Testosterone Formation and Metabolism During Male Sexual Differentiation in the Human Embryo

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ABSTRACT. The formation by the gonads of [^{3}H]testosterone from [7α-^{3}H]pregnenolone and [1,2-^{3}H]progesterone and the metabolism of [1,2-^{3}H]testosterone by various tissues have been studied in 33 human fetuses that varied in age from phenotypically undifferentiated stages (1–3 cm crown-rump length) to sexually differentiated male and female embryos greater than 21 cm in length. In the first series of studies utilizing thin-layer and celite column chromatography for quantification of the metabolic products following incubation of the gonads with the C_{21} steroids, it was concluded that testosterone is the principal androgen formed by the fetal testis at the time of male sexual differentiation. The capacity for testosterone formation from these precursors was shown to rise from undetectable levels at 1–3 cm of development to maximal rates of about 150 pmoles/10 mg tissue/2 hr in the testes obtained from embryos of 7.1–9 cm crown-rump length, a sequence that correlates closely with the androgen-mediated events in male sexual differentiation. No testosterone formation was demonstrated in the ovaries at any stage of development.

To characterize the mechanisms involved in androgen-mediated development of the male urogenital tract, the rate of conversion of [1,2-^{3}H] testosterone to [^{3}H]dihydrotestosterone was measured in a variety of tissues in these embryos. Dihydrotestosterone formation was most rapid in the tissues of the urogenital tract. In the urogenital sinus and urogenital tubercle the capacity for dihydrotestosterone formation was maximal prior to the onset of male differentiation, suggesting that dihydrotestosterone is probably the effective intracellular androgen that mediates male development in these tissues. In contrast, in the wolffian ducts the ability to form dihydrotestosterone could not be demonstrated until late in male differentiation, indicating that testosterone itself is the likely hormone that initiates the development of this anlage into the epididymis, vas deferens, and seminal vesicle. The implications of these findings for the elucidation of the pathogenesis of several disorders of male sexual differentiation have been discussed (*J Clin Endocrinol Metab* **38**: 113, 1974)

IN keeping with the concept of Jost that the development of the male phenotype depends upon androgen secretion by the fetal testis (2–4), it is now clear that testosterone[1] is formed by the fetal testis of several species (5–11). In the rabbit (9,10) and sheep (11), the onset of testosterone formation by the fetal testis occurs just prior to the onset of male differentiation of the urogenital tract. Although the regulatory factors that determine the timing of this process have not been elucidated, it is likely that in the rabbit C_{21} steroids from placenta and/or adrenal gland serve as substrate for androgen biosynthesis and that the onset of testosterone formation by the fetal gonad is dependent on maturation of enzyme system(s) capable of converting these C_{21} precursors to testosterone (10).

It is also clear from work done in animals that testosterone secreted by the fetal testes induces male differentiation of the urogenital tract by two fundamentally different mechanisms. Testosterone itself appears to be responsible for the initiation of differentiation of the wolffian duct into the epididymis, vas deferens and seminal vesicle (12–

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[1] The abbreviations used in this paper include: D, dehydroepiandrosterone; A, androstenedione; T, testosterone; 17-OH-P, 17α-hydroxy-progesterone; pregnenolone, 3β-hydroxy-pregn-5-ene-20-one; 17-OH-preg and 17-hydroxy-pregnenolone, 3β,17α-dihydroxy-pregn-5-ene-20-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; androstandione, 5α-androstan-3,17-dione; androstandiol, 5α-androstan-3α,17β-diol and 5α-androstan-3β,17β-diol.

14). In contrast, both in the urogenital sinus and urogenital tubercle, the capacity to convert testosterone to dihydrotestosterone is present prior to differentiation of these tissues into the prostate and external genitalia. Therefore, it is likely that dihydrotestosterone rather than testosterone itself is the effective intracellular mediator of this process (12–14).

The events in sexual differentiation in man are much more poorly understood. The fetal testis of the sexually differentiated male embryo does have the capacity to synthesize testosterone from radioactive precursors (5,8). Furthermore, it is clear that the testicular feminization syndrome in man (15,16) and in several animal species (16–19) represents an inherited resistance to testosterone action during embryogenesis that leads to male pseudohermaphroditism. However, neither the timing of the onset of fetal androgen synthesis, the nature of the androgens secreted at the time of male differentiation, nor the mechanisms by which the fetal androgens exert their effects have been characterized in man. Such information might provide explanatory insight into the pathogenesis of developmental disorders of male sexual differentiation. Therefore, a study was undertaken to chart the timing of the onset of testosterone synthesis by the gonads and to investigate the pathways of testosterone metabolism in various tissues of 33 male and female embryos that varied from 1 to more than 21 cm in crown-rump length.

Materials and Methods

Twenty male, 7 female, and 6 sexually undifferentiated embryos of both sexes were obtained from two sources. In the case of the younger embryos studied, the specimens were obtained from patients who because of some medical indication were subjected to hysterectomy during the first 14 weeks of pregnancy; the intact uterus was placed in chilled normal saline and transported to the clinical laboratory where it was dissected by a pathologist. The embryo was removed and stored in ice-cold normal saline until the time of study. In the case of the older embryos, specimens were obtained either from spontaneous abortions or from stillbirths. The material so utilized was grossly normal except for 1 stillborn infant that had a large meningomyelocele. In all instances about 2 hr elapsed between the time of death and the commencement of the study. Each embryo was carefully weighed and measured prior to dissection. The age of the fetus was estimated from the data of Patten, using the crown-rump (C-R) length as the reference (20). In the case of the younger embryos (1–3 cm) it was not possible to determine the sex by gross examination, and in several instances genetic sex was determined by fixing the chorionic membranes on a slide and examining for the presence of nuclear sex chromatin.

Incubation and extraction of gonads

The dissection of the urogenital tracts was performed under magnification as described (10,12,13,19). The gonads were blotted, weighed on a tissue balance, and sliced with a razor blade. The standard incubation mixture contained tissue slices (0.5–10 mg), Krebs-Ringer phosphate buffer, pH 7.4, glucose (11 mM) and radioactive [7α-^{3}H]pregnenolone or [1,2-^{3}H] progesterone (1 μM) in a total vol of 1 ml. The incubation tubes were gassed with 95% oxygen–5% carbon dioxide, capped and incubated at 37 C with shaking for 2 hr (10). Following completion of the incubation, the radioactive steroids were extracted with 4 ml of chloroform: methanol (2:1).

Chromatography and identification of the gonadal steroids

For thin-layer chromatography, a portion (0.1 ml) of the chloroform:methanol extract was combined with a carrier mixture of 10 μg each of 7 steroids, taken to dryness, and reconstituted in 25 μl of chloroform. The sample was spotted for 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 described previously (10,12). The identity of testosterone as an incubation product was established by comparing the results obtained by the thin-layer method with that obtained by column chromatography. In each instance the results of the two methods agreed within 15%.

For the column studies known amounts of 14C-labeled steroids were added to aliquots of the chloroform:methanol extracts prior to chro-

matography. Gradient elution chromatography on celite was performed as previously described (10) and as illustrated in the representative experiments shown in Fig. 1. In brief, 20 g of celite were mixed with 15 ml of ethylene glycol and packed into a 1.5 × 43 cm column in 12 equal portions. The samples were taken to dryness, reconstituted in 1 ml of ethylene glycol and 0.5 ml of isooctane, mixed with 2 g of celite, and then packed on top of the column. Elution was carried out in three steps, the first of which consisted of 130 ml of isooctane (Eluant 1). A linear gradient consisting of 200 ml isooctane and 200 ml isooctane-ethyl acetate (7:3) was then used (Eluant 2). Finally, polar metabolites were eluted using a linear gradient consisting of 200 ml of isooctane-ethyl acetate (7:3) and 200 ml of ethyl acetate (Eluant 3). Three ml fractions were collected, and 0.3 ml aliquots of alternate fractions were assayed for radioactivity. Since this procedure does not separate androstenedione from pregnenolone or 17α-hydroxyprogesterone from estrone, pooled aliquots from these combined fractions (Fractions 2,3 and 6,7 of Fig. 1) were then taken to dryness and separated by the thin-layer method described above.

The identity of the various incubation products obtained from the incubation of gonads with [7α-^{3}H]pregnenolone was established by subjecting appropriately pooled fractions from the celite chromatography procedure to acetylation (overnight at room temperature in pyridine: acetic anhydride 1:1) and sequential thin-layer chromatography in chloroform:ethyl acetate (13:1), chloroform:methanol (99:1), and isooctane:ethyl acetate (7:3). The acetylated sample or recovered androstenedione was then crystallized three times with carrier steroids in ether-petroleum ether or methanol-ethyl ether. As shown in Table 1, the constancy of the ^{3}H/^{14}C ratios following the various procedures indicates the validity of the celite procedure for the separation of most pregnenolone metabolites at periods of testicular development past the indifferent stage. Specifically, progesterone (peak 1), androstenedione (peak 2,3) dehydroepiandrosterone (peak 5), 17α-hydroxyprogesterone (peak 6,7) and testosterone (peak 8) were easily identified. Although no ^{14}C-labeled 17α-hydroxypregnenolone was available as a marker, peak 9 was shown to consist principally of 17α-hydroxypregnenolone as determined by chromatography with authentic material in the same thin-layer systems. Two steroids, estrone (peak 6,7) and dihydrotestosterone (peak 4), were shown to be present only in trace quantities by the same procedure. The ^{3}H in peak 10,11 of the [7α-^{3}H]pregnenolone incubations was shown not to be estradiol on recrystallization; the peak corresponded to the peak for androst-5-ene-3β,17β-diol, but the identity was not examined further.

The identity of the incubation products following the incubation of testes with [1,2-^{3}H] progesterone (Fig. 1) was similarly established by sequential acetylation and thin-layer chromatography (results not shown). In these incubations an additional metabolite (peak 12) was found and was tentatively identified as 21-hydroxyprogesterone by sequential thin-layer chromatography with carrier steroid.

Incubation and extraction of other tissues

The urogenital tracts and other tissues of embryos were dissected, blotted, and weighed on a tissue balance. The standard incubation mixture contained tissue fragments (1–12 mg), Krebs-Ringer phosphate buffer, pH 7.4, glucose (11 mM) and [1,2-^{3}H]testosterone (40 nM) in a total vol of 1 ml. The incubation tubes were gassed with 95% oxygen–5% carbon dioxide, capped and incubated with shaking at 37 C for 2 hr. Following the completion of the incubation the reaction was terminated by the addition of 5 ml of chloroform:methanol, 2:1. Lipids were extracted into the chloroform-methanol layer, the aqueous layer was aspirated, the chloroform-methanol layer was backwashed, and portions of the chloroform-methanol extract were taken for the separation of individual steroids by thin-layer chromatography (12). In brief, a carrier mixture of reference steroids was added to each sample, which was then taken to dryness and reconstituted in 25 μl chloroform. The sample was spotted on plates of silica gel H and developed in the double ascent system of chloroform: methanol, 98:2, at 4 C followed by benzene:ethyl acetate, 13:1, at room temperature. The plates were air dried, sprayed with an anisaldehyde mixture, and heated in an oven at 100 C for 20 min. The steroid areas were marked, and the individual sections constituting the entire channel from the origin to the front were then scraped into 10–12 counting vials and assessed for radioactivity. To determine the fraction of the total

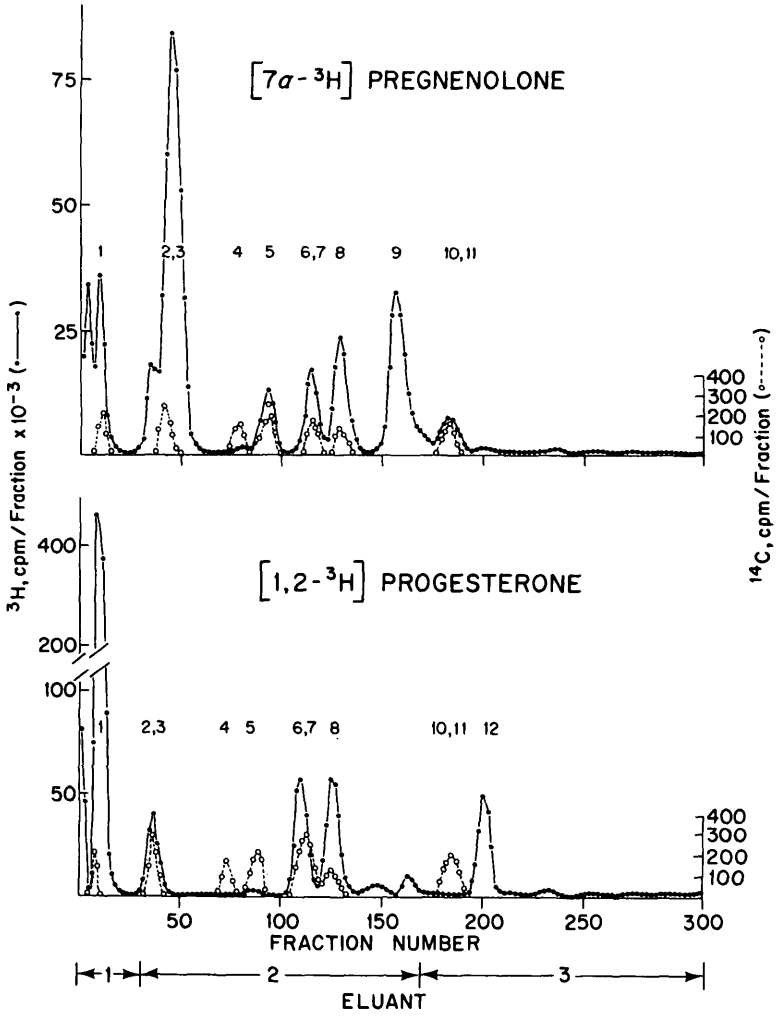

FIG. 1. Separation of [7α-^{3}H] pregnenolone and [1,2-^{3}H]progesterone metabolites by celite chromatography following incubation with testes. Slices (10 mg) from embryonic testes that weighed either 42 mg (pregnenolone incubation) or 18 mg (progesterone incubation) were incubated and chromatographed as described in the text. The numbers above the peaks refer to the identity as determined either by comparison with known ^{14}C steroid standard or by separate analysis. 1. progesterone, 2,3. androstenedione and pregnenolone, 4. dihydrotestosterone, 5. dehydroepiandrosterone, 6,7. estrone and 17-hydroxyprogesterone, 8. testosterone, 9. 17α-hydroxypregnenolone, 10,11. estradiol and unknown, 12. 21-hydroxyprogesterone (tentative).

radioactivity recovered in individual metabolites the amount recovered in the dihydrotestosterone area was divided by the amount recovered in the entire channel. In these studies, only trace quantities of radioactivity were recovered from areas of the chromatograms corresponding to androstenedione, androstandiol, androstandione, and 5β-dihydrotestosterone.

For the recrystallization studies, 6–10 different incubation mixtures from various portions of the urogenital tracts were combined, taken to dryness, streaked across chromatography plates, and developed for preparative chromatography as described above. Material tentatively identified as dihydrotestosterone was eluted from the gel by trituration with methanol, mixed with 200 mg of carrier steroid, and recrystallized to constant specific activity as described (21). As indicated in Table 2, the constancy of the specific activity following the various crystallizations demonstrates that at most the thin-layer procedure overestimates the rate of dihydrotestosterone formation by about 25%.

Sources

The radioactive steroids used for the incubation studies: [7α-^{3}H]pregnenolone (25 Ci/mmole), [1,2-^{3}H]progesterone (48 Ci/mmole), and [1,2-^{3}H]testosterone (50 Ci/mmole) and the radioactive steroids used as chromatographic markers: [4-^{14}C]progesterone (57 mCi/mmole), [4-^{14}C]pregnenolone (53 mCi/mmole), [4-^{14}C] androstenedione (51 mCi/mmole), [4-^{14}C]dehydroepiandrosterone (42 mCi/mmole), [4-^{14}C]dihydrotestosterone (59 mCi/mmole), [4-

TABLE 1. Confirmation by thin-layer chromatography and recrystallization of the identity of incubation products after incubation of fetal gonads with [7α-^{3}H]pregnenolone

Peak	Steroid	Type of gonad	Wt of gonads (mg)	$^3H/^{14}C$ ratio							
				Column pool	TLC-1	TLC-2	TLC-3	ML-1	ML-2	ML-3	Final crystals
1	Progesterone	Indifferent gonad	0.5	254	210	192	199	237	222	208	197
		Testis I	4	105	88	73	74	81	58	60	57
		Testis II	12	66	53	35	36	57	36	30	27
		Testis III	150	162	141	131	120	129	92	89	87
2,3	Androstenedione	Indifferent gonad	0.5	—	6.2	1.8	0.64	1.4	0.30	0.21	0.23
		Testis I	4	—	9.1	8.0	7.6	9.1	8.8	8.0	8.0
		Testis II	12	—	16	15	14	17	15	15	15
		Testis III	150	—	2.4	1.3	0.88	1.2	0.64	0.54	0.49
5	Dehydroepiandrosterone	Indifferent gonad	0.5	2.7	1.1	1.2	1.1	1.2	0.89	0.83	0.94
		Testis I	4	43	41	42	40	43	41	41	42
		Testis II	12	49	47	44	46	47	47	47	46
		Testis III	150	13	12	13	12	13	12	12	12
6,7	17α-OH Progesterone	Indifferent gonad	0.5	—	5.4	4.9	4.9	5.1	4.7	4.9	5.2
		Testis I	4	—	22	22	20	19	19	19	21
		Testis II	12	—	8.4	7.4	7.5	7.0	6.8	7.0	6.6
		Testis III	150	—	32	30	28	28	27	26	26
8	Testosterone	Indifferent gonad	0.5	12	2.0	1.8	1.5	2.1	1.8	1.5	1.8
		Testis I	4	163	169	168	165	163	167	177	173
		Testis II	12	163	176	169	166	199	171	171	173
		Testis III	150	47	36	37	32	35	33	33	33

TABLE 2. Confirmation by recrystallization of the identity of [^{3}H]dihydrotestosterone after incubation of embryonic tissue with [1,2-^{3}H]testosterone

#	Solvent	[^{3}H]dihydrotestosterone specific activity				
		Male urogenital sinus	Male urogenital tubercle	Male urogenital sinus	Female urogenital sinus	Female urogenital tubercle
				cpm/mg		
1	Original eluate	2726	2364	3965	3035	2828
2	Acetone	2377	1868	4093	2593	2368
3	Benzene-heptane	2365	1837	3904	2399	2183
4	Ethyl acetate-cyclohexane	2327	2009	3728	2388	2192
5	Ethyl ether-hexane	2185	1880	3740	2314	2262
6	Methanol-water	2046	1758	3544	2361	2194

Incubation mixtures from 6–10 experiments were combined, and material tentatively identified as [^{3}H] dihydrotestosterone was isolated by preparative thin-layer chromatography as described in the text, mixed with 200 mg carrier steroid, and recrystallized 5 times.

^{14}C]estrone (59 mCi/mmole), [17α-4-^{14}C] hydroxyprogesterone (50 mCi/mmole), [4-^{14}C] testosterone (51 mCi/mmole), and [4-^{14}C]estradiol (45 mCi/mmole) were obtained from New England Nuclear Co. The remainder of the steroids were obtained from Steraloids, Inc.

Results

The first experiment was designed to assess the ability of the fetal gonad to convert [7α-^{3}H]pregnenolone and [1,2-^{3}H]progesterone to testosterone at various stages of embryonic development (Fig. 2). For this study, the results from studies of 6 embryos prior to the onset of sexual differentiation of the urogenital tracts, 20 male embryos and five female embryos have been plotted in relation to the crown-rump length, the weight of the embryo, and the age of the embryo. The earliest embryos studied were 1–3 cm in length, and the oldest were more than 20 cm in length. During this time the average weight of the gonads increased from 0.5 mg at the youngest ages studied to 170 mg in the case of the oldest male embryos and 240 mg in the oldest female embryos (Fig. 2, top panel). Testosterone formation was not demonstrable following the incubation of the gonads from the earliest stages either with radioactive progesterone or pregnenolone whether the rate of formation was expressed per unit weight or projected to the weight of the entire organ. However, in all male embryos of 3.1–5 cm crown-rump length, a stage in which male phenotypic development is easily recognizable, testosterone synthesis from [7α-^{3}H]pregnenolone was demonstrable. On a unit weight basis this capacity was apparently maximal at the 7–9 cm stage and declined thereafter during fetal development. However, the weight of the testis continued to increase during the period encompassed in the study, and when the capacity for testosterone formation is projected to the weight of the whole organ, the rate increased until the 15–20 cm stage and then declined. The rate of conversion of [1,2-^{3}H]progesterone to testosterone showed a similar time course both when expressed in terms of unit weight or the whole organ. Approximately equal rates were observed following the incubation of the testes with either 1 μM [1,2-^{3}H]progesterone or [7α-^{3}H] pregnenolone. In this regard the embryonic testis of man resembles that of the newborn mouse testis (19) but differs from the fetal rabbit testes (10) in which the rates of testosterone formation from pregnenolone are about three times as rapid as from progesterone. The apparent lag in onset of synthesis from progesterone as compared with pregnenolone is probably the result of the small numbers of samples studied. At no time studied was significant testosterone formation demonstrated in ovaries incubated with either precursor.

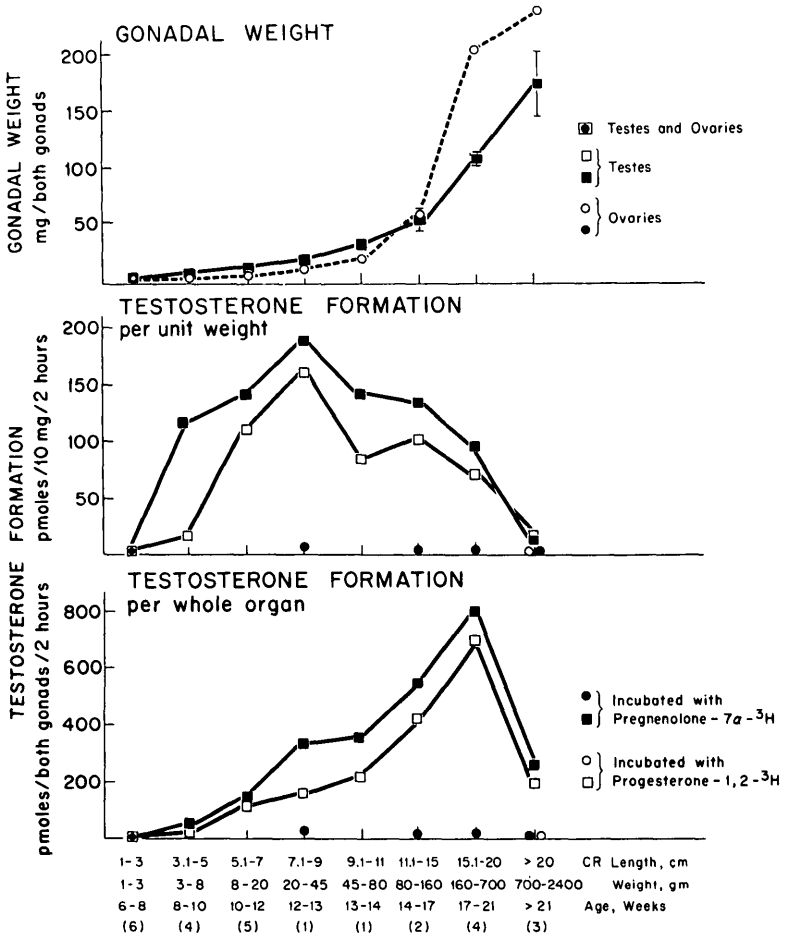

FIG. 2. Testosterone formation from [7α-^{3}H]pregnenolone by gonads of the male embryo as a function of the age of the embryos. The standard incubation conditions are described in the text, and the rate of testosterone formation was estimated by thin-layer chromatography. Each point represents the mean for the points where 2–6 male embryos were studied or single determinations at the stages in which only one analysis was performed. Crown-rump (CR) length and weight were measured directly; the estimated age of the embryos in weeks was taken from Pattern (20) using the CR length as the reference. The number in parentheses at the bottom of the chart refers to the number of male or sexually immature embryos studied at each stage.

The enzymatic process by which the capacity for testosterone biosynthesis is acquired by the testes of embryos between 1–3 and 3–5 cm in length was then studied. A combination of celite column and thin-layer chromatography was utilized to allow the separation and quantification of all the potential intermediates between progesterone or pregnenolone and testosterone (Fig. 1 and Table 1). Utilizing this procedure, the various metabolites formed following the incubation of embryonic testes with [7α-^{3}H] pregnenolone were analyzed in 11 experiments from embryos that varied from 2–29 cm in crown-rump length (Fig. 3). As before, testosterone formation was first demonstrable at the 4 cm stage, rose to a maximal rate at 19 cm and declined thereafter. In contrast to the sequence in the embryonic rabbit testis (10), no other metabolites were formed in significant amounts prior to the onset of testosterone formation. Since the earliest embryos studied were a mixture of male and female, in the later phases of the study the sex of two of the undifferentiated embryos was determined by analysis of the nuclear chromatin of the chorion. One was shown to be male, and in this preparation no pregnenolone metabolites were found. Thus, it was concluded that the onset of testosterone synthesis appears to be the result either of the appearance of one of the initial enzymes in pregnenolone conversion or the symmetrical development of the entire enzymatic machinery for the conversion to testosterone. The pattern of metabolites observed in the later stages of embryonic development was, however, strikingly dif-

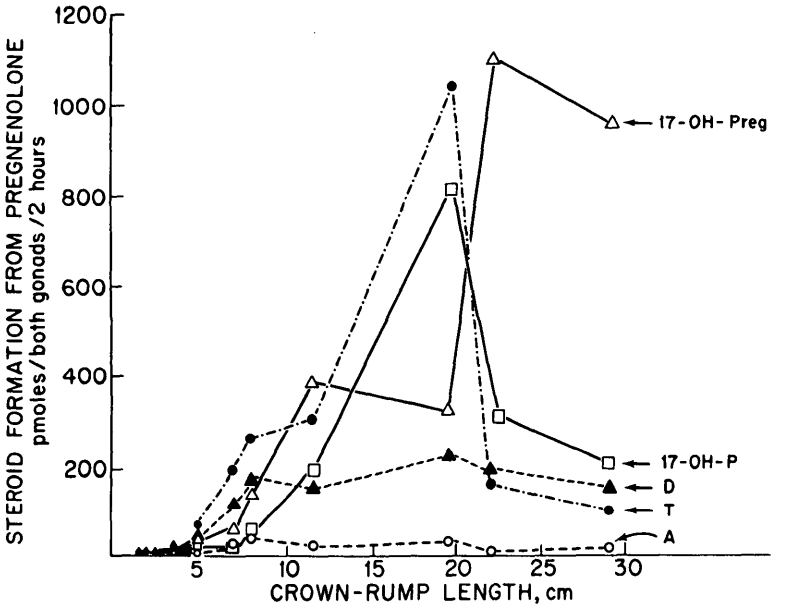

FIG. 3. The formation of dehydroepiandrosterone (D), 17α-hydroxy-pregnenolone (17-OH-preg), 17α-hydroxyprogesterone (17-OH-P), testosterone (T) and androstenedione (A) from [7α-^{3}H]pregnenolone by gonads as a function of the age of the embryos. The standard incubation conditions are described in the text, and the metabolites were separated and quantified by celite column and thin-layer chromatography.

ferent. As testosterone synthesis fell, 17α-hydroxypregnenolone accumulated suggesting that decreased testosterone formation was secondary to diminished activity of some enzyme subsequent to the 17-hydroxylation of the steroid.

A similar analysis of the metabolites formed following the incubation of five embryonic testes with [1,2-^{3}H]progesterone is shown in Fig. 4. As was the case with the pregnenolone incubations, no significant metabolite formation was demonstrated in the earlier gonads examined, but at the 5.2 cm stage, the formation of testosterone, androstenedione, and 17α-hydroxyprogesterone was evident. And, as before, in the oldest embryo studied testosterone formation was decreased at a time when 17α-hydroxyprogesterone formation was unimpaired, again indicating that the rate limiting step in testosterone formation in the late embryo is not the 17-hydroxylation reaction.

Thus, as the result of studies utilizing radioactive pregnenolone and progesterone as precursors, it was concluded that the appearance of the capacity for testosterone formation in the fetal testis coincides closely with the male differentiation of the urogenital tract.

The metabolism of testosterone by the tissues of the embryo was also studied. In Fig. 5 are summarized measurements of di-

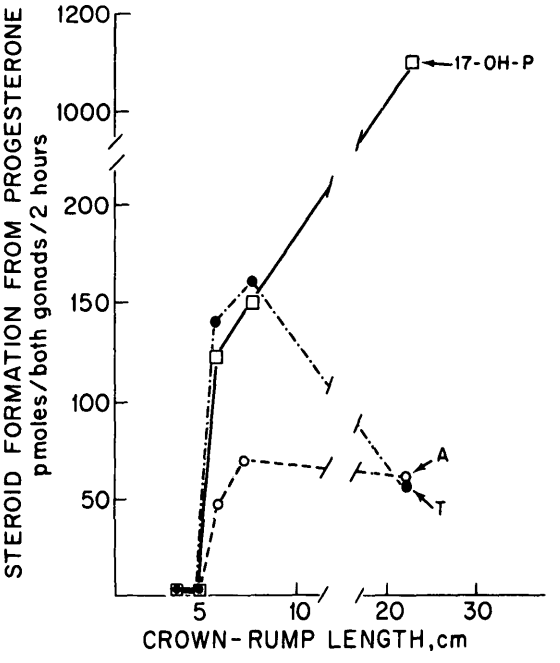

FIG. 4. The formation of androstenedione (A), testosterone (T), and 17α-hydroxyprogesterone (17-OH-P) from [1,2-^{3}H]progesterone by fetal tests as a function of the age of the embryos. The standard incubation conditions are described in the text, and the metabolites were separated and quantified by celite column and thin-layer chromatography.

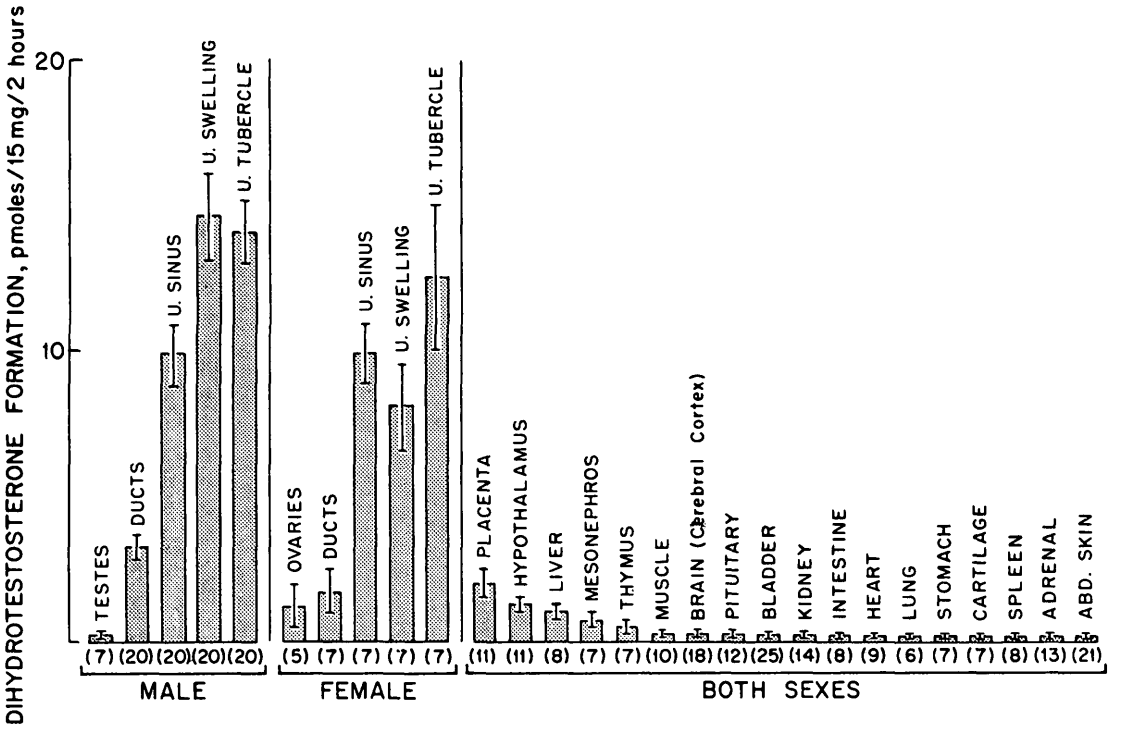

FIG. 5. Dihydrotestosterone formation by slices of 24 different tissues of the embryo. Each bar represents mean values for each tissue ±SEM, and the number of assays for each tissue is shown at the bottom of each bar.

hydrotestosterone formation in tissue slices of 24 tissues from 33 embryos of all stages of development. In this study, dihydrotestosterone was the only significant 5α-reduced steroid identified, although in some tissues such as liver and kidney a metabolite more polar than androstandiol was present but was not identified. As has previously been shown for rabbit (12,13), rat (12), and guinea pig embryos (14), dihydrotestosterone formation was much more rapid in the tissues of the urogenital tract and in particular in the urogenital sinus, urogenital swelling, and urogenital tubercle, than in the other tissues examined. Small amounts of dihydrotestosterone were also found in placenta, hypothalamus, liver, ovaries, mesonephros, and thymus, and the amounts were negligible in the other tissues examined.

Since these studies were performed in embryos in which the state of differentiation of the genitalia varied from the neuter stage to completely developed male and female phenotypes, the data for the müllerian and wolffian ducts, urogenital sinus, urogenital swelling, and urogenital tubercle were analyzed as a function of the age of the embryo (Fig. 6). In the urogenital sinus and urogenital tubercle dihydrotestosterone formation was approximately as high (8.5 ± 1.0 and 32.5 ± 9.0 pmoles/15 mg/2 hr, respectively) at the earliest stage examined and prior to differentiation as at any subsequent stage of development. Likewise, at the earliest stage in which the urogenital swelling could be dissected clearly (3.1–5 cm), dihydrotestosterone formation was close to 30 pmoles/15 mg/2 hr. In these three tissues the rates of formation remained high throughout embryonic development or fell slightly. Clearly, in the urogenital sinus and urogenital tubercle the capacity for dihydrotestosterone formation exists prior to the onset of testosterone secretion by the testis or the on-

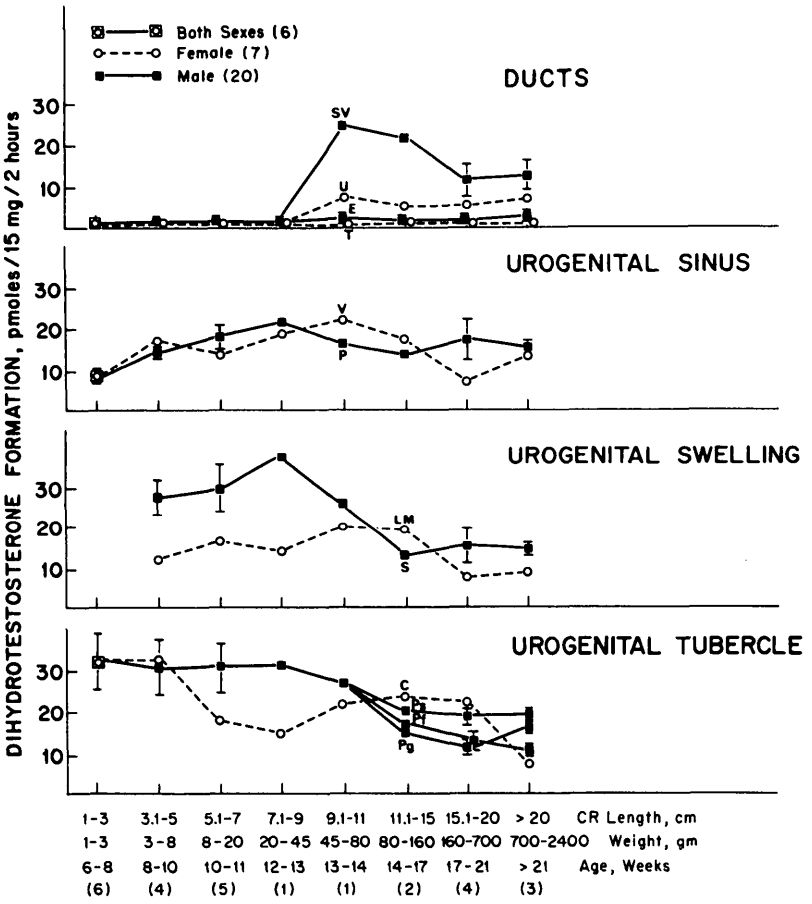

FIG. 6. Dihydrotestosterone formation by urogenital tracts of human embryos as a function of the age of the embryos. Each bar represents the mean value ±SEM for those stages in which 3–6 determinations were made or single measurements when 1 or 2 embryos were studied. The number of sexually undifferentiated on male embryos studied at each stage of development is shown in the parentheses at the bottom. One female embryo only was studied at each stage. SV, seminal vesicle; U, uterus; E, epididymis; V, vagina; P, prostate; LM, labia majora; S, scrotum; C, clitoris; Ps, shaft of penis; Pf, foreskin of penis; Pg, glans penis.

set of male sexual differentiation. In the müllerian and wolffian ducts, however, the situation is different since no dihydrotestosterone formation was demonstrable in either sex until the 9.1–11 cm stage of development, indeed until seminal vesicle and uterus formation were clear cut in both male and female embryos. Since in these tissues the ability to form dihydrotestosterone was acquired relatively late in the developmental process, it must be the consequence of some other hormone effect or of some nonhormonal process in differentiation. In this study, furthermore, no significant dihydrotestosterone formation was found in the epididymis at any stage examined whereas a very high rate of formation is present in the adult epididymis of man (22); thus, this capacity must be acquired at some later stage of development.

Discussion

The findings in human embryos are in general agreement with previous studies of male sexual differentiation in experimental animals (9–14). First, there is close correlation between the initiation of the development of the male phenotype and the acquisition of the capacity of the fetal testis to convert the C_{21} precursors pregnenolone and progesterone to testosterone. Such precursor steroids are known to be present in the fetal circulation late in pregnancy and may well serve as the physiological substrate for androgen synthesis prior to the development of the complete pathway for the conversion of cholesterol to testosterone. Second, on the basis of the recovery and quantification of all radioactive products following the incubation of fetal testes with labeled progesterone and pregnenolone, it is likely that the

effective testicular androgen synthesized by the testis at the time of onset of male differentiation is testosterone itself. It is of considerable interest that the onset of this capacity to form testosterone from C_{21} steroid precursors observed in this study (3.1–5 cm CR length) corresponds exactly to the phase in human embryonic development in which the interstitial cells of the testis undergo a striking cytodifferentiation and enlargement (3.1–5.4 cm) (23). Third, the embryonic anlage of the external genitalia and the prostate (the urogenital tubercle and swelling and the urogenital sinus) have the ability to form dihydrotestosterone at the time of onset of phenotypic development, and it is likely that in these tissues dihydrotestosterone is the intracellular androgen that initiates male development. Finally, in the wolffian ducts (the anlage of the epididymis, vas deferens, and seminal vesicle) no dihydrotestosterone formation could be demonstrated until development was far advanced, suggesting that in this process testosterone itself is the hormone responsible for male differentiation and that dihydrotestosterone formation is a secondary consequence of this developmental process.

On the basis of these findings, it can be deduced that at least three hormones are involved in the process of male sexual differentiation in the human. The testis itself is responsible for the secretion of testosterone and of müllerian regression factor, the substance responsible for the suppression of müllerian duct development at the time of male sexual differentiation (2–4). Testosterone acts directly to initiate virilization of the wolffian ducts and serves as a prohormone for the formation of dihydrotestosterone, which appears to be the effective androgen that mediates virilization of the urogenital sinus and external genitalia.

Several critical issues remain unexplained, however. Perhaps the most fundamental question is whether the initial testosterone formation by the fetal testis is regulated by other hormonal mechanisms or is instead an inherent capacity of the tissue. On the one hand it is possible that the enzymatic machinery for testosterone formation is regulated by differentiation that is genetically programmed and that the ability to form testosterone from circulating steroid precursors at this early and critical stage of male development is acquired prior to the maturation of the complete machinery required for de novo testosterone synthesis. If this is indeed the case, the initiation of male sexual differentiation by the fetal testis could be characterized as the result of enzymatic maturation that is programmed in testicular development and that is independent of control by trophic hormones. On the other hand, it is impossible at present to exclude the alternate possibility that even at the early stages of testicular development the tissue is under fundamental regulatory control by chorionic or pituitary gonadotropin.

It has also not been possible in these studies to determine whether the appearance of the enzymes that convert C_{21} steroids to testosterone appear asymmetrically or develop simultaneously. In the case of the rabbit embryo, in which advantage was taken of precisely timed pregnancies and the short gestation time, it was possible to show that the appearance of only one enzyme necessary for this process, 3β-ol-dehydrogenase-Δ^5-isomerase complex, correlated with the onset of testosterone synthesis by the fetal testis (10). In the human embryos studied, however, in which the gestation period is approximately 9 times as long and in which the sampling is random in nature it was not possible to show such an asymmetrical pattern of development. This could imply that the enzymatic capacities necessary for this process develop concurrently as a package in the human testis. However, it is also possible that a period of asymmetrical development of relatively short duration exists and was missed in the random specimens used in this study. It is also conceivable that the studies were not ideally designed to characterize the process. For example, if 17-hydroxylation were the rate limiting reaction, this could only be determined if a careful

time sequence study of the metabolism of all intermediates in the process in the early gonad were performed.

It is of real interest that testosterone synthesis appeared to decrease in the latest stages of embryonic development whether the results are expressed on a unit weight basis or projected to the whole organ. Such a fall might be real or artifactual. The latter could occur if in the later stages of development the testis begins to synthesis C_{21} steroids and consequently dilutes the specific activities of the exogenous precursors utilized for this study. However, the fact that 17-hydroxylation both of progesterone and pregnenolone was unchanged at this time suggests that at the late stage of development some reaction subsequent to 17-hydroxylation must be rate limiting in testosterone synthesis. It is also of interest that the decrease in testosterone formation is coincident with a regression in the size and numbers of the interstitial cells in the fetal testis that is pronounced by 29 cm of development (23). The coincidence of these two phenomenon suggests that the diminished testosterone formation observed in this study may be real and that the elucidation of the regulatory mechanisms involved might provide valuable insight into late events of sexual maturation in the embryo.

It is worthwhile emphasizing that an unstated assumption of these studies is that the capacity for testosterone synthesis reflects the actual secretion of the hormone by the testis. This assumption, which is unproven, seems reasonable in view of the fact that the timing of the appearance of the synthetic capacity correlates so closely with the onset of male sexual differentiation and with the known events in testicular development. It should be pointed out that measurement of blood secretory rates, even if technically feasible in the early embryo, would not necessarily yield a more appropriate assessment of testicular function at this stage of development since it is likely that some testosterone effects may be mediated by local diffusion of the hormones and would consequently not be reflected in blood secretory rates. Nevertheless, it is critical that the secretory processes and the protein carriers, if any, for the hormone be characterized.

Finally, certain conclusions of clinical importance can be deduced from these studies. It is clear that the processes that give rise to complete male pseudohermaphroditism must be operative in the human embryo by the 3–5 cm stage of development; for example, in the testicular feminization syndrome in which an inherited defect in the action of testosterone leads to the development of phenotypic females in affected XY individuals (15,16), the defect must be manifest by the time of onset of testosterone synthesis that occurs by 3.1 cm of development. Furthermore, the less severe malformations of sexual differentiation, of which hypospadias is the most common, must be the result of events that occur between the onset of testosterone formation and the anatomical completion of the male urethra, *e.g.*, between 3 cm and 6–8 cm of development (24). It is clear that a more precise definition of the quantitative aspects of testosterone synthesis during this critical period, the intracellular metabolism and the receptor mechanisms for testosterone, and the metabolic events involved in the differentiative process would yield valuable insight into the pathogenesis of these disorders.

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