J. Steroid Biochem. Molec. Biol. Vol. 39, No. 5A, pp. 735–740, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

REGULATION OF HUMAN PLACENTAL 17β-HYDROXYSTEROID OXIDOREDUCTASE: MECHANISM OF STIMULATION OF 17β-ESTRADIOL FORMATION FROM ESTRONE BY 5α-DIHYDROTESTOSTERONE IN HOMOGENATES AND VILLI *IN VITRO*

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(Received 18 March 1991)

Summary—The mechanism of stimulation of 17β-estradiol (E2) formation from estrone (E1) by 5α-dihydrotestosterone (5α-DHT) in placental villi was investigated by examining; (1) if dehydroepiandrosterone (DHA) was stimulatory, (2) if NAD(P)H-generating, non-steroidal substrates stimulated E2 formation, (3) the subcellular localization of the effect, (4) if NAD(P) or NAD(P)H was required and (5) rates of 5α-DHT oxidation by villi and microsomes. Although 5α-DHT and DHA both inhibited the E2 to E1 reaction in villi and microsomes, only 5α-DHT stimulated the conversion of E1 to E2. Glucose and lactate were slightly stimulatory when compared with 5α-DHT. Stimulation of E2 formation was observed with microsomes but not with cytosol, and NAD or NADP was required.

The results indicate that neither inhibition of the back reaction, E2 to E1, nor NADH or NADPH formation via the 3β-hydroxysteroid dehydrogenase/5-ene-3-ketosteroid isomerase reaction can account for the stimulation. It is proposed that the mechanism of stimulation involves one or more forms of membrane-bound 17β-hydroxysteroid oxidoreductase with NADH or NADPH formed as a product of 5α-DHT oxidation being used as the cofactor for E1 reduction. This may involve a direct transfer of reduced pyridine nucleotide between enzyme molecules without equilibration with intracellular coenzyme pools.

INTRODUCTION

By the 9th week of pregnancy in the human the placenta is the major source of 17β-estradiol (E2) and estrone (E1), which are formed from dehydroepiandrosterone (DHA) and dehydroepiandrosterone 3-sulfate (DHAS) of both maternal and fetal origin [1]. 17β-Hydroxysteroid oxidoreductase (17-HOR) catalyzes the pyridine nucleotide-dependent interconversion of E2 and E1 as well as testosterone (T) and androstenedione (A), and has been shown to be present in placental tissue by week 4 or 5 of gestation [2, 3]. A formed from DHAS and DHA by the action of steroid 3-sulfatase and 5-ene-3β-hydroxysteroid dehydrogenase/5-ene-3-ketosteroid isomerase (3-HSD/isomerase), is the precursor for E1 and E2 [4–6]. Since E1 is the product of the aromatization of A, the conversion of E1 to E2 by 17-HOR is not only a required step in E2 formation by that pathway, but would appear to be a potential regulatory site, as well.

Although the regulation of placental steroidogenesis has been a topic of long-standing interest [7, 8], our understanding of the control of 17-HOR is limited. Placental 17-HOR does not appear to be influenced at the level of mRNA or protein synthesis by pituitary gonadotropins or hCG. Regulation, instead, may occur at the level of catalysis by mechanisms involving endogenous steroids and pyridine nucleotides [9, 10].

Tseng *et al.* [11], in studies of placental slices, found that E2 and E1 were rapidly interconverted and that T stimulated the conversion of E1 to E2. Ling *et al.* [12, 13] reported that 5α-dihydrotestosterone (5α-DHT) stimulated E2 formation from E1 3-sulfate in explant cultures. In a series of studies in this laboratory in which the substrate and inhibitor specificity of placental 17-HOR were examined, evidence

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consistent with two forms of 17-HOR was obtained [14–16]. During the course of subsequent experiments to clarify the relative roles of these two forms in E2, E1 and T metabolism in placental villi *in vitro*, we confirmed that 5α-DHT stimulated the conversion of E1 to E2 [17]. The specificity of the effect suggested it was limited to steroid substrates for the dehydrogenase reaction catalyzed by 17-HOR.

A variety of mechanisms could account for the stimulation. Since the conversion of E2 to E1 is predominant in villi [11], inhibition of the E2 to E1 reaction could increase net E2 formation from E1. If the levels of reduced cofactor were rate-determining, increases in NADH or NADPH could also be stimulatory. As an approach to clarifying the mechanism of stimulation, this investigation was designed to examine; (1) if DHA, a 3-HSD/isomerase substrate, could stimulate E2 formation, (2) if non-steroidal, but NAD(P)H-generating metabolites were stimulatory, (3) the subcellular localization of the effect, (4) if the effect in subcellular fractions required reduced or oxidized pyridine nucleotides as cofactors and (5) rates of 5α-DHT oxidation by villi and microsomes.

EXPERIMENTAL

Materials

Reagents and supplies were obtained from the following sources: [6,7-^{3}H]E_2 (48.3 Ci/mmol), [6,7-^{3}H]E_1 (60.0 Ci/mmol), [1,2-^{3}H]T (52.5 Ci/mmol), [1,2-^{3}H]5α-DHT (51.6 Ci/mmol) and Liquifluor scintillation fluid, Dupont NEN Research Products (Boston, MA); unlabeled steroids, Steraloids Inc. (Wilton, NH); NAD, NADH, NADP and NADPH, Pharmacia P-L Biochemicals (Milwaukee, WI); Bicine (*N,N*-bis[2-hydroxyethyl]glycine) and Hepes (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), Sigma Chemical Co. (St Louis, MO); silica gel G plates with fluorescent indicator (Eastman Chromagram), Eastman Kodak Co. (Rochester, NY).

Placental tissue

Placentas were obtained as products of normal, term vaginal deliveries. The informed consent of the mother was obtained in each case in accordance with the policies of the Institutional Review Board at St Paul-Ramsey Medical Center. Placentas were placed on ice immediately after the delivery and transported to the laboratory within 4 h or less.

Isolation of villi and subcellular fractions

All procedures for the isolation of villi and the fractionation of placental homogenates were carried out with ice-cold reagents.

Villous tissue fragments (10–20 mg fresh weight) were dissected free of connective tissue and washed extensively in saline until free of blood, as described previously [17].

The procedure for preparing homogenates, microsomes and cytosol has been described in detail [16]. Briefly, 5- to 10-g portions of villous tissue were washed in saline and then homogenized (50 g total) in a Waring blender (3 × 10 s) in 160 ml of buffer containing 250 mmol/l sucrose, 10 mmol/l 2-mercaptoethanol and 100 mmol/l Bicine, pH 9.0. The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was filtered through 3 layers of cheesecloth and centrifuged at 105,000 *g* for 60 min at 4°C. The supernatant (cytosol) was drawn off and saved. The pellet (microsomes) was washed once by repeat centrifugation and suspended in buffer. Microsomes and cytosol were stored at 4°C.

17-HOR activity

To quantitate steroid interconversions in villous tissue, 50 mg of tissue was incubated at 22–23°C in 1.0 ml of phosphate-buffered saline containing 1.0 μmol/l [^{3}H]-labeled substrate and other additives. To quantitate 17-HOR activity of microsomes or cytosol, 0.5 ml buffered reaction mixtures contained labeled steroid substrate, cofactor, other additives and microsomes or cytosol, singly or in combination. Steroid stock solutions were prepared in methanol and added to reaction mixtures such that the final concentration of methanol was fixed at 20 or 40 ml/l. Methanol alone was added to controls. Further details of the composition and conditions for specific experiments are given in the figure legends.

In all cases, the reaction was terminated by the addition of 10 μl of 20 mmol/l hydrochloric acid and 3.0 ml of dichloromethane. After vortexing, reaction mixtures were filtered through Whatman P/S phase-separating filters and the organic phase taken to dryness in a stream of air. Residues were dissolved in 100 μl of dichloromethane containing unlabeled carrier steroids (100 mg/l). Aliquots of 50 μl were spotted on silica gel G sheets containing fluorescent indicator and chromatographed in benzene–acetone (4:1). The sheets were dried

and steroid-containing spots located with u.v. light. The spots were cut out and transferred to vials containing 10 ml of Liquifluor for liquid scintillation counting.

The extent of reaction was quantitated on the basis of cpm recovered in product steroid as percent of total cpm recovered in substrate and product [16].

Data analysis

Each experiment was repeated at least three times. In the majority of cases, individual reactions were run in triplicate. Mean values were compared by analysis of variance in combination with the Newman–Keuls multiple comparison test. Apparent Michaelis constants (K_m) and maximum velocities (V_{max}) were estimated graphically, as previously described [15, 16].

RESULTS

Effects of 5α-DHT and DHA on the conversion of E2 to E1 and E1 to E2 by villous tissue and microsomes

Previous findings with villi [17] indicate that 17-ketosteroid substrates of 17-HOR such as A and 5α-androstane-3,17-dione do not stimulate E2 formation. DHA is of particular interest in this regard because although it is a C_{19}-17-ketosteroid, it is a 3β-hydroxysteroid substrate for 3-HSD/isomerase, and would be expected to generate NAD(P)H in that reaction [18].

When 5α-DHT and DHA were compared at 100 μM, a concentration shown with 5α-DHT to maximally stimulate E2 formation [17], DHA inhibited the conversion of E2 to E1 by villous tissue (Fig. 1). 5α-DHT and DHA inhibited microsomal 17-HOR activity with both E2 and T, as well (Fig. 2), also consistent with earlier experiments. However, when 5α-DHT and DHA were compared as to their effects on the conversion of E1 to E2 by villous tissue, only 5α-DHT was stimulatory (Fig. 3). This suggests

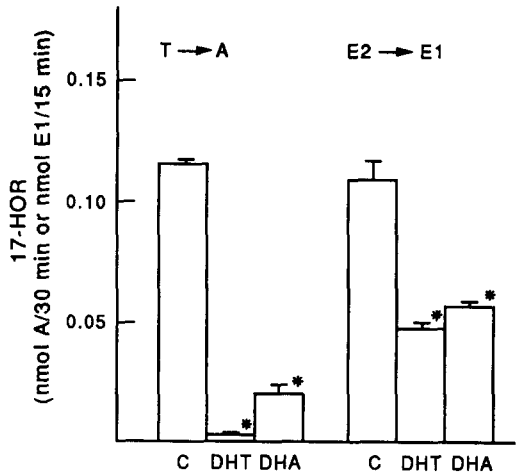

Fig. 2. A comparison of the effects of 5α-DHT and DHA on microsomal 17-HOR activity with T and E2. Reaction mixtures (0.5 ml) containing 1.0 mmol/l NAD, 1.0 μmol/l [^{3}H]T or [^{3}H]E2, 100 μmol/l 5α-DHT or DHA, 82.0 μg of microsomal protein and 80 mmol/l Bicine, pH 9.0, were incubated for 30 (T) or 15 min (E2). Control samples (C) lacked 5α-DHT or DHA. The values are means (±SD) of assays run in triplicate with microsomes from a single term placenta; $^*P \leq 0.01$, compared with controls.

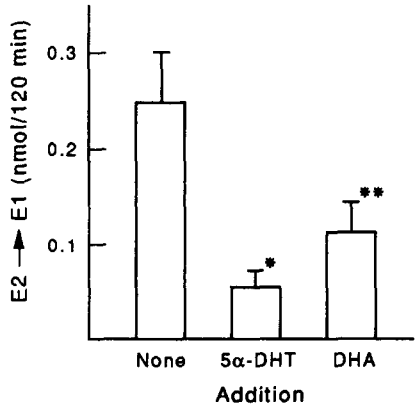

Fig. 1. Effects of 5α-DHT and DHA on the conversion of E2 to E1 by villous tissue. Villous tissue fragments (50 mg each) from a single term placenta were incubated in 1.0 ml of phosphate buffered saline containing 1.0 μmol/l [^{3}H]E2 and 5α-DHT or DHA at 100 μmol/l for 2 h. The activities are mean values (±SD) of assays run in triplicate; $^*P \leq 0.01$, $^{**}P \leq 0.05$, compared with controls.

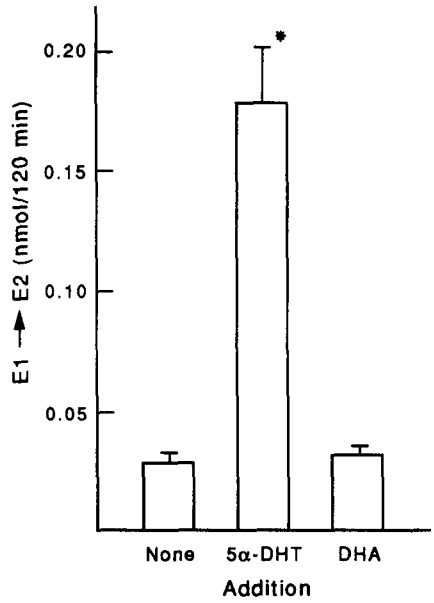

Fig. 3. Effects of 5α-DHT and DHA on the conversion of E1 to E2 by villous tissue. Tissue fragments (50 mg) from a single term placenta were combined with 1.0 ml of phosphate buffered saline containing 1.0 μmol/l [^{3}H]E1 and 5α-DHT or DHA at 100 μmol/l and incubated for 2 h. The values are means (±SD) of three samples; $^*P \leq 0.001$, compared with controls or reaction mixtures containing DHA.

that neither inhibition of back reaction of E2 to E1 nor generation of NADH or NADPH in the 3-HSD/isomerase reaction is the mechanism of stimulation.

Effects of glucose and lactate on the conversion of E1 to E2 by placental villous tissue

Glucose and lactate would also be expected to generate NADH or NADPH as products of their metabolism. As shown in Fig. 4, each had a slight stimulatory effect which did not reach statistical significance, and which was significantly less than the effect of 5α-DHT.

Effect of 5α-DHT on the conversion of E1 to E2 by microsomes and cytosol

To identify the subcellular site of stimulation, the effects of 5α-DHT on the conversion of E1 to E2 in microsomes and cytosol, singly and in combination, was examined (Fig. 5). When 5α-DHT was present, activity with microsomes was markedly stimulated with no effect on activity in the cytosol. DHA was not stimulatory. When microsomes and cytosol were combined, activity in the absence of 5α-DHT was approximately additive. However, no stimulation by 5α-DHT was detected with the combined fractions.

Pyridine nucleotide requirements for 5α-DHT-stimulated conversion of E1 to E2 by microsomes

Previous studies of the steroid specificity of stimulation suggested that the ability of 5α-DHT to generate NADH or NADPH might be the basis for stimulation [17]. When NAD(P) and NAD(P)H were compared, stimulation was seen only in the presence of oxidized pyridine nucleotide. No stimulation by 5α-DHT was seen in the absence of added nucleotide [Fig. 6(A)]. In parallel assays, when either NADH or NADPH was added as cofactor, the rate of conversion of E2 to E1 was not stimulated by 5α-DHT and was greater than the 5α-DHT-stimulated rate in the presence of oxidized pyridine nucleotide [Fig. 6(B)].

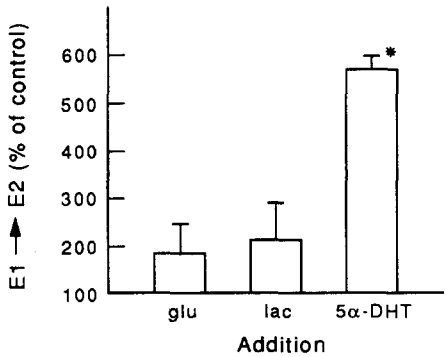

Fig. 4. Effects of glucose (glu), lactate (lac) and 5α-DHT on the conversion of E1 to E2 by placental villi. Tissue samples (50 mg) in 1.0 ml of phosphate buffered saline containing 1.0 μmol/l [^{3}H]E2 and supplemented with 5.0 mmol/l glucose, 3.2 mmol/l sodium lactate or 100 μmol/l 5α-DHT along with unsupplemented controls were incubated for 60 min. The results, expressed as percent of control, are mean values (±SD) from three separate experiments in which assays were run in duplicate. E2 formation in control samples was 1.3 ± 0.8 nmol/30 min. *$P \leq 0.01$, different from controls.

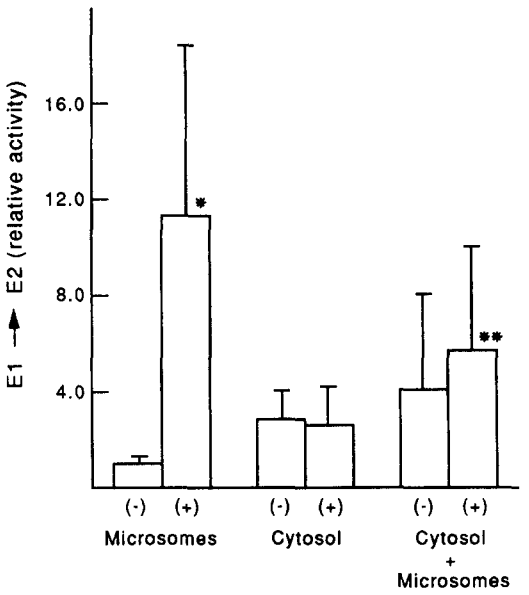

Fig. 5. Effect of 5α-DHT on the conversion of E1 to E2 by microsomes and cytosol, singly and in combination. Reaction mixtures (0.5 ml) containing 60 mmol/l Hepes, pH 7.2, 1.0 mmol/l NADP, 1.0 μmol/l [^{3}H]E1, microsomes (0.51–0.64 mg protein), cytosol (0.69–0.77 mg protein) or both, and with (+) or without (−) 100 μmol/l 5α-DHT. The values are means (±SD) from three separate experiments in which assays were run in duplicate. The values are expressed relative to that of microsomes in the absence of 5α-DHT which was assigned a value of 1.0. The actual activity was 19.3 ± 11.3 pmol/mg/30 min; *$P \leq 0.01$, different from microsomes minus 5α-DHT; **$P \leq 0.05$, different from microsomes plus 5α-DHT.

Rates of 5α-DHT oxidation by placental villous tissue and microsomes

To determine the relationship between the rates of E2 formation and 5α-DHT oxidation in villi and to characterize microsomal 17-HOR activity with 5α-DHT, ^{3}H-labeled 5α-DHT was examined as a substrate.

In three separate experiments in which duplicate 50-mg fragments of villous tissue were incubated with 100 μmol/l [^{3}H]5α-DHT, the mean (±SEM) rate of oxidation was 13.3 ± 10.0 pmol/50 mg fresh weight/min. The rate of E2 formation from 1.0 μM E1 under similar conditions was 1.3 ± 0.1 pmol/50 mg fresh weight/min ($n = 4$). Thus the mean rate of

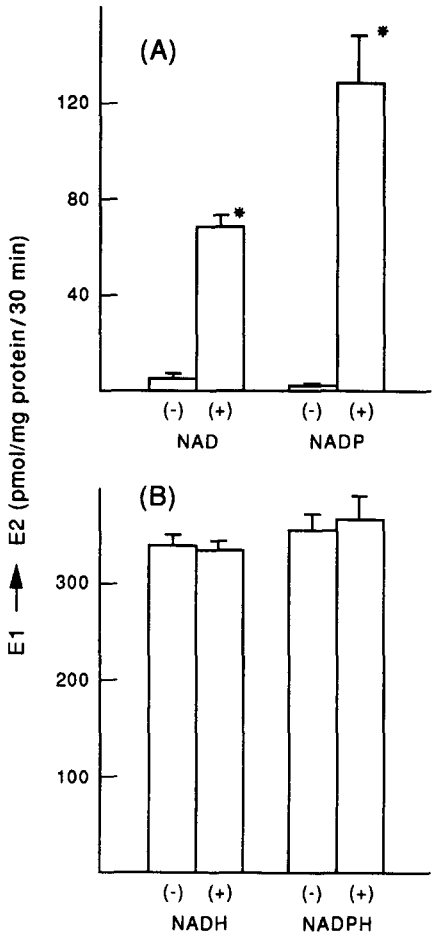

Fig. 6. Effect of 5α-DHT on microsomal 17-HOR activity with E1 in the presence of (A) oxidized or (B) reduced pyridine nucleotide. Reactions mixtures of 0.5 ml total volume containing 60 mmol/l Hepes, pH 7.2, 0.63 mg of microsomal protein, 0.5 mmol/l pyridine nucleotide, 1.0 μmol/l [^{3}H]E1 and with (+) or without (−) 100 μmol/l 5α-DHT were incubated for 30 min. The values are means (±SD) of triplicate assays with microsomes from a single term placenta: $^*P \leq 0.01$, (+) 5α-DHT vs (−) 5α-DHT samples.

5α-DHT oxidation equals or exceeds the rate of E2 formation under stimulatory conditions.

Cytosolic 17-HOR has a low affinity for 5α-DHT either as a substrate or inhibitor [14, 15, 19]. In contrast, 5α-DHT is a high affinity competitive inhibitor of microsomal 17-HOR activity with either E2 or T as substrate [15, 16]. It has not been examined in detail as a substrate. Microsomal 17-HOR activity as a function of 5α-DHT concentration is shown in Fig. 7. The results are suggestive of the presence of two forms of 17-HOR differing in affinity for 5α-DHT. The dashed line in Fig. 7(A) was calculated for two enzymes, with K_m and V_{max} values of 1.5 μmol/l and 0.87 nmol/mg protein/60 min and 197.0 μmol/l and 2.14 nmol/mg protein/60 min, respectively.

DISCUSSION

The objective of this investigation was to examine a number of possible mechanisms for 5α-DHT-stimulated conversion of E2 to E1 in placental villi *in vitro*. Considered in total, the lack of stimulation by DHA, glucose or lactate, the microsomal localization of stimulation along with the requirement for NAD or NADP, and the biphasic dependence of microsomal 17-HOR on 5α-DHT concentration are consistent with a mechanism based on pyridine nucleotide-mediated hydrogen transfer between 5α-DHT and E1 involving one or more forms of membrane-bound 17-HOR. The fact that cytosol reduced the extent of stimulation suggests enzyme–membrane binding may be unstable and affected by cytoplasmic factors. Pollow *et al.* [20, 21] and Wenzel *et al.* [22] were the first to present evidence of a direct hydrogen transfer between hydroxysteroids and ketosteroids mediated by microsomal 17-HOR. The results presented here extend that line of investigation

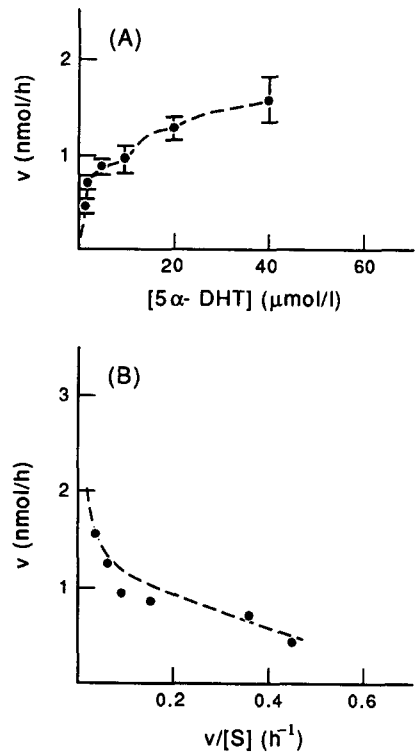

Fig. 7. Plots of (A) v vs [S] and (B) v vs v/[S] for microsomal 17-HOR activity with 5α-DHT. Reaction mixtures (0.5 ml) containing 1.0 mmol/l NAD, 87 μg protein and 1.0, 2.0, 5.0, 10.0, 20.0 or 40.0 μmol/l [^{3}H]5α-DHT in 80 mmol/l Bicine, pH 9.0, were incubated at room temperature for 60 min. The values are means (±SD) of triplicate assays at each substrate concentration with microsomes from a single term placenta. The dashed lines in the figures are calculated for two enzymes with K_m and V_{max} values of 1.5 μmol/l and 0.87 nmol/mg/60 min and 197.0 μmol/l and 2.14 nmol/mg/60 min, respectively.

to include 5α-DHT and support the concept of direct hydrogen transfer in intact villi.

The relevance of these effects to the regulation of placental 17-HOR *in vivo* remains to be established. Clearly the concentrations of 5α-DHT required to observe a stimulation of E2 formation *in vitro* are non-physiologic. However the fact that stimulation is observed only with microsomes suggests the phenomenon may involve enzyme molecules in close proximity to each other. If 17-HOR molecules are clustered on subcellular membranes, then direct transfer of NADH or NADPH among active sites may be a normal part of 17-HOR-catalyzed steps in steroid metabolism, in contrast to random diffusion and equilibration of reduced cofactor within intracellular pools. Evidence of such a direct transfer of NADH between α-glycerol phosphate dehydrogenase and lactate dehydrogenase from rabbit muscle has been presented [23]. The concept that random diffusion of substrates and cofactors in steroidogenesis is restricted and that the flow of intermediates is tightly controlled structurally has appeared to be most applicable to cytochrome *P*-450-catalyzed reactions [24]. The results of this investigation and previous kinetic studies suggest specificity of steroid recognition and close structural proximity of membrane-bound enzyme molecules may be fundamental aspects of the control of 17-HOR in term placenta, as well.

Acknowledgements—This work was supported by Ramsey Foundation Grants 8379 and 8407. The authors are grateful to Ms Chris Ann Hough and Ms Linda A. Feeney for skilful technical assistance and to Ms Linda Sackett-Lundeen for help with the figures.

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