that is close to zero indicating that daunorubicin aglycone causes almost no growth inhibition. At the highest amount of incorporation shown, daunorubicin aglycone was present in the microtitre plate well at a concentration equivalent to 0.18 μM. The error bars shown are SD's (n = 6).

concentration of daunorubicin that causes 50% growth inhibition ($IC_{50}$) in this assay is 0.10 ± 0.02 $\mu$M. The lack of cytotoxicity displayed by daunorubicin aglycone is in accord with another study[21] in which the $IC_{50}$ for daunorubicin aglycone was found to be approximately 1000 times larger than for daunorubicin.

## DISCUSSION

The reductive activation of the anthracyclines by enzymatic reductases such as xanthine oxidase, cytochrome P-450 reductase, b5-reductase and NADH dehydrogenase is thought to be partly responsible for their cardiotoxicity[6,7,22,23] through their ability to generate oxygen radicals. It is an interesting observation that the non-polar aglycone metabolite of daunorubicin that is incorporated in the cell membrane can be reduced to its semiquinone form. Our results also indicate the semiquinone can rapidly transfer its electron to oxygen. Thus, it seems likely that either superoxide or hydroperoxyl radical is formed directly in the cell membrane. The reductants (either non-enzymatic or enzymatic) that produce the daunorubicin aglycone semiquinone in our system are not known. The possibilities include reductases present in the cell[6,7,22,23] and in the plasma membrane itself.[24] If a cytosolic reductase is responsible for semiquinone formation, this would mean that even though the aglycone is incorporated in the membrane it must still be accessible enough on the inner plasma membrane surface to undergo reduction. If the reductases responsible are plasma membrane reductases,[24] then the daunorubicin aglycone would not have to be accessible to the cytoplasm. Daunorubicin has been shown previously[25] to inhibit plasma membrane NADH dehydrogenase, suggesting that it or its metabolites can interact with at least this membrane-bound enzyme.

The octanol-water partition coefficient of 180 measured for daunorubicin aglycone indicates that the daunorubicin aglycone would preferentially partition into the CHO cell membrane. The daunorubicin aglycone-derived EPR signals that were observed were invariably significantly asymmetric (Figure 3A), suggesting that the semiquinone observed was partly immobilized. This result is also consistent with daunorubicin aglycone being incorporated in the cell membrane. The loss of the EPR radical signal (Figure 3B) in the presence of the cell-impermeant line-broadening agent chromium(III) oxalate indicates that the semiquinone formed is accessible to the external chromium(III) oxalate. If the semiquinone is incorporated in the membrane, then on a time-averaged basis, at least, the chromium(III) oxalate comes in contact with the semiquinone. Because the semiquinone unpaired electron is delocalized over the whole aromatic ring system of daunorubicin aglycone, chromium(III) oxalate-induced line-broadening could occur if only a small part of the aromatic ring system was even transiently exposed to the external cell surface.

The peak-to-peak linewidth of the semiquinone signal (Figure 3A) ($\Delta H_{pp}$ of 5.1 G) is significantly larger than that found for daunorubicin free in solution ($\Delta H_{pp}$ 2.7 G) and for daunorubicin aglycone in DMSO ($\Delta H_{pp}$ of 0.8 – 1.0 G).[19] It has previously been shown by EPR for a number of semiquinones, including those of benzoquinone, vitamin K-1, ubiquinone and plastoquinone, that the $\Delta H_{pp}$ of the neutral protonated semiquinone formed under acidic conditions was invariably (factors of 1.6 to 2.9) larger than the anionic semiquinone formed under basic conditions.[26] Thus, the observation of a significantly larger $\Delta H_{pp}$ (by a factor of 1.9) relative to that of the anionic daunorubicin semiquinone, suggests that the semiquinone observed in the cell membrane is a neutral protonated semiquinone. The $pK_a$ values of a number of other semiquinones (including anthraquinone and substituted napthoquinones) have been measured and fall in the range 4.0 to 5.3.[27] Thus, in the more acidic environment of the cell membrane, the broadened semiquinone spectra that were seen is most likely due to a neutral protonated semiquinone species. Thus the

reactions for the formation of the semiquinone species ($QH^{\bullet}$), formed from daunorubicin aglycone (Q) by the action of a reductant (either enzymatic or non-enzymatic) donating an electron ($e^-$), and the subsequent formation of superoxide or the hydroperoxyl radical are shown below:

$$Q + e^- \rightarrow Q^{\bullet -}$$

$$Q^{\bullet -} + H^+ \rightleftharpoons QH^{\bullet}$$

$$QH^{\bullet} + O_2 \rightarrow Q + H^+ + O_2^{\bullet -}$$

$$QH^{\bullet} + O_2 \rightarrow Q + HO_2^{\bullet}$$

The spectrofluorometric and TLC results indicated that daunorubicin aglycone underwent extensive metabolism during the time (approximately 10–20 min) that daunorubicin aglycone was incorporated into the cells. The metabolites that were observed were all more polar than daunorubicin aglycone itself. Metabolites in which the C-13 keto side chain has been enzymatically reduced are among the major metabolites of daunorubicin[10] that are more polar than daunorubicin aglycone and may have been among the products formed in our system. These are, however, not products that might be expected to be derived from a semiquinone. The only other major metabolite formed both from a reductive activation and reduction of the C-13 keto group that is more polar than daunorubicin aglycone is daunorubicinol aglycone.[10] Thus, it cannot be concluded that the semiquinone that was observed led to the metabolites that were separated by TLC. It has been shown[28] that the NADH-cytochrome *c* reductase-induced doxorubicin semiquinone signal is correlated with 7-deoxy doxorubicin aglycone formation. This same study[28] also showed that both vitamin E and vitamin $K_3$ effectively quenched the semiquinone signal. The fact that we observed both extensive metabolism and semiquinone radical formation in the absence of any cell toxicity indicates that the cell membrane in our system was well protected from oxidative insult mediated by semiquinone formation.

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