Biochemical Genetics, Vol. 25, Nos. 1/2, 1987

Evidence that the Same Structural Gene Encodes Testicular and Adrenal 3β-Hydroxysteroid Dehydrogenase-Isomerase

John R. D. Stalvey,[1,4] Miriam H. Meisler,[2] and Anita H. Payne[1,3,5]

Received 16 July 1986—Final 29 Oct. 1986

Thermostability of 3β-hydroxysteroid dehydrogenase-isomerase (3βHSD) activity was examined in testes and adrenal glands from several inbred lines and feral mice. A thermolabile varant of 3βHSD was detected in the feral Brno mice. The thermostability ($t_{1/2}$) of 3βHSD was approximately 7 min for both testes and adrenal glands from C57BL/6J mice, compared with 4 min for both tissues from Brno mice. Comparison of testicular and adrenal 3βHSD thermostability in six kinds of mice indicated that the $t_{1/2}$ of 3βHSD was correlated in the two tissues and could be classified into two distinct types, thermolabile and thermostable. In contrast, quantitative variants in 3βHSD activity were not correlated in the two tissues. These data are consistent with the hypothesis that testicular and adrenal 3βHSD is encoded by the same structural gene but that expression of 3βHSD activity is independently controlled in testes and adrenal glands.

KEY WORDS: 3β-hydroxysteroid dehydrogenase-isomerase; structural gene; adrenal gland; testes; thermostability variant.

This work was supported by NICHHD National Research Service Award HD-06392 to J.R.D.S. and Grant HD-17916 to A.H.P.

[1] Department of Obstetrics and Gynecology, The University of Michigan, Ann Arbor, Michigan 48109.
[2] Department of Human Genetics, The University of Michigan, Ann Arbor, Michigan 48109.
[3] Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109.
[4] Present address: Department of Human Genetics, The University of Michigan, Ann Arbor, Michigan 48109.
[5] To whom correspondence should be addressed.

0006-2928/87/0200-0181$05.00/0

INTRODUCTION

Oxidation and isomerization of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids are reactions common to all steroidogenic tissues (Samuels *et al.*, 1951). This conversion is catalyzed by the microsomal enzyme Δ^5-3β-hydroxysteroid dehydrogenase-isomerase (3βHSD; Samuels *et al.*, 1951; Tamaoki, 1973). The activity of 3βHSD is essential for the production of testosterone by testes and corticosteroids by adrenal glands (Samuels *et al.*, 1951). It is not known, however, whether the enzyme in various steroidogenic tissues is encoded by the same gene.

The inherited human disorders of 3βHSD, which are characterized by a deficiency of testicular and/or adrenal 3βHSD activity, are clinically categorized as forms of adrenal hyperplasia (Bongiovanni, 1981). It has not been established yet whether these clinical deficiencies are due to defects in the structure of the enzyme molecule or, alternatively, to a reduced concentration of the enzyme molecules.

To examine whether testicular and adrenal 3βHSD is encoded by the same structural gene, the rate of heat inactivation of 3βHSD in these two tissues was measured in several inbred and feral lines of mice. In addition, we have evaluated whether quantitative differences in 3βHSD activity in testes are correlated with quantitative differences in adrenal glands from 10 different lines of mice.

MATERIALS AND METHODS

Materials

[7-(N)-^{3}H]Pregnenolone was purchased from Amersham Corp. (Arlington Heights, Ill.). [1,2-(N)-^{3}H]Progesterone, [4-^{14}C]progesterone, [4-^{14}C]17α-hydroxyprogesterone, [4-^{14}C]androstenedione, and [4-^{14}C]testosterone were purchased from New England Nuclear Corp. (Boston). The compounds were purified by thin-layer chromatography (TLC) before use. The radiochemical purity of all compounds was established by recrystalization of aliquots with authentic steroids. β-Nicotinamide adenine dinucleotide (NAD^+) and the reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Animals

Male mice (8–12 weeks of age) of the C57BL/6J, C3H/HeJ, DBA/2J, A/J, LP/J, 129/SvJ, FS/Ei, GL/Le, STX/Le, and WLH/Le strains were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice of the Brno line and the species *Mus molossinus* were the kind gift of Dr. Verne Chapman

(Roswell Park Memorial Institute, Buffalo, N.Y.). Animals were housed four to eight mice per cage; fighting was minimal and no animals were found to have wounds. The room in which the animals were housed was maintained at 23°C with a 14-hr light and 10-hr dark cycle. Animals were kept a minimum of 7 days before they were killed by cervical dislocation between 0800 and 0900 hr. Testes were taken out and decapsulated and adrenal glands were excised and adhering fat was removed.

Detection of Structural Variants

Testes and adrenal glands were homogenized in 50 mM potassium phosphate-buffered saline (KPBS; pH 7.4) containing 1.0% bovine serum albumin (BSA). Aliquots (400 μl) of homogenates were incubated in glass tubes at 43°C for 0, 5, 10, 15, and 20 min prior to assay of enzyme activity.

Evaluation of Quantitative Differences in 3βHSD

Testes and adrenal glands were homogenized in 50 mM KPBS containing 1.0% BSA. Enzyme activity was determined in aliquots of the homogenates.

Measurement of Steroidogenic Enzyme Activity

3β-Hydroxysteroid dehydrogenase-isomerase activity was determined by measuring the conversion of [^{3}H]pregnenolone to [^{3}H]progesterone, as described previously (Stalvey and Payne, 1984). Incubations were performed for 5 min at 37°C in glass tubes containing 0.5–1.0 μCi [^{3}H]pregnenolone with a saturating (2 μM) concentration (or increasing concentrations for determination of K_m) of nonradioactive pregnenolone, dissolved in 0.05 ml dimethylsulfoxide and 0.85 ml KPBS containing 0.5 mM NAD^+. The reaction was initiated by the addition of 0.1 ml of the appropriate homogenate and stopped by the addition of 0.1 ml of 1 N NaOH. Pregnenolone (50 μg) and [^{14}C]progesterone (50 μg; 1000 dpm) were added as carriers and to monitor recovery. Pregnenolone and progesterone were extracted with 10 ml toluene and separated by TLC in chloroform:ether (7:1). Radioactivity was quantitated by liquid scintillation counting. Representative samples were recrystallized to establish radiochemical purity.

17α-Hydroxylase activity was determined, as previously described (Stalvey and Payne, 1984), by measuring the conversion of [^{3}H]progesterone to [^{3}H]17α-hydroxyprogesterone, [^{3}H]androstenedione, and [^{3}H]testosterone. [^{3}H]Progesterone (0.5 μCi; 2 μM) was incubated in KPBS containing 0.5 mM NADPH for 5 min at 37°C. After stopping the reaction and adding the appropriate unlabeled and ^{14}C-labeled steroids as carriers to monitor recovery,

extraction and separation of steroids were carried as described above. The area representing androstenedione was cut out, eluted, and rechromatographed in the same system to remove progesterone contamination. Testosterone and 17α-hydroxyprogesterone cochromatographed and were quantitated together.

Statistical Analysis

Means derived from multiple strain comparisons were subjected to analysis of variance and Duncan's new multiple range test (Bliss, 1967) ($\alpha = 0.05$).

RESULTS

To detect structural variants of 3βHSD, the effect of pretreatment at 43°C on 3βHSD activity was determined in aliquots of testicular and adrenal homogenates from several inbred and feral lines of mice. In the feral Brno line, a thermolabile variant of 3βHSD was observed. The stability of the thermolabile enzyme in testes from Brno mice was compared with the common, thermostable form of testicular 3βHSD in C57BL/6J mice (Fig. 1A). At each time of

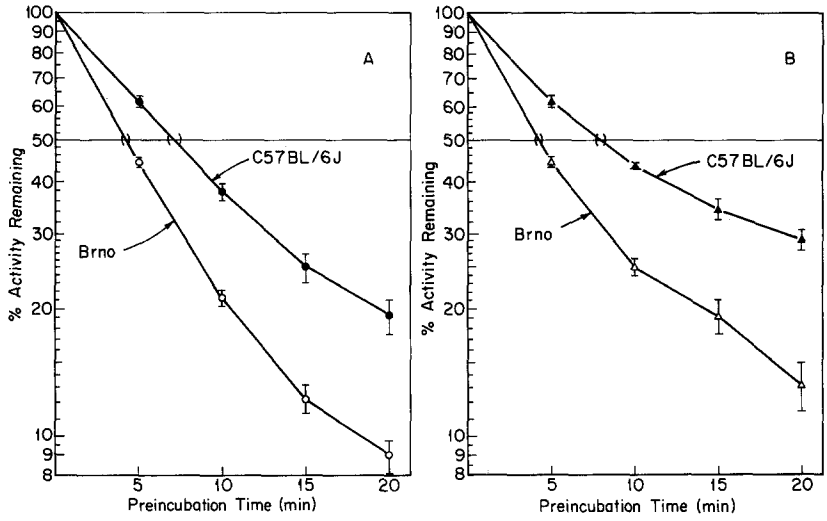

Fig. 1. Heat inactivation of 3β-hydroxysteroid dehydrogenase-isomerase activity at 43°C. Aliquots of homogenized testes (A) or adrenal glands (B) from Brno and C57BL/6J mice were preincubated at 43°C for 0, 5, 10, 15, and 20 min. To determine 3βHSD activity, aliquots from each time period were incubated in duplicate with a saturating concentration of [^{3}H]pregnenolone in the presence of NAD^+ at 37°C for 5 min. Values are expressed as a logarithm of the percentage of 3βHSD activity remaining after pretreatment with heat relative to 3βHSD activity at 0 min of pretreatment and represent the mean ± SE of four separate experiments.

treatment, a significantly lower percentage of 3βHSD activity remained in aliquots of testes from Brno mice than from C57BL/6J mice. To determine if the thermolabile enzyme is expressed in other steroidogenic tissues, the thermal denaturation of 3βHSD in adrenal glands from Brno and C57BL/6J mice was tested (Fig. 1B). A significantly lower percentage of 3βHSD activity remained in aliquots of adrenal glands from Brno than C57BL/6J mice. The thermostability ($t_{1/2}$) of testicular and adrenal 3βHSD was 7.1 ± 0.4 and 7.8 ± 0.2 min (mean ± SE; $N = 4$), respectively, for C57BL/6J and 4.2 ± 0.1 and 4.3 ± 0.1 min, respectively, for Brno mice. The results demonstrate that the same thermolabile variant was present in both tissues of Brno mice. Mixing aliquots of homogenized adrenal glands from Brno and C57BL/6J mice resulted in 3βHSD thermostability that was intermediate to that for either Brno or C57BL/6J. When the apparent K_m values for pregnenolone of the thermolabile

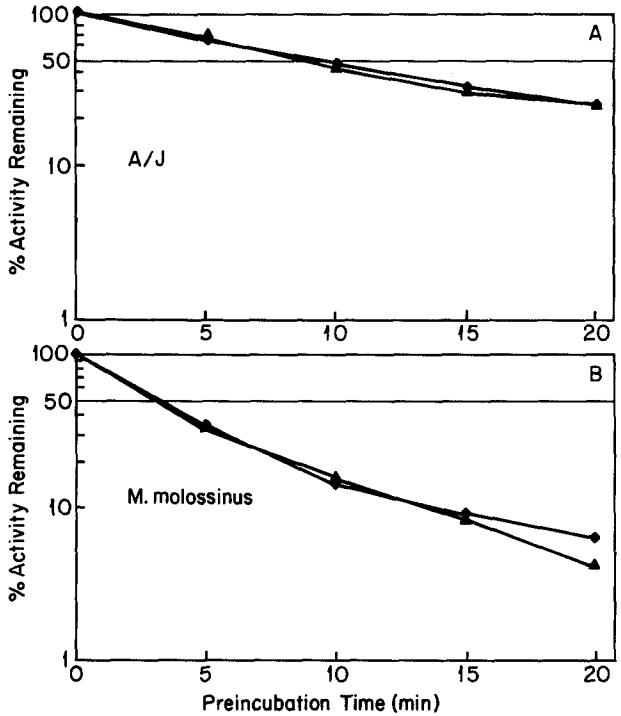

Fig. 2. Heat inactivation of 3β-hydroxysteroid dehydrogenase-isomerase activity at 43°C in testes and adrenal glands from A/J and *M. molossinus* mice. Aliquots of homogenized testes (●) or adrenal glands (▲) from A/J (A) or *M. molossinus* (B) mice were preincubated at 43°C for 0, 5, 10, 15, and 20 min. 3βHSD activity was determined as in the legend to Fig. 1. Data are representative of two separate experiments.

and thermostable forms of 3βHSD from both tissues were compared, the values obtained (0.1–0.15 μM) did not differ. To determine whether the thermolabile variant of 3βHSD in Brno mice was specific to the 3βHSD or a general effect of heat on the endoplasmic reticulum, the thermostability of another microsomal steroidogenic enzyme, 17α-hydroxylase, was compared in Brno and C57BL/6J mice. The thermostability of testicular 17α-hydroxylase activity did not differ between the two types of mice and 17α-hydroxylase was more thermostable than 3βHSD (data not shown). It should be noted that mouse adrenal glands have no 17α-hydroxylase activity and no immunoprecipitable 17α-hydroxylase (Perkins and Payne, unpublished).

Additional lines of mice were screened for thermostability of testicular and adrenal 3βHSD. Within each line, the thermostability of testicular and adrenal 3βHSD did not differ. The heat inactivation is illustrated for two of the lines in Fig. 2. In A/J mice, testicular and adrenal 3βHSD appeared to be of the thermostable type, similar to C57BL/6J mice, whereas the testicular and adrenal 3βHSD from *M. molossinus* mice was thermolabile, similar to Brno mice. The thermostability of testicular and adrenal 3βHSD in six strains was compared (Fig. 3). The 3βHSD could be classified into two distinct groups, thermolabile (Brno and *M. molossinus*) and thermostable (C57BL/6J, DBA/2J, A/J, and LP/J).

To determine whether quantitative differences in 3βHSD activity in testes and adrenal glands are correlated, enzyme activity in these two tissues was measured in several strains of mice (Fig. 4). The enzyme activity in the

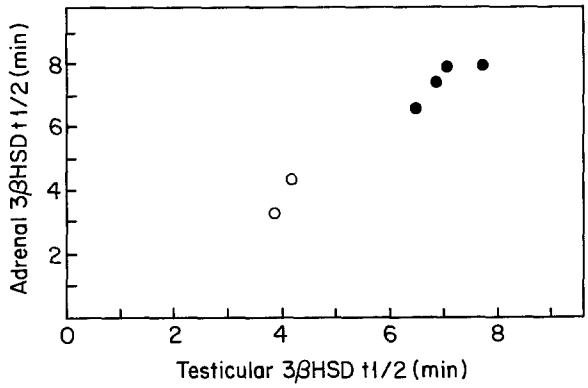

Fig. 3. Correlation of thermostability of testicular and adrenal 3β-hydroxysteroid dehydrogenase-isomerase in six lines and populations of mice. Thermostability ($t_{1/2}$ at 43°C) of testicular and adrenal 3βHSD was compared from the thermolabile Brno and *M. molossinus* mice (●) and from the thermostable lines C57BL/6J, A/J, DBA/2J, and LP/J (○). Values represent means from two to four animals per line or population.

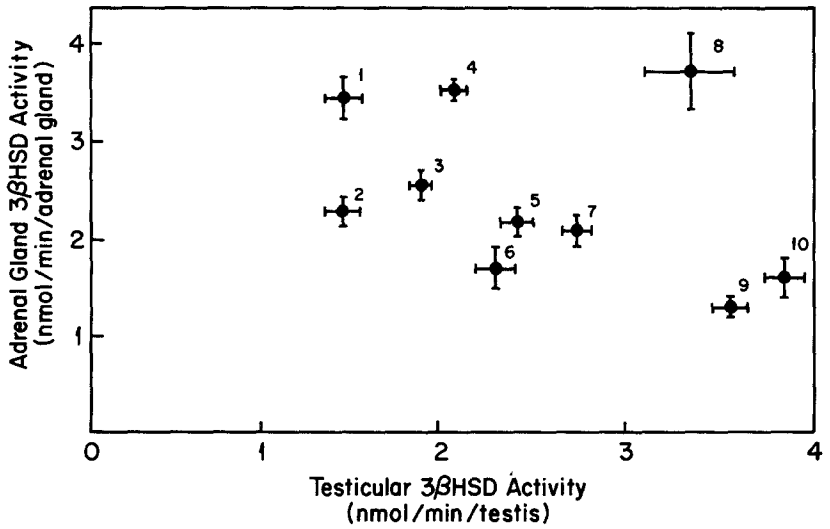

Fig. 4. 3β-Hydroxysteroid dehydrogenase-isomerase activity in testes and adrenal glands from 10 lines of mice. 3βHSD activity was determined in aliquots of homogenized testes and adrenal glands, as in the legend to Fig. 1. Lines utilized were (1) GL/Le, (2) C3H/HaJ, (3) STX/Le, (4) DBA/2J, (5) WLH/Le, (6) C57BL/6J, (7) Brno, (8) FS/Ei, (9) A/J, and (10) FSB/J. Values represent the mean ± SE from 3–10 mice, except Brno ($N = 2$).

two tissues varied independently in these strains. The lack of correlation between enzyme activity in the two tissues indicates that these strains differ with respect to tissue-specific determinants of expression of the common 3βHSD structural gene.

DISCUSSION

The results of this study are consistent with the hypothesis that testicular and adrenal 3βHSD is encoded by the same structural gene. Heat-induced loss of 3βHSD activity provides a sensitive method for the detection of structural variants. Analysis of the effect of serial alteration of single amino acids in β-galactosidase, by specific point mutations, indicated that approximately 70% of the amino acid substitutions alter the thermostability of the enzyme activity (Langridge, 1968). In light of this sensitivity, the finding that there are no differences between testes and adrenal glands of the same strain strongly indicates that testicular and adrenal 3βHSD is structurally identical. The future availability of a cloned probe for the 3βHSD gene would allow for more definitive proof of the structural identity of the 3βHSD gene in adrenal glands and testes.

The finding in the present study that the activity of another microsomal steroidogenic enzyme, testicular 17α-hydroxylase, is more thermostable than 3βHSD activity under identical heat pretreatment in both C57BL/6J and Brno mice indicates that the thermal inactivation loss of 3βHSD activity does not reflect a nonspecific instability of the smooth endoplasmic reticulum. Furthermore, it is unlikely that the rapid loss of 3βHSD activity in heat-pretreated tissues from Brno mice is due to increased protease activity in the Brno line, since mixing aliquots of homogenized testes from Brno and C57BL/6J mice results in 3βHSD activity with an intermediate thermostability. It is also of interest to note that the thermostability of 3βHSD activity is independent of the apparent K_m for pregnenolone, indicating that the structural variant detected in Brno mice probably does not affect the active site.

In contrast with results for structural variants, results for quantitative variants indicate that the amount of activity per tissue is under different genetic control in testes and in adrenal glands. When several strains were compared, it was evident that the amount of 3βHSD activity varied independently in the two tissues. Possible genetic mechanisms for regulating the quantitative expression of 3βHSD include regulation of circulating concentrations of trophic hormones and regulation of cellular responsiveness to these trophic hormones (Shire, 1979). Luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH) can increase steroid biosynthesis in testes and adrenal glands, respectively, by trophic stimulation of the steroidogenic enzymes as well as by acutely stimulating the metabolism of cholesterol. However, the mechanism and the extent to which LH and ACTH regulate 3βHSD activity in testes and adrenal glands, respectively, have not been resolved (Shaw *et al.*, 1979; O'Shaughnessy and Payne, 1982; Ruiz de Galarreta *et al.*, 1983; Payne *et al.*, 1985; Samuels and Helmreich, 1956; Liles and Ramachandran, 1977; Marston *et al.*, 1985).

Adrenal hyperplasia due to deficiency of 3βHSD activity in the human is characterized by elevated serum concentrations of Δ^5-3β-hydroxysteroids or urinary concentrations of appropriate metabolites (Bongiovani, 1981). In several cases of adrenal hyperplasia due to 3βHSD deficiency, close examination of patients revealed that 3βHSD activity in both the adrenal glands and the testes is essentially absent (Bongiovani, 1962; Goldman *et al.*, 1964). Familial inheritance patterns indicate that this form of 3βHSD deficiency is due to an autosomal recessive mutation (Grumbach and Conti, 1981). More recently heterogeneity in 3βHSD deficiency has become evident, and cases have been described in which the extent of deficiency differs in adrenal glands and gonads (Bongiovani, 1981; Rosenfeld *et al.*, 1980; Schneider *et al.*, 1975). In light of our evidence that the same structural gene encodes both testicular and adrenal 3βHSD, but that genetic regulation of the amount of 3βHSD activity is different in the two tissues, it is possible that severe forms of 3βHSD

deficiency which affect both adrenal glands and gonads equally may be due to structural gene defects, while the forms which affect one tissue more than the other may result from regulatory defects under separate genetic controls. Obvious care must be exercised, however, when extrapolating from animal models to the human.

In conclusion, we present evidence that testicular and adrenal 3βHSD is encoded by the same gene. The data on quantitative variants, however, indicate that expression of 3βHSD activity is independently controlled in testes and adrenal glands. C57BL/6J and Brno mice could prove useful models for studying the 3βHSD structural gene in the future.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Rosemary W. Elliott (Roswell Park Memorial Institute, Buffalo, N.Y.) for the kind gift of frozen testes from inbred mice which were used in an initial screen for quantitative variants in 3βHSD activity. We would also like to thank Louise M. Perkins for helpful discussion and Mary Dockrill and Margaret Snow for technical assistance.

REFERENCES

Bliss, C. I. (1967). *Statistics in Biology,* McGraw–Hill, New York, Vol. 1, p. 231.

Bongiovanni, A. M. (1962). Adrenogenital syndrome with deficiency of 3-beta-hydroxysteroid dehydrogenase. *J. Clin. Invest.* **41**:2086.

Bongiovanni, A. M. (1981). Acquired adrenal hyperplasia: With special reference to 3β-hydroxysteroid dehydrogenase. *Fertil. Steril.* **35**:599.

Goldman, A. S., Bongiovanni, A. M., Yakovac, W. C., and Practer, A. (1964). Study of Δ^5-3β-hydroxysteroid dehydrogenase in normal, hyperplastic and neoplastic adrenal cortical tissue. *J. Clin. Endocrinol. Metab.* **24**:894.

Grumbach, M. M., and Conte, F. A. (1981). Disorders of sex differentiation. In Williams, R. H. (ed.), *Textbook of Endocrinology,* ed. 6, W. B. Saunders, Philadelphia, p. 422.

Langridge, J. (1968). Genetic and enzymatic experiments relating to the tertiary structure of β-galactosidase. *J. Bacteriol.* **96**:1711.

Liles, S., and Ramachandran, J. (1977). Regulation of Δ^5-3β-hydroxysteroid dehydrogenase-isomerase activity in adrenocortical cell cultures by adrenocorticotropin. *Biochem. Biophys. Res. Commun.* **79**:226.

Marston, S. A., Waterman, M. R., and McCarthy, J. L. (1985). 3β-Hydroxysteroid dehydrogenase-isomerase activity in bovine adrenocortical cells in culture: Lack of response to ACTH stimulation. *J. Steroid Biochem.* **22**:789.

O'Shaugnessy, P. J., and Payne, A. H. (1982). Differential effects of single and repeated administration of gonadotropins on testosterone production and steroidogenic enzymes in Leydig cell populations. *J. Biol. Chem.* **257**:11503.

Payne, A. H., Quinn, P. G., and Rani, C. S. S. (1985). Regulation of microsomal cytochrome P-450 enzymes and testosterone production in Leydig cells. *Rec. Prog. Horm. Res.* **41**:153.

Rosenfeld, R. L., Rich, B. H., Wolfsdorf, J. L., Cassorla, F., Parks, J. S., Bongiovanni, A. M., Wu, C. H., and Shackleton, C. H. L. (1980). Pubertal presentation of congenital Δ^5-3β-hydroxysteroid dehydrogenase-isomerase deficiency. *J. Clin. Endocrinol. Metab.* **51**:345.

Ruiz de Galarreta, C. M., Fanjul, L. F., Meidan, R., and Hsueh, A. J. W. (1983). Regulation of

3β-hydroxysteroid dehydrogenase activity by human chorionic gonadotropin, androgens and anti-androgens in cultured testicular cells. *J. Biol. Chem.* **258:**10988.

Samuels, L. T., and Helmreich, M. L. (1956). The influence of chorionic gonadotropin on the 3β-ol dehydrogenase activity of testes and adrenals. *Endocrinology* **58:**435.

Samuels, L. T., Helmreich, M. L., Lasater, M. B., and Reich, H. (1951). An enzyme in endocrine tissue which oxidizes Δ^5-3-hydroxysteroids to α,β unsaturated ketone. *Science* **113:**490.

Schneider, G., Gend, M., Bongiovanni, A. M., Goldman, A. S., and Rosenfeld, R. L. (1975). Persistent testicular Δ^5-isomerase 3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) deficiency Δ^5-3β-HSD form of congenital adrenal hyperplasia. *J. Clin. Invest.* **55:**681.

Shaw, M. J., Georgopoulos, L. E., and Payne, A. H. (1979). Synergistic effect of follicle-stimulating hormone and luteinizing hormone on testicular Δ^5-3β-hydroxysteroid dehydrogenase-isomerase: Application of a new method for separation of testicular compartments. *Endocrinology* **104:**912.

Shire, J. G. M. (1979). The uses and consequences of genetic variation in hormone systems. In Shire, J. G. M. (ed.), *Genetic Variations in Hormone Systems*, CRC Press, Boca Raton, Fla., Vol. 1, p. 1.

Stalvey, J. R. D., and Payne, A. H. (1984). Maximal testosterone production in Leydig cells from inbred mice relates to the activity of 3β-hydroxysteroid dehydrogenase-isomerase. *Endocrinology* **115:**1500.

Tamaoki, B. (1973). Steroidogenesis and cell structure. Biochemical pursuit of sites of steroid biosynthesis. *J. Steroid Biochem.* **4:**89.