# Stereochemistry of Hydrogen Transfer by Rat Ovary 20α-Hydroxysteroid Dehydrogenase*

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ABSTRACT: Stereospecificity of hydrogen transfer by rat ovary 20α-hydroxysteroid dehydrogenase from reduced triphosphopyridine nucleotide (TPNH) to progesterone was studied using tritiated cofactors. (4-*S*)[4-$^3$H]TPNH was prepared from triphosphopyridine nucleotide (TPN), [1-$^3$H]glucose 6-phosphate, and glucose 6-phosphate dehydrogenase. (4-*R*)-[4-$^3$H]TPNH was prepared from [4-$^3$H]TPN, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase. When these specifically labeled cofactors were used in the reduction of progesterone with rat ovary 20α-hydroxysteroid dehydrogenase, the transfer of tritium to 20α-hydroxypregn-4-en-3-one from (4-*S*)[4-$^3$H]TPNH was 5.5% of the amount predicted from the stoichiometry of the reaction while the transfer from (4-*R*)[4-$^3$H]TPNH was 99% of the predicted amount. Rat ovary 20α-hydroxysteroid dehydrogenase mediates hydrogen transfer from the *pro-R* side of TPNH.

20α-Hydroxypregn-4-en-3-one is the major metabolite of progesterone in the laboratory rat (Berliner and Wiest, 1956). Rat ovaries contain a soluble TPN-specific 20α-hydroxysteroid dehydrogenase with a high activity toward progesterone[1] and 20α-hydroxypregn-4-en-3-one (Wiest, 1959). The properties and substrate specificity of this enzyme have been studied in some detail (Wiest and Wilcox, 1961; Wilcox and Wiest, 1966).

The direct, stereospecific transfer of hydrogen from a variety of substrates to the 4 position of the nicotinamide moiety of pyridine nucleotide coenzymes is well documented (Westheimer *et al.*, 1951; Fischer *et al.*, 1953; Pullman *et al.*, 1954; Levy *et al.*, 1962). If deuterated or tritiated substrates are used in the enzymic reduction of pyridine nucleotide coenzymes, an asymmetric carbon will be produced with the heavy isotope in the (4-*R*) or (4-*S*) configuration depending on the stereospecificity of the enzyme (Cornforth *et al.*, 1962). Enzymes which place hydrogen in the (4-*R*) configuration are designated class A dehydrogenases while those which place hydrogen in the (4-*S*) configuration are designated class B dehydrogenases since the earlier nomenclature referred to the (4-*R*) hydrogen as being on the A side and the (4-*S*) hydrogen as being on the B side of the nicotinamide ring (Hanson, 1966).

This paper presents evidence that rat ovary 20α-hydroxysteroid dehydrogenase transfers hydrogen to the *pro-R* position of TPN and is a class A dehydrogenase.

## Materials and Methods

*Chemicals.* TPN, ATP, hexokinase, glucose 6-phosphate dehydrogenase, and Tris were from Sigma Chemical Co., St. Louis, Mo. D-[1-$^3$H]Glucose and [4-$^3$H]TPN were purchased from New England Nuclear Corp., Boston, Mass., and used without further purification. DEAE-cellulose was Cellex-D from Bio-Rad Laboratories, Richmond, Calif. Progesterone was from Mann Research Laboratories, Inc., New York, N. Y. 20α-Hydroxypregn-4-en-3-one was generously provided by Dr. Walter G. Wiest, Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, Mo. All other reagents were of analytic grade.

*Preparation of the Enzyme.* Rat ovarian 20α-hydroxysteroid dehydrogenase was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography in a procedure similar to that previously described (Wilcox and Wiest, 1966). Ovaries were taken from sexually mature female albino rats from the Holtzmann Company, Madison, Wis. Protein was measured by the Lowry method (Lowry *et al.*, 1951).

*Measurement of Radioactivity.* Tritium was measured by liquid scintillation counting in a Beckman LS-250 liquid scintillation counting system (Beckman Instruments Inc., Fullerton, Calif.) in experiments with (4-*S*)[4-$^3$H]TPNH, and a Nuclear-Chicago series 720 system (Nuclear-Chicago Corp., Des Plaines, Ill.) in experiments with (4-*R*)[4-$^3$H]-TPNH. Samples were placed in glass counting vials with water, 0.5 ml, and Bray's solution, 10 ml (Bray, 1960).

*Preparation of (4-S)[4-$^3$H]TPNH.* Tritium was introduced into the (4-*S*) position of TPNH using [1-$^3$H]glucose 6-phosphate and glucose 6-phosphate dehydrogenase, a class B dehydrogenase (Stern and Vennesland, 1961). The reaction mixture had the following composition: D-[1-$^3$H]glucose, 25 μCi, 3 μmoles; ATP, 11 μmoles; hexokinase, 2 units; TPN, 3 μmoles; glucose 6-phosphate dehydrogenase, 1.25 units; $MgSO_4$, 24 μmoles; glycylglycine buffer (pH 8), 148 μmoles in a total volume of 6 ml. The reaction was run for 60 min at 37° in a Bausch and Lomb Spectronic 600 spectrophotometer (Bausch and Lomb, Rochester, N. Y.) equipped

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‡ Recipient of a Bank of America–Giannini Foundation Fellowship during a portion of the time this work was being carried out and to whom inquiries should be addressed.

[1] Abbreviations used are: progesterone, pregn-4-ene-3,20-dione; 20α-OH-P, 20α-hydroxypregn-4-en-3-one.

TABLE I: Distribution of Radioactivity.[a]

| Configuration of Tritium in [4-$^3$H]TPNH | Act. in [4-$^3$H]TPNH[b] | Sp Act.[c] | Act. in 20$\alpha$-OH-P[b] | Sp Act.[c] | Act. in Aqueous Phase[b] |
|---|---|---|---|---|---|
| (*S*) | 117,500 | 673,000 | 4,420 | 36,800 | 118,000 |
| (*R*) | 67,200 | 595,000 | 46,300 | 594,000 | 22,000 |

[a] Experimental details are given in the text. [b] Values for (*S*) are given in counts per minute, for (*R*) in disintegrations per minute [c] Values for (*S*) are given in cpm/$\mu$mole, for (*R*) in dpm/$\mu$mole.

with a constant-temperature cell holder and a Temptrol 151 circulating bath (Precision Scientific Company, Chicago, Ill.). The course of the reaction was followed by the change in $A_{340}$. At the end of the incubation 1.62 $\mu$moles of TPNH had been formed. The reaction mixture was chromatographed on DEAE-cellulose to separate the tritiated TPNH from other reaction components (Pastore and Friedkin, 1961).

*Reduction of Progesterone with (4-S)[4-$^3$H]TPNH.* The incubation mixture for this reaction had the following composition: (4-*S*)[4-$^3$H]TPNH, 0.6 ml, 0.175 $\mu$mole, 117,500 cpm; progesterone, 0.1 mg in 0.1 ml of methanol, 0.318 $\mu$mole; rat ovary 20$\alpha$-hydroxysteroid dehydrogenase, 0.1 ml, 0.2 mg of protein; potassium phosphate buffer (pH 6.5), 2.2 ml, 220 $\mu$moles; cysteine, 8.8 $\mu$moles. The reaction was run for 30 min in the spectrophotometer at 37° and the reaction was stopped by extracting the steroids into three successive 2-ml portions of ethyl acetate. The change in $A_{340}$ during the reaction indicated that 0.12 $\mu$mole of TPNH was oxidized. Since the reaction between TPNH and progesterone is stoichiometric (Wiest, 1959), 0.12 $\mu$mole of 20$\alpha$-hydroxypregn-4-en-3-one was formed. The combined ethyl acetate extracts were washed with four 2-ml portions of water, taken to dryness under a stream of dry nitrogen at 50°, and chromatographed on paper in the system petroleum ether–methanol–water (10:8:2) (Bush, 1952). The steroids were located by ultraviolet absorption and the 20$\alpha$-hydroxypregn-4-en-3-one eluted with methanol. The eluate was dried in a scintillation vial and counted. The aqueous phase of the reaction mixture was chromatographed on DEAE-cellulose in an attempt to separate TPN from unreacted TPNH. While an anomalous pyridine nucleotide elution pattern was obtained, all the radioactivity was associated with ultraviolet-absorbing material.

*Preparation of (4-R)[4-$^3$H]TPNH.* We prepared (4-*R*)[4-$^3$H]-TPNH using the same reaction system as in the preparation of (4-*S*)[4-$^3$H]TPNH, except in this case glucose 6-phosphate was the reducing agent and [4-$^3$H]TPN the cofactor. Since the unlabeled hydrogen from glucose 6-phosphate was introduced into the (4-*S*) position by glucose 6-phosphate dehydrogenase (Stern and Vennesland, 1961), tritium was retained in the (4-*R*) configuration. The reaction mixture had the composition: ATP, 5.8 $\mu$moles; $MgSO_4$, 12.2 $\mu$moles; glucose, 3 $\mu$moles; hexokinase, 4 units; [4-$^3$H]TPN, 1.5 $\mu$moles, 2.5 $\mu$Ci; glucose 6-phosphate dehydrogenase, 1 unit; glycylglycine buffer (pH 8), 58.5 $\mu$moles in a total volume of 3 ml. The reaction was run at 37° for 10 min in the spectrophotometer. At the end of this time the solution was diluted to 10 ml, the $A_{340}$ was measured, and the sample was applied to a DEAE-cellulose column; 1.2 $\mu$moles of TPNH was formed.

*Reduction of Progesterone with (4-R)[4-$^3$H]TPNH.* This reaction was carried out in an incubation mixture as follows: (4-*R*)[4-$^3$H]TPNH, 1.5 ml, 67,200 dpm, 0.113 $\mu$mole; progesterone, 0.318 $\mu$mole in 0.1 ml of methanol; rat ovary 20$\alpha$-hydroxysteroid dehydrogenase, 0.05 ml, 0.2 mg of protein; potassium phosphate buffer (pH 6.5), 1.35 ml, 135 $\mu$moles; cysteine, 5.4 $\mu$moles. The reaction was run for 25 min in the spectrophotometer at 37° and the reaction was stopped by extracting the steroids into three successive 2-ml portions of ethyl acetate. The change in $A_{340}$ during the reaction indicated 0.078 $\mu$mole of TPNH was oxidized, and thus an equivalent amount of 20$\alpha$-hydroxypregn-4-en-3-one formed. The ethyl acetate extracts were washed with three 2-ml portions of water and dried under a stream of dry nitrogen at 50°. The residue was chromatographed in the petroleum ether–methanol–water system and the 20$\alpha$-hydroxypregn-4-en-3-one eluted with hexane into a scintillation vial. The aqueous phase of the reaction mixture was concentrated to 2 ml under a stream of dry nitrogen at 50° and 0.5 ml was taken for liquid scintillation counting. The counting efficiency of each sample in this experiment was calculated by the channels–ratio method (Bush, 1963).

## Results

The radioactivity in various fractions of the reaction mixtures is shown in Table I. Assuming a stoichiometric transfer of hydrogen from 0.12 $\mu$mole of (4-*S*)[4-$^3$H]TPNH to steroid we should have found 80,800 cpm in the 20$\alpha$-hydroxypregn-4-en-3-one formed by progesterone reduction. The actual count in the 20$\alpha$-hydroxypregn-4-en-3-one was 4420 cpm, which is only 5.5% of the predicted value. On the other hand, assuming stoichiometric transfer of hydrogen from 0.078 $\mu$mole of (4-*R*)[4-$^3$H]TPNH to steroid there should have been 46,400 dpm in 20$\alpha$-hydroxypregn-4-en-3-one, and we found 46,300 dpm or 99.8% of the expected activity. Thus tritium was preferentially incorporated into substrate from (4-*R*)[4-$^3$H]TPNH (see Figure 1) and rat ovary 20$\alpha$-hydroxysteroid dehydrogenase is a class A dehydrogenase.

## Discussion

It has been suggested that there is a rough correlation between substrate size and the stereospecificity of hydrogen

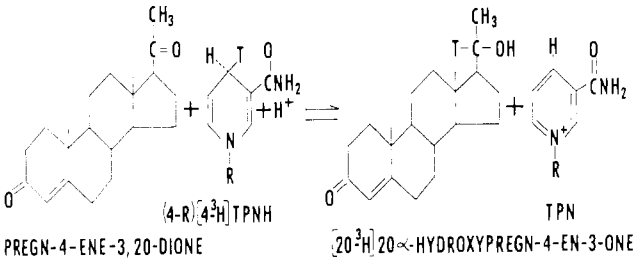


FIGURE 1: Tritium transfer in the reduction of pregn-4-ene-3,20-dione by rat ovary 20α-hydroxysteroid dehydrogenase.

transfer by pyridine nucleotide linked dehydrogenases (Krakow *et al.*, 1963), with class A dehydrogenases acting on smaller and class B dehydrogenases on larger substrates. To our knowledge all previously studied steroid dehydrogenases fall into class B, that is, they transfer hydrogen to the *pro-S* side of the pyridine nucleotide; thus steroid dehydrogenases appeared to fit this generalization. However, the TPN-linked 20α-hydroxysteroid dehydrogenase from rat ovary is a class A dehydrogenase and thus falls into that group of dehydrogenases for which no correlation can be found between substrate size and nucleotide stereospecificity (Krakow *et al.*, 1963).

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