Metabolism of Testosterone-^{14}C by Cultured Human Cells

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ABSTRACT The metabolism of ^{14}C-labeled testosterone by cultured human fibroblasts and amniotic fluid cells was investigated. Radiolabeled testosterone was incubated with the cultured cells for 48 hr, and the labeled metabolites present in the medium were subsequently identified. The major metabolic products of testosterone formed by cultured fibroblasts were Δ^4-androstenedione, dihydrotestosterone, androsterone, and androstanediol. The amount of testosterone metabolized through each of two pathways was calculated and used to form a ratio designated the 17β-hydroxyl/17-ketonic ratio. Fibroblasts from normal male and female children and adult females had high 17β-hydroxyl/17-ketonic ratios indicating testosterone metabolism occurred primarily through the 17β-hydroxyl pathway. There was a change in the pattern of testosterone metabolism with age in males, i.e., adult males had much lower 17β-hydroxyl/17-ketonic ratios than did male children.

The testosterone metabolism of fibroblast cultures derived from three children with testicular feminization and their mothers was compared to normal age and sex-matched controls. Fibroblasts of children with testicular feminization metabolized testosterone predominantly through the 17-ketonic pathway and manifested a pattern of testosterone metabolism distinctly different from their sex and age matched controls. The mothers of children with testicular feminization could be distinguished from normal females by their much lower 17β-hydroxyl/17-ketonic ratios. The much lower amounts of dihydrotestosterone and androstanediol produced by fibroblasts from patients with testicular feminization as compared with normals suggests there is a decrease in testosterone 5α-reductase activity in these patients.

Cultured amniotic fluid cells metabolized testosterone to the same four major metabolites found in fibroblast cultures, but their activity was much lower than that of fibroblasts. Most of the amniotic fluid cell cultures metabolized testosterone largely through the 17β-hydroxyl pathway as did fibroblasts from normal children.

INTRODUCTION

The technique of cell culture has been widely used in recent years to investigate the biochemical basis of many hereditary diseases. Cultured skin fibroblasts and cultured amniotic fluid cells have been used to detect genetic disorders of lipid, amino acid, mucopolysaccharide, and carbohydrate metabolism (1, 2). By use of the cell culture technique, homozygous and heterozygous states have been detected and distinguished for some genetic disorders.

Sweat et al. in 1958 and Berliner, Swim, and Dougherty in 1960 studied the metabolism of progesterone, cortisol, and corticosterone by cultured human fibroblasts (3, 4), thus demonstrating the feasibility of using the cell culture technique for the study of steroid metabolism. We wished to apply the technique of cell culture to the diagnosis of genetic disorders of steroid metabolism utilizing fibroblasts and amniotic fluid cells in culture. Recently a number of investigators have studied the metabolism of testosterone by human skin taken from normal subjects and patients with testicular feminization. Gomez and Hsia (5) studied the metabolism of testosterone-^{14}C by skin slices obtained from normal subjects. They identified androstenedione, dihydrotestosterone, androstanedione, androsterone, and epiandrosterone as metabolites of testosterone. Wilson and Walker (6) demonstrated a decreased conversion of testosterone to dihydrotestosterone in skin slices from the labia majora of patients with testicular feminization. Northcutt, Island, and Liddle (7) found that the amount of

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dihydrotestosterone formed by slices of suprapubic skin was lower in testicular feminization patients than in normal males. The mother of one of their testicular feminization patients could not be distinguished from a normal female when the reduction of testosterone to dihydrotestosterone by suprapubic skin slices was compared. The metabolism of testosterone by skin slices was studied by Jenkins and Ash (8) who reported that suprapubic skin slices from patients with testicular feminization metabolized testosterone in the same manner as did skin slices from normal males and females. In a study by Mauvais-Jarvis, Bercovici, and Gauthier (9), testosterone-^{14}C was administered i.v. while testosterone-^{3}H was applied percutaneously to normal subjects and patients with testicular feminization. They found that testosterone 5α-reductase was greatly decreased in the skin of patients with testicular feminization.

Since the metabolism of testosterone by human skin slices in vitro had been amply demonstrated, a study was undertaken of the metabolism of testosterone by cultured skin fibroblasts obtained from normal individuals, both male and female of different ages, as well as from patients with testicular feminization and their mothers. We hoped to be able to demonstrate a difference in the metabolism of testosterone by fibroblasts from testicular feminization patients as compared to normals for use in diagnosis and as a possible system by which to study this disease. Metabolism of testosterone by normal cultured amniotic fluid cells was also investigated in order to determine to what extent these fetal cells could be used to study steroid metabolism.

METHODS

Cell cultures

To establish skin fibroblast cultures, biopsies were taken without anesthesia from the extensor surface of the upper arm from all individuals studied. The method of Danes and Bearn (10) was used for the establishment of the fibroblast cultures. Cell lines were grown in the tissue culture laboratories of Dr. Hirschhorn and Dr. Danes. The medium used was Eagle's minimum essential medium (MEM) supplemented with 15% or 30% fetal calf serum, 1% penicillin (10,000 U/ml), 1% streptomycin (10,000 μg/ml), and 1% L-glutamine (200 mM). Studies of representative fibroblast cultures showed that there were no differences in testosterone metabolism whether 15% or 30% fetal calf serum was used. Fibroblast cultures were fed twice weekly and when confluent, were subcultured by treatment with 0.25% trypsin.

TABLE I

Chromatography Systems Used

I:M:W	Isooctane:methanol:water (10:10:1)
M:M:W	Mesitylene:methanol:water (10:10:1)
C:B:M:W	Cyclohexane:benzene:methanol:water (10:2.5:10:1.5)
D:N:M	Decalin:nitromethane:methanol (10:5:5)
C:M	Chloroform:methanol (98:2)

Amniotic fluid obtained by amniocentesis was centrifuged at 250 *g* for 15 min, and the cells were resuspended in McCoy's 5A medium (Grand Island Biological Co., Grand Island, N. Y.) and distributed to 5 ml sterile plastic flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The cell cultures were gassed with 10% CO_2 in air and incubated at 37°C. Cell cultures were fed twice a week and gassed after each feeding. The complete medium (5 ml) consisted of McCoy's 5A medium (containing 30% fetal calf serum) supplemented with 1% penicillin (10,000 U/ml), 1% streptomycin (10,000 μg/ml), and 1% L-glutamine (200 mM). When confluent, the amniotic fluid cells were subcultured by treatment with 0.25% trypsin. Sex of the amniotic fluid cells was confirmed by karyotypic analysis.

All cells used in these experiments had been subcultured one to six times. The average number of subcultures undergone by the fibroblasts was three and by the amniotic fluid cells, two.

Chemicals

Testosterone-4-^{14}C (SA approximately 58.8 mCi/mmole) was purchased from New England Nuclear Corp. (Boston, Mass.) and purified by chromatography on paper in the solvent system C:B:M:W for 16 hr. (See Table I). Specific activity of this testosterone-^{14}C was determined by acetylation with acetic anhydride-^{3}H whose specific activity had previously been determined (11). Tritium-labeled androgens obtained from New England Nuclear were checked for purity by paper chromatography in several different solvent systems and were used without further purification. Tritium-labeled 5α-androstanediols (3α + 3β) were a gift from Dr. Jean D. Wilson. Reference steroids were purchased from Mann Research Laboratories, Inc., N. Y. All solvents and reagents used were purified as previously described (11).

Incubation with testosterone-4-^{14}C

Purified testosterone-4-^{14}C (SA = 49.5 mCi/mmole) was dissolved in absolute ethanol for addition to the cell cultures. The final concentration of ethanol in the cell culture medium was usually 0.5% and never exceeded 1%. Previous control studies on alcohol toxicity had shown that these concentrations of ethanol in the media did not affect the growth or morphology of cultured fibroblasts or amniotic fluid cells. The volume of medium in each cell culture flask was 5 ml. Incubation of the culture with radioactive testosterone was carried out for 48 hr without cofactors. The medium was then removed for extraction of the radioactive steroids (Fig. 1). Preliminary experiments had shown that no significant amount of radioactivity remained in the cells after 48 hr incubation, therefore only the medium was subjected to steroidal analyses. The remaining cells were harvested by treatment with trypsin and disodium versenate, and their total DNA content was determined by the diphenylamine reaction (12). The medium and cells were stored at −20°C before determination of DNA content and extraction of steroids. Tritium-labeled testosterone, androsterone, Δ^4-androstenedione, dihydrotestosterone, and androstanediol were added to the medium before extraction to estimate losses during purification. Unlabeled androstanedione was added quantitatively to correct for losses of this metabolite. Extraction of steroids from the medium was carried out with 9 vol of dichloromethane-ethyl acetate (1:1, v:v in a separatory funnel). The upper aqueous layer was removed,

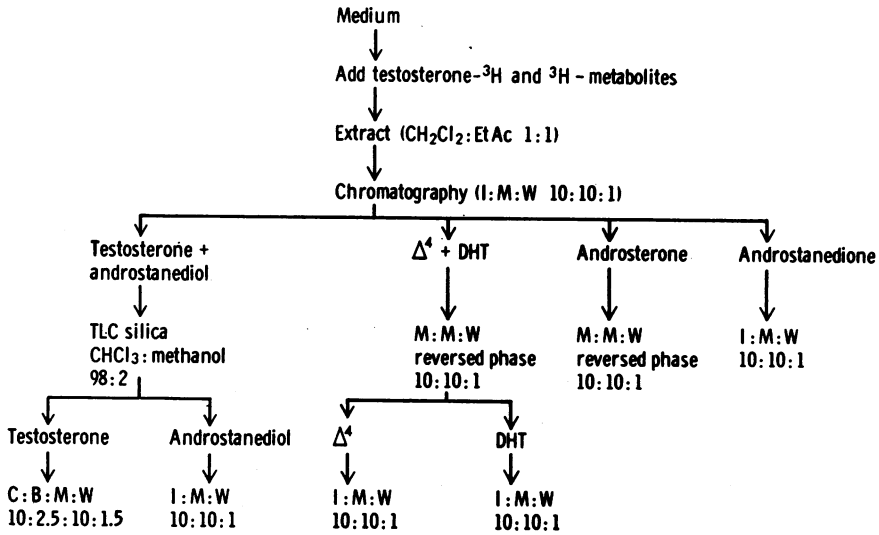

FIGURE 1 Purification of testosterone and its metabolites.

and the organic extract was washed once with a $\frac{1}{10}$ vol of 0.1 N NaOH and twice with a $\frac{1}{10}$ vol of water. The extract was evaporated to dryness and the residue was subjected to paper chromatography in the I: M: W system (Table I) for 16 hr. The radioactive steroids were located by scanning with a Packard Radiochromatogram Scanner (Model 7201; Packard Instrument Co., Inc., Downers Grove, Ill.) and comparison with reference steroids. Radioactive peaks were eluted with methanol and resubmitted to paper or thin-layer chromatography for further purification. Thin-layer chromatography which was used to separate labeled testosterone from the 5α-androstanediols (3α + 3β) was carried out on 10 × 40 cm precoated silica gel plates (MN Polygram Sil N-HR) obtained from Brinkmann Instruments, Inc., Westbury, N. Y. The solvent system used was chloroform: methanol (98:2), and the running time was approximately 3½ hr. Testosterone was detected by viewing the plate under a UV-scanner, and androstanediol was detected by staining reference steroids with 5% phosphomolybdic acid in ethanol and heating at 100°C.

TABLE II

*$^3H/^{14}C$ Ratios on Sequential Purification Steps (A-G)**

Testosterone		Androsterone		Δ^4-Androstenedione		Dihydrotestosterone		Androstanediol	
A	1.78	A	54.82	B	58.50	B	1.96	G	1.92
C	1.90	B	48.43	D	55.80	D	2.07	H	1.84
E	1.81	E	53.86	F	56.17	E	1.98		
A	26.87	A	29.30	D	10.27	B	36.0	G	1.21
C	28.81	B	30.80	F	10.35	D	35.8	H	1.28
E	27.85	E	29.62	E	10.58				
A	2.58	A	44.80	B	19.64	B	21.2	G	1.99
C	2.37	B	45.07	D	20.38	D	20.9	H	1.81
E	2.35			F	19.69				
A	9.65	A	79.50	B	60.16	B	91.8	G	13.30
C	9.27	B	78.94	D	62.50	D	91.9	H	14.52
E	9.42			F	61.28				

* A, after 1st paper chromatography (system I:M:W); B, after 2nd paper chromatography (system M:M:W); C, after 2nd paper chromatography (system C:B:M:W); D, after 3rd paper chromatography (system I:M:W); E, after acetylation and rechromatography (system D:N:M); F, after reduction with sodium borohydride and rechromatography (system C:B:M:W); G, after one paper chromatography (I:M:W) and one thin-layer chromatography (system C:M); H, after two thin-layer chromatographies (system C:M).

Each metabolite was chromatographed at least twice, and its $^{3}H/^{14}C$ ratio was determined by counting in a liquid scintillation counter (Packard Tri-Carb Model 3375). A minimum of 5,000 events was counted, resulting in a standard error of less than 1.4%. The efficiency for tritium was 41%, and for ^{14}C, 69%.

Evaluation of the method

Specificity. Purity of the labeled metabolites was demonstrated by constancy of the $^{3}H/^{14}C$ ratio during successive chromatographies and after derivative formation (acetylation or borohydride reduction) (Table II). Acetates of testosterone, dihydrotestosterone, androsterone, and etiocholanolone were formed by reacting dried eluates with 100% acetic anhydride (0.1 ml) and anhydrous pyridine (0.1 ml) overnight at room temperature. The borohydride reduction product of Δ^4-androstenedione was formed by the method of Gandy and Peterson (13). The acetylated derivatives were chromatographed in system D:N:M (Table I) for 16 hr. The Δ^4-androstenedione which was reduced to testosterone was chromatographed in the C:B:M:W system (Table I) for 16 hr.

After preliminary experiments revealed that no further purification was achieved by this step, derivative formation was eliminated from the method. In early experiments separation of 3α from 3β, 5α-androstanediol, indicated that all the radioactivity was in the 3α-fraction. No attempt was made subsequently to separate the 3α from the 3β-androstanediol. Epiandrosterone acetate was separated from dihydrotesterone (DHT) acetate in chromatography system D:N:M.

Precision. Results utilizing the same cell line but carrying out the incubation at separate times indicate good reproducibility between experiments (Table III).

Controls. Addition of testosterone at the end-time permitted us to estimate recovery of added unmetabolized substrate. These experiments showed 97–100% recovery of added testosterone.

Methodological losses. Labeled testosterone was incubated for 48 hr with 5 ml of complete medium but without cells. Recovery of approximately 94% of the added testosterone was achieved with no formation of metabolites detected.

TABLE III

Precision of Results in Two Separate Experiments Utilizing the Same Cell Strain from a 7 yr old Girl With Testicular Feminization

	Experiment I	Experiment II
Substrate (T-^{14}C), *cpm*	139,155	55,662
Amount of DNA, μg	18	21
Metabolities*		
Testosterone	38	33
Androsterone	0.61	0.65
Δ^4-Androstenedione	2.6	3.2
Dihydrotestosterone	1.6	1.5
Androstanediols	1.4	3.1
Ratio, 17β-hydroxyl/ 17-ketonic pathway	0.94	1.2

* Expressed as pmoles metabolite/μg DNA per nmole substrate.

After extraction and purification the following percentages of the tritiated metabolites were recovered: testosterone, 22%; androsterone, 32%; Δ^4-androstenedione, 33%; dihydrotesterone, 32%; and androstanediol, 13%. The preceding figures are the averages for recovery of these metabolites in 10 representative experiments.

RESULTS

Amount of testosterone metabolized by cultured cells. The amount of testosterone metabolized by the cultured cells was estimated from the amount of metabolic products formed. The amounts of testosterone metabolized in each cell culture experiment are listed in Table IV. The total amount of ^{14}C-radioactivity recovered consisted of the amounts of purified ^{14}C-metabolites plus the amount of unreacted testosterone-^{14}C recovered. All values were corrected for procedural losses. Table IV shows the recoveries of added radioactivity for each experiment. The average recovery of radioactivity for the fibroblast experiments was 86% and for the amniotic fluid cell experiments, 87%. It is clear, therefore, that the majority of the metabolic products of testosterone have been accounted for by our method and no significant quantity of testosterone or metabolites remained in the cell pellet. In preliminary experiments isolated dihydrotestosterone was acetylated and rechromatographed in system D:N:M (Table I). No change in the $^{3}H/^{14}C$ ratio of dihydrotestosterone acetate was observed after derivative formation. Since epiandrosterone acetate separates from dihydrotestosterone acetate in this system, it was concluded that no significant amount of epiandrosterone was formed by the cultured cells.

In order to compare the amounts of testosterone metabolized by each cell line, taking into account the variable cell number, the per cent testosterone metabolized per μgram of DNA was computed for each cell line (Table IV). There was no consistent correlation between the amount of testosterone metabolized and the age, sex, or clinical status of the person from whom the cells were derived.

Metabolites of testosterone formed by cultured cells. Preliminary experiments showed that the major metabolites of testosterone formed by fibroblast cell cultures were Δ^4-androstenedione, dihydrotestosterone, androsterone, and androstanediol (Table V). Androstanedione was sometimes found to be a minor metabolite of testosterone representing 1–3% of the added radioactivity. An attempt was made to identify etiocholanolone as a metabolite of testosterone, but no ^{14}C-radioactivity was associated with the tritium-labeled etiocholanolone isolated. In order to control variable cell number, DNA values were obtained for each flask of cells. Experimental results were thus expressed as pmoles of metabolite per μgram DNA per nmole of substrate used, thus taking into account the variability in cell number and in amount

TABLE IV

Amount of Testosterone-^{14}C Metabolized and Per Cent Added Radioactivity Recovered

Subjects*	Per cent testosterone metabolized‡	Per cent testosterone metabolized per μg DNA	Per cent added ^{14}C-radioactivity recovered (corrected for losses)
Fibroblast experiments			
2 yr old male (RL)	81.3	4.15	84.2
2 yr old male (ES)	92.9	19.35	99.6
2 yr old male (RS)	39.9	3.53	51.5
2 yr old TF (BS)	80.2	2.08	87.6
2 yr old TF (MP)	49.6	1.27	107.6
2 yr old TF (BS)	41.6	1.35	90.9
2 yr old TF (BS)	57.1	1.85	96.6
7 yr old TF (AC)	11.0	0.61	79.1
7 yr old TF (AC)	17.6	0.85	86.4
2 yr old female (BG)	51.0	5.93	93.9
2 yr old female (AR)	20.9	2.52	94.2
2 yr old female (ND)	24.7	1.52	59.7
Mother, TF (Mrs. C)	61.0	1.95	79.2
Mother, TF (Mrs. S)	31.1	1.88	91.1
Mother, TF (Mrs. P)	23.7	1.87	92.8
Mother, TF (Mrs. C)	40.8	2.20	81.8
Adult female (MD)	74.7	5.45	91.0
Adult female (JC)	104.3	3.24	106.0
Adult female (JM)	41	5.06	41.0
Adult female (MR)	92	2.45	104.4
Adult male (IL)	42.3	2.60	97.5
Adult male (LC)	11.2	1.87	89.9
Adult male (JG)	11.1	0.50	99.5
Adult male (DC)	12.5	2.16	56.5
Amniotic fluid cell experiments			
Male fetuses			
9 wk (BN)	22.4	0.50	95.2
9 wk (BN)	32.7	0.67	69.4
14 wk (BA)	8.5	0.33	63.3
15 wk (RP)	12.0	1.20	98.8
16 wk (MM)	7.4	0.24	82.0
16 wk (AB)	7.7	0.37	75.4
16 wk (FB)	17.7	0.98	95.0
16–19 wk (BM)	5.6	0.25	96.1
17 wk (FA)	10.1	0.35	95.7
22 wk (BC)	13.5	1.07	75.4
28.5 wk (BD)	10.5	0.46	85.1
Female fetuses			
14 wk (RC)	13.6	0.59	70.2
15 wk (MG)	11.6	0.43	113.8
15 wk (MG)	14.0	0.44	89
15 wk (DB)	9.1	0.23	86.4
16 wk (EL)	10.1	0.34	74.1
16 wk (RS)	28.8	0.62	91.7
16.5 wk (HN)	15.9	0.71	103.9
Female fetuses			
17 wk (PP)	11.3	0.52	81.7
17 wk (PP)	11.5	0.52	95.7
18 wk (BG)	4.7	0.29	95.7
20 wk (EV)	8.1	0.37	89.4

* TF, testicular feminization; letters in parentheses are the identifying initials of the subject from whom the sample was derived.

‡ Per cent testosterone metabolized as estimated from metabolic products formed.

of substrate used. In fibroblast cultures derived from normal male and female children dihydrotestosterone and androstanediol were the main metabolites of testosterone. Fibroblasts from normal adult females metabolized testosterone mainly to androstanediol and dihydrotestosterone, while those from normal adult males formed mainly Δ^4-androstenedione and dihydrotestosterone. The primary metabolite of testosterone in cultures from patients with testicular feminization and their mothers was Δ^4-androstenedione.

Metabolites formed from testosterone by cultures of amniotic fluid cells derived from 19 different male and female fetuses are shown in Table VI. The major metabolites of testosterone formed by cultured amniotic fluid cells are the same as those formed by fibroblasts, i.e., androsterone, Δ^4-androstenedione, dihydrotestosterone, and androstanediol. In seven amniotic fluid cell cultures derived from males, dihydrotestosterone was the major metabolic product of testosterone. Cultures from two slightly younger male fetuses and one older male fetus (9, 14, and 28½ wk of gestation) formed relatively more Δ^4-androstenedione. Amniotic fluid cell cultures derived from females formed mainly dihydrotestosterone, androstanediol, and Δ^4-androstenedione as metabolic products of testosterone. No one metabolite was consistently formed in greater amounts than any other. Androsterone was a relatively minor metabolic product in cultures from both male and female fetuses.

In an attempt to elucidate the relationship between substrate concentration and the formation of metabolites, the concentration of testosterone was varied in experiments using three different flasks of the same fibroblast cell line (derived from a 2 yr old testicular feminization patient). The DNA values for the three flasks were similar (38.5 μg, 30.9 μg, 30.9 μg). The fibroblasts were incubated for 48 hr with testosterone-4-^{14}C. The results

TABLE V

Testosterone Metabolism by Cultured Fibroblasts

Subjects*	Substrate	DNA	Metabolic products recovered						Ratio, 17β-hydroxyl/ 17-ketonic pathway	Per cent conversion of testosterone to DHT + adiol
			T	A	Δ⁴	DHT	Adiol	Adione		
	nmoles	*μg*	*pmoles metabolite/μg DNA/nmole substrate added*							
2 yr old male (RL)	3.8	20	1.5	0.92	0	13	28	0	45	82
2 yr old male (ES)	1.5	4.8	14	2.7	0	96	95	0	71	92
2 yr old male (RS)	2.6	11	10	2.8	1.5	16	15	1.3	7.2	34
2 yr old TF (BS)	3.9	39	1.9	4.1	15	0.91	0.73	2.2	0.086	6.2
2 yr old TF (MP)	3.9	39	15	0.46	12	0.46	0.21	0	0.056	2.6
2 yr old TF (BS)	0.50	31	16	2.6	9.6	0.71	0.55	1.0	0.11	4.0
2 yr old TF (BS)	1.2	31	13	3.1	13	0.91	1.5	1.0	0.15	7.4
7 yr old TF (AC)	1.9	18	38	0.61	2.6	1.6	1.4	—	0.94	5.4
7 yr old TF (AC)	0.74	21	33	0.65	3.2	1.5	3.1	—	1.2	9.6
2 yr old female (BG)	1.5	8.6	50	0.70	1.3	39	19	0	29	50
2 yr old female (AR)	3.8	8.3	88	0.24	1.6	16	7.2	0	13	19
2 yr old female (ND)	0.74	16	22	0.31	0.80	0.62	14	0	14	24
Mother, TF (Mrs. C)	1.9	31	5.8	7.2	4.6	2.9	4.8	1.2	0.64	24
Mother, TF (Mrs. S)	3.9	17	36	1.0	14	2.1	1.4	2.2	0.23	5.9
Mother, TF (Mrs. P)	3.9	13	54	2.5	11	1.7	3.4	0	0.36	6.5
Mother, TF (Mrs. C)	0.74	19	22	3.8	15	1.3	2.1	—	0.18	6.5
Adult female (MD)	1.5	14	12	1.2	0.29	19	34	0.22	35	74
Adult female (JM)	2.6	8.1	0.37	3.1	0.12	2.6	45	0	15	39
Adult female (JC)	1.2	32	0.52	3.1	0.10	0.87	28	0	9.1	93
Adult female (MR)	1.2	38	3.0	1.8	0.21	5.8	17	0	12	87
Adult male (IL)	3.9	16	34	2.2	22	1.2	0.86	1.0	0.087	3.4
Adult male (LC)	1.5	6.0	131	0.50	6.5	8.8	2.8	0	1.7	7.2
Adult male (JG)	1.2	22	40	0.63	2.0	1.1	1.3	0	0.92	5.3
Adult male (DC)	2.6	5.8	75	1.0	5.5	9.3	5.7	0	2.3	8.7

T, testosterone; A, androsterone; Δ⁴, Δ⁴-androstenedione; DHT, dihydrotestosterone; adiol, androstanediol; adione, androstanedione.

* See corresponding footnote to Table IV.

are shown in Fig. 2. As the concentration of testosterone in the incubation media was raised from 0.99 to 7.82 × 10^{-7} M, formation of the four major metabolites (androsterone, dihydrotestosterone, Δ⁴-androstenedione, and androstanediol) increased in a linear fashion. More Δ⁴-androstenedione was formed with increasing substrate concentration than any of the other three metabolites. These studies showed that the enzymes metabolizing testosterone were not saturated at the substrate concentrations utilized. There was no decline or plateau in the production of metabolic products with increasing substrate concentrations up to 7.82 × 10^{-7} M. All experiments with fibroblasts utilized substrate concentrations between 0.99 × 10^{-7} and 7.8 × 10^{-7} M.

To compare the relative importance of the two major pathways of testosterone catabolism (Fig. 3), the androsterone and the Δ⁴-androstenedione values were added together as were the dihydrotestosterone and androstanediol values. The former sum thus represented metabolites of the 17-ketonic pathway, and the latter, the 17β-hydroxyl pathway (14, 15). The ratio of these two pathways (17β-hydroxyl/17-ketonic) was then calculated for all experiments. The results for the fibroblast experiments are shown in Table V. The fibroblasts from the two 2 yr old patients with testicular feminization metabolized testosterone primarily through the 17-ketonic pathway. In two cultures derived from a 7 yr old patient with testicular feminization the 17-ketonic and the 17β-hydroxyl pathways were almost equally utilized. In contrast, cultures from normal 2 yr old male children metabolized testosterone almost exclusively through the 17β-hydroxyl pathway. Normal 2 yr old females also used the 17β-hydroxyl pathway primarily. Fibroblast cultures established from three mothers of children with testicular feminization showed predominance of the 17-ketonic pathway of testosterone metabolism. These mothers thus showed a significant reduction in the importance of the 17β-hydroxyl pathway when compared to normal adult

TABLE VI

Testosterone Metabolism by Cultured Amniotic Fluid Cells

Subjects*	Substrate	DNA	Metabolic products recovered						Ratio, 17β-hydroxyl/ 17-ketonic pathway	Per cent conversion of testosterone to DHT + adiol
			T‡	A	Δ⁴	DHT	Adiol	Adione		
	nmoles	*μg*	*pmoles metabolite/μg DNA/nmole substrate added*							
Male fetuses										
9 wk (BN)	3.6	45	16	0.21	4.0	0.30	0.68	0	0.23	4.2
9 wk (BN)	3.6	49	7.5	0.35	5.1	0.65	0.61	—	0.23	6.8
14 wk (BA)	5.0	26	21	0.13	1.4	0.78	0.93	—	1.1	4.5
15 wk (RP)	3.9	10	87	0.31	1.8	5.9	4.0	0	4.7	9.9
16 wk (MM)	3.6	31	24	0.05	0.53	0.76	1.0	0	3.1	5.6
16 wk (AB)	2.9	21	32	0.09	0.99	1.0	1.6	0	2.4	15.1
16 wk (FB)	1.1	18	44	0.26	1.2	6.6	2.0	0	5.7	5.5
16–19 wk (BM)	1.2	23	40	0	0.64	1.2	0.56	0	2.8	4.1
17 wk (FA)	3.6	29	30	0.24	0.80	1.5	0.98	0	2.5	7.1
22 wk (BC)	5.0	13	49	0.34	2.0	5.1	3.2	—	3.6	10.6
28.5 wk (BD)	0.74	23	32	0.12	3.2	0.60	0.64	0	0.36	2.9
Female fetuses										
14 wk (RC)	5.0	23	25	0.19	0.81	2.6	1.7	0.63	4.3	9.8
15 wk (MG)	2.9	27	38	0.15	1.1	0.96	2.1	0	2.6	8.2
15 wk (MG)	2.9	32	23	0.16	1.0	2.4	0.74	0	2.6	10.2
15 wk (DB)	1.9	41	19	0.08	0.83	0.88	0.41	0	1.4	5.3
16 wk (EL)	3.9	46	14	0.46	4.2	0.54	1.0	0	0.32	7.2
16 wk (RS)	5.0	30	21	0.17	0.48	2.0	1.1	—	4.8	8.1
16.5 wk (HN)	1.1	22	39	0.22	0.59	3.6	2.8	—	7.9	14.1
17 wk (PP)	3.6	22	32	0.17	1.2	2.3	1.5	—	2.7	8.2
17 wk (PP)	2.2	22	38	0.23	2.0	0.81	2.2	0	1.4	6.6
18 wk (BG)	1.2	16	56	0	1.6	0.88	0.40	0	0.81	2.1
20 wk (EV)	1.9	22	36	0.10	1.0	1.7	0.77	0	2.3	5.6

* See corresponding footnote to Table IV.

‡ See Table V for abbreviations.

females. In the case of normal adult males, the results are not consistent. Two experiments had ratios favoring the 17-ketonic pathway while two others utilized mainly the 17β-hydroxyl pathway. It is clear, however, that adult males can be distinguished from adult females; that children with testicular feminization can be distinguished from normal male children and that mothers of patients with testicular feminization can also be distinguished from normal adult females by virtue of their different 17β-hydroxyl/17-ketonic pathway ratios.

Amniotic fluid cell cultures from male fetuses, with three exceptions, metabolized testosterone mainly via the 17β-hydroxyl pathway (Table VI). One culture from a very young male fetus (BN, 9 wk of gestation) metabolized testosterone almost exclusively by the 17-ketonic pathway. Although these two experiments are hardly conclusive, the predominance of the 17β-hydroxyl pathway in the older fetuses may reflect maturation of the appropriate steroid enzymes. Amniotic fluid cells from seven female fetuses showed a 17β-hydroxyl/17-ketonic ratio greater than one, whereas the remaining two female fetuses showed a predominance of the 17-ketonic pathway.

DISCUSSION

It has been shown in this study that cultured skin fibroblasts metabolize testosterone actively. The metabolites of testosterone formed by cultured fibroblasts are the same as those reported for skin slices in vitro (5, 6, 8). Previous investigators who studied testosterone metabolism by skin slices in vitro did not, however, identify androstanediol as a metabolic product of testosterone (5, 8). No 5β-metabolites of testosterone were identified in the cell culture experiments which is in agreement with investigations (5, 6, 8) using tissue slices.

The metabolism of testosterone by fibroblasts derived from patients with testicular feminization was clearly

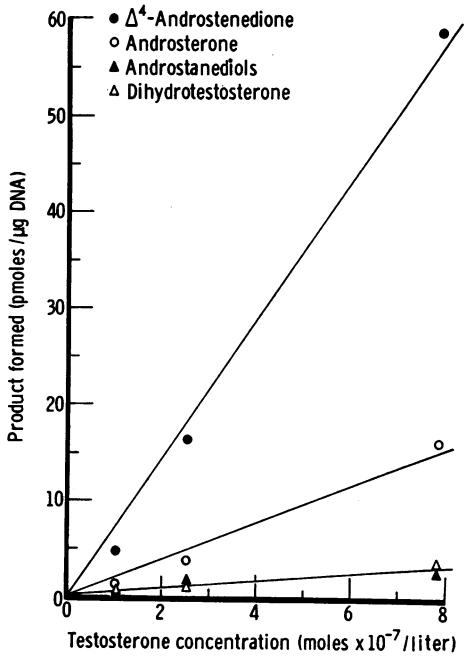

FIGURE 2 Relationship of substrate concentration to metabolic product formation in fibroblast cell cultures.

distinguished from that of fibroblasts derived from age-matched normal males. While normal male children utilized the 17β-hydroxyl pathway almost exclusively, the children with testicular feminization either favored the 17-ketonic pathway greatly or metabolized testosterone equally through both pathways. Cultured cells from children with testicular feminization produced less 5α-reduced metabolites (dihydrotestosterone and androstanediol) than did cells from normal children. These results suggest that there is a deficiency of testosterone 5α-reductase in fibroblasts of children with testicular feminization. It is, however, entirely possible that factors other than impaired enzymatic activity, e.g. decreased nuclear binding of testosterone, were responsible for the lower production of 5α-reduced metabolites by cultured cells from patients with testicular feminization. Wilson and Walker (6) and Northcutt, Island, and Liddle (7) have also found decreased activity of testosterone 5α-reductase in sex skin slices from patients with testicular feminization.

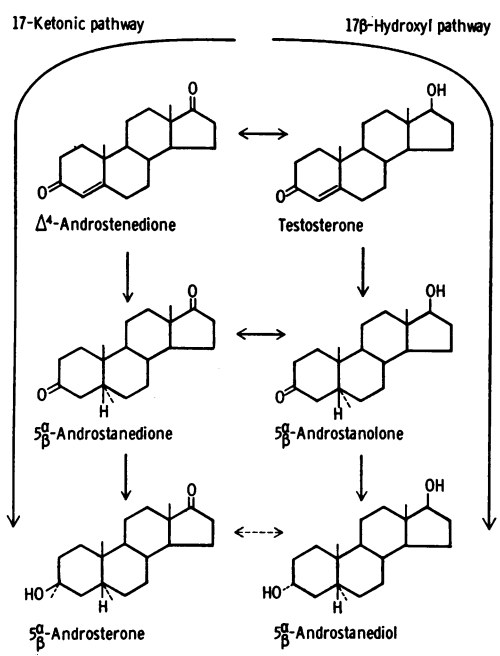

FIGURE 3 Different pathways of testosterone metabolism.

Cultured cells from adult females and adult males could be distinguished by the metabolic products of testosterone (17β-hydroxyl/17-ketonic ratios) but male and female children could not be clearly distinguished in this way. There was a change in the pattern of testosterone metabolism with age in fibroblasts from males but not in fibroblasts from females. Cultured fibroblasts from male children had much greater 17β-hydroxyl/17-ketonic ratios than did those from adult males suggesting a maturational change in testosterone metabolism. These in vitro results using cultured cells do not parallel the physiological in vivo metabolism of testosterone wherein the adult male would produce more of the biologically active 5α-reduced metabolites. However, the highly artificial experimental conditions of cultured cells may not permit extrapolation to in vivo metabolism.

It is apparent however, that the use of cultured skin fibroblasts in the study of testosterone metabolism provides a new diagnostic tool to distinguish children with testicular feminization from normal children. The metabolism of steroids other than testosterone by cultured skin fibroblasts may prove useful in the study of other steroid hormone disorders. Even organ-specific disorders such as the adrenogenital syndrome may be amenable to study by the cell culture technique since other organ-specific diseases such as Tay Sachs disease (16) and erythropoetic porphyria (17) have shown expression of their biochemical defects in cultured skin fibroblasts.

While the experiments presented here aid in the diagnosis of testicular feminization they do not shed light on the mechanism causing the difference in the metabolism of testosterone between normal cultured cells and cells from patients with testicular feminization nor do these observations elucidate the cause of the syndrome of testicular feminization. However cultured skin fibroblasts may provide a useful model system in which to study the target cell separate from

the host in a target cell disorder such as testicular feminization. At present, it is unclear whether the deficiency of testosterone 5α-reductase demonstrated in vivo is the basic defect in this condition. Strickland and French (18) provided evidence to the contrary in that patients with testicular feminization did not respond to dihydrotestosterone administration.

An important outcome of this study is the capacity to detect the carrier of the gene for testicular feminization. The mothers of patients with testicular feminization could be distinguished from normal women by reference to the pattern of testosterone metabolism of their skin fibroblasts. The testicular feminization syndrome in humans may be an X-linked disease since a presumably homologous mutation in rodents has been shown to be X-linked (19, 20). Results indicate that fibroblasts from the mothers of children with testicular feminization appear to show an abnormal testosterone metabolism. Therefore, it may be possible to clone the cells of a heterozygous mother and demonstrate the mode of inheritance of this disorder. According to the Lyon hypothesis (21), if the gene were X-linked in humans, two clonal populations would result; one normal and the other abnormal with respect to testosterone metabolism. This cloning technique has been used to demonstrate the mode of inheritance of Lesch-Nyhan syndrome (22).

Amniotic cells in culture have demonstrated a capacity to metabolize testosterone. Thus, a technique is now available to study fetal metabolism of testosterone without interruption of pregnancy. It may be possible to demonstrate a difference in the production of 5α-reduced metabolites of testosterone in amniotic cells from a fetus with testicular feminization thereby making the prenatal diagnosis of testicular feminization possible. Such studies could be extended to other steroidal hormones and would have broad scope in the prenatal diagnosis of other hormonal disorders and in the investigation of the effect of drugs and hormones on prenatal steroid metabolism. In conclusion, the study of the metabolism of steroids in cell culture has provided a new and powerful tool to study target cell disease and may yield a new approach to prenatal diagnosis and genetic counseling in hereditary diseases of steroid metabolism.

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REFERENCES

1. Hsia, D. Y. Y. 1970. Study of hereditary metabolic diseases using in vitro techniques. *Metabolism (Clin. Exp.)*. **19**: 309.
2. Milunsky, A., J. W. Littlefield, V. E. Shih, and L. Atkins. 1970. Prenatal genetic diagnosis (first of three parts). *N. Engl. J. Med.* **283**: 1370.
3. Sweat, M. L., B. I. Grosser, D. L. Berliner, H. E. Swim, C. J. Nabors, and T. F. Dougherty. 1958. The metabolism of cortisol and progesterone by cultured uterine fibroblasts, strain U 12-705. *Biochim. Biophys. Acta.* **28**: 591.
4. Berliner, D. L., H. E. Swim, and T. F. Dougherty. 1960. Metabolism of [4-^{14}C] corticosterone by fibroblasts, strain U 12-79. *Biochim. Biophys. Acta.* **38**: 184.
5. Gomez, E. C., and S. L. Hsia. 1968. In vitro metabolism of testosterone-4-^{14}C and Δ^4-androstene-3,17-dione-4-^{14}C in human skin. *Biochemistry.* **7**: 24.
6. Wilson, J. D., and J. D. Walker. 1969. The conversion of testosterone to 5α-androstan-17β-ol-3-one (dihydrotestosterone) by skin slices of man. *J. Clin. Invest.* **48**: 371.
7. Northcutt, R. C., D. P. Island, and G. W. Liddle. 1969. An explanation for the target organ unresponsiveness to testosterone in the testicular feminization syndrome. *J. Clin. Endocrinol. Metab.* **29**: 422.
8. Jenkins, J. S., and S. Ash. 1971. The metabolism of testosterone by skin in normal subjects and in testicular feminization. *J. Endocrinol.* **49**: 515.
9. Mauvais-Jarvis, P., J. P. Bercovici, and F. Gauthier. 1969. In vivo studies of testosterone metabolism by skin of normal males and patients with the syndrome of testicular feminization. *J. Clin. Endocrinol. Metab.* **29**: 417.
10. Danes, B. S., and A. Bearn. 1969. Cystic fibrosis of the pancreas. A study in cell culture. *J. Exp. Med.* **129**: 775.
11. New, M. I., J. M. Gross, and R. E. Peterson. 1968. Double isotope dilution derivative technique for testosterone glucuronoside in urine. *Acta Endocrinol.* **58**: 77.
12. Tedesco, T. A., and W. J. Mellman. 1967. Desoxyribonucleic acid assay as a measure of cell number in preparations from monolayer cell cultures and blood leucocytes. *Exp. Cell Res.* **45**: 230.
13. Gandy, H. M., and R. E. Peterson. 1968. Measurement of testosterone and 17-ketosteroids in plasma by the double isotope dilution derivative technique. *J. Clin. Endocrinol. Metab.* **28**: 949.
14. Baulieu, E. E., and P. Mauvais-Jarvis. 1964. Studies on testosterone metabolism. I. Conversion of testosterone-17α-^{3}H to 5α-androstane-3α, 17β-diol-17α-^{3}H: a new "17β-hydroxyl pathway." *J. Biol. Chem.* **239**: 1569.
15. Mauvais-Jarvis, P., J. P. Bercovici, O. Crepy, and F. Gauthier. 1970. Studies on testosterone metabolism in subjects with testicular feminization syndrome. *J. Clin. Invest.* **49**: 31.
16. Kolodny, E. G., B. S. Uhlendorf, J. M. Quirk, et al. 1969. Gangliosides in cultured skin fibroblasts: accumulation in Tay-Sachs disease. *12th Int. Conf. Biochem. Probl. Lipids.* 39.

17. Romeo, G., M. M. Kaback, and E. Y. Levin. 1970. Uroporphyrinogen III cosynthetase activity in fibroblasts from patients with congenital erythropoietic porphyria. *Biochem. Genet.* **4**: 659.
18. Strickland, A. L., and F. S. French. 1969. Absence of response to dihydrotestosterone in the syndrome of testicular feminization. *J. Clin. Endocrinol. Metab.* **29**: 1284.
19. Ohno, S., R. Dofuku, and U. Tettenborn. 1971. More about X-linked testicular feminization of the mouse as a noninducible (i^s) mutation of a regulatory locus: 5α-androstan-3α-17β-diol as the truce inducer of kidney alcohol dehydrogenase and β-glucuronidase. *Clin. Genet.* **2**: 128.
20. Bardin, C. W., J. E. Allison, A. J. Stanley, and L. G. Gumbreck. 1969. Secretion of testosterone by the pseudohermaphrodite rat. *Endocrinology.* **84**: 435.
21. Lyon, M. F. 1968. Chromosomal and subchromosomal inactivation. *Ann. Rev. Genet.* **2**: 31.
22. Migeon, B. R., V. M. Der Kaloustian, W. L. Nyhan, W. J. Young, and B. Childs. 1968. X-linked hypoxanthine-guanine phosphoribosyl transferase deficiency: heterozygote has two clonal populations. *Science (Washington).* **160**: 425.