Dehydrogenation of Androsterone by Purified 3 α -Hydroxy Steroid-Dependent Nicotinamide-Adenine Dinucleotide (Phosphate)-Transhydrogenating Enzyme of Rat Liver

By REGINA PIETRUSZKO AND D. N. BARON

Department of Chemical Pathology, Royal Free Hospital and School of Medicine, London, W.C.1

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1. An enzyme from rat liver, catalysing 3 α -hydroxy steroid-dependent NAD(P) transhydrogenation and NAD-linked and NADP-linked dehydrogenation of 3 α -hydroxy steroids, has been purified 100-fold by chromatography on DEAE-cellulose and calcium phosphate gel. 2. No separation of these activities into different protein fractions has been achieved. 3. The properties of the enzyme in catalysing NAD-linked and NADP-linked dehydrogenation have been compared, with androsterone as substrate. Differences were found in pH optima, affinity for coenzyme and steroid, equilibrium constants and effects of salts. 4. NAD-linked dehydrogenation is inhibited by NADPH₂ but is protected from this inhibition by chloride, which alone is itself an inhibitor. 5. The relevance of these findings to the problem of the number of enzymes involved in catalysis of 3 α -hydroxy steroid-dependent transhydrogenation is discussed.

Tomkins (1956) first demonstrated the occurrence in rat liver of 3 α -hydroxy steroid-NAD(P) oxidoreductase (EC 1.1.1.50). Hurlock & Talalay (1958) partially purified this enzyme and showed that it catalysed 3 α -hydroxy steroid-dependent NAD(P) transhydrogenation. Talalay & Williams-Ashman (1958), on the basis of their work with 17 β -hydroxy steroid dehydrogenase of human placenta, supported by the results of work with the rat-liver enzyme, suggested that steroid-dependent NAD(P) transhydrogenation may in general proceed via a hydroxy steroid dehydrogenase with dual nucleotide specificity.

The sensitivity to anions of 3 α -hydroxy steroid-dependent transhydrogenation and of NAD-linked but not of NADP-linked steroid dehydrogenation catalysed by rat-liver enzyme (Baron, Gore, Pietruszko & Williams, 1963) justified further investigation of the interdependence of these reactions. In the present work further purification of the rat-liver enzyme that catalyses 3 α -hydroxy steroid-dependent NAD(P) transhydrogenation has been achieved, and the conditions under which this preparation catalysed dehydrogenation of androsterone with NAD and NADP have been investigated.

MATERIALS AND METHODS

Reagents. NAD, NADP, disodium glucose 6-phosphate, yeast glucose 6-phosphate dehydrogenase [A.R. grade, 5 mg./ml. in 3.3 M-(NH₄)₂SO₄ solution; specific activity 70 μ moles/mg./min.] and yeast alcohol dehydrogenase

[A.R. grade in 2.4 M-(NH₄)₂SO₄ solution; specific activity 180 μ moles/mg./min.] were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. NAD and NADP solutions were adjusted to the required pH with NaOH before use. Glutathione reductase [2 mg./ml. of suspension in (NH₄)₂SO₄ solution; specific activity 80 μ moles/mg./min.] was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Androsterone and androstanedione were obtained from Organon Laboratories Ltd., Morden, Surrey, and used after two recrystallizations from acetone. Methanol, used as solvent for steroids, was distilled from DNP-hydrazine and then redistilled twice. The (NH₄)₂SO₄ ('enzyme grade') was from Mann Research Laboratories, New York, N.Y., U.S.A. Serum albumin (British Drug Houses Ltd., Poole, Dorset, or Armour Laboratories, Eastbourne, Sussex) was dialysed in water before use, then adjusted to neutrality with NaOH. DEAE-cellulose and CM-cellulose were from W. and R. Balston, Maidstone, Kent. Sephadex G-25 was obtained from Pharmacia, Uppsala, Sweden. Cellophan dialysis tubing, from Solmedia Ltd., London, E.17, was soaked before use for 1-2 hr. in a solution containing 2 g. of EDTA/l., 1.37 g. of NaHCO₃/l. and 0.5 ml. of β -mercaptoethanol/l. (Jarabak, Adams, Williams-Ashman & Talalay, 1962) and then washed in water. Carbowax 20M was obtained from G. T. Gurr Ltd., London, S.W.6. All chemicals were A.R. grade, except where stated otherwise. Glass-distilled water was used throughout. The buffers generally employed were: tris-HCl; H₃PO₄-tris; H₂SO₄-tris; glycine-NaOH; acetic acid-tris; H₂PO₄-NaOH. The stated molarity of the buffer solutions used was that of the first-named component, adjusted to the stated pH with the second component.

Enzyme assays. Transhydrogenase and 3 α -hydroxy steroid-dehydrogenase activities were assayed by following the reduction of either NAD or NADP at 340 m μ , at 25°,

in micro-cuvettes of 1 cm. light-path and 0.27 ml. total volume, in a Hilger Uvispek spectrophotometer with Gilford attachment for automatic reading. Enzyme activities are expressed either as changes in extinction (ΔE), or as specific activities.

(a) Transhydrogenase. The assay system for transhydrogenase was as described by Baron *et al.* (1963) except that steroid was added in methanol, and that 55 mM-tris-HCl buffer, pH 7.5, and 0.1 M-(NH₄)₂SO₄ were replaced by 0.1 M-H₂SO₄-tris buffer, pH 7.5.

(b) Steroid dehydrogenases. These were assayed by measuring the initial rate of reduction of NAD or NADP with androsterone (added in 0.005 ml. of methanol/0.27 ml., unless stated otherwise), catalysed by 0.1–0.5 μ g. of purified rat-liver enzyme, in the presence or absence of 1% (w/v) bovine serum albumin. NAD-linked dehydrogenation was determined in 0.1 M-H₃PO₄-tris buffer, pH 8.0, and NADP-linked dehydrogenation in 0.1 M-glycine-NaOH buffer, pH 9.6. Controls contained all components except steroid. The reaction was initiated by the addition of enzyme.

Determination of NAD, NADP and androsterone. Concentrations were calculated from the extinctions at 340 m μ of the enzymically reduced nucleotides, in cuvettes of 1 cm. light-path and 2.7 ml. total volume, and from the molar extinction coefficient, 6220, at 340 m μ for NADH₂ and NADPH₂.

(a) NAD was reduced in 0.1 M-tris-HCl buffer, pH 10.1, with ethanol (0.42 M) and yeast alcohol dehydrogenase (0.3 mg.) (Ciotti & Kaplan, 1957).

(b) NADP was reduced in 0.033 M-tris-HCl buffer, pH 8.5, with glucose 6-phosphate (5.5 mM), MgCl₂ (13 mM) and yeast glucose 6-phosphate dehydrogenase (50 μ g.) (modified from Horecker & Kornberg, 1957).

(c) Androsterone was determined by reduction of NAD with androsterone, catalysed by 3 α -hydroxy steroid dehydrogenase from *Pseudomonas testosteroni* in 0.033 M-pyrophosphate buffer, pH 10.0, with NAD (1.25 mM) and androsterone (approx. 40 μ g.) (J. Boyer, D. N. Baron & P. Talalay, unpublished work).

Michaelis constants (K_m). These were determined graphically by plotting $1/v$ against $1/s$, s/v against s , and v against v/s (v =velocity, s =substrate concentration), as described by Dixon & Webb (1964). The arithmetic means of the results obtained by these methods are quoted.

Equilibrium constants. These were determined at 25° in 2.7 ml. total volume in 0.1 M-H₃PO₄-tris buffer for NAD-linked dehydrogenation and 0.1 M-glycine-NaOH buffer for NADP-linked dehydrogenation, 0.063 M-androsterone, added in 0.05 ml. of methanol, and various concentrations of nucleotides, in the presence of 22 μ g. of purified enzyme [in 0.05 ml. of 0.004 M-H₃PO₄-NaOH buffer, pH 7.5, containing 20% (v/v) of glycerol]. Controls contained all components except steroids. The reaction was followed spectrophotometrically until equilibrium. Androstanedione (in 0.05 ml. of methanol) was then added to tests and controls and the reaction was followed until a second equilibrium was reached. At the end of the reaction the H⁺ concentration was determined with a micro-glass electrode and a Vibron electrometer, model 33 B (Electronic Instruments Ltd., Richmond, Surrey). Apparent equilibrium constants (K values) were obtained from:

$$K = \frac{[\text{NAD(P)H}_2][\text{androstanedione}]}{[\text{NAD(P)}][\text{androsterone}]}$$

The K_H values were obtained by multiplying K by the respective H⁺ concentration.

Protein estimation. Protein concentrations of enzyme preparations were estimated from the difference in E at 215 and 225 m μ of a suitable aqueous dilution (Waddell, 1956) and calculated from a standard curve based on human serum albumin.

Preparation of chromatographic media and columns. (a) CM-cellulose (150 g.) was suspended in 2 l. of 0.5 N-NaOH. After stirring, NaOH was removed by suction. The CM-cellulose was then washed with 2 l. of water and suspended in 3 l. of 0.5 N-HCl. After stirring, HCl was removed by suction, the CM-cellulose was washed with 3 l. of water, transferred into a column-shaped separating funnel and washed further with water until no chloride was present in the eluate. The CM-cellulose was then suspended in 1 l. of 0.1 M-acetic acid-tris buffer, pH 6.0, packed in a column and washed with 0.02 M-acetic acid-tris buffer, pH 6.0, until conductivity of the column eluate was constant. The slurry, in 0.02 M-acetic acid-tris buffer, pH 6.0, was packed into columns 15 cm. long $\times$ 2 cm. diam. and washed with 20–100 ml. of cold 0.02 M-acetic acid-tris buffer, pH 6.0, before use.

(b) DEAE-cellulose was prepared as described by Baron *et al.* (1963), except that the material was finally suspended in 0.02 M-tris-HCl buffer, pH 7.5, and packed into columns 10 cm. long $\times$ 2 cm. diam.

(c) Calcium phosphate gel was prepared as described by Levin (1962). The protein was applied to a column 8 cm. long $\times$ 1 cm. diam. in 0.01 M-H₃PO₄-NaOH buffer, pH 6.8, or 0.1 M-tris-HCl buffer, pH 7.5, and eluted in H₃PO₄-NaOH buffer, pH 6.8.

(d) Sephadex G-25 was soaked in water containing EDTA for a minimum of 24 hr. The columns were packed by gravity and washed first with water, followed by overnight washing in 0.01 M-tris-HCl buffer, pH 7.5. The column of Sephadex used for desalting was 30 cm. long $\times$ 2 cm. diam.

Preparation of 3 α -hydroxy steroid dehydrogenase. The partially purified enzyme obtained by (NH₄)₂SO₄ fractionation and DEAE-cellulose chromatography of rat-liver protein (Baron *et al.* 1963) was purified further as follows. Active eluates of DEAE-cellulose columns were concentrated with Carbowax 20M until the concentration of protein was about 5 mg./ml. Saturated (NH₄)₂SO₄ solution was added to give 62% saturation, and the precipitate was removed by centrifugation. Solid (NH₄)₂SO₄ was added to the supernatant to give 100% saturation. After centrifuging, the precipitated protein was dissolved in about 3 ml. of 0.01 M-tris-HCl buffer, pH 7.5, and desalted by chromatography on Sephadex G-25 in 10 mM-tris-HCl buffer, pH 7.5. After adjustment of the pH to 6.0 with acetic acid, the Sephadex eluate was applied directly to a CM-cellulose column (CM-cellulose in 0.02 M-acetic acid-tris buffer, pH 6.0) and the column was washed with about 15 ml. of 0.02 M-acetic acid-tris buffer, pH 6.5, followed by the same volume of 0.02 M-acetic acid-tris buffer, pH 6.8. The active material was eluted with 0.02 M-acetic acid-tris buffers, pH 7.0 and 7.2. The pooled fractions eluted at pH 7.0 and 7.2, which contained the enzyme, were applied directly (after adjusting the pH with tris to 7.5) to the DEAE-cellulose column (DEAE-cellulose in 0.02 M-tris-HCl buffer, pH 7.5). The column was washed with about 30 ml. of 0.04 M-tris-HCl buffer, pH 7.5, and the enzyme was eluted with 0.1 M-tris-HCl buffer, pH 7.5. The combined fractions containing the

activity were concentrated with Carbowax 20M to 1-2 ml. and the concentrated material was applied to a calcium phosphate column (calcium phosphate in 0.01M-H₃PO₄-NaOH buffer, pH 6.8). The calcium phosphate column was washed successively with its own volume of 0.01M-, 0.03M-, 0.06M-, 0.09M- and 0.12M-H₃PO₄-NaOH buffers. The enzyme was eluted in 0.15M-H₃PO₄-NaOH buffer and concentrated to about 1 ml. with Carbowax 20M. It was, when necessary, reactivated by incubation in 0.01% (v/v) β -mercaptoethanol solution at 0-2° for 5 hr. under N₂. After reactivation the enzyme was dialysed against N₂-saturated 0.004M-H₃PO₄-NaOH buffer, pH 7.5, containing 20% (v/v) of glycerol. The enzyme has been stored at -40° under N₂ for 8 months without apparent loss of activity.

RESULTS

Purification of the enzyme catalysing 3 α -hydroxy steroid-dependent transhydrogenation

The progress of purification and yield of the enzyme, assayed as transhydrogenase, are shown in Table 1. These results were obtained with one preparation from four rat livers. Preparations from four to six rat livers gave about 20% yield and 100-200-fold purifications; the specific activity in the finally purified enzyme varied from 0.1 to 0.3 μ mole of NADH₂ formed/mg./min., the activity of most preparations falling within the range 0.1-0.15 μ mole/mg./min.

Comparison of 3 α -hydroxy steroid-dependent transhydrogenation with NAD-linked and NADP-linked dehydrogenation during enzyme purification

No separation of transhydrogenase activity from NAD-linked or NADP-linked dehydrogenase activities was observed during purification of the enzyme. During chromatography on CM-cellulose the proteins exhibiting transhydrogenase activity separated into two peaks (Fig. 1): the ratio of transhydrogenase

activity to NAD-linked and NADP-linked dehydrogenase activities (Table 2) was the same in both protein peaks. Results of preliminary experiments to investigate this phenomenon suggest that the two peaks consist of enzyme bound to different amounts of coenzyme. In eluates of the calcium phosphate column, the ratio of activities of transhydrogenase to NAD-linked and NADP-linked dehydrogenase was also constant. Subsequent electrophoresis on starch gel in 0.5M-tris-borate-EDTA buffer (60.5g. of tris, 4g. of boric acid and 6g. of EDTA/l.), and on cellulose acetate in 0.5M-tris-borate-EDTA buffer and 0.5M-acetic acid-tris buffer, pH 6.0, also failed to achieve separation. The single protein band that was obtained on starch-gel electrophoresis was shown, on elution, to catalyse androsterone-dependent transhydrogenation and NAD-linked and NADP-linked dehydrogenation of androsterone (M. B. R. Gore, unpublished work).

Deactivation and reactivation. During the process of purification the enzyme gradually became inactivated. Reactivation could be achieved by incubation with freshly diluted 0.05M- β -mercaptoethanol for 10-20 min. at 25° under nitrogen. In Table 3, the initial rates of hydrogen transfer from androsterone to NAD and NADP, and of steroid-dependent transhydrogenation, are compared before and after reactivation with β -mercaptoethanol. These results are at variance with those of Koide (1964), who noticed inhibition of the enzyme with β -mercaptoethanol. In our experience, solutions of β -mercaptoethanol prepared just before the experiment always activated the enzyme. However, on one occasion, when old β -mercaptoethanol solution was used [a 10% (v/v) solution of β -mercaptoethanol in water, which had been standing on the bench for about 2 months], inhibition of NAD-linked and NADP-linked dehydrogenation was observed.

Table 1. *Purification of the enzyme catalysing 3 α -hydroxy steroid-dependent transhydrogenation*

Transhydrogenase activity was assayed as described in the Materials and Methods section. One unit of enzyme activity is represented by $\Delta E_{340}=0.001/\text{min.}$ in 0.27 ml. reaction volume.

Purification procedure	Total protein (mg.)	Total activity (units)	Specific activity (units/mg.)	Overall yield (%)
(1) Homogenization and centrifugation	1860	49000	26	100
(2) 50-80% Satd. (NH ₄) ₂ SO ₄ precipitation and dialysis	530	45000	85	92
(3) DEAE-cellulose chromatography	268	52000	195	106
(4) Second fractionation with (NH ₄) ₂ SO ₄ and chromatography on Sephadex G-25	88.5	22000	260	44
(5) CM-cellulose chromatography	17.5	19000	1080	39
(6) Second DEAE-cellulose chromatography and concentration with Carbowax 20M	7.2	18600	2550	38
(7) Calcium phosphate chromatography and concentration with Carbowax 20M	3.1	9300	3000	19

Fig. 1. Chromatography on CM-cellulose column. ●, Concn. of protein (mg./ml. of eluate); ○, transhydrogenase activity (units/ml. of eluate). Eluate was collected in 5 ml. fractions. Eluting solutions are 0.02M-acetic acid-tris buffers of increasing pH: (1) pH 6.0; (2) pH 6.5; (3) pH 6.8; (4) pH 7.2; (5) pH 7.5. Total protein and units of activity added to the column were 120 mg. and 65 000 units respectively.

Dehydrogenation of androsterone with NAD and NADP

Effect of pH on enzyme activities. The optimum pH for NAD-linked dehydrogenation is about pH 8.0 and that for NADP-linked dehydrogenation about pH 9.6, in 0.1M-phosphoric acid-tris buffer and in 0.1M-glycine-sodium hydroxide buffer respectively (Fig. 2). When the activity of the enzyme was measured by oxidation of NADPH₂ and NADH₂ with androstanedione [NADPH₂ (0.13 mM) or NADH₂ (0.145 mM), androstanedione (0.16 mM in 0.05M-phosphoric acid-tris buffer) and bovine serum albumin (1%)] as a function of pH, the rate of oxidation of NADPH₂ and of NADH₂ was constant between pH 6.5 and 8.5. Increase of this reaction rate was noticed with both nucleotides at pH values below 6.5, and decrease at pH values above 8.5.

Effect of pH on Michaelis constants. The Michaelis

Table 2. Comparison of the rate of androsterone-dependent NAD(P) transhydrogenation with the rate of NAD-linked and NADP-linked dehydrogenation in combined eluates from a CM-cellulose column

Transhydrogenase assayed as described in the Materials and Methods section. The rates of NAD-linked and NADP-linked dehydrogenation are expressed as maximal velocities at pH 8.0 and 9.6 respectively.

Combined fractions	Total protein (mg.)	NAD(P)H ₂ formed (μmoles/mg. of protein/min.)			Proportions a:b:c
		Transhydrogenation (a)	NAD-linked dehydrogenation (b)	NADP-linked dehydrogenation (c)	
6-14	107.5	0.004	0.035	0.069	1:9:17
15-46	10	0.113	0.87	1.74	1:8:15
47-60	<0.1	None	None	None	
61-68	9	None	None	None	

Table 3. Reactivation of purified rat-liver enzyme with β-mercaptoethanol

To 0.26 ml. enzyme [380 μg./ml. in 0.004M-H₃PO₄-NaOH buffer, pH 7.5, containing glycerol (20%, v/v)], was added 0.01 ml. of water or 0.01 ml. of 10% (v/v) β-mercaptoethanol in water. The enzyme was incubated under N₂ at 25° for 10 min. and further at 1-2° in an ice bath. NAD-linked dehydrogenation was estimated with 0.01 ml. of enzyme/2.7 ml. total volume. NADP-linked dehydrogenation was estimated with 0.005 ml. of enzyme/2.7 ml. total volume. Transhydrogenase was estimated with 0.01 ml. of enzyme/0.27 ml. total volume, as described in the Material and Methods section. The rates of NAD-linked and NADP-linked dehydrogenation expressed here as maximal velocities were determined at nucleotide concentrations corresponding to their respective *K_m* values and the maximal velocities were calculated by doubling this rate. The reaction mixtures contained: NAD (0.8 mM) and androsterone (0.064 mM) in H₃PO₄-tris buffer, pH 8.0 (0.1M), or NADP (0.14 mM) and androsterone (0.064 mM) in glycine-NaOH buffer, pH 9.6 (0.1M).

Time of incubation in ice bath (hr.)	β-Mercaptoethanol	NAD(P)H ₂ formed (μmoles/mg. of protein/min.)			Proportions a:b:c
		Transhydrogenation (a)	NAD-linked dehydrogenation (b)	NADP-linked dehydrogenation (c)	
2	—	0.06	0.9	1.9	1:15:32
2	+	0.15	2.7	3.3	1:18:22
4	—	0.05	0.9	1.6	1:18:32
4	+	0.14	2.3	3.4	1:16:24

constants for NAD and NADP increased with pH (Table 4). At pH 7.5, K_m for NADP was less than 0.02 mm (which was the lowest NADP concentration at which measurement could be made in cuvettes of 1 cm. light-path), and it increased to 0.14 mm at pH 9.6. At their respective pH optima the ratio of K_m for NAD to K_m for NADP was about 5:1, but at pH 8.1 it was about 30:1. From a preliminary study it appeared that K_m for androsterone did not vary appreciably with pH, but varied according to the nucleotide used as hydrogen acceptor. K_m for androsterone at saturating concentrations of NAD was 0.02 mm at pH 8.0 and 8.5. When NADP at pH 9.6 was used as hydrogen acceptor, K_m for

androsterone could not be determined as no decrease in velocity was observed with the lowest feasible concentration of androsterone (6 μ M). The ratio of V_{NAD} (maximum velocity of NAD-linked dehydrogenation) to V_{NADP} (maximum velocity of NADP-linked dehydrogenation) also varied with pH. V_{NADP} at pH 7.5 was 40% of V_{NAD} , and at pH 8.1 it was 26%. At the respective pH optima, however (NADP-linked dehydrogenation in 0.1 M-glycine-sodium hydroxide buffer, pH 9.6; and NAD-linked dehydrogenation in 0.1 M-phosphoric acid-tris buffer, pH 8.0), V_{NADP} was about twice the value of V_{NAD} . Fig. 2 does not represent a true ratio of activity of NAD-linked to NADP-linked dehydrogenation: whereas 1.0 mM-NADP completely saturates the enzyme, 1.2 mM-NAD does not.

Effect of pH on the equilibrium constants. The value of $\log K$ (Talalay & Marcus, 1956) increased linearly with pH with both nucleotides (Fig. 3), thus obeying the general rule for reactions in which H^+ takes part. Equilibrium constants K_H ($K \times [H^+]$) were calculated for all K values and averaged. K_H for NAD-linked dehydrogenation was 7.5 (s.d. 1.28) $m\mu$ M and K_H for NADP-linked dehydrogenation was 1.5 (s.d. 0.38) $m\mu$ M. Standard free energy (from $\Delta F^0 = -RT \ln K_H$) for the reaction of androsterone with NAD is +11 kcal./mole, and that of androsterone with NADP +12 kcal./mole, at 25°. The values for equilibrium constants quoted apply to the stated buffers only. In one experiment, equilibrium constant of NADP-linked dehydrogenation of androsterone was determined in 0.1 M-phosphoric acid-tris buffer at pH 8.86 and at pH 9.20. The K_H values so obtained were 3.5 and 3.9 $m\mu$ M before the addition of androstanedione and 3.46 and 2.8 $m\mu$ M after equilibration with androstanedione, and therefore at least twice those obtained in glycine-sodium hydroxide buffer.

Fig. 2. NAD-linked and NADP-linked dehydrogenation of androsterone as a function of pH. O, NAD-linked dehydrogenation [NAD (1.2 mm), androsterone (0.34 mm) and serum albumin (1%) in 0.1 M-H₃PO₄-tris buffer]. ●, NADP-linked dehydrogenation [NADP (1.0 mm), androsterone (0.17 mm) and serum albumin (1%) in 0.1 M-glycine-NaOH buffer].

Table 4. K_m values for NAD and NADP at various pH values

All values quoted are results of single experiments.

Final pH	Temp.	Buffer	1% Bovine serum albumin	Concn. of androsterone (mm)	K_m for NAD (mm)	K_m for NADP (mm)
7.5	26.5°	0.1 M-H ₃ PO ₄ -tris	+	0.113	0.46	Very small (probably about 0.005)
8.0	25	0.1 M-H ₃ PO ₄ -tris	—	0.064	0.64	—
8.1	24	0.1 M-H ₃ PO ₄ -tris	+	0.113	0.75	0.02
8.9	25	0.1 M-H ₃ PO ₄ -tris	—	0.064	—	0.074
8.9	25	0.1 M-Glycine-NaOH	—	0.064	—	< 0.01
9.6	26	0.1 M-Glycine-NaOH	+	0.017	—	0.085
9.6	26	0.1 M-Glycine-NaOH	+	0.034	—	0.112
9.6	26	0.1 M-Glycine-NaOH	+	0.085	—	0.134
9.6	26	0.1 M-Glycine-NaOH	+	0.17	—	0.135
9.6	26	0.1 M-Glycine-NaOH	+	0.34	—	0.126
9.6	25	0.1 M-Glycine-NaOH	—	0.064	—	0.136

Effect of temperature on enzyme activities. The enzyme in 1% bovine serum albumin was stable to heat at temperatures below 40°. After heating at 45° for 10 min. in 0.035M-phosphoric acid-tris buffer, pH 8.0, containing bovine serum albumin (1%), the initial rates of NAD-linked and NADP-linked dehydrogenation were 85 and 83% respectively of those of the controls before heat treatment.

Fig. 3. Relation between apparent equilibrium constant and pH for rat-liver enzyme, with NAD and NADP. Δ , NAD, forward reaction; $\blacktriangle$, NAD, back reaction; $\circ$, NADP, forward reaction; $\bullet$, NADP, back reaction. NAD-linked dehydrogenation was determined in 0.1M- H_3PO_4 -tris buffer and NADP-linked dehydrogenation in 0.1M-glycine-NaOH buffer.

After 10 min. at 50° the activities were 28 and 34% of those of the controls. All attempts to produce differential inactivation of dehydrogenation of androsterone with NAD and NADP by heat treatment were unsuccessful.

NAD-linked and NADP-linked dehydrogenase activities were determined in the presence of 1% serum albumin and 0.17mM-androsterone, between 15° and 39°. In the former reaction 2mM-NAD and 0.1M-phosphoric acid-tris buffer, pH 8.0, were used; in the latter reaction 0.8mM-NADP and 0.1M-glycine-sodium hydroxide buffer, pH 9.6. Temperature coefficients between 20° and 30° (Q_{10}) for NAD-linked and NADP-linked dehydrogenation of androsterone, calculated from the slopes of plots of the logarithm of the rate against reciprocals of absolute temperature, were 2.3 for both reactions.

Effect of inhibitors on enzyme activities. For these experiments (see Table 5) inhibitors were added to the reaction mixtures together with substrates. NAD-linked dehydrogenation shows greater susceptibility to thiol reagents and metals of Group IIB than does NADP-linked dehydrogenation. However, when inhibitors at concentrations shown in Table 5 were preincubated with the enzyme both NAD-linked and NADP-linked dehydrogenation were completely abolished; at lower concentrations of inhibitors NAD-linked and NADP-linked dehydrogenation were inhibited to the same extent. Differential inhibition, obtained in the presence of substrates, may be due to a protective effect of NADP. Inhibition by heavy-metal ions and *p*-chloromercuribenzoate could be partially reversed by subsequent incubation of the enzyme with 0.05M- β -mercaptoethanol. GSSG (1.5mM) completely abolished NAD-linked and NADP-linked

Table 5. *Effect of inhibitors on NAD-linked and NADP-linked dehydrogenation*

The reaction system contained: tris-HCl buffer, pH 8.5 (0.055M), $(\text{NH}_4)_2\text{SO}_4$ (0.1M), NAD (0.34mM), NADP (0.30mM), androsterone (0.09mM) and enzyme (approx. 3.0 $\mu\text{g.}/0.27\text{ ml.}$). Enzyme for this experiment was purified by DEAE-cellulose chromatography followed by calcium phosphate chromatography.

Inhibitor	Final concn. (mM)	Activity in the presence of inhibitor (% of uninhibited rate)	
		NAD-linked dehydrogenation	NADP-linked dehydrogenation
ZnCl ₂	0.22	2	53
CdSO ₄	0.22	0	75
HgCl ₂	0.22	0	0
CuSO ₄	0.22	70	92
CoCl ₂	0.22	92	100
FeCl ₃	0.22	95	—
FeSO ₄	0.22	72	100
MgCl ₂	10.0	75	100
K ₂ Cr ₂ O ₇	0.11	90	85
Iodoacetamide	1.0	62	100
<i>p</i> -Chloromercuribenzoate	0.006	0	100

dehydrogenation, if it was preincubated with the enzyme, and this effect could not be reversed with β -mercaptoethanol; preincubation of 0.015 mM-GSSG with the enzyme for 15 min. at 0–4° depressed the rate of NAD-linked and NADP-linked dehydrogenation by 75%.

Effect of salts on enzyme activities. The effects of 0.1 M-glycine-sodium hydroxide and 0.1 M-phosphoric acid-tris buffers on the rate of NADP-linked dehydrogenation were compared at pH 8.9, with 60 μ M-androsterone at various NADP concentrations. At low NADP concentrations the initial rate of NADP-linked dehydrogenation was higher in glycine-sodium hydroxide buffer than in phosphoric acid-tris buffer (in the ratio 1:0.3 at 0.03 mM-NADP); at high NADP concentrations, however, the rates were approximately equal. At the same pH, K_m for NADP appeared to depend on the buffer used: thus K_m for NADP was higher in phosphoric

acid-tris buffer than in glycine-sodium hydroxide buffer at pH 8.9 (Table 4).

We have confirmed the activation of NAD-linked dehydrogenation by sulphate and phosphate (Baron *et al.* 1963): maximum reaction rates were obtained at 0.01–0.02 M salt concentrations. The effect of various salts and buffers on the rate of NAD-linked dehydrogenation is shown in Table 6. The fact that buffers (other than sulphate and phosphate) either activated or inhibited the enzyme introduced considerable difficulty in interpretation of results. Because of this difficulty and because very low concentrations of sulphate and phosphate (0.01 M) produced maximum activation, experiments on the effect of variation of NAD and androsterone on the rate of NAD-linked dehydrogenation in the presence or absence of activators (which might have thrown some light on the mechanism of activation) could not be carried out. The rate of NAD-linked dehydro-

Table 6. *Effect of salts on the rate of NAD-linked dehydrogenation*

The reaction system at pH 8.5, in 0.27 ml. total volume, contained: NAD (0.36 mM), androsterone (0.13 mM), serum albumin (1%) and enzyme (0.5 μ g.).

Salts added in excess of 0.1 M-H ₃ PO ₄ -NaOH buffer		Use of different buffers at pH 8.5	
Added salt (final concn.)	Initial rate (% of the rate in 0.1 M-H ₃ PO ₄ -NaOH buffer)	Buffer (final concn.)	Initial rate (% of the rate in 0.1 M-H ₃ PO ₄ -NaOH buffer)
0.1 M-(NH ₄) ₂ SO ₄	88	0.01 M-H ₃ PO ₄ -NaOH	100
0.1 M-NaCl	45	0.08 M-Barbitone	7
0.01 M-Sodium acetate	65	0.02 M-Tris-acetic acid	32
0.1 M-Sodium acetate	20	0.05 M-Tris-H ₃ PO ₄	122
0.1 M-Tris acetate	59	0.1 M-Tris-H ₃ PO ₄	128
0.008 M-Barbitone	80	0.05 M-Tris-H ₂ SO ₄	97
0.08 M-Barbitone	16	0.1 M-Tris-H ₂ SO ₄	93
0.02 M-Tris hydrochloride	89	0.1 M-Tris-H ₂ SO ₄ containing NaCl (0.05 M)	55
0.04 M-Tris hydrochloride	78	0.1 M-Tris-H ₂ SO ₄ containing NaCl (0.1 M)	25
0.05 M-Tris hydrochloride	75		

Table 7. *Effect of chloride concentration on the rate of NAD-linked dehydrogenation*

Rates are expressed as percentages of the initial uninhibited rate at respective phosphate concentrations. The reaction system at pH 7.8, in 0.27 ml. total volume, contained: NAD (0.36 mM), androsterone (0.13 mM), serum albumin (1%) and enzyme (0.5 μ g.).

Concn. of NaCl (M)	Rate in 0.05 M-H ₃ PO ₄ -tris buffer (%)	Rate in 0.1 M-H ₃ PO ₄ -tris buffer (%)	Rate in 0.18 M-H ₃ PO ₄ -tris buffer (%)
0	(100)	(100)	(100)
0.005	93	90	90
0.01	84	80	83
0.025	69	66	—
0.05	50	48	56
0.075	42	40	46
0.1	32	31	37
0.2	13	14	22

Table 8. *Effect of chlorides on the rate of NAD-linked dehydrogenation*

The reaction system at pH 8.0, in 0.27 ml. total volume, contained: NAD (0.36 mM), androsterone (0.17 mM), serum albumin (1%) and enzyme (0.25 μ g.).

Salts added in excess of 0.1 M-H ₃ PO ₄ -tris buffer (final concn.)	Rate (ΔE /min.)	Relative activity
0	5.3	[100]
0.05 M-LiCl	2.75	52
0.05 M-NH ₄ Cl	3.05	57
0.05 M-NaCl	2.8	53
0.05 M-KCl	2.8	53
0.08 M-H ₃ PO ₄ -tris	4.0	75

genation decreased with the increase of concentration of tris-EDTA and tris-borate buffers at pH 8.5. Some of the experimental results in Table 6 show that the addition of tris-hydrochloric acid buffer to the system in phosphoric acid-sodium hydroxide buffer also decreased the rate of NAD-linked dehydrogenation. The degree of inhibition of NAD-linked dehydrogenation increased with the increase in concentration of tris-hydrochloric acid buffer (Table 6), or of sodium chloride (Table 7), whereas the extent of inhibition of NAD-linked dehydrogenation by the same concentrations of alkali-metal chlorides and ammonium chloride was very similar (Table 8). This suggests that it is the anions that are inhibitory. Sodium chloride, thrice recrystallized from water containing EDTA, inhibited NAD-linked dehydrogenation to the same extent as did A.R. grade reagent. This inhibitory effect of sodium chloride was of special interest as in a number of our previous experiments the rate of NAD-linked dehydrogenation was measured in tris-hydrochloric acid buffer, which, of necessity, contained chloride. Table 7 shows that the degree of inhibition of the rate of NAD-linked dehydrogenation by sodium chloride is independent of the concentration of phosphoric acid-tris buffer in the range 0.05–0.18 M. The K_m for androsterone determined in 0.1 M-phosphoric acid-tris buffer, pH 8.0, is the same in the presence of 0.05 M-sodium chloride as in its absence. The effect of varying NAD concentration on the initial rate of NAD-linked dehydrogenation in the presence and absence of sodium chloride shows that chloride increases the K_m for NAD (Fig. 4). It therefore appears that inhibition of NAD-linked dehydrogenation by chloride may be due to competition of chloride with NAD for the enzyme.

Interaction of nucleotides in the dehydrogenase reaction, and the effect of chloride. Fig. 5 shows that, when NAD and NADP are present together at equimolar concentrations, only NADP-linked dehydrogenation of androsterone proceeds; in these

Fig. 4. Effect of NaCl on NAD-linked dehydrogenation of androsterone at varied concentrations of NAD. s =NAD concentration (mM); v =rate/min. (arbitrary units). The reaction system was NAD, bovine serum albumin (1%), androsterone (0.17 mM) and enzyme (0.25 μ g./0.27 ml.) in 0.1 M-H₃PO₄-tris buffer, pH 7.8. O, $1/v$ in the absence of chloride; ●, $1/v$ in the presence of 0.05 M-NaCl.

Fig. 5. Interaction of nucleotides in dehydrogenase reactions. Reactions were carried out in 0.1 M-H₃PO₄-tris buffer, pH 8.1, in the presence of bovine serum albumin (1%), at androsterone concentration of 0.34 mM and 0.75 μ g. of enzyme/0.27 ml. □, Reaction system contained 1.0 mM-NAD; ○, reaction system contained 1.0 mM-NADP; ●, reaction system contained 1.0 mM-NAD and 1.0 mM-NADP. Acetaldehyde (0.01 M) and yeast alcohol dehydrogenase were added to all cuvettes at the time shown by the arrow. Formation of NADPH₂ was confirmed by the use of GSSG and glutathione reductase.

circumstances the rate of NADP-linked dehydrogenation is not influenced by the presence of NAD. The effect of low concentrations of NADPH₂ on NAD-linked dehydrogenation of androsterone, in experimental conditions where NADPH₂ concentration was maintained by glucose 6-phosphate

Fig. 6. Effect of NaCl on nucleotide interaction in the dehydrogenase reaction. The reaction system, in 0.27 ml. final volume, contained: H_3PO_4 -tris buffer, pH 8.0 (0.1M), serum albumin (1%), androsterone (0.17mM), NAD (2.0 mM), glucose 6-phosphate (5.5 mM), dialysed glucose 6-phosphate dehydrogenase (2.5 $\mu\text{g.}$) and enzyme (0.25 $\mu\text{g.}$). O, In the absence of chloride; ●, in the presence of 0.1M-NaCl.

dehydrogenase and glucose 6-phosphate, is shown in Fig. 6. The rate of NAD-linked dehydrogenation was depressed to half the control value by 3.7 μM -NADPH₂ in the presence of 2.0mM-NAD. In the presence of 1.0mM-NAD, 1.6 μM -NADPH₂ was sufficient to produce the same effect. It therefore appears that NADPH₂ may compete with NAD. The inhibitor constant, K_i , for NADPH₂ in 0.1M-phosphoric acid-tris buffer, pH 8.0, calculated from K_m for NAD, is approx. 1.5 μM . Although 0.1M-sodium chloride inhibits the rate of NAD-linked dehydrogenation in the absence of NADPH₂, in the presence of NADPH₂ chloride protects the enzyme against NADPH₂ inhibition (Fig. 6).

Effect of serum albumin on enzyme activities. The purified rat-liver enzyme was unstable at high dilutions and also susceptible to oxidation in air. In some experiments the enzyme was stabilized with 1% bovine serum albumin. Eik-Nes, Schellman, Lumry & Samuels (1954) found that, in a solution containing serum albumin, reversible combination between steroids and serum albumin occurred and that solubility of steroids was greatly enhanced by the presence of albumin. We have observed that serum albumin affected the availability of androsterone for the rat-liver enzyme, increasing the apparent K_m values with respect to androsterone. The K_m values with respect to NAD and NADP were not affected by serum albumin, provided that conditions of saturation of the enzyme by androsterone were satisfied. The availability of

androsterone for 3 α -hydroxy steroid dehydrogenase from *Pseudomonas testosteroni*, unlike that for the rat-liver enzyme, was not affected by serum albumin.

DISCUSSION

Talalay & Williams-Ashman (1958) and Jarabak *et al.* (1962) consider that a single protein from human placenta (and by inference from rat liver) catalyses NAD-linked hydroxy steroid dehydrogenation, NADP-linked hydroxy steroid dehydrogenation and steroid-dependent transhydrogenation. This view is not shared by Hagerman & Vilee (1959). In the present work, no separation of NAD-linked from NADP-linked 3 α -hydroxy steroid-dehydrogenase activity or from steroid-dependent NAD(P)-transhydrogenase activity was achieved, and it seems probable that a single protein is responsible for the three enzymic activities. Jarabak *et al.* (1962) and Koide (1964) accepted the absence of variability of the ratio of NAD-linked to NADP-linked dehydrogenase activity to steroid-dependent NAD(P)-transhydrogenase activity as evidence for a single enzyme. In our system, considerable alteration of the activity ratio could be produced by several means. Thus, by the addition of low concentrations of NADP, NAD-linked dehydrogenation could be completely abolished, leaving the other two activities practically unaffected. Numerous differences between NAD-linked and NADP-linked dehydrogenation, such as pH-activity relationships, K_m , equilibrium constants and susceptibility to inhibitors, have been observed. It is, however, not necessary to postulate the presence of more than one enzyme to explain these differences. One catalytic centre would probably behave differently with respect to substrates of different structure and charge. Talalay & Marcus (1956), using NAD-specific β -hydroxy steroid dehydrogenase of *Pseudomonas testosteroni* with NAD and various steroids as substrates, showed that K_m for NAD varied with the steroid used. It is likely that K_m for steroid may also vary with different nucleotides. Differences in reaction rates and various constants determined during this work with NAD and NADP cannot therefore serve as means of establishing the individuality of enzymes.

Opposing effects of salts on NAD-linked and NADP-linked dehydrogenation of androsterone appear to present a problem that is more difficult to explain on the basis of one catalytic centre. Phosphate activates NAD-linked dehydrogenation and inhibits NADP-linked dehydrogenation of androsterone. The structural difference between NAD and NADP would probably not alone account for these opposing effects. Salt inhibition is possibly brought about as a result of competition of salts with nucleotides for the nucleotide-binding site(s)

of the enzyme, and this appears to be the way in which inhibition of NADP-linked dehydrogenation by phosphate and inhibition of NAD-linked dehydrogenation by chloride is effected. The mechanism of phosphate activation of NAD-linked dehydrogenation is not clear. It seems likely that phosphate increases in some ways the affinity of the enzyme for NAD, but experimental evidence is lacking at present. If phosphate increases the affinity of the enzyme for NAD and decreases the affinity of the enzyme for NADP, postulation of two catalytic sites, one specific for NAD and another for NADP, would probably be necessary to explain these effects.

Pietruszko (1965) showed that anion activation of 3α -hydroxy steroid-dependent transhydrogenation catalysed by rat-liver enzyme may have been due to competition with NADPH₂ for the enzyme and consequent removal of the inhibitory effect of NADPH₂ on the enzyme. The possibility that this mechanism might operate in the activation of NAD-linked dehydrogenation of androsterone was suggested to us by the findings of Dalziel & Dickinson (1964), who showed that commercial preparations of NADP contained about 0.1% of NAD. The presence of a small amount of NAD in NADP would not affect our results because NAD has a much lower affinity for the rat-liver enzyme than NADP. If, however, commercial NAD contained approx. 0.1% of NADP (no information about this is at present available), salts might activate NAD-linked dehydrogenation by abolishing the inhibition by NADP or NADPH₂.

Protection of the enzyme by NADP against inhibition by thiol reagents suggests that the NADP-binding site includes thiol groups. On this hypothesis inhibition of NAD-linked and NADP-linked

dehydrogenation by GSSG and by 'old' mercapto-ethanol solutions could be due to the formation of compounds with the enzyme by exchange with the hydrogen of the thiol groups (Frater & Hird, 1963).

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