An Improved Method for Evaluating Testosterone Biosynthetic Defects

FRED I. CHASALOW,[23] SANDRA L. BLETHEN, HARRY B. MARR, AND FRANK S. FRENCH

Edward Mallinckrodt Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri USA, the Chemistry and Life Science Group, Research Triangle Institute, Research Triangle Park, North Carolina USA and the Department of Pediatrics, University of North Carolina, Chapel Hill, North Carolina USA

Summary

A double-label, double-substrate incubation technique has been developed and used to study the conversion of progesterone to testosterone in testes extracts from incompletely virilized males. The procedure involves separation of the microsomes from a testicular homogenate, incubating the microsomes with 1 μM [7-^{3}H]progesterone, 1 μM 17-hydroxy[4-^{14}C]progesterone, and 0.25 mM NADPH in pH 7.4 phosphate buffer at 37°C. Steroid precursors and products are separated by column chromatography on Sephadex LH-20 with a solvent system of isoctane:ethyl acetate:methanol (4:1:1 by volume). These procedures can be completed in 2 days, and thus the method represents an improvement in time, reproducibility, and simplicity when compared to techniques based on thin layer or paper chromatography.

The method has been used to distinguish the biochemical abnormality in three cases with XY sex chromatin, posterior labial fusion, clitoromegaly, and hypospadias. The abnormalities identified were: Case 1, no defect in testosterone synthesis (probable androgen insensitivity); Case 2, 17-ketosteroid reductase deficiency; and Case 3, steroid-17,20-lyase deficiency.

Abbreviation

hCG, human chorionic gonadotropin

Enzymatic defects in the biosynthesis of testosterone can be tentatively distinguished by hCG stimulation tests (8, 15) and the diagnosis can be confirmed by *in vitro* testicular incubation after surgery. However, the classical techniques based on thin layer (11) or paper chromatography (6) are laborious and time consuming. We have developed a method based on double-substrate, double-label incubation and Sephadex LH-20 chromatography that can confirm these diagnoses simply and rapidly. The method depends for its success on the reproducibility of the Sephadex LH-20 columns and on the double label method of incubation of the tissue with the steroid substrates. A preliminary report of the procedure and results has appeared (3).

MATERIALS AND METHODS

The procedures for the isolation of microsomes from human testes and the incubation techniques are similar to those described in detail elsewhere using rat testes (2). Briefly, fresh testicular tissue was homogenized in 3 volumes (w/v) of 0.25 M sucrose and the microsomes were collected by differential centrifugation between 11,000 and 105,000 $\times$ g, resuspended at a dilution of 1 ml/g original fresh weight of tissue, and incubated with (a) 1 μM [7-^{3}H]progesterone; (b) 1 μM 17-hydroxy[4-^{14}C] progesterone; (c) 0.25 mM NADPH; and (d) 0.05 M potassium dihydrogen phosphate adjusted to pH 7.4 with 2 M potassium

hydroxide. The total volume of each incubation mixture was 1 ml; the incubation was at 37°C in a shaking water bath in tubes open to the air. The reaction was started by the addition of 0.05 ml of microsome suspension and quenched at selected times by the addition of 2 ml of methanol containing 20 mg of one or more of the following steroids: 17-hydroxyprogesterone, androstenedione, or testosterone. Although the corresponding cytosol stimulates androstenedione formation by rat testes microsomes, cytosol from adult rats prestimulated with hCG had no effect on testicular microsomes from humans who had been stimulated with hCG. In testes extracts prepared from Case 1, addition of the human cytosol fraction to the human microsomal fraction also had no effect on either the time course of testosterone synthesis or on the pattern of steroids produced. Consequently, cytosol was not routinely added during the incubations. Samples were stored at −20°C until assayed. At the time of assay, each sample was diluted with water (30 ml) and extracted 3 times with ethyl acetate (30 ml each, 90 ml total). The organic phases were combined, evaporated to dryness with a rotary evaporator, and chromatographed on a 30 $\times$ 1.9 cm Sephadex LH-20 column with the solvent isoctane:ethyl acetate:methanol (4:1:1 by volume) (2). The material in each peak was acetylated with a mixture of pyridine and acetic anhyride (3:1) as previously described (2), and rechromatographed on the same column with the same solvent system. This column procedure has baseline separation for steroids differing in polarity by as little as the difference between an alcohol and a ketone with the same carbon skeleton. The capacity of the column is limited only by the solubility of the steroid in the solvent mixture. In representative samples, the carrier steroids were reisolated and crystallized to constant specific activity. In each case tested, the assignments of steroid identity were correct, and more than 90% of the activity in each chromatographic peak could be accounted for in the final specific activity of the reisolated steroid.

Serum steroid levels before and after hCG administration were measured by Dr. C. Migeon in Case 1 and 2 and by Endocrine Science, Tarzana, CA for Case 3.

Subjects. The clinical history of the children is shown in Table 1. Each had incomplete labial fusion, clitoromegaly, and hypospadias. Serum testosterone was determined before and after the administration of four daily doses of hCG (5000 IU/m^2 per dose) (19). While Case 1 had a marked increase in testosterone, neither Case 2 nor Case 3 showed a normal testosterone response (>300 ng/dl after hCG). The appearance of the external genitalia in these children was such that the female sex of rearing was deemed the more appropriate. Twenty-four to 48 h following the last dose of hCG, reconstructive surgery was performed and testicular tissue obtained (0.5–1.0 g of tissue was used to prepare the microsomes). Histologic examination showed normal testicular histology in each case. Informed consent for the procedures was obtained from the parents.

Fig. 2. Sephadex LH-20 column chromatograms from Case 1. *A* is the original chromatogram obtained after [7-³H]progesterone and 17-hydroxy[4-¹⁴C]progesterone were incubated for 10 min with microsomes prepared from testes of Case 1. *B*, *C*, and *D* show the rechromatography after acetylation of *Peaks III*, *IV*, and *II*, respectively. The steroids are: *Peak I*, progesterone; *II*, androstenedione; *IIIa*, testosterone acetate; *IIIb*, 17-hydroxyprogesterone; *IVa*, 16α-hydroxyprogesterone; and *IVb*, 17α,20ξ-dihydroxy-4-pregnen-3-one.

have been shown to produce 16α-hydroxyprogesterone (11), the greater peak has been attributed to that compound. The lesser of the two peaks was also labeled with both carbon-14 and tritium and also formed an acetate under these mild conditions. One possible identity is 17α,20ξ-dihydroxy-4-pregnen-3-one which has previously been shown to be synthesized in the testes (5) but there was insufficient labeled material to confirm this identification. Thus, in Case 1 (Fig. 2), testosterone was produced from both substrates, and this child did not have a defect in the enzymes that produce testosterone. The most likely cause of her phenotypic appearance is one of the androgen resistance syndromes but these can't be distinguished on the basis of testicular incubations (18).

Figure 3 shows the chromatograms obtained following testicular incubation in Case 2. Androstenedione (Peak II) was synthesized from both precursors but was not converted to testosterone. This finding confirmed a deficiency of 17-ketosteroid reductase which had been suggested by the observation that testosterone levels in this child did not increase normally following hCG treatment (16, 17).

The chromatographic results from Case 3 are shown in Figure 4. Neither androstenedione nor testosterone nor 17-hydroxy-

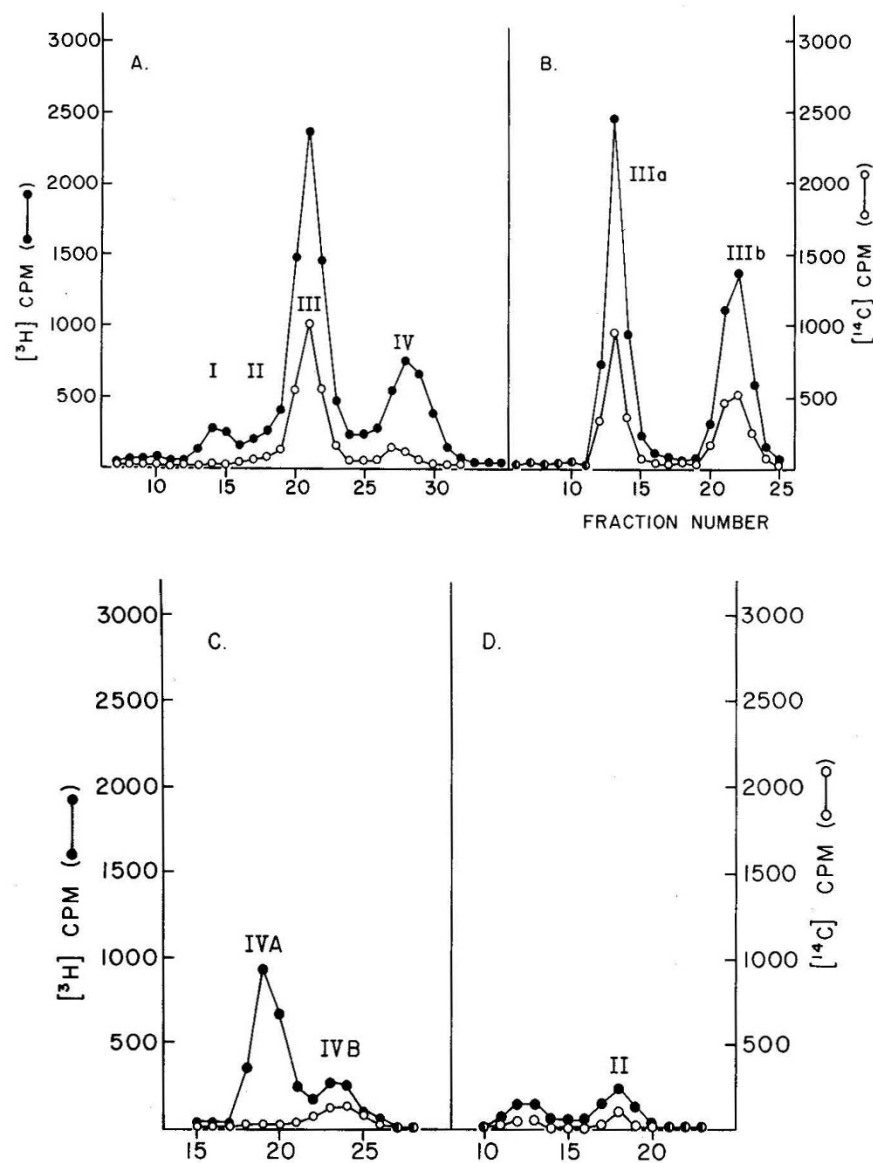progesterone increased following hCG treatment. The chromatogram shows no conversion of either progesterone or 17-hydroxyprogesterone to either testosterone or androstenedione. These results suggested that the patient had a defect in steroid-17,20-lyase (20). In addition, although there was some conversion of progesterone to 17-hydroxyprogesterone, there was substantially less tritium in peak III B than was seen in the two other cases, and therefore there was also a considerable decrease in 17-hydroxylase activity. The decrease in both 17-hydroxylase activity and steroid-17,20-lyase activity is consistent with her lack of stimulation of serum 17-hydroxyprogesterone levels in response to hCG shown in Table 1 and reported in a similar case by Kaufman *et al.* (7). The two defects are frequently closely associated because in the testes the two reactions are catalyzed by the same protein complex (2, 9). The defect in this enzyme complex can be distinguished from 17-hydroxylase deficiency on the basis of cortisol levels since the latter are extremely low in 17-hydroxylase deficiency (4). Our Case 3 had normal cortisol levels and thus has inadequate steroid-17,20-lyase catalytic activity. As there was little accumulation of radioactivity in the region of Peak IV, its nature could not be investigated with these samples.

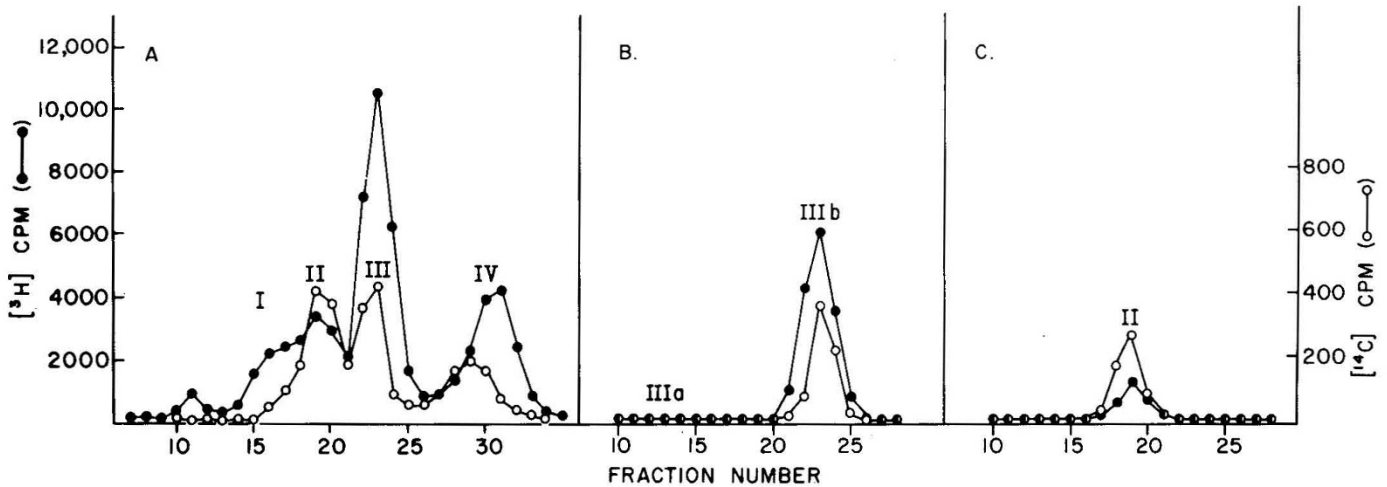Although our data are not complete, the same procedures have

Fig. 3. Chromatograms from Case 2. *A* is the original chromatogram. *B* and *C* show the rechromatography after acetylation of *Peaks III* and *II*, respectively. See Figure 2 for details.
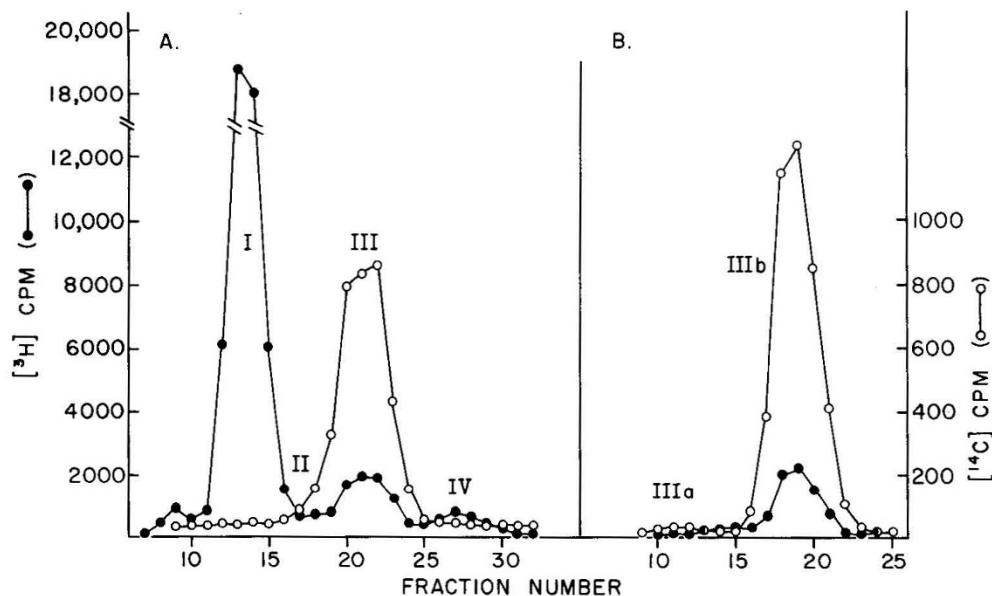

Fig. 4. Chromatograms from Case 3. *A* shows the original chromatogram from Case 3. *B* shows the rechromatography of *Peak III*. See Figure 2 for details. Note the differences in scale for ³H and ¹⁴C.

been used to define the biosynthetic defect in four additional children. They had 46 XY karyotypes, clitoromegaly, hypospadias, and labial fusion just as the three cases described in detail in Table 1 and Figures 1–4. Of the additional cases, two made testosterone *in vitro*, one accumulated androstenedione, and one synthesized only 17-hydroxyprogesterone. The serum steroid levels in response to hCG were consistent with the diagnosis of 17-ketosteroid reductase and steroid-17,20-lyase deficiency, respectively.

DISCUSSION

The double-label, double-substrate mode of incubation has several advantages over single-label incubation procedures. When the reaction is allowed to proceed to only 70–80% of completion (10 min), the unreacted starting materials serve as internal references to aid the identification of the products. The labeling pattern for each peak (³H, ¹⁴C, or both) also aids the recognition of metabolites and pathways.

As long as sufficient products for characterization have been formed and have not all been metabolized by contaminating enzymes, the exact extent of reaction does not confuse the

interpretation of the data. Since testosterone was present in Case 1 at the 2-min time point and remained until the 20-min time point, which was the last time point studied, additional optimization of the incubation conditions is not necessary. Rodriguez-Rigau *et al.* (14), who studied testosterone biosynthesis in biopsy samples from adults with oligospermia, have also found that the pattern of steroids produced is relatively persistent, and thus changes in the Leydig cell density do not limit the interpretation of the metabolic pattern. However, it should be noted that our method might not discriminate subtle partial enzymatic defects such as these authors reported (14).

Each of the defects in the conversion of progesterone to testosterone produces a characteristic chromatographic pattern. A defect in 17-hydroxylase would result in androgens labeled only from the carbon-14-containing substrate, 17-hydroxyprogesterone. A defect in steroid-17,20-lyase would lead to an accumulation of tritiated 17-hydroxyprogesterone but no conversion of either tritium or carbon-14 to androgens. A defect of 17-ketosteroid reductase would lead to the accumulation of androstenedione labeled with both carbon-14 and tritium but without the conversion of androstenedione to testosterone. When these studies were started, the appearance of testosterone in the chro-

matograms after incubation with testicular microsomes was a surprise because microsomes from rat testes catalyze the formation of androstenedione from progesterone and require the interaction of the 17-ketosteroid reductase from the cytosol to produce testosterone (2). Thus, the situation in humans is different in two ways: first, 17-ketosteroid reductase is present in the newborn period (10) and second, during cell fractionation, it is isolated as a component of the microsomes. The combination of these two factors allows us to evaluate 17-ketosteroid reductase activity with our method of incubation. Thus, in a single incubation with two chromatograms, each of these three enzyme defects can be identified.

There are other steroid enzyme defects that can result in a failure of testosterone biosynthesis. Defects in the synthesis of progesterone from cholesterol are known and would not be detected with this *in vitro* assay system (1, 13). These children usually show evidence of defective salt regulation at birth. However, a recent report describes a family with 3β-hydroxysteroid dehydrogenase deficiency without salt loss (12). The male sibling did not respond to hCG with a rise in serum testosterone or progesterone levels. However, in such an individual, the pathway from progesterone to testosterone in the testes studied *in vitro* would be intact. Thus, a defect in this enzyme, whether or not it was associated with salt loss, would give a characteristic result: synthesis of testosterone *in vitro* but not *in vivo*. Therefore, 3β-hydroxysteroid dehydrogenase deficiency also would give a characteristic result. Of the cases we studied, Cases 1 and 2 were able to synthesize progesterone in response to hCG. While progesterone levels were not measured in Case 3, testes from this individual were unable to metabolize progesterone to androgens *in vitro* and thus a cause of her problem was apparent.

One problem which has arisen when testosterone biosynthesis has been studied in testicular slices or homogenates is the production of 5α-reduced substances. These are difficult to separate from their unreduced precursors. However, since in the human testes, the 5α-reductase is found in the nucleus, it is separated from the microsomes by the low speed centrifugation step (unpublished observation). This eliminates the problems associated with separation and identification of the 5α-reduced steroids.

In our laboratory, when we perform an incubation to identify a defect, we routinely (a) use several time points, (b) acetylate and rechromatograph each peak, and (c) crystallize at least a few samples to constant specific activity. However, such an extensive protocol is not necessary as study of the 10-min time point usually provides all the information needed to confirm the presence or absence of an enzymatic defect.

The technique described here is useful in documenting androgen biosynthetic defects. The steroid pattern in serum following hCG stimulation and the pattern of steroids produced *in vitro* provide complementary approaches to the diagnosis of the various possible defects. Children with these problems are generally subjected to gonadectomy as the gonads are nonfunctional and prone to develop malignancies. Once the testes have been removed, the nature of the defect which caused the failure to virilize can never be reexamined. Therefore, we believe that a study of *in vivo* and of *in vitro* steroid responses to hCG should be undertaken at the time of surgery in order to obtain the most accurate diagnosis possible. As androgen insensitivity and defects in testosterone synthesis have different modes of inheritance, an incorrect diagnosis in such a child would lead to inaccurate counseling.

REFERENCES AND NOTES

1. Camacho AM, Kowarski A, Migeon CJ, Brough AJ 1968 Congenital adrenal hyperplasia due to a deficiency of one of the enzymes involved in the biosynthesis of pregnenolone. J Clin Endocrinol Metab 28:153
2. Chasalow F 1979 Mechanism and control of rat testicular steroidogenesis. J Biol Chem 254:3000
3. Chasalow F, Blethen SL, French FS 1982 An improved method for evaluating testosterone biosynthetic defects. Pediatr Res 16:345
4. D'Armiento M, Reda G, Kater C, Shackleton CHL, Biglieri EG 1983 17α-Hydroxylase deficiency: mineralocorticoid hormone profiles in an affected family. J Clin Endocrinol Metab 56:697
5. Fan DF, Oshima H, Troen BR, Troen P 1974 Studies of the human testis. IV. Testicular 20α-hydroxysteroid dehydrogenase and steroid 17α-hydroxylase. Biochim Biophys Acta 360:88
6. Goebelsmann U, Hall TD, Paul WL, Stanczyk FZ 1975 *In vitro* steroid metabolic studies in testicular 17β-reduction deficiency. J Clin Endocrinol Metab 41:1136
7. Kaufman FR, Costin G, Goebelsmann U, Stanczyk F, Zachmann M 1983 Male pseudohermaphroditism due to 17,20-desmolase deficiency. J Clin Endocrinol Metab 57:32
8. Kershnar AK, Borut D, Kogut MD, Biglieri EG, Schambellan M 1976 Studies in a phenotypic female with 17-alpha-hydroxylase deficiency. J Pediatr 89:395
9. Nakajin S, Shively JE, Yuan P-M, Hall PF 1981 Microsomal cytochrome P-450 from neonatal pig testis: two enzymatic activities (17α-hydroxylase and C-17,20-lyase) associated with one protein. Biochemistry 20:4037
10. Oshima H, Ishiwata D, Hatakeyama S, Yokokawa M 1980 *In vitro* steroidogenesis in testes of three infants, two with ambiguous external genitalia and one with true precocious puberty: evidence for the presence of active 17β-hydroxysteroid oxidoreductase in immature human testes. Endocrinol Jpn 27:59
11. Oshima H, Sarada T, Ochi-Ai K, Tamaoki B-I 1967 Intracellular distribution and properties of steroid 16α-hydroxylase in human testes. J Clin Endocrinol Metab 27:1249
12. Pang S, Levine LS, Stoner E, Opitz JM, Pollack MS, DuPont B, New MI 1983 Non-salt-losing congenital adrenal hyperplasia due to 3β-hydroxysteroid dehydrogenase deficiency with normal glomerulosa function. J Clin Endocrinol Metab 56:808
13. Parks GA, Bermudez JA, Anast CS, Bongiovanni AM, New MI 1971 Pubertal boy with the 3β-hydroxysteroid dehydrogenase defect. J Clin Endocrinol Metab 33:269
14. Rodriguez-Rigau LJ, Weiss DB, Smith KD, Steinberger E 1978 Suggestion of abnormal testicular steroidogenesis in some oligospermic men. Acta Endocrinol 87:400
15. Rosenfield RL, Rich BH, Wolfsdorf JI, Cassorla F, Parks JS, Bongiovanni AM, Wu CH, Shackleton CHL 1980 Pubertal presentation of congenital Δ5-3β-hydroxysteroid dehydrogenase deficiency. J Clin Endocrinol Metab 51:345
16. Saez JM, Frederich A, Bertrand J 1971 Endocrine and metabolic studies in children with male pseudohermaphroditism. J Clin Endocrinol Metab 32:611
17. Saez JM, Morera AM, DePeretti E, Bertrand J 1972 Further *in vitro* studies in male pseudohermaphroditism with gynecomastia due to a testicular 17-ketosteroid reductase defect (compared to a case of testicular feminization). J Clin Endocrinol Metab 34:598
18. Wilson JD, Griffin JE, Leshin M, MacDonald PC 1983 The androgen resistance syndromes: 5α-reductase deficiency, testicular feminization, and related disorders. In "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, eds.), pp. 1001–1026. McGraw-Hill Book Company, New York, 1983.
19. Zachmann M 1974 Evaluation of gonadal function in childhood and adolescence. Helv Paediatr Acta Suppl 34:53
20. Zachmann M, Vollmin JA, Hamilton W, Prader A 1972 Steroid -17,20-desmolase deficiency: a new cause of male pseudohermaphroditism. Clin Endocrin 1:369
21. This work was supported in part by Grant HD 11678 from the National Institute for Child Health and Human Development and Grant RR36 from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health. Presented in part at the combined meeting of the American Pediatric Society and the Society for Pediatric Research, May 6, 1982, Washington, D. C.
22. We would like to thank Dr. Claude Migeon for his help with the steroid assays on the serum samples of Case 1 and 2 and Aileen Derhake and Kim Kendall for help in the preparation of the manuscript.
23. Requests for reprints may be addressed to Dr. Fred Chasalow, Schneider Children's Hospital, Division of Endocrinology, Long Island Jewish-Hillside Medical Center, New Hyde Park, NY 11042.
24. Received for publication December 7, 1983.