Studies on the Pathogenesis of the Pseudohermaphroditism in the Mouse with Testicular Feminization

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ABSTRACT The pathogenesis of the male pseudohermaphroditism in the mouse with X-linked testicular feminization (*Tfm*) has been investigated by comparing testosterone formation, the effects of androgen administration, and the metabolism of testosterone-1,2-^{3}H in normal mice and *Tfm* mice of varying ages. First, it was established that the adult *Tfm* animal, in contrast to the human with testicular feminization, has both a low serum testosterone and a low rate of testosterone formation as assessed in slices of testes utilizing a variety of precursors. However, the formation of testosterone from pregnenolone-7α-^{3}H was shown to be normal in newborn *Tfm* testes, suggesting that a defect in testosterone synthesis may not be primary to this mutation. Second, to establish that the pseudohermaphroditic state is due to androgen resistance rather than to diminished androgen biosynthesis during fetal life, the effect of the administration of dihydrotestosterone to pregnant animals was studied in male, female, and *Tfm* offspring. Whereas normal and carrier female littermates demonstrated striking virilization of the internal genital tract after such treatment, there was no sign of virilization in the *Tfm* animals. This finding provides direct experimental evidence in support of the view that male pseudohermaphroditism in testicular feminization is the result of resistance to androgen action during androgen-mediated sexual differentiation in embryos. Third, the metabolism of testosterone-1,2-^{3}H was investigated both in tissue slices and in functionally hepatectomized animals. Dihydrotestosterone formation in tissue slices of the fetal anlage of the male organs of accessory reproduction is normal in the *Tfm* animal, suggesting that the primary defect in this disorder involves an intracellular event subsequent to this step and that the deficient dihydrotestosterone formation observed in the adult genital tract of the *Tfm* mouse is secondary to the failure of differentiation in these tissues. Finally, deficient binding of testosterone in the nuclei of the submandibular gland of adult *Tfm* animals, a known testosterone target tissue, was demonstrated in functionally hepatectomized mice. This finding could either be a manifestation of the primary genetic defect in this disorder or might reflect another acquired abnormality due to incomplete differentiation of adrogen-sensitive cell lines.

INTRODUCTION

An inherited syndrome of testicular feminization has now been described in several species in addition to man (1), including the cow (2), the dog (3), the rat (4, 5), and the mouse (6, 7). In those species in which the disorder has been characterized in detail, the affected male pseudohermaphrodites manifest several features in common, namely external genitalia and breasts characteristic of phenotypic females, an absence of internal genitalia other than the testes, and striking resistance to endogenous and exogenous androgens (5, 7–9). Indeed, in accordance with the theories formulated by Wolff (10), by Jost (11), and by others (12, 13) that the differentiation of the male internal and external genitalia is mediated by androgen secreted by the fetal testes, it has been assumed that resistance to the action of testosterone [1] is the primary defect in this condition and that

Dr. Goldstein is the recipient of a Special National Institutes of Health Fellowship GM 47841-01.

Received for publication 1 November 1971 and in revised form 14 February 1972.

[1] *Abbreviations and trivial names used in this paper:* androstenedione, androst-4-ene-3,17-dione; androsterone, 3α-hydroxy-5α-androstan-17-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 17-hydroxy-pregnenolone 3β,17α-dihydroxy-preg-5-en-20-one; 17-hydroxy-progesterone, 17α-hydroxy-preg-4-ene-3,20-dione; pregnenolone, 3β-hydroxy-preg-5-en-20-one; progesterone, preg-4-en-3,20-dione; *Ta*, Tabby gene; testosterone, 17β-hydroxyandrost-4-en-3-one; *Tfm*, testicular feminization.

the failure of male differentiation of the internal and external genitalia during embryogenesis is secondary to this abnormality in testosterone action (5–9). In keeping with this hypothesis, a number of recent studies have appeared attempting to explain the biochemical basis of the androgen resistance in testicular feminization. For example, in testicular feminization in man the conversion of testosterone to its active derivative dihydrotestosterone is deficient in perineal skin (14–16). In affected rats (17) and in affected mice (18, 19) it has been reported that the intracellular binding of androgen in target tissues, such as the preputial gland and the kidney, is diminished despite normal rates of dihydrotestosterone formation.

These conflicting results can be explained in one of several possible ways. First, genetic heterogeneity of the testicular feminization phenotype might exist among the different species or even within the same species, and this would be expected to result in different biochemical abnormalities in the various affected animal or human subjects despite similarities in the resulting phenotypes. Second, all studies to date both in man and in affected animals have been performed on tissues taken either from sexually mature subjects or from humans several years after the completion of sexual differentiation, and although these accessory sex tissues are normally dependent on androgen for growth and function, they may have differentiated in these mutants without androgen dependent elements. Consequently, it is not known whether any of the various defects reported in this syndrome in man and in animals are primary manifestations of the mutant gene(s) or whether they represent secondary effects of some earlier failure of androgen action which occurred during embryonic sexual differentiation. In fact, at the present time it is unclear whether the cellular mechanisms that mediate androgen action physiologically in the differentiating embryo are identical to those that are thought to mediate growth and function of mature tissues. It is also not known which of the features of testosterone metabolism presumed to be essential to the action of the hormone in the adult might be the consequence of some earlier androgen action in the embryo.

A series of studies was designed, therefore, to investigate the pathogenesis of the male pseudohermaphroditism in the mouse with testicular feminization (*Tfm*) by examining systematically the effect of the *Tfm* mutation on the synthesis of androgen, on the action of androgen during differentiation, and on the metabolism of radioactive testosterone. These processes were studied in the anlage of the internal and external genitalia of affected newborn mice before complete tissue atrophy and compared with those of the analogous differentiated tissues of normal newborn and adult mice in order to distinguish between primary and secondary manifestations of the genetic defect in the testicular feminization syndrome. The results outlined below indicate that in this mutant the failure of male sexual differentiation is indeed the result of a primary resistance to the action of androgen during embryogenesis; that several of the features of the syndrome observed in the adult mouse, including diminished dihydrotestosterone formation and decreased testosterone biosynthesis, are secondary to this fundamental defect; and that the basis of the androgen resistance appears to involve an intracellular event that occurs after the conversion of testosterone to dihydrotestosterone.

METHODS

Treatment of animals. The mice used in these experiments were from a colony developed from two carrier *Tfm* females that were the gift of Dr. S. Ohno. One of these females carried the tabby mutation (*Ta*) on the other X chromosome (*Tfm/Ta*). Since the tabby gene is closely linked to the *Tfm* mutation in these animals (6), matings between *Ta/Tfm* females and *Ta*/Y males were used in experiments in which it was essential to identify carrier female (*Tfm/Ta*), affected male (*Tfm*/Y), and normal male and female (*Ta*/Y and *Ta/Ta*) offspring. In the remaining experiments animals were derived from matings between *Tfm/Ta* or *Tfm*/X females and normal BALB/c males (X/Y).

For studies of the effect of androgen administration on the differentiation of the male and female genital tracts, i.m. injections of dihydrotestosterone (2 mg dissolved in triolein) were begun between days 9 and 11 postcopulation and continued daily until delivery; after delivery, the newborn animals were given 0.2 mg dihydrotestosterone subcutaneously every other day and were killed on day 10.

To study the effects of ligation of the vas deferens and surgically induced cryptorchidism on testosterone synthesis in the testes, normal male littermates of *Tfm* mice were operated at 3 wk of age under ether anesthesia. After ligation of the vas deferens and/or suturing the testes to the abdominal wall, the animals were allowed to recover, and at 6 wk of age the testes were removed for study. All other experiments were performed either on animals 2–5 days after birth or on animals that were between 6 and 8 wk of age as indicated.

Tissue slice studies. For the tissue slice studies, tissues were dissected shortly after death, stored in ice-cold Krebs-Ringer phosphate buffer, pH 7.4, and combined for incubation experiments. In order to obtain adequate tissue for analyses, specimens from 2–4 newborn animals were combined for study. On the average about 60 min elapsed between the time of death and the beginning of the incubations. For the incubation studies tissue slices, approximately 0.2 mm thick, were prepared by hand and weighed on a tissue balance.

The method used for the assessment of rates of dihydrotestosterone formation in small amounts of tissue has been described in detail (20). The standard incubation mixture contained tissue slices (5–15 mg in weight), Krebs-Ringer phosphate buffer, pH 7.4, testosterone-1,2-^{3}H (3.5×10^{-8} M containing 9.5×10^5 cpm), and glucose (1.1×10^{-2} M) in a total volume of 1 ml. The tubes were gassed with 95% oxygen-5% carbon dioxide, capped, and incubated with shaking at 37°C for 2 hr. At the end of the incubation

TABLE I

*Recrystallization of Dihydrotestosterone-^{3}H from Mouse Tissues**

Solvent	Dihydrotestosterone-^{3}H specific activity									
	Adult							Newborn		
	Epididymis and seminal vesicle	Prostate	Uterus and fallopian tubes	Vagina	*Tfm* urogenital sinus	Bladder	Stomach	Urogenital sinus	Bladder	Stomach
	cpm/mg				*cpm/mg*			*cpm/mg*		
Methanol	828	524	438	211	151	740	292	300	452	114
Benzene + heptane	819	504	286	199	108	500	88	253	43	52
Ethyl acetate + pentane	868	680	305	291	143	275	41	268	37	19
Ethyl ether + heptane	894	631	342	302	154	148	59	267	43	13

* Material tentatively identified as dihydrotestosterone-^{3}H was isolated by preparative thin-layer chromatography as described in the text, mixed with 100 mg carrier steroid and recrystallized four times.

period the radioactive metabolites were extracted, chromatographed, and assayed as described (20). To establish the validity of these incubation conditions for mouse tissues, a series of recrystallization experiments were performed in which extracts from 3–5 different incubation mixtures were combined, and material tentatively identified as dihydrotestosterone-^{3}H was isolated by preparative thin-layer chromatography, combined with carrier steroid, and recrystallized four times as described previously (21). In the case of urogenital tissues from adult and newborn animals of both sexes, the final specific activities of the crystals were similar to that of the starting material, as the result of which it was concluded that the thin-layer chromatographic system is valid for estimation of the rates of dihydrotestosterone formation in these tissues (Table I). In two tissues (bladder and stomach), however, some radioactive material chromatographed with dihydrotestosterone but was lost during recrystallization (this substance has been identified tentatively as androsterone), and the apparent rates of dihydrotestosterone formation for these two tissues were suitably corrected.

For studies of the rates of testosterone formation by slices of gonadal tissues, a preliminary series of experiments were performed to establish reliable conditions for this assay (Table II). Testosterone synthesis was shown to be reasonably linear with incubation time between 15 min and 2 hr and to be linear with amounts of tissue slices that varied between 5 mg and 20 mg in weight. At substrate concentrations of pregnenolone-7α-^{3}H and progesterone-1,2-^{3}H that varied between 4×10^{-7} and 2×10^{-6} M no plateau in testosterone formation was observed. The standard incubation mixture contained gonadal slices (10 mg), Krebs-Ringer phosphate buffer, pH 7.4, glucose (1.1×10^{-2} M) and substrate (either pregnenolone-7α-^{3}H, progesterone-1,2-^{3}H, 17α-hydroxy-progesterone-1,2-^{3}H, or androstenedione-1,2-^{3}H) at a concentration of 1×10^{-6} M (and containing $2.5–5.0 \times 10^7$ cpm) in a total volume of 1.0 ml. The latter concentration was chosen arbitrarily after a series of exploratory experiments because it allowed linear rates of synthesis without depletion of substrate during the incubation experiments. The tubes were gassed with 95% O_2-5% CO_2 and incubated at 37°C for 1 hr with shaking. At the end of the incubation the radioactive steroids were extracted with 4 ml of chloroform: methanol (2:1). A 0.1 ml portion of the chloroform: methanol extract (containing about 40,000 cpm ^{3}H) was then combined with a carrier mixture of 10 μg each of seven steroids, taken to dryness, and reconstituted in 25 μl of chloroform. The sample was spotted for thin-layer chromatography on plates of silica gel H and developed in the cold in chloroform: methanol (97:3). The plates were air-dried, stained, and assessed for radioactivity as previously described (22). As is illustrated in Fig. 1, this chromatographic system allows separation of a variety of metabolites that are intermediates in the conversion of pregnenolone to testosterone.

Evidence for the validity of this thin-layer chromatographic technique of assessing testosterone formation in slices of testis is demonstrated in Table III in which material tentatively identified as testosterone-^{3}H after incubation of the slices with pregnenolone-7α-^{3}H or progesterone-1,2-^{3}H was mixed with 200 mg carrier testosterone and recrystallized four times. Since there was no significant decrease in the specific activity it was concluded that this

TABLE II

Establishment of Conditions for the Assessment of Testosterone Formation from Pregnenolone-7α-^{3}H and Progesterone-1,2-^{3}H by Slices of Mouse Testes

Weight of slices	Incubation time	Substrate concentration	Testosterone formation from	
			Pregnenolone-7α-^{3}H	Progesterone-1,2-^{3}H
mg		*M*	*pmoles*	*pmoles*
10	1 hr	4×10^{-7}	77	68
10	1 hr	8×10^{-7}	148	86
10	1 hr	1.2×10^{-6}	192	233
10	1 hr	1.6×10^{-6}	221	299
10	1 hr	2.0×10^{-6}	286	—
10	15 min	1×10^{-6}	88	38
10	30 min	1×10^{-6}	123	51
10	1 hr	1×10^{-6}	162	121
10	2 hr	1×10^{-6}	229	168
5	1 hr	1×10^{-6}	60	52
10	1 hr	1×10^{-6}	162	121
15	1 hr	1×10^{-6}	182	139
20	1 hr	1×10^{-6}	229	208

Slices were incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing glucose (1.2×10^{-2} M) and substrate as indicated in a total volume of 1 ml.

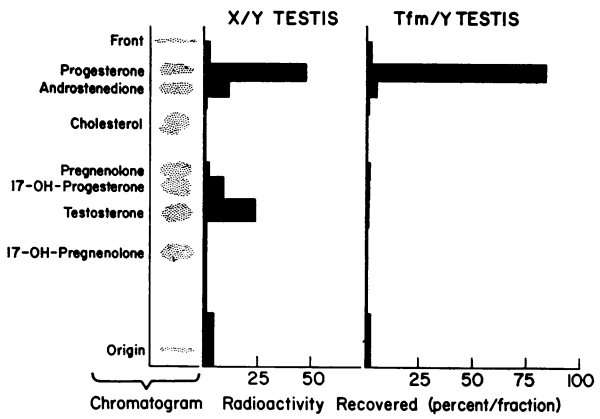

FIGURE 1 Separation by thin-layer chromatography of the metabolites of progesterone-1,2-^{3}H after incubation with slices of testes. Slices of adult testes (15 mg) were incubated with progesterone-1,2-^{3}H (1×10^{-6} M containing 5×10^7 cpm), glucose (1.1×10^{-2} M) and Krebs-Ringer phosphate buffer, pH 7.4, in a total volume of 1.0 ml. After incubation for 1 hr at 37°C, the steroids were extracted into chloroform: methanol (2:1), and portions were spotted on thin-layer chromatograph plates. The plates were developed in chloroform: methanol (97:3), sprayed with anisaldehyde, scraped into counting vials, and assayed for radioactivity.

separation does provide a suitable means for the assessment of testosterone formation in mouse testes. For the assessment of rates of conversion the percentage of the total radioactivity recovered in the testosterone area was multiplied by the amount of substrate steroid added to the original mixture.

Intranuclear localization studies. For studies of the intranuclear localization of radioactive testosterone the mice were subjected to functional hepatectomy under ether anesthesia using the technique described by Hotta and Chaikoff (23). Some animals were subjected to castration 1–2 weeks before study, and some received 2 mg testosterone in oil every other day for 7–10 days before death. In a preliminary series of experiments it was found to be necessary in this procedure to ligate the hepatic artery; otherwise, almost no unmetabolized testosterone could be recovered from the blood at the end of 1 hr. Testosterone-1,2-^{3}H (50 μCi dissolved in saline as described previously (22)) was administered i.v. The animals were killed 1 hr later, and the submandibular glands were removed, carefully dissected free of connective tissue, sliced into small pieces by hand with a razor blade, and homogenized with 10 vol of 0.88 M sucrose-1.5 mM $CaCl_2$ in a specially constructed micro Dounce homogenizer with 20 strokes of the plunger. The homogenates were filtered through cheese cloth, and nuclei were isolated by a micromodification of the Chauveau procedure (24). The homogenates were centrifuged at 800 *g*; the pellets from this centrifugation were rehomogenized in 4.5 ml 2.0 M sucrose-0.5 mM $CaCl_2$ and centrifuged at 63,000 *g* (23,000 rpm) in a SW 39.1 rotor for 60 min. The pellet was rehomogenized in 2.0 M sucrose-0.5 mM $CaCl_2$ and centrifuged again for 1 hr at 63,000 *g*. The pellet from this second centrifugation was suspended in 0.88 M sucrose and assayed for radioactivity and DNA, as were the starting homogenates. By light microscopy (21) these nuclei were 97% pure, the remainder consisting of occasional whole cells or nuclei with large cytoplasmic tags. In a preliminary series of experiments 1 hr was shown to provide better recovery of nuclear radioactivity than 30 min or 1½ hr, and concentrations of sucrose greater than 2.0 M were shown to result in small yields of recovered DNA in the nuclear pellet. DNA was measured by the diphenylamine method of Burton (25), using salmon sperm DNA as the standard. In one study the nuclei from several experiments were pooled, and the radioactive steroids were extracted into chloroform: methanol, subjected to thin-layer chromatography and assayed for ^{3}H as before.

Blood hormone assays. Blood was collected from the inferior vena cava of anesthetized animals. The serum from 2–5 animals was separated, pooled, and frozen for subsequent studies. Serum luteinizing hormone was assayed by Doctors Robert Eskay and John Porter using a radioimmunoassay technique and NIAMD Rat LH-RP-I (National Institute of Arthritis and Metabolic Diseases) as the reference standard. Serum testosterone assay was performed by the Inter Science Institute, Los Angeles, Calif., utilizing a protein binding method, without knowledge of the identity of individual samples.

Materials. Dihydrotestosterone was obtained from Steraloids, Inc., Pawling, N. Y. Male BALB/c and Tabby mice were purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine. The various radioactive steroids (testosterone-1,2-^{3}H (5 mCi/32 μg), pregnenolone-7α-^{3}H (5 mCi/68.5 μg), progesterone-1,2-^{3}H (5 mCi/32.5 μg), 17α-hydroxyprogesterone (5 mCi/33.6 μg), and androstenedione-1,2-^{3}H (5 mCi/28.5 μg) were purchased from the New England Nuclear Corp., Boston, Mass., and were demonstrated to be at least 95% pure by thin-layer chromatography before use.

TABLE III

Recrystallization of Testosterone-^{3}H from Mouse Testes Incubated with Progesterone-1,2-^{3}H and Pregnenolone-7α-^{3}H

	SA of testosterone-^{3}H recovered after incubation of testes with			
	Pregnenolone-7α-^{3}H			Progesterone-1,2-^{3}H
Solvent	Normal newborn testes	*Tfm* newborn testes	Normal adult testes	Normal adult testes
	cpm/mg			*cpm/mg*
Methanol	380	1050	3820	5200
Benzene + heptane	460	1130	4000	4910
Ethyl acetate + pentane	420	1300	4300	4630
Ethyl ether + heptane	500	1320	4600	5082

* Material tentatively identified as testosterone-^{3}H was isolated by preparative thin-layer chromatography, mixed with 200 mg carrier testosterone and recrystallized four times.

RESULTS

Identification of the phenotypes in the newborn. To determine whether the testicular feminization phenotype could be identified in newborn animals, a series of dissections were performed in animals 2 days of age. As is illustrated in the photographs of whole mounts of the

mouse urogenital tracts in Fig. 2*A*, in newborn mice three phenotypes can easily be distinguished—female, male, and *Tfm;* the *Tfm* animal has a distinct testis but lacks the accessory organs of male reproduction (the epididymis, vas deferens, seminal vesicle, and prostate), which are replaced with strands of fibrous connective tissue. It has not been possible to distinguish between carrier (*Tfm*/X) and normal (X/X) females on the basis of dissection of the urogenital tracts either at this age or later; however in offspring of *Ta*/Y males and *Tfm/Ta* females it is possible to distinguish between the *Tfm/Ta* and *Ta/Ta* offspring since the *Ta/Ta* phenotype in the mouse is manifest clearly by day 3 (26).

Characterization of the hormonal status of the Tfm mouse. It has been reported that the rat with inherited male pseudohermaphroditism (27), in contrast to the testicular feminization syndrome in man (9, 28, 29), has a defect in testosterone biosynthesis and low testosterone blood levels. Since early castration in the rat has been reported to lead to a variety of secondary changes, including partial androgen insensitivity (30, 31), it was necessary to characterize the hormonal status of the *Tfm* mice. Therefore, testosterone values were compared in pooled samples of serum from normal male, normal female and *Tfm* mice, all between 6 and 8 wk of age (Fig. 3). The average serum testosterone value in normal male animals was 800±150 ng/100 ml (±2 SEM), and that in normal females was 50±10 ng/100 ml (±2 SEM); in *Tfm* animals the concentration was intermediate between that of males and females, averaging 150±100 ng/100 ml (±2 SEM), a value which was on an average distinctly lower than in the normal male and in some animals in the castrate range. In keeping with low circulating testosterone values was the finding that serum LH concentration in the *Tfm* rat was higher (averaging 470 ng/ml serum) than in normal male littermates (averaging 160 ng/ml serum).

Evidence that the abnormality in the circulating levels of testosterone is secondary to a defect in testosterone synthesis is summarized in Fig. 4 in which testosterone formation from four precursors was compared in tissue slices of male, female, and *Tfm* gonads from animals 6–8 wk of age. In each instance, testosterone formation was low in the tissues from the *Tfm* animals; furthermore, if the average rates of biosynthesis per unit weight are projected to the weight of the entire organ (averaging 80 mg per testis in the normal male and 9 mg per testis in the *Tfm*), it is apparent that the testis from the *Tfm* animal has a profound defect in testosterone biosynthesis. It is also striking that the defective synthesis is almost equally low from all four precursors studied—androstenedione, 17α-hydroxyprogesterone, progesterone, and pregnenolone.

That the defect in testosterone biosynthesis, however, is not a primary defect in this mutation is suggested by the findings reported in Fig. 5, for clearly the testis from the newborn *Tfm* mouse has a normal capacity to synthesize testosterone from pregnenolone as compared with the testis from normal male animals. This finding implies that the capacity for testosterone formation may be normal at birth and that the subsequent failure in this capacity is an indirect consequence of some other defect.

The possibility was then investigated that the failure of testosterone biosynthesis in these animals might in part be the result either of the inevitable cryptorchidism that occurs in this condition or the fact that the excretory ducts are atrophic. As shown in Table IV, testosterone biosynthesis and testicular weight were assessed in normal males, *Tfm* animals, and littermate males that had been subjected to vas duct ligation and/or made surgically cryptorchid at 3 wk of age. Neither surgical cryptorchidism nor vas duct ligation resulted in a significant decrease in the capacity for testosterone biosynthesis as compared with the normal animal, but a significant retardation in growth of the testes resulted from these procedures. It was concluded that the inability of the testis to form testosterone at a normal rate in the *Tfm* animal cannot be explained by these anatomical abnormalities and may well be the result of some loss in the capacity of the testes to grow in response to androgen (32).

Androgen resistance during sexual differentiation. In order to establish with certainty that deficient androgen synthesis does not play a role in the failure in differentiation of male internal and external genitalia in this

TABLE IV

Effect of Vas Duct Ligation and Cryptorchidism on Testicular Weight and Testosterone Biosynthesis in Mouse Testes

Group	Animals	Operation	Mean weight of testes	Testosterone formation from pregnenolone-7α-^{3}H
			mg	*pmoles/10 mg/hr* ±SEM
1	Male (12)	None	90.4	187.8±15.4
2	*Tfm* (12)	None	10.1	15.0±5.2
3	Male (7)	Vas duct ligation	94.6	155.0±14.2
4	Male (4)	Cryptorchid	49.8	146.5±18.5
5	Male (4)	Vas duct ligation plus cryptorchid	30.0	124.0±69.5

The animals were operated at 3 wk of age, and the experiments were performed at 6 wk of age. The number of animals in each experiment is shown in parentheses.

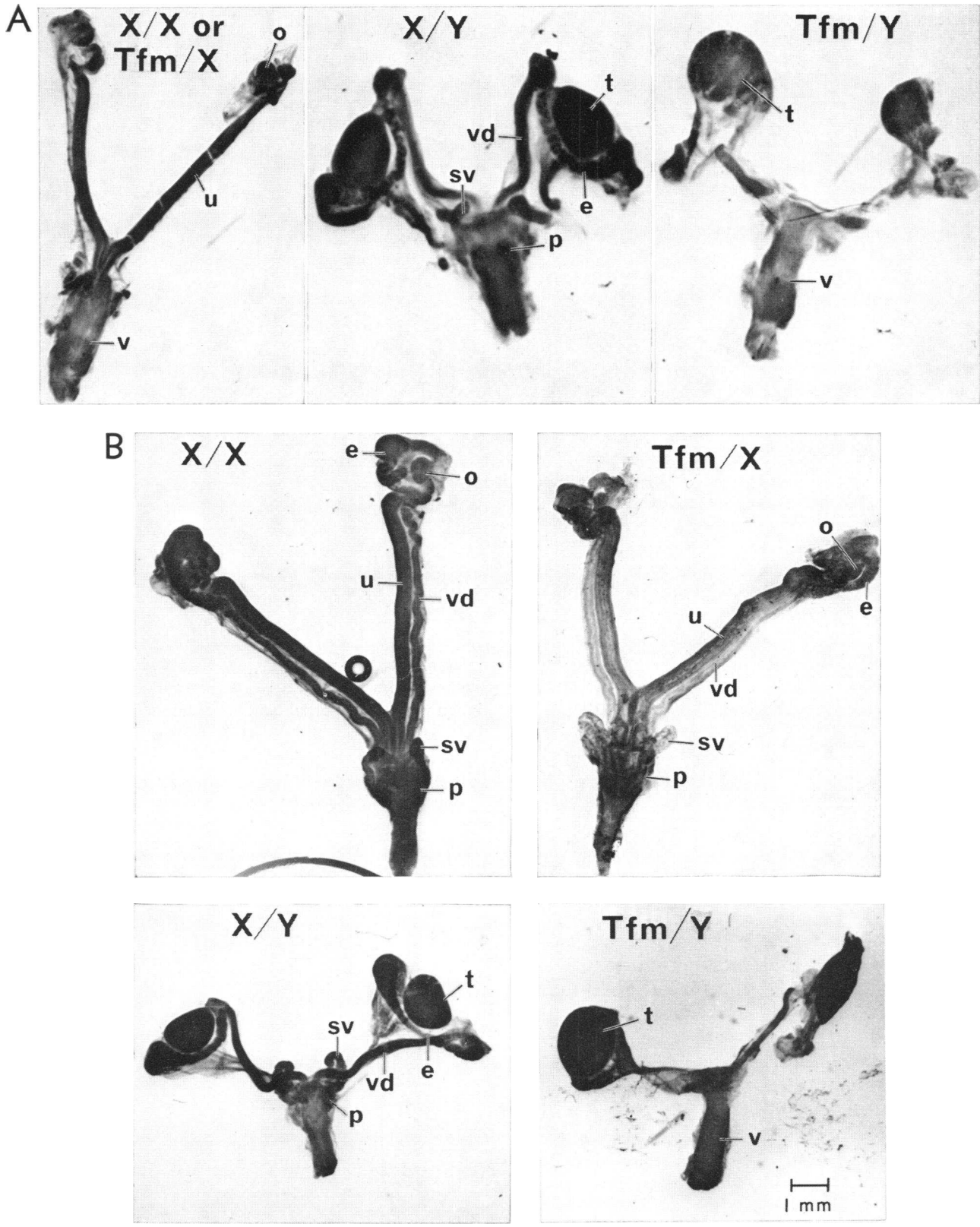
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TABLE V

Effect of Dihydrotestosterone on the Development of the Epididymis, Seminal Vesicles, and Prostate in the Mouse Embryo

Genotype	Total number of animals	Animals with: Masculine Wolffian duct and urogenital sinus	Animals with: Intact Mullerian system
Ta/Y	4	4	0
Tfm/Y	3	0	0
Ta/*Ta*	5	5	5
Ta/*Tfm*	2	2	2

Dihydrotestosterone dissolved in oil (2 mg) was administered subcutaneously to pregnant animals beginning 9–11 days postcopulation and continued until delivery; after delivery the newborn were given 0.2 mg dihydrotestosterone subcutaneously every other day and were killed on day 10. Photographs of whole mounts of the urogenital tracts of four of the animals are shown in Fig. 2*B*.

condition, a series of experiments were performed in which dihydrotestosterone was administered to pregnant carrier females (*Tfm*/*Ta*) that had been mated to *Ta*/Y males, beginning on the 9–11th day postcopulation and continuing up until 10 days after birth of the animals (Fig. 2*B* and Table V). Since virilization of the male fetus in the mouse does not begin until day 13 (12), such treatment should result in virilization of female embryos. As is illustrated in Fig. 2*B*, both the female (*Ta*/*Ta*) (X/X in Fig. 2*B*) and carrier (*Tfm*/*Ta*) (*Tfm*/X in Fig. 2*B*) offspring exhibited striking virilization, including male development of the Wolffian duct into epididymis, vas deferens, and seminal vesicle and the development of a prostate. As would be expected from the studies of Jost (11), such androgen treatment did not cause Mullerian regression in these animals and may have actually stimulated its development (13). The male urogenital tract appears normal, whereas there is no evidence of virilization of the *Tfm* urogenital tract in comparison with the untreated specimens shown in Fig. 2*A*. This study was interpreted as providing the first direct experimental proof of the view (8) that the failure of male sexual differentiation in the testicular feminization syndrome is due to androgen resistance during embryogenesis.

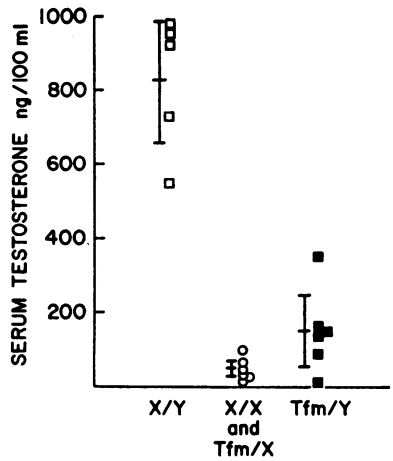

FIGURE 3 Serum testosterone in male, female, and *Tfm* mice. Serum from four to six 6–8 wk old mice was pooled for each assay. The bars represent mean values ±2 SEM.

Dihydrotestosterone formation in the Tfm animal. The next aspect of testosterone action that was examined was the conversion of testosterone to dihydrotestosterone, the testosterone metabolite that has been postulated to be the active intracellular androgen both for certain testosterone mediated functions in the adult (21, 33) and for some aspects of sexual differentiation in embryos as well (20). As is clearly demonstrated in Fig. 6, the rate of dihydrotestosterone formation in the tissues of the normal adult male mouse is similar to that previously reported in the rat, namely high rates of formation in the male organs of accessory reproduction—the epididymis, seminal vesicle, prostate, and phallus—and low rates in the remaining tissues (34). In adult normal and carrier females, however, the rates of formation are low in all tissues other than for the Fallopian tubes and uterus and the external genitalia. In the adult *Tfm* animal, the pattern of formation resembles closely that of the female animal. However, it is clear that it is impossible to assess the importance of this formation properly under conditions in which the tissues in question such as epididymis, prostate, and seminal vesicles have failed to develop. To attempt to determine whether the failure of male differentiation was the cause rather than the result of deficient dihydrotestosterone formation, therefore, dihydrotestosterone formation was assessed in tis-

FIGURE 2 Whole mounts of dissected urogenital tracts of infant normal and *Tfm* mice. (*A*) Control mice. Dissections were performed 3 days after delivery. (*B*) Dihydrotestosterone-treated mice. Dihydrotestosterone (2 mg/day) was administered to pregnant animals beginning 9 days postcopulation, and after delivery the newborn were given dihydrotestosterone (0.2 mg every other day). The animals were killed and dissected on day 10. The results of studies in 14 animals so treated are summarized in Table V. o, ovary; u, uterus; v, vagina; t, testis; vd, vas deferens; sv, seminal vesicle; e, epididymis; p, prostate.

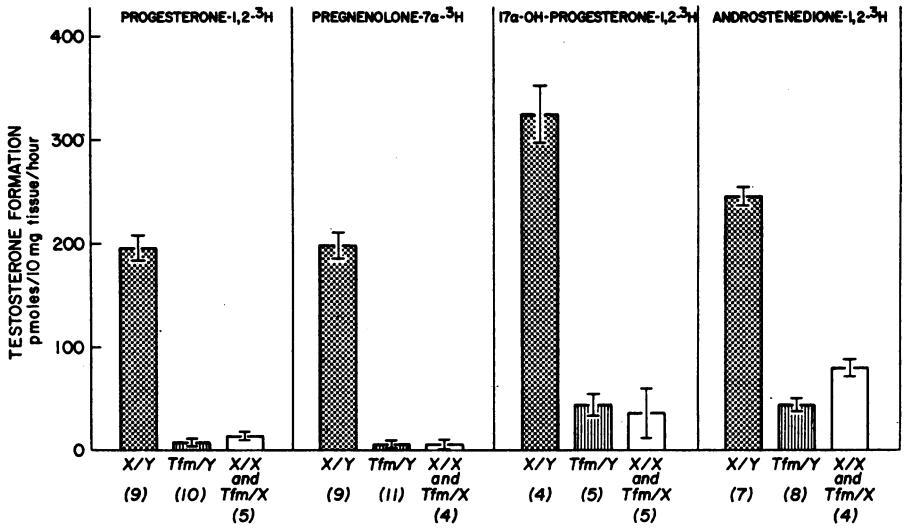

FIGURE 4 Testosterone formation from progesterone, pregnenolone, 17α-OH-progesterone, and androstenedione by slices of normal mouse testes and ovaries and of testes from *Tfm* mice. 6–8 wk old animals were used for these experiments. The figures in parentheses indicate the number of experiments, and the bars represent mean values ±1 SEM.

sues of newborn animals (Fig. 7). In these tissues, there was no difference between male and *Tfm* animals in the rates of formation of dihydrotestosterone in the urogenital sinus, the urogenital tubercle, or the Wolffian ducts, which represent the embryologic anlage of the prostate, the external genitalia, and the epididymis and seminal vesicles respectively. Clearly in the *Tfm* mouse, deficient dihydrotestosterone formation is a result rather than the cause of the male pseudohermaphroditism.

Intranuclear localization of testosterone in the submandibular gland. In order to determine whether the reported defect in the intranuclear localization of testosterone in the kidney of the adult *Tfm* animal (18, 19) was a university aspect of this disorder, the intracellular localization of testosterone-1,2-³H was assessed in the submandibular gland, a tissue in which secretion is testosterone-dependent (35–37) and in which the testosterone-mediated secretion of nerve growth factor in the *Tfm* animal is deficient.[2] As is shown in Table VI the intranuclear localization of radioactivity in submandibular gland nuclei following the administration of testosterone-1,2-³H was compared in the nuclei of normal, testosterone-treated, and castrated animals. The uptake of radioactivity by the glands in male, female, and *Tfm* animals was similar, but there was a significant difference between the male and female mice and the *Tfm* animals in the nuclear uptake, both in the gross recovery of radioactivity in the nuclear preparations and the recovery of radioactivity when corrected for the percentage of total DNA recovered in the purified nuclei fraction. Nuclear preparations from six experiments were pooled, and the steroids were extracted into chloroform: methanol (2:1) and chromatographed by thin-layer chromatography. In nuclei from male and female animals, 76% of the recovered radioactivity corresponded to testosterone and 9% to dihydrotestosterone, and in the nuclei from the *Tfm* animals 36% of the recovered ³H corresponded to testosterone and 23% to dihydrotestosterone; the remainder of

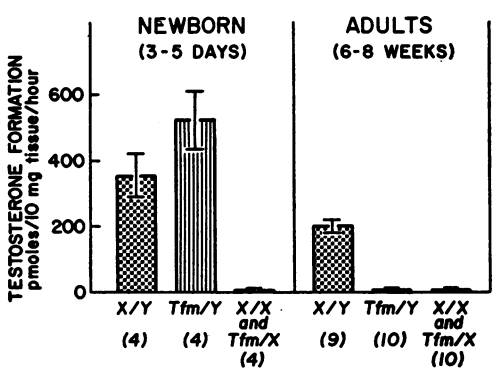

FIGURE 5 Comparison of the ability of newborn and adult testes to convert pregnenolone-7α-³H to testosterone. The incubation conditions are described in the text and in Fig. 1. The figures in parentheses indicate the number of experiments, and the bars indicate the mean value ±1 SEM.

[2] Lyon, M. F. Personal communication.

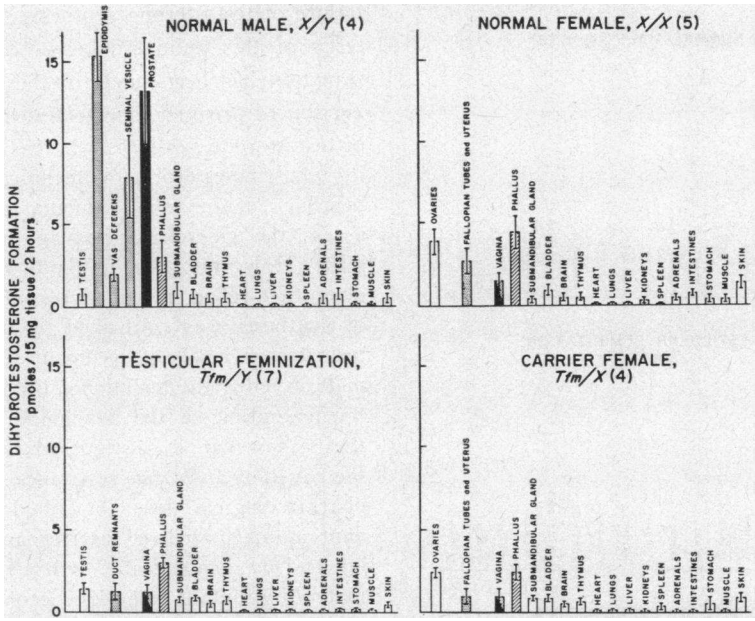

FIGURE 6 Dihydrotestosterone formation by tissue slices from adult mice. The standard incubation mixture contained tissue slices (5–15 mg), Krebs-Ringer phosphate buffer, pH 7.4, testosterone-1,2-^{3}H (3.5×10^{-8} M), and glucose (1.1×10^{-2} M) in a total volume of 1 ml. After incubation for 2 hr at 37°C, the steroids were extracted, chromatographed, and analyzed for radioactivity as described in the text. The stippled bars indicate tissues which were derived from the embryonic Mullerian or Wolffian ducts, the solid black bars tissues derived from the embryonic urogenital sinus.

the radioactivity in each case was spread uniformly across the plates. These experiments were interpreted as confirming the recent reports of defective intranuclear binding of androgen in kidney nuclei (18, 19) and are compatible with the possibility that such a defect may be primary in this condition.

TABLE VI

Intracellular Localization of Testosterone-1,2-^{3}H in Submandibular Glands of Functionally Hepatectomized Mice

Group	Treatment	Number of experiments	Average submandibular gland weight	Average DNA recovery		^{3}H recovery			
				Whole homogenate	Nuclei	Whole homogenate	Nuclei	Nuclei	Nuclei % ^{3}H/DNA recovery ×100
			mg	*mg/organ*	%	*cpm* × 10^{-5}	*cpm*	%	
Male	None	4	132	0.317	45.6	6.3±0.7	8350±2500	1.4±0.3	3.1±0.6
	Castration	4	96	0.419	25.0	5.5±0.1	7710±940	1.4±0.2	5.7±0.6
	Testosterone	2	155	0.351	30.4	7.8	6820	0.9	2.8
Female	None	4	105	0.295	24.6	7.1±0.4	4440±760	0.6±0.1	2.9±0.6
	Castration	4	88	0.334	24.4	7.0±0.9	5828±1160	0.9±0.2	4.5±1.7
	Testosterone	3	87	0.330	22.7	5.7	5520	1.0±0.2	5.7
Tfm	None	4	111	0.448	40.3	6.5±0.8	800±370	0.2±0.0	0.4±0.3
	Castration	4	98	0.417	33.7	5.9±0.6	1184±390	0.1±0.0	0.6±0.2
	Testosterone	3	104	0.256	19.0	5.0	1280	0.2	1.2

6–8-wk old mice were functionally hepatectomized and given 50 μCi testosterone-1,2-^{3}H i.v. 1 hr later the animals were killed, and the submandibular glands from two animals were pooled, homogenized, and subjected to differential centrifugation as described in the text. Some animals were castrated 1–2 wk before study, and in some cases 2 mg testosterone in oil was administered i.m. every other day for 7–10 days before death. The ± figures indicate ±SEM.

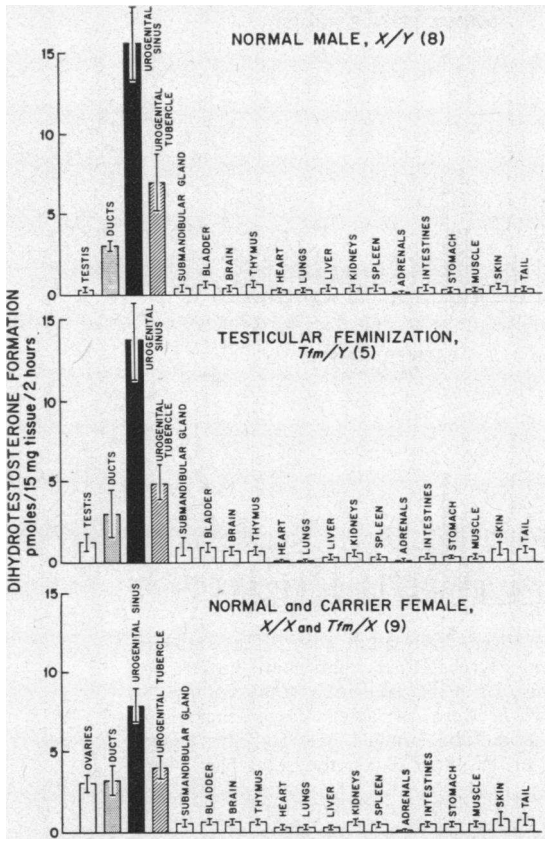

FIGURE 7 Dihydrotestosterone formation by tissue slices from newborn mice. The incubation conditions are described in Fig. 6 and the text.

DISCUSSION

On theoretical grounds male pseudohermaphroditism can be the result of defects at any of several critical steps in androgen action in the fetus—a failure of testosterone biosynthesis by the fetal testis, an inability to convert testosterone to dihydrotestosterone or other active androgen within the target cells, defective binding of androgens to cellular receptors, or some other defect in the ability of cells to respond to androgenic stimulus. The studies reported here in newborn and adult *Tfm* mice clearly establish that the failure of differentiation of the male accessory organs of reproduction in this mutation cannot be the consequence either of defective androgen synthesis or of diminished dihydrotestosterone formation but must instead be the result of a defect in some later aspect of androgen action.

Indeed, these findings suggest that two striking features of the *Tfm* syndrome in the adult mouse—namely deficient testosterone biosynthesis and diminished conversion of testosterone to dihydrotestosterone—are secondary consequences of some other primary defect. The failure of testosterone synthesis that occurs in the adult testis of this animal deserves comment. First, the assumption has been made in these studies that the conversion of pregnenolone to testosterone is a valid index of testosterone synthesis whereas the initial precursor is, in fact, cholesterol; for technical reasons, it was not possible in the small amount of tissues available to assess the side-chain cleavage reaction of cholesterol (38). However, it seems likely that the latter reactions do in fact reflect the in vivo conditions since the failure of synthesis was confirmed by measurements of actual testosterone levels of serum in affected and normal animals. Even if the techniques used here do underestimate the magnitude of the deficiency in testosterone biosynthesis, however, it is still clear that such a defect is not the cause of androgen resistance since administration of pharmacological doses of dihydrotestosterone to pregnant animals virilized carrier and normal female mice but had no demonstrable androgenic effect on the littermate *Tfm* mutant embryos. Second, the failure in testosterone biosynthesis and growth of the adult testis is probably not the consequence of cryptorchidism in these animals and must be the result of some other defect since it is not present in the newborn animal. Thus, the defect in the rat and mouse is different than in the human variety of testicular feminization, in which both testicular size and testosterone secretory rates are normal in the adult (9, 28, 29, 39). Third, it is not possible at present to assign the defect of testosterone biosynthesis to a specific enzymatic defect in the biosynthetic pathway for testosterone, since the rate of formation was uniformly depressed for all substrates tested and since no consistent pattern of accumulation of intermediates was observed in the tissue slice studies. Since the enzymes that are involved in the conversion of pregnenolone to testosterone are thought to exist in a distinctive microsomal unit (38), the possibility exists that this enzyme package has been lost symmetrically in the *Tfm* testes. Finally, it is striking that testosterone biosynthesis is diminished under circumstances in which the Leydig cells have been said to be hypertrophic (6). This apparent discrepancy is probably due to at least three factors—the remarkably small size of the *Tfm* testis, the fact that underdevelopment of the tubules may cause an apparent rather than a real increase in number of Leydig cells per testis, and finally the fact that in the rodent testis testosterone biosynthesis from pregnenolone may not take place exclusively in the Leydig cells (40).

The findings in the present study that dihydrotestosterone formation was normal in the anlage of the male organs of accessory reproduction at birth may have implications for the interpretation of the pathogenesis of the human variety of the disease, where it has been reported that dihydrotestosterone formation is deficient

in the perineal skin and other tissues (14–16). On the basis of the present study it is clear that deficient dihydrotestosterone formation in human testicular feminization could result either from a defect that involves primarily the enzyme that performs this conversion or from a developmental defect that prevents the differentiation of the cell lines that contain this enzymatic function. Furthermore, the finding that dihydrotestosterone formation was normal in the newborn urogenital sinus in the *Tfm* animal despite a total lack of androgen effect on this tissue is strong confirmation of the previous conclusion in the embryos of rats, rabbits, and guinea pigs that the ability to form dihydrotestosterone by the 5α-reductase enzyme system is an inherent capacity of this tissue, rather than induced by the action of fetal androgen (20, 41). The finding in the present studies that the rate of dihydrotestosterone formation is lower in the normal newborn Wolffian ducts than in the normal adult is also in keeping with the view that this enzymatic capacity is acquired during the development of the tissues derived from the Wolffian duct, namely the seminal vesicles and the epididymis (20, 41).

The demonstration in these studies of diminished intranuclear binding of testosterone in the nuclei of the submandibular gland of the adult *Tfm* animals, together with the previous reports of Gehring, Tomkins, and Ohno (18) and Bullock, Bardin, and Ohno (19) that defective nuclear retention of androgen occurs in the kidney of the adult *Tfm* mouse, raise the possibility that the fundamental defect in androgen action in this abnormality involves an interaction between hormone and receptor sites. An alternative interpretation of these data is that the abnormalities observed in androgen binding in adult *Tfm* tissues do not represent the primary action of the mutant *Tfm* gene but are an example of another acquired, secondary abnormality of the testicular feminization syndrome in the adult. The resolution of this important issue will require the development of a highly sensitive method for assessing directly the ontogeny of androgen receptors in sexually differentiating tissues of the mouse embryo.

ACKNOWLEDGMENTS

James E. Unger, Mary B. Neal, and Frances E. York have provided able technical assistance in the performance of these studies.

This work has been aided by Grant AM3892 from the National Institutes of Health.

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