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ISOLATION AND CHARACTERIZATION OF RABBIT LIVER XENOBIOTIC CARBONYL REDUCTASES

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Abstract—Xenobiotic carbonyl reductases have been isolated from rabbit liver by ammonium sulfate fractionation and isoelectric focusing. Although these enzymes are very heterogeneous, the above procedures resolve the majority of the reductases in good yield. Most of the carbonyl reduction of oxisuran, 3,7-dimethyl-l-(5-oxyhexyl)-xanthine, metyrapone and daunorubicin (pH 6.0) was accomplished by two distinct enzymes of pI 4.84 and 4.98. Other reductases with lesser activities toward these same substrates also occurred at higher pI values. Also resolved were several forms of enzymes that reduced daunorubicin (pH 8.5) (previously identified as aldehyde reductase), naloxone and naltrexone (dihydromorphinone reductases), and the model compounds, *p*-nitrobenzaldehyde and *p*-nitroacetophenone. The hydrogen stereospecificity of each of the rabbit liver carbonyl reductases, as well as rat liver aldehyde reductase, was determined by reducing the carbonyl substrates with A- and B-labeled [4-^{3}H]NADPH and examining transfer of label to alcohol products and retention of label in the resulting oxidized cofactors. All of the oxisuran, metyrapone and daunorubicin (pH 6.0) reductases displayed B-hydrogen stereospecificity. Some enzymes that reduce 3,7-dimethyl-l-(5-oxyhexyl)-xanthine, *p*-nitroacetophenone and *p*-nitrobenzaldehyde were also B-stereospecific while other forms of these same enzymes were A-stereospecific. Only daunorubicin (pH 8.5) (rabbit and rat), naloxone and naltrexone reductases were exclusively A-stereospecific. Apparent deuterium isotope effects of A- and B-labeled [4-^{2}H]NADPH with daunorubicin (pH 6.0) reductases, daunorubicin (pH 8.5) reductase and naloxone reductases confirm the above hydrogen stereospecificity assignments. The results confirm the hydrogen specificity of aldehyde reductases as A-stereospecific and the majority of ketone reductases as B-stereospecific. In addition, several significant A-stereospecific ketone reductases appear to represent exceptions to the generalization that enzymes which catalyze the same reaction have the same stereospecificity. Finally, the binding of rat liver aldehyde reductase to NADPH produced a red shift in the cofactor 340 nm absorbance maximum which is opposite to that predicted on the basis of its hydrogen stereospecificity.

Carbonyl-containing xenobiotic compounds are reduced in mammals by pyridine nucleotide-dependent reductases. Of these reductases, aldehyde-specific enzymes are well studied and described [1–3]. Although a class of reductases with specificity for ketone-containing drugs also is known, these enzymes have been largely neglected. Recent studies have revealed the existence of extremely active carbonyl-drug reduction potential in rabbits and humans [4,5]. In fact, evidence suggests that this potential occurs as a very heterogeneous mixture of drug-specific ketone and aldehyde reductases which display many similar properties. Although the *in vivo* role(s) of these enzymes is unclear, their constitutive and ubiquitous distribution suggests their involvement in important physiological processes. Therefore, a better understanding of their comparative properties and mechanisms is desired.

We have devised a simple procedure to resolve this complex group of enzymes into several separate and distinct aldehyde and ketone reductase pools. We report here the stereospecificity of hydrogen transfer from NADPH in the carbonyl reduction of several important carbonyl-containing xenobiotic compounds. Our results demonstrate clear differences between the otherwise very similar classes of aldehyde and ketone reductases. In addition, isotope effects show that the transfer of hydrogen contributes to the rate-limiting step. Finally, several exceptions are found to the generalizations which states that (1) enzymes which catalyze the same reaction have the same stereospecificity, and (2) the direction of shift of the absorption spectra of pyridine nucleotide on binding to enzymes is predicted by the stereospecificity of the oxidoreductase.

* Abbreviations used are: 3,7-DMX, 3,7-dimethyl-l-(5-oxyhexyl)-xanthine; daunorubicin (pH 6.0) reductase daunorubicin reductase activity as measured at pH 6.0; daunorubicin (pH 8.5) reductases, daunorubicin reductase activity as measured at pH 8.5; A-[^{3}H]NADPH, 4-(R)-[4-^{3}H]NADPH; A-[^{2}H]NADPH, 4-(R)-[4-^{2}H]NADPH: B-[^{3}H]NADPH, 4-(S)-[4-^{3}H]NADPH; and B-[^{2}H]NADPH, 4-(S)-[4-^{2}H]NADPH.

EMPERIMENTAL PROCEDURE

Materials

Yeast alcohol dehydrogenase (EC 1.1.1.1), isocitrate dehydrogenase (EC 1.1.1.42) (type IV), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glutamate dehydrogenase (EC 1.4.1.2), α-glycerol phosphate dehydrogenase (EC 1.1.1.72), hexokinase (EC 1.7.1.1) and NADP$^+$ were puchased from the Sigma Chemical Co. (St. Louis, MO). D-[1-^{3}H] Glucose(18 Ci/mmole) and sodium [^{3}H]borohydride (227.5 mCi/mmoles) were from New England

Nuclear (Boston, MA). Sodium [d_4-^{2}H]borodeuteride (98 atom per cent D) was from ICN Chemical and Radioisotopes Division (Irvine, CA). Daunorubicin was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, and purified by the method of Bachur and Cradock [6]. NADPH was obtained from P-L Biochemicals (Milwaukee, WI) and carrier ampholytes were from LKB (Bromma, Sweden). All drugs were gifts from the respective manufacturers: metyrapone, Ciba Geigy Corp. (Ardsley, NY); oxisuran and [1-^{14}C]oxisuran (0.95 μCi/mg), Warner Lambert Research Institute (Morris Plains, NJ); naloxone and naltrexone, Endo Laboratories Inc. (Garden City, NY); and 3,7-DMX*, Chemische Werke Albert A. G. (Frankfurt, F.R.G.).

Triethyloxalosuccinate was synthesized by the method of Bottorff and Moore [7]. The [2-^{3}H]isocitrate and [2-^{2}H]isocitrate were prepared by reducing triethyloxalosuccinate with sodium [^{3}H]borohydride or sodium[^{2}H]borodeuteride, respectively, followed by hydrolysis and purification by ion exchange chromatography by the methods of Lowenstein [8]. The [2-^{3}H]isocitrate and [2-^{2}H]isocitrate were assayed by the method of Ochoa [9].

Enzyme preparations–rabbit liver carbonyl reductases

New Zealand White male rabbits (1.8–2.2 kg) were killed by a blow to the head, and their livers were excised and immediately chilled on ice. All subsequent manipulations were performed at 0–4°.

Step 1: *Extraction.* Livers were minced with scissors, then homogenized by two full passes in a glass Teflon Potter–Elvehjem homogenizer in 3 vol. of 2.5 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose and 0.5 mM dithiothreitol, and centrifuged at 27,000 *g* for 15 min. The 27,000 *g* supernatant solution was collected and centrifuged 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 per cent saturation for 15 min and the resulting precipitate was removed by centrifugation and discarded. The supernatant solution was fractionated to 70 per cent saturation by the addition of ammonium sulfate. Following centrifugation of the suspension, the second precipitate was dissolved in a minimal volume of 1 per cent glycine containing 0.5 mM dithiothreitol and dialyzed overnight against two changes of 4 liters of the same buffer. After dialysis, the contents of the dialysis bag were centrifuged at 27,000 *g* for 5 min and the clear supernatant fraction was retained.

Step 3: *Isoelectric focusing.* Electrofocusing was performed in an LKB 8102 electrofocusing column (440 ml) with 2 per cent ampholyte (either pH 4–7 or pH 4–8) and a stabilizing sucrose gradient containing 0.5 mM dithiothreitol. The dialyzed ammonium sulfate fraction from the previous step was mixed into the sucrose gradient by addition to the less dense solution used in formation of the sucrose gradient. Any precipitation of protein caused by the presence of ampholyte was removed by centrifugation before gradient formation. Electrofocusing lasted about 64 hr at 0–4° with an initial power of 10.0 W. After the electrofocusing was complete, the column contents were collected in 5-ml fractions, and pH measurements were performed immediately at 0–4°. All reductase assays were completed within 48 hr.

Re-electrofocusing was accomplished by using a LKB 8101 electrofocusing column (110 ml). The sucrose and ampholyte concentrations of the appropriate pool from the first electrofocusing column were estimated and then incorporated into a new sucrose gradient containing a fresh equivalent of 0.5 mM dithiothreitol and a final ampholyte concentration of about 1 per cent. Re-electrofocusing lasted about 65 hr at 0–4° with an initial power of 3.4 W. The column contents were collected in 1.5-ml fractions, and pH and activity measurements performed as above.

Rat liver aldehyde reductase

Homogeneous rat liver aldehyde reductase was prepared as described previously [10].

Enzyme assays

Reductases were assayed spectrophotometrically by following the oxidation of NADPH at 340 nm in 1 cm pathlength quartz cuvettes with a Cary 118 spectrophotometer. The reaction mixtures contained 0.16–0.18 mM NADPH in 0.08 M potassium phosphate buffer, pH 6.0, and appropriate substrate concentrations at 25° in a final volume of 1.0 ml. The substrate in each assay mixture at the following indicated concentrations are at least $10 \times K_m$:oxisuran(10 mM),3,7-DMX(10 mM), metyrapone (10 mM), naloxone (10 mM), naltrexone (10 mM), *p*-nitroacetophenone (2.8 mM) and *p*-nitrobenzaldehyde (2.5 mM). Oxisuran and *p*-nitrobenzaldehyde solutions were prepared fresh daily. Daunorubicin (0.375 mM), assayed at pH 6.0 as indicated above, is refered to as the daunorubicin (pH 6.0) reductase activity. Daunorubicin (0.75 mM) is also assayed as above in 0.25 M Tris–HCl buffer, pH 8.5, and is referred to as the daunorubicin (pH 8.5) reductase activity. Reactions were initiated by the addition of the enzyme and corrected for background absorbance changes at pH 6.0 due to nonspecific acid destruction of cofactor, and for nonspecific NADPH oxidation in the absence of carbonyl-containing substrates. The unit of enzyme activity is defined as the μmoles of cofactor oxidized per min.

Preparation of A-[^{3}H]NADPH and A-[^{2}H]NADPH

A-[^{3}H]NADPH (4-(R)-[4-^{3}H]NADPH) or A-[^{2}H]NADPH (4-(R)-[4-^{2}H]NADPH) were prepared by the method of Walton [11] with isocitrate dehydrogenase. The reaction mixtures included 0.9 μmole $NADP^+$, 0.25 μmole $MnCl_2$, 0.2 units of isocitrate dehydrogenase and 1 μmole L-[2-^{3}H]isocitrate (2.12–135 μCi/μmole) or 0.8 μmole L-[2-^{2}H]isocitrate (98 atom per cent D) in 0.1 M Tris–HCl buffer, pH 7.5, and a final volume of 1.2 ml. The reactions were monitored at 340 nm and stopped when no further increase in absorbance was observed. Approximately 0.77 μmole of NADPH was formed at equilibrium. The [^{3}H]NADPH or [^{2}H]NADPH was purified by

addition of the reaction mixtures to a DEAE–cellulose column (Whatman DE-52) (1.5 × 7 cm) equilibrated with 0.1 M Tris–HCl, pH 8.1, buffer [12]. The column was washed with 25 ml of equilibration buffer containing 0.06 M NaCl and the labeled reduced cofactors eluted with about 50 ml of equilibration buffer containing 0.2 M NaCl. Fractions (2 ml) were collected and A-[^{3}H]NADPH or A-[^{2}H]NADPH was recovered in fraction numbers 15–20.

Preparation of B-[^{3}H]NADPH and B-[^{2}H]NADPH

B-[^{3}H]NADPH (4-(S)-[4-^{3}H]NADPH) and B-[^{2}H]NADPH (4-(S)-[4-^{2}H]NADPH were prepared by the method of Walton [11] using glucose-6-phosphate dehydrogenase. The reaction mixtures included 1.0 μmole $NADP^+$, 17 μmoles ATP, 20 μmoles $MgCl_2$, 4 units of hexokinase, 2 units of glucose-6-phosphate dehydrogenase and 1.0 μmole D-[1-^{3}H]glucose (3.16–53.8 μCi/μmole) or 1.0 μmole D-[1-^{2}H]glucose (98 atom per cent D) in 0.1 M Tris–HCl buffer, pH 7.5, in a final volume of 3.0 ml. When the increase in absorption at 340 nm leveled off (about 0.82 μmoles of labeled reduced cofactor), the B-[^{3}H]NADPH and B-[^{2}H]NADPH were purified as indicated above.

Assays for stereospecificity

Method 1: Immediately before use, the freshly prepared and chromatographed labeled reduced cofactors were adjusted to pH 7.0 with 0.1 M HCl with a Radiometer titrator model II with care that the pH never dropped below pH 7.0. The concentration of [^{3}H]NADPH was increased to approximately 0.16 mM with unlabeled NADPH (final sp. act. 22–34 × 10^6 d.p.m./μmole). Reaction mixtures contained either daunorubicin (0.633 mM), oxisuran (10 mM), 3,7-DMX (10 mM), metyrapone (10 mM), naloxone (10 mM), naltrexone (10 mM), *p*-nitroacetophenone (2.4 mM), *p*-nitrobenzaldehyde (1.5 mM) and 80 μmoles of either A-[^{3}H]NADPH or B-[^{3}H]NADPH in 0.2 M potassium phosphate buffer, pH 6.55, 0.1 M potassium phosphate buffer, pH 6.00, or 0.2 M Tris–HCl buffer, pH 8.49, and a final volume of 1.0 ml in disposable plastic cuvettes. Reactions were initiated with aliquots of appropriate enzyme and incubated at room temperature until the decrease in absorption had ceased, indicating reaction equilibrium (60–90 min). After equilibrium had been achieved, the reactions were transferred to test tubes and placed in a boiling water bath for 5 min to denature proteins. The denatured protein was removed by centrifugation, and supernatant solutions were extracted as follows. Daunorubicin, oxisuran and 3,7-DMX were extracted by the addition of 0.4 ml isopropanol after saturating with solid ammonium sulfate; metyrapone, naloxone, naltrexone, *p*-nitrobenzaldehyde and *p*-nitroacetophenone were extracted with 0.4 ml ethylacetate after the addition of 1.0 ml of 1 M sodium carbonate buffer, pH 10. For all compounds except daunorubicin, the extracted drug alcohol products were isolated by thin-layer chromatography on silica gel containing fluorescent indicator (Merck) and detected by quenching of the fluorescent background. Daunorubicinol was isolated by chromatography on silica gel without fluorescent indicator (Merck) and detected by its natural fluorescence.

Chromatography solvents for the various drugs and their respective alcohol products were as follows: daunorubicin (chloroform–methanol–acetic acid–water, 80:20:14:6), oxisuran (ethylacetate–methanol, 4:1), 3,7-DMX and metyrapone (benzene–acetone, 70:30 with an ammonia atmosphere), naloxone and naltrexone (chloroform–methanol–ammonium hydroxide, 90:10:4), and *p*-nitrobenzaldehyde and *p*-nitroacetophenone (benzene–ethylacetate, 3:1). The products were scraped from the plates and in every case except daunorubicin were added directly to scintillation vials. The daunorubicinol was extracted from the silica gel with 2.0 ml of acid alcohol according to the method of Bachur and Cradock [6], and an aliquot was counted. The remainder of the extracted eluate was assayed fluorometrically to determine directly the amount of danuorubicinol formed [13]. Quantitation of other alcohol products was obtained from the change in absorption at 340 nm (1 to 1 stoichiometry and extinction coefficient of 6.22 × 10^3). In the case of daunorubicin, the validity of this approach was confirmed by comparing the amount of daunorubicinol formed as calculated from the absorbance change to the daunorubicinol formed as determined directly by fluorescence assay.

Method 2. Low specific activity A- or B-[^{3}H]NADPH was prepared and adjusted to approximately 0.16 mM (3 × 10^5 d.p.m./μmole) as described above. Reaction mixtures were exactly as described in Method 1, except that approximately 0.15 μmole of A- or B-labeled cofactor was used. The deproteinized reaction mixtures were then directly applied to DEAE–cellulose columns and eluted as described above except that each elution buffer was continued until the appropriate substrate or product was completely eluted before changing to the next elution buffer.

Method 3. A- and B-[^{2}H]NADPH were synthesized, chromatographed, and neutralized as described above. Because of the sensitivity of the reductases to different buffers and ionic strengths [4,14], it was necessary that all enzymes tested be assayed under identical ionic environments. Therefore, unlabeled NADPH was similarly chromatographed and neutralized so that it would be in a solvent of identical ions and ionic strength (verified with a Radiometer CDM3 conductivity meter). Buffer (0.1 M potassium phosphate, pH 6.55, containing 0.2 M NaCl) used to elute the reduced cofactors from the ion exchange column was also neutralized and was used to adjust cofactor concentrations for subsequent kinetic analysis which was performed within 24 hr. Kinetic measurements were made at a pH removed from the respective enzyme pH optima [i.e. pH 6.6 for both daunorubicin (pH 8.5) and daunorubicin (pH 6.0) reductases] so that the isotope effect might be enhanced [15].

Difference spectra

Difference spectra were recorded on a Cary 118 spectrophotometer with tandem quartz cuvettes in 0.1 M Tris–HCl buffer, pH 8.5, and 0.1 M potassium

phosphate buffer, pH 6.6 (each solution compartment had a pathlength of 0.438 cm).

Analytical methods

Scintillation counting was performed in 10 ml Aquasol (New England Nuclear) by a Searle Mark III liquid scintillation counter. Protein estimations were made by the method of Lowry *et al.* [16]. Enzyme aliquots (1 ml) from isoelectric focusing were first chromatographed on Sephadex G-50 fine (Pharmacia) columns (1.5 × 20 cm) equilibrated in 0.1 M potassium phosphate buffer, pH 7.4, to remove ampholytes before protein determination. Solutions of cold NADPH were prepared fresh daily and concentrations were determined from a molar extinction coefficient of 6.22×10^3 at 340 nm. Daunorubicin concentrations were determined with a molar extinction coefficient of 11.4×10^3 at 485 nm. Kinetic constants were calculated from computer programs as described [14,17]. Isotope effects were calculated from kinetic data by the computer programs of Cleland [18].

RESULTS

Resolution of carbonyl reductases

After ammonium sulfate fractionation of the rabbit liver soluble extract, carbonyl reductases for oxisuran, daunorubicin (pH 6.0), metyrapone, daunorubicin (pH 8.5), and 3,7-DMX were resolved by isoelectrofocusing on a sucrose gradient from pH 4 to 7 (Fig. 1A). Major enzymatic species were detected and analyzed as pools: I, II and III. Most of the daunorubicin (pH 6.0), oxisuran, 3,7-DMX and metyrapone reductase activities occurred in pool I and pool II. The major daunorubicin (pH 8.5) reductase activity, as well as minor amounts of the other reductase activities, were resolved in pool III. Although low levels of naloxone and naltrexone reductases were also found in pools I, II and II, better resolution was obtained on gradients from pH 4 to 8 (Fig. 1B). Also analyzed in the pH 4–8 gradient were the reductases of *p*-nitroacetophenone and *p*-nitrobenzaldehyde, as well as daunorubicin (pH 8.5) and 3,7-DMX reductases which are included for easier reference to Fig. 1A. The enzyme pools I–III in the pH 4–8 gradient (Fig. 1B) correspond to similarly labeled enzyme pools of the pH 4–7 gradient (Fig. 1A). Several additional enzyme pools (IV–VII) which include distinct forms of naloxone, naltrexone, 3,7-DMX, *p*-nitroacetophenone and *p*-nitrobenzaldehyde reductases were detected by appropriate analysis and collected for study.

Although reductase levels in the enzyme pools varied, the general activity profiles shown in Fig. 1 were reproduced in six similar experiments. As a result, the enzyme species present in pools I–VII are distinct and well defined with respect to isoelectric points (Table 1). The reproducible isoelectric points as well as the heterogeneity is further demonstrated by re-isoelectrofocusing pool I (Fig. 1A) over a gradient of pH 4.5–5.1 (Fig. 2). Although only daunorubicin (pH 6.0) and daunorubicin (pH 8.5) reductases are presented in Fig. 2 (for clarity), oxisuran,

Table 1. Summary of apparent isoelectric points of rabbit liver carbonyl reductases

Enzyme pools	Substrates	Range of apparent isoelectric points (pI)*
I	Daunorubicin (pH 6.0) Oxisuran 3,7-DMX Metyrapone *p*-Nitrobenzaldehyde *p*-Nitroacetophenone	4.8–5.0
II	3,7-DMX Metyrapone Naloxone Naltrexone *p*-Nitroacetophenone	5.4–5.5
III	Daunorubicin (pH 8.5) *p*-Nitrobenzaldehyde	6.0–6.3
IV	3,7-DMX Naloxone Naltrexone *p*-Nitrobenzaldehyde	6.4–6.6
V	Naloxone Naltrexone 3,7-DMX *p*-Nitroacetophenone	6.6
VI	Naloxone Naltrexone 3,7-DMX *p*-Nitroacetophenone	6.9–7.2
VII	*p*-Nitrobenzaldehyde	7.8

* Determined at 0–4°.

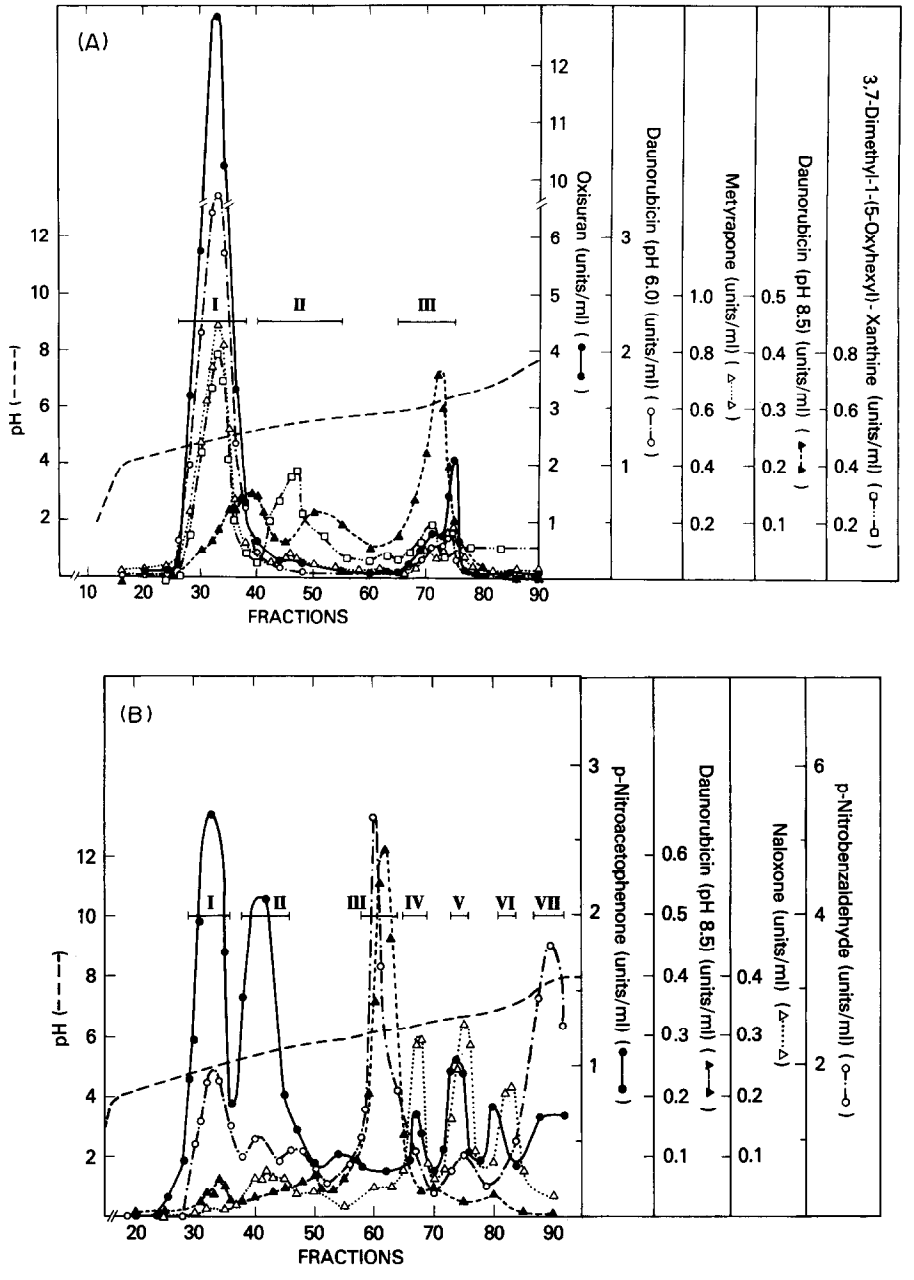

Fig. 1. Electrofocusing of rabbit liver dialyzed 30–70 per cent ammonium sulfate fraction in a pH gradient (– – –) of 2 per cent ampholyte containing 0.5 mM dithiothreitol and stabilized by a sucrose gradient. Carbonyl reductases were assayed with the following substrates in panel A (pH gradient 4–7): oxisuran (●——●), daunorubicin (pH 6.0) (○—·—○), metyrapone (△····△), daunorubicin (pH 8.5) (▲ – – – ▲); and 3,7-dimethyl-l-(5-oxyhexyl)-xanthine (□—·—□); in panel B (pH gradient 4–8): *p*-nitroacetophenone (●——●), daunorubicin (pH 8.5) (▲ – – – ▲), naloxone (△····△) and *p*-nitrobenzaldehyde (○—·—○). Pooled fractions are indicated by brackets and designated I–VII with increasing pH.

3,7-DMX and metyrapone give activity profiles similar to daunorubicin (pH 6.0). The two major daunorubicin (pH 6.0) reductase activities occurred at pI 4.84 and pI 4.98 (Fig. 2). Reproducibility is dependent on the use of freshly prepared cell extract. Although pools from the first isoelectric focusing experiment (Fig. 1A) stored in 0.5 mM dithiothreitol for 2 weeks at 5° (or for 2 months at −15°) retained 100 per cent of reductase activities, re-isoelectrofocusing showed increasingly diffuse and ill-defined reductase patterns.

A summary of the recoveries and purifications of reductases for one typical experiment is present in Table 2. Recoveries of activities was 50 per cent or

Table 2. Summary of purification of rabbit liver carbonyl reductases

Substrate		High speed supernatant fraction	Ammonium sulfate	Electrofocusing pools I	II	III	IV	V	VI	VII
Daunorubicin* (pH 6.0)	Units†	107	115	92	—‡	11	—	—	—	—
	Sp. act.§	0.014	0.023	0.19	—	0.11	—	—	—	—
	Purification	1.0	1.6	13.5	—	7.7	—	—	—	—
Daunorubicin* (pH 8.5)	Units	12.7	30.2	4.5	7.4	8.3	—	—	—	—
	Sp. act.	0.0017	0.0061	0.0094	0.040	0.081	—	—	—	—
	Purification	1.0	3.5	5.5	2.3	4.8	—	—	—	—
Oxisuran*	Units	411	292	265	—	31	—	—	—	—
	Sp. act.	0.055	0.059	0.555	—	0.30	—	—	—	—
	Purification	1.0	1.1	10	—	5.4	—	—	—	—
3,7-DMX*	Units	52	48	19	11	4.2	—	—	—	—
	Sp. act	0.0069	0.0096	0.038	0.061	0.042	—	—	—	—
	Purification	1.0	1.4	5.5	8.8	6.1	—	—	—	—
Metyrapone*	Units	66	35	19	3.2	—	—	—	—	—
	Sp. act.	0.0089	0.0071	0.040	0.017	—	—	—	—	—
	Purification	1.0	0.79	4.5	1.9	—	—	—	—	—
Naloxone‖	Units	19.5	17.8	—	—	—	5.2	5.6	3.1	—
	Sp. act.	0.0024	0.0033	—	—	—	0.031	0.042	0.025	—
	Purification	1.0	1.4	—	—	—	13.1	17.6	10.6	—
p-Nitrobenzaldehyde‖	Units	2100	610	60	—	74	—	—	—	131
	Sp. act.	0.26	0.11	2.4	—	3.1	—	—	—	3.2
	Purification	1.0	0.42	9.2	—	12	—	—	—	12
p-Nitroacetophenone‖	Units	248	193	58.1	51.3	—	8.1	154	—	15.3
	Sp. act.	0.031	0.036	2.34	1.02	—	0.49	1.2	—	0.38
	Purification	1.0	1.2	75	33	—	16	38	—	12

* Data from experiment shown in Fig. 1A.
† Expressed as μmoles/min.
‡ Not determined.
§ Expressed as μmoles/min/mg of protein.
‖ Data from experiment shown in Fig. 1B.

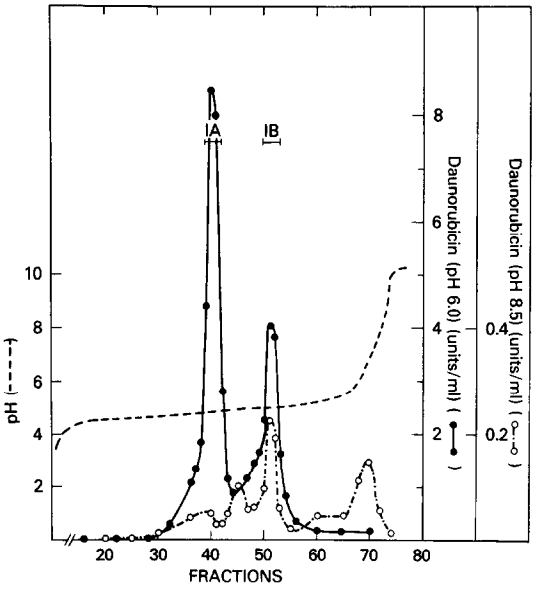

Fig. 2. Electrofocusing of pool I (Fig. 1A) in approximately 0.89 per cent ampholyte pH gradient (– – –) (pH 4.5–5.1) containing at least 0.5 mM dithiothreitol. Carbonyl reductases were assayed with the following substrates: daunorubicin (pH 6.0) (●—●) and daunorubicin (pH 8.5) (○—·—○). Pooled fractions are indicated by brackets and designated 1A and 1B.

greater for most of the enzymes. The isoelectrofocusing step, however, is essentially quantitative when all minor peaks and shoulders are combined.

Hydrogen stereospecificity of carbonyl reductases

The hydrogen stereospecificity of rabbit liver carbonyl reductases was examined by incubating saturating concentrations of each carbonyl substrate in the presence of A- or B-labeled NADPH and the appropriate isoelectrofocused enzyme pool. Stereospecificity was established by the following procedures: Method 1–isolation of alcohol products and comparison of specific radioactivities resulting from A- or B-[^{3}H]NADPH; Method 2–isolation of oxidized cofactor ($NADP^+$) and comparison of retention of label from A- or B-[^{3}H]NADPH; and Method 3—comparison of deuterium isotope effects on V_{max} and V_{max}/K_m of carbonyl reduction using A-[^{2}H]NADPH, B-[^{2}H]NADPH and unlabeled NADPH.

Method 1. The stereospecificities of a large number of carbonyl reductases were determined by isolating and analyzing the alcohol products. Essentially all reductase activities located in pool I exhibited a preference for the transfer of label from B-[^{3}H]NADPH (i.e. B-specificity) (Table 3). In addition, minor reductases in pools II and III showed B- or mixed specificity. In contrast, nearly all the major enzymatic activities of the more alkaline pools (II–VII) preferred A-[^{3}H]NADPH (i.e. A-specificity).

Table 3. Hydrogen stereospecificity of rabbit liver carbonyl reductases*

Substrates	Enzyme pools†	Total products formed‡ (μmoles)		Specific activity of products (d.p.m./μmoles)		Relative radioactivity of products	
		A§ (10^{-6})	B§ (10^{-6})	A§ (10^{-6})	B§,‖ (10^{-6})	A§	B§
Daunorubicin (pH 6.0)	I(III¶)	0.074**	0.065**	0.224	5.4	1	24.1
Oxisuran	I(II,III)	0.073††	0.064††	0.030	3.94	1	131
3,7-DMX	I(III¶)	0.072	0.064	0.31	2.9	1	9.7
Metyrapone	I(II, III)	0.056	0.057	0.322	1.94	1	6.0
p-Nitroacetophenone	I(II)	0.054	0.069	0.100	9.8	1	98
p-Nitrobenzaldehyde	I	0.082	0.079	0.12	8.6	1	72
3,7-DMX	II(IV, V, VI)	0.062	0.054	8.0	1.9	4.2	1
Daunorubicin (pH 8.5)	III (I¶)	0.091**	0.090**	8.43	1.6	5.3	1
p-Nitrobenzaldehyde	III (VII)	0.074	0.083	9.9	0.48	20.6	1
Naloxone	IV (II, V, VI)	0.083	0.089	5.6	0.27	20.7	1
Naltrexone	IV (II, V, VI)	0.079	0.088	5.3	0.27	19.6	1
p-Nitroacetophenone	V (IV)	0.076	0.079	13.5	0.42	32.1	1

* Method 1.

† Results typical of one experiment using enzyme from indicated isoelectric focusing enzyme pools (see Fig. 1). Similar results were also obtained from enzyme pools indicated in parentheses (not included in table).

‡ Quantified by total change in absorbance at 340 nm. Includes all diastereoisomer alcohol products.

§ A refers to A-[^{3}H]NADPH (i.e. 4-(R)-[4-^{3}H]NADPH), and B refers to B-[^{3}H]NADPH (i.e. 4-(S)-[4-^{3}H]NADPH).

‖ Normalized to be directly comparable to the specific activity of product formed from A-[^{3}H]NADPH. Normalized specific activity of B-[^{3}H]NADPH labeled product =

$$\frac{\text{(specific activity of product formed from B-[}^3\text{H]NADPH) (specific activity of A-[}^3\text{H]NADPH)}}{\text{(specific activity of B-[}^3\text{H]NADPH)}}.$$

¶ Mixed stereospecificity (i.e. the ratio of alcohol products was close to 1:1 or variable).

** Also quantified by fluorescence of isolated products.

†† Also quantified by ^{14}C-label of isolated products.

It is clear that, within the same tissue, some of the substrates are reduced by carbonyl reductases of opposite hydrogen stereospecificity. For example, 3,7-DMX, *p*-nitroacetophenone and *p*-nitrobenzaldehyde reductases were B-specific in pool I but A-specific in the other pools. Only oxisuran, metyrapone and possibly daunorubicin (pH 6.0) were reduced exclusively by B-specific enzymes. In contrast, only naloxone, naltrexone and possibly daunorubicin (pH 8.5) were exclusively reduced by A-specific enzymes. Individual diastereoisomer alcohol products of oxisuran, naloxone and naltrexone exhibited the same hydride stereospecificity (Table 3). In some cases, the ratio of specific radioactivities of alcohol products is nearly 1:1 or so variable that a definitive stereospecificity assignment is not possible.

In pool I, while the ratio of daunorubicinol from A- and B-[^{3}H]NADPH to daunorubicin (pH 6.0) was 1:24 (Table 3) and indicates a B-stereospecificity, the ratio for daunorubicin (pH 8.5) was 3:1, suggesting an A-stereospecificity. This apparent change in specificity with pH is actually the contribution of multiple reductases of opposite stereospecificity. After re-isoelectrofocusing of pool I, daunorubicin (pH 6.0) gave ratios of 1:800 for pool 1A and 1:310 for pool 1B, while daunorubicin (pH 8.5) gave ratios of 1:5 for pool 1A and 1:35 for pool 1B. Apparently, re-isoelectrofocusing over a very narrow pH range was sufficient to resolve most of the contaminating daunorubicin (pH 8.5) of opposite A-stereospecificity. The remaining daunorubicin (pH 6.0) then gave a B-stereospecificity even when assayed at pH 8.5 [2.5 pH units from its optimum where its activity was relatively low in comparison to fully active contaminating A-specific daunorubicin (pH 8.5) reductase].

The daunorubicin (pH 8.5) reductase from rat liver has been purified previously to homogeneity and identified as an aldehyde reductase [10,14,19]. The stereospecificity of this carbonyl reductase was similarly examined with the substrates *p*-nitrobenzaldehyde and daunorubicin (Table 4). The pH optima of aldehyde reductase for *p*-nitrobenzaldehyde and daunorubicin are pH 6.0 and pH 8.5, respectively [10,14]. Therefore, the two substrates were examined at both pH values. An A-specificity was maintained for both substrates at both pH values.

Method 2. Hydrogen stereospecificities were also determined for selected reductases by isolating the resulting oxidized cofactors ($NADP^+$) and analyzing them for retention of label from A- or B-[^{3}H]NADPH. This was done for rat liver daunorubicin (pH 8.5) and rabbit liver daunorubicin (pH 6.0) reductases (Fig. 3). Both substrates were reduced separately with A-[^{3}H]NADPH and B-[^{3}H]NADPH plus appropriate enzyme. After completion of reduction, the reaction mixtures were applied to separate DEAE-cellulose columns, and then daunorubicin and daunorubicinol, $NADP^+$ and residual NADPH were eluted as separate fractions. The reduction of daunorubicin with rat liver daunorubicin (pH 8.5) reductase and A-[^{3}H]NADPH resulted in a quantitative recovery of radioactive label daunorubicinol in the flow-through peak, while the $NADP^+$ was completely devoid of radioactivity, indicating an A-hydrogen stereospecificity for the rat liver enzyme (Fig. 3A). This conclusion was confirmed by performing the same reaction with B-[^{3}H]NADPH. In this case, negligible radioactivity was recovered as daunorubicinol, but a quantitative recovery of radioactivity occurred in $NADP^+$ (Fig. 3B). A similar analysis of rabbit liver daunorubicin (pH 6.0) reductase (pool I) confirmed the previous identification of this enzyme as a B-specific reductase (Figs. 3C and 3D). Several additional substrates and enzyme pools were assayed similarly. Each substrate was reduced with A-[^{3}H]NADPH and B-[^{3}H]NADPH, and the percentage tritium $NADP^+$ (expressed as a

Table 4. Hydrogen stereospecificity of rat liver aldehyde reductase*

Substrate	pH of Assays	Total products formed† (μmoles)		Specific activity of products (d.p.m./μmoles)		Relative radioactivity of products	
		A‡ (10^{-6})	B‡ (10^{-6})	A‡ (10^{-6})	B‡, § (10^{-6})	A‡	B‡
p-Nitrobenzaldehyde	6.5	0.046	0.036	38.6	0.04	965	1
	8.5	0.243	0.260	0.47	0.004	118	1
Daunorubicin (pH 8.5)	6.0	0.056‖	0.038‖	15.3	0.20	76	1
	6.5	0.057‖	0.056‖	20.4	0.079	258	1
	8.5	0.046‖	0.053‖	42.0	0.12	350	1

* Method 1.

† Quantified by total change in absorbance at 340 nm. Includes all diastereoisomer alcohol products.

‡ A refers to A-[^{3}H]NADPH (i.e. 4-(R)-[4-^{3}H]NADPH and B refers to B-[^{3}H]NADPH (i.e. 4-(S)-[4-(S)-[4-^{3}H]NADPH).

§ Normalized to be directly comparable to the specific activity of product formed from A-[^{3}H]NADPH. Normalized specific activity of B-[^{3}H]NADPH labeled product =

$$\frac{\text{(specific activity of product formed from B-[}^3\text{H]NADPH) (specific activity of A-[}^3\text{H]NADPH).}}{\text{(specific activity of B-[}^3\text{H]NADPH)}}$$

‖ Also quantified by fluorescence of isolated products.

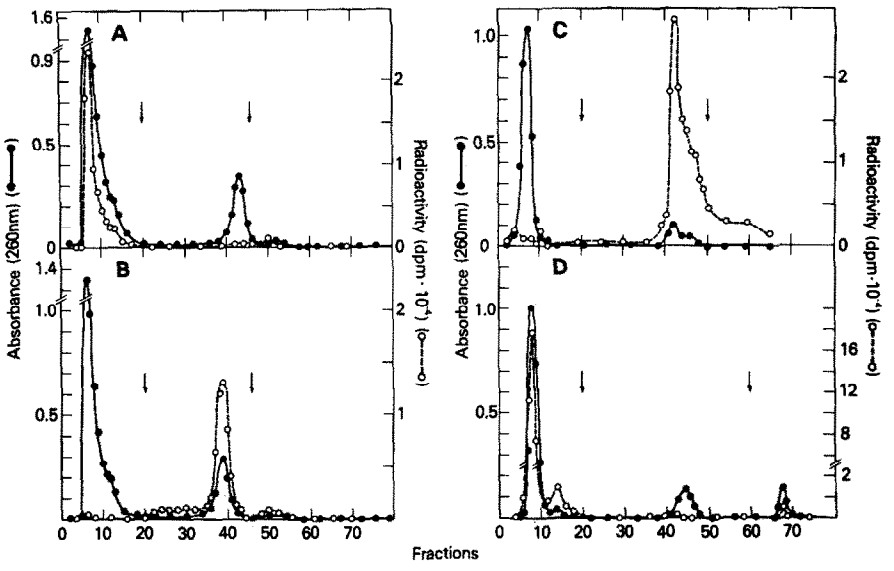

Fig. 3. Determination of hydrogen stereospecificity of reductases. Daunorubicin plus daunorubicinol, oxidized cofactor and reduced cofactor were separated on DEAE-cellulose after the reduction of daunorubicin by rat liver daunorubicin (pH 8.5) reductase and (A) A-[^{3}H]NADPH or (B) B-[^{3}H]NADPH and after reduction of daunorubicin by rabbit liver daunorubicin (pH 6.0) reductase (pool I) and (C) A-[^{3}H]NADPH or (D) B-[^{3}H]NADPH. The columns (1.5 × 6 cm) were equilibrated in 0.1 M Tris–HCl, pH 8.1. After addition of reaction mixtures, the columns were washed with equilibration buffer to elute daunorubicin plus daunorubicinol. At the first arrow, the solution was changed to equilibration buffer containing 0.06 M NaCl to elute oxidized cofactor. At the second arrow, the solution was changed to equilibration buffer containing 0.2 M NaCl to remove reduced cofactor. Key: absorbance at 260 nm (●——●) and radioactivity (○- - -○).

percentage of the total tritium in the pyridine nucleotide plus alcohol product) was recorded for each reaction (Table 5). For each substrate the sum of these percentages for A- or B-labeled cofactors was very nearly 100 per cent and the conclusion of stereospecificity is in agreement with Method 1. In addition, the reactions which exhibited lower stereospecificity by Method 1 were also of low stereospecificity by Method 2.

Method 3. If hydrogen transfer contributes to the rate-limiting step of the reductase mechanism, then substitution of deuterium for the stereospecified hydrogen might result in an isotope effect during carbonyl reduction. On the other hand, substitution

Table 5. Hydrogen stereospecificity of carbonyl reductases*

Substrate	pH‡	Enzymes	Per cent radioactivity as NADP$^+$†	
			A-[^{3}H]NADPH	B-[^{3}H]NADPH
Daunorubicin	8.5	Aldehyde reductase§	5	94
Daunorubicin	6.5	Pool I‖	93	3
Daunorubicin	6.5	Pool IA¶	99	1
3,7-DMX	6.5	Pool I‖	87	7
p-Nitrobenzaldehyde	6.5	Pool I‖	88	14
p-Nitrobenzaldehyde	6.5	Pool III‖	31	70
Naloxone	6.5	Pool IV‖	43	84
3,7-DMX	6.5	Pool V‖	39	62
p-Nitrobenzaldehyde	6.5	Pool VII‖	13	79

* Method 2.

† Individual substrates and enzymes were incubated with A- or B-[^{3}H]NADPH. After completion, the reaction mixtures for each cofactor were separated into daunorubicin plus daunorubicinol, NADP$^+$ and NADPH. The per cent radioactivity as NADP$^+$ is obtained from the total radioactivity in all substrate and products.

‡ Reaction mixture pH.

§ Rat liver.

‖ Rabbit liver (from experiment similar to that shown in Fig. 1).

¶ Rabbit liver (from experiment shown in Fig. 3).

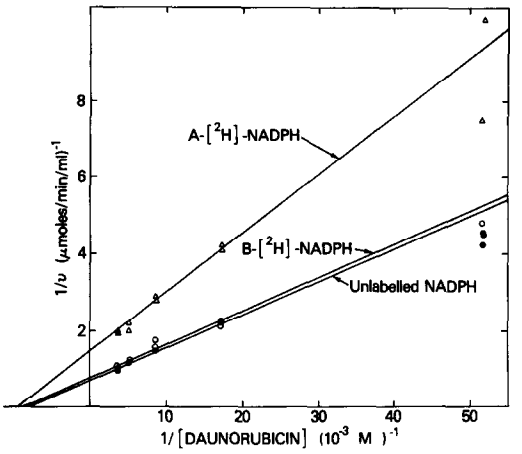

Fig. 4. Lineweaver–Burk plot of daunorubicin reduction by rat liver aldehyde reductase [daunorubicin (pH 8.5) reductase] at pH 6.6. Cofactor concentrations were 10 mM. Key: A-[²H]NADPH (△), B-[²H]NADPH (○), and unlabeled NADPH (●).

of isotopic hydrogen in the epimeric position should give no isotope effect. A comparison of the effects of A-[²H], B-[²H] and unlabeled NADPH on the kinetics of daunorubicin and naloxone reduction was made by steady state kinetics (Fig. 4). The calculated kinetic constants were compared (Table 6). Daunorubicin (pH 6.0) reductase exhibited an isotope effect on V_{max} and V_{max}/K_m with B-labeled cofactor, whereas A-labeled and unlabeled cofactors gave nearly identical kinetic constants, In contrast, daunorubicin (pH 8.5) and naloxone reductases displayed an isotope effect with A-labeled cofactor

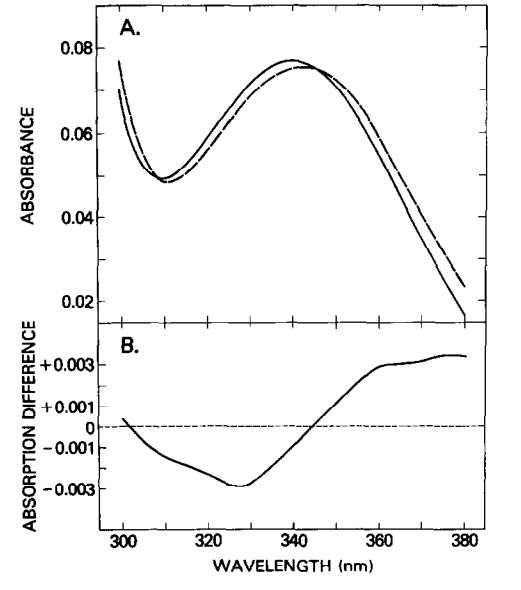

Fig. 5. Panel A. Absorption spectra of 12.4 μM NADPH in the presence (- - -) or absence (——) of 3.7 μM rat liver aldehyde reductase in 0.1 M potassium phosphate buffer, pH 6.6. Panel B. Different spectra determined directly from the above mixtures, using a full scale chart expansion of 0.02 absorbance units.

Table 6. Apparent deuterium isotope effects of carbonyl reduction

Enzyme	Cofactor	V_{max} (μmoles/min)	$\frac{[V_{max}]_H}{[V_{max}]_D}$	V_{max}/K_m (μmoles/min/M)	$\frac{[V_{max}/K_m]_H}{[V_{max}/K_m]_D}$
Daunorubicin (pH 8.5) reductase*	Unlabeled NADPH	1.4 ± 0.071	1.0	1.1 ± 0.077 (× 10^4)	1.0
	A-[²H]NADPH	0.71 ± 0.045	2.02	0.65 ± 0.060 (× 10^4)	1.78
	B-[²H]NADPH	1.3 ± 0.094	1.09	1.1 ± 0.11 (× 10^4)	1.03
Daunorubicin (pH 6.0) reductase†	Unlabeled NADPH	0.99 ± 0.068	1.0	1.6 ± 0.11 (× 10^5)	1.0
	A-[²H]NADPH	0.98 ± 0.065	1.01	1.8 ± 0.13 (× 10^5)	0.91
	B-[²H]NADPH	0.60 ± 0.11	1.67	0.76 ± 0.11 (× 10^5)	2.21
Naloxone reductase‡	Unlabeled NADPH	0.15 ± 0.0075	1.0	7.9 ± 0.76 (× 10^2)	1.0
	A-[²H]NADPH	0.065 ± 0.0027	2.36	2.8 ± 0.20 (× 10^2)	2.91
	B-[²H]NADPH	0.13 ± 0.0062	1.15	7.1 ± 0.67 (× 10^2)	1.12

* Rat liver aldehyde reductase.
† Rabbit liver pool I (Fig. 1A).
‡ Rabbit liver pool IV (Fig. 1A).

Table 7. Correlation between enzyme hydrogen stereospecificity and enzyme-dependent cofactor absorbance shift

Enzyme	Source	Enzyme conc. (μM)	Cofactor	Enzyme hydrogen stereo-specificity	Shift (340 nm)	Reference
Aldehyde reductase*	Rat liver	3.7	NADPH	A	Red (3)†	This paper
Aldehyde reductase	Human liver	21	NADPH	—‡	Red (12)	21
Glutamate dehydrogenase*	Bovine liver	2.7	NADH	B	Red (31)	17
α-Glycerophosphate dehydrogenase*	Rabbit muscle	14.5	$NADP^+$	B	Red (31)	17
Aldehyde reductase	Pig kidney	60.3	NADPH	A	Blue (10)	20
Alcohol dehydrogenase*	Yeast	14.8	NADPH	A	Blue (2)	17
Isocitrate dehydrogenase*	Pig heart	6.5	NADH	A	Blue (2)	17

* Direction of each ultraviolet absorbance shift was determined or confirmed in this study.
‡ Not reported.
† Absorbance shift measured in nanometers.

but not with B-labeled or unlabeled cofactors. These results confirm daunorubicin (pH 6.0) reductase as B-specific and daunorubicin (pH 8.5) and naloxone reductase as A-specific.

Ultraviolet absorbance shift on binding of NADPH to rat liver aldehyde reductase

The ultraviolet absorption spectra of reduced pyridine nucleotide coenzymes are shifted toward the red or blue spectrum upon binding with specific dehydrogenases. It has been proposed that the direction of that shift can be predicted by the dehydrogenase hydrogen stereospecificity [20]. We verified the absorbance shift at 340 nm on binding of reduced cofactor to several dehydrogenases (Table 7). The B-specific α- glycerophosphate and glutamate dehydrogenases had expected red shifts and the A- specific alcohol and isocitrate dehydrogenases gave expected blue shifts. Similarly, it had been reported that A-specific, pig kidney aldehyde reductase produces a blue shift, and human liver aldehyde reductase of unknown specificity produces a red shift. Rat liver aldehyde reductase was A-specific and gave a red shift on binding of NADPH (Fig. 5). Rat liver aldehyde reductase, therefore, represents an exception to the correlation of hydrogen stereospecificity and cofactor absorbance shifts.

DISCUSSION

Because of differences in general chemical reactivity of aldehydes and ketones, we have found it convenient to subdivide enzymatic reduction of carbonyl-containing compounds into a consideration of aldehyde reductases and ketone reductases. Although aldehyde reductases have been studied for many years and recently purified to homogeneity from several sources [10,14,21–26] ketone reductases have not been adequately purified and studied.

The lack of information on ketone reductases is partially explained by low recoveries of activity on storage and during purification. The procedures presented in this study include 0.5–1.0 mM dithiothreitol as an effective and practical means of stabilizing the reductase activities over at least a 2-month period. The presence of dithiothreitol during storage does not, however, appear to prevent the time-dependent 'aging' phenomenon of unknown mechanism which increases the isoelectrofocusing complexity of an already heterogeneous enzyme distribution.

The heterogeneity and 'aging' phenomenon also contribute to the difficulties encountered when applying conventional procedures for the purification of these enzymes. Obviously, a rapid and specific affinity absorption approach offers the ultimate solution. However, the procedure of isoelectric focusing described in this study provides a rapid and quantitative means of resolving the major classes of carbonyl reductases. The majority of the ketone reductases [as defined by daunorubicin (pH 6.0), oxisuran, metyrapone and 3,7-DMX reductases] are essentially resolved from the major aldehyde reductase [as defined by daunorubicin (pH 8.5) activity]. On the other hand, except for some overlap, the reductases of naloxone and naltrexone are separate and distinct from both of the other groups. The separation of

these groups of enzymes is defined by rather narrow isoelectric point ranges. The possibility of ampholyte-induced heterogeneity has been considered and is not excluded entirely. Nevertheless, similar enzyme patterns were obtained at ampholyte concentrations ranging from 0.8 to 4 per cent, and prompt re-electrofocusing of peak 1 gave activity peaks with isoelectric points entirely within the activity peak of the first broad range experiment. Heterogeneity also was seen previously when the enzymes were separated by gel filtration and ion exchange chromatography only [4,5].

Pool I retained the majority of the daunorubicin (pH 6.0), oxisuran, 3,7-DMX, metyrapone and *p*-nitroacetophenone reductase activities (although minor activity peaks were observed in other pools). Pool I was subsequently re-electrofocused into two peaks (Pool 1A and Pool 1B) over a very narrow pH gradient. With minor exceptions, most of the above activities were coincidentally distributed between these two peaks. It appears, therefore, that the majority of the total ketone reductase activity resides in two enzymes of pI values 4.84 and 4.98 (Fig. 2).

Because of the greater chemical reactivity of aldehydes relative to ketones and assuming otherwise similar structures, it might be expected that ketone reductases react readily with the highly reactive aldehyde substrates. This prediction could cause a serious limitation in a study of aldehyde reductases in the presence of ketone reductases. Consider the reduction of a typical aldehyde reductase substrate, *p*-nitrobenzaldehyde, by rabbit liver (Fig. 2). The distribution of a *p*-nitrobenzaldehyde reduction suggests an extremely heterogeneous population of aldehyde reductases. However, only a small shoulder of this total *p*-nitrobenzaldehyde reductase activity is coincident with aldehyde reductase as determined with daunorubicin at pH 8.5 (see below). In addition, except for pool III, there is considerable overlap of *p*-nitrobenzaldehyde reduction with *p*-nitroacetophenone reduction throughout the pH gradient. Therefore, it would appear that *p*-nitrobenzaldehyde is reacting with ketone as well as aldehyde reductases.

By the same reasoning, it might be expected that aldehyde reductases do not normally cross-react with the relatively less reactive ketones. This was confirmed for rat liver aldehyde reductase which is very active with *p*-nitrobenzaldehyde but has negligible activity with *p*-nitroacetophenone [14]. The rat liver aldehyde reductase, however, does reduce the methyl ketone antibiotic daunorubicin [10,14]. Although an exception, this latter reaction is nevertheless catalyzed by an aldehyde reductase and daunorubicin is the only ketone known to be reduced by this enzyme. In addition, the reduction occurs at an alkaline pH unique to all other aldehyde and ketone reductases. In other words, although daunorubicin is reduced by ketone reductases at pH 6.0, it is reduced only by aldehyde reductases at pH 8.5 [14]. Therefore, the reduction of daunorubicin at pH 8.5 is suggested as a specific assay of aldehyde reductases in the presence of ketone reductases.

Since aldehyde reductases apparently do not react with most ketones, the specific assay for ketone reductases should be straightforward. The individual rabbit liver ketone reductases show considerable selectivity for individual ketones. For example, the dihydromorphinone reductases (pools IV, V and VI) show low activity with daunorubicin (pH 6.0) and metyrapone. Nevertheless, most pools exhibit significant activity for *p*-nitroacetophenone (Fig. 2) as would be expected for a model ketone reductase substrate.

The hydrogen stereospecificity of carbonyl reductases has been studied previously for a number of aldehyde reductases and closely related enzymes. Rat intestine retinal reductase [21], rabbit muscle glycerol dehydrogenase [11], rat liver mevaldate reductase [28], human placental aldose reductase [29], kidney aldehyde reductases from rabbit [20], rat [3], chicken [3] and aldehyde reductases from fruit fly [3] and yeast [3] have all been shown to utilize the A-side (pro-4R) hydrogen of the dihydronicotinamide ring of NADPH to reduce their appropriate aldehyde or aldose substrate. To this list we can now add rat and rabbit liver aldehyde reductase as well as the rabbit liver dihydromorphinone reductases and minor forms of 3,7-DMX and *p*-nitroacetophenone reductases. All of the above-mentioned enzymes, therefore, have the same A-specificity as alcohol dehydrogenase [30].

In contrast, a B-side (pro-4S) hydrogen stereospecificity occurs for the aromatic aldehyde ketone reductase(s) from rabbit kidney [31]. In our study, most of the rabbit liver ketone reductases also have a B-hydrogen stereospecificity. Since these B-specific enzymes make up the bulk of the ketone reductase activity, a general distinction between aldehyde and ketone reductases can be made on the basis of their hydrogen stereospecificity. This difference may be an important criterion of identity because these enzymes have very similar physical, chemical and kinetic properties.

A major difficulty with some of our observations is the mixed or low stereospecificities of certain enzyme fractions. Whereas most of the reactions show a stereospecificity preference of 95 per cent or greater by Method 1, a few of the reactions had specificities as low as 75 per cent for Method 1 or 60 per cent for Method 2. There was, however, agreement as to which reactions were of high specificity and which were of low specificity between these two methods. The most likely explanation for low and sometimes variable specificities is undoubtedly the use of partially purified enzyme pools. For example, when homogeneous rat liver aldehyde reductase was examined at its optimum pH, better than 99 per cent stereospecificity was obtained. In addition, after re-electrofocusing of pool I over a narrower pH gradient, there was an apparent increase in stereospecificity (see Results). Finally, when purified A- and B-[^{3}H]NADPH were examined with commercially purified yeast alcohol dehydrogenase, A/B ratios as high as 300 to 1 were obtained. The low stereospecificities of partially purified enzyme pools may have resulted from the presence of drug-specific reductases of opposite specificities. The apparently low stereospecificities might also have resulted from the presence of contaminating enzymes such as pyridine nucleotide transhydrogen-

ases which would mix the label and/or lower the intitial cofactor specific radioactivities. The importance of using highly purified enzymes for precise stereospecificity determinations was emphasized previously for nitrate reductase [32].

Generally, if stereospecificities were to be determined by Method 1 or 2 the carbonyl reductions were done at pH 6.5 (rather than at the optimum of pH 6.0) to avoid acid destruction of NADPH. The quantitation of most alcohols used to determine specific radioactivities was based on the change in absorption at 340 nm of NADPH during carbonyl reduction, assuming 100 per cent recovery. The validity of this assumption was verified for daunorubicin reduction by quantifying the formation of daunorubicinol by an independent fluorescence assay, and for oxisuran reduction by utilizing ^{14}C-labeled oxisuran.

A wide range of specific radioactivities of starting tritium-labeled cofactors was used with identical results by Method 1. In Method 2, however, the use of very high specific radioactive cofactors resulted in an apparent radiation decomposition product(s) which appeared in the flow-through peak of the DEAE-cellulose column. This difficulty was eliminated by using a 100-fold lower cofactor radioactive specific activity of that used in Method 1. In both Methods 1 and 2, the reactions were run to equilibrium or completion. The results, therefore, should not be influenced by isotope rate effects.

Method 3 differed from the other methods in that stereospecificity was determined by an isotope rate effect on the intitial steady state enzyme kinetics. In addition, in order to accentuate the effect, carrier-free deuterium-labeled cofactors were used instead of tritium-labeled cofactors. Since the conclusions were based on initial rate effects, this procedure was probably less sensitive to the presence of low levels of contaminating enzymes which might otherwise obscure the results of reactions which had run to equilibrium. Agreement among the different procedures gives confidence to the final conclusions.

Besides establishing the stereospecificities of aldehyde and ketone reductases, the present study demonstrates an exception to the generalization that the direction of a shift of the pyridine nucleotide absorption when binding to dehydrogenases is predicted by the stereospecificity of the enzymes. Although all the aldehyde reductases examined to date show A-specificity, only pig kidney aldehyde reductase exhibited a blue shift as predicted [23]. Contrary to expectations, we found that rat liver aldehyde reductase exhibits a red shift. Human liver aldehyde reductase also gives a red shift on binding the pyridine nucleotide; however, its stereospecificity has not yet been reported [25].

A number of generalized rules regarding the stereospecificity of pyridine nucleotide catalyzed reactions have been established [30, 33]. One states that enzymes which catalyze the same reaction have the same stereospecificity regardless of the enzyme source. Only 3-α-hydroxysteroid dehydrogenase and ketopantoyl lactone reductase are reported to be exceptions to this rule [30, 32]. Several of the reactions described in this study represent additional exceptions to this rule. For example, *p*-nitroacetophenone and *p*-nitrobenzaldehyde occur with different specificities depending on which enzyme pool is considered. 3,7-DMX is another possible exception. Also, the reduction of daunorubicin is accomplished by enzymes of opposite specificity. This latter example was clearly demonstrated by all three methods. The exceptions are even more striking because they occur within the same animal.

Naloxone, naltrexone and oxisuran were each reduced to two diastereoisomers. The stereospecificities of reduction of naloxone and naltrexone α-OH and β-OH alcohols by pools II and IV and α- and β-oxisuranols by pool I were analyzed by Method 1. All naloxol and naltrexol diastereoisomers incorporated label only from A-[^{3}H]NADPH, whereas both oxisuranol diastereoisomers used label exclusively from B-[^{3}H]NADPH. Using A-labeled cofactor, we found the ratio of specific radioactivities of α-OH/β-OH-naloxol and naltrexol diastereoisomers was 1/10 in Pool II and 1/100 in Pool IV. Whether these pools contained separate α-OH or β-OH reductases or a single enzyme with different stereoselectivities is presently unknown.

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