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FURTHER *IN VITRO* STEROID METABOLIC STUDIES OF TESTICULAR 17β-REDUCTION DEFICIENCY

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SUMMARY

The conversion of androstenedione (A) to testosterone (T) by homogenates of testes from a male pseudohermaphrodite with a testicular 17β-reduction deficiency (17RD) was examined in relation to time while using individual cofactors, NADH or NADPH. Little T was produced when NADPH was added, whereas NADH effected a significant increase in T formation, in contrast to testes from a control which reduced A to T nearly maximally with NADPH but formed very little T with NADH. The conversion of A to T by the 17RD testes was then studied using varying concentrations of NADH and of NADPH. Similar studies were carried out for the conversion of dehydroepiandrosterone (DHA) to 5-androstene-3β,17β-diol (A-DIOL), estrone (E_1) to estradiol (E_2), as well as for the reverse reactions including T to A. The results indicate that in the control testes, NADPH was the preferred cofactor for 17β-reduction of A and E_1, but NADH effected a greater conversion of DHA to A-DIOL. In the 17RD testes, however, there was a sizable reduction of A and DHA only in the presence of NADH. In the reverse reactions in the control testes, NAD^+ was the preferred cofactor for the oxidation of T to A, whereas $NADP^+$ rather than NAD^+ effected a greater conversion of E_2 and A-DIOL to E_1 and DHA, respectively. In contrast, the 17RD testes oxidized T, E_2 and A-DIOL primarily in the presence of NAD^+.

The above findings are consistent with the assumption that the reduction defect may be due to decreased affinity of a single 17β-hydroxysteroid oxidoreductase (17β-HSOR) or that two or more substrate and/or cofactor specific 17β-HSORs are present in normal testes and that the one requiring NADPH is deficient or has decreased substrate and/or cofactor affinity in the 17RD testes.

INTRODUCTION

Recently we reported a detailed *in vitro* study of steroid biosynthesis in testicular tissue from a male pseudohermaphrodite with a testicular 17β-reduction deficiency (17RD) [1, 2]. The results obtained from incubations of control and 17RD testicular minces with dehydroepiandrosterone (DHA), 4-androstene-3,17-dione (A) and estrone (E_1) showed that only minimal quantities of these substrates were converted to their 17β-reduced counterparts by the 17RD testes. 17β-Reduction increased slightly but was not restored to control levels upon addition of NADH plus NADPH. However, oxidation of testosterone (T) to A by 17RD testicular minces was comparable to that by the control, with and without the addition of NAD^+ plus $NADP^+$.

Since the observation that 17β-reduction in the 17RD testicular tissue was only slightly increased upon addition of NADH plus NADPH, but that oxidation of T to A was normal could not be explained, it was considered of interest to study further the conversion of A to T by this tissue. Consequently, we first examined the conversion of A to T in relation to time while using individual cofactors, NADH or NADPH. Furthermore, since there have been no reports of studies evaluating the effect of varying cofactor concentrations on steroid transformations by 17RD testicular tissue, it seemed important to investigate this aspect of steroid metabolism in the enzymatic conversion of DHA, A and E_1 to their 17β-reduced products as well as in the reverse reactions.

MATERIALS AND METHODS

Patients. Testicular tissue was obtained from 2 male pseudohermaphrodites who underwent gonadectomy. One patient was a 46-year-old phenotypic female with gynecomastia and virilization whose disorder, based on *in vivo* endocrine studies [3] as well as *in vitro* studies of testicular steroid biosynthesis [2], was compatible with a testicular 17β-reduction deficiency. The other patient was a 20-year-old phenotypic female with virilization but no breast development. The *in vivo* endocrine findings and the *in vitro* steroid metabolic results with testicular minces indicate that this patient may represent a case of 5α-reductase deficiency with apparently normal testicular testosterone biosynthesis [2]. For these reasons and because no hormones were given during the 4 weeks preceding gonadectomy, this patient's testes were used as control tissue for this study.

Solvents and reagents. All solvents and reagents were of analytical grade. Solvents were distilled prior to use, with the exception of diethyl ether (USP) and

absolute ethanol (USP), which were tested prior to use to ascertain the absence of peroxides. NAD^+, $NADP^+$ NADH and NADPH were purchased from Sigma Chemical Co.

Steroids. Non-radioactive steroids were purchased from Sigma Chemical Co. or Steraloids, Inc. and were recrystallized twice prior to use. The following radioactive steroids were obtained from New England Nuclear Corp. or Amersham/Searle Corp. and were checked for purity by subjecting aliquots of each to paper partition chromatography (PPC) in 2 solvent systems and recrystallization to constant S.A.: [4-^{14}C]-DHA (S.A. 52 mCi/mmol), [4-^{14}C]-A (S.A. 60 mCi/mmol), [4-^{14}C]-E_1 (S.A. 58 mCi/mmol), [4-^{14}C]-T (S.A. 56 mCi/mmol), [4-^{14}C]-estradiol-17β (E_2, S.A. 58 mCi/mmol), [7-^{3}H]-DHA (S.A. 14 Ci/mmol), [1,2-^{3}H]-A (S.A. 48 Ci/mmol), [6,7-^{3}H]-E_1 (S.A. 54 Ci/mmol), [7-^{3}H]-T (S.A. 40 Ci/mmol) and [6,7-^{3}H]-E_2 (S.A. 49 Ci/mmol). Tritiated as well as ^{14}C-labeled 5-androstene-3β,17β-diol (A-DIOL) were prepared by reduction of [7-^{3}H]-DHA and [4-^{14}C]-DHA, respectively, as described previously [2].

Incubation procedure. At each operation the blood supply of that testis which was dissected free first was left intact until the second testis could be removed almost simultaneously with the first one. Specimens for biopsy and karyotyping were removed at once and the remaining tissue was placed on ice and then homogenized in calcium-free Krebs–Ringer phosphate buffer (KRPB), pH 7.4, using a Potter–Elvehjem homogenizer.

In the time study, 100 mg of homogenized testes (wet weight) was suspended in 4 ml of KRPB. Individual cofactors were added in 1 ml of KRPB to give a final cofactor concentration of 0.5 mM. Approximately 0.5 μCi of [4-^{14}C]-A (8.3 nmol) was added to each incubation flask in 50 μl of absolute ethanol. The samples were then incubated for 0, 5, 10, 30 and 60 min at 37°C in air in a Dubnoff metabolic shaker.

In the studies in which the effect of various cofactor concentrations was evaluated, 35 mg of homogenized testes (wet weight) was suspended in 2 ml of KRPB. Individual cofactors were added in 0.5 ml KRPB to give the following final concentrations: 0.05 mM, 0.1 mM, 0.5 mM or 1.0 mM. The following tritiated substrates were then added in 50 μl of absolute ethanol: DHA, A, E_1, A-DIOL, T or E_2 in amounts ranging from 16 to 228 pmol. Also, with each of the 6 precursors incubated, a blank was run. Blanks were prepared by adding the appropriate radioactive substrate and cofactor to 35 mg of homogenized testes which were first boiled. All samples were incubated for 2 h at 37°C.

In both studies, incubation was terminated by adding 10 ml of boiling ethanol to each flask.

Extraction, isolation and identification of labeled steroids. Prior to extraction, metabolic precursors and principal expected metabolites were added as unlabeled and tritiated or carbon-14 steroids to all incubation flasks in order to facilitate detection of steroids on paper chromatograms and correct for procedural losses. The incubate was then centrifuged and the supernatant, which contained the steroids, was removed. The homogenate was washed 3 times with 10 ml of 80% ethanol and was centrifuged each time. The pooled supernatants were evaporated to dryness and residues of the extracts were subjected to ethyl acetate/water partition. The pooled ethyl acetate fraction was then washed with water, dried over anhydrous sodium sulfate and evaporated to dryness.

Table 1. Paper chromatographic systems employed

Systems	Solvents (by vol)
A	Isooctane–t-butanol–methanol–water (10:2:7:1)
B	Isooctane–toluene–methanol–water (25:75:80:20)
C	Skellysolve C–methanol–water (5:4:1)

Individual labeled steroids were isolated by paper partition chromatography in 2 different solvent systems (Table 1) as shown in Table 2. Radioactivity on the chromatograms was detected with a Packard Radiochromatogram Scanner (Model 7201). The isolated steroids were identified by recrystallization to constant ^{3}H to ^{14}C ratio after being diluted with the appropriate authentic steroid.

Calculation of percent conversion. Liquid scintillation counting was carried out in a Mark II Nuclear-Chicago Liquid Scintillation Counter. Background, counting efficiencies and the percentage of c.p.m. appearing in the opposite channel were monitored carefully throughout this study, using internal standards. All data were were corrected for the half-life of tritium. Conversion of c.p.m. to d.p.m., calculation of ^{3}H/^{14}C ratios and counting precision estimates were carried out on an IBM 360/50 computer.

Recovery of labeled substrates and conversion to labeled metabolites (c) was calculated as percent of labeled substrate according to the following formula in which p represents d.p.m. of precursor incubated, t the d.p.m. of the precursor or metabolite added as internal standard and r either the ^{3}H/^{14}C (when ^{14}C-labeled precursors were used) or ^{14}C/^{3}H (when

Table 2. Paper partition chromatography (PPC) of isolated steroids

Isolated steroid	Solvent system (duration of run) PPC 1	PPC 2
Dehydroepiandrosterone	C (8 h)	A (12 h)
4-Androstene-3,17-dione	C (8 h)	A (8 h)
Estrone	B (S.F.*)	C (20 h)
5-Androstene-3β,17β-diol	A (24 h)	C (40 h)
Testosterone	C (10 h)	A (21 h)
Estradiol-17β	B (S.F.*)	A (30 h)

* S.F. = Solvent front to end of paper.

^{3}H-labeled precursors were used) ratio of the final crystals of the particular precursor or metabolite isolated:

$$c = (t/p) \times (100/r)$$

RESULTS

Time study

In the control, A to T reduction was nearly maximal (30.3% conversion) after 5 min incubation when NADPH was added (Fig. 1), whereas A to T conversion with NADH was only 1.1% after 60 min. In the 17RD testes, to the contrary, there was an approximately 4-fold greater formation of T from A with NADH than with NADPH after 60 min. In order to demonstrate that our findings did not result from the particular cofactor concentration used (0.5 mM), the conversion of A to T was studied at a constant incubation time (2 h) using varying concentrations of NADPH and of NADH. The results of the latter study as well as the effect of varying cofactor concentrations on the other reactions are described below.

Effect of varying cofactor concentrations

Figures 2–4 show the results of conversion studies of 17β-reduction of A, E_1 and DHA to T, E_2 and A-DIOL, respectively, with varying concentrations of NADH or of NADPH. With A as precursor (Fig. 2), the highest conversion in the control was observed in response to NADPH, in contrast to the 17RD testes which showed only minute conversions to T with this cofactor. However, with increasing concentrations of NADH, the 17RD testes increased the conversion of A to T similar to that produced by the control in response to NADH. Reduction of E_1 to E_2 (Fig. 3) was greater with NADPH than with

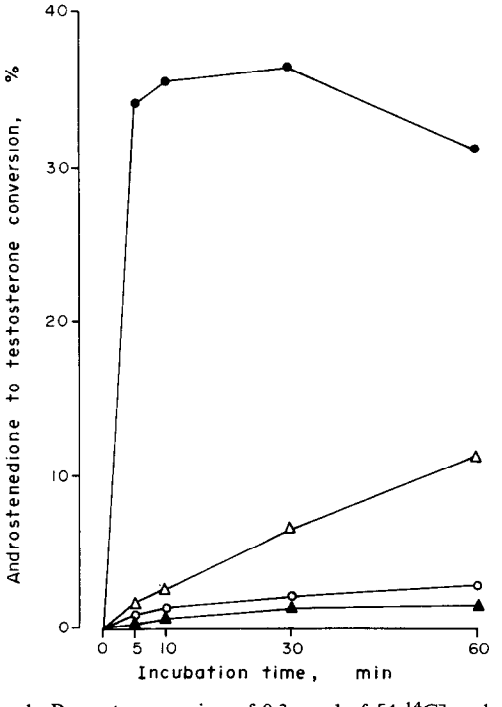

Fig. 1. Percent conversion of 8.3 nmol of [4-^{14}C]-androstenedione to testosterone by 100 mg of testicular homogenate from a male pseudohermaphrodite with testicular 17β-reduction deficiency (open symbols) and from a control (filled symbols) incubated at 37°C for different time intervals with 0.5 mM NADH (triangles) and NADPH (circles).

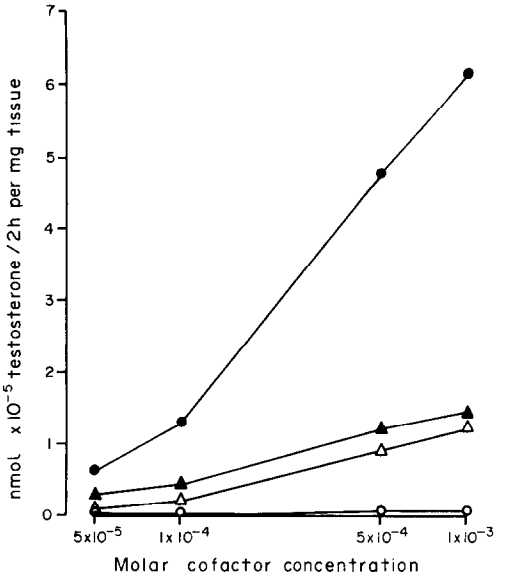

Fig. 2. Reduction of androstenedione to testosterone during 2 h incubation at 37°C with testicular homogenate from a male pseudohermaphrodite with testicular 17β-reduction deficiency (open symbols) and from a control (filled symbols) at various concentrations of NADH (triangles) and NADPH (circles).

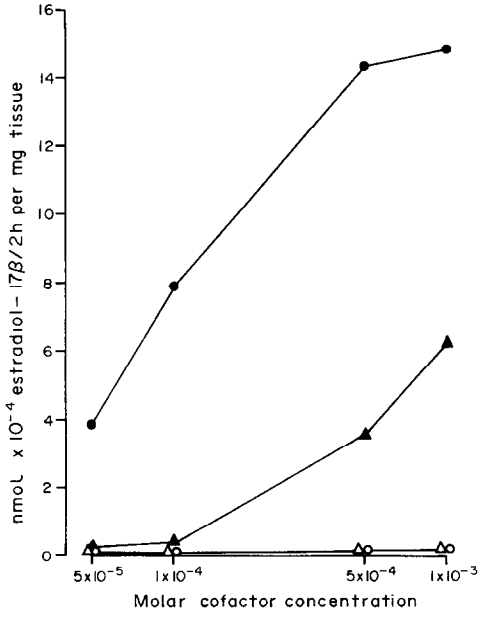

Fig. 3. Reduction of estrone to estradiol-17β during 2 h incubation at 37°C with testicular homogenate from a male pseudohermaphrodite with testicular 17β-reduction deficiency (open symbols) and from a control (filled symbols) at various concentrations of NADH (triangles) and NADPH (circles).

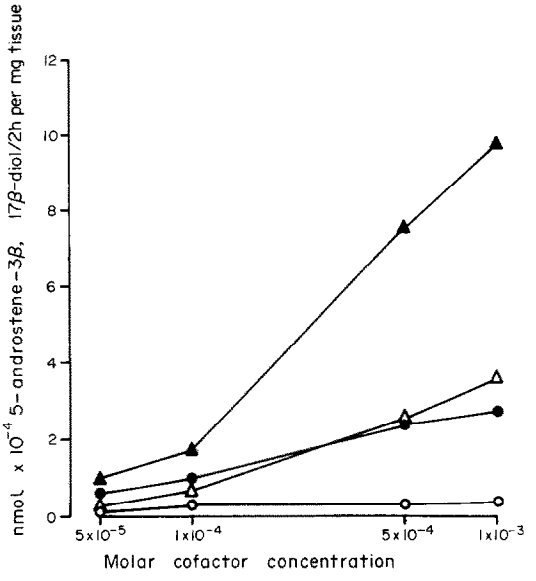

Fig. 4. Reduction of dehydroepiandrosterone to 5-androstene-3β,17β-diol during 2 h incubation at 37°C with testicular homogenate from a male pseudohermaphrodite with testicular 17β-reduction deficiency (open symbols) and from a control (filled symbols) at various concentrations of NADH (triangles) and NADPH (circles).

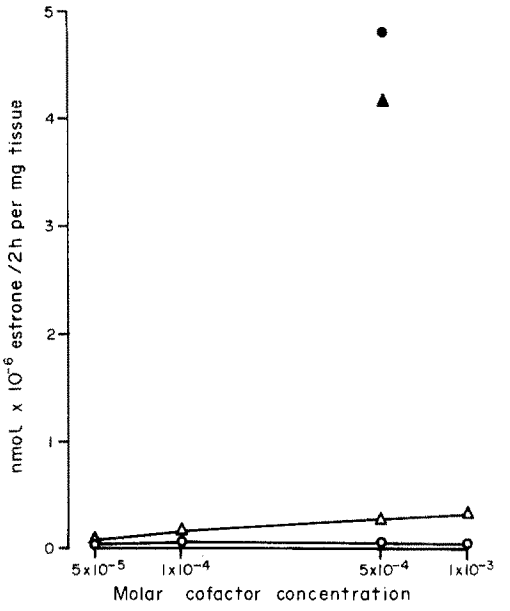

Fig. 6. Oxidation of estradiol to estrone during 2 h incubation at 37°C with testicular homogenate from a male pseudohermaphrodite with testicular 17β-reduction deficiency (open symbols) and from a control (filled symbols) at various concentrations of NAD^+ (triangles) and $NADP^+$ (circles).

NADH in the control. The 17RD testes, on the other hand, reduced only minute amounts of E_1 with either cofactor. When DHA was used as precursor (Fig. 4), a much greater conversion was observed in the control tissue with NADH than with NADPH. In the 17RD testes, NADH effected a sizable conversion of DHA to A-DIOL, but only a minute conversion was observed with NADPH.

Figures 5–7 show the results of conversion studies of the oxidation of T, E_2 and A-DIOL to A, E_1 and DHA, respectively, with varying concentrations of NAD^+ and of $NADP^+$. In the control, only one concentration of each cofactor was used since we were limited by tissue availability.

The results of the oxidation of T (Fig. 5) by the control tissue showed extensive conversion to A with

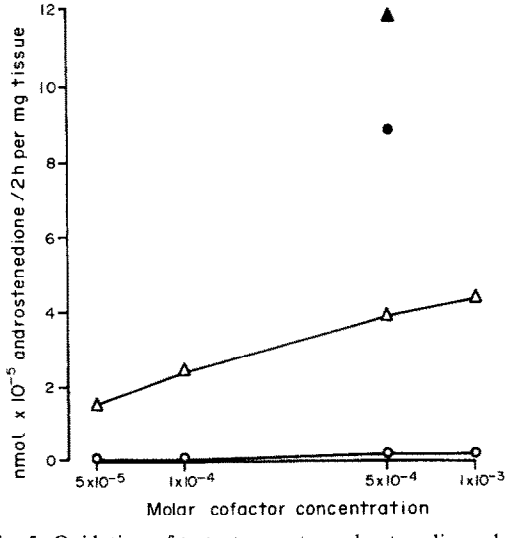

Fig. 5. Oxidation of testosterone to androstenedione during 2 h incubation at 37°C with testicular homogenate from a male pseudohermaphrodite with testicular 17β-reduction deficiency (open symbols) and from a control (filled symbols) at various concentrations of NAD^+ (triangles) and $NADP^+$ (circles).

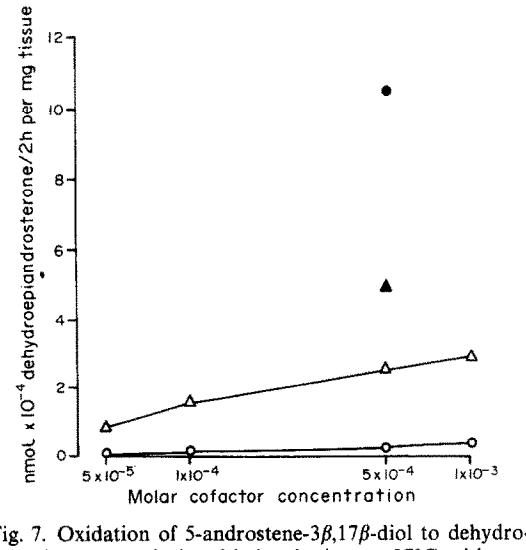

Fig. 7. Oxidation of 5-androstene-3β,17β-diol to dehydroepiandrosterone during 2 h incubation at 37°C with testicular homogenate from a male pseudohermaphrodite with testicular 17β-reduction deficiency (open symbols) and from a control (filled symbols) at various concentrations of NAD^+ (triangles) and $NADP^+$ (circles).

either 0.5 mM NAD^+ or $NADP^+$, but NAD^+ appeared to be the preferred cofactor at that concentration. The 17RD testes, in contrast, showed a sizable conversion of T to A only with NAD^+. In the formation of E_1 from E_2 (Fig. 6) by the control, 0.5 mM $NADP^+$ rather than 0.5 mM NAD^+ effected a slightly higher conversion of E_2 to E_1 in contrast to the 17RD testes which produced a greater conversion with NAD^+ than with $NADP^+$. Finally, the oxidation of A-DIOL to DHA (Fig. 7) by the control testes showed that 0.5 mM $NADP^+$ effected a much greater conversion than 0.5 mM NAD^+. With the 17RD testes, only NAD^+ produced a sizable conversion of A-DIOL to DHA.

DISCUSSION

This study extends our earlier *in vitro* steroid metabolic results [2] which demonstrated a 17β-reduction deficiency in the 17RD testes. The findings in the present study show decreased activity of the 17β-hydroxysteroid oxidoreductase (17β-HSOR) in the 17RD testes in the presence of the preferred cofactor, NADPH, but not NADH, with either DHA or A as substrate. In addition, our results demonstrate decreased 17β-HSOR activity in the 17RD testes with $NADP^+$, and to a smaller extent with NAD^+, following incubation of either of the 17β-reduced counterparts of the above steroids, namely, A-DIOL and T. Very recently, Pittaway *et al.* [4] reported similar *in vitro* studies in which they found decreased 17β-HSOR activity in the presence of NADPH, but not NADH, using DHA or A as substrates with the microsomal fraction of testicular tissue from a male pseudohermaphrodite. This independent demonstration of impaired NADPH-dependent 17β-HSOR activity indicates that this defect may be a common characteristic in male pseudohermaphroditism with testicular 17RD.

The results obtained from the incubations of control and 17RD testicular homogenates with E_1 as substrate show that there was decreased activity of 17β-HSOR in the 17RD testes with either cofactor, NADH or NADPH. Pittaway *et al.* [4] obtained similar results when they incubated E_1 with the microsomal fraction of testicular tissue from a male pseudohermaphrodite using either NADH or NADPH as cofactor. These findings differ from those reported by Schneider and Bardin [5] who found only a small diminution of 17β-reduction of E_1 but a significant testicular deficiency in 17β-reduction of A in 17RD testes from male pseudohermaphrodite rats.

Although in the control a single cofactor concentration (0.5 mM) was used in the study of the oxidation of A-DIOL, T and E_2 due to a limited amount of control testes, this concentration was near optimal for this conversion. Comparisons between control and 17RD testes for the reverse reactions were made only at the 0.5 mM concentration.

The finding that there is decreased 17β-HSOR activity in the 17RD testes with $NADP^+$, and to a smaller extent with NAD^+, when incubated with either A-DIOL or T is surprising in view of our previous *in vitro* data [2]. These results showed that the 17RD testes oxidized proportions of T to A which were comparable to the control, without and with additional NAD^+ plus $NADP^+$. One explanation for this discrepancy could be the existence of a rapid transhydrogenation between the NADH, formed by oxidation of the substrate, and $NADP^+$, making more NAD^+ available for further oxidation of substrate.

Incubation of E_2 with the 17RD testes gave results similar to those found for the forward reaction, i.e., there was decreased activity of 17β-HSOR with either NAD^+ or $NADP^+$. These findings show that in the 17RD testes there is impaired 17β-HSOR activity for the interconversion of E_1 and E_2 with either cofactor.

The exact molecular defect in the 17RD testes cannot be ascertained from the present study. The reduction defect may be due to decreased affinity of a single 17β-HSOR for NADPH. However, our data would also be consistent with the assumption that two or more substrate and/or cofactor specific 17β-HSORs are present in normal testes, and that the one requiring NADPH is deficient or has decreased substrate and/or cofactor affinity in the 17RD testes. Very recently, Murono and Payne [6] reported data which demonstrates the existence of 2 distinct 17β-HSORs in adult rat testes, one in the interstitial tissue and the other in seminiferous tubules. Further studies must be carried out to define the exact molecular defect in the 17RD testes.

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