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PARTIAL PURIFICATION OF A NADPH-DEPENDENT 21-HYDROXYSTEROID DEHYDROGENASE FROM HUMAN PLACENTA

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SUMMARY

A NADPH-dependent 21-hydroxysteroid dehydrogenase was purified 70- to 117-fold from term human placenta. Properties of the enzyme were studied using 21-dehydrocortisol synthesized by an improved procedure. Final specific activity ranged from 0.026 to 0.048 μmoles NADPH oxidized per min per mg of protein. Optimum pH was at 6.7 in sodium phosphate and 6.3 in imidazole buffers. K_m values for several 21-dehydrosteroids were approx. $1 \cdot 10^{-3}$ M. In addition, methylglyoxal and phenylglyoxal, but not glyoxal, were reduced. K_m value for NADPH was $1.2 \cdot 10^{-5}$ M. Excess NADPH inhibited 21-dehydrocortisol reduction. The inhibition constant, K_{iB}, was $1.3 \cdot 10^{-4}$ M. NAD^+ and $NADP^+$ also inhibited the enzyme. Androgens, estrogens and progestagens were inactive as substrates, and did not inhibit reduction of 21-dehydrocortisol. The 21-hydroxysteroids were neither reduced nor oxidized by the enzyme system, and were weak competitive inhibitors of the reduction of 21-dehydrocortisol. Sulfhydryl groups on the enzyme appeared to be necessary for activity. Molecular weight was estimated at 33 000 to 35 000 by gel filtration. Oxidation of 21-hydroxysteroids by the dehydrogenase could not be demonstrated. It is suggested that 21-hydroxysteroid dehydrogenase functions to control the level of 21-dehydrosteroids in the placenta formed by other pathways.

INTRODUCTION

Dehydrogenases which catalyze the reduction of 21-dehydrocorticosteroids* to 21-hydroxycorticosteroids according to the equation:

$$\text{21-Dehydrocorticosteroids} + NADH + H^+ \rightarrow \text{21-hydroxycorticosteroids} + NAD^+$$

have been found in sheep liver[1,2], bovine adrenal[3], and in several rat tissues[4]. The

* The following trivial terms are used: 11-deoxycortisol, 17α,21-dihydroxy-Δ^4-pregnene-3,20-dione; androstenedione, Δ^4-androstene-3,17-dione; isoandrosterone, 3α-hydroxy-5β-androstane-17-one; dehydroepiandrosterone, 3β-hydroxy-Δ^5-androstene-17-one; 21-dehydrocortisol, 11β,17α-dihydroxy-3,20-dioxo-Δ^4-pregnene-21-al. Other 21-dehydrosteroids are named in a corresponding way.

sheep liver contained, in addition to the NADH-requiring enzymes, some NADPH-dependent activity. This latter minor enzyme could be separated from the NADH-dependent ones only with great difficulty and with serious loss in activity.

Human placenta, on the other hand, contained predominantly NADPH-dependent 21-hydroxysteroid dehydrogenase activity with little or no NADH-dependent enzyme[5]. A readily available source of NADPH-dependent dehydrogenase is therefore established. This paper describes the partial purification and characterization of the enzyme from human placenta.

MATERIALS AND METHODS

Fresh term human placentas were obtained from the Department of Obstetrics of the Mount Sinai Hospital, New York. "Enzyme grade" $(NH_4)_2SO_4$ was bought from Mann Research Laboratories. All solutions were prepared in water purified in a Corning water-distillation apparatus, model AG-2 (Corning Glass Works, Corning, New York). Pyridine nucleotides were obtained from P-L Biochemicals, Milwaukee, Wisc. All were reported to be more than 95% in the β form. Corticosteroids were purchased from Steraloids, New York. Various 21-dehydrosteroids were synthesized in this laboratory by a modification of a method described by LEWBART AND MATTOX[6] as follows:

To 2 g of cortisol dissolved in 250 ml methanol in a 1-liter round-bottom flask was added a solution of 500 mg of cupric acetate in 250 ml methanol. The mixture was aerated with bubbling for 2 h. 1 gram of Na_2EDTA suspended in 100 ml of 1% sodium carbonate and adjusted to pH 9 was poured into the steroid solution. Methanol was removed *in vacuo* at a temperature below 45°, and the concentrated solution was diluted to 1 l with water. Steroid was extracted into ethyl acetate. The organic layer was washed with 2% sodium bicarbonate and with water, then evaporated to dryness *in vacuo*. The residue was dissolved in a minimum of acetone and poured into 500 ml of 0.01 M sodium phosphate (pH 7.5). Needles of steroid crystallized overnight at 4°. Yield ranged from 1.4 to 1.7 g. The compound had a m.p. of 175–180° (decomp); 2-quinoxaline derivative melted at 265°; Sulfuric acid chromogen: maxima at 470 (shoulder), 400, 325 and 285 mμ; minima at 355 and 237 mμ; light absorption max. in ethanol, 240 mμ (ε_M 15 200) (Found: C, 66.8; H, 7.99. C_{21} $H_{28}O_5$ H_2O requires C, 66.7; H, 7.99). Purity of the steroids was confirmed by paper chromatography[7].

Kinetic studies were performed with a Gilford multiple-sample absorbance recorder, model 2000. The temperature of the cuvette carrier was thermostatically controlled at 28°. Hydrogen ion concentration was measured with a Beckman Expandomatic pH meter. Sephadex G-100 (140–400 mesh beads, water regain 10 g/g) and Sephadex G-200 (140–400 mesh beads, water regain 20 g/g) were purchased from Pharmacia, Uppsala, Sweden. The gel suspensions were permitted to swell for about one week in 0.02 M sodium phosphate buffer (pH 7.35) at 4°. The gels were than washed free of "fines" and transferred to columns (Pharmacia). Subsequent treatments are described under the appropriate experiments.

Enzyme activity was measured in a standard assay system containing 1.1 μmoles of 21-dehydrosteroid in 0.1 ml 50% aq. propylene glycol, enzyme, and 60 μmoles of imidazole buffer (pH 6.5) in a volume of 0.8 ml. The reaction was initiated by addition of 1.05 μmoles of NADPH in 0.2 ml of buffer. Later, it was found that such

high levels of pyridine nucleotide were inhibitory, and in subsequent determinations, 0.39 μmole of pyridine nucleotide was used. Oxidation of NADPH was measured spectrophotometrically at 340 mμ and 28° in quartz cuvettes with a 10-mm light path. Controls contained 0.1 ml of 50% aq. propylene glycol in place of the 21-dehydrosteroid.

RESULTS

Enzyme purification

Human placentae were packed in ice immediately after delivery and brought to the laboratory where they were washed clean of blood clots. The umbilical cord was discarded and the rest of the organ was homogenized in a Waring Blendor for 2 min with 4 vol. of acetone at −20°. Acetone was removed on a Buchner funnel with suction, and the packed cake was homogenized in a second portion of chilled acetone. Acetone was removed by suction on a Buchner funnel, the dehydrated tissue was crumbled and residual acetone was evaporated in the air at room temperature overnight. Twenty grams of acetone powder were extracted into 200 ml of 0.1 M sodium phosphate (pH 7.2) with gentle stirring at room temperature for 60 min. The suspension was cooled to 4° (at which temperature all subsequent steps were performed) and centrifuged at 12 000 × *g* for 15 min. Solid $(NH_4)_2SO_4$ was added to the supernatant solution to 35% saturation (1.37 M). 15 min later, the precipitate was centrifuged down at 12 000 × *g* and discarded. To the supernatant fluid was added solid $(NH_4)_2SO_4$ to a final concentration of 2.0 M (50% of saturation). The precipitate was collected by centrifugation and redissolved in a minimum volume of 0.05 M sodium phosphate (pH 7.2). Gel filtration was performed on a Sephadex G-200 column having a bed volume of 280 ml and a void volume of 80 ml with 0.05 M sodium phosphate (pH 7.2) as eluant. 5-ml fractions were collected and those having the highest specific activities were combined. Enzyme activity was concentrated by adding solid $(NH_4)_2SO_4$ to 2.8 M final concentration and redissolving the resulting precipitate in a smaller volume of buffer. The solution was desalted by passing through a Sephadex G-25 column, eluting with 0.05 M sodium phosphate (pH 7.2). A solution of $BaCl_2$ was used to detect the emergence of $(NH_4)_2SO_4$. Alumina C_γ was added to the $(NH_4)_2SO_4$-free enzyme solution at a level of solids equal to that of the protein and gently stirred for 15 min. The solid matter was centrifuged down leaving

TABLE I

FRACTIONATION OF HUMAN PLACENTAL NADP-21-HYDROXYSTEROID DEHYDROGENASE

Treatment	*Total vol. (ml)*	*Total protein (mg)*	*Total activity (μmoles/min)*	*Specific activity (μmole/min per mg protein) × 10³*
Acetone powder extract	134	2600	0.97	0.37
35%–50% $(NH_4)_2SO_4$ precipitate	12	758	2.46	3.2
Sephadex G-200 effluent	5.0	63	0.70	11
Alumina C_γ supernatant	5.0	23	0.60	26

the enzyme activity in the supernatant fluid. A purification is summarized in Table I. Placentae varied considerably in activity. In one instance no activity was detected in the crude acetone powder extract, and no attempts were made to purify enzyme from this placenta. No NADH-dependent 21-hydroxysteroid dehydrogenase was detected in crude extracts of any placental acetone powders examined. Passage of the crude extracts through a Sephadex G-100 column revealed the presence of a small amount of NADH-dependent activity in the early effluent fluid, accompanied by a similar amount of an NADPH enzyme. Activity of these enzymes was apparently suppressed in the unfractionated extract. The major NADPH-dependent activity followed soon after. A profile is reproduced in Fig. 1. It is the latter enzyme which is described in this paper. The minor NADH and NADPH enzymes were readily re-

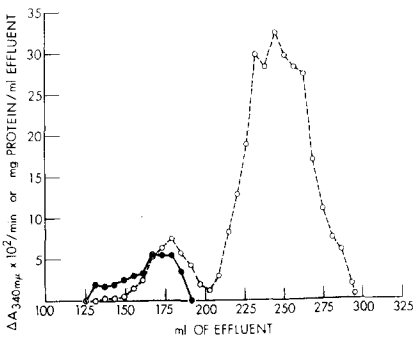


Fig. 1. Fractionation of extracts of acetone powder of human placenta on Sephadex G-100. 10 ml of extract were passed through the column using 0.05 M sodium phosphate (pH 7.2) as eluant. The column was 81.6 cm high and 3.5 cm diameter. Void volume was 125 ml. 5-ml fractions were collected. The assay system consisted of 1.1 μmoles of 21-dehydrocortisol in 0.1 ml 50% aq. propylene glycol, 0.1 ml of column effluent, 60 μmoles of imidazole buffer (pH 6.5) and 0.39 μmoles of NADPH in a final volume of 1.0 ml. The ordinate indicates initial rate of change in absorbance at 340 mμ. ○— — ·○, NADPH-dependent activity; ●—●, NADH- dependent activity; shaded area, protein.

moved in the $(NH_4)_2SO_4$ fractionation step. In general, increase in specific activity ranged from 70- to 117-fold, and the final specific activity varied from 0.026 to 0.048 μmole NADPH oxidized per min per mg of protein. Since placenta is a highly vascular organ, the possibility that the enzyme was derived from blood was explored. Freshly drawn human blood and clotted blood from the placentae had no activity.

Enzyme stability

Although the dehydrogenase was stable at 3° in 0.05 M sodium phosphate (pH 7.2), it was completely inactivated within 30 min at the same temperature in 0.005 M buffer. All activity was lost when the enzyme solution was dialyzed against distilled water for 2 h or when it was passed through a Sephadex G-25 column previously washed with water, a procedure which took less than 10 min. Addition of sodium phosphate, NaCl or $(NH_4)_2SO_4$ to the turbid solutions caused them to clear but did not restore potency. Table II shows that the purified enzyme was fairly stable at pH 10 at 3°, losing 40% of its activity in 20 h, but was 90% inactivated in 15 min at pH 4. At pH 7.1 no activity was lost in 20 h. Fig. 2 shows the effects of temperature on the stability of the enzyme. Activity was maintained without change at 37° for

TABLE II

EFFECT OF pH ON STABILITY OF PLACENTAL 21-HYDROXYSTEROID DEHYDROGENASE

Time interval (h)	*% of initial rate*		
	pH 4.0	*pH 7.1*	*pH 10.0*
0	100	100	100
0.25	13	100	84
1.0	8.4	100	92
4.0	1.3	100	71
20.0	0.0	100	58

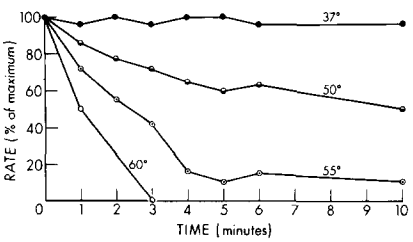
at least 10 min. When the temperature was raised to 50° and higher, the decrease in activity accelerated with increased incubation temperature. No active enzyme was left after 3 min of heating at 60°.

pH optimum

The pH optimum varied with the nature of the buffer. Fig. 3 shows that this value was 6.7 in phosphate and 6.3 in imidazole. At their respective pH optima, activity in imidazole was usually, though not invariably, higher than in phosphate. Despite this, the positions of the pH optima remained constant for all placentae examined.

Kinetics of steroids and related compounds

In Fig. 4 is presented a double reciprocal plot of cortisol concentration against initial velocity corresponding to the equation:



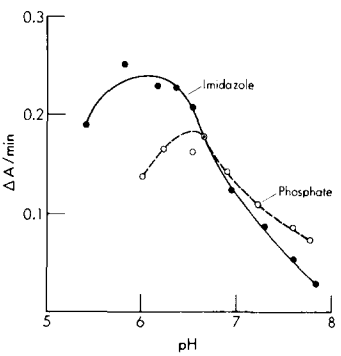


Fig. 2. Heat inactivation of placental 21-hydroxysteroid dehydrogenase. Purified enzyme giving an absorbance change of 0.234 absorbance units per mg protein per min was diluted to contain 5.3 mg/ml. Enzyme (2.0 ml) was brought to the indicated temperature within 10 sec by immersion in a water bath. Aliquots were removed at intervals, chilled in an ice bath, and assayed as described in MATERIALS AND METHODS. The reaction was started by the addition of 0.39 μmoles of NADPH in 0.1 ml of water. Control cuvettes contained 50% propylene glycol in place of 21-dehydrocortisol.

Fig. 3. pH–activity curves of placental 21-hydroxysteroid dehydrogenase. The system contained 1.1 μmoles of 21-dehydrocortisol in 0.1 ml 50% aq. propylene glycol, enzyme (0.23 absorbance units per mg protein per min in 0.06 M imidazole), 60 μmoles of imidazole or sodium phosphate buffer in 0.9 ml volume. Reaction was initiated by addition of 0.39 μmoles NADPH in 0.1 ml water. Initial velocities were measured as decrease in absorbance at 340 mμ per min.

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A][B]}}$$

where v and V are initial measured and maximum velocities, respectively; A and B refer to 21-dehydrocortisol and NADPH. The resulting linear plots intersected on the (21-dehydrocortisol)$^{-1}$ axis at a value of $K_{AB}/K_B = 1.0 \cdot 10^{-3}$. This value, representing the dissociation constant of the steroid–enzyme complex is also equivalent to the Michaelis constant, K_m (ref. 8).

Table III summarizes the Michaelis constants for several 21-dehydrocortico-

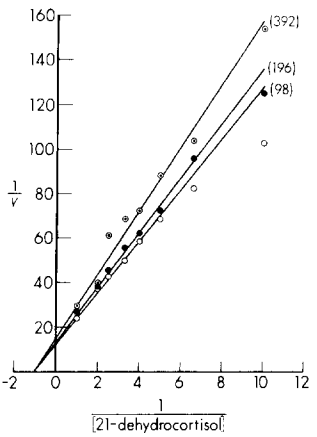


Fig. 4. Kinetics of 21-dehydrocortisol reduction. Buffer was 0.1 M imidazole (pH 6.5). Ordinate, (μmoles of 21-dehydrocortisol per ml per min)$^{-1}$. Numbers in parentheses indicate μmolar concentration of NADPH.

TABLE III

K_m VALUES OF KETOALDEHYDES

Substrate	*Number of enzyme preparations*	*Concn. range (M) × 10⁴*	*K_m (M) × 10³*	*S.E.*	*Rel. v_{max} (F21A = 1.00)*
21-Dehydrocortisol	4	1.10–5.50	1.02	0.25	1.00
21-Dehydrocortisone	3	1.10–5.50	1.36	0.51	1.13
21-Dehydro-11-deoxycortisol	4	1.15–4.60	0.74	0.37	0.95
21-Dehydrocorticosterone	4	0.48–4.60	0.91	0.48	1.03
21-Dehydro-11-deoxycorticosterone	3	0.38–3.30	0.55	0.10	0.56
Methylglyoxal	3	1.0 –4.8	2.30	1.10	2.16
Phenylglyoxal	3	0.64–5.81	0.75	0.07	3.70
Glyoxal	3	1.0 –4.00	—	—	0.00
Hydroxypyruvaldehyde	3	1.0 –4.00	—	—	0.00
Benzaldehyde	1		—	—	0.00
Pyruvic acid	1		—	—	0.00

* Determined at 0.196 mM NADPH.

steroids. The values fall within a narrow range, although the value for 21-dehydro-11-deoxycorticosterone is somewhat low. Therefore, the presence of substituents on the steroid nucleus had little influence on the action of the enzyme. The observation is further supported by the fact, also shown in Table III, that the maximum velocities of reduction were similar. Methylglyoxal, a simple keto-aldehyde homologue of corticosterone devoid of the steroid nucleus, and phenylglyoxal were reduced at faster maximum rates than any steroid, though their K_m values were not appreciably different from those of the steroids. Glyoxal and hydroxypyruvaldehyde were not reduced by the enzyme even at final substrate concentrations of $4 \cdot 10^{-2}$ M. These data suggest that though the concentration of substrate of which half maximal velocity is attained was about the same for active substrates, the nature of the substituent group may play some role in determining the rate of reduction at any given substrate concentration.

Unlike phenylglyoxal, benzaldehyde was not reduced by the enzyme. Since pyruvate was not reduced, lactate dehydrogenase was absent, and it was therefore unlikely that the reduction of glyoxals was due to the action of this enzyme.

The following steroids were not substrates for the dehydrogenase: cortisol, cortisone, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone, progesterone, testosterone, androstenedione, isoandrosterone, dehydroepiandrosterone, estriol, estrone and aldosterone.

The effect of NADPH concentrations on the rate of 21-dehydrocortisol reduction is presented in Fig. 5. The inset shows that inhibition by excess pyridine

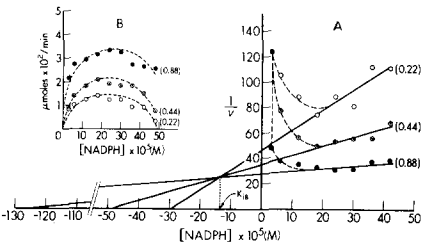


Fig. 5. Effect of NADPH concentration on human placental 21-hydroxysteroid dehydrogenase. Buffer was 0.1 M imidazole (pH 6.5). Ordinate of main graph (A) represents initial reduction rate in (μmoles per ml per min)$^{-1}$; abscissa represents concentration of NADPH. Numbers in parentheses represent μmolar concentration of steroid. The inset (B) shows the NADPH–velocity relationships from which chart A was derived.

nucleotide occurred at relatively low levels of nucleotide. An estimate of the kinetic and inhibitory constants was made from the relationship for competitive inhibition,

$$V = v + v\frac{K_B}{B} + v\frac{B}{K_{iB}}$$

where K_{iB} is the dissociation constant of the complex of NADPH as inhibitor and enzyme, and the other symbols are as above. The values for V and K_B were obtained from the equation[9]

$$v_{max} = \frac{V}{1 + 2\sqrt{K_B/K_{iB}}}$$

TABLE IV

KINETIC CONSTANTS FOR NADPH

21-Dehydrocortisol concn. $(M) \times 10^4$	v_{max}	V	K_{iB} $(M) \times 10^4$	K_B $(M) \times 10^5$	K_B/K_{iB}
2.2	0.08	0.135	1.3	1.53	
4.4	0.116	0.179	1.3	0.96	
8.8	0.192	0.228	1.3	1.13	
				Av. $1.21 \cdot 10^{-5}$	0.093

where v_{max} is the maximum velocity attained. A graph was made of $1/v$ *vs.* NADPH concentration at three levels of 21-dehydrocortisol (Fig. 5) yielding an estimate of K_{iB} from the intersection in the second quadrant of the linear portions of the curves at high NADPH, and of V from their intersection on the $1/V$ axis. These results are summarized in Table IV. The ratio of the Michaelis constant of $1.2 \cdot 10^{-5}$ M and the inhibitor constant of $1.3 \cdot 10^{-4}$ M was 0.093, a value inconsistent with noncompetitive inhibition[9]. In the same concentration range, NADH was totally ineffective in reducing 21-dehydrocortisol to cortisol.

TABLE V

INHIBITION OF PLACENTAL 21-HYDROXYSTEROID DEHYDROGENASE BY NAD^+ AND $NADP^+$

NAD+ (μmoles)	*Rate* $(\Delta A_{340\ m\mu}/min)$	*Inhibition (%)*	*NADP+ (μmoles)*	*Rate* $(\Delta A_{340\ m\mu}/min)$	*Inhibition (%)*
1.08	0.606	19	1.00	0.380	49
0.54	0.682	9	0.50	0.437	42
0.27	0.704	6	0.25	0.630	16
0.00	0.750	0	0.00	0.750	0

Table V shows the effects on enzyme activity of adding increasing amounts of $NADP^+$ or NAD^+ to the NADPH-dependent reaction. Both oxidized pyridine nucleotides inhibited the reaction. $NADP^+$ appeared to be considerably more effective than NAD^+. The mechanism of the inhibition by pyridine nucleotide could not be investigated, because such a study could only be performed at low concentrations of NADPH in order to avoid inhibition by excess co-factor. Under these conditions, it was not possible to measure low reaction rates with sufficient confidence to unequivocally distinguish between competitive and noncompetitive inhibition.

Inhibitors

The reduction of 21-dehydrocortisol in the range $1.1 \cdot 10^{-4}$ to $5.5 \cdot 10^{-4}$ was not inhibited by the following steroids at 10^{-5} M: progesterone, androsterone, testosterone, androstenedione, estriol. Estrone at 10^{-6} M was also not inhibitory. Cortisol, deoxycorticosterone and corticosterone were extremely weak competitive inhibitors, as shown in Fig. 6. Inhibitor constants ($K_i \times 10^3$ M) were 2.10 ± 0.10, 1.19 ± 0.40 and 0.87 ± 0.25 for cortisol, corticosterone and 11-deoxycorticosterone, respectively.

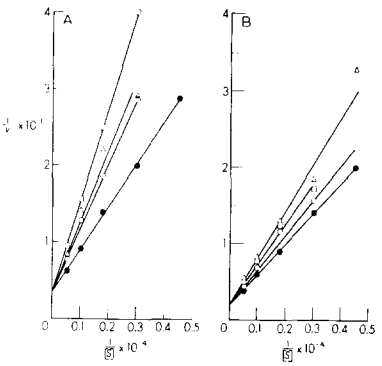


Fig. 6. Inhibition of placental 21-hydroxysteroid dehydrogenase by steroids. Ordinate, reciprocal of absorbance change/min; abscissa, reciprocal of molar 21-dehydrocortisol concentration. The system contained 0.39 μmole of NADPH, 120 μg of enzyme (specific activity, 0.043 μmole/min per mg protein), 60 μmoles of imidazole buffer (pH 6.5), 21-dehydrocortisol, and inhibitor as indicated. The reaction was started after 3 min of prior incubation of other components at 28° by addition of 21-dehydrocortisol in 0.1 ml of 50% aq. propylene glycol. Controls received propylene glycol in place of steroid. Final volume was 1.0 ml. A. ●—●, no addition; ○—○, 1.10 mM cortisol; △—△, 1.15 mM corticosterone; □—□, 1.21 mM deoxycorticosterone. B. ●—●, no addition; ○—○, 0.27 mM cortisol; △—△, 0.288 mM corticosterone; □—□, 0.302 mM deoxycorticosterone.

The effects of a number of organic inhibitors and metal ions on enzyme activity are summarized in Table VI. Several reagents that react with sulfhydryl groups, including *o*-iodosobenzoate, *p*-mercuribenzoate, Ag^+ and Hg^{2+} were effective inhibitors of the dehydrogenase. In contrast, iodoacetate and *N*-ethylmaleimide were

TABLE VI

INHIBITION OF PLACENTAL 21-HYDROXYSTEROID DEHYDROGENASE ACTIVITY

Inhibitor	*Concn.* $(M) \times 10^6$	*Inhibition* (%)
Sodium bisulfite	100	0
Sodium bisulfite	1 000	79
p-Mercuribenzoate	1	38
p-Mercuribenzoate	10	89
p-Mercuribenzoate	100	96
o-Iodosobenzoate	10	92
o-Iodosobenzoate	100	90
Diethyldithiocarbamate	100	0
Iodoacetate	100	0
N-Ethylmaleimide	100	0
Nicotinamide	1 000	0
Ag^+	1	5
Ag^+	10	50
Hg^{2+}	1	82
Hg^{2+}	10	72
Hg^{2+}	100	79
Al^{3+}, Ca^{2+}, Co^{2+}, Fe^{3+}, Pb^{2+}, Li^+, Mn^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}	100	0
Na^+, K^+, Cs^+, Li^+	10 000	0

ineffective. These results indicate that sulfhydryl groups on the enzyme required for activity were readily oxidized or reacted easily to form metal mercaptides, but were alkylated with difficulty if at all. Sodium bisulfite probably inhibited by forming an addition compound with the carbonyl groups of the substrates.

Effect of enzyme concentration

Using 21-dehydrocortisol as substrate, the rate of reduction was proportional to the amount of enzyme present. No deviation from linearity was noted over a wide range of enzyme concentration, as may be seen in Fig. 7.

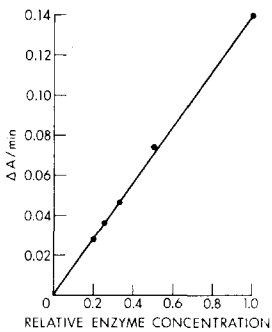


Fig. 7. Relationship between enzyme level and rate of reduction of 21-dehydrocortisol. Initial rate of reaction was measured as decrease in absorbance at 340 mμ in the assay system described under MATERIALS AND METHODS using enzyme (specific activity, 0.026 μmole/min per mg protein) diluted appropriately.

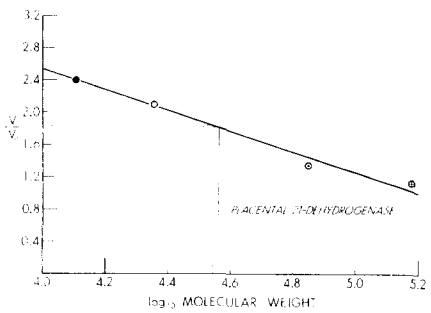


Fig. 8. Estimate of mol. wt. of partially purified placental 21-hydroxysteroid dehydrogenase. Method used was gel filtration through Sephadex G-100. Column was 105 cm high and 1.5 cm in diameter, eluting buffer was 0.1 M sodium phosphate (pH 7.2). Flow rate was 0.4 ml/min, temperature was 4°. Emergence of protein was detected with a recording flow monitor set at 280 mμ. Void volume, V_0, was determined by passing Blue Dextran 2000 (Pharmacia) through the column. Elution volume, V, was the peak of the elution curve for a given protein. Marker proteins were cytochrome *c* (●), trypsin (○), serum albumin (○), alcohol dehydrogenase (⊕). Grey area indicates elution of placental 21-hydroxysteroid dehydrogenase. Total dehydrogenase protein place on column was 13 mg.

Molecular weight

The mol. wt. of the partially purified enzyme was estimated by measuring the rate of filtration through Sephadex G-100 (ref. 10). Standards used were alcohol dehydrogenase, serum albumin, trypsin and cytochrome *c*. The results illustrated in Fig. 8 showed that the activity was eluted at a position relative to the protein standards corresponding to a mol. wt. between 33 000 and 35 000. A preparation of lamb liver NADP-21-hydroxysteroid dehydrogenase, treated in the same way, gave an estimated mol. wt. value in the range of 41 000 to 43 000.

Reversal of reaction

Conditions described previously[1] were set up to demonstrate the oxidation of cortisol to 21-dehydrocortisol with the NADP enzyme. It was not possible to demonstrate reversal of the reduction reaction, nor was it possible to demonstrate the oxidation of corticosterone or 11-deoxycorticosterone to their corresponding 21-dehydrosteroids.

DISCUSSION

This paper describes the partial purification and properties of a NADP-dependent 21-hydroxysteroid dehydrogenase from human placenta. The enzyme differs from the NADP-dependent enzyme of calf liver[2] in several respects. Its mol. wt. of 33 000 to 35 000 is lower than the estimated value of 41 000 to 43 000 for the liver enzyme. It should be emphasized that calculation of these values, determined by the use of gel filtration, is based on the assumption that the molecular shapes of the two proteins are very similar, and are closely similar to those of the proteins used as standards[11]. Since the enzyme is not yet pure enough to obtain precise values for the sedimentation coefficient, a direct test of this assumption is not possible. It has a pH optimum in phosphate buffer of 6.7, contrasted with that of 6.0 for liver. The K_m with respect to 21-dehydrocortisol is about 5 times higher than for the liver NADP enzyme ($1 \cdot 10^{-3}$ M *vs.* $1.8 \cdot 10^{-4}$ M), and this relationship appears to hold for all steroid substrates. In other respects, including heat stability, inhibition by reagents which react with sulfhydryl groups, substrate specificity and K_m for NADPH, the two enzymes appear to be similar.

The specificity of the placental enzyme is somewhat broader than that of the other 21-dehyroxysteroid dehydrogenases, and it might therefore be suggested that it be named ketol dehydrogenase or glyoxal reductase. However, the various enzymes studied in the past acted in common on glyoxals derived from corticosteroids. Of the five enzymes which have been described so far, only two, the NADPH-dependent enzyme from sheep liver and the one described in this paper, reduced other glyoxals. Even with these, only some nonsteroidal ketoaldehydes were substrates. Because the characteristic actions of the various enzymes are on steroids, it appears logical that the original designation of 21-hydroxysteroid dehydrogenase be retained.

How the enzyme influences steroid metabolism in the placenta is obscure, but some possibilities can be eliminated by the following considerations. Enzymes which participate in the metabolism of numerous steroids are found in the feto-placental unit[12], but those which are involved in the oxidative metabolism of corticosteroids seem to be absent from the placenta[13,14] except for 11β-hydroxysteroid dehydrogenase[15–17]. Although corticosteroids are found in the placenta[16,18], it is not clear if human placenta can synthesize corticosteroids. The early clinical evidence that pregnant women have remission of arthritis[19] and that pregnant Addisonian women have normal blood corticosteroid levels[20], and evidence for the synthesis of corticosteroids by the placenta *in vitro* suggested that the human placenta can synthesize corticosteroids[21,22]. Later work, however, indicated that the presence of corticosteroids in placenta is due to accumulation from the blood[15,23] reflecting the ability of corticosteroids to pass through the feto-placental barrier[24], and that the placenta probably did not synthesize corticosteroids in physiologically significant amounts[25].

If the prevailing interpretation that corticosteroids are not synthesized to any appreciable degree in the human placenta is accepted, then it must be concluded that placental 21-hydroxysteroid dehydrogenase is not involved in corticosteroid synthesis. Since the equilibrium constant of the reaction catalyzed by this enzyme is so strongly in the direction of reduction of 21-dehydrocorticoids to 21-hydroxycorticoids[1], it can safely be assumed that the enzyme is also not involved in the oxidation of the corticosteroid side chain. With the elimination of these two possibilities it must be

asked if the dehydrogenase prevents the net oxidation of corticosteroids to 21-dehydrosteroids. In model systems the oxidation of corticosteroids to 21-dehydrocorticosteroids has been shown to occur spontaneously, catalyzed by traces of Cu^{2+} and possibly other metals normally present in aqueous media[26] or coupled to physiological oxidation–reduction systems[27]. If 21-dehydrosteroids, like other ketoaldehydes, are toxic to the cell, then perhaps the role of 21-hydroxysteroid dehydrogenase is to prevent their accumulation and maintain the steroids in the physiologically active 21-hydroxy form. Alternatively, if 21-dehydrosteroids have physiological significance, then 21-hydroxysteroid dehydrogenase may function to control their level in the cell.

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REFERENCES

1 C. MONDER AND A. WHITE, *J. Biol. Chem.*, 238 (1963) 767.
2 C. MONDER AND A. WHITE, *J. Biol. Chem.*, 240 (1965) 71.
3 C. MONDER AND C. S. FURFINE, in J. M. LOWENSTEIN AND R. S. CLAYTON, *Methods in Enzymology*, Academic Press, New York, N.Y., in the press.
4 C. MONDER AND A. WHITE, *Biochim. Biophys. Acta*, 46 (1961) 410.
5 C. MONDER AND F. A. E. MARTINSON, *Excerpta Medica Intern. Congr. Ser.*, 157 (1968) 197.
6 M. L. LEWBART AND V. R. MATTOX, *J. Org. Chem.*, 28 (1963) 2001.
7 C. MONDER, *Biochem. J.*, 90 (1964) 522.
8 C. FRIEDEN, *J. Am. Chem. Soc.*, 79 (1957) 1894.
9 B. H. J. HOFSTEE, *J. Biol. Chem.*, 216 (1955) 235.
10 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
11 G. K. ACKERS, *J. Biol. Chem.*, 242 (1967) 3237.
12 S. SOLOMON, C. E. BIRD, W. LING, M. IWAYAMA AND P. C. M. YOUNG, *Recent Progr. Hormone Res.*, 23 (1967) 297.
13 D. M. HART, *J. Endocrinol.*, 35 (1966) 255.
14 M. M. FERGUSON AND G. A. CHRISTIE, *J. Endocrinol.*, 38 (1967) 291.
15 S. SYBULSKI AND E. H. VENNING, *Can. J. Biochem. Physiol.*, 39 (1961) 203.
16 D. L. BERLINER, E. JONES AND H. A. SALHANICK, *J. Biol. Chem.*, 223 (1956) 1043.
17 P. A. OSINSKI, *Nature*, 187 (1960) 777.
18 R. NEHER AND G. STARK, *Experientia*, 17 (1961) 510.
19 P. S. HENCH, *Proc. Staff Meetings Mayo Clinic*, 13 (1938) 161.
20 J. W. JAILER AND A. I. KNOWLTON, *J. Clin. Invest.*, 29 (1950) 1430.
21 B. LITTLE AND E. ROSSI, *Endocrinology*, 61 (1957) 109.
22 P. TROEN, *J. Clin. Endocrinol. Metab.*, 21 (1961) 1511.
23 H. A. SALHANICK, L. M. NEAL AND J. P. MAHONEY, *J. Clin. Endocrinol.*, 16 (1956) 1120.
24 C. J. MIGEON, H. PRYSTOWSKY, M. M. GRUMBACH AND M. C. BRYON, *J. Clin. Invest.*, 35 (1956) 488.
25 E. E. BAULIEU, H. BRICAIRE AND M. F. JAYLE, *J. Clin. Endocrinol. Metab.*, 16 (1956) 690.
26 C. MONDER, *Endocrinology*, 82 (1968) 318.
27 C. MONDER, *Biochim. Biophys. Acta*, 164 (1968) 369.