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METABOLISM OF ADRIAMYCIN IN HEPATOCYTES ISOLATED FROM THE RAT AND THE RABBIT*

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Abstract—Metabolism of adriamycin, an anthracycline antibiotic, was characterized in both rat and rabbit hepatocytes under aerobic conditions. Adriamycin was the predominant fluorescent species within the cell in hepatocytes from both the rat and the rabbit. In the rat hepatocyte, the primary intracellular metabolite was deoxyadriamycin aglycone, while significant levels of deoxyadriamycinol aglycone were also synthesized; little adriamycinol was observed in the cells or the incubation medium. In contrast, in the rabbit hepatocyte, significant levels of adriamycinol as well as deoxyadriamycinol aglycone were formed; deoxyadriamycinol aglycone was the primary intracellular metabolite while low levels of deoxyadriamycin aglycone were observed. The relative formation of deoxyadriamycinol aglycone and deoxyadriamycin aglycone suggests that adriamycinol may be metabolized more effectively to the deoxyaglycone derivative than the parent drug. Conjugates of adriamycin were not observed in hepatocytes from either the rat or the rabbit or in the incubation medium.

The anthracycline antibiotics adriamycin and daunorubicin have proven to be effective in the clinical treatment of various malignancies [1]. These compounds differ in the C-9 position of the anthracycline nucleus where the hydroxyacetyl group on adriamycin is replaced by an acetyl group on daunorubicin. This structural difference results in enhanced lipophilicity of daunorubicin, more rapid metabolism and elimination of daunorubicin from the organism [2] as well as differing clinical utilities for daunorubicin and adriamycin [3].

The pharmacokinetics of adriamycin have been characterized in humans [4, 5] and in various animal models [2, 6]. While the primary circulating metabolite of adriamycin is the alcohol, adriamycinol, a number of other metabolites (including aglycones and conjugated derivatives) have been reported in the blood, bile and urine of both animals and humans [6–10]. In addition, various hepatic preparations have been shown to metabolize adriamycin to adriamycinol and deoxyaglycone derivatives [11, 12].

A previous paper describes the metabolism of daunorubicin in rat and rabbit hepatocytes [13]. The present studies were also designed to utilize the hepatocyte in suspension to characterize and compare the metabolic pathways for adriamycin in rat and rabbit liver cells, since the liver is the primary site for excretion of the anthracyclines and may be a primary site for their metabolism as well [14]. Furthermore, in view of the structural difference between these anthracyclines, it was of interest to determine whether metabolism of adriamycin would differ from that of daunorubicin in the hepatic cells.

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METHODS

Materials

All compounds used were of analytical grade. Adriamycin and collagenase were obtained from the Sigma Chemical Co., St. Louis, MO. [^{14}C]-Adriamycin was obtained from The Pharmaceutical Resources Branch of the Developmental Therapeutics Program (Division of Cancer Treatment) at the National Institutes of Health and purified prior to use by high pressure liquid chromatography. Chromatographic solvents were HPLC grade (Bodman Chemical Co., Media, PA) and were filtered through 0.45 μm filters before use.

Isolation of hepatocytes and incubation conditions

Techniques for isolation of hepatocytes, incubation conditions, and extraction of the anthracyclines and metabolites have been presented previously [13]. The density of the cell suspension was approximately 2×10^6 cells/ml. Purity of the adriamycin was verified by high pressure liquid chromatography prior to the initiation of the experiment. [^{14}C]Inulin was used as a marker of the extracellular water. Intracellular water space was determined from the difference between total cell water (cell pellet wet weight − dry weight) and the [^{14}C]inulin space, as reported previously [15].

Experiments were performed with concentrations of adriamycin ranging between 25 and 100 μM. The pattern of drug metabolism was essentially the same over this concentration range, and *identical* metabolites were synthesized in *similar* ratios with each individual cell preparation. Each experiment presented in Results is representative of a series of three

to four experiments using five different concentrations of adriamycin.

Analytical techniques

Two independent techniques were utilized in the high pressure liquid chromatographic (HPLC) analysis. The first technique, which is a modification of the procedure reported by Andrews *et al.* [16], involves gradient elution of adriamycin and its metabolites from a 10 μm C-18 (250 × 4.5 mm) Partisil ODS column (Chromanetics Corp., Jessup, MD) using 22.5 to 50% acetonitrile (in 0.1% ammonium formate buffer, pH 4) for 10 min followed by 50% acetonitrile for 5 min. Elution of drug and metabolites was monitored by their fluorescence (excitation at 485 nm, emission at 580 nm) using a fixed wavelength Gilson model 121 fluorometer. Peak areas were determined using a Shimadzu CR3A Chromatopac integrator. Sensitivity of fluorometric detection for adriamycin and adriamycinol was approximately 5 pmol; and for deoxyadriamycin aglycone and deoxyadriamycinol aglycone, 2 pmol. This analytical technique failed to distinguish between the *aglycones* and the deoxy-alcohol aglycones. Consequently, HPLC analysis was performed, utilizing an isocratic procedure as reported by Bolanowska *et al.* [17], eluting from a Waters μ-phenyl-Bondapak C-18 reverse phase column preceded by a phenyl-Bondapak pre-column from Alltech Associates, Deerfield, IL. The elution buffer was 25% acetonitrile (in 0.1% ammonium formate buffer, pH 4). This approach failed to discriminate between adriamycin aglycone and deoxyadriamycinol aglycone, as previously reported by others [16, 17].

To identify the cellular adriamycin metabolite eluting coincident with standards of adriamycin aglycone and deoxyadriamycinol aglycone, the column elutant was collected, lyophilized, redissolved in methanol, and re-analyzed by isocratic elution from a 10 μm Partisil C-18 ODS-3 column (Chromanetics Corp.) using 40% acetonitrile in 0.01 M phosphoric acid (pH 3) [18]. While this analytical procedure did not allow for a precise determination of the relative amounts of adriamycin aglycone and deoxyadriamycinol aglycone when both compounds were simultaneously present in a given sample, it did allow for discrimination between these two compounds, i.e. adriamycin aglycone eluted at 7.48 min, whereas deoxyadriamycinol-aglycone eluted at 7.19 min (in a selected series of analyses). Since no detectable adriamycin aglycone was present in the experimental samples, the adriamycin metabolite in question was identified as deoxyadriamycinol aglycone.

Synthesis of metabolite standards

Metabolite standards for calibration of the column were synthesized as follows [10–12]:

(1) *Adriamycinol.* Aliquots (1 ml) of the 100,000 *g* supernatant fractions of rabbit liver were incubated with adriamycin (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 adriamycinol was dissolved in 0.2 M Tris–HCl buffer, pH 8.4.

(2) *Deoxyadriamycinol aglycone.* The adriamycinol synthesized by the preceding procedure was incubated for 1 hr at 37° with microsomal preparations from mouse liver in 0.2 M Tris–HCl buffer (pH 8.4) that had been evacuated of oxygen and purged with nitrogen. A 1.5 mM concentration of NADPH was included. The deoxyadriamycinol aglycone was extracted with toluene, the toluene was evaporated under nitrogen, and the deoxyadriamycinol aglycone was dissolved in ethanol.

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

(4) *Adriamycin aglycone.* Adriamycin was subjected to acid hydrolysis, as described above for the synthesis of adriamycinol aglycone, and extracted with toluene.

(5) *Deoxyadriamycin aglycone.* This compound was synthesized from adriamycin using the mouse liver microsomal preparations (nitrogen atmosphere) as described above for deoxy adriamycinol aglycone. This preparation also produced significant quantities of the deoxyadriamycinol aglycone, as a consequence of residual aldo-keto reductase activity from adherent cytoplasmic fluid.

The elution times of the standard metabolites from a μ-phenyl-Bondapak C18 reverse phase column using 25% acetonitrile in 0.1% ammonium formate buffer, pH 4, at a flow rate of 2 ml/min, are presented below:

Adriamycinol	5.8 min
Adriamycinol aglycone	7.2 min
Adriamycin	9.3 min
Deoxyadriamycinol aglycone	11.6 min
Adriamycin aglycone	11.6 min
Deoxyadriamycin aglycone	20.5 min

Correction for fluorescent yield

Data are presented in terms of adriamycin fluorescent equivalents and, where appropriate, after correction for enhanced fluorescent yield of the deoxyaglycone derivatives. Relative fluorescence was determined by simultaneous assessment of metabolite and parent drug fluorescence and radioactivity after hepatocytes had been incubated with [^{14}C]adriamycin, and the drug and metabolites had been extracted and resolved chromatographically. The ratio of fluorescence (in relative fluorescent units) to radioactivity (in cpm) of the parent compound was used as a baseline for determination of the relative fluorescent yield of metabolites. By determining the ratio of fluorescence to radioactivity of metabolites and comparing these values to the ratio determined for adriamycin, it was possible to assess the enhanced fluorescent yield of the deoxyaglycone derivatives.

RESULTS

Relative fluorescent yield of adriamycin metabolites

The relative fluorescent yields of the metabolites

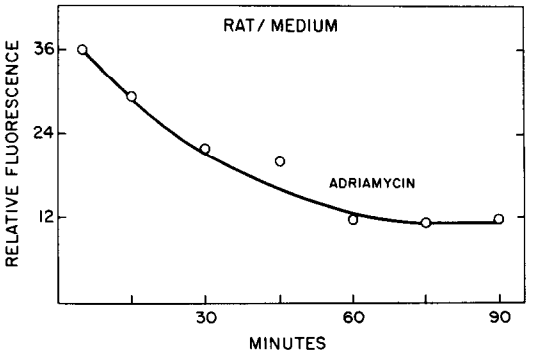

Fig. 1. Decline in adriamycin levels in incubation medium: Rat hepatocytes. Rat hepatocytes in suspension at a density of approximately 2×10^6 cells/ml were incubated with 25 μM adriamycin. The incubation medium was analyzed by high pressure liquid chromatography.

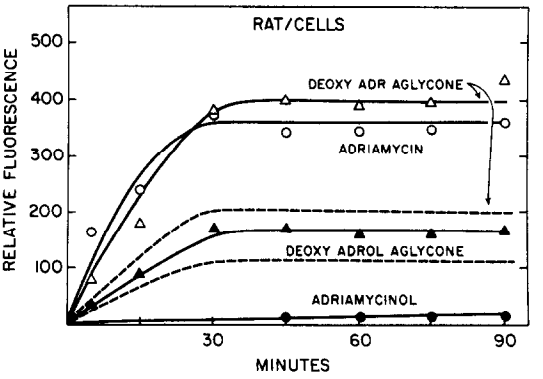

Fig. 2. Metabolism of adriamycin in the rat hepatocyte. Incubation conditions correspond to those given in the legend of Fig. 1. Cellular adriamycin and metabolites were extracted with chloroform/methanol. The cell extract was evaporated to dryness and redissolved in methanol for injection onto the HPLC column for determination of metabolites. Levels of deoxyadriamycin aglycone and deoxyadriamycinol aglycone are presented in terms of relative fluorescence (solid lines) as well as after correction for enhanced control fluorescent yield (broken lines). Abbreviations: ADR, adriamycin; and ADROL, adriamycinol.

of adriamycin formed in the hepatic cell were determined by comparing the levels of adriamycin and its metabolites in terms of both fluorescence and radioactivity. The peaks of radioactivity, representing adriamycin and metabolites eluting from the HPLC column, coincided with peaks of fluorescence. Although the alcohol, adriamycinol, appears to have the same fluorescent yield as the parent compound, reliance on fluorescence alone resulted in an overestimation of the levels of deoxyaglycone metabolites. The level of deoxyadriamycinol aglycone is overestimated by 47.6 ± 11.5% (based on seventeen determinations) and the level of deoxyadriamycin aglycone is overestimated by 101 ± 17% (based on ten determinations). Using the terminology of Cummings and Stuart [19], adriamycinol has a relative molar fluorescence (RMF) of 1; deoxyadriamycinol aglycone, a RMF of 1.5; and deoxyadriamycin aglycone, a RMF of 2. Ratios of metabolite to parent compound presented in this manuscript have been corrected for fluorescent yield of metabolite.

Adriamycin metabolism in the rat hepatocyte

Figure 1 presents the decline in adriamycin fluorescence in the incubation medium that resulted from uptake (and metabolism) of the anthracycline in the rat hepatocyte (in a representative experiment). This decline in medium fluorescence was slower than observed previously using daunorubicin [13], since about 40% of the original adriamycin remained in the medium after 1 hr; only minute amounts of the adriamycin metabolite, adriamycinol, were observed in the incubation medium. The deoxyaglycone metabolites produced within the cell (see below) did not appear in the incubation medium.

Figure 2 presents the concomitant profiles of adriamycin and its metabolites with the rat hepatocyte. Although the absolute *amount* of drug accumulation varied with different cell preparations, the *pattern* of adriamycin metabolism was essentially identical in all preparations.

The level of adriamycin within the cell was maintained relatively constant throughout the course of the experiment. With an initial drug concentration of 25 μM, the intracellular steady-state adriamycin concentration was approximately 1 mM based on the presumption of homogenous distribution of drug in the cell water. While the absolute concentrations of metabolites varied between experiments as a consequence of differences in the level of parent drug accumulated, the ratios of metabolite to parent compound were consistent in the different cellular preparations.* The primary intracellular metabolite was deoxyadriamycin aglycone. Measurable levels of deoxyadriamycinol aglycone were routinely observed as well. The level of adriamycinol formed was low and generally limited to the experiments in which the highest concentration of adriamycin (approximately 100 μM) was utilized.

The maintenance of steady-state levels of adriamycin and metabolites, while extracellular adriamycin continued to decline, is difficult to explain. It is possible that "non-fluorescent" drug metabolites are formed, as recently reported for the anthracycline antibiotic marcellomycin [20].

In various *in vivo* studies, conjugated derivatives of adriamycin have been reported in urine, blood and bile [2, 6, 8, 16]. Since the liver is a primary site for drug conjugation [21], it was expected that conjugates of adriamycin would be synthesized by these hepatocytes. However, no conjugates of adriamycin were observed within the hepatocytes or the incubation medium in the present studies. An early eluting peak, which was thought previously to represent a drug conjugate [22], proved to be an endogenous fluorescent compound which was lost from the cells into the incubation medium.

* In four experiments, the ratio of metabolites to parent compound (after correction for enhanced fluorescent yield) was (±SE): deoxyadriamycin aglycone/adriamycin, 0.65 ± 0.15; deoxyadriamycinol aglycone/adriamycin, 0.27 ± 0.05; and adriamycinol/adriamycin, 0.037 ± 0.039.

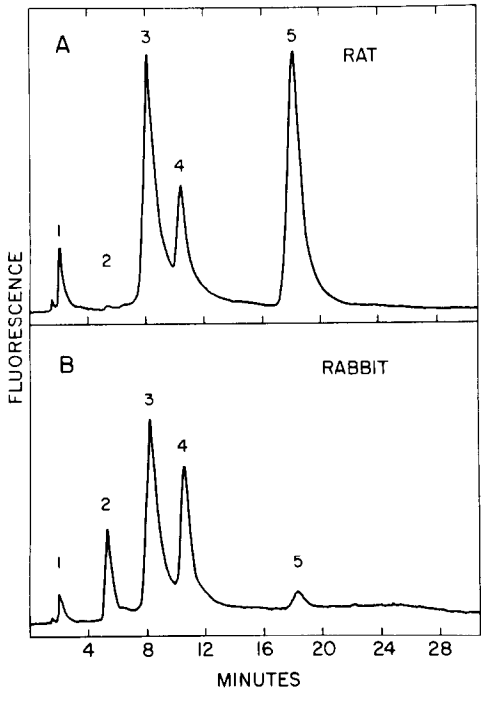

Fig. 3. Fluorescence monitoring of HPLC elution of cell extracts. Chromatographs were generated by isocratic elution from a μ-phenyl-Bondapak column using 25% acetonitrile in 0.1% ammonium formate buffer, pH 4, as described in Methods. Fluorescence eluting from the HPLC column was monitored with excitation at 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 25 μM adriamycin; panel B represents an extract from rabbit hepatocytes incubated with 25 μM adriamycin. Peak 1, fluorescent artifact; peak 2, adriamycinol; peak 3, adriamycin; peak 4, deoxyadriamycinol aglycone; and peak 5, deoxyadriamycin aglycone.

Figure 3 presents a representative high pressure liquid chromatographic analysis of cell extracts from the rat and the rabbit after incubation with 25 μM adriamycin. Metabolites were eluted isocratically from a μ-phenyl-Bondapak C-18 reverse phase column with 25% acetonitrile in 0.1% ammonium formate buffer (pH 4) at a flow rate of 2 ml/min. The early eluting (non-adriamycin-derived) fluorescent peak was utilized as an internal standard to verify the reproducibility of the extraction procedure between samples.

Adriamycin metabolism in the rabbit hepatocyte

Figure 4 shows the decline in adriamycin levels and the concomitant appearance of low levels of adriamycinol in the medium in a representative experiment where rabbit hepatocytes were incubated with 25 μM adriamycin. The apparent rate of drug depletion from the medium was slow compared to

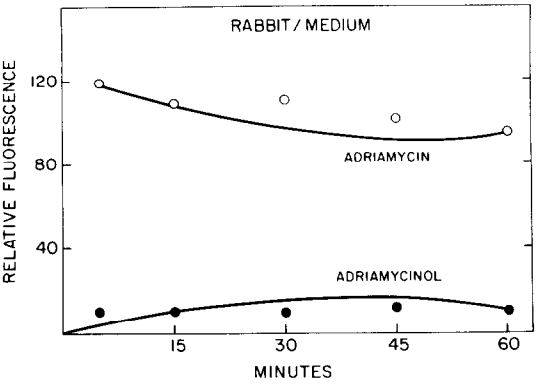

Fig. 4. Decline in adriamycin levels in incubation medium with rabbit hepatocytes. Rabbit hepatocytes in suspension at a density of 2×10^6 cells/ml were incubated with 25 μM adriamycin. The incubation medium was sampled at various times and analyzed by high pressure liquid chromatography.

that observed with rat hepatocytes; the bulk of the adriamycin appeared to remain in the incubation medium. The deoxyadriamycin aglycone metabolites that formed in the hepatocyte and were less polar than adriamycin or adriamycinol did not exit from the cell into the medium.

Figure 5 presents the concomitant time-dependent profiles of the appearance of adriamycin and its metabolites in the rabbit hepatocyte. The parent compound, adriamycin, was the predominant fluorescent species within the cell and achieved intracellular concentrations on the order of 250 μM. The primary intracellular metabolite was deoxyadriamycinol aglycone. The levels of deoxyadriamycin aglycone in the rabbit hepatocyte were much lower than those in the rat hepatocyte. Significant levels of the alcohol, adriamycinol, were formed in the rabbit hepatocyte as well.* In some studies, low levels of adriamycinol aglycone were observed. As with the rat hepatocytes, conjugates of adriamycin were not formed in the rabbit hepatocyte, or observed in the incubation medium.

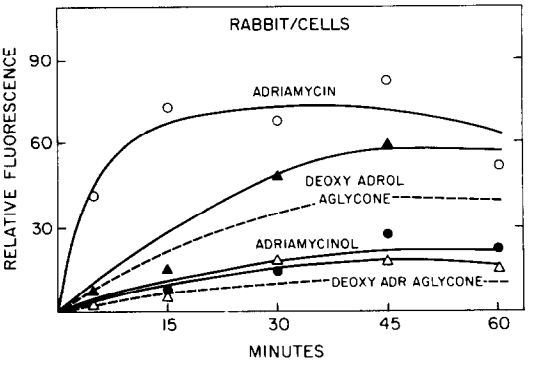

Fig. 5. Metabolism of adriamycin in the rabbit hepatocyte. Incubation conditions correspond to those given in the legend of Fig. 4. Experimental protocols are described in Methods and in the legend of Fig. 2. Levels of deoxyadriamycin aglycone and deoxyadriamycinol aglycone are presented in terms of relative fluorescence (solid lines) as well as after correction for fluorescent yield (broken lines).

* In three experiments, the ratio of metabolites to parent compound (after correction for enhanced fluorescent yield) was (±SE): deoxyadriamycin aglycone/adriamycin, 0.06 ± 0.04; deoxyadriamycinol aglycone/adriamycin, 0.72 ± 0.35; and adriamycinol/adriamycin, 0.26 ± 0.05.

DISCUSSION

Comparative metabolism of adriamycin and daunorubicin in rat and rabbit hepatocytes

The metabolism of adriamycin displayed a markedly different pattern in the rat and the rabbit hepatocyte. Whereas the rabbit hepatocyte effectively synthesized the alcohol, adriamycinol, little adriamycinol was formed in the rat hepatocyte; this observation is consistent with previous reports of low aldoketo reductase affinity for adriamycin in the rat liver [11] and low levels of adriamycinol circulating in the intact animal [6, 23]. Conversely, metabolism of adriamycin to deoxyadriamycin aglycone in rat hepatocytes was more extensive than in rabbit hepatocytes.

Metabolism of adriamycin is substantively different from that of daunorubicin in the *rat* hepatocyte [13]. Whereas relatively little adriamycinol is formed in the rat hepatocyte, daunorubicin is clearly a preferred substrate for conversion to its corresponding alcohol, daunorubicinol. Furthermore, little deoxydaunorubicin aglycone is formed from daunorubicin, whereas adriamycin is readily transformed (by reductive deglycosidation) to deoxyadriamycin aglycone. In the case of both adriamycin and daunorubicin, the level of the deoxyaglycone metabolite of the alcohol exceeded that of the alcohol, its presumed precursor, whereas the level of the deoxyaglycone metabolites of adriamycin or daunorubicin was low compared to that of the parent compound. This observation may indicate that the alcohol is superior to the parent drug as a substrate for metabicinol aglycone) may be synthesized from deoxyadriamycin aglycone (and deoxydaunorubicin aglydeoxyadriamycinol aglycone (and deoxydaunorubicinol aglycone) may be synthesized from deoxyadriamycin aglycone (and deoxydaunorubicin aglycone, respectively) as well as from adriamycinol (and daunorubicinol). This concept is consistent with studies by Hartman *et al.* [24] and Bachur *et al.* [25], indicating the formation of deoxyadriamycinol aglycone even in the presence of low levels of the alcohol, adriamycinol (or in the absence of adriamycinol).

The distribution of adriamycin metabolites in the *rabbit* cell was generally similar to that for daunorubicin [13]; that is, daunorubicin is transformed to daunorubicinol and deoxydaunorubicinol aglycone, while levels of deoxydaunorubicin aglycone are low.

The metabolism of adriamycin in the isolated rat hepatocyte has been described previously by Hartman *et al.* [24]. Studies by Hartman *et al.* were performed under anaerobic conditions and demonstrated that rat hepatocytes contain adriamycin, deoxyadriamycin aglycone and deoxyadriamycinol aglycone. Our studies, performed under aerobic conditions, are basically in agreement with the reports of this group, although the transformation of adriamycin to its metabolites was more extensive in our studies—even at the lowest concentration of adriamycin used (which corresponds to the 20 μM concentration reported by Hartman *et al.*). Hartman and coworkers report that metabolism of adriamycin is similar under anaerobic and aerobic conditions. These observations suggest rapid utilization and depletion of local oxygen by the hepatocyte in suspension.

Our observations in the isolated hepatocyte of the metabolism of adriamycin (and daunorubicin) are basically in agreement with previous studies by Asbell *et al.* [26] and Schwartz and Parker [27] using liver microsomes or homogenates. The determination of fluorescent yield for deoxyadriamycin aglycone is in agreement with the report by Cummings and Stuart [19]. However, we calculate a "relative molar fluorescence" of 1 for adriamycinol in agreement with previous reports [2, 28], whereas Cummings and Stuart indicate a value of 1.5. For deoxyadriamycinol aglycone, we calculate a relative molar fluorescence of 1.5, whereas Cummings and Stuart reported a value of 2.

Lack of drug conjugate formation

The metabolism of the anthracyclines to deoxyaglycone derivatives, which are relatively water insoluble, suggests the necessity of a subsequent conjugative step, to allow for elimination of these compounds from the liver. However, in this report, in the study by Hartman *et al.* [24], and in our previous report on the metabolism of daunorubicin in the hepatocyte [13], conjugated derivatives of the anthracycline antibiotics were not observed within the hepatocyte or in the incubation medium. (The possibility that the presence of serum proteins in the incubation medium would facilitate efflux of the deoxyaglycone derivatives by allowing for formation of drug–protein complexes was investigated; however, the inclusion of serum protein in the incubate did not result in extrusion of the deoxyaglycone derivatives from the cell.) The lack of formation of drug conjugates raised the possibility that the hepatocyte is an inappropriate model for analysis of hepatic metabolism of the anthracyclines.

Consequently, despite evidence that the hepatocyte in suspension maintains the hepatic capacity to conjugate substrates such as harmol, acetaminophen, and naphthol [29], it was important to substantiate the metabolic capacity for conjugation reactions in our own hepatocyte preparation. As reported in the previous study [13], isolated hepatocytes in suspension, incubated under the same conditions used for analysis of anthracycline metabolism, were shown to maintain a capacity for formation and excretion of glucuronide conjugates of bilirubin. The presence of the anthracycline antibiotics did not interfere with conjugation of the bilirubin substrate. It has been demonstrated further that the capacity of the liver cell to sustain conjugation reactions is not compromised as a consequence of ether anesthesia for liver perfusion and cell isolation [30]. The potential problem of ether anesthesia interfering with conjugative capacity does not arise for the rabbit hepatocytes, which are generated using phenobarbitol as an anesthetic agent.

The possibility that anthracycline conjugates were formed but inefficiently extracted from the cell must be considered. However, since the liver generally conjugates drugs in order to increase their polarity and facilitate their excretion, the bulk of conjugates formed could be found in the incubation medium (as is the case with glucuronides of bilirubin [31]). As

reported, the incubation medium did not contain drug conjugates. It therefore seems likely that, over the time frame of these experiments, conjugate formation was minimal. If drug conjugates were formed, the level(s) might have been insufficient to assess by our analytical techniques.

Minimal accumulation of anthracycline conjugates in the liver is not inconsistent with work by other investigators. In the study by Cradock *et al.* [32], the level of polar metabolites of DNR in the liver (presumed to be drug conjugates) ranged between 0.5 and 1.47% of total fluorescent equivalents. Studies of adriamycin metabolism in rabbits by Bachur *et al.* [2] indicate that levels of adriamycin polar metabolites in the rabbit liver were equivalent to only 1.5% of total fluorescence after 8 hr. Bolanowska and Gessner [10] do not report conjugates of the anthracyclines in the livers of mice 24 hr after treatment with adriamycin. Tavoloni and Guarino [6] report adriamycin conjugates in rat bile, but not in the liver itself. Consequently, although anthracycline conjugates appear in the bile of rats and rabbits over extended periods of time [2, 6], it appears that conjugate synthesis in the liver cell is extremely slow compared to the metabolic processes that convert adriamycin to adriamycinol and to deoxyaglycone derivatives.

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