Initial Biotransformations of Daunorubicin to Aglycones by Rat Liver Microsomes

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ABSTRACT

Anaerobic incubations of rat liver microsomes convert daunorubicin into at least six or seven aglycones in the presence of reduced nicotinamide adenine dinucleotide phosphate or a reduced nicotinamide adenine dinucleotide phosphate-generating system. The partial identification of some of the aglycones is based on cochromatographs of chemically synthesized derivatives by isocratic high-pressure liquid chromatography. The major reductive reaction is the reductive cleavage of daunorubicin to 7-deoxydaunorubicin aglycone which is converted to another product that cochromatographs with 7-deoxydaunorubicinol aglycone. In this reaction, NADPH-supplemented rat liver and kidney homogenates. Aerobically, biotransformation of DM is limited to the formation of DMOL (2, 9).

Takanashi and Bachur (27) identified from urine 8 different aglycones, 3 of which were isolated as conjugates. Except for the formation of 7-hydroxy- and 7-deoxyaglycones from DM and DMOL, the further metabolic transformation of these agents by microsomes has not been described. In this communication, an isocratic method of HPLC was used to show that rat hepatic microsomes transform DM into at least 6 or 7 aglycones. The reactions of DM are likely to involve direct formation of at least 2 aglycones, and a further sequential change for one of these (7-deO-DMA) is demonstrated.

INTRODUCTION

DM* and AM (Table 1) are both metabolized in animal tissues by 2 interacting routes. Aldoketoreductase is found as a soluble enzyme and forms DMOL and AMOL, respectively, by reduction of the C-13 carbonyl to the respective alcohol (2, 9). Both products are biologically active and are furthercleaved to aglycones as are the parent compounds (3). Aglycone formation provides a second major route of biotransformation of these anthracyclines which may be implicated as an activation pathway by free radical mechanisms (8, 15) as well as one of inactivation by conjugation mechanisms (27).

Insofar as is presently known, the pathways for the biotransformations of AM and DM are qualitatively identical, although differing quantitatively from each other in different animal species, tissues, and subcellular preparations.

The initial description of DM biotransformation in rat liver extracts by DiMarco and Rusconi in 1967 (12, 13) suggested formation of 2 aglycones by reduction. Following these observations, Bachur and Gee (5) proposed structures for 3 metabolites in homogenates of rat liver and kidney: D2, D3, and D4, respectively. D2 was identified as DMOL, formed by the soluble aldoke reductase with NADPH. Microsomal preparations formed 7-HO-DMA (D4; daunorubicinone) directly from DM and the corresponding 7-HO-DMOLA (D5) from DMOL. Asbell et al. (2) and Bullock et al. (10) also identified the 7-hydroxy- and 7-deoxyaglycones of both DM and AM after incubation with NADPH-supplemented rat and hamster liver and kidney homogenates. Aerobically, biotransformation of DM is limited to the formation of DMOL (2, 9).

MATERIALS AND METHODS

Chemicals. The following agents were kindly provided as gifts: DM and AM from Professor F. Arcamone (Farmitalia, Milan, Italy); [14-methyl-14C]DM from Dr. R. Maral (Rhone-Poulenc, Vitry-sur-Seine, France); prepared at the French Atomic Energy Commission: 49 mCi/mmol), and carminomycin from Dr. A. Syrkin (Soviet Academy of Medical Science, Moscow, U.S.S.R.). Agents were chemically reduced and converted to 7-deoxyaglycones by methods described by Smith et al. (26). The cleavage of DM to 7-HO-DMO by mild acid hydrolysis was previously described by Arcamone et al. (1). DMOL was prepared enzymatically by aerobic incubation of DM with rat liver supernatant containing NADPH (9) and converted to 7-deO-DMOLA (28). Agents and derivatives were purified by silicic acid column chromatography and thin-layer chromatography (4) to >97% homogeneity as determined by HPLC.

Chromatography. Samples are injected (usually 20 μl) into a Dupont Model 830 high-pressure liquid chromatograph and chromatographed isocratically on Zorbax ODS columns (25 cm x 4.6 mm) at room temperature. The mobile phase contains 85% CH3OH and 15% 8.5 mM ammonium formate buffer adjusted to pH 4.0 with HCI. Flow rates are adjusted to 1.1 ml/min, and eluates are detected with the Aminco Fluoromonitor (Corning No. 47B primary filter; Wratten 61 secondary filter). Peak times, height, and area (mV) are computed on a Columbia Supergrator 3-A. Dilution curves were linear with respect to mV for compounds shown in Chart 1 (r² > 0.99) for the range of concentrations used in these studies (10 to 100 mV).

Microsomal Preparation and Incubations. Microsomes from perfused (0.85% NaCl solution) liver of Sprague-Dawley rats are prepared by standard procedures at 0–4°, homogenized...
in 0.075 M KCl; 0.025 M Tris (pH 7.4), and centrifuged for 15 min at 15,000 × g, and the supernatant was recentrifuged for 90 min at 100,000 × g. The pellet is washed twice and stored at −70°C. Protein is determined by the method of Lowry et al. (19).

Incubations are carried out at 37° under N2. The mixtures (usually 1.2 ml) contain 1.6 to 1.8 mg microsomal protein in 0.05 M Tris (pH 7.4). The NADPH-generating system contains (final concentrations): MnCl2 3 mm; isocitric acid, 25 mm; isocitric acid dehydrogenase (Sigma), 0.5 unit; and NADP+ (as indicated in “Results”). Concentrations of NADPH (when used in place of the NADPH-generating system) and DM are also indicated below. Incubations are terminated by addition of 2 volumes of CHCl3:CH3OH (2:1). The mixtures are extracted at 4°C; the organic phase is dried under N2 and usually redissolved in 200 μl of CH3OH for HPLC. Dimethyl sulfoxide (10%, v/v) is sometimes added to solubilize aglycones. Tissue blanks (not shown) were essentially flat, and variations were below levels of sensitivity of the integrator settings used here.

Calculations. Methods used in computing results are described in the “Appendix” and were programmed on a Hewlett-Packard 9825 A. Theoretical regressions for a linear sequential pathway were drawn by hand from computed values derived at 0.2-min intervals between 0 and 15 min with a 1-min latent period.

RESULTS

HPLC Separations. Chart 1 shows the separation of 3 purified aglycones, DMOL, and DM by HPLC as described in “Materials and Methods.” The RT’s relative to DM (RT 1.0) of these and other agents in this series are shown in Table 1.

Microsomal Formation of DM Aglycones. Anaerobic microsomal metabolism of DM is shown in Chart 2. After preincubation of the isocitric dehydrogenase-generating system with 0.5, 1.0, and 2.0 mM NADP+ for 15 min, anaerobic metabolism was carried out for 60 min. In this study, NADPH (Chart 2, a to c) was used for a comparison with results obtained with the generating system (Chart 2, d to f). The elution profiles show positions of DM (Chart 2a) and aglycone metabolites formed during the incubations.

It will be convenient to consider the aglycones in groups as I, II, and III for discussion of the kinetics of transformation (see below). Group I, insofar as we are presently able to determine, consists of a single agent and cochromatographs with 7-deO-DMA (RT 0.25) as indicated in Chart 2f. Group II is seen as 2 (Chart 2, a and b) or 3 bands (Chart 2, c to e). One of the major peaks in Group II cochromatographs with 7-HO-DMA (RT 0.19 to 0.20) as indicated in Chart 2f; the other major band in Group II (RT 0.21) cochromatographs with 7-deO-DMOLA. A small peak sometimes eluting as a shoulder (RT 0.18) in front of 7-HO-DMA and metabolites seen as 3 bands in Group III (RT 0.33, 0.36, 0.42) are unidentified at the present time. In 7 experiments (with NADPH or the generating systems; 15-, 30-, and 60-min of incubations), the S.E.’s of retention times of the 7 aglycones were 0.59 to 0.69% of the average RT for each band. Coelutions (at concentrations approximately equal, half, and twice the fluorescent area of unknowns) were judged by elution times (RT within the S.E. of unknown bands) and curve shape (absence of shoulder and of changes in symmetry). It should be noted that the 6 or 7 aglycones shown

![Chemical structures of DM and related agents and aglycones](image)

<table>
<thead>
<tr>
<th>R1 (ring carbon 4)</th>
<th>R2 (ring carbon 7)</th>
<th>R3 (ring carbon 12)</th>
<th>Compound</th>
<th>RT</th>
</tr>
</thead>
<tbody>
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<td>C—CH3</td>
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<td>OH</td>
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<td>H—C—CH3</td>
<td>7-deO-DMOLA</td>
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</table>

*S. daunomycin moiety.

**Table 1**

**Chemical structures of DM and related agents and aglycones**

S. daunorubicinone.

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Biotransformations of DM to Aglycones

ANAEROBIC (N\textsubscript{2})

Fig. 2. Anaerobic biotransformations of DM (0.16 mM) by rat liver microsomes with varying concentrations of NADPH (top) and NADP\textsuperscript{+} (bottom) with the isocitric acid dehydrogenase-generating system. See text for description of conditions and groups.

Kinetics of Microsomal Biotransformation of DM. The overall kinetics of aglycone formation from [14-\textsuperscript{14}C]DM are shown in Chart 3. Standard conditions (anaerobic incubation; 1 mM NADPH) were used in this experiment. The loss of DM is rapid, with 7-deO-DMA (I) reaching maximal concentration by 5 to 10 min. Concentration of the latter then decreases slowly. The aglycones in Group II also increase rapidly; the first appears at RT 0.21, and then from 15 to 60 min others in Group II also increase. The total radioactivity in Group III reaches a maximum under these conditions at about 15 min. Similar types of kinetics are obtained (using only fluorescence) with the NADPH-generating system. The major components in Group II increase with time while those in Group I decrease, and after 15 min the fluorescence remains constant in the 3 bands in Group III (data not shown).

In the above studies with [\textsuperscript{14}C]DM, we estimated the eluting fluorescence (i.e., area under the curve) per unit of radioactivity for Groups I and II. Relative to DM, fluorescence of Group I was enhanced 2.5-fold, and that of Group II (at RT 0.21) was enhanced 2.8-fold. These relative fluorescence values are used for the kinetic studies described below.

Chart 4 shows relative initial rates of DM loss and formation in Chart 2 appear in only 2 fluorescent bands in several thin-layer chromatographic systems (4).

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Chart 3. Biotransformation of [14-\textsuperscript{14}C]DM aglycones (Groups I, II, and III) by microsomes with NADPH (1.0 mM). Labeled drug (1 \times 10^6 dpm) was added to 0.16 mM DM and incubated anaerobically as described in the text. Recoveries of radioactivity after extractions and HPLC separations (6.5 to 7 \times 10^3 cpm) were >95% complete.

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Chart 4. Initial biotransformation of (0.16 mM) DM to 7-deO-DMA and to RT 0.21 (Group II) by microsomes and 1.0 mM NADP* plus generating system. The points shown are experimental values; the regressions are drawn from values calculated for a linear sequential pathway as described in the “Appendix.”

of Groups I and II during anaerobic incubation with the NADPH-generating system and microsomes. There may be a variable latent period initially up to about 3 min, presumably due to residual traces of O2 which inhibit the loss of DM. During the incubations from 3 to 9 min, there is little or no formation of aglycones in Group III, and only traces of 7-HO-DM (RT 0.20) and the preceding shoulder material (RT 0.18) are seen in Group II. Group II, in these studies, consists primarily of the major peak (RT 0.21), and Group I again cochromatographs with 7-deO-DMA. The points shown in Chart 4 are experimental values; the solid lines are drawn from theoretical values for a linear sequential reaction (Ref. 23; see “Appendix”). The correspondence between observed and theoretical values is consistent with the assumption of a sequential reaction; i.e., RT 0.21 forms from 7-deO-DMA (I) rather than from DM.

Estimation of Apparent Rate Constants (see “Appendix”). The rates of DM depletion and product formation are proportional to microsomal protein concentration, and they approach linearity after the lag period with the NADPH-generating system and initial drug concentrations up to 1.0 mM. With lower drug concentrations (0.08 mM), there are losses of linearity with respect to depletion of DM and formation of Group I. These data are not shown.

Chart 5 shows the velocities of transformation for the interval from 3 to 9 min with varying initial concentrations of DM. As noted above, the decrease in DM concentration (indicated as negative velocity) is concurrent with formation of 7-deO-DMA (I). With the reservation in mind that Group II may be heterogeneous, double-reciprocal plots (Chart 6) for DM disappearance and Group I and II (RT 0.21) formation are estimated for apparent K_m and V_max values as shown in Table 2. The apparent K_m and V_max values for Group II are estimated from the initial concentrations of DM. This is a reasonable approximation because the loss of substrate is closely paralleled by the formation of Group I.

DISCUSSION

The reductive cleavage of DM to Product I (7-deO-DMA) is the first reaction that occurs under anaerobic conditions, and it confirms previous observations (2, 10). Kinetic studies provide evidence that, after DM is cleaved, Product I is further metabolized to one or more compounds in Group II. This is suggested by the build-up of the RT 0.21 product (which cochromatographs with 7-deO-DMA) as concentrations of Product I decline and is confirmed by the kinetics of a linear sequential pathway (Charts 3 and 4). In experiments not shown here, incubations of 7-deO-DMA converted to RT 0.21 product (27 to 36%) with microsomes incubated under the same conditions used in Chart 5.

One of the products in Group II appears to be 7-HO-DM. The studies of others (11, 27) indicate that this is a metabolic product of DM and has been identified in plasma, urine, and bile. The kinetics of formation of 7-HO-DM has not yet been fully investigated, although studies not shown here presently suggest that 7-HO-DM probably derives directly from DM and not from 7-deO-DMA (I). This is consistent with hypothesized pathways for DM biotransformation (3).

The products in Group III are formed optimally under anaer-
were 0.10 to 0.95 mM, and incubation times were 3 to 9 min.

obic conditions with the isocitric dehydrogenase-NADPH-generating system. Products in this group are unidentified, and we are at this time uncertain of the pathway of formation.

The material at RT 0.21 may be the 7-deoxyaglycone of DMOL (i.e., 7-deO-DMOLA) which would be consistent with other studies (2, 3). It is possible that the twice-washed microsomes used here contain an enzyme with ketoreductase activity which transforms the 13-ketoaglycones to 13-hydroxyaglycones (2, 9, 22). This possibility seems quite likely but has not been tested yet. We have found no accumulations of DMOL during aerobic (O2) microsomal incubations, indicating that supernatant aldoketoreductase, if present, is below our limits of detection. The relative amounts in the RT 0.21 band are increased with unwashed microsomes (N2), whereas the RT 0.21 material formed with microsomes washed 5 times is about the same as that with twice-washed microsomes (data not shown). Thus, we are unable to rule out a possible direct nonmicrosome enzyme conversion of the 7-deO-DMOLA or other 13-ketoaglycones to the respective 13-hydroxy forms, and we tentatively assign RT 0.21 to 7-deO-DMOLA.

The implications of the inhibition of biotransformation of DM by O2 are worthy of further consideration. Anaerobic reductive activation was proposed in 1963 to account for the antitumor potency of mitomycin C (25). It was further suggested that selective cytotoxicity against tumors is due to the relatively low pO2 under which sensitive tumors grow (24, 25); strong experimental evidence for the concept of reductive activation has been obtained recently by Lin, Sartorelli, and coworkers (16-18), who have extended the earlier studies. In recent years, the concepts of reductive activation with free radical participation also have been extended to a number of other agents, including AM and DM (20, 21).

The free radical mechanisms for biotransformation of AM, DM, and other quinone-containing antitumor agents have contributed additionally to our understanding of anaerobic reductive activation, suggesting that O2 may be transformed into an O2- radical through a cyclic reduction-oxidation of DM (6-8, 14). Although in vitro biotransformations are inhibited by O2 in microsomes, there is considerable evidence that reductive activation does occur in normal and anesthetized animals and in patients. The evidence for this is based on identification of reduction products excreted in urine and bile (3, 27). Some of these include 7-deoxyaglycones of DM, DMOL, AM, and AMOL, which have been identified as microsomal products formed only anaerobically in vitro. Thus, formation of such metabolites of DM (and AM) in oxygenated organs occurs but probably at greatly reduced rates.

In the present study, we have provided evidence for the formation of at least 6 or 7 aglycones from DM, although only 2 are currently identified with another tentatively assumed. In anticipation of future studies, we may expect that other microsomal products will include aglycones of DM which are 4-demethylated, 4-demethyloxylated, and perhaps reduced at C-13 to form the DMOL aglycones. Such metabolites, alone and as conjugated species, have been identified as excretory products of DM and AM (3, 11, 27).

APPENDIX: Computations for Corrected Fluorescent Emissions and Sequential Kinetics

Herbert S. Schwartz

The following quantitative expressions used in this paper are as follows:
1. Corrections for Fluorescent emission by aglycones of DM. At 0 time, the fluorescence area (a) under the DM elution curve (in MV) is a linear function of DM concentration:

\[ [DM] = k \cdot a_{DM} \]

where \( k \) is the slope of the regression for area as a function of concentration. At this time, initial \( a_{DM} \) is approximately equal (i.e., >99%) to the total fluorescence (\( a_t \)). When DM is transformed, \( a_t \) increases due to increased fluorescence of aglycones in Groups I and II (\( a_i \) and \( a_{II} \), respectively) as described above. To correct for this effect, the following relationship is used:

\[ a_{DM} + (a_t/2.5) + (a_{II}/2.8) = a_t \]

After correcting for metabolic fluorescence, the concentration of DM lost is equal to the sum of amounts of aglycones in Groups I and II (primarily RT 0.21) and the rates are:

\[ \frac{d[DM]}{dt} = k_1[DM] - k_2[I] \]
\[ \frac{d[I]}{dt} = k_2[I] - k_1[DM] \]

2. Linear Sequential Reactions (23). For the conversion of DM into Products I and II sequentially, the reaction is assumed to follow first-order kinetics:

\[ -\frac{d[DM]}{dt} = k_1[DM] - k_2[I] \]

After correction of \( a_i \) and \( a_{II} \) for respective fluorescence coefficients (above), [DM] at time \( t \) is:

\[ [DM] = [DM]_0 e^{-k_2t} \]

[I] at time \( t \) is:

\[ [I] = \frac{k_1[DM]_0}{(k_2 - k_1)} (e^{-k_1t} - e^{-k_2t}) \]

and [II] is:

\[ [II] = [DM]_0 \left(1 - \frac{1}{(k_2 - k_1)} \right) e^{-k_1t} \]

where \( k_1 = 0.069 \text{ min}^{-1} \) and \( k_2 = 0.036 \text{ min}^{-1} \), and corrected for a 1 min latent period.
REFERENCES


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