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METABOLISM OF THE ANTHRACYCLINE ANTIBIOTIC DAUNORUBICIN TO DAUNORUBICINOL AND DEOXYDAUNORUBICINOL AGLYCONE IN HEPATOCYTES ISOLATED FROM THE RAT AND THE RABBIT*

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Abstract—In the rat and rabbit hepatocyte in suspension, daunorubicin was metabolized primarily to deoxydaunorubicinol aglycone and daunorubicinol. Little deoxydaunorubicin aglycone was observed in either species. High levels of daunorubicinol in the rabbit hepatocyte reflected the greater affinity of rabbit hepatic aldo-keto reductase for the substrate, daunorubicin. Conjugates of the anthracyclines were not observed with hepatocytes from either species. The relative formation of deoxydaunorubicinol aglycone and deoxydaunorubicin aglycone suggests that daunorubicinol is a preferred substrate for reductive deglycosidation. Consequently, the presence of daunorubicinol in the circulation of patients undergoing chemotherapy with daunorubicin may be a factor in the therapeutic efficacy of this antineoplastic agent.

Anthracycline antibiotics such as adriamycin and daunorubicin have proven to be clinically effective in the treatment of a number of malignancies [1]. While the primary site for excretion (and presumably metabolism) of the anthracycline antibiotics is the liver [2–6], little is known of the mechanisms by which the liver accumulates, metabolizes and secretes these compounds. A number of reports have appeared in the literature describing the presence of anthracycline metabolites in the blood, urine and bile of man as well as various animal models [3–9]; in addition, various hepatic preparations have been shown to metabolize the anthracyclines [10, 11]; however, only one report has addressed the metabolism (of adriamycin) in an intact hepatic cell preparation [12]. To develop a more comprehensive understanding of the contributions of the liver cell to the elimination of the anthracyclines from the organism (as well as potential effects on therapeutic efficacy), we have assessed the metabolism of daunorubicin in freshly isolated hepatocytes derived from the rat and the rabbit.

METHODS

Isolation of hepatocytes/incubation

Hepatocytes were isolated from male Sprague–Dawley rats weighing between 175 and 275 g and from male New Zealand rabbits weighing between 4 and 6 pounds by a modification of the method of Berry and Friend [13] as described previously [14]. Rats were anesthetized with ether and rabbits with sodium pentobarbital injected intravenously. Hepatocytes were suspended in Krebs–Henseleit bicarbonate buffer [15] containing 0.25% gelatin and 10 mM glucose, and the pH of the buffer was maintained by a continuous flow of warm and humidified 95% oxygen and 5% carbon dioxide. Cells were maintained in suspension by a motor-driven stirring device. The purity of daunorubicin was verified by HPLC‡ prior to the initiation of the experiment. Drug concentration was determined from absorbance at 485 nm.

Extraction and analysis of metabolites

After incubation (in a darkened room) for appropriate times with daunorubicin (in flasks that were covered with foil to prevent photolytic degradation of the anthracyclines), aliquots of the cell suspension were layered onto 200 μl of inert silicone oil in 1.5-ml microcentrifuge tubes and centrifuged at 11,000 *g* for 15–30 sec in an Eppendorf microcentrifuge. Pellets were quickly frozen in a dry ice and acetone mixture, and the supernatant fraction was removed and frozen for HPLC analysis. The anthracyclines and metabolites in the cell pellet were extracted using 4:1 (v/v) chloroform–methanol. The chloroform–methanol was evaporated under nitrogen, and the extract was redissolved in methanol for HPLC analysis. To control for endogenous cellular compounds which fluoresce at the excitation and emission wavelengths utilized in our studies, hepatocytes were incubated in the absence of drug, and both cell extracts and medium were chromatographed. To control for spontaneous drug decomposition, daunorubicin was incubated in the absence of cells and subsequently chromatographed. While

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‡ Abbreviations: HPLC, high pressure liquid chromatography; DNR, daunorubicin; and DNROL, daunorubicinol; UDPGA, uridine diphosphoglucuronic acid.

the daunorubicin was stable under our experimental conditions, the liver cell extracts contained an early eluting fluorescent compound peak which was also excreted into the incubation medium (see Results).

Experiments were performed with concentrations of daunorubicin ranging between 2 and 25 μM. While the concentrations of daunorubicin used in these studies exceed the levels of drug in the circulation of patients during the terminal phase of elimination in clinical protocols (~0.1 μM), high concentrations of daunorubicin were required to assure that cellular drug levels were sufficient for fluorescent detection (~10–20 pmoles for a discrete signal over noise). The concentrations of anthracyclines used in these studies are generally not cytotoxic to the liver cell. Babson *et al.* [16] have demonstrated that high levels of glutathione protect the liver cell from anthracycline-mediated damage.

The pattern drug of metabolism was essentially identical over the concentration range utilized, i.e. there appeared to be a direct relationship between the concentration of daunorubicin used and the formation of metabolites. Experiments presented in the Results are representative of the general pattern of drug metabolism. As a consequence of inherent variability in the accumulation and metabolism of the anthracyclines in different cellular preparations, it would be inappropriate to present mean levels of drug and metabolite fluorescence. The pattern of drug metabolism may be substantiated by determining the ratios of metabolite to parent drug at a given time point. For the rat hepatocyte incubated with 25 μM daunorubicin for 1 hr, the fluorescence ratio (uncorrected for fluorescent yield) in seven experiments was DNROL/DNR: 0.28 ± 0.05; deoxyDNROL aglycone/DNR: 0.94 ± 0.32; and deoxyDNR aglycone/DNR: 0.098 ± 0.056. These data are presented as means ± standard deviation. Levels of deoxyDNROL aglycone were approximately equivalent to that of the parent compound; the level of DNROL was approximately 25% of the DNR level and the deoxyDNR aglycone level approximately 10% of DNR. For the rabbit hepatocyte, there was greater inherent variability in the metabolic profile. Consequently, ratios of metabolite to DNR are presented for individual experiments (after incubation for 30 min with 25 μM DNR): DNROL/DNR: 49.7, 10.66 and 6.23; deoxyDNROL aglycone/DNR: 6.56, 13.22 and 10.17; deoxyDNR aglycone/DNR: 0.42, 0.10 and 0. Levels of DNROL and deoxyDNROL aglycone far exceeded those of the parent compound, while the level of deoxyDNR aglycone was insignificant.

Two independent techniques were utilized in the high pressure liquid chromatographic analysis. The first technique, which is a modification of the procedure reported by Andrews *et al.* [17], involves gradient elution of daunorubicin and its metabolites from a 10 μm C_{18} Partisil ODS column (250 × 4.6 mm, Chromanetic, Jessup, MD) using 22.5 to 75% acetonitrile (in 0.1% ammonia formate buffer, pH 4) for 10 min (at a flow rate of 2 ml/min) followed by 75% acetonitrile for 5 min. This technique, however, failed to distinguish between the aglycones and the deoxy-alcohol aglycones. We therefore utilized an isocratic procedure as reported by Bolanowska [8] eluting from a Waters μBondapak Phenyl C_{18} reverse phase column preceded by a phenyl-bondapak pre-column from Alltech Associates, Deerfield, IL. The elution buffer was 29% acetonitrile (in 0.1% ammonia formate buffer, pH 4).

Synthesis of metabolite standards

Metabolite standards for calibration of the column were synthesized as follows [9, 18].

Daunorubicinol. Aliquots (1 ml) of the 100,000 *g* supernatant fractions of rat kidney were incubated with daunorubicin (1–2 mM initial concentration) in a final volume of 5 ml in 0.2 M Tris–HCl buffer, pH 7.4, with 1.5 mM NADPH for 1 hr at 37°. At the termination of the incubation, the tubes were placed on ice and the alcohol was extracted with *n*-butanol (three consecutive extracts using 2 ml, 1 ml and 1 ml). The *n*-butanol was evaporated under nitrogen and the daunorubicinol was dissolved in 0.2 M Tris–HCl buffer, pH 8.4.

Deoxydaunorubicinol aglycone. The daunorubicinol synthesized by the previous procedure was incubated for 1 hr at 37° with microsomal preparations from rat or mouse liver in 0.2 M Tris–HCl buffer (pH 8.4), which had been evacuated of oxygen and purged with nitrogen. A 1.5 mM concentration of NADPH was included. The deoxydaunorubicinol aglycone was extracted with toluene; the toluene was evaporated under nitrogen, and the deoxydaunorubicinol aglycone was dissolved in ethanol.

Daunorubicinol aglycone. Daunorubicinol was subjected to acid hydrolysis in 0.1 M HCl at 95° for 1 hr. Daunorubicinol aglycone was extracted with toluene, as described above.

Daunorubicin aglycone. Daunorubicin was subjected to acid hydrolysis as described above for the synthesis of daunorubicinol aglycone.

Deoxydaunorubicin aglycone. This compound was synthesized from daunorubicin using the microsomal preparations (in a nitrogen atmosphere) as described above for deoxydaunorubicinol aglycone. This preparation also produced significant quantities of the deoxydaunorubicinol aglycone.

The elution times of the standard metabolites by the HPLC technique are presented below:

Daunorubicinol	6.5 min
Daunorubicinol aglycone	7.9 min
Daunorubicin	10.4 min
Deoxydaunorubicinol aglycone	12.9 min
Daunorubicin aglycone	14.2 min
Deoxydaunorubicin aglycone	24.8 min

Correction for fluorescent yield

Data are presented in terms of daunorubicin fluorescent equivalents. Previous studies have indicated that the metabolites of daunorubicin generally display a similar fluorescent yield to the parent compound [3, 19, 20]. However, while utilization of (purified) radiolabeled daunorubicin ([^{3}H]daunorubicin from New England Nuclear, Boston, MA) for verification of metabolite formation demonstrated that the peaks of radioactivity co-eluted with peaks of fluorescence, levels of deoxydaunorubicinol aglycone were overestimated approximately 2-fold

[209 ± 45% (N = 12 samples)] based on fluorescence, while levels of the deoxydaunorubicin aglycone were overestimated 2.5-fold (249 ± 45% in 5 samples). Levels of radioactivity were very low for deoxyDNR aglycone; consequently these reported values are inherently less certain than those for the deoxyDNROL aglycone. However, Schwartz and Parker [21] also determined a 2.5-fold enhancement of fluorescence (as compared to parent drug) of deoxyDNR aglycone, and a 2.8-fold enhancement of the fluorescence of a mixture of aglycone metabolites which includes deoxyDNROL aglycone. The dashed lines in Figs. 2 and 5 represent the cellular levels of deoxydaunorubicinol aglycone when corrected for the apparently enhanced fluorescent yield of these derivatives. This correction also proved necessary for calculation of total fluorescence (cells + medium) during the course of the experiment; neglecting to correct for enhanced fluorescent yield of the deoxydaunorubicinol aglycone resulted in a determination of total fluorescence (of parent compound and metabolites) which exceeded the total fluorescence (of daunorubicin) added at the initiation of the experiment.

Glucuronidation of bilirubin

Bilirubin was dissolved in a 4 mM solution of Na_2CO_3 in 0.17 M NaCl. Rat hepatocytes in suspension were incubated with 25 μM bilirubin under the same experimental conditions as described for the anthracyclines, and bilirubin and its metabolites were extracted from the cells with chloroform–methanol, as described for the anthracyclines. Bilirubin and its glucuronides were resolved by elution from a reverse phase column (Spherisorb ODS-2, 5 μm, 250 × 4.6 mm, Thompson Instrument Co., Springfield, VA) using a linear gradient of 55–100% methanol (at 2 ml/min) in 0.1 M sodium acetate buffer (pH 4) containing 5 mM heptane-sulfonic acid [22]. Absorbance of bilirubin in the effluent was monitored at 436 nm using a Knauer variable wavelength u.v. detector. Quantitation of bilirubin glucuronides was performed using a Spectra-Physics integrator.

Materials

Bilirubin, daunorubicin, collagenase (Type V) and heptane-sulfonic acid were obtained from the Sigma Chemical Co., St. Louis, MO. Silicone oil was formulated using 16% of Dow Corning 200 fluid and 84% of Dow Corning 550 Fluid (William F. Nye, Inc., Bedford, MA). [^{3}H]DNR (sp. act. 4585 dpm/pmole) was obtained from New England Nuclear and purified by high pressure liquid chromatography.

All chromatographic solvents were HPLC grade (obtained from Bodman Chemicals, Media, PA) and were filtered through 0.45 μm filters before use.

RESULTS

Daunorubicin metabolism in the rat hepatocyte

Figure 1 shows the decline in daunorubicin fluorescence in the incubation medium as a result of the uptake and metabolism of the daunorubicin by the rat hepatocyte. After 1 hr of incubation, less than 10% of the parent drug remained in the incubation medium. The depletion in extracellular daunorubicin was accompanied by the appearance of daunorubicinol in the medium.

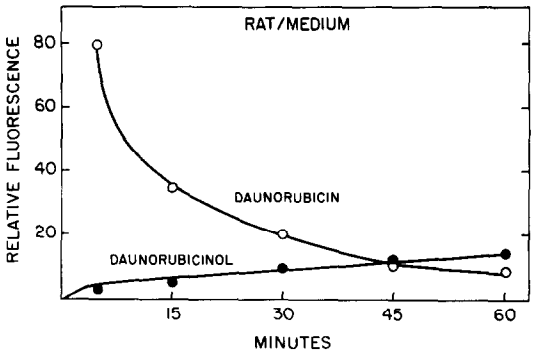

Fig. 1. Depletion of daunorubicin from the incubation medium by rat hepatocytes. Rat hepatocytes in suspension at a cytocrit of approximately 2.5% were incubated with 25 μM daunorubicin. The incubation medium was analyzed by high pressure liquid chromatography. Initial levels of daunorubicin in the medium were equivalent to 200 fluorescence units.

Figure 2 presents the concomitant profile of daunorubicin and its metabolites within the hepatic cell during the course of a 1-hr incubation. There was significant accumulation of the unaltered parent compound within the cell and conversion of daunorubicin to daunorubicinol. The primary hepatic cell metabolite was deoxydaunorubicinol aglycone. When assessing relative fluorescence, the levels of deoxydaunorubicinol aglycone generally equalled or exceeded the cellular levels of daunorubicin. However, after correction for the apparent enhanced fluorescent yield of the deoxydaunorubicinol aglycone, the levels of this metabolite appeared not to exceed the levels of daunorubicin in the cell. Figure 2 presents the level of deoxydaunorubicinol aglycone in terms of relative fluorescence (solid line) as well as after correction for enhanced fluorescent yield (dashed line). The level of cellular deoxydauno-

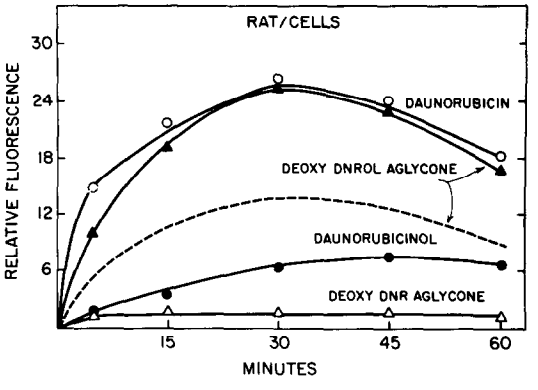

Fig. 2. Metabolism of daunorubicin in the rat hepatocyte. Incubation conditions correspond to those of Fig. 1. Cellular daunorubicin and metabolites were extracted with chloroform/methanol. The cell extract was evaporated to dryness and redissolved in methanol for injection onto the HPLC column for determination of metabolites. The dashed line represents the level of deoxydaunorubicinol aglycone when corrected for its enhanced fluorescent yield.

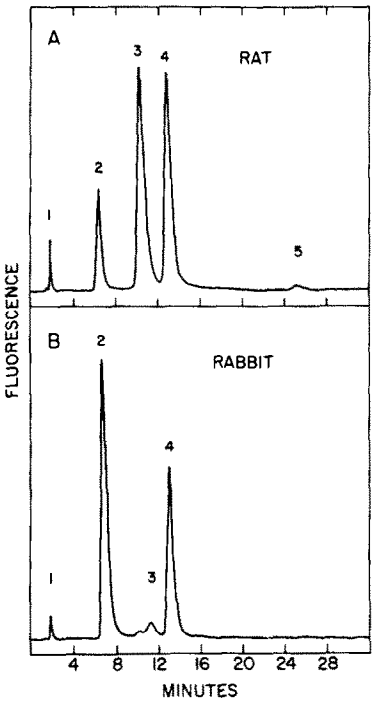

Fig. 3. Fluorescence monitoring of HPLC elution of cell extracts. Fluorescence eluting from the HPLC column was monitored with excitation of 485 nm and emission at 580 nm. Peak areas were determined by integration using a Shimadzu Chromatopac integrator. Panel A represents an extract from rat hepatocytes incubated with daunorubicin; panel B represents an extract from rabbit hepatocytes incubated with daunorubicin. Peak 1, fluorescent artifact; peak 2, daunorubicinol; peak 3, daunorubicin; peak 4, deoxydaunorubicinol aglycone; and peak 5, deoxydaunorubicin aglycone.

rubicinol aglycone declined with time, an observation that was reproduced in other experiments using both rat and rabbit hepatocytes. Low levels of deoxydaunorubicin aglycone were also observed in the rat hepatocyte. The aglycone derivatives, which are less water soluble than the parent drug, did not appear in the incubation medium.

It has been reported that conjugates of the anthracyclines are observed in the bile, urine and plasma of rats, rabbits, and humans [3, 6, 7]. Since the rat hepatocyte in suspension maintains the capacity for drug conjugation [23], it was expected that daunorubicin conjugates (e.g. glucuronides) would be synthesized. However, no daunorubicin conjugates were observed in these studies. An early eluting peak, which was previously thought to represent a polar drug conjugate [24], proved to be an endogenous fluorescent compound in the hepatocytes which was also lost into the incubation medium. Figure 3 presents a representative high pressure liquid chromatographic analysis of cell extracts from rat and rabbit hepatocytes after incubation with daunorubicin for 45 min, where the parent compound, metabolites, and the early eluting (non-daunorubicin-derived) fluorescent peaks are apparent. A small peak of fluorescence which eluted before the daunorubicin in the rabbit hepatocyte has not been identified.

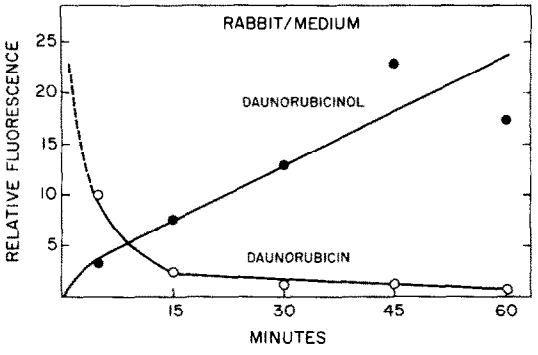

Fig. 4. Depletion of daunorubicin from the incubation medium by rabbit hepatocytes. Rabbit hepatocytes in suspension at a cytocrit of approximately 2.5% were incubated with 25 μM daunorubicin. The incubation medium was sampled at various times and analyzed by high pressure liquid chromatography. The dashed line indicates that initial levels of daunorubicin in the medium were significantly higher than indicated (i.e. 50 fluorescence units).

Rabbit hepatocytes

Figure 4 presents the time-dependent depletion of daunorubicin from the incubation medium by rabbit hepatocytes, and the concomitant appearance of daunorubicinol. Again, as with rat hepatocytes, within the course of 1 hr, approximately 90% of extracellular drug was depleted, and significant levels of daunorubicinol were observed in the incubation medium. Other daunorubicin metabolites formed in the cell did not appear in the medium.

Figure 5 shows the profile of cellular daunorubicin and metabolites during the course of a 1-hr incubation with rabbit hepatocytes. Little unmetabolized daunorubicin was observed within the cell, which tends to reflect more rapid metabolism of daunorubicin in the rabbit than rat hepatocyte. The primary metabolic products were daunorubicinol and deoxydaunorubicinol aglycone. Levels of deoxydaunorubicinol aglycone are presented in terms of relative fluorescence (solid lines) as well as after correction for the fluorescent yield of this compound (dashed line). Clearly, the aldo-keto reductase in the rabbit hepatocyte is more effective at utilizing

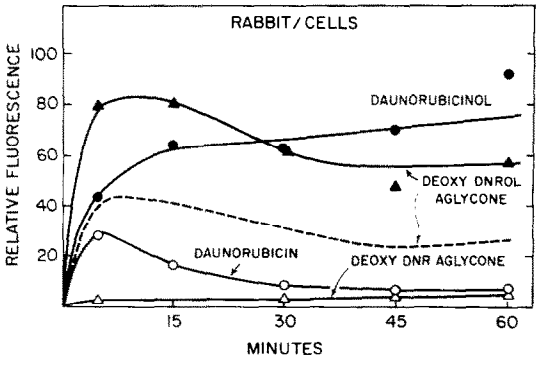

Fig. 5. Metabolism of daunorubicin in the rabbit hepatocyte. Incubation conditions correspond to those of Fig. 4. Experimental protocols are described in Methods and in the legend to Fig. 2.

daunorubicin as a substrate for conversion to daunorubicinol than is that in the rat. As in the studies with the rat hepatocyte, the levels of deoxydaunorubicinol aglycone declined with time. Low levels of deoxydaunorubicin aglycone were also observed in these hepatocytes. Daunorubicin conjugates were not evident in these studies.

Formation of bilirubin glucuronides

To establish that the experimental conditions used in these studies permit the formation of drug conjugates in the hepatocyte in suspension, the conjugation of bilirubin was assessed in rat hepatocytes. Using 25 μM bilirubin, the rat hepatocyte demonstrated the capacity for time-dependent formation of monoglucuronides and diglucuronides of bilirubin (data not shown). In contrast to the report by Sing and Schwarz [25], exogenous uridine diphosphoglucuronic acid was not required by the hepatocytes to form glucuronides of bilirubin which were secreted into the medium. Furthermore, the presence of 25 μM daunorubicin in the incubation medium did not compromise the synthesis of bilirubin glucuronides.

DISCUSSION

The present studies demonstrate that hepatocytes in suspension metabolized daunorubicin via two primary enzymatic pathways which have been reported previously in other hepatic preparations [10, 11]. The activity of aldo-keto reductase resulted in the formation of daunorubicinol, while hepatic cytochrome P-450 reductase (mixed-function oxidase), xanthine oxidase and cytochrome *c* reductase are thought to play a role in the formation of deoxydaunorubicin and derivatives [26]. These metabolic products are formed in both rat and rabbit hepatocytes; however, because of reduced activity of the aldo-keto reductase in the rat [27], significant levels of unmetabolized daunorubicin accumulate in the rat hepatocyte, while daunorubicin in the rabbit hepatocyte is observed at low and continuously declining levels. Overall, however, the rat and rabbit hepatocytes appear to be comparable models for study of hepatic metabolism of daunorubicin.

It is important to attempt to place these studies of hepatic metabolism of daunorubicin in perspective in terms of other reports of daunorubicin metabolism in the intact animal. While daunorubicin is generally agreed to be the primary circulating metabolite of daunorubicin, daunorubicin aglycones and daunorubicin conjugates have been reported in the blood, urine and bile of patients and in various animal models [3, 5–7]. Hepatocytes in suspension form deoxyaglycone derivatives; however, these compounds do not readily enter the incubation medium from the hepatic cell because of their low aqueous solubility, and would presumably require either conjugation or association with circulating proteins to allow for their appearance in the blood, urine and/or bile. Hepatocytes in suspension maintain the capacity to form drug conjugates [23], and would be expected to form conjugates of the deoxyaglycones; however, conjugated derivatives were not formed in the rat or rabbit hepatocytes, nor was there any evidence of such derivative in the incubation medium. The decline in the level of deoxydaunorubicinol aglycone with time, which would be expected to reflect its cellular conjugation and subsequent excretion, is not readily explained.

Previously it was thought that exposure of the rat to ether would compromise the ability of the rat hepatocytes to form drug conjugates [28]; however, a recent report has demonstrated that the liver cells recover from the short exposure to ether during anesthesia and that conjugation capacity is restored in the isolated cell in suspension [29]. This was confirmed in our laboratory in studies demonstrating the conjugation of bilirubin to glucuronide derivatives which are extruded into the medium, even in the absence of exogenous UDPGA.

Since the rat liver cells retain the capacity for drug conjugation [29], it appeared possible that the anthracyclines might inhibit the glucuronidation reaction in the hepatocytes, thereby preventing their own conjugation. However, daunorubicin failed to interfere with bilirubin conjugation, and may, in fact, enhance this biosynthetic reaction. Consequently, it appears likely that the anthracyclines do not serve as effective substrates for conjugation in the hepatic cell and that the anthracycline conjugates reported by others are synthesized at tissue sites other than the liver in the intact animal. In this regard, it should be noted that studies by Hartman *et al.* in the isolated rat hepatocyte [12], by Bolanowska *et al.* in mice and humans [8, 9] and by Bachur and colleagues in microsomal preparations [30] have failed to report the biosynthesis of anthracycline conjugates.

The formation of deoxydaunorubicinol aglycone derivatives in the liver cell is consistent with the enzymatic activities (aldo-keto reductase and cytochrome P-450 reductase) known to be present in the hepatic cell. The existence of this metabolic step indicates low oxygen tension in the hepatic cell despite continuous gassing of the incubation medium with 95% O_2/5% CO_2 [31]. This observation indicates that the hepatic cell depletes its local environment of O_2 more rapidly than the O_2 can be replaced. In this context, Hartman *et al.* [12] have reported that hepatocytes in suspension display a similar metabolic profile for adriamycin under aerobic and anaerobic conditions. Further, it is important to reiterate that an obligatory intermediary step in the formation of the deoxyaglycone derivative is a free radical semiquinone intermediate of the anthracyclines [32–34]. Therefore, the formation of the deoxyaglycone derivative indirectly supports the studies by Babson *et al.* [16] indicating the existence of free radicals in liver cells treated with anthracycline antibiotics. Free radical intermediates have been implicated in the general cytotoxicity as well as the cardiotoxicity of the anthracycline antibiotics [33–36].

The present studies do not readily distinguish between daunorubicinol and deoxydaunorubicin aglycone as precursors for the formation of the deoxydaunorubicinol aglycone. The rabbit hepatocyte forms high levels of daunorubicinol (and low levels of deoxydaunorubicin aglycone), and it is logical to infer that daunorubicinol is converted to its deoxyglycone derivative. However, in the rat hepa-

tocyte, where relatively low levels of daunorubicinol are formed, the deoxydaunorubicinol aglycone derivative represents the primary cellular metabolite. The level of deoxydaunorubicin aglycone is extremely low, despite the presence of high levels of daunorubicin in the cell. It appears that conversion of daunorubicinicol to its deoxyglycone derivative is more rapid than for daunorubicin. Alternatively, deoxydaunorubicin aglycone may be formed at a sufficient rate to provide a substrate for the aldo-keto reductase mediated conversion to deoxydaunorubicinol aglycone as previously suggested by Schwartz and Parker [21].

Daunorubicinol, the primary circulating metabolite of daunorubicin, may be superior to daunorubicin as a substrate for the hepatic mixed-function oxidase activity (since little deoxydaunorubicin aglycone is formed in these cells). This hypothesis (which awaits further verification using purified hepatic cytochrome P-450 reductase) as well as previous findings indicating differential accumulation of daunorubicin and daunorubicinol in the P388 leukemic cells [37–39] suggest that it would be worthwhile to further investigate the role of daunorubicinol in the cytotoxicity of the anthracycline. For instance, P388 leukemic cells have been reported to display mixed-function oxidase activity [40]; it therefore appears reasonable to expect that daunorubicinol would be metabolized to deoxydaunorubicinol aglycone in this cellular model or in other cell lines with enzymatic capacities for similar electron transfer reactions. In this context, two recent abstracts by Gessner *et al.* [41, 42] have reported metabolism of anthracyclines to deoxyaglycones in tumor cell models.

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