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ANDROGENS IN TESTES AND ADRENAL GLANDS OF THE FETAL PIG

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SUMMARY

Androgen content of fetal testes and adrenal glands was measured for late stages of pregnancy in the domestic pig. Androgens were separated by partition chromatography on Celite. Testosterone and 5-androstene-3β, 17β-diol were measured directly by competitive protein-binding to human late pregnancy plasma; androstenedione and dehydroepiandrosterone were reduced to testosterone and 5-androstene-3β, 17β-diol, respectively, for estimation. Testosterone was determined in 76 pairs of fetal testes obtained at Caesarian section in 18 sows, between days 99 and 113 of gestation. Testosterone was also estimated in testes from pigs at parturition and during early postnatal life. The amounts of testosterone were similar in testes of 99- to 106-day-old fetuses (229 ± 100 to 248 ± 28 ng per pair of testes). Levels were higher at day 110 (811 ± 229 ng), remained high until term (731 ± 371 ng), and were unchanged at day 3 of postnatal life. Appreciable amounts of androstenedione, 5-androstene-3β, 17β-diol and dehydroepiandrosterone were also present in fetal and neonatal pig testes. At all stages, however, testosterone was the androgen present in highest amounts. Lower levels of androgens were found in adrenal glands than in testes. Androstenedione was the principal androgen present in male and female fetal adrenals.

INTRODUCTION

The development of more sensitive methods in recent years has made possible the measurement of testosterone and androstenedione in testes of human, sheep, rhesus monkey and rat fetuses [1–5]. Both morphological [6] and histochemical studies [7, 8] have suggested that fetal pig testes are capable of synthesizing steroid hormones at certain periods in both early and late stages of pregnancy. Biological evidence for androgen secretion by the early fetal pig testes in organ culture has also been provided [9]. To our knowledge there have been no reports on the content of androgens in the testes of fetal pigs. In view of the importance of androgens in sexual development [10, 11], we have measured the amounts of testosterone and other androgens in the testes, and in adrenal glands, of fetuses at late stages of pregnancy in the pig.

EXPERIMENTAL

Solvents and reagents. Chemicals used were reagent grade. Solvents and the Celite for chromatography were purified as described by Bauld and Greenway [12]. Florisil (Fisher Scientific, 60–100 mesh) was washed several times with distilled water [13].

Steroids. Steroids were obtained from Steraloids Inc. [7α-^{3}H]-Androstenedione (7·7 Ci/mmol), [1,2,6,7-^{3}H]-testosterone (87 Ci/mmol) and [4-^{14}C]-dehydroepiandrosterone (57 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. [4-^{14}C]-5-Androstene 3β, 17β-diol was prepared by reduction of [4-^{14}C]-dehydroepiandrosterone (DHA). All radioactive standards were purified by thin-layer chromatography (t.l.c.).

Biological samples for assay. All sows used were Yorkshire or Yorkshire crossbreds. Fetal testes and adrenal glands were taken at surgery during the last two weeks of pregnancy in 18 sows. Testes were also removed from piglets in 4 litters within 3 days of birth. Testes from each individual were processed as a pair. Adrenals from fetuses within the same litter were pooled, according to sex. Heart tissue from female fetuses only was collected for use as control tissue. All material was stored at −20°C.

Extraction. Fetal testes and adrenal glands were homogenized in 3 ml of 0·9% saline after addition of about 1500 c.p.m. of ^{3}H-testosterone to each sample, as internal standard. Similarily, about 2000 c.p.m. of [^{14}C]-5-androstene-3β, 17β-diol, [^{14}C]-dehydroepiandrosterone and [^{3}H]-androstenedione each were added together to 0·5 g of heart which served as control tissue, in triplicate, with every batch of samples being extracted. Recovery values for the radioactive steroids in analyses of the fetal heart tissue were used for correction of the estimations of steroids, excluding testosterone, in testes and adrenals. Each homogenate was extracted twice with 10 ml of methylene dichloride, centrifuged each time, and the combined extracts were washed with 3 ml of 0·1 M NaOH and twice with 15 ml of water before evaporating to dryness.

Thin-layer chromatography. Radioactive steroid standards, tissue extracts and acetate derivatives of steroids were purified by t.l.c., using silica gel G (E. Merck AG). A system of chloroform–ethyl acetate (2:1 v/v) was used for unconjugated steroids, and

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benzene–ethyl acetate (7:3 v/v) for their acetate derivatives. Radioactive steroid standards were detected with a radioscanner (Varian Aerograph/Berthold, Model LB 2722). Steroids were then recovered from the plates by transferring the silica gel to small columns and eluting with 12 ml of methanol.

Partition column chromatography. A modification of the partition method of Butt *et al.*[14] was used to separate the androgens on small columns (0·5 × 7·0 cm.) containing about 1·0 g of Celite. The stationary phase was 80% aqueous methanol and elution was carried out in two steps, using 11 ml of hexane–benzene (4:1 v/v) followed by 14 ml of hexane–benzene (3:2 v/v). In initial work, column fraction volumes of 0·5 ml were taken to determine elution patterns (Fig. 1). When extracts from tissue samples were being eluted, androstenedione and DHA were collected in the first fraction (2–7 ml); then, both testosterone (8–13 ml) and 5-androstene-3β, 17β-diol (15–22 ml) were obtained separately. Aliquots were taken for recovery values of radioactive testosterone and 5-androstene-3β, 17β-diol.

Preparation of androstenedione and DHA for assay. Column eluate fractions which contained both androstenedione and DHA were all combined for each litter and evaporated to dryness. The residues were acetylated[15] and steroids separated by t.l.c. Dehydroepiandrosterone acetate was then hydrolyzed in methanolic potassium hydroxide solution[16].

Androstenedione and DHA were reduced to testosterone and 5-androstene-3β, 17β-diol, respectively[17, 18]; and the reduction products were purified further by partition chromatography. Recovery values for radioactive androstenedione and DHA added to heart tissue were obtained at this point in the analyses.

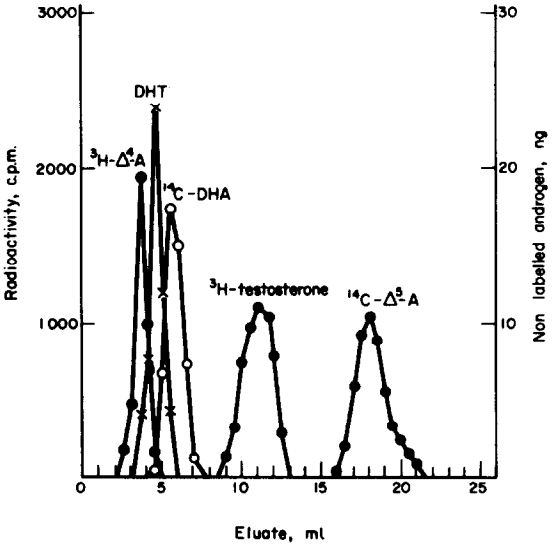

Fig. 1. Elution pattern of androgens on Celite partition column. Abbreviations used are: Δ^4-A, androstenedione; DHT, 5α-dihydrotestosterone; DHA, dehydroepiandrosterone; Δ^5-A, 5-androstene-3β, 17β-diol.

Competitive protein-binding (CPB) radioassay. Testosterone and 5-androstene-3β, 17β-diol were measured by a CPB assay similar to that described by Frick and Kincl[13]. [^{3}H]-Testosterone was added to a 1% solution of human late pregnancy plasma (HLPP) in distilled water, to give approximately 26,000 c.p.m. per ml, and the solution was stored overnight at 4°. Standard curves were prepared with duplicate tubes containing 0, 1, 2, 3, 4 and 5 ng of the appropriate steroids. Florisil (65 mg) was used to separate free from bound steroids. An aliquot of 0·5 ml of the supernatant was taken to determine the amount of bound radioactivity.

Measurement of radioactivity. Radioactivity was measured in a Packard liquid-scintillation spectrometer, model 3375, in 10 ml of Bray's solution[19], with efficiencies of 29% for ^{3}H and 77% for ^{14}C. Samples were counted until the standard deviation was <2%.

Statistical analysis. The Linear regressions of fetal age and size on the amounts of the various steroids measured in the study were analyzed using the method of least squares[20], and correlation studies were also done[20].

Validation of the method

(1) *Sensitivity.* The lowest value significantly ($P < 0{\cdot}01$) different from the control value (zero) was 0·50 ng for testosterone and 0·75 ng for 5-androstene-3β, 17β-diol. Blank values from solvents and reagents were determined by extracting 4 ml of distilled water. Fetal heart (0·5 g) served as control tissue. Tissue and solvent blanks were usually zero and always <0·15 ng, for both steroids.

(2) *Accuracy.* The quantity of testosterone estimated by the method showed a close correlation ($r = 0{\cdot}99$; $P < 0{\cdot}01$) with the expected values when 0, 2, 4, 6 and 8 ng of steroid, in triplicate, were extracted from 4 ml of distilled water. Similarly, when 50 ng quantities of steroid were added to fetal heart tissue, the values obtained were in accord with the expected values. Coefficients of variation for 4 estimations of testosterone and 5-androstene-3β, 17β-diol were 5·0 and 4·5, respectively.

(3) *Precision.* A pool of plasma was made from blood collected from the umbilical arteries of male pig fetuses. From two-ml aliquots ($n = 25$), assayed in batches of 5 aliquots each, the coefficients of variation for within and between assays were found to be 6·7% and 11·9%, respectively, with a mean value of 1·18 ± 0·14 ng of testosterone per ml of pooled plasma.

(4) *Specificity.* The specificity of the method owed much to the chromatographic separation of steroids (Fig. 1). When [^{3}H]-testosterone was chromatographed on Celite with an estimated 120 ng of testosterone from fetal pig testes, the specific activities of testosterone in the fractions

forming the elution peak were similar (26·0, 26·8, 25·7 c.p.m./ng).

Further evidence was obtained in the following way. Approximately 5000 c.p.m. of [^{3}H]-testosterone and [^{14}C]-5-androstene-3β, 17β-diol were added to the appropriate chromatographic fractions of extracts from each of four pairs of fetal testes, as internal standards. After acetylation and chromatography on t.l.c., the steroid acetates were hydrolyzed and the resulting steroids were then chromatographed on separate Celite columns. The amounts of testosterone and 5-androstene-3β, 17β-diol found were in agreement with the initial estimations (Table 1).

In addition, plasma levels of testosterone were determined in five normal men (25–45-years-old). The mean value of 5·78 ± 1·96 ng per ml of plasma is within the range reported by other investigators using radioassay methods [13, 21–23].

RESULTS

Levels of androgens in late fetal and early neonatal testes. The levels of testosterone in fetal pig testes between days 99 and 113 of pregnancy are summarized in Table 2. The mean recovery for testosterone was 67·6 ± 5·1%. All estimations were corrected for procedural losses. Total amounts of testosterone were similar in testes of 99- to 106-day-old fetuses, then rose to day 110 and remained high until term. Concentrations of testosterone showed no definite changes during the last two weeks of pregnancy. No differences in testosterone content or concentration were seen between testes at term and at 1 to 3 days of postnatal life (Fig. 2).

The total testosterone content was significantly correlated ($r = 0{\cdot}4$, $P < 0{\cdot}01$) with weight per pair of testes, and also with fetal age ($r = 0{\cdot}8$, $P < 0{\cdot}01$). Analysis of the regression of fetal age on total testosterone content indicated that the amount of

Table 1. Testosterone and androstenediol levels in fetal testes before and after further purification by acetate derivative formation

	Testosterone (ng)*		5-androstene-3β, 17β-diol (ng)*	
Sample**	Before acetylation	After acetylation	Before acetylation	After acetylation
1	1,097	1,080	81	75
2	716	707	243	261
3	1,057	1,008	92	101
4	536	559	103	99

* All estimations were corrected for procedural losses.

** One pair of fetal pig testes was used for each sample.

Table 2. Testosterone levels in fetal testes during the last two weeks of pregnancy

					Testosterone*	
No. of fetuses	No. of litters	Age (days)	Size (C.R. length, cm)	Testes weight (mg per pair)	Total (ng per pair)	Concentration (ng per 100 mg)
5	1	99	-	348 ± 45	229 ± 100	66 ± 29
4	1	101	-	629 ± 166	348 ± 66	56 ± 20
4	1	103	24.2 ± 1.3**	180 ± 11	216 ± 55	121 ± 41
4	1	105	-	304 ± 28	227 ± 39	75 ± 17
2	1	106	-	305 ± 45	248 ± 28	84 ± 21
4	1	107	26.0 ± 0.5	243 ± 33	378 ± 68	151 ± 21
8	1	108	26.8 ± 1.9	399 ± 77	488 ±185	113 ± 50
3	1	109	28.5 ± 0.4	488 ± 67	646 ± 91	135 ± 29
11	2	110	26.5 ± 1.5	493 ±121	811 ±229	176 ± 72
13	3	111	28.7 ± 1.3	534 ±176	812 ±252	50 ± 71
13	3	112	28.4 ± 1.2	489 ±155	456 ±231	93 ± 39
5	2	113	29.9 ± 1.3	722 ±139	855 ±194	103 ± 52
76	18					

* All estimations were corrected for procedural losses.

** Mean ± standard deviation.

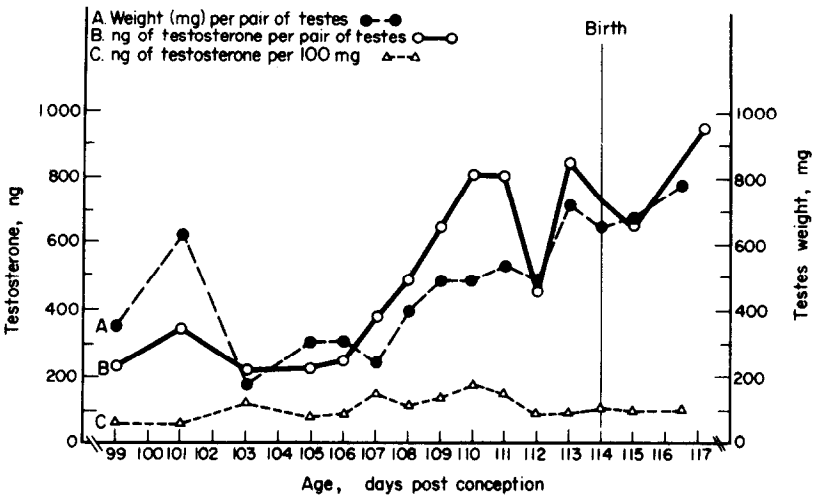

Fig. 2. Testosterone concentration and content of pig testes in relation to gestational age and early postnatal life.

testosterone per pair of testes was dependent on fetal age ($m = 44$, $P < 0{\cdot}01$); testosterone concentrations were not affected by fetal age ($m = 4{\cdot}4$, $P < 0{\cdot}01$).

Levels of 5-androstene-3β, 17β-diol in the testes of 56 fetuses from 13 litters are summarized in Table 3. The mean recovery for 5-androstene-3β, 17β-diol was $61{\cdot}2 \pm 10{\cdot}7\%$. All estimations were corrected for procedural losses. The total content and, to a lesser degree, the concentrations of 5-androstene-3β, 17β-diol per pair of fetal testes showed an increase and reached highest levels at the end of pregnancy.

Large quantities of androstenedione and DHA were found in fetal testes at late stages of pregnancy (Table 4). Since the method was not completely evaluated for these two steroids, the values given for their measurement must be regarded as semi-quantitative. All estimations were corrected for the considerable procedural losses. The mean recovery rates for androstenedione and DHA were $26{\cdot}4 \pm 17{\cdot}0$ and $43{\cdot}0 \pm 15{\cdot}0\%$, respectively. Five α-Dihydrotestosterone was not present in measurable amounts.

A comparison of the levels of the various androgens measured during late pregnancy and early postnatal life is presented in Fig. 3. It is apparent that testosterone is the major androgen present in fetal and in neonatal testes.

Androgen content of fetal adrenal glands. The amounts of testosterone, 5-androstene-3β, 17β-diol, androstenedione and DHA in adrenal glands of male and female fetuses during late pregnancy are given in Table 5. Recovery rates for these four androgens were $62{\cdot}5 \pm 9{\cdot}6$, $59{\cdot}4 \pm 12{\cdot}8$, $38{\cdot}5 \pm 11{\cdot}4$ and $18{\cdot}0 \pm 8{\cdot}1\%$, respectively. All estimations were corrected for procedural losses. Relatively small amounts of these steroids were recorded for adrenal glands in both sexes; the quantities of androstenedione were greatest in all instances.

Table 3. Androstenediol levels in fetal testes in late pregnancy

No. of fetuses	No. of litters	Age (days)	Size (C.R. length, cm)	Testes weight (mg per pair)	5-androstene-3β, 17β-diol* Total (ng per pair)	5-androstene-3β, 17β-diol* Concentration (ng per 100 mg)
5	1	99	-	348 ± 45	24.0 ± 6.5	6.9 ± 2.1
4	1	105	-	304 ± 28	25.5 ± 6.2	8.5 ± 2.4
4	1	107	26.0 ± 0.5**	243 ± 33	29.0 ±17.5	10.4 ± 3.5
3	1	109	28.5 ± 0.4	488 ± 67	52.7 ±30.0	11.1 ± 7.0
11	2	110	26.5 ± 1.5	493 ±121	90.5 ±45.1	17.4 ± 6.3
13	3	111	28.7 ± 1.3	534 ±176	176.1 ±72.3	30.7 ±22.1
13	3	112	28.4 ± 1.2	489 ±155	71.2 ±39.1	12.6 ± 5.0
3	1	113	29.2 ± 1.2	704 ±103	456.0 ±64.0	61.1 ±14.1
56	13					

* All estimations were corrected for procedural losses.

** Mean ± standard deviation.

Table 4. Androstenedione and dehydroepiandrosterone levels in fetal testes in late pregnancy

No. of fetuses	Age (days)	Testes weight (mg per pair)	Androstenedione*		Dehydroepiandrosterone*	
			Total (ng per pair)	Concentration (ng per 100 mg)	Total (ng per pair)	Concentration (ng per 100 mg)
3	109	488 ± 67**			82	17
6	110	437 ± 53	504	115		
5	110	560 ±143	210	37	152	27
4	111	580 ±212	529	91	302	52
5	112	482 ±105	200	41	341	71
2	113	749 ±174	280	37		

* All estimations were corrected for procedural losses.

** Mean ± standard deviation.

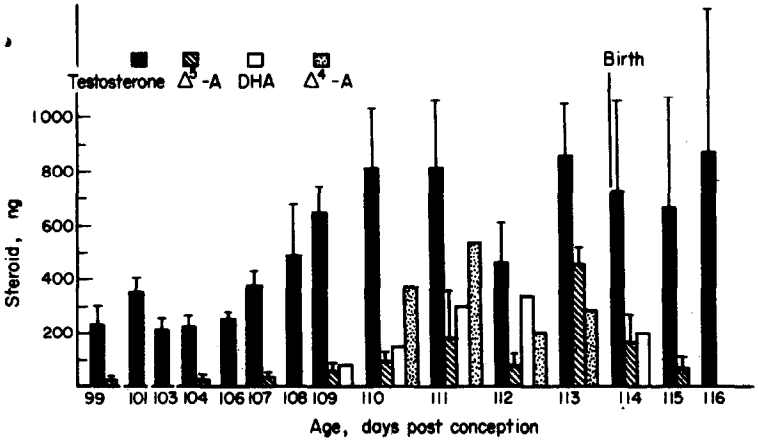

Fig. 3. Total content of testosterone and other androgens (per pair of testes) in relation to gestational age and early postnatal life. Abbreviations used are the same as given for Fig. 1. Mean values ± S.D. are shown for testosterone and 5-androstene-3β, 17β-diol only.

Table 5. Androgen content of fetal adrenal glands in late pregnancy

Sex of fetus	No. of fetuses	Size (C.R.length,cm)	Age (days)	Adrenal weight (mg)**	Testosterone	Androstenedione (ng per pair of adrenal glands)	5-androstene -3B, 17B-diol	DHA
Male	8	26.8	108	129 ± 14	3.9	24.3	4.9	6.7
Female	9	26.7	108	146 ± 28	2.2	18.0	3.2	5.3
Male	5	26.7	110	152 ± 31	5.0	42.0	4.0	4.2
Female	4	26.8	110	138 ± 28	3.4	39.0	6.0	6.0
Male	3	29.0	111	202 ± 65	1.9	18.3	3.3	13.3
Female	2	29.2	111	200 ± 40		15.0	3.0	7.1
Male	7	28.1	112	166 ± 15	5.3	28.5	3.8	4.5
Female	4	28.8	112	172 ± 31	3.5	33.2		3.3
Male	6	28.1	112	175 ± 29	6.9		8.4	8.1
Female	8	28.1	112	174 ± 24	2.5		4.2	7.5
Male	2	31.0	113	186 ± 16	11.7	40.0	5.7	

* All estimations were corrected for procedural losses.

** Mean ± standard deviation

DISCUSSION

Testosterone was the principal component in the large amounts of androgens found in fetal pig testes during late pregnancy. The findings for testicular testosterone levels during the last two weeks of pregnancy correlated well with the histological appearance [6, 24] and histochemical studies [7, 8] previously reported for the fetal pig. Testosterone concentrations, however, remained unaffected by fetal age and size. The increase in testosterone

content was, therefore, a reflection of an increase in the size of the fetal testes, which has been recorded by Ullrey *et al.*[25].

Smaller amounts of androstenedione were present; and the ratio of androstenedione to testosterone ranged from 0·35 to 0·67, in litters between days 110 and 113 of pregnancy. Similar results have been reported for the testes of fetal sheep and rhesus monkeys during late stages of pregnancy[1, 3].

Relatively high levels of 5-androstene-3β, 17β-diol and DHA were found in fetal pig testes suggesting that androgen biosynthesis might occur by a 5-ene-steroid pathway. Various biochemical studies have indicated that DHA can be formed from sodium acetate and pregnenolone in mammalian fetal testes[26–28]. It has been demonstrated also that both DHA and 5-androstene-3β, 17β-diol are good substrates for 5-ene and 17β-hydroxysteroid dehydrogenase activity in the fetal and neonatal pig testes[7, 8]. These compounds might, therefore, serve as possible precursors for testosterone synthesis in the testes of fetal pigs.

Large quantities of testosterone, 5-androstene-3β, 17β-diol and DHA were noted in pig testes taken between days 1 to 3 of postnatal life. In contrast, testosterone in testes from lambs[29], rhesus monkeys[3] and rats[18] decreased considerably after the first day of postnatal life. Mechanisms regulating the endocrine activity of fetal and neonatal testes remain largely unknown[30].

Histochemical evidence for steroidogenic activity in adrenal glands of pig fetuses during late pregnancy has been given[7]. In the present study small amounts of testosterone were detected in the adrenal glands of pig fetuses during late pregnancy, but androstenedione was present in larger quantities in both sexes. Davies and Ryan[31] reported that andorstenedione is produced in the adrenal gland of fetal pigs *in vitro*. Resko[3] also found androstenedione but little, if any, testosterone in the adrenal glands of fetal rhesus monkeys. Only small amounts of DHA and 5-androstene-3β, 17β-diol were detected in adrenal tissues of both male and female pig fetuses.

Meusy-Dessolle[32] measured testosterone in the umbilical cord blood of pigs from day 44 to birth. Plasma concentrations of testosterone in female pig fetuses were lower than those in males until the last day or two before parturition. From this observation and our findings it seems clear that the testis, rather than the fetal adrenal gland, is the principal site of testosterone biosynthesis in the late stages of fetal development in the pig.

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