Rat Liver Daunorubicin Reductase

AN ALDO-KETO REDUCTASE

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SUMMARY

Daunorubicin, a cancer chemotherapeutic antibiotic, is converted by rat liver extracts to daunorubicinol by daunorubicin reductase. This enzyme is purified from rat liver by ammonium sulfate fractionation, DEAE-cellulose, hydroxypatite, and Bio-Gel P-200 column chromatography with an over-all recovery of 46%. On the basis of polyacrylamide electrophoresis with and without sodium dodecyl sulfate, gel filtration chromatography, and high speed ultracentrifugation, the final preparation is judged homogeneous. Sedimentation equilibrium analysis and sodium dodecyl sulfate polyacrylamide electrophoresis give molecular weights of 38,796 ± 369 and 39,800, respectively, and suggest a single polypeptide chain. In agreement is the molecular weight of 39,541 calculated from the proposed amino acid composition. The enzyme has an isoelectric point of 6.3 and a strict requirement for NADPH. The daunorubicin-NADPH interaction occurs with a 1:1 stoichiometry. The apparent equilibrium constant at pH 8.5 for reduction is 2.91 (± 0.26) × 10^9 M⁻¹, and the apparent Kₚ for daunorubicin is 57 μM. Several common sulphydryl reactants inhibited the enzyme. A closely related antibiotic, adriamycin, is reduced at about 5% of the rate of daunorubicin reduction. In addition to the antibiotics, the enzyme reduces some aldo sugars, particularly, D-glucuronolactone, D-glyceraldehyde, and the isomers D-glucuronate and D-galacturonate. D-Glucuronolactone and daunorubicin were the preferred substrates. This reactivity with D-glucuronolactone suggests a normal role of daunorubicin reductase in ascobic acid synthesis or in the glucuronic acid cycle, or both.

The cancer chemotherapeutic antibiotic, daunorubicin, is metabolized by various mammalian cells and cell extracts (1-4). One step in the metabolic sequence is the conversion of daunorubicin to daunorubicinol (Fig. 1). Occurring primarily in the cellular cytosol, this enzymatic reaction is sustained by NADPH (2).

The in vivo activity of the reductase is well documented by the large amounts of the enzymatic product, daunorubicinol, that are excreted by humans and rats treated with daunorubicin (5, 6). In fact, daunorubicinol is the major excretion form of daunorubicin and is a major form of the drug in tissues. One significant aspect of this metabolic conversion is that the enzymatic product, daunorubicinol, has cytotoxic properties and is an important component in the action of daunorubicin (7).

Since daunorubicin reductase appears to be a constitutive and ubiquitous mammalian enzyme, even occurring in erythrocytes and platelets, it is doubtful that the natural substrate for this enzyme is an antibiotic arising from a mold. In order to answer, ultimately, the basic questions of the enzyme's identity and its role in drug metabolism we have purified the enzyme to apparent homogeneity from rat liver and have determined a number of its physical and chemical properties. These studies are the subject of this report.

METHODS AND MATERIALS

Daunorubicin was obtained from the Drug Development Branch, Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health and adriamycin was generously provided by Farmitalia, Milan, Italy. Both substrates were purified according to the method of Bachur and Craddock (1). NADPH was obtained from P-L Biochemicals. Aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease were from calibration kits obtained from Pharmacia Fine Chemicals. Crystallized bovine serum albumin, cytochrome e type III, two times crystallized and lyophilized pepsin, glucose 6-sulfate, α-l-pyruvate, γ-1-glucuronic acid, α-γ-glucuronic acid (grade I), D-glucuronic acid lactone, α-glyceraldehyde, β-glyceraldehyde (70% pure), α-lactose, β-lactose, maltose, estrone, cortisone, androsten-3,17-dione, corticosterone, D(+) galactose, α-glucose 6-phosphate, D(−) ribose, D(+) glucose, D(−) galactose, α-d-glucosaminic acid, β-hydroxybutyrate (Bio-Gel HTP) were from Bio-Rad Laboratories. DEAE-cellulose (DE-11) was from Whatman. All other chemicals were of the highest grade commercially available and all solutions were made in glass-distilled water.

Measurement of Daunorubicin Reductase Activity

Daunorubicin reductase was assayed in the presence of approximately 0.18 mM NADPH and either 0.75 or 1.5 mM daunorubicin in 0.2 M Tris-HCl, pH 8.5, at 25°. We monitored reaction progress spectrophotometrically in 1-cm light path quartz cuvettes by following the oxidation of NADPH at 340 nm using a Cary 14 spectrophotometer equipped with a 0 to 0.2 optical density slide wire. Any background rates of NADPH reduction encountered during purification were subtracted from the rate observed with the standard assay. The reaction was initiated by addition of enzyme. At daunorubicin concentrations of 0.75 and 1.5 mM, the assays were first order in enzyme concentration to at least 0.32 and 0.50 μM of purified enzyme, respectively. Regardless of the
substrate concentration, the rates measured in a typical assay never exceeded an equivalent purified protein concentration of 0.08 to 0.13 μM. This same basic assay was utilized with other substrates in place of daunorubicin.

In certain cases, such as monitoring columns with low levels of enzyme activity, a more sensitive but time-consuming fluorescence assay (2) was utilized. This assay involved the reduction of daunorubicin under conditions similar to those described above. After an appropriate incubation time, the reaction was terminated by extraction of substrate and product with 1-butanol followed by their separation by thin layer chromatography. The product, daunorubicinol, was extracted from the plate and its fluorescence was quantified against standards. Enzyme activity units were defined as micromoles of NADPH oxidized per min at 25°C.

Daunorubicin reductase also was tested for isocitrate dehydrogenase (NADP) (1.1.1.42) and malate dehydrogenase (decarboxylating) (NADP) (1.1.1.42) activity by previously described methods (8, 9).

Concentrations of stock NADPH and daunorubicin solutions were determined from molar extinction coefficients of 6.22 × 10² and 11.4 × 10² at 340 and 485 nm, respectively.

Kinetics

Under conditions of saturating cofactor and the assumption that daunorubicin reductase follows a Michaelis-Menten mechanism, if $S \ll K_m$, then the typical kinetic expression reduces to

$$v = \frac{V_{\text{max}}}{K_m} S = \frac{k_{\text{cat}}}{K_m} E_0 S$$

where $v$ is the rate of the reaction, $V_{\text{max}}$ is the maximum rate, $E_0$ is the enzyme concentration, $S$ is the substrate concentration, and $k_{\text{cat}}$ and $K_m$ are the catalytic rate and Michaelis constants of the enzyme, respectively. $K_m$ and $V_{\text{max}}$ values were determined by Lineweaver-Burk plots and $V_{\text{max}}$ values converted to $k_{\text{cat}}$ values by dividing by $E_0$. $E_0$ was determined by dividing the protein concentration (10) by the assumed molecular weight of 39,000. Relative pseudo-second order rate constants were estimated by dividing the apparent $k_{\text{cat}}$ by the respective apparent $K_m$ value. The apparent $V_{\text{max}}$ values for daunorubicin reduction were independent of initial NADPH concentrations down to 2.5 μM.

Equilibrium Constants

The apparent thermodynamic equilibrium constant, $K_{eq}$, and the stoichiometry were determined with 0.18 to 0.36 mM NADPH and 0.075 to 0.75 mM daunorubicin in 0.1 M potassium phosphate buffer (pH 7.0), 0.2 mM Tris-HCl (pH 8.5), and 0.1 M glycine-NaOH (pH 10.1) at 25°C. These reactions were initiated with enough enzyme to reach equilibrium within 1 hour. The decrease in absorbance at 340 nm was followed continuously with a Cary 14 spectrophotometer equipped with a 0 to 2.0 optical density slide wire. Equilibrium concentrations of NADP⁺ and NADPH were calculated from the change in 340 nm absorbance and the known initial concentrations. Substrate and product were quantified by the fluorescence assay, and it was assumed that the hydrogen ion concentration remained constant at the pH of the buffer throughout the reaction.

Polyacrylamide Electrophoresis

Proteins were subjected to electrophoresis in 7% (w/v) acrylamide gels at 4 × 10⁵ according to the method of Davis (11) or Hedrick and Smith (12) except that the concentrating gels were sometimes omitted in the latter method. Proteins in a solution of 10 to 20% glycerol were layered on the gels, and the gels were stained with Coomassie brilliant blue and destained according to the method of Weber and Osborn (13). Bromphenol blue was used as a tracking dye and its position was indicated with a marker wire before staining.

After polyacrylamide electrophoresis, enzyme activity of the freshly run polyacrylamide gels were measured. The gels were removed from the glass tubes, chilled in a freezer for 10 min, and cut into 1-mm slices. Each slice was suspended in 0.285 M Tris-HCl, pH 7.4, at 37°C for about 30 min. NADPH and daunorubicin were added to 0.2 mM and 1 mM, respectively, and the incubation was continued for 16 min at 37°C. The reactions were terminated and quantified by the fluorescence assay.

Sodium dodecyl sulfate electrophoresis was done on 10% acrylamide-0.1% sodium dodecyl sulfate gels according to the method of Weber and Osborn (13).

Electrofocusing

The enzyme isoelectric point was determined on an LKB 8101 column at 4°C with pH 5 to 8 Ampholine in a sucrose gradient (0 to 1.9 M). The focusing process averaged 65 hours and started with a maximum load of about 1.3 watts.

Gel Filtration

The molecular weight of daunorubicin reductase was estimated by column gel filtration at 4°C with Sephadex G-150 (1.5 × 85 cm), Sephadex G-100 (1.5 × 79 cm), and Bio-Gel P-150 (1.5 × 83 cm). All columns were packed and run in 0.01 or 0.001 M potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl by descending chromatography. Proteins were applied in 3% sucrose by layering on top of the gel bed. As suggested by Pharmacia, the calibration curves were constructed by plotting the logarithm of molecular weight against $K_{sv}$, where $f = V_s - V_0/V_s - V_b$ ($V_s$ = elution volume for the protein, $V_b$ = elution volume for blue dextran, and $V_0$ = total bed volume).

Sedimentation Equilibrium

Sedimentation equilibrium of the purified reductase was carried out in a Spinco model E analytical ultracentrifuge equipped with
interference optics according to the method of Yphantis (14). Enzyme was dialyzed for 28 hours against 0.001 M potassium phosphate buffer, pH 7.4, and 0.15 M NaCl at 4°C; and the dialysate was used for enzyme dilutions and reference solution. One hundred micrograms of dialyzed daunorubicin reductase at initial protein concentrations of 0.29, 0.48, and 0.97 mg per ml were analyzed simultaneously with a Yphantis centipiece. FC-43 oil was not used here as possible protein denaturation (15). After an initial 60-min overspeed at 24,630 rpm, equilibrium was established at 19,160 rpm after 65 hours. The speed was then increased to 29,495 rpm and equilibrium was established again after 29 hours. Final equilibrium was established 17 hours after increasing the speed to 35,739 rpm. Total elapsed time for all three equilibria was 112 hours and the temperature varied from 7.2 to 7.4°C. Equilibria at each speed were verified by comparing photos at least 4 hours apart. Blanks were obtained by taking zero time photos for each equilibrium speed. Rayleigh patterns were measured with a Nikon model 6C microcomparator. The data were analyzed with a modified FORTRAN IV high speed equilibrium ultracentrifuge computer program of Roark and Yphantis (16). The molecular weights reported were obtained by extrapolation of the reciprocal weight-average molecular weight versus protein concentration data to zero protein concentration by a linear regression analysis. A density of 1.0068 g per ml was calculated for the solvent from the International Critical Tables (17). A partial specific volume was calculated from the amino acid composition (18).

Amino Acid Analysis

The Worthington Corporation performed the amino acid analyses using a Beckman 120 C amino acid analyzer. Samples were hydrolyzed at the indicated times and the amounts of labile amino acids were extrapolated to zero time. Methionine and half-cystine were determined as methionine sulfone and cysteic acid, respectively, after performic acid oxidation (19). Tryptophan was estimated from the protein absorption and by the method of Edelhoch (20). Based on the Lowry protein, a recovery of 77% was calculated from one complete amino acid analysis.

Purification of Rat Liver Daunorubicin Reductase

All operations were carried out at 0-5°C unless otherwise specified.

Step 1: Extraction—The livers from adult Sprague-Dawley rats were frozen and stored at -15°C. Frozen or fresh rat livers were homogenized with 3 volumes of 0.05 M Tris-HCl, pH 8.6, and centrifuged at 12,000 X g for 30 min. The 12,000 X g supernatant solution was further clarified by centrifugation at 80,000 X g for 75 min. These high speed supernatant solutions were frozen sometimes as long as 2 years before the purification.

Step 2: Ammonium Sulfate Fractionation—The frozen microsome-free supernatant solutions were allowed to thaw in the cold room overnight, and the preparation was fractionated by the addition of solid (NH₄)₂SO₄. The 0.35- to 0.65-(NH₄)₂SO₄ precipitate which contained most of the activity was dissolved in a fresh protein solution (at zero time) and centrifuged at 4°C. Methionine and half-cystine were determined as methionine sulfone and cysteic acid, respectively, after performic acid oxidation (19). Tryptophan was estimated from the protein absorption and by the method of Edelhoch (20). Based on the Lowry protein, a recovery of 77% was calculated from one complete amino acid analysis.

Storage

The purified enzyme obtained from ultrafiltration in Step 5 was separated into small aliquots and stored frozen at -15°C.

RESULTS

Enzyme Purification—The purification of daunorubicin reductase presented no insurmountable problems. In fact, the purification was facilitated by the low retention of this protein to DEAE-cellulose and hydroxyapatite (Table I).

Purity of the enzyme was established by a number of criteria. One protein band resulted from polycarboxylamide electrophoresis at pH 8.4 (Fig. 4A) and pH 9.5 (Fig. 4B) and after polycarboxylase activity were determined on each 4-ml fraction, and tubes having a constant activity to absorbance ratio were combined and concentrated by ultrafiltration (Amicon UHF-2 membrane) (Fig. 3).

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FIG. 2. Elution of daunorubicin reductase from DEAE-cellulose by a linear salt gradient. The gradient was started at Fraction 19 and consisted of 0.01 M Tris-HCl, pH 8.6, 0.01 M Tris-HCl, pH 8.6, and 0.1 M NaCl. Protein was approximated by absorption at 280 nm (- - -) and enzyme activity was determined as described under "Methods and Materials" (• - - -). The bar represents those fractions pooled for subsequent purification.

FIG. 3. Gel filtration of daunorubicin reductase from a Bio-Gel P-200 column equilibrated with 0.01 M potassium phosphate buffer, pH 7.4. Protein was followed by measuring absorption at 280 nm (- - -) and enzyme was assayed as described under "Methods and Materials" (• - - -). The specific activity (O-O) was estimated by dividing enzyme activity by absorbance at 280 nm. The void and inclusion volumes of the column are indicated by blue dextran and vitamin B₆, respectively. The bar represents those fractions pooled for subsequent characterization.
TABLE I
Purification of rat liver daunorubicin reductase

| Step | Volume | Protein | Activity | Specific activity | Yield | Purification |
|------|--------|---------|----------|-------------------|-------|--------------|
| 1. High speed supernatant | 750 | 28,800 | 31.3 | 0.00109 | 100 | 1 |
| 2. (NH₄)₂SO₄ (0.35-0.65) | 133 | 9,380 | 22.6 | 0.00241 | 72 | 2.2 |
| 3. DEAE-cellulose | 10.9 | 265 | 20.3 | 0.0707 | 65 | 71 |
| 4. Hydroxyapatite | 1.34 | 9.4 | 15.7 | 1.67 | 50 | 1,540 |
| 5. Bio-Gel P-200 | 1.47 | 5.7 | 14.4 | 2.33 | 46 | 2,330 |

Fig. 4 (left). Polyacrylamide electrophoresis of daunorubicin reductase from Bio-Gel P-200 column (A), 7.2 µg of enzyme subjected to electrophoresis at pH 8.4; (B) 0.3 µg of enzyme subjected to electrophoresis at pH 9.5; and (C) 11 µg of enzyme subjected to electrophoresis in the presence of 0.1% sodium dodecyl sulfate. Electrophoresis was from top (cathode) to bottom (anode) and the dye fronts are marked with wire.

Fig. 5 (right). Coincidence of daunorubicin reductase activity with protein stain after polyacrylamide electrophoresis was obtained by running two identical gels in parallel at pH 9.5; one was stained for protein and the other sliced for subsequent enzyme assay (see "Methods and Materials").

Ultracentrifugation provided strong additional evidence for size homogeneity. The plots of reciprocal number- and weight-average molecular weights versus protein concentration gave nearly straight lines throughout the cell channels for each analysis (Fig. 8).

Physical and Chemical Characteristics—Total amino acid analysis was performed on two different purified preparations of daunorubicin reductase after acid hydrolysis at 24, 48, and 72 hours. The proposed amino acid composition is summarized in Table II. No neutral sugars (<1%) were detected when the protein was tested by the phenolsulfuric acid reaction.

When the purified enzyme was electrofocused on a pH gradient, a single peak of enzyme activity occurred at pH 6.3. This activity was coincident with the single protein peak from the isoelectric column.

The absorption spectrum of the purified enzyme exhibited a maximum at 280 nm with a shoulder at 292 nm. The 280:260 nm ratio was 1.84.

The molecular weight was estimated by several methods. The results from gel filtration on calibrated columns of Bio-Gel P-150 and Sephadex G-150 are presented in Fig. 6. Rat liver daunorubicin reductase elutes as a protein of molecular weight 43,500 and 30,500, respectively. When rat liver daunorubicin reductase was similarly chromatographed on Sephadex G-100, a molecular weight of 29,700 was obtained.

Polyacrylamide electrophoresis of rat liver daunorubicin reductase in the presence of sodium dodecyl sulfate yielded a single protein band of molecular weight 39,800 (Fig. 7).

The molecular weight of daunorubicin reductase was also determined by high speed sedimentation equilibrium. Fig. 8 is an example of a typical computer-plotted reciprocal number- and weight-average molecular weights versus protein concentration (in millimeters of fringe displacement) across one cell channel. The weight-average molecular weights from such data were obtained by linear regression analysis of the reciprocal weight-average molecular weight plot followed by extrapolation to zero protein concentration. The molecular weight obtained from five similar analyses at two different rotor speeds and three different initial protein concentrations was 28,786 ± 369. In the equilibrium experiments the reciprocal molecular weights were nearly constant across each cell channel. The average slope of the best fit reciprocal weight-average molecular weight versus protein concentration plots was -0.0458 ± 0.0086.

The molecular weight calculated from the proposed amino acid composition was 39,541. A partial specific volume of 0.742 ml per g was calculated.

Effect of pH on Enzyme Activity—Optimal enzyme activity with daunorubicin as substrate was observed between pH 8.5 and 9.0. Tris-HCl buffer exhibited a slight inhibition relative...
to glycine and phosphate buffers (Fig. 9). The enzyme exhibited a similar pH optimum for reduction of the related anthracycline antibiotic, adriamycin.

**Cofactor, Substrates, Equilibrium Constant, and Stoichiometry**

Table II

**Amino acid composition of daunorubicin reductase**

| Amino acid | Amino acids per molecule |
|------------|-------------------------|
| Aspartic acid | 29.4 (1.76) |
| Threonine | 14.9 |
| Serine | 21.5 |
| Glutamic acid | 36.3 (0.71) |
| Proline | 28.1 (1.44) |
| Glycine | 21.0 |
| Alanine | 31.3 (0.44) |
| Half-cystined | 4.0 |
| Valinee | 20.1 |
| Methionined | 3.8 |
| Isoleucinee | 13.5 (0.79) |
| Leucinee | 39.3 (2.30) |
| Tyrosine | 13.7 (0.78) |
| Phenylalanine | 8.7 (0.44) |
| Lysinee | 22.6 (1.66) |
| Histidine | 11.5 (0.94) |
| Arginine | 16.1 (0.68) |
| Tryptophanf | 9.4 |

**Notes:**
- Mean values determined from six analyses. Analysis performed on 24-, 48-, and 72-hour hydrolysates of two different enzyme preparations. Standard deviations are expressed in parentheses.
- Based on 21.0 moles of glycine per mole of daunorubicin reductase.
- Mean values determined from two sets of 24-, 48-, and 72-hour hydrolysates by extrapolation to zero time.
- Mean values determined from two enzyme preparations and determined as cysteic acid and methionine sulfone after performic acid oxidation.
- Average of two 72-hour hydrolysates.
- Determined spectrophotometrically.

NADPH was an absolute requirement for reduction of daunorubicin, and no reduction occurred when NADPH was replaced with 170 μM NADH. This confirms previous specificity studies carried out on the unpurified enzyme (2).

The apparent thermodynamic equilibrium constant, $K_{eq}$, and stoichiometry for the reaction in Fig. 1 at pH 8.5 was established by allowing the reaction to go to completion and measuring the concentrations of reactants and products (see "Methods and Materials"). From three such experiments $K_{eq}$ was calculated to be $2.91 (\pm 0.26) \times 10^9 \text{M}^{-1}$. Also, at equilibrium the ratio

![FIG. 6 (left). Molecular weight estimation of daunorubicin reductase by gel filtration on Bio-Gel P-150 (A) and Sephadex G-150 (B). Columns were equilibrated with 0.01 M potassium phosphate (pH 7.4) and 0.15 M NaCl, 35,732 rpm, 7.4°C; concentration is given in millimeters of fringe displacement. One milligram per ml of bovine serum albumin gives a displacement of about 1.16 mm. Number-average, 0; weight-average, A; and Z-average, 0.](http://www.jbc.org/)

![FIG. 7 (right). The molecular weight of daunorubicin reductase as determined by sodium dodecyl sulfate polyacrylamide electrophoresis. The mobility of the unknown enzyme relative to standards is indicated by the arrow. The standard proteins and molecular weights were: pepsin (Pep), 35,000; cytochrome c (Cyto C), 11,700; and the other proteins listed in Fig. 6.](http://www.jbc.org/)
of NAD\textsuperscript{+} to daunorubicinol formed was 1.003 ± 0.044 confirming a 1:1 stoichiometry for the reaction. Similar duplicate experiments at pH 7.0 and 10.1 gave $K_{eq}$ values of $1.2 \times 10^7$ M\textsuperscript{-1} and $2.1 \times 10^9$ M\textsuperscript{-1}, respectively. Despite the increase in $K_{eq}$ with increased pH, the reverse reaction was detected spectrophotometrically only above pH 9.0.\textsuperscript{1}

Under the standard assay conditions, the apparent $K_m$ of the reductase for daunorubicin was 57 $\mu$M (Table III). The apparent $K_m$ for NADPH at 1.5 $\mu$M daunorubicin was about 1 $\mu$M. The closely related anthracycline antibiotic, adriamycin, was also reduced by the enzyme; but only at about 5% of the daunorubicin rate (Table III).

**Enzyme Substrates**—In addition to daunorubicin and adriamycin, a number of naturally occurring biological compounds were tested as possible alternative substrates for daunorubicin reductase. The reductase readily reduced D-glucuronolactone, D-glyceraldehyde, D-glucuronate, and D-galacturonate and a lower level of reactivity was displayed toward D-glucose 6-sulfate, D-ribose, D-galactose, D-glucose, and D-glucose 6-phosphate (Table III). In addition to those substrates, the purified enzyme exhibited very slight activity toward maltose, β-lactose, α-lactose, and pyruvate when assayed at high substrate (0.14 M) and enzyme (0.24 $\mu$M) concentrations. Acetone (0.135 M), α-ketoglutarate (1 mM), and the steroids (0.1 mM), estrone, cortisone, androsten-3,17-dione, and corticosterone, were not reduced under similar conditions.

The enzyme was tested for dehydrogenase activity with isocitric acid and malic acid as substrates under previously described assay conditions (8, 9). No reaction with these substrates was detected.

**Inhibitors**—A number of chemicals were tested for their ability to inhibit daunorubicin reductase (Table IV). In addition to being sensitive to the classical sulfhydryl reagents, p-chloromercuribenzoysulfonate, mercaptoethanol, and dithiothreitol, the enzyme was also inhibited by cyanide.

**DISCUSSION**

With the purification of daunorubicin reductase from rat liver cytosol, we have found this enzyme capable of catalyzing the reduction of several endogenous substrates. Depending on which compounds are compared, both loose and broad specificity might be inferred. Since D-glucuronolactone, D-glyceraldehyde, D-glucuronate, and daunorubicin all function as substrates, it appears that the enzyme is a general aldo-keto reductase. However, several other closely related aldoses, such as D-ribose, D-glucose, D-galactose, and D-glucose 6-phosphate, do not func-

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1. R. L. Felsted and N. R. Bachur, unpublished observations.

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### Table III

**Apparent kinetic constants toward various substrates**

| Substrate       | $K_m$ (mM) | $k_{cat}$ (sec\textsuperscript{-1}) | $k_{cat}/K_m$ |
|-----------------|------------|-----------------------------------|---------------|
| Daunorubicin    | 0.027      | 1.5                               | 27,000        |
| Adriamycin      | 0.190      | 0.074                             | 360           |
| D-Glucuronolactone | 0.69      | 1.9                               | 2,800         |
| D-Glyceraldehyde | 0.73       | 1.2                               | 1,600         |
| D-Galacturonate  | 1.1        | 1.9                               | 1,700         |
| D-Glucuronate    | 1.8        | 1.9                               | 1,000         |
| D-Glucose 6-sulfate | 63        | 0.80                              | 13.0          |
| D-(−)-Ribose    | 100        | 0.41                              | 4.1           |
| D-(+)-Galactose | 750        | 0.26                              | 0.34          |
| D-(+)-Glucose   | 950        | 0.52                              | 0.55          |
| D-Glucose 6-phosphate | 1,000    | 0.38                              | 0.38          |

### Table IV

**Effect of some common chemicals on daunorubicin reductase activity**

| Additions      | Concentration* (mM) | Relative enzyme activity (%) |
|----------------|----------------------|------------------------------|
| None           | 1.0                  | 100                          |
| p-CMBS\textsuperscript{a} | 0.1                  | 26                           |
| DTT\textsuperscript{a}  | 0.1                  | 50                           |
| KCN            | 1.0                  | 46                           |
| Merscaptoethanol | 1.0                  | 79                           |
| Iodoacetamide  | 1.0                  | 77                           |
| Iodoacetate    | 1.0                  | 80                           |
| NaF            | 1.0                  | 91                           |
| NaAsO\textsubscript{4} | 1.0                  | 97                           |
| MgCl\textsubscript{2} | 1.0                  | 92                           |
| CaCl\textsubscript{2} | 1.0                  | 96                           |
| NaN\textsubscript{3} | 1.0                  | 98                           |
| EDTA           | 1.0                  | 93                           |

* The enzyme was preincubated for 10 min in the presence of the indicated concentrations of inhibitors at 24° and 0.25 M Tris-HCl, pH 8.5. After the addition of NADPH and daunorubicin, the remaining enzyme activity was determined under conditions of the standard assay.

\textsuperscript{a} p-CMBS, p-chloromercuribenzoysulfonate.

\textsuperscript{b} DTT, dithiothreitol.
tion well as substrates. Also, Adriamycin which differs from daunorubicin only by the replacement of a hydrogen with a hydroxyl is a poor substrate. Thus, the enzyme displays a reactivity for substrates which have little similarity, i.e., \( n \)-glucuronolactone versus daunorubicin whereas compounds closely related to both of these active substrates are less or unreactive.

Besides \( n \)-glucuronolactone and \( n \)-glyceraldehyde, the endogenous substrates which have a high degree of reactivity are \( n \)-glucurate and \( n \)-galaconurate, both of which have acidic moieties at the sixth position. However, the alcohol conjugates of these uronic acids, \( n \)-glucose and \( n \)-galactose, have little reactivity. Two other substituted acid sugars, \( n \)-glucose 6-sulfate and \( n \)-glucose 6-phosphate, also have very low substrate activity. It therefore appears that the aldose activity is enhanced with a carboxylic acid group at the sixth position but, as is also illustrated by \( n \)-glucuronolactone, charge alone is not enough to determine substrate activity.

The most effective endogenous substrate tested was \( n \)-glucuronolactone. The ability of daunorubicin reductase to reduce \( n \)-glucuronolactone as well as \( n \)-glucurate suggests that the enzyme may be involved in \( L \)-ascorbic acid synthesis and in the glucuronic acid cycle in vivo. Recommendations (1964) of the International Union of Biochemistry (21) list two enzymes which fill these roles, namely, \( L \)-gulonate: NADP oxido-gluconate dehydrogenase \((1,1,1,19)\) and \( L \)-gulono-\( \gamma \)-lactone:NADP oxido-gluconate dehydrogenase \((1,1,1,30)\). A partially purified preparation catalyzing both of these reactions was previously described from rat liver and was characterized extensively with respect to substrate specificity (22). Our purified enzyme catalyzes both reactions and may normally function at the bifurcation of the \( L \)-ascorbic acid synthesis and the glucuronic acid cycle.

Purification of daunorubicin reductase activity yielded a single protein with enzymatic activity. This conclusion was supported by the results from electrophoresis, gel filtration, and sedimentation equilibrium data. Confidence that the major protein isolated represented the active enzyme was obtained from the coincidence of reductase activity with protein stain after polyacrylamide electrophoresis. Since all criteria examined indicated homogeneity, the reactivity of \( n \)-glucuronolactone, \( n \)-glyceraldehyde, and the uronic acids with daunorubicin reductase apparently represent true multiple substrate specificity.

When the purified reductase was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, a single protein band of molecular weight 39,800 was found. This value was in agreement with the molecular weight of 38,800 obtained from sedimentation equilibrium and indicates that the active enzyme consists of a single polypeptide chain.

Chromatography of the reductase on a calibrated Bio-Gel P-150 column gave a molecular weight of 43,500 in reasonable agreement with electrophoresis and ultracentrifugation data. However, gel filtration on Sephadex columns consistently yielded lower molecular weight values around 30,000. Since the enzyme had reactivity with certain sugars, this slower elution of reductase from Sephadex relative to Bio-Gel columns probably reflected specific interactions of the enzyme with the dextran support.

Inhibition of the reductase by sulphydryl reagents demonstrates both reactive free sulphydryl and disulfide bonds. Since amino acid analysis indicated 4 half-cystine residues per mole of enzyme, this suggests that 2 of the half-cystines exist as free cysteines and the other two are linked via a disulfide bond. Cyanide inhibition might reflect the presence of susceptible metal(s) as part of the active enzyme structure. If metal(s) are present, the stability of the enzyme to dialysis or treatment with chelators imply a very tight or restricted association between metal and enzyme. Conjugation of cyanide with oxidized pyridine nucleotide would not normally be expected to effect the initial rates used as a measure of inhibition. The precise mechanism of the cyanide inhibition is unknown.

When the in vivo metabolism of daunorubicin and Adriamycin in rats and other mammals was studied, it was apparent that the aldo-keto reductase functioned in situ. In the 8 hours after treatment, daunorubicin-treated rats excreted from 50 to 60% of their drug as daunorubicinol in urine and bile (6) whereas Adriamycin-treated rats excreted about 15 to 20% of their drug as Adriamycinol (3). Similarly, rat tissues contained high levels of the reduced product of daunorubicin and lesser amounts of reduced Adriamycin. This degree of in vivo conversion agrees with the reactivity of these substrates with the purified rat liver enzyme. The in vivo experiments also suggest that this reversible reaction favors the reductive pathway. The apparent thermodynamic equilibrium constant obtained from the purified enzyme in the present work verifies this conclusion.

Since daunorubicin reductase has been observed in all of the tissues of several mammalian species tested, it is likely that the characteristics of this enzyme differ both on an interspecies basis as well as on an interorgan basis. More importantly, we wonder if this broad ability of mammalian tissues to reduce these compounds may indicate active cytoplasmic systems which have a yet wider substrate receptivity for exogenous aldehydes and ketones. We are presently investigating this possibility.

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