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HETEROGENEITY OF ANTHRACYCLINE ANTIBIOTIC CARBONYL REDUCTASES IN MAMMALIAN LIVERS

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Abstract—The anticancer antibiotics, daunorubicin and adriamycin, are reduced to their corresponding alcohol and glycol metabolites by cytoplasmic carbonyl reductases occurring in human, rabbit, rat and mouse tissues. Our data indicate that at least two different groups of pH-dependent daunorubicin reductases occur in rabbit and human liver: (1) the pH activity profile for daunorubicin reductases shows distinct optima or significant activity at both pH 6.0 and 8.5, and (2) pH 8.5-dependent daunorubicin reductases are resolved from pH 6.0-dependent daunorubicin reductases by ion exchange chromatography on DEAE-cellulose columns, gel filtration chromatography on BioGel P150, and isoelectric focusing. Ion exchange chromatography and isoelectric focusing also resolve multiple forms of each class of activities. A similar analysis suggests that a single type of pH-dependent daunorubicin reductase occurs in rat and mouse livers: (1) rat and mouse livers show a single pH optima for daunorubicin at pH 8.5, and (2) isoelectric focusing of rat and mouse preparations confirms the existence of a pH 8.5 daunorubicin and the absence of significant pH 6.0 daunorubicin activity. Although total adriamycin reduction is lower than daunorubicin reduction at any pH, significant adriamycin reduction also occurs in rabbit liver at pH 6.0 and 8.5; however, neither of these activities can be distinguished from pH 6.0 daunorubicin reductase activity by ion exchange and gel filtration chromatography and isoelectric focusing. In comparison, very low levels of pH 6.0 optimum adriamycin reductase activity are seen in human, rat and mouse livers. Thus, all species have pH 8.5 daunorubicin reductase and probably pH 8.5 adriamycin reductase, whereas rabbit and human also have pH 6.0 daunorubicin reductase(s) and rabbit has a pH 6.0 adriamycin reductase(s), which accounts for the bulk of the active anthracycline antibiotic metabolism in these species.

The cancer chemotherapeutic antibiotics, daunorubicin and adriamycin, are metabolized in mammals and by cell extracts to their respective alcohol or glycol glycosides [1–4]. The enzymes responsible for these reductions are constitutive, occur primarily in the cellular cytosol, and require the cofactor NADPH [5]. Indications from *in vivo* anthracycline metabolism and *in vitro* tissue extract studies are that the reduction of the methylketone group of daunorubicin occurs in preference to the reduction of the hydroxymethyl ketone group of adriamycin in all species; however, enhanced rates of daunorubicin and adriamycin reduction do occur in rabbits and humans.

Daunorubicin reductase has been purified to homogeneity previously from rat liver [6] and identified as an aldehyde reductase [7, 8]. A comparison of the kinetics of daunorubicin and adriamycin reduction in several mammalian tissues suggested that rabbit liver might contain several anthracycline antibiotic reductases [9]. In the present study, a detailed comparison of the properties of the anthracycline antibiotic reductases from several mammalian species confirms the existence of enzymes previously unknown in rabbit and human liver in addition to aldehyde reductase. The presence of these new enzymatic activities probably accounts for the elevated anthracycline antibiotic drug metabolism seen in rabbits and humans. These observations also confirm the widespread occurrence as well as the variety of aldo-keto reductases in mammalian tissues.

EXPERIMENTAL PROCEDURE

Materials

Daunorubicin was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD, and adriamycin was provided by Adria Laboratories, Milan, Italy. Daunorubicin was purified by the method of Bachur and Cradock [10]. NADPH was obtained from P-L Biochemicals, Milwaukee, WI, and DEAE cellulose (DE-11) from Whatman, Clifton, NJ. Carrier ampholytes and Bio Gel P150 were from LKB, Richmond, CA, and Bio Rad Laboratories, Rockville, MD respectively.

Livers were obtained from adult male DBA2 mice, Sprague–Dawley rats and New Zealand white rabbits. Adult human liver was obtained at autopsy within 24 hr of accidental death. Enzyme preparations were prepared from fresh liver except for some human liver which was stored at −15° for up to 6 months prior to use.

Enzyme preparations

All solutions for enzyme preparations were maintained at 0–4°.

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Step 1: *Extraction.* After the livers were homogenized in a Potter–Elvehjem homogenizer with 3 vol. of 2.5 mM potassium phosphate buffer, pH 7.5, containing 0.25 M sucrose, and 5 mM β-mercaptoethanol, the homogenates were centrifuged at 27,000 g for 20 min. The 27,000 g supernatant solutions were further clarified by centrifugation at 80,000 g for 90 min.

Step 2: *Ammonium sulfate fractionation.* The 80,000 g supernatant solution was mixed with solid ammonium sulfate to 30% saturation and the resulting precipitate was discarded. The supernatant solution was fractionated further by the addition of ammonium sulfate to 60% saturation. This second precipitate, which contained most of the activity, was dissolved in a minimal volume of 0.01 M Tris–HCl, pH 7.5, containing 5 mM β-mercaptoethanol and dialyzed overnight against two changes of a total of 100 vol. of the same buffer. This dialyzed fraction was used in all experiments, unless stated otherwise.

DEAE-cellulose chromatography

DEAE cellulose was washed twice with 0.1 M HCl, twice with 0.1 M NaOH, and equilibrated with 0.1 M Tris–HCl, pH 7.5, containing 5 mM β-mercaptoethanol. The dialyzed ammonium sulfate fraction was applied to this column (26 × 4 cm) and washed with the equilibration buffer. The enzymatic activity was eluted by a linear salt gradient made from 4 l. of the equilibration buffer and 4 l. of equilibration buffer containing 0.3 M NaCl. Fractions (25 ml) were collected, monitored for protein at 280 nm, assayed for both adriamycin and daunorubicin reductase activity at pH 6.0 and 8.5.

Calibrated gel filtration chromatography

The molecular weights of adriamycin and daunorubicin reductases were estimated by gel filtration on a column (2.5 × 36 cm) of BioGel P150, equilibrated in 0.05 M potassium phosphate buffer, pH 7.4, containing 5 mM β-mercaptoethanol. The reference proteins used for calibration of the column included bovine serum albumin, ovalbumin, chymotrypsinogen and ribonuclease. Standard curves were established by plotting the logarithm of molecular weights vs K_{av} where $K_{av} = (V_e - V_o/V_t - V_o)$ and where V_e is the elution volume for the protein, V_o is the elution volume for blue dextran and V_t is the total bed volume.

Isoelectric focusing

Isoelectric focusing was performed in an LKB 8101 electrofocusing column (110 ml capacity) with 1% carrier ampholyte (pH range 4–7) and a stabilizing sucrose gradient containing 0.5 mM dithiothreitol. Dialyzed ammonium sulfate fractions were mixed into the sucrose gradient of the column by addition of the enzyme solution to the less dense solution. Electrofocusing lasted about 70 hr at 4° with an initial power of 3.0 W. After electrofocusing was completed, the column contents were collected in 1-ml fractions at a flow rate of 60 ml/hr, and pH measurements were performed immediately at 4°. Reductase assays followed within 48 hr.

Daunorubicin reductase assay at pH 6.0 *and* 8.5

Daunorubicin reductase was assayed spectrophotometrically by following the oxidation of NADPH at 340 nm in a Cary 118 spectrophotometer. The reaction mixture contained 0.16–0.18 mM NADPH and 0.75 mM daunorubicin and either 0.25 M Tris–HCl buffer, pH 8.5, or 0.08 M potassium phosphate buffer, pH 6.0, at 25° in a final volume of 1.0 ml. At these concentrations both substrates were at least ten times their K_m. The reaction was initiated by the addition of the enzyme; background rates of NADPH oxidation in the absence of daunorubicin were subtracted from the rate observed in the presence of the antibiotic.

Adriamycin reductase assay at pH 6.0 *and* 8.5

Adriamycin reductase was assayed in a final volume of 0.5 ml with 1 mM NADPH and 2.7–2.8 mM and 1.5–1.6 mM adriamycin at pH 8.5 and 6.0, respectively, as described above. The mixtures were first incubated at 60° for 5 min to dissolve precipitated adriamycin, and then reincubated at 37° for 5 min before starting the reactions by the addition of enzyme. The reaction mixtures were incubated at 37° for 5–20 min and stopped by the addition of 0.25 ml isopropanol, mixing and placing on ice. The reaction mixtures were then saturated with ammonium sulfate and centrifuged at room temperature to separate the phases. Aliquots (10–40 μl) of the isopropanol phase were removed and applied to silica gel thin-layer plates and the adriamycinol was quantified as described previously [4]. Cofactor and adriamycin concentrations were saturating for all tissues [9]. Relative adriamycin reductase activites for the gel filtration, DEAE cellulose, isoelectric focusing and pH optimum profiles were assayed as above, except that adriamycin was 0.13–0.15 mM. At this lower concentration, adriamycin is apparently saturating for rabbit liver but it is possibly less than saturating for human liver [9]. In any case, the reactions were linear with protein and incubation times for all assays.

pH Profile assays

The pH profiles of adriamycin and daunorubicin reductases were determined in the buffers indicated, at buffer concentrations giving conductivity readings of 14–17 milliSiemens (Radiometer Conductivity Meter CDM 3C). Otherwise assays were identical to enzyme assays above.

Analytical methods

Protein estimations were made by the method of Lowry *et al.* [11]. Solutions of NADPH were prepared fresh daily and concentrations were determined from a molar extinction coefficient of 6.22×10^3 at 340 nm. Daunorubicin and adriamycin concentrations were determined with a molar extinction coefficient of 11.4×10^3 at 485 nm.

RESULTS

Effect of pH on anthracycline antibiotic reductase activites

Dialyzed ammonium sulfate preparations were used to examine the pH activity profile of rabbit, human, rat and mouse liver daunorubicin reductases (Fig. 1, panels A and B). Optimal daunorubicin reduction in

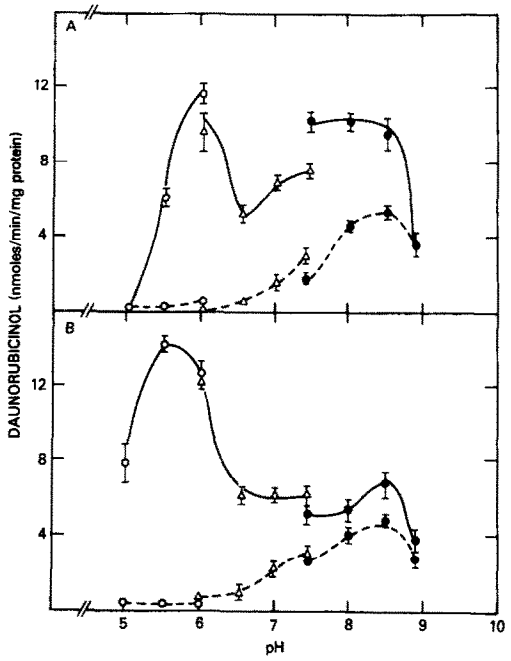

Fig. 1. Activity pH profiles of dialyzed ammonium sulfate fractions of liver daunorubicin reductase. Panel A: human (——) and rat (– – –); panel B: rabbit (——) and mouse (– – –). Buffers used were citrate phosphate (pH 5.0, 5.5 and 6.0, O——O); potassium phosphate (pH 6.0, 6.6, 7.0 and 7.5, △——△); and Tris–HCl (pH 7.5, 8.0, 8.5 and 8.8, ●——●). Assays were run within 48 hr of enzyme preparation as described in Experimental Procedure.

crude mouse and rat liver preparations occurs at pH 8.5 as observed previously for purified rat liver daunorubicin reductase [6]. In contrast, rabbit and human liver enzyme preparations show two distinct daunorubicin reductase optima with maximum activity occurring at pH 6.0 and lower but with a significant activity optimum occurring around pH 8.5. The results for rabbit liver are dependent upon the use of fresh enzyme preparations because, after 48 hr, the pH activity profile changes to a broad plateau between pH 6.0 and 8.5 (data not shown).

Dialyzed ammonium sulfate fractions of rabbit, human, rat and mouse livers were similarly assayed for pH-dependent adriamycin reductase activity (not shown). The rabbit and human livers show optimal or significant activity at pH 8.5. In addition, rabbit liver adriamycin reductase shows a second optimum at pH 6.0. Total adriamycin reduction is consistently higher in rabbit liver than in human liver preparations. In comparison, rat and mouse liver adriamycin reduction is very low at pH 8.5 and negligible at pH 6.0.

The specific activites of rabbit, human, mouse and rat liver daunorubicin and adriamycin reductases were determined at pH 6.0 and 8.5 (Table 1). Significant pH 8.5 daunorubicin reduction occurs in rabbit, human, rat and mouse. High levels of pH 6.0 daunorubicin reduction also occurs in rabbit and human but are completely absent in rat and mouse. Similarly, although total adriamycin reduction is much lower than daunorubicin reduction in all species, significant pH 6.0 adiramycin reduction is measured in rabbit but not in human, rat and mouse livers. In addition, compared to the other species, adriamycin reduction in rabbit and human livers is elevated relative to daunorubicin reduction.

These results confirm the enhanced daunorubicin and adriamycin reductase activities previously reported for rabbit and human livers [9]. The results also confirm the presence of a pH 8.5 optimum anthracycline antibiotic reductase in all four species, which is probably identical or similar to the pH 8.5 daunorubicin reductase previously purified to homogeneity from rat liver [6]. The data also suggest that the increased anthracycline antibiotic reduction in rabbit and human is explained, at least partially, by the presence of a previously unknown pH 6.0 anthracycline antibiotic reductase(s). Therefore, at least two classes of pH-dependent antibiotic reductases occur in rabbit and human livers whereas only a single class of antibiotic reductase(s) is found in mouse and rat liver.

Isolation of multiple molecular forms of anthracycline antibiotic reductases by gel filtration and ion exchange chromatography and by isoelectric focusing

Human and rabbit liver dialyzed ammonium sulfate fractions were chromatographed on calibrated columns of BioGel P150. Gel filtration chromatography resolves pH 8.5 daunorubicin and pH 6.0 daunorubicin reductases into two closely eluting but distinct symmetrical activity peaks (Fig. 2). The pH 8.5 daunorubicin reductase(s) consistently elutes as a higher molecular weight species slightly before the pH 6.0 daunorubicin reductase (s). In addition, the pH 8.5 and 6.0 adriamycin reductases elute as a single

Table 1. Specific activities of daunorubicin and adriamycin reductases at pH 6.0 and 8.5

Species	Daunorubicin*		Adriamycin		Daunorubicin / Adriamycin	
	pH 6.0†	pH 8.5†	pH 6.0†	pH 8.5†	pH 6.0	pH 8.5
Rabbit	12.3 ± 0.28	6.8 ± 0.65	0.492 ± 1.109	0.737 ± 0.093	25	9.2
Human	12.2 ± 0.59	9.8 ± 0.42	0.083 ± 0.0035	1.01 ± 0.19	147	9.7
Rat	‡	5.8 ± 0.35	0.07 ± 0.01	0.19 ± 0.07	§	30
Mouse	‡	5.1 ± 0.00	0.05 ± 0.01	0.35 ± 0.05	§	15

*Specific activity is expressed as nmoles/min/mg of protein.
†For standard assays see Experimental Procedures.
‡Not detectable under assay conditions used.
§Not calculated.

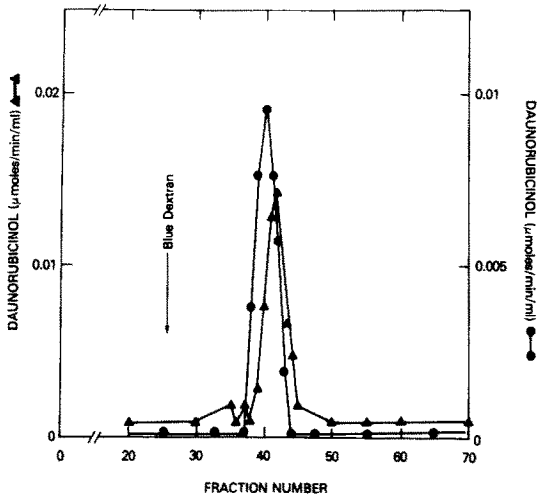

Fig. 2. Gel filtration on BioGel P150 of the human dialyzed ammonium sulfate fraction. The following activites were measured as described in Experimental Procedure: (▲—▲ and ●—●) daunorubicin reductase at pH 6.0 and pH 8.5 respectively.

activity peak nearly coincident with the pH 6.0 daunorubicin reductase(s) (not shown). The apparent molecular weights of these reductases were determined by comparison with standard proteins of known size (Table 2). Although the pH 8.5 and 6.0 daunorubicin reductases apparently have different molecular weights, both enzymes fall within a rather narrow molecular weight range similar to previously described aldehyde reductases [6]. The elution behavior on gel filtration also provides evidence for at least two classes of daunorubicin reductase(s).

DEAE-cellulose chromatography resolves the dialyzed ammonium sulfate fraction of rabbit liver into four and perhaps five separate pH 6.0 daunorubicin reductase activity peaks and two or three additional pH 8.5 daunorubicin reductase activity peaks (Fig. 3). Although the bulk of pH 6.0 daunorubicin reductase activity is clearly separated from and completely devoid of pH 8.5 activity, a significant amount of pH 6.0 daunorubicin reductase activity is coincident with the major pH 8.5 daunorubicin reductase peak. The elution of the adriamycin reductases is very similar to the elution of pH 6.0 daunorubicin reductase

Table 2. Apparent molecular weights of anthracycline antibiotic reductase

Enzyme	Species	Apparent molecular weight*
pH 8.5 Daunorubicin reductase	Human	38,700
	Rabbit	36,000
pH 6.0 Danuorubicin reductase	Human	34,500
	Rabbit	32,300
pH 6.0 and pH 8.5 Adriamycin reductases	Human	34,500
	Rabbit	32,800

*Molecular weight was determined on calibrated columns of BioGel P150 as described in Experimental Procedure.

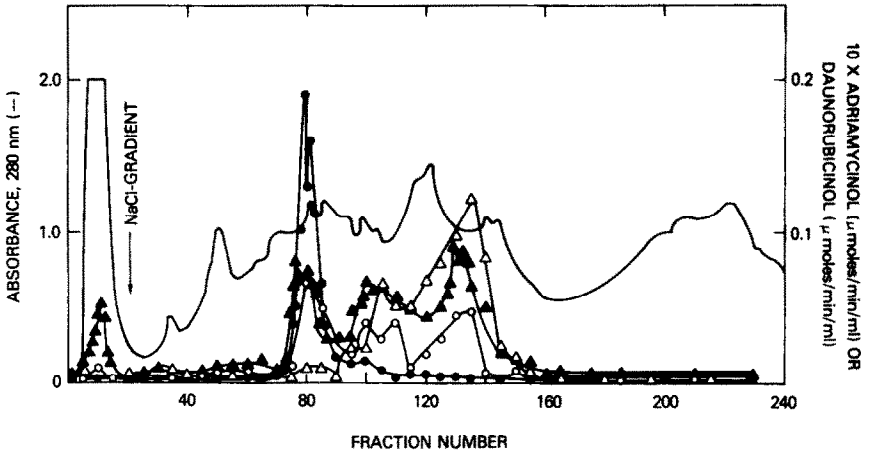

Fig. 3. DEAE-cellulose chromatography of rabbit liver dialyzed ammonium sulfate fraction. Elution was accompanied by a linear NaCl gradient made from 4 l. of 0.01 M Tris–HCl buffer, pH 7.5, containing β-mercaptoethanol and 4 l. of this buffer containing 0.3 M NaCl. Gradient started at tube 20. The following activities were measured as described in Experimental Procedure: (▲——▲ and ●——●) daunorubicin reductase at pH 6.0 and 8.5 respectively; (△——△ and ○——○) adriamycin reductase at pH 6.0 and 8.5 respectively.

and is essentially resolved from the pH 8.5 daunorubicin reductase. This technique, therefore, not only resolves two major classes of daunorubicin reductase but further demonstrates that considerable heterogeneity exists within each type of enzyme.

The complexity of anthracycline antibiotic reduction in rabbit and human livers is illustrated further by isoelectric focusing (Fig. 4 panels A and B). Isoelectric focusing over a pH gradient from 4 to 7 resolves the rabbit liver reductases into at least three major pH 6.0 daunorubicin reductase activities (pI values 4.8, 5.3 and 6.3) and two major pH 8.5 daunorubicin reductase activities (pI values 5.9 and 6.3) (Fig. 4A). In addition, numerous minor forms are detected for both pH classes. The adriamycin reductase activities are similarly resolved into two major pH 6.0 and 8.5 activites which are coincident primarily with the pH 6.0 daunorubicin peaks. Isoelectric focusing of the human liver enzymes is similar to that of rabbit, with multiple forms of both pH 8.5 and 6.0 daunorubicin and adriamycin reductases (Fig. 4B). In contrast to the rabbit, although numerous minor forms are similarly resolved from human liver extracts, the prominent four anthracycline antibiotic reductase activites are coincident (pI 5.4). Isoelectric focusing of mouse and rat liver enzyme preparations shows a single broad pH 8.5 daunorubicin reductase activity and a smaller coincident pH 8.5 adriamycin activity (not shown). As expected, no reductase activities are seen at pH 6.0 for daunorubicin or adriamycin in either mouse or rat. Therefore, isoelectric focusing demonstrates the exis-

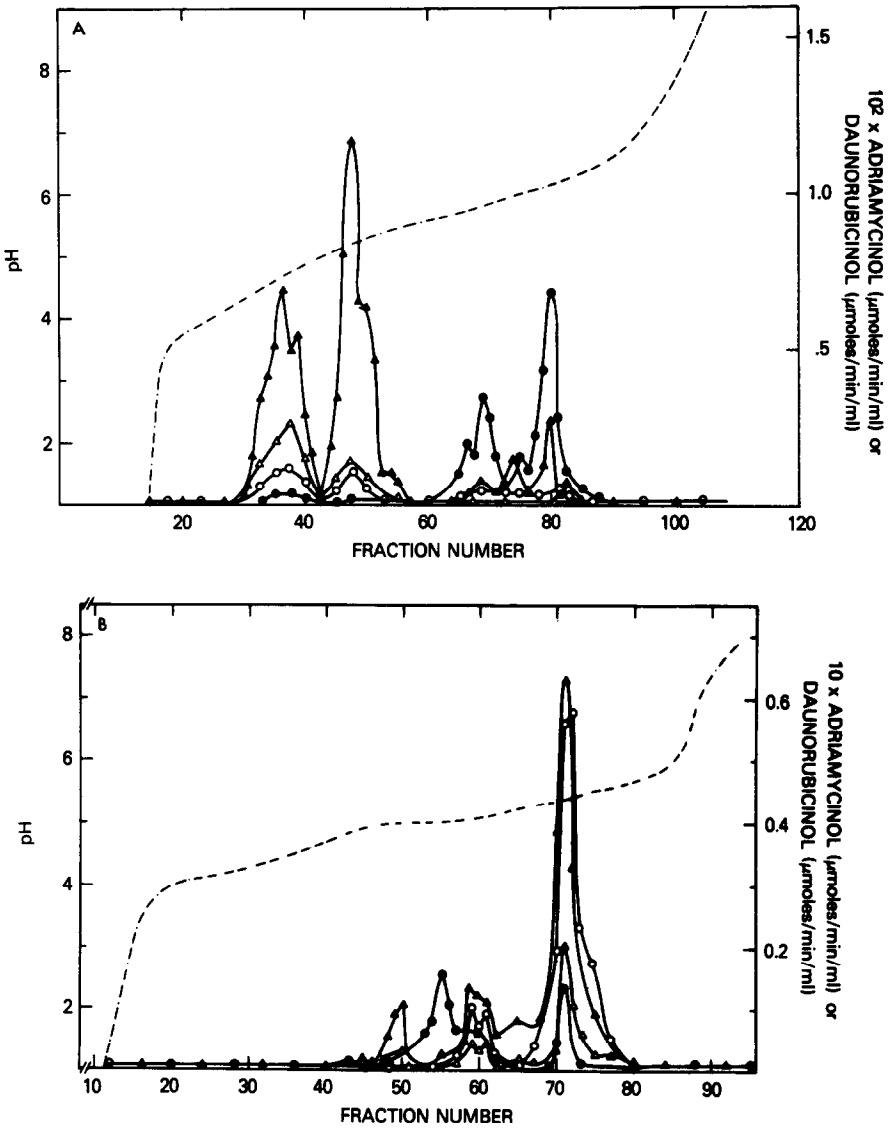

Fig. 4. Isoelectric focusing of liver reductases over the pH range of 4–7. Panel A: rabbit; panel B: human. The following activities were measured as described in Experimental Procedure: (▲—▲ and ●—●) daunorubicin reductase at pH 6.0 and 8.5 respectively; (△—△ and ○—○) adriamycin reductase at pH 6.0 and 8.5 respectively. pH gradient (—·—·—).

tence of previously unknown pH 6.0 daunorubicin and adriamycin reductases in rabbit liver and pH 6.0 daunorubicin reductase in human liver and the absence of these enzymes in mouse and rat livers. In addition, the heterogeneity of these enzymes is established.

DISCUSSION

Carbonyl reductions are catalyzed by what appears to be a class of drug-metabolizing enzymes located in the cytoplasm of mammalian cells [2, 5, 12]. This class of enzymes includes aldehyde reductases (EC 1.1.1.2) which catalyze the reduction of xenobiotic as well as naturally occurring aldehydes and aldoses, and ketone reductases, a similar yet distinct group of enzymes which appear to be specific for xenobiotic ketones. Typical ketone reductase substrates include oxisuran [13, 14], methadone [15], naloxone and naltrexone [16], bunolol [17], warfarin [18], and *p*-nitroacetophenone [19]. Although the aldehyde reductases have been purified extensively and characterized [6, 7, 20–23], ketone reductases have not undergone this scrutiny nor have their properties been described adequately.

Daunorubicin reductase has been purified to homogeneity from rat liver [6]. This enzyme displays broad specificity for typical aldehyde and aldose substrates, but has limited activity for ketone substrates [7]. In fact, daunorubicin is the only ketone known to be reduced significantly by this enzyme. This limited ketone specificity is somehow facilitated by the unique alkaline optimum (pH 8.5) of the enzyme for anthracycline antibiotic reduction compared to its more typical acid optimum (pH 6.0) for other aldehyde and ketone substrates. Based on this rather restricted ketone specificity, as well as its inhibition by barbiturates, this enzyme as classified as an aldehyde rather than a ketone reductase [7, 8].

In a survey of the enzymatic reduction of daunorubicin and adriamycin by several mammalian tissues, we reported recently the probable presence of multiple carbonyl reductases in rabbit tissues [9]. It was suggested that these additional enzymic species might be important to drug efficacy and toxicity in these animals. The results presented here confirm that rabbit liver, in fact, contains multiple anthracycline antibiotic reductases and provide evidence for similar heterogeneity in human liver. More importantly, a new class of anthracycline antibiotic reductase activities with acidic pH optima (approx pH 6.0) is identified in rabbit and human livers, which is essentially absent in rat and mouse livers. This new reductase activity is distinguished clearly from the pH 8.5 daunorubicin reductase by ion exchange and gel filtration chromatography and by isoelectric focusing. It is, therefore, now clear that daunorubicin reduction is mediated by at least two classes of enzymes in rabbit and human livers (1) the pH 8.5 optimum daunorubicin reductase, and (2) a previously unknown pH 6.0 optimum daunorubicin reductase. In addition, the rapid reduction of adriamycin seen *in vivo* in the rabbit [4] also is accomplished by an enzyme(s) distinct from the pH 8.5 reductase. In rabbit liver, the pH 6.0 optima daunorubicin and adriamycin reductase activities provide the bulk of the total anthracycline reductase activity *in vitro*. The exact number of enzyme forms within each group, and whether the adriamycin reductase(s) are distinct from the pH 6.0 daunorubicin reductase(s) must await further purification and characterization of these enzymes.

The high *in vivo* metabolism of anthracycline antibiotics in rabbit relative to other mammalian species (i.e. rat and mouse) is consistent with the high reductase activities demonstrated *in vitro*. Similarly, the *in vivo* drug metabolism in humans is intermediate between rabbit and other species and correlates with the intermediate levels of *in vitro* reduction demonstrated in this study. However, the effect of post-mortem changes on total reductase activity or reductase heterogeneity in humans is presently unknown.

It has been reported [24] that isoelectric focusing can induce multiple enzyme forms. This effect was attributed to artifacts formed by interaction of the protein with ampholyte. Although this effect may account for some of the minor enzyme species observed in our studies, the existence of the two major classes of anthracycline antibiotic reductases is also evident by DEAE cellulose and gel filtration chromatography and by pH profiles. By analogy with the rat liver daunorubicin reductase [6–8], the rabbit liver pH 8.5 daunorubicin reductase described in this study probably will be identified as a typical aldehyde reductase. Since the other reductases are similar in substrate specificities and molecular weights, their identity as closely related aldehyde and/or ketone reductases is also probable. Specific conclusions on the origins and inter-relationships of these anthracycline antibiotic reductases as well as their respective multiple forms must await further purification and detailed study of the physical, chemical and kinetic features of the individual enzymes.

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