SHORT COMMUNICATIONS

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Stereospecificity of hydrogen transfer by bovine adrenal 3β-hydroxysteroid dehydrogenase

WESTHEIMER *et al.*[1], FISHER *et al.*[2] and LOEWUS *et al.*[3] first demonstrated that pyridine nucleotide-linked dehydrogenases effect a stereospecific transfer of hydrogen between substrate and the 4 position of the cofactor nicotinamide ring.

Several steroid dehydrogenases have been shown to transfer to the 4β position: 17β-hydroxysteroid dehydrogenase from human placenta[4,5], 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) from *Pseudomonas testosteroni*[4] and 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*[6]. Similar results were obtained with the 3(or 17)β-hydroxysteroid dehydrogenase (EC 1.1.1.51) from *P. testosteroni*[4,7].

We have previously studied the site specificity of 3β-hydroxysteroid dehydrogenase from bovine adrenal microsomes which uses NAD^+ as cofactor and is specific for the 3β position of C_{19} and C_{21} steroids[8]. This investigation was designed to evaluate this mammalian enzyme in terms of stereospecificity of cofactor hydrogenation. We used the method described by WARREN, GONZALEZ AND SORIA[5]. It basically involves synthesis of [3α-^{3}H]androstane-3β, 17β-diol and its use to enzymatically generate [^{3}H]NADH. Steroids are extracted and the reaction mixture containing the reduced cofactor is then used to reduce substrates of enzymes known to accept the 4α- and 4β-H. The resulting NAD^+ (or [^{3}H]NAD^+) and products are separated by chromatography and analyzed for radioactivity. It will be shown that 3β-hydroxysteroid dehydrogenase from bovine adrenal microsomes transfers the steroid 3α-^{3}H to the 4β position of the nicotinamide ring of the [^{3}H]NADH generated.

Generation and consumption of NADH was monitored in a Beckman DU spectrophotometer. Radioactivity was quantitated in a Nuclear-Chicago 6801 liquid scintillation spectrometer as previously described[5]. Chromatograms were scanned for radioactivity in a Nuclear-Chicago gas-flow counter.

The steroid substrate was synthesized as described by ROBERTS AND WARREN[9], using 12 mg of 17β-hydroxyandrostane-3-one obtained from Sigma Chemical Co., St. Louis, Mo. and 20 mg of sodium borotritide (5.25 mC/mg) obtained from New England Nuclear Corp., Boston, Mass. The steroid residue was taken up in 40 ml of chloroform which was washed exhaustively with distilled water until radioactivity in the aqueous phases was minimal and constant. Aliquots of the product retained radioactivity on acetylation[10] but lost it on oxidation[10] to androstane-3,17-dione.

Reduction of cofactor was carried out in 12.0 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 5 mM NAD^+, 0.17 mM [3α-^{3}H]androstane-3β, 17β-diol (added in 0.25 ml of propylene glycol), 1.0 mM KCN and 3β-hydroxysteroid dehydrogenase. The enzyme preparation, equivalent to 5.0 mg of protein, was a triton sonicated supernatant of microsomes from adrenal cortex, prepared as previously described[6], equivalent to 3.2 g of adrenal, and lacking the capacity of spontaneously oxidizing NADH.

After incubation with gentle agitation at 37° for 75 min, the reaction mixture was cooled and extracted with 1.5 vol. of chloroform to remove stereoid. A 0.5-ml

aliquot of the aqueous phase was chromatographed on Dowex 1-X10 (Baker Chemical Co., Phillipsburg, N.J.) as described by SILVERSTEIN[11]. Elution pattern of NAD^+, NADH and radioactivity are shown in Fig. 1. The ^3H is localized in the NADH fraction indicating that [^{3}H]NADH of high specific activity has been produced.

Aliquots of the aqueous solution (2.6 ml) were used for enzymatic oxidation of the [^{3}H]NADH. Reduction of α-ketoglutarate was carried out in a mixture containing [^{3}H]NADH solution, 10 μmoles of α-ketoacid and 20 μmoles of NH_4Cl (both adjusted to pH 7.0) and 200 μg of crystalline glutamate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) in a total volume of 3.0 ml. Reduction of pyruvate was carried out in a mixture containing [^{3}H]NADH solution, 10 μmoles of pyruvate (adjusted to

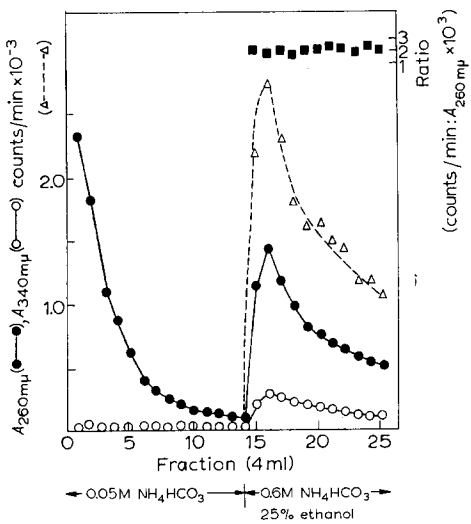

Fig. 1. Column chromatography of NAD^+ and [^{3}H]NADH after generation of the latter by adrenal 3β-hydroxysteroid dehydrogenase with [3α-^{3}H]androstane-3β,17β-diol as substrate ●—●, absorbance at 260 mμ; ○—○, absorbance at 340 mμ; △ ---△, counts/min per 0.10 ml. Fractions containing less than 60 counts/min are not shown.

pH 7.0) and 20 μg of crystalline lactate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.). Spectrophotometrically, approximately one half of the total NADH present was oxidized in each instance. Control incubations were carried out with [^{3}H]NADH solution, 0.3 μmole of lactate or glutamate (adjusted to pH 7.0) but with no added enzyme. After 2 h, all incubations were immersed in a boiling-water bath for 2 min.

Aliquots of 100 μl from each of the incubation mixtures above were chromatographed in triplicate in butanol–acetic acid with 1.5 μmoles of carrier lactate or glutamate and subsequently treated exactly as previously described[5]. Scanning of the chromatograms revealed counts with carrier glutamate but not with carrier lactate in those situations where enzyme was included. Carrier spots of lactate and glutamate, as well as the origin area (NAD^+ and NADH), were eluted and radioactivity quantitated. Results are shown in Table I. It can be seen that reduction of pyruvate yields [^{3}H]-NAD^+ and unlabeled lactate. Reduction of α-ketoglutarate results in transfer of tritium from [^{3}H]NADH to glutamate by glutamate dehydrogenase, a known β ac-

TABLE I

LOCALIZATION OF RADIOACTIVITY AFTER ENZYMATIC REDUCTION OF α-KETOGLUTARATE AND PYRUVATE WITH [^{3}H]NADH

Values for counts/min are amounts eluted from the NAD$^+$:NADH, glutamate and lactate areas of chromatograms and represent 0.75% of the reaction which was used to oxidize [^{3}H]NADH. Glutamate and lactate fractions are corrected for nonspecific radioactivity by subtraction of counts in the comparable fractions of control incubations (less than 1% of total radioactivity).

Incubation	*Counts/min in chromatography fractions*		
	NAD$^+$: NADH	*Glutamate*	*Lactate*
Pyruvate	1825	—	2
	1510	—	3
	1300	—	1
α-Ketoglutarate	437	446	—
	535	392	—
	545	465	—

ceptor. Further, the amount of tritium transferred is what one would expect from a reaction which spectrophotometrically resulted in oxidation of one half of the total reduced cofactor present.

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1 F. H. WESTHEIMER, H. F. FISHER, E. E. CONN AND B. VENNESLAND, *J. Am. Chem. Soc.*, 73 (1951) 2403.
2 H. F. FISHER, E. E. CONN, B. VENNESLAND AND F. H. WESTHEIMER, *J. Biol. Chem.*, 202 (1953) 687.
3 F. A. LOEWUS, P. OFNER, H. F. FISHER, F. H. WESTHEIMER AND B. VENNESLAND, *J. Biol. Chem.*, 202 (1953) 699.
4 J. JARABAK AND P. TALALAY, *J. Biol. Chem.*, 235 (1960) 2147.
5 J. C. WARREN, M. C. GONZALEZ AND J. SORIA, *Endocrinology*, 80 (1967) 784.
6 G. BETZ AND J. C. WARREN, submitted for publication.
7 P. TALALAY, F. A. LOEWUS AND B. VENNESLAND, *J. Biol. Chem.*, 212 (1955) 801.
8 S. G. CHEATUM, A. DOUVILLE AND J. C. WARREN, *Biochim. Biophys. Acta*, 137 (1967) 172.
9 J. D. ROBERTS AND J. C. WARREN, *Endocrinology*, 74 (1964) 746.
10 J. C. WARREN AND H. A. SALHANICK, *J. Clin. Endocrinol.*, 21 (1961) 1218.
11 E. SILVERSTEIN, *Anal. Biochem.*, 12 (1965) 199.

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