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An Improved Method for Evaluating Testosterone Biosynthetic Defects

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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 × *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 × 1.9 cm Sephadex LH-20 column with the solvent isooctane: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.

RESULTS

In order to select the appropriate time of incubation for recognition of defects, detailed time studies were performed for both Case 1 and Case 2. The results from Case 2 are shown in Figure 1. The material in Peak I (identified as progesterone) was almost completely metabolized during the first 10 min. The Peak III steroids (17-hydroxyprogesterone and testosterone) reached a maximum at 5 min and then declined as the 17-hydroxyprogesterone was further metabolized to androstenedione which chromatographed separately in Peak II. Peak IV increased until progesterone was no longer present and was not further metabolized. The results from Case 1 differed from these only in that androstenedione did not accumulate. The amount of testosterone present in Peak III was quantitated after acetylation and rechromatography.

Table 1. *Clinical presentation of the cases studied*

	Case 1	Case 2	Case 3
Age (yr)	1.93	9.0	0.04
Karyotype	46 XY	46 XY	47 XY (+21)
Clitoromegaly	+	+	+
Labial fusion	+	+	+
Hypospadias	3°	3°	3°
Testosterone (ng/dl)			
Pre-hCG*	32	42	31
Post-hCG	642	125	31
Androstenedione (ng/dl)			
Pre-hCG	36	NM†	98
Post-hCG	181	249	91
17-hydroxyprogesterone (ng/dl)			
Pre-hCG	30	NM	56
Post-hCG	398	334	31
Progesterone (ng/dl)			
Pre-hCG	36	NM	NM
Post-hCG	106	89	NM

* 5000 IU hCG/m^2/day for 4 days; serum obtained 24 h after the late dose of hCG.

† NM, not measured.

In both cases almost all of the [^{3}H]progesterone and a large portion of the 17-hydroxy[4-^{14}C]progesterone had been metabolized after 10 min. Because the microsomes were resuspended in a volume according to the weight of tissue obtained, suspensions of microsomes from patients without enzyme blocks would have about the same enzymatic activity. Although the absolute amounts of each of the products changed with the time course, after 2 min of incubation, substantial amounts of testosterone were formed with Case 1 and with Case 2 after 5 min of incubation, substantial amounts of androstenedione were formed. Thus, the metabolic pattern was established within 5 min and in both cases more products accumulated at the 10- and 20-min time points. To avoid the consequences of any possible alterations in Leydig cell density, the 10-min time point was selected for presentation.

The chromatograms obtained following incubation of the testicular microsomes of Case 1 are shown in Figure 2. The pattern of tritium and carbon-14 label in each peak helps to confirm structural assignments based on chromatographic mobility and on cochromatography with each specific carrier. Peaks labeled only with tritium must be derived solely from progesterone and not from 17-hydroxyprogesterone. Thus, Peak I is labeled only with tritium and is progesterone, the starting material. The material in the region of the chromatogram labeled Peak II is not altered by attempted acetylation and contains both carbon-14 and tritium labels; it has been identified as androstenedione on the basis of its label pattern and crystalization to constant specific activity with authentic material (2). In this instance, there is no definite peak of radioactivity in the region of Peak II as the androstenedione is rapidly metabolized and does not accumulate. The material in Peak III which contains both carbon-14 and tritium labels can be resolved into two peaks on rechromatography after acetylation as shown in Figure 2*B*. The peaks are testosterone acetate and 17-hydroxyprogesterone. The identity of the steroids in Peak IV is less clear. Acetylation and rechromatography resolve this material into two peaks, both of which have decreased polarity (Fig. 2*C*). The greater of these peaks contains only tritium label and cochromatographs with 16α-hydroxyprogesterone (or 16β-hydroxyprogesterone) both as the free compound and as the acetate. As testicular homogenates

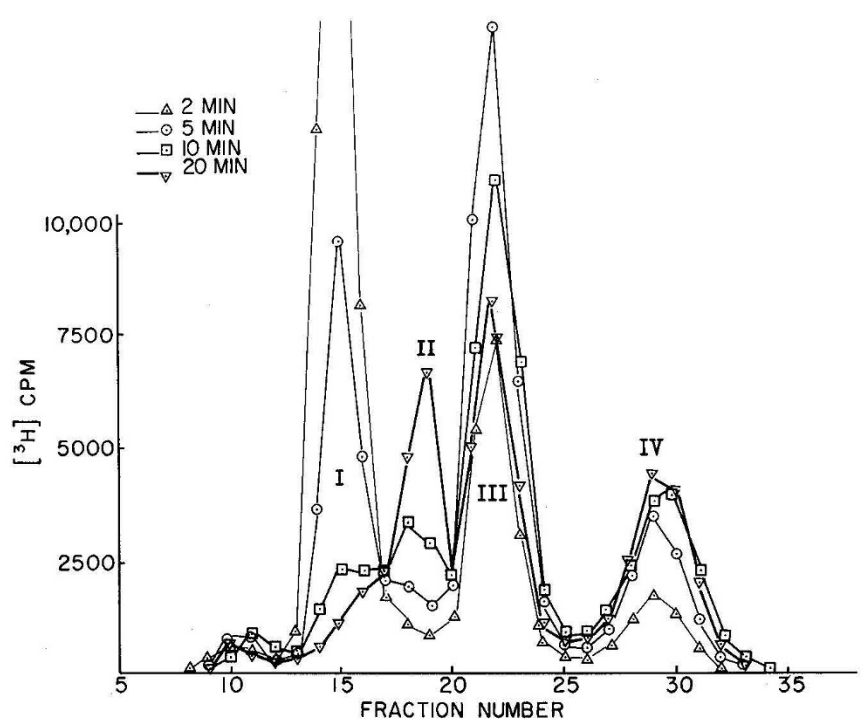

Fig. 1. Time course of [7-^{3}H]progesterone metabolism. Sephadex LH-20 column chromatograms obtained from Case 2 after different periods of incubation. The peaks represent: *I*, progesterone; *II*, androstenedione; *III*, testosterone and 17-hydroxyprogesterone; *IV*, 16α-hydroxyprogesterone and 17α,20ξ-dihydroxy-4-pregnen-3-one.

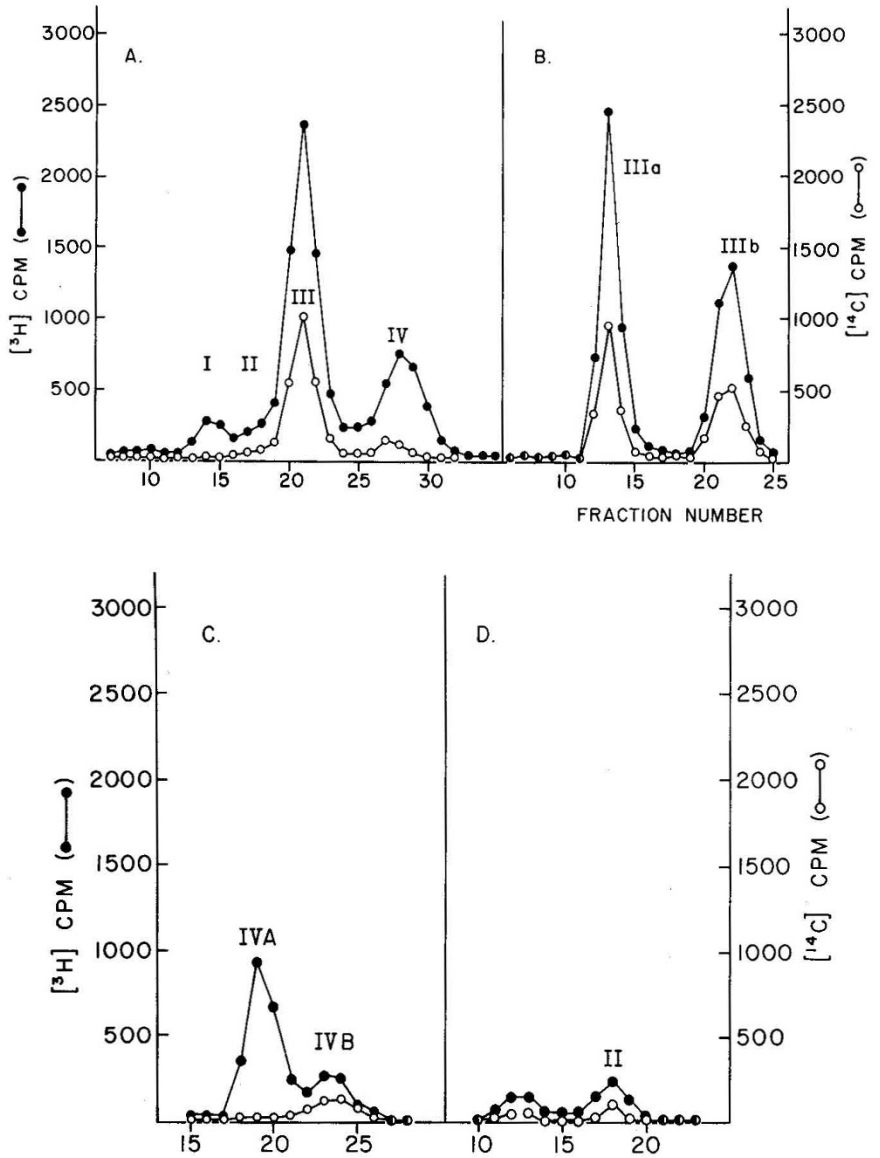

Fig. 2. Sephadex LH-20 column chromatograms from Case 1. *A* is the original chromatogram obtained after [7-^{3}H]progesterone and 17-hydroxy[4-^{14}C]progesterone were incubated for 10 min with microsomes prepared from testes of Case 1. *B*, *C*, and *D* show the rechromatography after acetylation of *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-hydroxyprogesterone 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

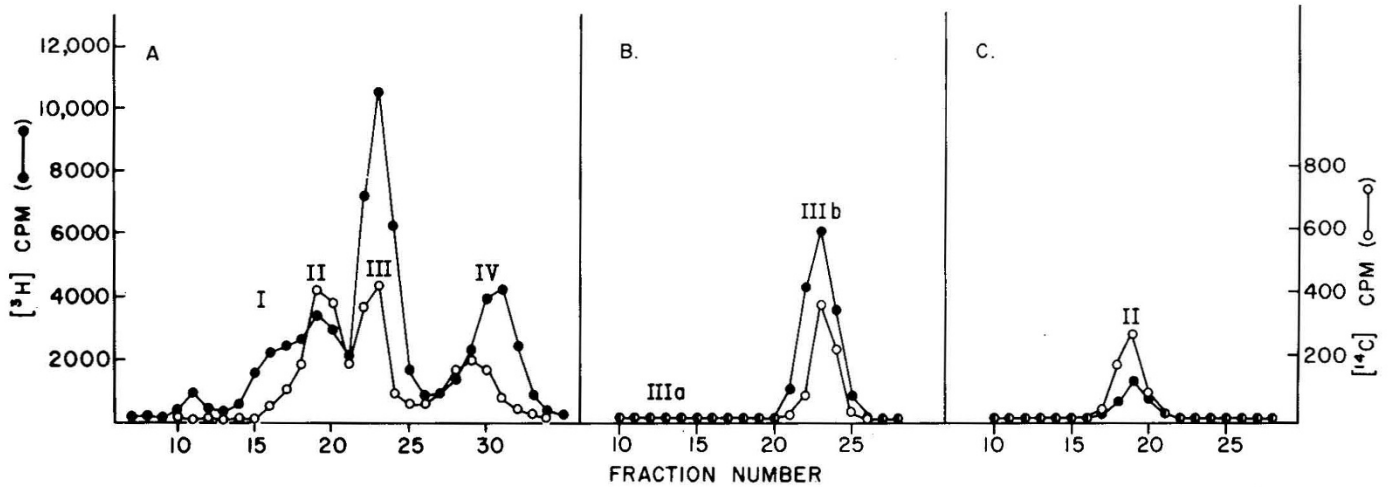

Fig. 3. Chromatograms from Case 2. *A* is the original chromatogram. *B* and *C* show the rechromatography after acetylation of *Peaks III* and *II*, respectively. See Figure 2 for details.

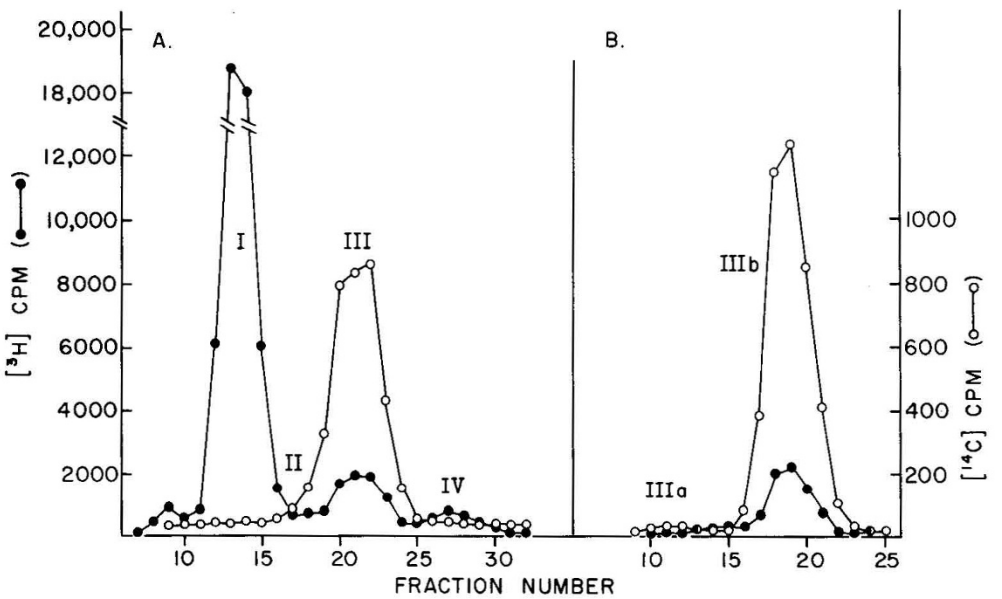

Fig. 4. Chromatograms from Case 3. *A* shows the original chromatogram from Case 3. *B* shows the rechromatography of *Peak III*. See Figure 2 for details. Note the differences in scale for ^{3}H and ^{14}C.

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 (^{3}H, ^{14}C, 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.

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