Stereospecificity of Hydrogen Transfer by Pyridine Nucleotide-linked Hydroxysteroid Dehydrogenases

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The present studies are concerned with the determination of the steric course of the transfer of hydrogen catalyzed by hydroxysteroid dehydrogenases. The classic work of Vennesland et al. (1-3) has demonstrated that pyridine nucleotide-linked dehydrogenases catalyze a direct and stereospecific transfer of hydrogen between the substrate and the para position of the nicotinamide ring of the nucleotide. With the aid of deuterium labeling, it was possible to distinguish two classes of dehydrogenases which transferred the isotope respectively to give one or the other of the two diastereomeric forms of reduced diphosphopyridine nucleotide-nicotinamide-4-d. The first three enzymes to be studied (the dehydrogenases for alcohol, l-lactate, l-malate) all had the same stereospecificity of hydrogen transfer, which was designated as (Y (or side 1)' in the absence of information on the absolute configuration (4-6). In 1955, two other pyridine nucleotide-linked enzymes were shown likewise to catalyze a direct transfer of hydrogen, but to utilize the other diastereomic form of reduced DPN in which the reacting hydrogen was designated as $\beta$ (side II). These enzymes were the $\beta$-hydroxysteroid dehydrogenase of Pseudomonas testosteroni which catalyzed the DPN-dependent interconversion of testosterone and 4-androstene-3,17-dione (7)) and the pyridine nucleotide transhydrogenase of Pseudomonas Juorescens (8). Subsequently, other dehydrogenases which transfer hydrogen to one or the other side of the nicotinamide ring have been described. These studies have been recently extended to several TPN-linked enzymes where analogous stereospecificity with respect to the pyridine nucleotide was observed (9-11). The basic significance of the two types of stereospecificity among these hydrogen transferring reactions has remained obscure. It has been suggested that metabolic coupling of reactions between enzymes of opposite stereospecificity without dissociation of DPN might be facilitated (12). This argument is strengthened by the finding that, for instance, the bound DPN of triosephosphate dehydrogenase (side II stereospecificity) reacts more rapidly with lactic dehydrogenase (side I stereospecificity) than does free DPN (13).

Recently, specific dehydrogenases which catalyze the oxidation of either member of an enantiomorphic pair of substrates have been studied. Separate DPN-linked n- and l-lactic dehydrogenases from Lactobacillus arabinosus were shown to remove hydrogen from the same side (side I) of the pyridine ring of DPNH and to add it stereospecifically to pyruvate (14). The present studies likewise show that there is no apparent relation between the stereospecificity for substrate and for the pyridine nucleotide for reactions catalyzed by hydroxysteroid dehydrogenases.

$\beta$-Hydroxysteroid dehydrogenase of Pseudomonas testosteroni is a highly purified steroid-induced enzyme which promotes the DPN-linked interconversion of 3$\beta$- and 17$\beta$-hydroxysteroids and their respective ketones (15, 16). The stereospecificity of hydrogen transfer by this enzyme during the reduction of 4-androstene-3,17-dione to testosterone has been previously reported to involve side II (7). The present experiments show that side II stereospecificity is also observed for the oxidation-reduction of 3$\beta$-hydroxysteroids, in the following reaction:

\[
\text{Androstane-3,17-dione} + \text{DPNH} + \text{H}^+ \rightarrow \text{Androsterone} + \text{DPN}^+ + \text{H}_2
\]

(1)

3$\alpha$-Hydroxysteroid dehydrogenase has been obtained as a highly purified induced enzyme from Pseudomonas testosteroni (15, 16). It will be shown that the stereospecificity of the latter enzyme also involves side II of the nicotinamide ring of DPN:

\[
\text{Androsterone} + \text{DPN}^+ \rightarrow \text{Epiandrosterone} + \text{DPNH} + \text{H}^+
\]

(2)

* Supported by grants from the American Cancer Society.
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1 The designations side I and side II are used in the text in preference to $\alpha$ and $\beta$, respectively, in order to avoid confusion with the symbols of steroid nomenclature.
A 17β-hydroxysteroid dehydrogenase for estradiol-17β has been characterized and purified from human placenta (17, 18). This enzyme interconverts estradiol-17β and estrone. The rate of reaction is comparable when either DPN or TPN is used. This enzyme may also act as a pyridine nucleotide transhydrogenase in the presence of catalytic quantities of estradiol-17β. This process may be visualized as follows (18, 19):

\[
H^+ + DPNH + estrone \rightleftharpoons DPNP + estradiol-17β
\]

\[
TPNP + estradiol-17β \rightleftharpoons TPNNH + estrone + H^+
\]  

(3)  
\[
DPNP + TPNP \rightleftharpoons TPNNH + DPNN
\]  

The stereospecificity of the placental enzyme for pyridine nucleotides has been studied in the following reactions:

\[
\text{Estradiol-17β + DPNN} \rightarrow \text{estrone + DPNN + H}^+ 
\]

(4)  
\[
\text{Estradiol-17β + TPNP} \rightarrow \text{estrone + TPNN + H}^+ 
\]  

(5)  
\[
\text{DPPN + 3-acetylpyridine-DPNN} \rightarrow DPNN + 3-acetylpyridine-DPNN
\]  

These reactions were selected for study because they could be carried to completion in the indicated direction.

In all of the experiments, tritium was employed as a tracer, and for this purpose DPN-nicotinamide-4-t and TPN-nicotinamide-4-t were prepared by decomposing the alkaline cyanide complexes of the nucleotides in the presence of tritium oxide enriched water, according to the procedure described by San Pietro (20) for preparing DPN-nicotinamide-4-d. The use of tritium-labeled pyridine nucleotides in conjunction with liquid scintillation counting has substantially simplified the experiments on enzyme stereospecificity, since sample burning and mass spectrometry which are necessary for deuterium tracer studies are avoided. Moreover, the radioactive tracer provides potentially greater sensitivity. Isotope discrimination effects probably introduced little, if any, error in these experiments because all oxidations and reductions of the nucleotides were carried to completion. Vennesland and colleagues (21) have recently established the stereospecificity of malic enzyme with the use of TPN-nicotinamide-4-t.

**EXPERIMENTAL PROCEDURE**

**Steroids**—Estradiol-17β (m.p. 176–177°) and epialdrosterone (m.p. 172–173°) were purified from commercial preparations. Androsterone (m.p. 183–185°) was synthesized from epialdrosterone.

**Enzymes**—3a-Hydroxysteroid dehydrogenase (specific activity 30,600 units per mg of protein) and (3 and 17) β-hydroxysteroid dehydrogenase (specific activity 94,200 units per mg of protein) were purified from *Pseudomonas testosteroni*, by the procedure of Marcus and Talalay (15). The placental 17β-hydroxysteroid dehydrogenase was prepared by slight modifications of the procedure of Talalay et al. (18), and had specific activities of 96 to 112 units per mg of protein. Twice crystallized yeast alcohol dehydrogenase was obtained as a suspension in 50% ammonium sulfate from the Sigma Chemical Company. Crystalline liver glutamic dehydrogenase was likewise a suspension in ammonium sulfate obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Diphosphopyridine nucleotidase was prepared from zinc-deficient *Neurospora* by the method of Nason et al. (22).

**Other Materials**—Nicotinamide (m.p. 127–128°) was recrystallized from benzene. L-Glutamic acid (m.p. 200 202°) was recrystallized from hot water. α-Ketoglutaric acid (m.p. 114–115°) was recrystallized from ether and from benzene, and was a gift of Dr. B. K. Stern. DPN, TPN, and the 3-acetylpyridine analogue of DPN were purchased from the Pabst Laboratories.

**Preparation of Tritium-labeled Pyridine Nucleotides**—DPN-nicotinamide-4-t was prepared by decomposition of the basic cyanide addition complex of DPN in the presence of water enriched with tritium oxide, as described by San Pietro (20) for the preparation of the corresponding deuterium containing pyridine nucleotide. Five millimoles of KCN were dissolved in 3.0 ml of water containing about 150 mc of HTO. Then, 952 μmole of DPN and 0.1 ml of 5 × KOH were added. The resulting yellow solution was allowed to stand at room temperature for 2.25 hours and then 1.5 g of KH₂PO₄ (in 10 ml of H₂O) were added. The mixture was acidified by addition of 8 ml of 2 N H₂SO₄ to a pH below 3. More N₂ was bubbled through the solution mixture until the odor of HCN was no longer detectable (4 hours were adequate). The reactions were carried out with precautions to trap radioactive water vapors. The volume was then increased to 48 ml by the addition of 35 ml of H₂O. The mixture was acidified by addition of 2.8 ml of 2 N HCl to a pH below 3. More N₂ was bubbled through the solution, but no HCN was detected. The addition of 5.5 volumes of cold acetone (−20°) resulted in the formation of a silky tan precipitate which was permitted to accumulate overnight at −20°. The supernatant liquid was decanted and the precipitate washed three times with cold acetone. The precipitate was then dissolved in water and the pH raised to 8.0 by addition of 0.25 N NaOH. This material was then chromatographed on a Dowex 1-formate column as described by Kornberg (23).

**Hydrolysis of Nucleotides and Isolation of Nicotinamide**—The nicotinamide-ribose bond of DPN and TPN may be readily and quantitatively cleaved by *Neurospora* diphosphopyridine nucleotidase, and this procedure was utilized in one of the experiments. In most instances, this bond was cleaved by a slight modification of the method of Marcus et al. (24) which is based on earlier observations of Colowick et al. (25) on the heat lability of the nicotinamide-ribose linkage in the presence of phosphatase. Quantitative release of nicotinamide was obtained by heating for 20 minutes at 100° at pH 10.2 in the presence of 0.22
m phosphate. Appropriate and accurately known quantities of nicotinamide were then added, the pH was lowered to 7 by addition of HCl, and the volume of the solution was increased to about 80 ml. The solution was extracted with ether for 40 to 45 hours in a continuous liquid-liquid extractor of the Kutscher-Stauden type. Preliminary experiments indicated that under these conditions, quantitative extraction of the nicotinamide was obtained. The ether extract was evaporated to dryness on a rotary evaporator, and the nicotinamide residue was recrystallized twice from benzene. In some instances, the nicotinamide was crystallized once and then sublimed at 80° under reduced pressure (less than 1 micron Hg pressure) in a micro-sublimator.

**Determination of Tritium Content of Nicotinamide**—The radioactivity of the nicotinamide was determined in a Packard Tri-Carb liquid scintillation spectrometer. Accurately weighed samples of the isolated nicotinamide (4 to 14 mg) were dissolved in 0.3 ml of H2O in a counting bottle. Five milliliters of absolute ethanol and 10 ml of scintillator solution were added. The scintillator solution had the following composition: 7.5 g of 2,5-diphenyloxazole, 75 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene, 120 g of recrystallized naphthalene, 500 ml of dioxane (distilled from calcium hydride), and 500 ml of xylene (26). The counting efficiency was determined from an aliquot of an HTO standard obtained from the National Bureau of Standards. The efficiency of counting was of the order of 10 to 15%. The counting was carried out for a sufficient time to give an accuracy of at least ±5%.

**Isolation and Determination of Tritium Content of Glutamic Acid**—A direct measure of the transfer of tritium from reduced pyridine nucleotide to L-glutamic acid was obtained by isolating the l-glutamate produced by the reaction between reduced pyridine nucleotide, α-ketoglutarate, NH4+, and crystalline glutamic dehydrogenase (side II stereospecificity). Both nicotinamide and glutamic acid were isolated from the same reaction mixture, and thus a measurement obtained of both the tritium remaining in the pyridine nucleotide and that transferred to the α-ketoglutarate to give glutamate. Nicotinamide was cleaved from the oxidized pyridine nucleotide by heating in 0.2 m phosphate of pH 10 to 10.2, as described above. Carrier nicotinamide and glutamic acid were then added. Ether extraction of the alkaline aqueous phase removed the nicotinamide. When this was completed, the pH of the aqueous phase was reduced to 3.0 with HCl. The solution was concentrated to 1 to 5 ml on a rotary evaporator under reduced pressure. The residue was filtered and the glutamic acid permitted to crystallize at 4°. The product was recrystallized twice from hot water.

Because of the extremely low solubility of the glutamic acid in scintillation fluids, it was not easy to count the acid directly. Accordingly, the glutamic acid was oxidized to nitrogen, carbon dioxide, and water by the procedure of Jacobson et al. (26). The resultant water was counted and its radioactivity determined of both the tritium remaining in the pyridine nucleotide and that transferred to the α-ketoglutarate to give glutamate. Nicotinamide was cleaved from the oxidized pyridine nucleotide by heating in 0.2 m phosphate of pH 10 to 10.2, as described above. Carrier nicotinamide and glutamic acid were then added. Ether extraction of the alkaline aqueous phase removed the nicotinamide. When this was completed, the pH of the aqueous phase was reduced to 3.0 with HCl. The solution was concentrated to 1 to 5 ml on a rotary evaporator under reduced pressure. The residue was filtered and the glutamic acid permitted to crystallize at 4°. The product was recrystallized twice from hot water.

**Experimental Results and Discussion**

**Oxidation of Androsterone and Epianandrosterone**

The stereospecificity of hydrogen transfer for DPN was determined during the course of the oxidation of epianandrosterone by β-hydroxysteroid dehydrogenase (equation 1) and of androsterone by α-hydroxysteroid dehydrogenase (Equation 2). In experiments with each enzyme, limiting quantities of DPN-nicotinamide-4-t were completely reduced in the presence of excess steroid. The hydroxysteroid dehydrogenase was heat inactivated, and the reduced nucleotide was then completely reoxidized by either: (a) crystalline yeast alcohol dehydrogenase (which removes hydrogen from side I), or (b) crystalline glutamic dehydrogenase (which removes hydrogen from side II). The nicotinamide was isolated from the oxidized nucleotide in each case and its radioactivity determined.

In a typical experiment, the reaction system contained in a final volume of 20 ml: 4.5 mmoles of sodium phosphate buffer of pH 10.2, 2 μmoles of androsterone or epianandrosterone in 0.3 ml of dioxane, approximately 1 μmole of DPN-nicotinamide-4-t and 300 units of α- or 1880 units of β-hydroxysteroid dehydrogenase. A control system contained all components except the steroid. The total change in absorbancy at 340 μm resulting from the enzymatic reaction was determined. This change in absorbancy was used to calculate the precise quantity of DPN-nicotinamide-4-t originally added to the reaction system. When the nucleotide was completely reduced, the mixture was heated with agitation at 100° for 2 minutes to inactivate the hydroxysteroid dehydrogenases. After cooling, the absorbancy at 340 μm was reetermined and had usually changed by only 1 or 2%.

The reoxidation of the reduced nucleotide by alcohol dehydrogenase was accomplished by adding 10 μmoles acetaldehyde, lowering the pH to 7 by the slow addition of 1.2 N HCl and by adding 0.02 ml of a 1:10 dilution of the crystalline yeast alcohol dehydrogenase suspension. The reoxidation of the reduced nucleotide by glutamic dehydrogenase occurred upon the addition of 1.4 mmoles of NH4Cl and 60 μmoles of α-ketoglutarate, lowering the pH to 7 with 1.2 N HCl, and adding 0.1 ml of a 1:50 dilution of crystalline glutamic dehydrogenase. The reoxidations by alcohol and glutamic dehydrogenases were followed by absorbancy measurements at 340 μm. When the reduced nucleotide was completely reoxidized, the pH of the reaction mixtures was raised to 10.2 by addition of NaOH, and the system heated with agitation at 100° for 20 minutes. Carrier nicotinamide was then added and the nicotinamide isolated and purified as described.

The results of representative experiments are shown in Table I. The results show clearly that the bacterial enzymes transfer the 3β-hydrogen of androsterone and the 3α-hydrogen of epianandrosterone to side II of the pyridine ring, thus shifting the tritium to side I. In the subsequent reoxidation of the reduced nucleotide, alcohol dehydrogenase (side I stereospecificity) removes at least 95% of the tritium, whereas reoxidation with glutamic dehydrogenase (side II stereospecificity) results in retention of the major part (73.6 to 95.6%) of the isotope in the nicotinamide. It may also be concluded that the tritium in the original DPN-nicotinamide-4-t prepared by the San Pietro procedure (26), is virtually all located at the para position of the nicotinamide ring, since it has been removed almost completely by appropriate enzymatic reactions. Several factors may contribute to the apparently incomplete tritium retention (73.6 and 83.0%) in one pair of the reoxidations with glutamic dehydrogenase. The most likely error is an underestimate of the specific activity of the isolated nicotinamide, possibly due to the presence of impurities. This nicotinamide had a m.p. of 127–127.5°, but was not sublimed. Subsequent samples were sublimed and more complete retention of the isotope was observed. Androsterone and epianandrosterone are diasteromers containing a single epimeric hydroxyl group, yet the same side of the
### Table I

**Stereospecificity of hydrogen transfer by Pseudomonas α- and β-hydroxysteroid dehydrogenases**

<table>
<thead>
<tr>
<th>Method of oxidation of DPNT</th>
<th>Specific activity</th>
<th>Amount of nicotinamide counted</th>
<th>Radioactivity of nicotinamide count</th>
<th>Retention of isotope in nicotinamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase</td>
<td>4270</td>
<td>0.915</td>
<td>261</td>
<td>56</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>4220</td>
<td>0.960</td>
<td>252</td>
<td>8</td>
</tr>
<tr>
<td>Glutamic dehydrogenase</td>
<td>4200</td>
<td>0.945</td>
<td>252</td>
<td>762</td>
</tr>
<tr>
<td>Glutamic dehydrogenase</td>
<td>4200</td>
<td>0.943</td>
<td>253</td>
<td>855</td>
</tr>
</tbody>
</table>

* Corrected for decay and self-absorption, but not for counter efficiency, which accounts for variations in specific activity.

### Table II

**Stereospecificity of hydrogen transfer by placental 17β-hydroxysteroid dehydrogenase**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Method of oxidation of DPNT or TPN</th>
<th>Product isolated</th>
<th>Specific activity of DPNT (+)</th>
<th>Amount of DPNT (+) in system</th>
<th>Dilution of isolated product</th>
<th>Amount of product counted</th>
<th>Total radioactivity of product</th>
<th>Retention of isotope in product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glutamic dehydrogenase</td>
<td>Nicotinamide</td>
<td>3340</td>
<td>0.758</td>
<td>315</td>
<td>34.6</td>
<td>407</td>
<td>111.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutamic acid</td>
<td>3340</td>
<td>0.758</td>
<td>1347</td>
<td>250</td>
<td>44</td>
<td>3.9</td>
</tr>
<tr>
<td>2</td>
<td>Glutamic dehydrogenase</td>
<td>Nicotinamide</td>
<td>3340</td>
<td>0.770</td>
<td>311</td>
<td>37.8</td>
<td>408</td>
<td>100.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutamic acid</td>
<td>3340</td>
<td>0.770</td>
<td>1326</td>
<td>240</td>
<td>2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* Corrected for decay and self-absorption, but not for counter efficiency, which accounts for variations in specific activity.

† Sample calculation: % retention = 

\[
\text{Retention} = \left( \frac{\text{radioactivity of sample, c.p.m.} \times \text{dilution}}{\text{amount of product counted}} \right) \times 100
\]

### Table III

**Stereospecificity of transhydrogenase function of placental 17β-hydroxysteroid dehydrogenase**

<table>
<thead>
<tr>
<th>Method of oxidation of DPNT</th>
<th>Product isolated</th>
<th>Specific activity of DPNT (+)</th>
<th>Amount of DPNT (+) in system</th>
<th>Dilution of isolated product</th>
<th>Amount of product counted</th>
<th>Total radioactivity of product</th>
<th>Retention of isotope in product</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-Hydroxysteroid dehydrogenase</td>
<td>Nicotinamide</td>
<td>3320</td>
<td>0.933</td>
<td>256</td>
<td>102.7</td>
<td>1252</td>
<td>95.5</td>
</tr>
</tbody>
</table>

- Estradiol + DPNT (+) → estrone + DPNT (+) + H+
- Estradiol + TPN (+) → estrone + TPN (+) + H+

* Corrected for decay and self-absorption, but not for counter efficiency, which accounts for variations in specific activity.

† Sample calculation: % retention = 

\[
\text{Retention} = \left( \frac{\text{radioactivity of sample, c.p.m.} \times \text{dilution}}{\text{amount of product counted}} \right) \times 100
\]
Androsterone + DPN+ → androstane-3,17-dione + DPNH + H+
Epiandrosterone + DPN+ → androstan-3,17-dione + DPNH + H+

3. The 17β-hydroxysteroid dehydrogenase for estradiol-17β of human placenta also utilizes side II in oxidations carried out with diphasophopyridine nucleotide and triphosphopyridine. The transhydrogenase function of this enzyme in the presence of catalytic quantities of estradiol-17β was studied in the following reaction:

DPNH + 3-acetylpyridine-DPN+ →
DPN+ + 3-acetylpyridine-DPNH

and was found likewise to involve side II of the donor nucleotide.

REFERENCES
Stereospecificity of Hydrogen Transfer by Pyridine Nucleotide-linked Hydroxysteroid Dehydrogenases
Joseph Jarabak and Paul Talalay


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