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Species and strain differences in the epimerization of 3β, 17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene to the 3α-hydroxy epimer*

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THE CONVERSION of 3β,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene to 3α,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene, both intermediary metabolites of norethynodrel (17α-ethynyl-17β-hydroxy-$\Delta^{5(10)}$-estrene-3-one), is catalyzed by a 3β-hydroxy-$\Delta^{5(10)}$-steroid epimerase found in the soluble fraction of rat liver.[1] Since much new information has been published on the effects of drugs on different strains of the same species and on different species of animals,[2,3] it is of great importance that all the biochemical pathways of each species be determined, especially if it is unique to one species. The importance of species differences in drug metabolism is shown by the number of recent reviews[4–6] and symposia[7,8] correlating species differences in drug metabolism with the mechanism of action of many drugs.

A study was undertaken to determine whether the 3β-hydroxy-$\Delta^{5(10)}$-steroid epimerase was unique to the Charles River rat, CD strain, in which it was originally found,[1] or whether it exists in other rat strains, in other rodent species commonly used in laboratory experimentation, and in man. In addition, the Michaelis constant (K_m) for the epimerization reaction and the inhibitor constant (K_i) for the 3α-hydroxy epimer were determined.

NADP was purchased from Sigma Chemical Co. The animals and their source follow: Golden Syrian Hamster, Petterson Hamstery; New Zealand White Rabbit, Franklin's Rabbitry; Mongolian Gerbil, Chick Line Co., Guinea Pig, Hartley strain, Flow Laboratories; CF-1 mouse, Carworth Farms; Charles River CD strain rat, Charles River Breeding Laboratory; Blue Spruce Farms hood rat (LE); Blue Spruce Farms Sprague–Dawley rat (SD); Holtzman rat; Carworth CFN rat; Carworth CFE rat. The 3β,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene was synthesized by Dr. Ivy Carroll of this laboratory's organic synthesis group using the method of Palmer *et al.*[9]

Two young adult male and two young adult female animals from each rodent species or strain were used in this study. The animals were killed by decapitation, the livers quickly removed and a 1-g section from each liver was homogenized in 10 ml of 0·1 M potassium phosphate buffer, pH 7·4, using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant obtained by centrifuging the 10,000 *g* supernatant in a Beckman L2-65B at 105,000 *g* for 60 min was used as the enzyme source.

All incubations were performed in duplicate. A ratio of 1 mg of the 3β-hydroxy steroid substrate per gram animal liver was consistently used in all experiments except in those incubations used to determine the kinetic constants. Thus, 1 mg of substrate dissolved in 0·5 ml ethanol and 1·0 mM NADP were incubated with the 105,000 *g* supernatant from 1 g rat liver for 20 min in a 50-ml Erlenmeyer flask (total fluid vol. 10 ml) using a Dubnoff metabolic shaker (37°). The reaction was

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stopped by the addition of 20 ml redistilled ethyl acetate to each incubation flask. Rat liver was used as the enzyme source for the kinetic studies.

After extraction with ethyl acetate, the solvent was removed *in vacuo* and the residue partitioned between hexane and aqueous (10%) methanol. A considerable amount of endogenous lipid material remained in the hexane fraction while the remaining substrate and its metabolite were recovered quantitatively from the methanol fraction.

The initial identification of the steroids extracted from the incubation media was by both gas–liquid chromatography (GLC) and GLC–Mass spectrometry (GLC–MS) using procedures similar to those described by Palmer *et al.*[9] The absolute separation of the 3α- and 3β-hydroxy epimers by GLC techniques can be obtained by first forming the trimethylsilyl (TMS) ethers.[9] The steroidal extraction from every incubation was routinely reacted with hexamethyldisilazone and trimethylchlorosilane in pyridine at 100° to form the TMS ethers. The routine GLC analysis was carried out on a column of 1·9% OV-17 on acid-washed, silanized Chromosorb W at 215° in a Varian Aerograph Series 1200 flame ionization chromatograph.

The Michaelis constant for the epimerization reaction with the rat liver epimerase was determined using the double reciprocal plot of Lineweaver and Burk.[10]

Table 1 lists the relative amounts of epimerase activity in 1 g of liver from six different animal species. Although rat liver demonstrates the highest epimerase activity, the activity from the Mongolian Gerbil liver is appreciable. The results obtained from the guinea pig, hamster, and mouse indicate

TABLE 1. COMPARISON OF EPIMERASE ACTIVITY IN LIVER FROM SIX ANIMAL SPECIES*

Species	% Conversion
Charles River Rat, CD strain	91
Mongolian Gerbil	53
New Zealand White Rabbit	20
Guinea Pig, Hartley strain	3
Golden Syrian Hamster	2
CF-1 Mouse	0

* Standard incubation conditions: 1 mg 3β-hydroxy steroid substrate, 105,000 *g* supernatant from 1 g liver, 1·0 mM NADP, 0·1 M potassium phosphate buffer, pH 7·4, incubated at 37° for 20 min. The enzyme from both young adult male and female animal liver of the same species gave identical results.

that a 3β-hydroxy-$\Delta^{5(10)}$-steroid epimerase does not exist in the liver of these species. Although a significant difference exists between different species, no intraspecies sex difference was seen.

Although a large species variation exists in the ability to epimerize the 3β-hydroxy-$\Delta^{5(10)}$-steroid substrate, there appears to be no difference in epimerase content in livers from different rat strains (Table 2). Both male and female rat liver enzyme gave identical results.

TABLE 2. COMPARISON OF EPIMERASE ACTIVITY IN LIVER FROM DIFFERENT RAT STRAINS

Rat strain	% Conversion*
Blue Spruce Farms—LE (Long–Evans)	91
Blue Spruce Farms—SD (Sprague–Dawley)	90
Carworth—CFE (Sprague–Dawley-derived)	91
Carworth—CFN (Wistar-derived)	91
Charles River—CD	91
Holtzman	91

* For experimental details, see Table 1. Both male and female rat liver from each strain gave identical results.

A Lineweaver–Burk plot of the reciprocal of the velocity of 3α,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene formation versus the reciprocal of the concentration of 3β,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene in the presence and absence of the 3α-hydroxy steroid shows that the product inhibits competitively (Fig. 1). The K_m and K_i, as determined from the plot, are $1{\cdot}1 \times 10^{-4}$ M and $1{\cdot}8 \times 10^{-4}$ M respectively.

A number of recent studies have shown significant differences between species and strains in the metabolism and response to specific drugs. Low *et al.*[11] have shown a variation in enzymatic activity between strains of mice and Cram *et al.*[12] demonstrated a 10-fold variation in the basal metabolic rate of rabbits. However, the wide variation in drug response and drug metabolism seen between strains of one species and between various species has not been demonstrated in the rat.[3]

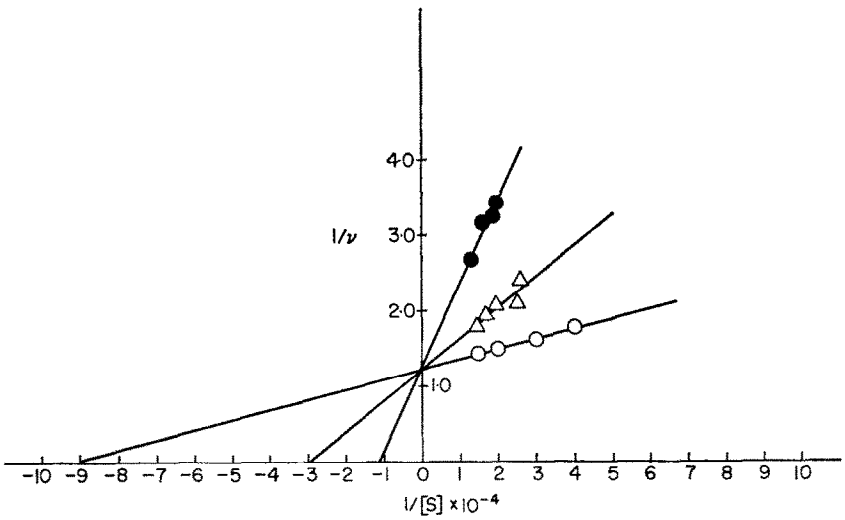

FIG. 1. Lineweaver–Burk plot of reciprocal velocity of 3β-hydroxy steroid epimerization versus the reciprocal of the substrate concentration in the absence (○) and presence (△, 4×10^{-4} M; ●, 10×10^{-4} M) of the 3α-hydroxy steroid product. The reaction mixture contained 0·1 M potassium phosphate buffer, pH 7·4, 1·0 mM NADP and enzyme from 1 g rat liver. The incubation was performed at 37° for 20 min in a Dubnoff Incubator.

The enzyme responsible for the epimerization of 3β,17β-dihyroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene to the 3α-hydroxy epimer is highly active in livers from three different species. There was no obvious sex difference found among the six species studied or in the six rat strains. It has been shown in the literature that a sex difference exists for the microsomal metabolism of several drugs,[13] but that all of the sex dependent rat liver enzymes are localized in the microsomal fraction.[14] It is therefore not unusual not to find a sex difference in the soluble enzymes.

It is of considerable interest that mouse liver is unable to epimerize the 3β-hydroxy steroid in light of the recent finding that 3β, 17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-esterene shows greater teratogenic activity in mice than the corresponding 3α-hydroxy epimer.[15]

That the dissociation constants are very similar in the presence and absence of the 3α-hydroxy steroid is indicative of an almost identical binding affinity by both epimers for the enzyme. However, although both steroids have the same dissociation constant for the substrate binding site, only the 3β-hydroxy epimer has the stereospecificity required for recognition by the catalytic site. It has been previously shown that in $\Delta^{5(10)}$ steroids the conformation of the 3α-hydroxyl is equatorial, the corresponding 3β-proton being axial. In the epimeric 3β-hydroxy steroids, the 3β-hydroxyl is axial and the corresponding 3α-proton is equatorial.[16] It has previously been shown that the reaction involves the formation and subsequent reduction of an enzyme-bound 3-keto intermediate.[1] If the rate determining step in the enzymatic epimerization corresponds to that of a chemical oxidation, then the removal of a proton at C-3 is required for the formation of the 3-keto steroid. In general the abstraction of an equatorial proton is considerably easier than the removal of the more sterically

hindered axial proton. Since the 3β-hydroxy steroid contains a 3α-equatorial proton, it is most probable that the equatorial proton is a steric requirement by the catalytic site for catalysis.

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