Development of Δ^5, 3β-Hydroxysteroid and Glucose-6-Phosphate Dehydrogenase in the Testes, Adrenals and Ovaries of the Rabbit Fetus[1] (36701)

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The classical work of Jost demonstrating the fetal testicular control of masculine differentiation was performed in the rabbit (1). Orchiectomy produced testosterone-reversible blockade of androgen-dependent masculine development between days 19 and 22 of gestation but had no effect after day 22. Decapitation, performed on male fetuses before day 22, resulted in a gonadotropin-reversible partial block in masculine differentiation manifested by hypospadias, suggesting that hypophysectomy induced deficient testicular function. The fetal ovary did not play a role in sexual differentiation of the rabbit fetus. Jost has also shown that there is a pituitary-dependent early period of growth of the rabbit fetal adrenal cortex between days 20 and 25 (2). From day 25 to day 28 he has demonstrated a cortisone- and ACTH-dependent increase in fetal liver glycogen, which does not occur in adrenalectomized or decapitated fetuses in pregnant rats whose corticosteroid production is eliminated by adrenalectomy.

Conversion of Δ^5, 3β-hydroxysteroids to the corresponding Δ^4, 3-ketones is necessary for biosynthesis of virtually all biologically active steroid hormones (3). These reactions are catalyzed by two steroidogenic enzymes, 3β-hydroxysteroid dehydrogenase and Δ^{5-4}, 3-ketosteroid isomerase. Activity of glucose-6-phosphate dehydrogenase provides NAD PH which is the coenzyme required for steroid hydroxylations and cleavage of cholesterol and C_{17-20} side-chains. Although previous sequential histochemical analyses of these enzymes in the adrenals and testes of the human (4) and rat fetus (5) have suggested a temporal correspondence of enzymatic activity to embryonic function or dysfunction of these tissues, the semiquantitation of the histochemical method has been questioned (6). Moreover, the validity of the observation that development of these ovarian enzymes begins prenatally in the human but postnatally in the rat has been challenged because of the use of human postmortem tissue (6).

In this report we have studied the development of these enzymes in the fetal testes, adrenals and ovaries of the rabbit with the semiquantitative histochemical technique and with a quantitative method sensitive enough to detect the low level of activity of the dehydrogenase system in individual rabbit fetal tissues.

Materials and Methods. Animals. New Zealand albino female rabbits which had been mated by the supplier (Camm Research Co.) were used for these experiments. This species has a gestation period of 31 days. Pregnant females were killed by intravenous pentobarbital on days 14 through 31 of gestation. The fetuses were removed by cesarean section, sealed in plastic bags, flash frozen at —80° and stored at —20°. The gonadal sex of each fetus was determined by hematoxylin and eosin staining of sections of each gonad as described below.

Activity of 3β-hydroxysteroid dehydrogenase. The production of androstenedione-7-^{3}H (and testosterone-7-^{3}H) from dehydroepiandrosterone-7-^{3}H by 1–2 mg of a sin-

[1] Supported by research grants from the National Foundation "March of Dimes" and from the U.S. Public Health Service (AM-10,521, HD-4683).

[2] Recipient of Career Development Award (HD-13,628) from the U.S. Public Health Service.

gle fetal testis, adrenal or ovary was determined by our ultramicromodification (7) of the method of Shikita, Ogiso and Tamaoki (8). The testis, adrenal or ovary was dissected from each frozen fetus, and a 1–2 mg portion was cut, weighed and placed in an Eppendorf microcuvette (Brinkman Instruments) at 0°. The fetal gonadal tissues were incubated for 30 min at 37° in 10 μl, 66 μM potassium phosphate buffer–incubation mixture containing 20 ng (20,000 cpm) dehydroepiandrosterone-7-^{3}H (10.4 Ci/μmole) in 0.5 μl dimethylsulfoxide (DMSO), 30 μg NAD, 4 μg NADP, 6.25 μg glucose-6-phosphate and 1.25 units of glucose-6-phosphate dehydrogenase (Sigma Chemical Co.). Components of the NADPH generating system were omitted from the incubations of fetal adrenal tissues.

The reaction was terminated by the addition of 100 μl of a (1:1, v/v) mixture of absolute ethanol and acetone, shaken well, and centrifuged at 17,000g to precipitate protein. Unlabeled carrier dehydroepiandrosterone, androstenedione and testosterone in ethanol were added to each sample.

These conditions of incubation were chosen as optimum after kinetic studies had shown that production of androstenedione by fetal adrenals and ovaries and of androstenedione and testosterone by fetal testes was propor tional to time of incubation up to 1 hr as well as to the wet weight of the tissue.

Chromatography. The percentage conversion of substrate to androstenedione (or testosterone) was determined in each supernate in two thin-layer chromatographic (TLC) systems (I: chloroform/acetone, 96:4, v/v; or II: benzene/acetone, 90:10, v/v) and also on propylene glycol-impregnated Whatman No. 1 paper with hexane as mobile phase (9) and presented as an average value of the two systems. The unlabeled standards were visualized on TLC by iodine vapor and UV light and those on paper by the Zimmerman reaction.

The chromatograms were cut into 0.5 cm segments which were immersed in toluene scintillation media containing 4 g PPO and 100 mg bis-MSB (p-bis [1-metylstyryl]-benzene)/liter and counted in a Packard Tricarb 3375 liquid scintillation spectrometer. The instrument gave an absolute counting efficiency of 60% for ^{3}H. Counting time was adjusted to give a standard deviation of no more than ±7.5%. Recovery of the radioactivity added to the incubation medium was between 84 and 93%.

Radio-gas–liquid chromatography. Chloroform extracts of the supernates remaining from incubations of the tissues of 31 day fetuses were spotted on TLC and developed twice in TLC (I) system. Radio-gas–liquid chromatography (GLC) was performed on ethanolic eluates of the centers of individual label peaks corresponding to the area of standard androstenedione (or testosterone) from the thin-layer chromatograms. Standards added to the eluates included dehydroepiandrosterone, androstenedione, testosterone, 3α, 17β-dihydroxy-5α-androstan, 3β, 17β-dihydroxy-5α-androstan, 3β-hydroxy-5α-androstan-17-one, 3α-hydroxy-5α-androstan-17-one, 17β-hydroxy-5α-androstan-3-one and 5α-androstan-3, 17-dione. Samples were run directly from the eluates or, after evaporation of the ethanol, under N_2, and conversion to trimethylsilylethers (TMS-derivatives) by reacting the residue with 1:1 mixture of $CHCl_3$ and Regisil (bis[trimethylsilyl]trifluoracetamide) containing 1% trimethylchlorosilane (TMCS) for 30 min at 56°. Separation and identification was achieved on a 6 ft 1/4 in. diameter stainless steel column packed with 3% OV-225 on Supelcoport 80/100 mesh (Supelco-Inc.) in a Perkin-Elmer 900 gas chromatography. Carrier gas was helium at a flow of 130 ml/min. The effluent was split 2 to 1, one-third passing through a flame ionization detector, two-thirds being collected on p-terphenyl crystals in a Packard GLC fraction collector, Model 852. Crystals of p-terphenyl give 95–100% recovery of label in GLC effluents (10). Average recovery of standard testosterone-1,2-^{3}H not corrected for the 2 to 1 split, determined at various times during the experimental period was 56.4% (range: 45–47%).

Statistics. Significance was calculated according to the method of analysis of variance: ± one standard deviation.

Histochemical procedure. The activities of

3β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase was demonstrated histochemically by previously described methods (4, 11). The method of semiquantitation has also been descibed previously (12). The adrenals and gonads were located by serial sectioning. At various levels in these tissues, three consecutive sections were used for staining, one with hematoxylin and eosin and the other two for the two enzymes being studied.

Results. Histochemical. Testis. Significant staining of the fetal testis by activity of both dehydrogenases is confined to the interstitial cells (Fig. 1). Activity of 3β-hydroxysteroid appears first on day 19 and rapidly reaches a peak on day 21, thereafter declining, but rising again to a second more sustained level of intense activity on days 26 through 31 (Fig. 3). The levels of glucose-6-phosphate dehydrogenase have a roughly similar time of appearance and biphasic pattern of activity.

Ovary. In contrast to the testis, 3β-hydroxysteroid activity appears rather late in ovarian interstitial cells, with minimal staining first becoming evident on day 29 and remaining at very low levels for the remaining 2 days of gestation (Fig. 3). Activity of glucose-6-phosphate dehydrogenase can be seen in interstitial cells as early as day 19 (Fig. 3). This activity falls from maximum intensity on day 19 steadily until day 28 when it resembles that of Δ^5,3β-hydroxysteroid dehydrogenase.

Adrenal cortex. Activity of Δ^5,3β-hydroxysteroid dehydrogenase begins in the adrenal cortex of both sexes on day 16 (Figs. 2, 3). The pattern of both dehydrogenases also appears to be biphasic and activity of each enzyme is maximal first at day 21 and later on days 29 and 30 as in the testis.

Activity of 3β-hydroxysteroid dehydrogenase in vitro. There was no significant difference in percentage of recoveries of androstenedione and testosterone as products from dehydroepiandrosterone between the two thin-layer systems and that of the paper chromatography system. Analysis of the TLC label peak corresponding to androstenedione in incubations of fetal tissues on day 31 by

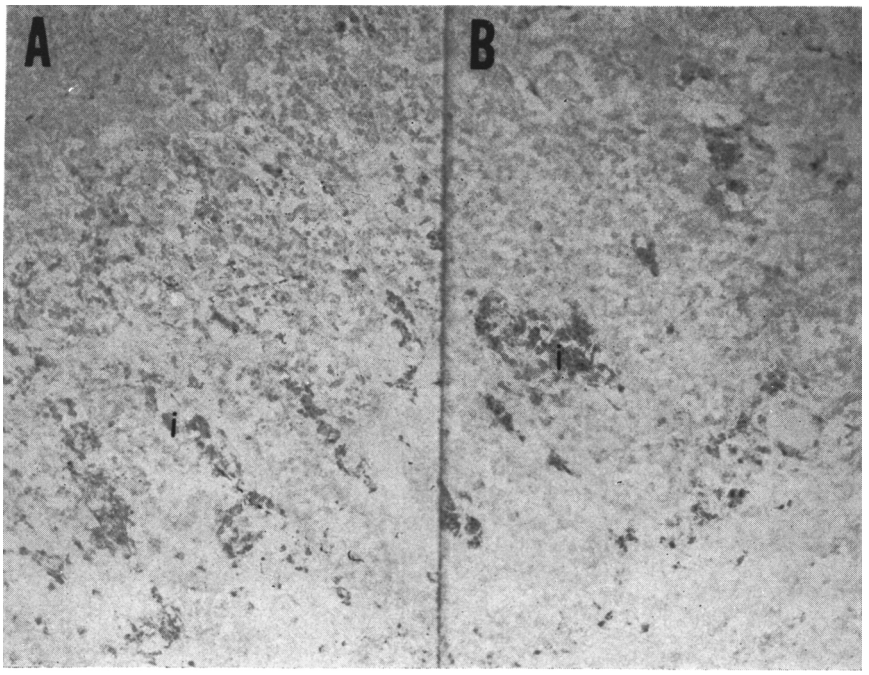

FIG. 1. Activity of 3β-hydroxysteroid dehydrogenase (A) and glucose-6-phosphate dehydrogenase (B) in interstitial cells of fetal rabbit testis on day 28. Note darker staining interstitial cells (i). ×150.

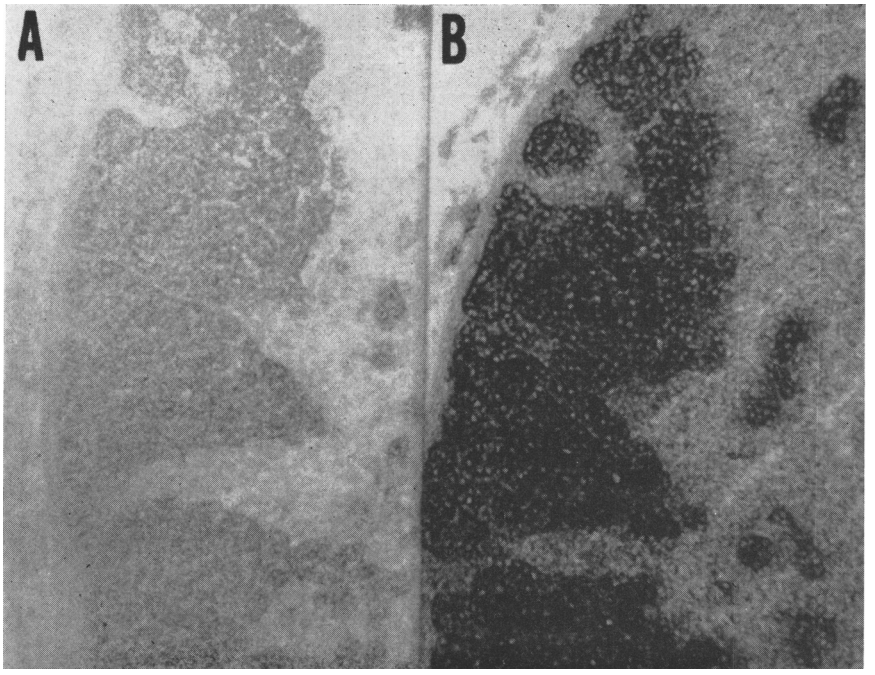

FIG. 2. Activity of 3β-hydroxysteroid dehydrogenase (A) and glucose-6-phosphate dehydrogenase (B) in fetal rabbit adrenals on day 31 of gestation. ×150.

radio-gas–liquid chromatography showed that 100% of the label had the same retention time as the authentic standard and was not contained by any of the other standards. Significant amounts of testosterone were produced only by incubations of fetal testis, only trace amounts resulting in incubations of other tissues (Table 1). Radio-gas–liquid chromatography showed that all of the label in the testosterone area had the same retention time as standard testosterone-TMS and was not contaminated by the other metabolites. Thus, the products of the incubations were demonstrated to be primarily androstenedione (and testosterone) by all three methods. There is a good correlation of the semiquantitative patterns of activity of 3β-hydroxysteriod dehydrogenase determined histochemically and the quantitative pattern of activity determined by labeled product generation *in vitro* (Fig. 3). Essential agreement can be seen in the two approaches on the time of onset of activity, biphasic pattern in the adrenal and definite but slight activity of the enzyme in the ovary just before birth.

Discussion. In the past others have relied upon pooling of large numbers of fetal glands of various species to determine steroidogenic enzyme activity (13–17). The present quantitative procedure allows for sensitive measurement of the dehydrogenase enzymes in the endocrine tissue of a single rabbit fetal gland by using an incubation volume of only 10 μl. We have utilized a similar micromethod with slightly larger incubation volumes to determine different enzymes involved in the synthesis of testosterone from progesterone by a single fetal rat testis (7). Thus, this method may have wide application for the various fetal enzymes.

The present observations indicate a good correlation between the histochemical and quantitative method in time of beginning of activity of Δ^5,3β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase in the fetal adrenal, ovaries and testes and biphasic pattern in activity in the adrenal. Although the quantitative method does not show a biphasic pattern in the testis

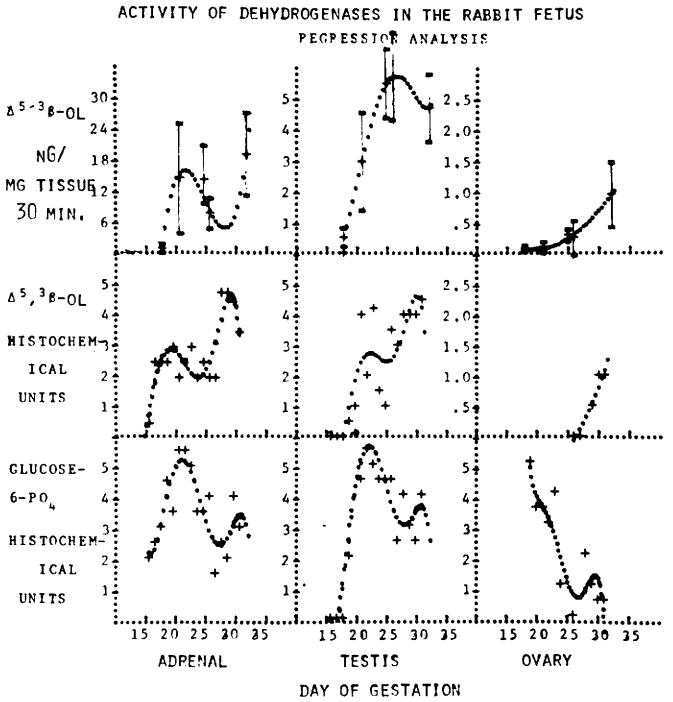

FIG. 3. Comparison of development of activity of 3β-hydroxysteroid dehydrogenase determined *in vitro* by the conversion of labeled substrate (upper) to that determined semiquantitatively by histochemistry of 3β-hydroxysteroid dehydrogenase (middle) and of glucose-6-phosphate dehydrogenase (lower). Columns: adrenal (left); testis (middle); ovary (right). Regression curves were plotted according to the equation: $Y = b(0) + b(1) + b(2)x^2 + b(3)x^3 + b(4)x^4 + b(5)x^5 + b(6)x^6$. Vertical bar: ± one standard deviation.

as does the histochemical method, Lipsett and Tullner (13), assaying testes on several more days of gestation, have demonstrated a biphasic pattern of synthesis of testosterone from pregnenolone by rabbit testis similar to the present histochemical results. The presently observed times of appearance of histochemical activity of the Δ^5, 3β-hydroxysteroid dehydrogenase in the fetal adrenal and testis agree essentially with those in the previous report of Tramontana, Botte and Chieffi (18) of day 15 in the adrenals and day 21 in the testis. Though these authors found no activity of this enzyme in the fetal ovaries on days 20 or 30, Lipsett and Tullner (13) reported the synthesis of testosterone from pregnenolone by the rabbit fetal ovary on day 29.

The development of activities of the 3β-hydroxysteroid dehydrogenase system and glucose-6-phosphate dehydrogenase system in the testis are completely consistent with the critical periods of testicular steroidogenesis indicated by the castration experiments of Jost (1, 2). Activity of the enzyme in the testis begins before the time of development of the prostatic buds and seminal vesicles on days 22 and 23 at which time the urethral fold fusion is also occurring (1). The time of the second rise in histochemical activity of Δ^5,3β-hydroxysteroid dehydrogenase activity in the testis correlates well with that of the development of the hypothalamus and stabilization of other portions of the male genital tract (11). It may be that this second rise is, as in the rat, a reflection of the increased pituitary trophic activity occurring in the last period of gestation (4).

The first peak of activity of the adrenal enzymes correlates with the early period of growth of the rabbit fetal adrenal cortex itself (2). The presence of the pituitary is

TABLE I. Conversion (%) of DHA by Fetal Tissues.

Day	No.	Tissue/incubation (mg)	Androstenedione (%)	Testosterone (%)
Adrenal				
18	6	1.7 ± 0.42	2.7 ± 0.53	1.1 ± 0.26
21	6	0.7 ± 0.17	66.2 ± 14.7	1.1 ± 0.34
25	4	1.1 ± 0.35	89.0 ± 4.0	1.4 ± 0.47
26	6	1.8 ± 0.41	62.8 ± 15.4	1.5 ± 0.36
28	4	1.2 ± 0.61	58.5 ± 15.4	1.0 ± 0.20
31	6	1.4 ± 0.13	95.6 ± 1.3[a]	0.5 ± 0.2
Testes				
18	4	3.5 ± 0.21	5.1 ± 4.4	9.1 ± 4.7
21	4	1.5 ± 0.3	19.0 ± 1.4	9.9 ± 5.7
25	2	2.1 ± 0.50	23.6 ± 8.7	24.8 ± 3.7
26	6	2.4 ± 0.47	6.6 ± 4.8	61.1 ± 13.9
31	4	2.5 ± 1.1	31.4 ± 6.7[a]	28.4 ± 18.8[a]
Ovary				
18	4	1.2 ± 0.3	0.47 ± 0.32	0.53 ± 0.32
21	4	2.1 ± 1.6	1.2 ± 1.3	1.1 ± 0.81
25	2	1.1 ± 0.8	0.8 ± 0.8	1.3 ± 0.8
26	6	1.0 ± 0.2	1.3 ± 1.0	0
31	4	4.6 ± 1.0	23.2 ± 10.9[a]	0.80 ± 0.8

[a] No major contamination by another metabolite indicated by radio-gas–liquid chromatography.

critical for this growth and it may be that ACTH leads to the increased enzyme activity, and most especially of the glucose-6-phosphate dehydrogenase (19). It cannot be surmised when this enzymatic activity is reflected in an increase in corticosteroid synthesis as is indicated by enzymatic studies of the rat (5). The second increase in activity, however, does correlate well with changes in the thymus and with increased glycogen deposition in the liver and myocardium which are observed from day 25 onward, and which have been shown to be dependent on the presence of the adrenal cortex and its production of glucocorticoids (2).

The time of appearance of the adrenal steroidogenic enzymes relative to that of the testicular enzymes varies in the rabbit, rat and man. In the rabbit, enzymatic differentiation occurs in the adrenal 3 days before it occurs in the testis, whereas in the human, the enzymes occur in the adrenal 1 month after they occur in the testes (4) and in the rat they appear concurrently (11, 20).

The development of 3β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase activity in the interstitial cells in the ovary toward the end of gestation would appear to indicate some capacity of hormone synthesizing activity at this time. Neither the ovary nor estrogens are required for female development (1), but there remains the possibility that fetal ovaries may have steroidogenesis occurring *in utero*. Although Bloch (14) did not identify testosterone synthesis in two human fetal ovaries, and no Δ^5,3β-hydroxysteroid dehydrogenase activity was found by us in the rat fetal ovary until age 10 days after birth (21), Roberts and Warren (22) showed that fetal bovine ovaries synthesize testosterone from androstenedione and progesterone.

Activity of glucose-6-phosphate dehydrogenase was determined histochemically and activity of Δ^5,3β-hydroxysteroid dehydrogenase was determine both histochemically and by a new microquantitative assay in 1 mg of endocrine tissue of the rabbit fetus beginning on day 16 of gestation. In the adrenal and testis, there is a biphasic histochemical pattern of the two enzyme systems with an early peak around days 20–22 and a

later one around days 28–30. In the fetal ovary slight histochemical activity of Δ^5, 3β-hydroxysteroid dehydrogenase appears in the interstitial cells on day 29 while that of glucose-6-phosphate dehydrogenase appears in these cells first on day 19, falls to zero by day 26 and reappears on day 28. The quantitative method demonstrates the same time of appearance of activity of 3β-hydroxysteroid dehydrogenase in all three glands (adrenals: day 17; testis: day 19; ovary: day 28) as well as the biphasic pattern of adrenal activity. The time of appearance and development of these testicular and adrenal enzymes demonstrates that these tissues are capable of steroidogenesis during the critical periods of their embryologic functioning as demonstrated by Jost, and further suggests that the rabbit ovary is capable of steroidogenesis during the last few days of gestation.

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Received Jan. 7, 1972. P.S.E.B.M., 1972, Vol. 140.