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RAT LIVER ALDEHYDE REDUCTASE

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Abstract—Rat liver aldehyde reductase is a soluble constitutive enzyme having the ability to catalyze the reduction of several natural aldehydes such as lactaldehyde, glyceraldehyde, glyceraldehyde-3-phosphate, glucuronate, glucuronolactone, and succinic semialdehyde. However, on the basis of apparent K_m, V_{max} and relative pseudo-second order rate constants (k_{cat}/K_m), 4-carboxybenzaldehyde is the best substrate and a number of other xenobiotic aldehydes are excellent substrates for the enzyme. Low or neglible activity is seen for short chain aliphatic aldehydes, 4-hydroxyphenylglycolaldehyde, and myristic aldehyde. The ketones, *p*-nitroacetophenone, D-2,3-boranedione, adriamycin, and *N*-acetyldaunorubicin are poor substrates. The only significant ketone substrate is the antibiotic, daunorubicin, which is reduced with the unique alkaline optimum of pH 8.5. All other substrates (except adriamycin) were reduced at an acid optimum of pH 6.0. The *N*-acetylation of the free amino group of daunorubicin eliminates the alkaline activity suggesting the involvement of this group in the unique alkaline optimum. Reductase activity is noncompetitively inhibited by barbital and phenobarbital, as well as other pharmacologic compounds. Inhibitors of the classical alcohol dehydrogenase do not inhibit the aldehyde reductase. These and other properties suggest that this enzyme belongs to the class of reductases ubiquitously distributed among animal tissues and classified as alcohol:$NADP^+$ oxidoreductases (E.C. 1.1.1.2).

Numerous important carbonyl containing drugs undergo carbonyl reduction as a major biotransformation step in mammals [1–6]. These carbonyl reductions are catalyzed by a group of reductases which are ubiquitous among mammalian tissues and which may have fundamental roles in cellular metabolism [7, 8]. Unfortunately, most studies have utilized crude extracts or partially purified enzymes, and information about these reductases is limited [8].

Daunorubicin is a keto containing cancer chemotherapeutic antibiotic which is reduced to the alcohol, daunorubicinol, by the cytoplasmic enzyme daunorubicin reductase (Fig. 1). Daunorubicin reductase has been purified to homogeneity from rat liver and shown to reduce several naturally occurring compounds. However, the best natural substrate was at least an order of magnitude less reactive than the antibiotic which made the identification of the purified enzyme uncertain [9].

In order to identify the rat liver reductase, "daunorubicin" reductase, we have studied the substrate specificity and kinetic characteristics for a diverse array of aldehyde and ketone substrates under several assay conditions. We have also characterized the enzyme by inhibitor classification and ionic effects.

Surprisingly, we find that daunorubicin reductase has very limited ketone reductase activity but exhibits extensive activity towards xenobiotic and endogenous aldehydes. As a result we identify this enzyme as an aldehyde reductase (E.C. 1.1.1.2) and suggest its possible involvement in numerous physiological processes as well as general drug metabolism.

MATERIALS AND METHODS

Daunorubicin, adriamycin, and *N*-acetyldaunorubicin were obtained from the Drug Development Branch, Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health. NADPH and NADH were obtained from P-L Biochemicals. Bovine serum albumin, $NADP^+$, warfarin, *p*-nitrobenzaldehyde, D-glyceraldehyde (70% pure), D,L-glyceraldehyde-3-phosphate, D-glucuronate, D-glucuronolactone, D-glucose-6-sulfate, D-ribose, D-glucose, D-galactose, oxytetracycline, 1,10-phenanthroline, α,α′-dipyridyl, biliverdin (80% pure), octopamine, indoleacetaldehyde (sodium bisulfite), retinal (trans), γ-hydroxybutyric acid and nicotinic acid were purchased from Sigma. Propionaldehyde, acetaldehyde, pyrazole, isobutrylamide, cyclohexanone, D-2,3-boranedione, *m*-hydroxybenzaldehyde (practical), *p*-nitroacetophenone (practical), *n*-butrylaldehyde, *p*-nitroacetophenone were from Eastman Chemicals. Valeraldehyde, 4-carboxybenzaldehyde, 2-carboxybenzaldehyde, *p*-aminoacetophenone were obtained from Aldrich. Caproic acid was from Applied Science Lab, Inc. and L-glyceraldehyde from Fluka. Trifluorperazine dihydrochloride and chloropromazine hydrochloride were donated by Smith Kline and French Labs. Oxisuran and bunolol were provided by the Warner Lambert Research Institute. Tetramethylene glutamic acid and 1,3-dioxo-1H-benz[de]-isoquinoline-2(3H) acetic acid (AY 22,284) were donated by Dr. Kinoshita. Methadone and phenobarbital were obtained from the U.S. Public Health Service Hospital.

The *p*-Nitroacetophenone was recrystallized three times from ethanol–water and *m*-hydroxybenzaldehyde was recrystallized three times from warm water. D,L-Lactaldehyde was prepared by the reaction of ninhydrin with D,L-threonine [10]. Succinic semialdehyde was prepared from hydroxybutyrolactone as described by Taberner *et al.* [11]. The concentrations of D,L-lactaldehyde and succinic semialdehyde were

determined just before use by their bisulfite binding capacity [12]. L- and D-glyceraldehyde were prepared and the concentration of D-glyceraldehyde was determined by the method of Kormann *et al.* [13]. The 4-hydroxyphenylglycolaldehyde was prepared from octopamine by treatment with monoamine oxidase and the free indoleacetaldehyde was prepared from its sodium bisulfite addition product as previously described [14]. Myristic aldehyde from Pfaltz and Bower, Inc. was purified by silica gel thin layer chromatography which was developed in hexane–chloroform–methanol (75:25:1.5, v/v/v). The major aldehyde spot was extracted with ether, the ether removed with nitrogen gas, and the residue diluted in hexane. Where necessary, aldehydes were visualized on plates with 2,4-dinitrophenylhydrazine [14]. The concentrations of 4-hydroxyphenylglycoaldehyde, indoleacetaldehyde and myristic aldehyde were also determined by reaction with 2,4-dinitrophenylhydrazine as described by Lappin and Clark [15]. Daunorubicin and adriamycin were purified according to the method of Bachur and Cradock [16]. At least 95% of the *N*-acetyldaunorubicin migrated as a single spot ahead of daunorubicin on silica gel thin layer chromatography. Daunorubicinol was prepared enzymatically from daunorubicin with the purified rat liver aldehyde reductase [9] and excess NADPH in 0.2 M Tris–HCl buffer, pH 8.5. The reaction mixture was then desalted on an Amberlite XAD-2 column (Rohm and Haas, Philadelphia, PA) or extracted with *n*-butanol and then purified by counter current chromatography.

The rat liver aldehyde reductase was prepared as previously described [9].

Assays. The standard assay for rat liver aldehyde reductase activity was determined spectrophotometrically in 0.2 M Tris–HCl buffer, pH 8.5 or in 0.1 M potassium phosphate buffer, pH 6.0. The rat liver dehydrogenase activity was determined spectrophotometrically in 0.2 M glycine–NaOH buffer, pH 10.1. Unless otherwise specified, all reductase and dehydrogenase assays included either about 0.18 mM NADPH or about 2.8 mM $NADP^+$, respectively. Assays also included either 0.75 mM daunorubicin, 3.5 mM nitrobenzaldehyde, 37.5 mM n-butrylaldehyde, 70 mM D-glucuronate, 200 mM D-glucuronolactone, 0.53 mM daunorubicinol, 1.54 M glycerol or 1.4 M sorbital as substrates in a final vol. of 1.0 ml. All assays were monitored at 25° in 1 cm light path quartz cuvettes by following the oxidation of NADPH or reduction of $NADP^+$ at 340 nm using a Cary 14 spectrophotometer equipped with a full scale expansion of 0–0.2 absorbance units. Background rates under acid assay conditions were substracted from rates observed after the addition of enzyme. Apparent kinetic constants were determined under the same assay conditions at appropriate substrate concentrations.

Strong ultraviolet absorbing inhibitors of reductase activity were examined by a modification of a previously described fluorescence assay [17]. Daunorubicin was incubated under conditions of the standard pH 8.5 assay in a total vol. of 0.5 ml. After a 20 min incubation, the reaction was terminated by the addition of 0.5 ml of 95% ethanol and an aliquot chromatographed on thin layer silica gel [17]. Reaction rates were determined by extracting the product, daunorubicinol from the silica gel and determining its fluorescence against standards.

Enzyme activity units were defined as μmole of NADPH oxidized (or $NADP^+$ reduced) per min at 25°.

Yeast and horse liver alcohol dehydrogenase (E.C. 1.1.1.1) were assayed spectrophotometrically in 0.2 M Tris–HCl buffer, pH 8.5 containing 1.3 mM NAD^+ and 1.67 M ethanol in a final vol. of 1.0 ml. The reaction was monitored as described for the rat liver enzyme. The ability of alcohol dehydrogenase to metabolize the anthracycline antibiotics was tested by incubating each of the above commercial enzymes in 0.2 M Tris–HCl buffer, pH 8.5 with (1) 0.41 mM daunorubicin and either 21.2 mM NADPH or 10.3 mM NADH and (2) 1.06 mM daunorubicinol and 7.75 mM NAD^+. After 1 hr incubation at 24°, the antibiotics were extracted with *n*-butanol and conversion examined by the fluorescence assay [17].

Solutions of pyridine nucleotides were made fresh daily and concentrations of the reduced and oxidized cofactors determined from molar extinction coefficients of 6.22×10^3 at 340 nm and 18.0×10^3 at 260 nm, respectively. Daunorubicin, daunorubicinol, *N*-acetyldaunorubicin, and adriamycin concentrations were determined with a molar extinction coefficient of 11.4×10^3 at 485 nm.

Emulsification of insoluble substrates in Tween-20 was accomplished by dissolving substrates in 2–3 ml of diethyl ether, adding appropriate amounts of Tween-20, mixing, evaporating the ether under N_2 and dissolving the residue in phosphate buffer.

The effects of ionic strength on the reductase were determined in (1) increasing concentrations of potassium phosphate buffer (pH 6.0); (2) increasing concentrations of glycine–NaOH buffer (pH 8.5); and (3) in 0.05 M glycine–NaOH buffer (pH 8.5) supplemented with increasing concentrations of NaCl or Na_2SO_4. Each salt containing buffer was made up individually and adjusted to pH 8.5. The pH optima was determined similarly to the standard spectrophotometric assays in 0.05 M potassium phosphate or 0.05 M glycine-NaOH buffers. Correction for ionic strength effects do not change the optima indicated in these buffers.

Kinetics. The enzyme displays a K_m for NADPH of 1 μM or less in both the standard pH 8.5 and pH 6.0 assays. All kinetics were therefore carried out in the presence of saturating cofactor. Under these conditions, the enzyme reaction is described by equation (1).

$$v = \frac{V_{max} S}{K_m + S} \quad (1)$$

where v is the rate of the reaction, V_{max} is the maximum rate. S is the substrate concentration and K_m is the Michaelis constant. Kinetic constants were determined by fitting the data to equation (1) by using the least-squares method and assuming equal variance for the velocities [18]. Calculations were performed on the Tymshare computer network using FORTRAN programs [19] which provided K_m, V_{max}, K_m/V_{max}, $1/V_{max}$, and the standard deviations of their estimates. The type of inhibition was determined by

graphically plotting $1/v$ against $1/s$ and the slopes (K_m/V_{max}) and the intercepts ($1/V_{max}$) against the inhibitor concentration. For inhibition experiments, data were then fit to equation (2)

$$v = \frac{V_{max}\, S}{K_m(1 + I/K_{IS}) + S(1 + I/K_{II})} \quad (2)$$

which describes a linear noncompetitive type of inhibition and where I is the inhibitor concentration and K_{IS} and K_{II} are the inhibition constants obtained from secondary plots of slopes and intercepts against I, respectively. All fits assumed equal variances for the observed velocities and used the computer program of Cleland [19].

Assays of different experiments were generally performed in duplicate or triplicate at from 5 to 8 substrate concentrations over at least a 10-fold substrate concentration range. The velocity data from all experiments were then normalized to the same enzyme concentration (2.15 units/mg) by comparing the rates of the different enzyme dilutions or preparations used. Most kinetic constants were calculated from data obtained from at least two different experiments each with freshly prepared substrates and/or inhibitors.

Under conditions where $S \ll K_m$ equation (1) reduces to

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

Here k_{cat} is the overall catalytic rate constant, and E_0 is the enzyme concentration. Protein concentration was determined by the method of Lowry [20] and E_0 calculated using a mol. wt of 39,000 [9]. V_{max} values were determined as described above and converted to k_{cat} values by divinding by E_0. Relative pseudo-second order rate constants were estimated by dividing the apparent k_{cat} by the respective apparent K_m.

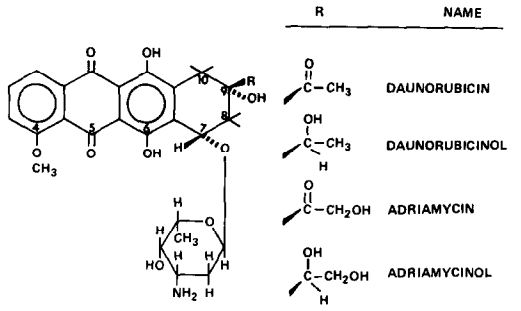

Fig. 1. Structures of daunorubicin, daunorubicinol, adriamycin, and adriamycinol.

RESULTS

Substrate dependent pH optima. When rat liver aldehyde reductase was assayed under conditions of optimal daunorubicin reduction (pH 8.5), other aldehyde and ketone substrates were at least an order of magnitude less reactive [9]. Since most aldehyde and ketone reductases described in the literature function optimally from pH 5.5 to 8.0 [13, 21–35], several aldehydes were compared as possible substrates of the reductase over this lower pH range. Figure 2A shows the apparent pH optima for the reduction of daunorubicin and three aldehyde substrates. The aldehydes were optimally reduced near pH 6.0 with a velocity up to seven times faster than at pH 8.5, the optimum for daunorubicin reduction. Except for the antibiotics, daunorubicin and adriamycin (Fig. 1), all other substrates tested possessed the acid pH optima. The unique reduction of daunorubicin at alkaline pH was eliminated by *N*-acetylation of the amino group on the sugar of the antibiotic suggesting involvement of the free amino group in the preferential alkaline reduction of the drug (See Table 2, below).

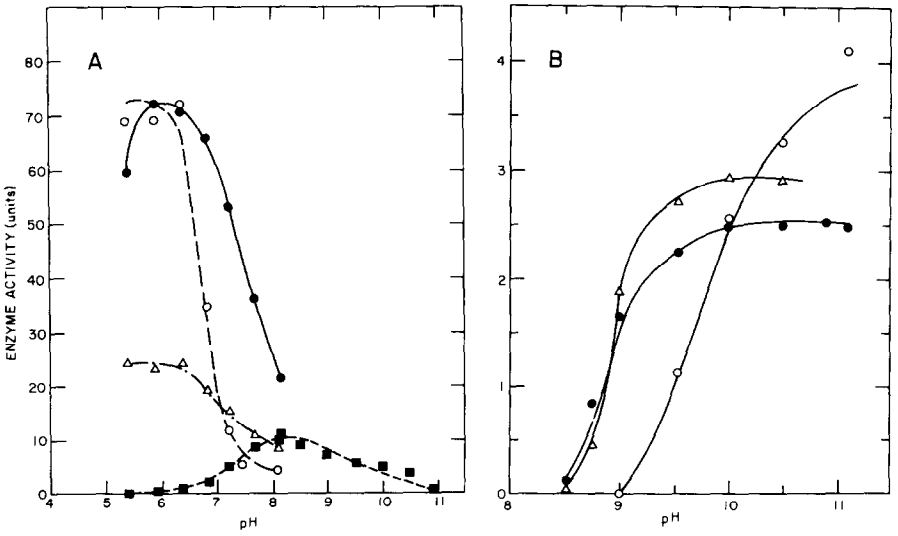

Fig. 2. Apparent pH optima for rat liver reductase; A, reduction of several substrates determined in 0.05 M potassium phosphate buffers (pH 5.4–8.2) and 0.05 M glycine–NaOH buffers (pH 8.1–10.9). The substrates determined were D-glucuronolactone, ○; *n*-butrylaldehyde, △; *p*-nitrobenzaldehyde, ●; and daunorubicin ■; B, pH optima for the enzymatic oxidation of several substrates determined in 0.05 M glycine–NaOH buffers. The substrates determined were sorbitol, △; glycerol, ●; and daunorubicinol, ○.

Table 1. Apparent kinetic constants for substrate oxidation*

Substrate	K_m (mM)	V_{max} (μmole/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
Sorbitol	1,070 ± 70†	4.33 ± 0.187	0.612	0.572
Glycerol	576 ± 32	3.14 ± 0.078	0.444	0.770
Daunorubicinol	0.108 ± 0.012	3.81 ± 0.169	0.538	4,980

* Kinetic constants determined at pH 10.1 as described in Methods Section.
† Standard deviations.

The enzyme also displayed a pH dependent dehydrogenase activity. Despite the thermodynamic equilibrium favoring reduction [9], the reverse reaction occurred at elevated pH values (Fig. 2B). Analogous with the reductase activity, the oxidation of the antibiotic, daunorubicinol, proceeded under more alkaline conditions relative to the polyglycols sorbital and glycerol. Kinetic constants determined for the reverse reaction with these alcohols were obtained at pH 10.1 (Table 1). The reversal of other aldehyde and ketone reductases have been demonstrated under similar conditions [22, 23, 25, 27, 35].

Ketone substrate specificity. In addition to its unique alkaline reaction optimum, daunorubicin is the only ketone significantly reduced by this enzyme. Even the closely related ketone antibiotics, *N*-acetyldaunorubicin and adriamycin, are one and two orders of magnitude less reactive, respectively. A comparison of kinetic parameters of other ketone substrates were determined by the standard pH 8.5 and pH 6.0 assays (Table 2). All ketones examined, except daunorubicin and adriamycin, exhibited acid pH optima. The ketones *p*-nitroacetophenone and *d*-2,3-boranedione exhibited high K_m and low V_{max} values and have solubilities which precluded reliable estimations of kinetic constants. Therefore, they were compared at specified substrate concentrations. Other drugs and ketones which are inactive with 0.1–0.2 μM enzyme in the standard pH 6.0 assay at the indicated ketone concentrations include oxisuran (10 mM), bunolol (1 mM), retinal (0.04 mM in 2% ethanol), cyclohexanone (0.65 M) and *p*-aminoacetophenone (0.5 mM).

The ketones warfarin and methadone are not reduced. In fact, they are moderate inhibitors of daunorubicin reduction (see below). Since warfarin is actively reduced by crude extracts of rat liver, the presence of a reductase other than daunorubicin reductase is therefore suggested [36].

Aldehyde specificity. In contrast to the low activity of ketone substrates, a variety of aldehydes, including xenobiotic as well as naturally occurring compounds, function well as substrates for daunorubicin reductase. The apparent kinetic constants of these substrates were determined at pH 6.0 (Table 3). Among the compounds examined in Table 3, as the K_m value increases, the second order rate constant (k_{cat}/K_m) generally decreases, reflecting decreasing catalytic activity.

Benzaldehydes containing electron withdrawing groups in the para position were the best substrates tested. As measured by the relative second order rate constants (k_{cat}/K_m) the optimum reduction of 4-carboxybenzaldehyde (pH 6.0) was about 23 times greater than the optimum daunorubicin reduction (pH 8.5). Surprisingly, 2-carboxybenzaldehyde was not reduced.

From the kinetic constants (Table 3), it is apparent that rat liver aldehyde reductase is capable of reducing a number of physiologically occurring compounds. For example, indoleacetaldehyde which is a model compound for the physiological metabolite of serotonin was very efficiently reduced. In contrast, 4-hydroxyphenylglycolaldehyde (1 mM), a metabolite of the biogenic amine, octopamine, was slowly

Table 2. Comparison of apparent kinetic constants of ketone substrates

Substrate	K_m (mM)	V_{max} (μmole/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)	Specific activity (μmole/min/mg)
pH 6.0*					
Daunorubicin	0.257 ± 0.0504†	1.03 ± 0.115	0.145	566	0.206‡ (0.178)§
N-Acetyldaunorubicin	0.0508 ± 0.00358	1.21 ± 0.0255	0.171	3,370	0.175¶ (0.174)§
p-Nitroacetophenone	—‖	—	—	—	0.0343‡
d-2,3-Boranedione	—	—	—	—	0.121‡,**
pH 8.5*					
Daunorubicin	0.0800 ± 0.00521	10.6 ± 0.173	1.50	18,700	-
Adriamycin	0.220 ± 0.0213	0.546 ± 0.0169	0.0771	351	-
N-Acetyldaunorubicin	0.0688 ± 0.00511	0.858 ± 0.0172	0.121	1,760	-

* See Methods Section for standard assay conditions.
† Standard deviations.
‡ Determined at a substrate concentration of 1 mM.
§ Calculated using the K_m and V_{max} from the Table, the indicated substrate concentration, and equation (1) (See Methods Section).
‖ Not determined.
¶ Determined at a substrate concentration of 0.1 mM.
** Determined in presence of 1% Tween-20.

Table 3. Apparent kinetic constants for reduction of aldehyde substrates*

Substrate	K_m (mM)	V_{max} (μmole/min)	k_{cat}/K_m ($s^{-1}M^{-1}$)
4-Carboxybenzaldehyde	0.0262 ± 0.00378†	79.3 ± 5.23	427,000
p-Nitrobenzaldehyde	0.211 ± 0.0118	98.9 ± 1.94	66,000
Indoleacetaldehyde	0.488 ± 0.0478	82.7 ± 5.58	23,900
D,L-Lactaldehyde	1.27 ± 0.0973	37.7 ± 1.39	4,180
D-Glyceraldehyde	2.43 ± 0.317	68.6 ± 3.99	3,980
L-Glyceraldehyde	4.21 ± 0.312	43.7 ± 1.25	1,470
D,L-Glyceraldehyde-3-phosphate	4.60 ± 0.668	64.7 ± 3.57	1,990
D-Glucuronate	6.23 ± 0.806	89.1 ± 5.18	2,020
Succinic semialdehyde	10.9 ± 4.77	26.0 ± 9.48	337
D-Glucuronolactone	26.3 ± 4.03	78.5 ± 4.82	421
n-Butyraldehyde	34.8 ± 4.17	55.6 ± 3.75	226
Propionaldehyde	36.2 ± 4.15	51.6 ± 2.35	201
D-Glucose-6-sulfate	46.1 ± 7.65	5.85 ± 0.490	17.9
D-Ribose	107 ± 31.4	5.57 ± 1.11	7.35
Acetaldehyde	130 ± 49.8	2.10 ± 0.45	2.30
D-Glucose	8,000 ± 1,702	19.2 ± 3.52	0.340
D-Galactose	20,700 ± 15,900	106 ± 81	0.722

* Kinetic constants determined at pH 6.0 as described in Methods Section
† Standard deviation.

reduced with an initial rate of 0.742 ± 0.089 μmole/min/mg of enzyme in the standard pH 6.0 assay. 4-Hydroxyphenylglycolaldehyde and other biogenic aldehydes are readily reduced by rat brain aldehyde reductase [14]. Low but measurable activity was also found with short chain length aliphatic aldehydes. Reactivity generally increased with increasing chain length. However, the long chain fatty aldehyde, myristic aldehyde (1 mM), was not reduced in the standard pH 6.0 assay in the presence of 1% Tween-20. *m*-Hydroxybenzaldehyde and valeraldehyde were significantly reduced but low solubilities precluded reliable estimations of kinetic constants.

Cofactor requirements for different substrates. One distinguishing characteristic of aldehyde and ketone reductases are their nearly universal preference for NADPH as a cofactor. The cofactor preference of the rat liver reductase for daunorubicin, D-glucuronate, and *p*-nitrobenzaldehyde were determined by assaying in the presence of 0.277 mM NADH or 0.183 mM NADPH. Whereas daunorubicin (pH 8.5 assay) was reduced exclusively with NADPH confirming previous observations [9], D-glucuronate and *p*-nitrobenzaldehyde (pH 6.0 assay) were reduced by NADH at 7–8% of the rate sustained by NADPH. This slow reduction was non-linear, however, suggesting nonsaturating dinucleotide concentrations. Considering the low K_m of the reductase for NADPH ($\leq 1\ \mu M$) the rates sustained by NADH may reflect contamination of the commercial dinucleotide with traces of NADPH. Daunorubicin reductase clearly prefers NADPH as a cofactor, however, the possibility that the enzyme exhibits an elevated K_m toward NADH is not excluded.

Multiple substrate specificity. Although multiple substrate specificities have been described for many aldehyde and ketone reductases most enzyme preparations are either partially purified or contain demonstrated multiple enzymatic species. In such systems apparent multiple substrate specificity is still possibly the result of the action of several enzymatic species. To assess the purity of the rat liver reductase preparations, we tested the ability of the enzyme to reduce daunorubicin, *p*-nitrobenzaldehyde and D-glucuronate at each step of a typical enzyme purification (Table 4). Whereas the *p*-nitrobenzaldehyde reduction dropped relative to daunorubicin reduction over the first two steps and then remained constant, the D-glucuronate to daunorubicin reduction ratio remained essentially constant over the entire 1300 fold purification. It seems reasonable to assume that if different enzymes are responsible for all or part of the multiple

Table 4. Copurification of reductase activities

Purification Step	Reductase Activity Toward Various Substrates*			Ratio of Substrate Activity		
	Daunorubicin	*p*-Nitrobenzaldehyde	D-glucuronate	*p*-nitrobenzaldehyde daunorubicin	D-glucuronate daunorubicin	Purification (-fold)
1. High Speed Supernate	0.063	2.9	0.53	46	8.3	1
2. $(NH_4)_2SO_4$ 0.35–0.65	0.31	14.0	1.1	46	3.7	2.5
3. DEAE-Cellulose	0.81	6.2	3.8	7.6	4.7	25.0
4. Hydroxylapatite	7.7	72.0	39.0	9.4	5.1	1100
5. Gel Filtration	6.1	31.0	47.0	5.2	7.7	1300

* Activities toward *p*-nitrobenzaldehyde and D-glucuronate were determined at pH 6.0 and activities toward daunorubicin determined at pH 8.5 (see Methods Section) and expressed as μmole/min/ml.

substrate specificity, that the distinct classes of compounds represented by these three substrates (a ketone, aromatic aldehyde and aldo sugar) would reveal some differential purification. Apparent removal of a *p*-nitrobenzaldehyde reductase activity over the first two steps in fact confirm that multiple aldehyde reductases do exist *in vivo*. The constant ratios of *p*-nitrobenzaldehyde and D-glucuronate to daunorubicin reduction over the latter steps of the purification, however, strongly support a one enzyme-multiple substrate proposal and confirm the broad substrate specificity.

Since the classical alcohol dehydrogenase is also capable of reducing several of the substrates [37] reduced by the rat liver enzyme, its ability to reduce daunorubicin was checked. Commercial preparations of horse liver or yeast alcohol dehydrogenase did not reduce daunorubicin with either NADPH or NADH and did not oxidize daunorubicinol with NAD^+.

Inhibitors. Like other aldehyde reductases the rat liver reductase is inhibited by a number of pharmacologically active compounds (Table 5). Barbiturate inhibition of aldehyde reductases have previously been used to distinguish this class of enzymes from the similar alcohol dehydrogenases [38]. Barbiturates inhibit daunorubicin reductase in a linear noncompetitive fashion typical of other aldehyde reductases [38]. Barbital and phenobarbital inhibit daunorubicin reduction in the standard pH 8.5 assay with K_i values of 19.1 (± 2.4) and 59.1 (± 5.9) μM, respectively (Fig. 3). Pyrazole, which is a potent inhibitor of *p*-nitrobenzaldehyde reduction with NADPH by rat liver alcohol dehydrogenase [39] did not significantly affect daunorubicin reduction. Similar to other reductases [21, 25, 30], classical metal chelators were also without significant effect on this enzyme (Table 5).

Other linear non competitive inhibitors include

Table 5. Inhibition of daunorubicin reduction

Additions*	Concentration mM	Relative enzyme activity %
None		100
Barbital	0.1	19
Warfarin	1.0	9
Phenobarbital	1.0	12
Nicotinic	1.0	57
Oxytetracycline†	1.0	62
AY22,284†,‡	1.0	64
Methadone	1.0	67
Tetramethylene glutamic acid†	1.0	83
1,10-Phenanthroline	1.0	90
α,α'-Dipyridyl	1.0	90
Pyrazole	10.0	82
Isobutyramide	10.0	86

* Except where otherwise stated, 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. Remaining enzyme activity was then determined by the addition of NADPH and daunorubicin and assayed with the standard pH 8.5 spectrophotometric assay.

† Assayed by fluorescence assay without a preincubation with enzyme.

‡ 1,3-dioxo-1H-benz[de]-isoquinoline-2(3H) acetic acid.

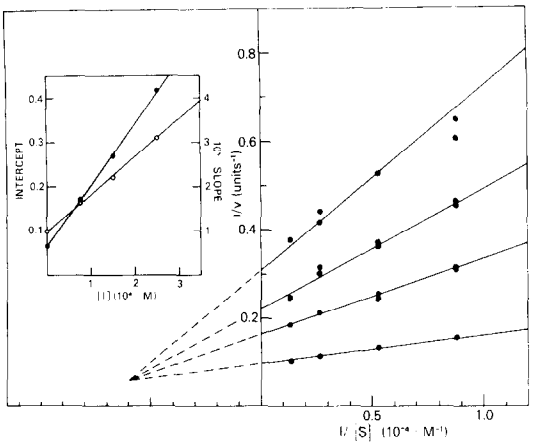

Fig. 3. Inhibition of rat liver daunorubicin reductase activity by phenobarbital in the standard pH 8.5 assay (see Methods Section). Inhibitor concentrations were 75, 150, and 250 μM in phenobarbital. The insert is a replot of the intercepts (–○–) and the slopes (–●–) against the respective inhibitor concentrations.

warfarin and methadone with K_i values of 0.079 (± 0.018) and 8.8 (± 6.2) mM, respectively, in the standard pH 8.5 assay. Rat liver aldehyde reductase was also sensitive to tetramethylene glutamic acid and 1,3-dioxo-1H-benz[de]-isoquinoline-2(3H) acetic acid (Table 5) which are potent inhibitors of lens aldose reductase [39, 40].

Although aldehyde reductases from brain and kidney are not affected by nonionic detergents and organic solvents [22, 23], Triton-X100, Tween 20, ethanol, and methanol, at a concentration of 1 per cent inhibited daunorubicin reduction about 20 per cent. Sensitivity to Tween 20 was also reported for bovine cardiac muscle aldehyde reductase [41].

Anions also affect the activity of rat liver aldehyde reductase. Low concentrations of NaCl and Na_2SO_4 stimulate daunorubicin reduction from 30–50 per

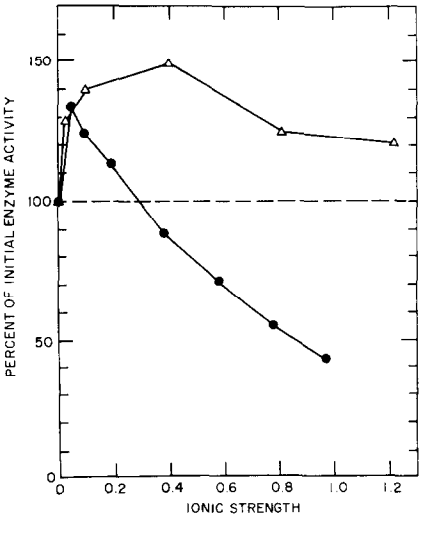

Fig. 4. Effect of salts on rat liver reductase activity. Assays performed in 0.05 M glycine NaOH buffers, pH 8.5, supplemented with increasing concentrations of NaCl (–●–) and Na_2SO_4(–△–) using daunorubicin as substrate.

cent. Further increases in ionic strength result in a rapid decrease of activity in the case of NaCl, 50 per cent of control values at an ionic strength of about 0.8. Higher concentrations of Na_2SO_4 resulted in a slight drop but even at an ionic strength of 1.2 the reductase activity is still 120 per cent of control (Fig. 4). Increasing concentrations of glycine–NaOH buffer (pH 8.5) did not stimulate daunorubicin reduction, but resulted in a 10 per cent inhibition at ionic strengths around 0.5. On the other hand, increasing concentrations of potassium phosphate buffer (pH 6.0) resulted in a 2.5-fold stimulation of *p*-nitrobenzaldehyde reduction at an ionic strength of 0.3–0.4. The enzyme was also sensitive to other organic anions. Caproate, citrate and ascorbate at 1 mM concentrations inhibit daunorubicin reduction 63, 15, and 10 per cent respectively. Similar behavior has been noted with lens aldose reductase [26], rat liver L-hexonate dehydrogenase [30] and kidney aldehyde reductase [25].

Cardiac and brain aldehyde reductases are inhibited by phenothiazine derivatives [35, 41, 42]. The reduction of *p*-nitrobenzaldehyde in the standard pH 6.0 assay was not affected by 1 mM of either chloropromazine or trifluorperazine. This difference between the brain and cardiac reductases and rat liver reductase is consistent with the low activity of the liver enzyme toward 4-hydroxyphenylglycolaldehyde and long chain fatty aldehydes, respectively (above), and imply a role-dependent specificity of the aldehyde reductases from different tissues.

DISCUSSION

Daunorubicin reductase is ubiquitously distributed among mammalian tissues, and we previously reported its purification from rat liver [9]. Although the ketone antibiotic was the most active substrate known at that time, several naturally occurring aldose sugar substrates were also described. As a result the enzyme was described as a mixed aldehyde and ketone reductase with a possible role in normal glucuronate metabolism.

In our continuing study of this and similar enzymes, we have tested other substances as substrates with the rat liver enzyme. We found the enzyme's substrate specificity to include a wide range of aldehydes. In addition, we have found that all aldehyde, aldose, and ketone substrates (except daunorubicin and adriamycin) are preferentially reduced with an acid optima pH of about 6.0. On the basis of the relative second order rate constants (k_{cat}/K_m) the enzyme has the highest reductive efficiency toward substituted aromatic aldehydes.

Although the rat liver reductase actively reduces aldehydes, ketones are less acceptable as substrates. For example, the enzyme has negligible activity toward the ketones, oxisuran, retinal, bunolol, *p*-aminoacetophenone, acetone, pyruvate, cyclohexanone, and some steroid hormones [9]. Low but measurable activity was found for *p*-nitroacetophenone and *d*-2,3-boranedione although the cyclic ketones methadone and warfarin actually inhibited. Of particular interest is the preference for *p*-nitrobenzaldehyde over *p*-nitroacetophenone which illustrates the enzyme aldehyde specificity. Another important comparison is the reactivity of the reductase with *p*-nitroacetophenone, *p*-aminoacetophenone, warfarin, and daunorubicin. Since all four compounds are methyl ketones, daunorubicin's high rate of reduction and much lower K_m over the other compounds suggest that other structural characteristics enhance it as a substrate. This methyl ketone is uniquely reduced by an enzyme which is otherwise specific for aldehyde substrates. Whatever the reasons for daunorubicin's substrate activity it is the exception rather than the rule, and the enzyme functions primarily as an aldehyde reductase.

Aldehyde and ketone reductases have been reported from various mammalian tissues including brain [21, 22, 43], kidney [23–25, 44], placenta and seminal vesicles [45], skeletal muscle [13], lens [26], liver [27–32], erythrocytes [33, 34], and heart [35, 41]. These enzymes are implicated in the metabolism of biogenic aldehydes [14], succinic semialdehyde [46], fatty aldehydes [41], lactaldehyde [23], aldo-sugars [13, 26, 27], as well as numerous xenobiotic aldehydes and ketones [13, 21–23, 25, 26, 30, 33, 34, 41, 46, 47].

Bosron and Prairie [48] suggested that aldehyde reductases were similar since they reduced a wide range of aromatic aldehydes, uncyclized aldoses and D-glucuronate, were NADPH linked, had low mol. wt, and were inhibited by barbiturates. They recommended that enzymes possessing these characteristics be reclassified as alcohol·$NADP^+$ oxidoreductases (E.C. 1.1.1.2) or trivially as NADPH linked aldehyde reductases. The rat liver aldehyde reductase would fit in this classification. In addition, the property of low affinity of daunorubicin reductase to DEAE-cellulose and hydroxylapatite allowed an independent purification of this enzyme which is remarkably similar to purifications developed by other investigators for other aldehyde and ketone reductase activities [22, 25, 43].

The classical alcohol dehydrogenase also reduces aldehydes and ketones and is implicated in the physiological metabolism of carbonyl containing substrates [49]. A distant relationship has been suggested for the general class of NADPH linked aldehyde reductases and classical alcohol dehydrogenase from [50]. Although this relationship remains possible, alcohol dehydrogenase from horse liver and yeast are clearly unable to oxidize or reduce the antibiotics. In addition, since most properties of the rat liver aldehyde reductases are distinct from those analogous properties ascribed to rat liver alcohol dehydrogenase [51, 52], they are clearly separate and distinct enzymes.

The rat liver reductase has intermediate activity toward a number of physiological substrates including D,L-lactaldehyde, D-glyceraldehyde, D,L-glyceraldehyde-3-phosphate, succinic semialdehyde, D-glucuronate, and D-glucuronolactone. Although most of these naturally occurring substrates have high V_{max} values, their high K_m values result in intermediate apparent second order rate constants (Table 3). Based on these kinetic constants, no single physiological compound can be identified as the best substrate. The reductases probably fulfill a broad spectrum of functions depending on tissue or origin. For example, it has been established that the biogenic monoamines are converted in the brain by monoamine oxidase to

their aldehyde derivatives [53]. Brain tissue reductases metabolize some of these aldehyde intermediates to their respective alcohol products [14]. The specific roles in other tissues are not as well defined although with nearly a universal activity toward glyceraldehyde, aldehyde reductases are probably involved in glycerol metabolism [glycerol:$NADP^+$ oxidoreductase (E.C. 1.1.1.27)] [13, 28]. Other possible physiological roles for aldehyde reductase type enzymes are (1) involvement as aldose reductases in sugar metabolism for example in lens [26], liver [30], placental and seminal vesicles [45], and other tissues [54], [alditol:$NADP^+$ oxidoreductase (E.C. 1.1.1.21); or L-gulonate:$NADP^+$ oxidoreductase (E.C. 1.1.1.19)]; (2) the reduction of fatty aldehydes for the synthesis of glycerol ethers in cardiac tissues (fatty aldehyde reductase) [41]; (3) the reduction of mevaldic acid to mevalonic [mevalonate:$NADP^+$ oxidoreductase (E.C. 1.1.1.33)] [55]; and (4) vitamin A metabolism in rat intestinal mucosa [retinol:NAD^+ oxidoreductase (E.C. 1.1.1.105)] [56]. Even with these possibilities as roles of physiological function, the ubiquitous distribution and constitutive nature of the reductases and their potential role in aldehyde detoxification and drug metabolism constitute an important justification for the existence of this class of enzymes.

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Note added in proof. Since the submission of this work, a study by A. J. Turner and P. E. Hick has appeared with similar conclusions (*Biochem. J.* **159**, 819 (1976).

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