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A Colorimetric Assay Method for 3β-Hydroxy-Δ^5-steroid Dehydrogenase[1]

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3β-Hydroxy-Δ^5-steroid dehydrogenase is an important enzyme of steroid hormone biosynthesis present in steroidogenic tissues like adrenal, testis, and ovary of vertebrates. The enzyme is assayed mainly by radiochemical substrates. Spectrophotometric assay is not adequately sensitive to detect the enzyme activity since it is often present in low levels. We have developed a simple colorimetric assay based on formazan formation due to the reduction of the tetrazolium salt. The reaction mixture containing the substrate, pregnenolone or dehydroepiandrosterone, NAD and iodonitrotetrazolium in 0.1 M Tris–HCl buffer (pH 7.8), and the enzyme extract is incubated for 1 h at 37°C. Absorbance at 490 nm is read in a spectrophotometer. The enzyme activity was linear with time and protein concentration. The assay works well with adrenal tissue extract, whereas in the case of testis, Leydig cell preparation may be required. We have assayed the enzyme activity in the adrenal of rat, mouse, and gerbil. The method is two- to threefold more sensitive than the spectrophotometric assay.

The conversion of 3β-hydroxysteroids to their keto-forms is an important step in the biosynthesis of steroid hormones which involves two enzyme systems, viz. 3β-hydroxysteroid dehydrogenase and Δ^5 ketoisomerase (1–3). 3β-Hydroxy-Δ^5-steroid dehydrogenase (3β-HSDH,[3] EC 1.1.1.145) is present in steroidogenic tissues such as adrenal, ovary, testis, and placenta. It is a microsomal NAD-dependent enzyme that catalyzes the conversion of pregnenolone to progesterone, a rate-limiting step in the biosynthesis of hormonal steroids (4). HSDH activity has been assayed mainly by the reduction of NAD spectrophotometrically (340 nM) (11) or fluorometrically (465 nM) and by the quantitation of the product by extraction and TLC (5–8). In recent years, a radiochemical assay for HSDH has been available (9, 10). The spectrophotometric method suffers from poor sensitivity

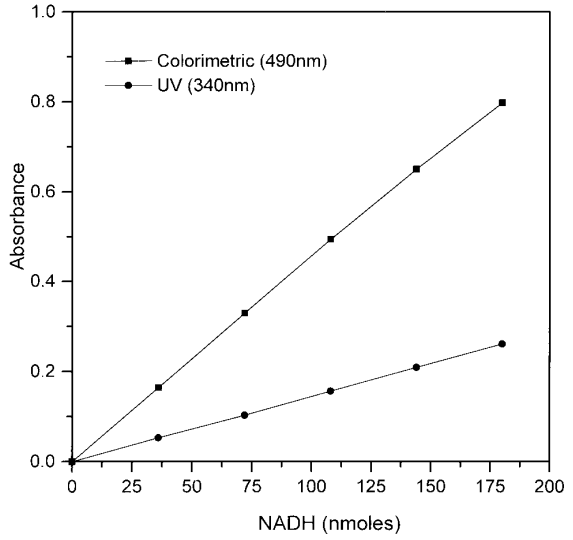

FIG. 1. Comparison of the standard curve of NADH measured by tetrazolium reduction with that of UV absorbance. Graded concentrations of NADH were reacted with iodonitrotetrazolium in the presence of PMS in Tris buffer (0.1 M, pH 7.4). The color was measured at 490 nm and plotted with the absorbance of NADH at 340 nm. Each point is the mean of triplicate values.

[1] This paper is dedicated to Professor H. B. Devaraj Sarkar.

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[3] Abbreviations used: 3β-HSDH, 3β-hydroxy-Δ^5-steroid dehydrogenase; INT, iodonitrotetrazolium chloride, PMS, phenazene methosulfate; DMF, dimethyl formamide.

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TABLE 1

Comparison of Sensitivity between the Colorimetric Assay and the Spectrophotometric Assay for the Adrenal 3β-HSDH Activity

Sample (mg protein)	Enzyme activity[a]	
	Spectrophotometric method	Colorimetric method
0.07	Not detectable	0.1 ± 0.01
0.13	Not detectable	0.38 ± 0.12
0.26	Not detectable	1.04 ± 0.18
0.52	Not detectable	1.03 ± 0.02
1.01	1.41 ± 0.18	1.5 ± 0.02

[a] Values (nmol of NAD reduced/min) are means ± SD of at least three determinations.

and often is not usable for assaying the enzyme activity in the tissue extracts of the laboratory rat and mice. The radiochemical assay, although sensitive, is cumbersome in addition to being expensive, requiring radioactive chemicals and sophisticated instrumentation.

We describe, in this paper, a simple quantitative colorimetric assay for 3β-HSDH in the rat adrenal based on the well-known histochemical reaction of Wattenberg (12). The assay is simple, inexpensive, and two- to threefold more sensitive than the spectrophotometric method and is applicable to other steroidogenic tissues such as the ovary, testis, and placenta.

MATERIALS AND METHODS

Chemicals. Pregnenolone (5-pregne-3β-ol-2one), DHEA (5-androsten-3β-ol-17 one), and testosterone (17β-hydroxyandrostan-4en-3one) were purchased from Sigma Chemical Co.(St. Louis, MO); nicotinamide adenine dinucleotide sodium salt (NAD), nicotinamide adenine dinucleotide reduced form (NADH), iodonitrotetrazolium chloride (INT), nicotinamide adenine dinucleotide phosphate (NADP), and phenazene methosulfate (PMS) were purchased from Sisco Research Labs, Bombay. Other chemicals were purchased locally.

Reagents. Reagents consisted of (a) phthalate buffer (50 mM, pH 3.0): 2.55 g of potassium hydrogen phthalate dissolved in a mixture of 51 ml N/10 HCl and 2.5 ml Tween 20; pH was adjusted to 3.0 and the volume made up to 250 ml with distilled water; (b) Tris–HCl buffer (0.1 M, pH 7.8); (c) NAD (5 mM); and (d) color reagent: 40 mg INT, 10 mg PMS, and 0.5 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. (e) The substrate (pregnenolone or DHEA) was first dissolved in 0.3 to 0.5 ml of

TABLE 2

Activity of 3β-HSDH in Steroidogenic Tissues of the Wistar Rat

Tissue	Enzyme activity[a]	
	Spectrophotometric method	Colorimetric method
Adrenal	0.48 ± 0.07	1.61 ± 0.12
Testis	0.49 ± 0.02	1.20 ± 0.00
Ovary	Not detectable	0.48 ± 0.03

[a] Values (nmol of NAD reduced min^{-1} mg^{-1}) are means ± SD of at least three determinations.

TABLE 3

Species Difference in the Adrenal 3β-HSDH Activity

Species	Enzyme activity[a]	
	Spectrophotometric method	Colorimetric method
Rat	0.48 ± 0.07	1.61 ± 0.12
Gerbil	0.25 ± 0.00	1.40 ± 0.04
Mouse	Not detectable	0.41 ± 0.007

[a] Values (nmol of NAD reduced min^{-1} mg^{-1}) are means ± SD of at least three determinations.

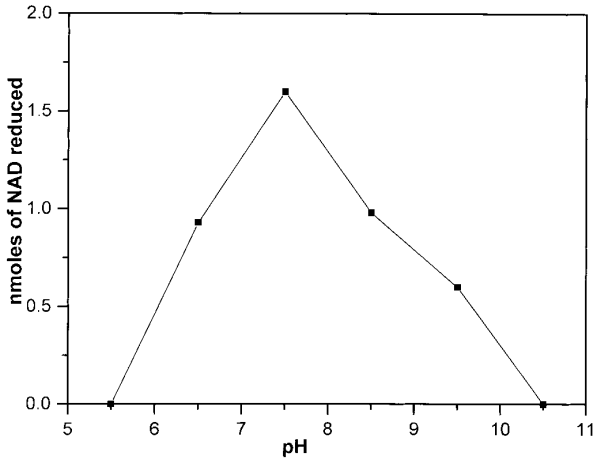

FIG. 2. Effect of pH on the activity of 3β-hydroxysteroid dehydrogenase in the rat adrenal extract. The enzyme activity was assayed colorimetrically as described under Materials and Methods.

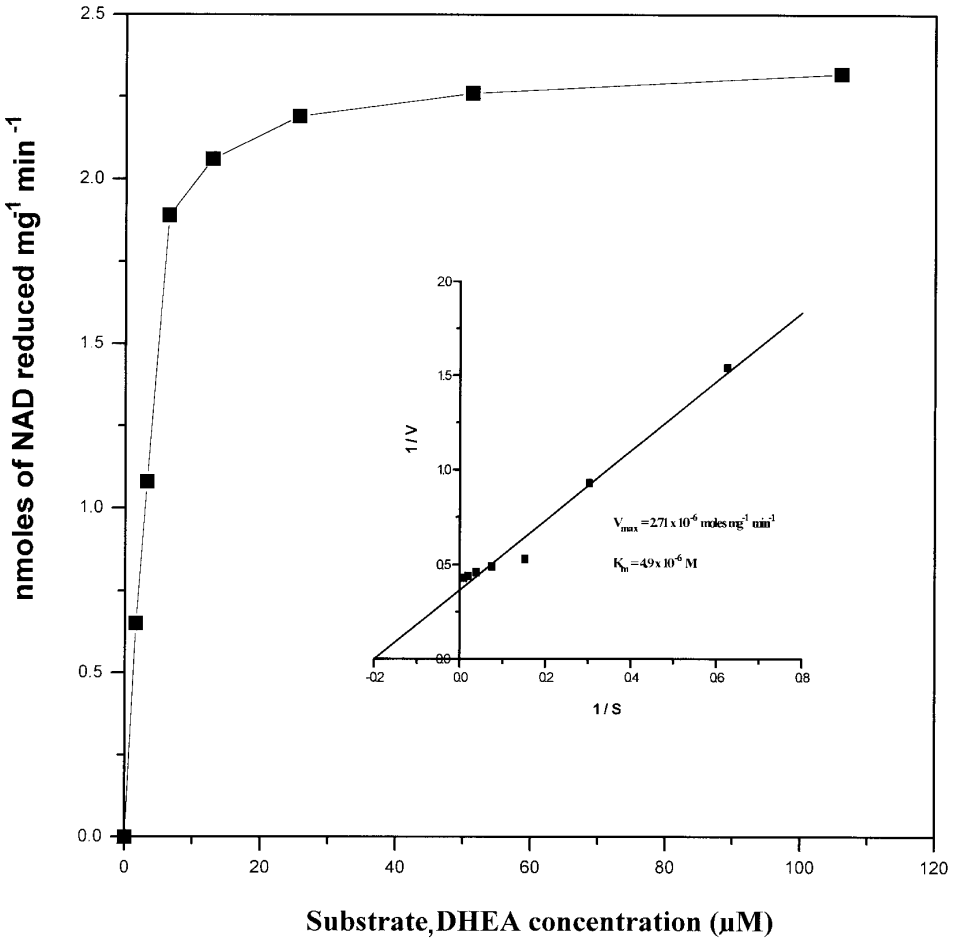

FIG. 3. Determination of apparent K_m for 3β-hydroxysteroid dehydrogenase of the rat adrenal extract. Saturation curve was obtained by varying the concentration of the substrate (DHEA) in the standard assay system as described under Materials and Methods. Inset shows the double-reciprocal plot.

dimethyl formamide (DMF) and the stock solution (1 mM) prepared in 50 or 100 ml Tris–HCl buffer (0.1 M, pH 7.8).

Standard curve. A 1 mM solution of NADH was freshly prepared in distilled water. Aliquots of graded concentrations of NADH (0 to 150 nmol) were reacted with the color reagent (0.5 ml) and after color formed, 2.0 ml of phthalate buffer was added to each tube and the absorbance read at 490 nm. A standard curve was prepared by plotting NADH concentration vs absorbance.

Enzyme assay. Adult male rats were killed by ether anaesthesia and the adrenals excised. At least four to six adrenals from two to three rats were pooled as the gland size was small (20 mg), homogenized in 5.0 ml of 0.1 M Tris–HCl buffer (pH 7.8), and centrifuged at 12,000*g* at 4°C in a refrigerated high-speed centrifuge (Sorval RC 5B). The supernatant was used as the enzyme extract. For other steroidogenic tissues such as the testis and ovary, a 10% homogenate was prepared and centrifuged as above and the supernatant used for the assay. In the case of adrenal, microsomal preparation is not feasible and not essential for the assay.

The enzyme was assayed in 0.1 M Tris–HCl buffer (pH 7.8) containing NAD (500 μM), and the substrate, DHEA or pregnenolone (100 μM), in a total volume of 3.0 ml. The reaction was started by adding the enzyme (50 μl) and incubated at 37°C for 60 min. The reaction was stopped by the addition of 2.0 ml of phthalate buffer (pH 3.0). The turbidity was removed by centrifugation at 3000 rpm for 20 min and the supernatant was read at 490 nM in a spectrophotometer. The enzyme activity was calculated from the standard curve of NADH and expressed as nmol NADH formed h^{-1} mg^{-1} protein.

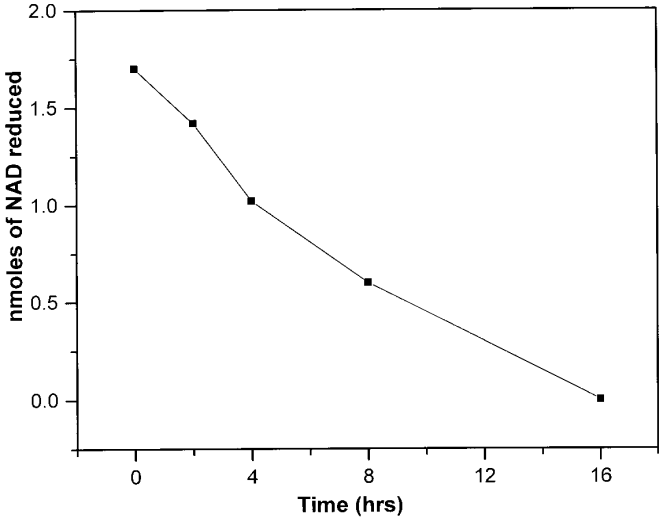

FIG. 4. Stability of 3β-hydroxysteroid dehydrogenase activity of the rat adrenal homogenate stored at 4°C. Aliquots of the adrenal extract were assayed for enzyme activity colorimetrically at various time points as described under Materials and Methods.

RESULTS AND DISCUSSION

The original histochemical reaction of Wattenberg (1958) for 3β-HSDH is based on the reduction of the soluble nitroblue tetrazolium salt into insoluble formazan coupled to NAD reduction deposited in the cell showing the presence of the enzyme. Nitroblue tetrazolium, which upon reduction gives a diformazan which absorbs at two wavelengths, is not suitable for a biochemical assay. Substituting INT, a monotetrazolium salt, has resulted in the use of this principle to develop assays for other dehydrogenases (12). In the case of 3β-HSDH, enzyme activity is rather low in steroidogenic tissues and often not assayable with the UV method. The present assay measures enzyme activity not detectable by UV assay because of the higher absorbance of the formazan and therefore gives a two- to threefold higher sensitivity (Fig. 1, Table 1). The enzyme activity is linear up to 0.8 mg protein concentration. The reaction is also linear with time up to 1 h.

The assay is based on the formation of NADH due to enzymatic oxidation of the steroid alcohol which is coupled to the reduction of the tetrazolium via diaphorase present in the tissue (13). Since the enzyme diaphorase is quite high in steroidogenic tissues, it is not necessary to use an intermediate electron acceptor like PMS in the reaction mixture. To make a standard curve, however, PMS is required. Because of the low activity of the enzyme and due to its restricted distribution to specific cell types in steroidogenic tissues, assaying the activity by measuring NADH formed at 340 nM is often not possible; longer incubation leads to oxidation of NADH but shorter incubation time does not produce measurable NADH by its absorbance at 340 nm. This contributes to the difference in the sensitivity between the spectrophotometric and colorimetric methods and higher activity in the latter (Tables 2 and 3).

The adrenal 3β-HSDH is NAD-dependent and no activity was demonstrable with NADP. Maximum activity was seen for the adrenal enzyme at pH 7.8 under the conditions of the assay (Fig. 2). It is recommended that assays be carried out at pH 7.8 since higher pH can promote nonspecific reduction of INT. The adrenal 3β-HSDH showed similar activity with both the substrates, pregnenolone and DHEA. Results of substrate saturation kinetics show that the adrenal enzyme has a K_m of 4.9×10^{-6} M (DHEA) and V_{max} of 2.71×10^{-6} mol min^{-1} mg^{-1} protein (Fig. 3). Under these assay conditions, no product inhibition was seen. It is important to keep the DMF level at 1 μl/ml in the reaction mixture since the enzyme activity is inhibited at higher concentrations. Other solvents like acetone or alcoholic solvents (ethanol, methanol, propyl glycol) should not be used to dissolve the steroid substrate since they themselves contribute to the nonspecific reduction of NAD or NADP (14).

Adrenals of adult male rats consistently showed higher activity than those of females. Among the steroidogenic tissues, 3β-HSDH activity was in the order adrenal > testis > ovary (Table 2). A comparison of the adrenal 3β-HSDH activity in the three mammalian species showed that the laboratory rat (Wistar strain) has the highest activity followed by gerbil (*Meriones hurrianae*) and least in Swiss mice (Table 3). For assaying the testicular 3β-HSDH, a Leydig cell preparation may be required in order to enrich the activity. In tissues like adrenal no microsomal preparation is required as the postmitochondrial supernatant (containing microsomes) will suffice. Our study shows that 3β-HSDH activity is rapidly lost on storage at 4°C (Fig. 4) and therefore fresh sample should be used for enzyme assays. The method is applicable, in principle, to other NAD- or NADP-dependent steroid dehydrogenases such as 17β- and 21α-HSDH. In summary, we have developed a simple and rapid colorimetric assay for 3β-HSDH in the rat adrenal that is applicable to other steroidogenic tissues. Although radiochemical assay is more sensitive, routine assays in tissue sample are easily performed with the colorimetric method and can be relied upon.

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