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Development of a quantitative assay method for 3 beta-hydroxy-delta 5-steroid dehydrogenase in the rat testis

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Abstract

We investigated the first step of the sex steroid hormone biosynthesis pathway by assaying the activities of 3 beta-hydroxy-delta 5-steroid dehydrogenase, the rate-limiting enzyme in this pathway. We have developed a simple and rapid colorimetric assay for 3 beta-hydroxy-delta 5-steroid dehydrogenase in rat testis. The supernatant from rat testis tissue homogenates were used for the enzyme assay. The enzyme activity was determined by measuring the absorbance at 570 nm which indicates the rate of conversion of pregnenolone into progesterone in the presence of NAD, using phenazine methosulfate and nitro blue tetrazolium as the color reagent. The activity of this enzyme ranged from 4.57 ± 1.34 to 10.56 ± 2.13 nmol/mg protein/min with a mean activity of 8.96 ± 1.27 nmol/mg protein/min. The K_m of the enzyme at an optimum pH of 7.25 was about 4.7 ± 0.12 nM.

Keywords: 3β-Hydroxysteroid-delta 5-dehydrogenase; Testis; Steroid hormone; Colorimetric

1. Introduction

3β-Hydroxysteroid dehydrogenase (3β-HSDH) is an enzyme complex involved in the synthesis of steroid hormones [1,2]. A key step in the biosynthesis of steroid hormones by the gonads or adrenal glands is the conversion of the relatively inert Δ5-3β-ol steroids into the biologically active 3,5-oxo-steroids, such as progesterone and androst-4-ene-3,17-dione, which can undergo further transformation into androgens, estrogens, or corticosteroids. This conversion, which is a two-stage reaction, is catalyzed by the 3β-HSDH enzyme complex consisting of 3-5β-hydroxysteroid dehydrogenase (EC 1.1.1.51) and 3,5-oxo-steroid isomerase (EC 5.3.3.1). The initial reaction, which requires oxidized nicotinamide-adenine dinucleotide as an electron acceptor, is the rate-limiting step [3].

Cholesterol taken up from the maternal circulation is the precursor for human placental progesterone synthesis [4]. Two enzyme systems are involved in the enzymatic conversion of cholesterol to progesterone. The cholesterol side chain cleavage enzyme is necessary for splitting the C6 fragment from the side chain of the cholesterol molecule and thus, also for formation of pregnenolone. This enzymatic reaction is thought to be the slowest and therefore, rate-limiting step by which the steroidogenesis of pregnenolone and progesterone can be controlled.

The enzyme system that converts pregnenolone to progesterone itself consists of two different enzymes: 3 beta-hydroxysteroid: NAD (P) oxidoreductase (EC 1.1.1.145) and the 3-ketosteroid-delta-isomerase (EC 5.3.3.1). The conventional abbreviation for the latter enzyme system is 3β-HSDH, which consists not only of the dehydrogenase, but also the isomerase. Dehydrogenation is the initial and rate-limiting step of this reaction [5,6]. In mammalian tissues this enzyme is associated with a subcellular fraction [7–9] and converts C19 and C21 steroids with the delta-3 beta-hydroxyl structure to those with the delta-3-ketone group characteristic of most of the active steroid hormones. It apparently differs from an enzyme present in liver that catalyzes the reduction of 3-keto-steroids and that has been named 3β-HSDH [10].

3β-HSDH and steroid delta-isomerase were co-purified as a single protein from human placental microsomes [11]. It has been suggested that the 3 beta-hydroxy-delta 5-steroid dehydrogenase may be involved in the control of biosynthesis of steroid hormones, but hitherto, largely the lack of adequate technical tools has hindered verification of this hypothesis. Three beta-hydroxy-delta 5-steroid dehydrogenase activity has been assayed mainly by the reduction of NAD spectrophotometrically. In recent years, a radiochemical assay for 3 beta-hydroxy-delta 5-steroid

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dehydrogenase activity has been available. Because of the low activities of 3 beta-hydroxy-delta 5-steroid dehydrogenase, the spectrophotometric method suffers from poor sensitivity. The radiochemical assay, although sensitive, is cumbersome in addition to being expensive, requiring radioactive chemicals and sophisticated instrumentation. The purpose of this investigation was to develop a new colorimetric, rapid, and sensitive method for the determination of 3 beta-hydroxy-delta 5-steroid dehydrogenase activity in rat testis by use of nitro blue tetrazolium (NBT).

2. Materials and methods

2.1. Chemicals

Pregnenolone (5-pregne-3β-ol-2-one) and testosterone (17β-hydroxyandrostan-4-en-3-one) were purchased from Sigma Chemical Co. (St. Louis, MO). Nicotinamide adenine dinucleotide (NAD) and the reduced form of nicotinamide adenine dinucleotide (NADH) were obtained from Boehringer Mannheim (Germany). NBT, nicotinamide adenine dinucleotide phosphate (NADP), and phenazine methosulfate (PMS) were purchased from Merck (Darmstadt, Germany). All other reagents were of highest purity, and available solvents were freshly redistilled prior to use.

2.2. Reagents

Phthalate buffer (70 mmol, pH 4.25): 3.57 g of potassium hydrogen phthalate was dissolved in a mixture of 50 ml of 0.15 M HCl and 3.0 ml of Tween 20, and the pH was adjusted to 4.25. The volume was made up to 300 ml with distilled, deionized water. Then, Tris–HCl buffers (0.15 M, pH 7.25) and NAD (10 mm) were added, and the contents were mixed.

2.3. Color reagent

Fifty milligram NBT, 15 mg PMS, and 1.0 ml Tween 20 were dissolved in 50 ml distilled water for the standard curve. For the enzyme assay, PMS was omitted from the reagent. The reagent containing PMS was stored in a dark bottle. The substrate (Pregnenolone, 50 mM) was first dissolved in 0.5 ml of dimethyl formamide, and the stock solution (1 mm) was prepared in 50 or 100 ml Tris–HCl buffer (0.15 M, pH 7.25).

2.4. Standard curve

A 1 mM solution of NADH was freshly prepared in distilled water, and the contents were mixed. Aliquots of graded concentrations of NADH (0–2 nm) were reacted with the color reagent (0.5 ml), and after the color formed, 2.0 ml of phthalate buffer was added to each tube, and the absorbance of standard against blank was read at 570 nm. The coloration was stable for 30 min. A standard curve was prepared by plotting NADH concentration versus absorbance.

2.5. Animals

The animals used in these studies were adult male rats, which were fed an estrogen free diet ad libitum. The rats weighed between 250 and 300 g. All animals survived the study without signs of illness. All experimental manipulation was carried out with the animals under ether inhalation anesthesia.

2.6. Preparation of homogenates

Adult male rats were killed by ether anesthesia, and their testes were excised. Excess fat was removed from the testes. They were then chilled on ice, blotted, and weighed. At least 10–12 testes from five to six rats were pooled as the gland size was small (70 mg), homogenized using ground glass homogenizers with 10.0 ml of 0.15 M Tris–HCl buffer (pH 7.25), and centrifuged at 10,000 × *g* at 5 °C. The lipid layer was removed by aspiration, and the supernatant was used as the enzyme extract.

2.7. Assay procedure

3β-HSDH activity was determined by measuring the rate of conversion of pregnenolone into progesterone. The isomerase reaction is rapid, so it is customary to refer to the assay in terms of the rate-limiting dehydrogenation step.

The enzyme was assayed in 1.5 ml of 0.15 M Tris–HCl buffer (pH 7.25) containing 1.5 ml of 400 μM NAD and 0.2 ml of 200 μM pregnenolone in a total volume of 3.20 ml. The reaction was started by adding the enzyme (100 μl) and incubated at 37 °C for 60 min. A control incubation of the rat testis homogenate and NAD, without addition of pregnenolone was carried out. At the end of the incubation period, the reaction was stopped by the addition of 2.0 ml of phthalate buffer (pH 4.25). The turbidity was removed by centrifugation at 5000 × *g* for 30 min, and the supernatant was read at 570 nm in a spectrophotometer. Enzyme activity was calculated from the standard curve of NADH and expressed as nmol NADH formed/h mg protein. An enzyme unit is defined as the amount of enzyme that catalyzes the release of 1 nmol NADH/min at 37 °C. Specific activity is expressed in terms of units per mg of protein. The protein contents of various enzyme extracts relative to standard solution of bovine serum albumin were determined by the Lowry et al. method [12].

2.8. Measurement of the conversion of radioactive pregnenolone to progesterone

3β-HSDH activity was measured by a modification of the procedure described by Armstrong and Wells [13]. A solution of the substrate, [4-^{14}C] pregnenolone (200 μM;

activity: 54 mCi/mmol; Radiochemical Center, Amersham) in 7 μl redistilled ethanol, was mixed with 250 μl 0.14 M phosphate-buffered saline, pH 7.25 containing 0.30 μmol NAD. This mixture was warmed to 37 °C and the homogenate (100 μl) was added to start the reaction. After incubation for 15 min the reaction was stopped by adding 90 μl of acetone containing 45 μg pregnenolone and 55 μg [^{3}H] progesterone (specific activity: 312 μCi/mmol; Radio Chemical Center, added as an internal standards). This mixture was extracted with 4 ml methylene dichloride which was separated and evaporated to dryness. The radioactive progesterone was separated from pregnolone by partition chromatography on thin-layer silica gel and counted. The yield of product was calculated from the ^{3}H/^{14}C ratio for each homogenate. Duplicate measurements were made with and without the addition of 0.85 mM NAD. The expression of enzyme activity was based on the difference between these two measurements, one unit was defined as 1 nmol product produced/min/mg protein at 37 °C.

3. Results

Fig. 1 shows the standard curve of NADH measured by NBT reduction. Various concentrations of NADH were reacted with NBT in the presence of PMS in Tris–HCl buffer (0.15 M, pH 7.25). The color was measured at 570 nm. The three groups of animals assayed on three separate occasions yielded mean testis levels within 5% of one another, indicating that the method was reproducible (Table 1).

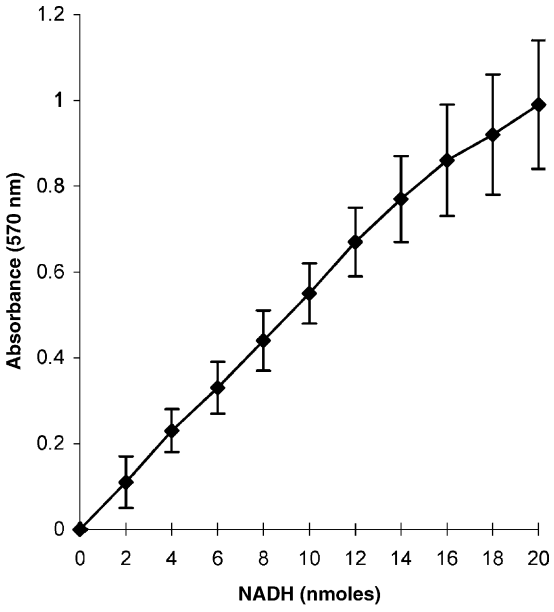

Fig. 1. Standard curve of NADH measured by NBT reduction. Various concentrations of NADH were reacted with NBT in the presence of PMS in Tris–HCl buffer (0.15 M, pH 7.25). The color was measured at 570 nm. Each point represents the mean ± S.E. of six separate experiments.

Table 1
Kinetic characterization of 3β-HSDH in rat testis

Substrate	Pregnenolone
K_m (with respect to NAD in nM)	4.7 ± 0.12
V_{max} (nmol/min/mg protein)	3.83 ± 0.26
Activity (nmol NAD reduced/min/mg protein)	8.96 ± 1.27

Values (nmol NAD reduced/min/mg protein) are the mean ± S.E. of at least three determinations.

3.1. Determination of optimum pH

The pH dependence of 3β-HSDH was analyzed using Tris–HCl buffer: pH 6.5–9.5 in a 0.2 M concentration (Fig. 2). The optimum pH for the 3β-HSDH was found to be at pH 7.25.

3.2. Kinetic studies

Fig. 3 shows 3 beta-hydroxy-delta 5-steroid dehydrogenase activity at various substrate concentrations. Enzyme kinetics were studied in relation to both protein concentration (Fig. 4) and time (Fig. 5). First-order enzyme kinetics (initial velocity) was observed for an incubation period up to 60 min using a protein concentration of the enzyme solution less than 10 μg/ml. A linear correlation between 3β-HSDH

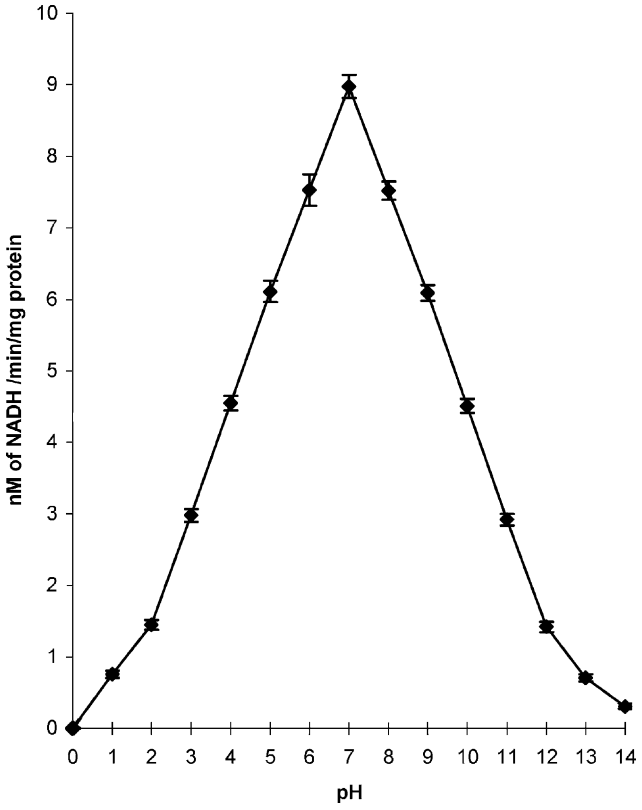

Fig. 2. Effect of pH on the activity of 3β-HSDH in rat testis. Each point represents the mean ± S.E. of six separate experiments. Substrate concentration and homogenate volume were 200 μM and 100 μl, respectively.

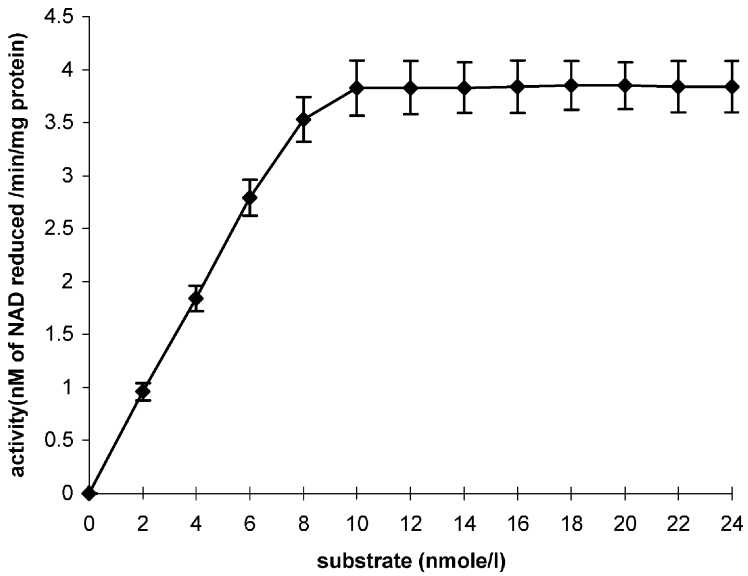

Fig. 3. Effect of substrate concentration on the activity of 3β-HSDH in rat testis. Each point represents the mean ± S.E. of six separate experiments. Homogenate volume was 100 μl.

activity and protein concentration of the enzyme solution was demonstrated up to a protein concentration of 10 mg/ml (Fig. 4). All enzymatic tests were performed using 0–40 μg of 3β-HSDH protein per test and an incubation period of 20 min. Fig. 5 shows that the utilization of pregnenolone was found to be a linear function of time for up to 60 min.

3.3. Effect of substrate level on the kinetic parameters

The K_m value for pregnenolone was determined by Michaelis–Menten plot using concentration range from 0 to 40 μM. The apparent K_m of pregnenolone was calculated to be 0.47 μM (Fig. 3).

3.4. Determination of the optimum temperature

Influence of temperature on the activity of 3β-HSDH was investigated in various separate test series at different temperatures between 20 and 55 °C (Fig. 6). There was a maximum 3β-HSDH activity at 37 °C which will be referred to as the temperature optimum (100% enzyme activity) as shown in Fig. 6. A gradual enhancement of 3β-HSDH

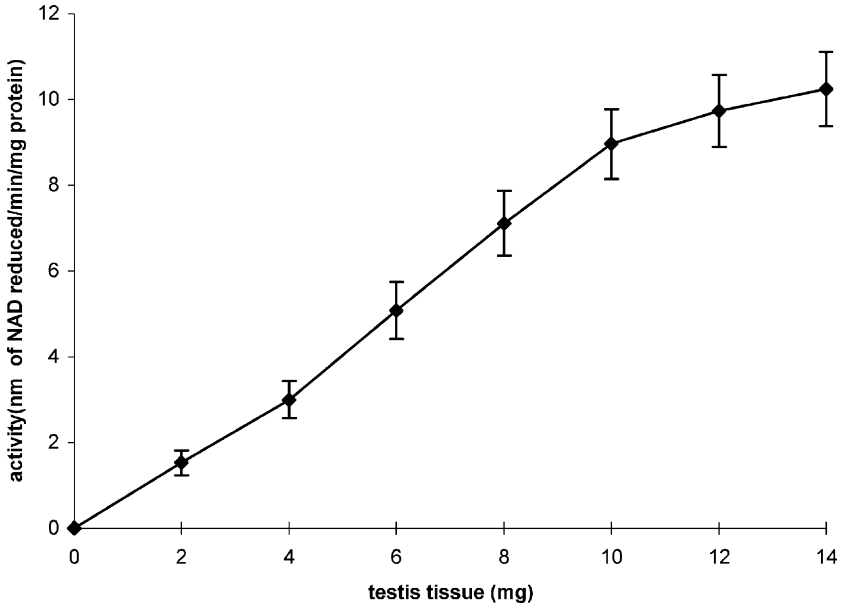

Fig. 4. 3β-HSDH activity in different levels of homogenates of rat testis tissue. Each point represents the mean ± S.E. of six separate experiments. Substrate concentration was 200 μM.

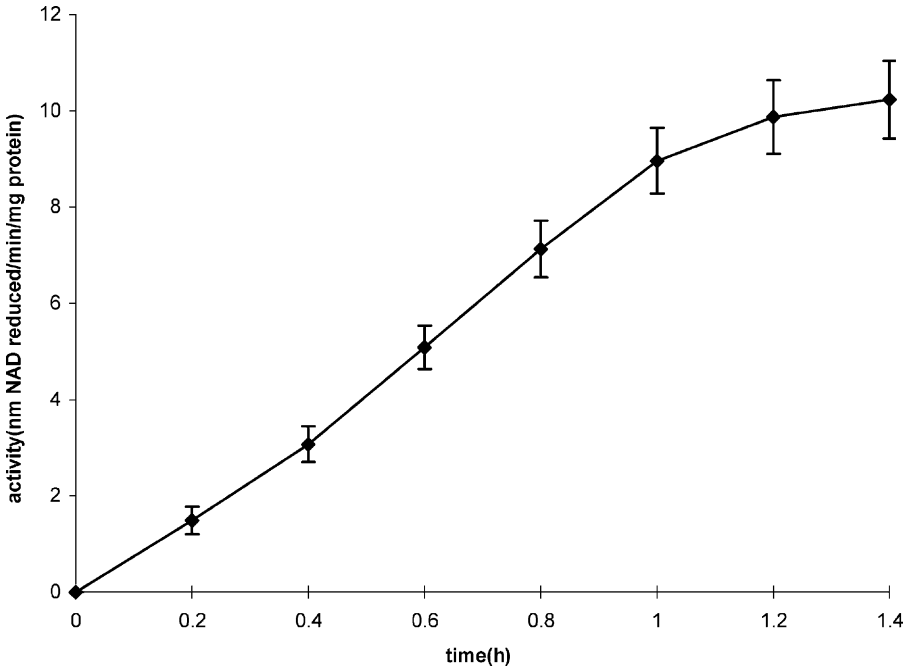

Fig. 5. 3β-HSDH activity at various time points, in rat testis. Each point represents the mean ± S.E. of six separate experiments. Substrate concentration and homogenate volume were 200 μM and 100 μl, respectively.

activity (25–100%) was found when the temperature was increased from 20 to 40 °C. When incubations were carried out at higher than optimal temperatures (37 °C), the activity of 3β-HSDH rapidly decreased until no enzymatic conversion could be measured.

3.5. Validity of analysis

Comparison of 3β-HSDH activity measured by conversion of radioactive pregnenolone to progesterone with that of colorimetric assay was shown in Fig. 7.

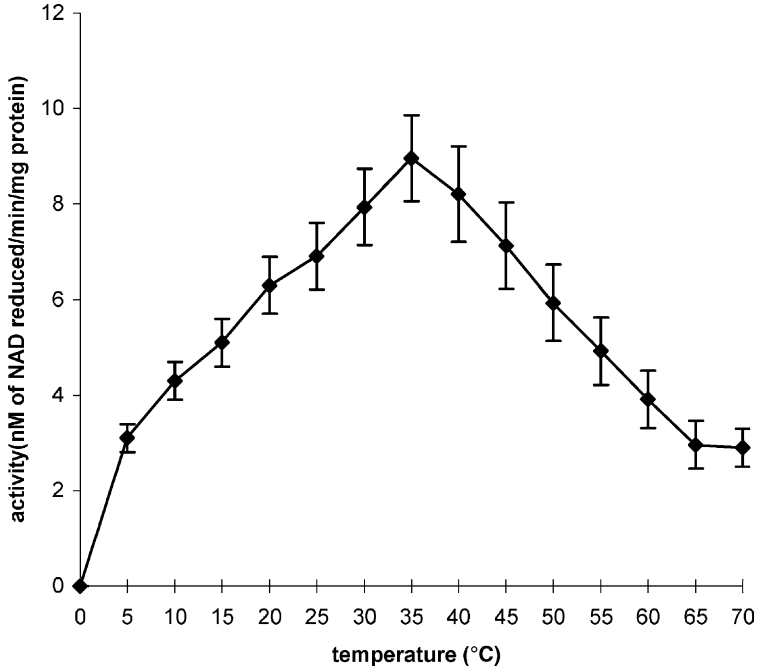

Fig. 6. Effect of various temperatures on the activity of 3β-HSDH at various time, in the rat testis. Each point represents the mean ± S.E. of six separate experiments. Substrate concentration and homogenate volume were 200 μM and 100 μl, respectively.

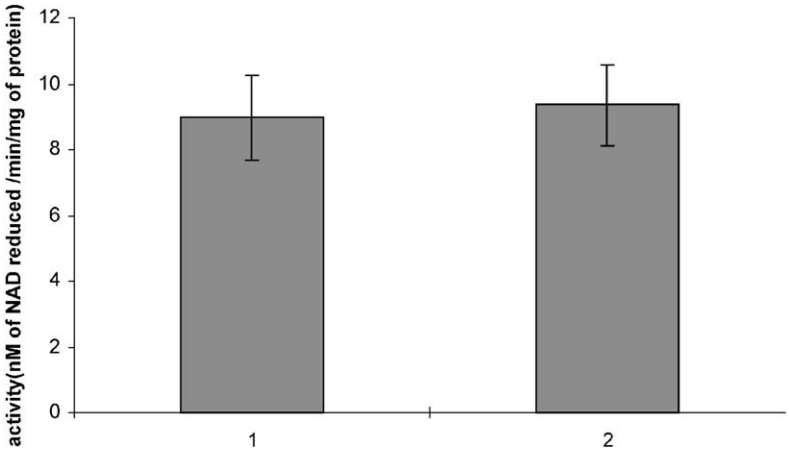

Fig. 7. Comparison of 3β-HSDH activity measured by conversion of radioactive pregnenolone to progesterone with that of colorimetric assay. Each column represents the mean ± S.E. of six separate experiments (column 1: colorimetric assay; column 2: measurement of the conversion of radioactive pregnenolone to progesterone). Substrate concentration and homogenate volume were 200 μM and 100 μl, respectively.

4. Discussion

The importance of 3 beta-hydroxy-delta 5-steroid dehydrogenase in steroidogenic tissues derives, from the fact that its activity is responsible for production of steroid hormones. We have developed a simple and rapid colorimetric assay for 3 beta-hydroxy-delta 5-steroid dehydrogenase activity in the rat testis that is applicable to other steroidogenic tissues. Although the radiochemical assay is more sensitive, routine assays can be easily performed with our colorimetric method and are reliable.

The method described is easily performed and is sensitive enough to assay steroid-3β-dehydrogenase activity in rat testis. The biochemical reaction for 3β-HSDH activity is based on the reduction of the NBT salt into formazan coupled to NAD reduction, which results in deposits in the tube that show enzyme activity. To eliminate the other NAD, NADH interconverting enzyme systems that coexist with crude preparation of steroid oxidoreductases, a control incubation of the testis homogenate and NAD, without addition of pregnenolone was carried out. The higher absorbance of formazan gives a two to threefold higher sensitivity.

A parallel measurement of the conversion of radioactive pregnenolone to progesterone with the 3β-HSDH was carried out to confirm conversion of NAD to NADH. The basic kinetic properties of 3 beta-hydroxy-delta 5-steroid dehydrogenase activity obtained in this study were somewhat different from those previously reported [5,6,15]. The specific activity of the enzyme was independent of the protein concentration and the apparent K_m values for pregnenolone was similar to that previously reported. Also, the optimum pH, was similar to that previously reported [10,11,16]. The value of the Michaelis constant found for pregnenolone indicated that the rate of the 3 beta-hydroxy-delta 5-steroid dehydrogenase activity reaction in vivo in the testis was not limited by the concentration of this substrate, which presumably is higher than the K_m value in most parts of the testis. The concentration of pregnenolone in testis is, however, probably somewhat lower than the K_m value for this substrate. This might be of importance for the activity of the enzyme in vivo. The K_m for pregnenolone as well as the optimum pH, were almost identical to the values reported for the testicular, adrenal, and ovarian enzymes. Our results were in good agreement with those reported previously [14,15].

In conclusion, the importance of this study lies in its establishment of a new methodology. This assay approach offers a simple, inexpensive, and sensitive testing procedure for 3 beta-hydroxy-delta 5-steroid dehydrogenase activity that can be applied to the measurement of dehydrogenase enzyme in other tissues. Also, the method is applicable for use in routine clinical chemistry laboratories.

Acknowledgments

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References

[1] Armstrong DG, Davidson MF, Gilbert AB, Wells JW. Activity of 3β-hydroxysteroid dehydrogenase in the postovulatory follicle of the domestic fowl. J Reprod Fertil 1977;49:253–9.

[2] Tang PZ, Tsai Morris CH, Dufau ML. Regulation of 3 beta-hydroxysteroid dehydrogenase in gonadotropin induced steroidogenic desensitization of leydig cells. Endocrinology 1998;139:4496–505.

[3] Rubin BL, Deane HW. Effects of superovulation on ovarian Δ5-3β-hydroxysteroid dehydrogenase activity in rats of different ages. Endocrinology 1965;76:382–9.

[4] Diezfalusy E. Steroid metabolism in the human foetoplacental unit. Acta Endocr Copen 1969;61:649–64.

[5] Neville AM, Orr JC, Engel LL. The delta 5-3-beta-hydroxysteroid dehydrogenase of bovine adrenal microsomes. J Endocr 1969;43: 599–608.
[6] McCune RW, Roberts S, Young PL. Competitive inhibition of adrenal delta-3beta-hydroxysteroid dehydrogenase and delta-ketosteroid isomerase activities by adenosine-3,5-monophosphate. J Biol Chem 1970;245:3859–67.
[7] Beyer KF, Samuels LT. Distribution of steroid-3beta-ol-dehydrogenase in cellular structures of the adrenal gland. J Biol Chem 1956;219:69–76.
[8] Koide SS, Torres MT. Distribution of 3beta-hydroxysteroid dehydrogenase and delta-3-oxosteroid isomerase in homogenate fractions of human term placenta. Biochim Biophys Acta 1965;105:115–20.
[9] Basch RS, Finegold MJ. 3Beta-hydroxysteroid dehydrogenase activity in the mitochondria of rat adrenal homogenase. Biochem J 1971;125:983–9.
[10] Rubin BL, Leipsner G, Deane HW. A rapid, sensitive assay procedure for adrenal steroid-3beta-ol-dehydrogenase activity. Endocrinology 1961;69:619–25.
[11] Thomas JL, Frieden C, Nash WE, Strickler RC. An NADH-induced conformational change that mediates the sequential 3 beta-hydroxysteroid dehydrogenase/isomerase activities is supported by affinity labelling and the time-dependent activation of isomerase. J Biol Chem 1995;270:21003–8.
[12] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:256–7.
[13] Armstrong DG, Wells JW. The measurement of 3βHSD in ovaries of fowls (*Gallus domsticus*). Gen Comp Endocr 1976;29:313–8.
[14] Rubin BL, Dorfman RI. Quantitative assay of the 3β-ol-dehydrogenase system in whole adrenal homogenate. Endocrinology 1957; 61:601–10.
[15] Geldof AA, Dijkstra I, Newling DW, Rao BR. Inhibition of 3 beta-hydroxysteroid dehydrogenase: an approach for prostate cancer treatment? Anticancer Res 1995;15:1349–54.
[16] Datta M, Nagendra Prasad RJ, Bhattacharya S. Thyroid hormone regulation of perch ovarian 3 beta-hydroxysteroid dehydrogenase delta 5-delta 4-isomerase activity: involvement of a 52-kDa protein. Gen Comp Endocrinol 1999;113:212–20.