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The Stereochemistry of Hydrogen Transfer to $NADP^+$ by Enzymes Acting upon Stereoisomeric Substrates[1]

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The stereochemistry of hydrogen transfer from estradiol-17α and estradiol-17β to $NADP^+$ in the presence of chicken liver estradiol-17α and estradiol-17β dehydrogenases was found in both cases to involve the 4-pro-S proton of the pyridine nucleotide. One of these enzymes must therefore use the stereochemically less favorable mode of interaction of steroid with coenzyme.

INTRODUCTION

Estradiol-17α and estradiol-17β differ only in the chirality of their substituents at C-17. In their interaction with optically active reagents the two estradiols should therefore interact preferentially with reagents of opposite stereochemistry. Such an effect is well documented (*1*) and is the basis (*2*) of the Horeau method (*3*) of determination of stereochemistry of an alcohol.

If a close fit of the oxidized cofactor ($NADP^+$) with the steroid alcohol is of overriding importance in the enzymic transfer of hydrogen on oxidation of the two estradiols to estrone, then they should interact with opposite sides of the nicotinamide ring. If the enzyme plays an important role in directing the orientation of the substrates and cofactor, no such prediction can be made. The occurrence in chicken liver of two soluble, $NADP^+$-dependent and stereospecific enzymes, one of which catalyzes the oxidation of estradiol-17α and the other the oxidation of estradiol-17β has been reported (*4*). The separation of the two activities demonstrates that two enzymes are indeed present and thus the stereochemical problem can be attacked.

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EXPERIMENTAL

Enzyme Preparation

The $NADP^+$-dependent estradiol dehydrogenases of chicken liver were prepared according to Renwick and Engel (*4*). Samples of the stock ammonium sulfate fraction were subjected to gel filtration on a column (2.5 × 40 cm) of Sephadex G-100 in 1 mM phosphate buffer (pH 7.0) with added glycerol (20% by vol). The active fractions were pooled to yield a preparation that contained 3.84 mU of estradiol-17β dehydrogenase, 1.1 mU of estradiol-17α dehydrogenase and less than 0.20 mU of $NADP^+$ reductase activity per milligram of protein (*4*).

Preparation of Deuterated Substrates

Reduction of estrone in isopropanol with sodium borodeuteride gave [17α-^{2}H]-estradiol-17β (97%) and the 17-epimer (2%); both were obtained pure by direct crystallization. They were shown by gas chromatography–mass spectrometry to contain 81% deuterium at C-17. NMR (pyridine) confirmed the location of the deuterium as 17α. Oxidation with CrO_3 in pyridine gave estrone containing no excess deuterium. [17β-^{2}H]estradiol-17α was obtained from the 17-epimer by treatment of the toluene *p*-sulfonate in refluxing *N*-methylpyrrolidone with tetra-*n*-butylammonium acetate (*5*) followed by hydrolysis of the [17β-^{2}H]estradiol-17α acetate. The product, which contained 79% ^{2}H at C-17β, was identical to the minor product obtained from the borodeuteride reduction.

Physical Methods

Gas chromatography–mass spectrometry was performed in an LKB-9000 instrument modified for solid sample introduction (*6*). Estrone and the estradiols were chromatographed on 2% OV-1 as acetates or trimethylsilyl ethers at 180 and 210°C, respectively. Nicotinamide was chromatographed at 170°C on 3% OV-225. The He flow in all cases was 30 ml per min. For mass spectral measurements the molecular separator was kept at 250°C and the ion source at 270°C. The ionizing current was 50 μA and ionizing energy 70 eV during scans.

Incubations and Isolation of Products

Incubations were carried out at 25°C in duplicate. Each flask contained 80 ml of 0.225 M potassium phosphate buffer (pH 9.2) and either 155 mU of estradiol-17β dehydrogenase activity or 80 mU of estradiol-17α dehydrogenase activity. To each flask was added 4 μmoles of the appropriate deuterated estradiol and 2 μmoles of $NADP^+$. The reaction was initiated with substrate, and was allowed to proceed until there was no further increase in absorbance at 340 nm (about 30 min). The flasks were then heated to 100°C and cooled; denatured protein was removed by centrifugation.

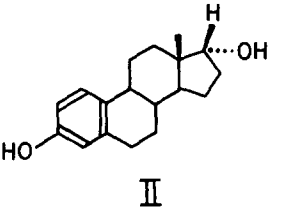
To one flask was added EDTA (33 μmoles), magnesium sulphate (200 μmoles), oxalosuccinate (200 μmoles) and water (20 ml). The pH was lowered to 7.4 with HCl and the oxidation of NADPH initiated with 380 U of isocitric dehydrogenase and allowed to proceed until there was no further decrease in absorbance at 340 nm. The pH was raised to 10.2 with NaOH and the reaction mixture heated to 100°C for 20 min with stirring to cleave the nicotinamide–ribose bond of $NADP^+$.

To the second flask was added ammonium chloride (5.6 mmoles), and α-oxoglutarate (0.24 mmoles). The pH was lowered to 7.0 with HCl and the reaction initiated with 350 U of glutamic dehydrogenase. The reaction reached equilibrium in about 3 min and was quenched as described above.

After cooling, absolute ethanol was added to the flasks to make the solutions 80% in ethanol; the precipitated protein was removed by centrifugation. The supernatant was concentrated at 50°C under reduced pressure to about 10 ml and extracted three times with 1 vol of methylene chloride to recover the steroids. The methylene chloride extract was washed with one-tenth its volume of water. Estrone and the estradiols were identified by gas chromatography–mass spectrometry. The aqueous solutions and water washes were concentrated further. Nicotinamide was isolated by gel filtration on a column of Sephadex G-10 (1.5 × 32 cm) eluting with water. Nicotinamide (260 nm absorption) was eluted at 2.6 times the void volume of 53 ml. The solutions were evaporated to dryness at 50°C under reduced pressure and analyzed by gas chromatograph–mass spectrometry.

RESULTS AND DISCUSSION

The analyses of the four samples of nicotinamide and the recovered estradiols are shown in Table 1. Although the hydrogen transfer was by no means stoichiometric, it is evident that the dehydrogenases of chicken liver catalyze the transfer of hydride from both estradiol epimers to the 4-pro-S-position[5] of reduced NADP as is the case with the majority of steroid alcohol dehydrogenases thus far examined (7).



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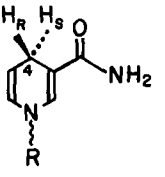

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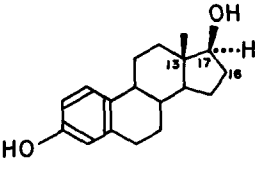

III

The preferred orientation of cofactor and substrate can be predicted if it is assumed that the transfer is directed only by their steric interaction. In both estradiols, the largest group attached to C-17 is C-13; the 17α-hydroxyl group is bulkier than the 16-CH_2. C–$CONH_2$ is the largest substituent on C-4 of the dihydronicotinamide

[5] The designation pro-S is used in place of the less precise B-side of the dihydronicotinamide moieyt of NADPH. Pro-R is equivalent to A-side.

TABLE 1

HYDROGEN TRANSFER FROM 17-DEUTERATED ESTRADIOLS TO NADP+

Estradiol dehydrogenase	Dehydrogenase oxidation of [4-^{2}H]NADPH	Percentage ^{2}H in nicotinamide	Percentage ^{2}H in recovered estradiol[a]
17β	Isocitrate (4R)[b]	31	80
17β	Glutamate (4S)[b]	3	80
17α	Isocitrate (4R)	11	73
17α	Glutamate (4S)	1	77

[a] The [17α-^{2}H]estradiol-17β contained 81% deuterium and the 17α-[17β-^{2}H]-estradiol, 79%.

[b] Isocitrate dehydrogenase transfers the 4-pro-R proton of NADPH; glutamate dehydrogenase transfers the 4-pro-S-proton.

moiety (I) of the cofactor; CH is larger than the pro-R hydrogen. Direct transfer of hydride between cofactor and estradiol-17α (II) [an as yet unproved but likely (*8*) assumption] would be as in Figs. 1 and 2 [which is a Newman Projection of 1 along the

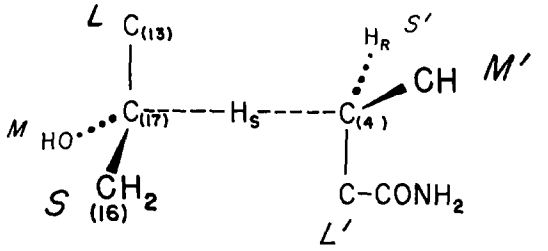

FIG. 1. Stereochemistry of the transition state postulated for oxidoreduction of estradiol-17α.

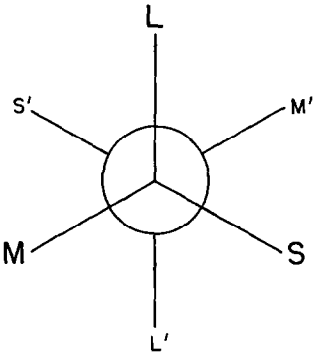

FIG. 2. Newman projection of the transition state postulated for oxidoreduction of estradiol-17α.

steroid C-17-H_S–nicotinamide C-4 axis]. These figures correspond to the optimal condition, with each "small" substituent between "large" and "medium." Thus it might be expected that 17α-estradiol would be formed by transfer of the pro-S-hydrogen of dihydronicotinamide, and 17β-estradiol (III) by transfer of the pro-R hydrogen. This argument depends upon the further assumptions that (1) the 17-OH is not solvated

and is intermediate in size between the other two nontransferring substituents on C-17, and (2) no dipole interactions occur between the 17-OH and the amide group of NADPH (*9*).

A similar situation exists in the case of the 3α- and 3β-hydroxysteroid dehydrogenases of *Pseudomonas testosteroni*; both utilize the 4-pro-S proton of NADPH. However, in this case the stereochemical requirements are less exacting because of the greater distance of the reaction site from the closest asymmetric center (*10–12*). In contrast, the oxidoreduction at C-20 (adjacent to the asymmetric center at C-17) by rat ovary 20α-hydroxysteroid dehydrogenase (*13*) involves the 4-pro-R proton of NADPH, while the 20β-hydroxysteroid dehydrogenase of *Streptomyces hydrogenans* uses the 4-pro-S proton of NADH (*14*). In this case the fact that both reactions correspond to optimal fit for hydrogen transfer may be coincidence.

Another case in which stereochemistry of hydrogen transfer may be related to stereochemistry of substrate attack is that of the Δ^4-reductases of rat liver. The soluble 5β-reductase transfers the 4-pro-R hydrogen of NADPH to C-5 of the steroid nucleus (*15*), while the microsomal 5α-reductase transfers the 4-pro-S hydrogen (*16*). Recently Akhtar *et al.* (*17*) listed a series of nine NADPH dependent steroid reductases of mammalian origin in which the hydride transferred to the α-face of the steroid was the 4-pro-S hydrogen of the coenzyme and that transferred to the β-face was the 4-pro-R hydrogen. The avian enzymes also employ $NADP^+$ as cofactor but do not follow this rule.

Since both estradiols are formed by transfer of the pro-S hydrogen, it would appear that the stereochemistry of the estradiol does not play the dominant role in determining the stereochemistry of hydride transfer, at least in the case of estradiol-17β dehydrogenase. Indeed, regardless of the assumptions made concerning any interaction of steroid and coenzyme, in one case, the stereochemically awkward mode is selected by the enzyme; one of these enzymes is just a little clumsy.

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