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Corticosteroidogenesis and the Response to Stress in the Developing Fetal Rabbit (41357)

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Abstract. This study examined the *in vitro* synthesis and secretion of corticosteroids by the adrenal glands of fetal rabbits on Days 21, 24, 27, and 30 of gestation and in the neonate, as well as in Day 30 fetuses obtained from pregnant rabbits who had been either dehydrated or restrained. The results (ng steroid/mg protein/hr) demonstrate that the synthesis and secretion of aldosterone were low on Day 21 and gradually increased as development progressed, while comparable parameters of corticosterone were initially high and then decreased. Fetal cortisol production was quite low in relation to corticosterone and changed very little throughout gestation. The neonates' adrenal glands exhibited marked increases in aldosterone production but not in corticosterone or cortisol. These data suggest that those enzymes related to glucocorticoid production are present prior to those for aldosterone production. Moreover, since maternal dehydration or restraint caused increases or slight diminutions, respectively, in the fetal production of both aldosterone and corticosterone, these data indicate that the fetal adrenal gland is responsive to maternal stressors. Factors implicated in this fetal adrenal response include fetal ACTH and maternal glucocorticoids.

The ability of the adrenal glands of fetuses (1–7) or neonates (6, 8, 9) to synthesize and secrete glucocorticoids (e.g., corticosterone, cortisol) has been demonstrated in numerous species either by direct examination of glandular activity or by detection of glucocorticoids in plasma. Although the fetal adrenal gland has also been shown to be capable of aldosterone production in some species (1–3, 7, 10), little information, however, is available concerning the developmental pattern of aldosterone synthesis and secretion in the rabbit, as well as the fetal adrenocortical response to maternal perturbations. Thus, the present investigation was undertaken not only to describe the *in vitro* rates of aldosterone and glucocorticoid synthesis and secretion through the later phase (Days 21–30) of gestation and in the neonate (12 hr), but also to ascertain the effects of the maternal stresses of dehydration and restraint on fetal corticosteroid production.

Materials and Methods. New Zealand white rabbits of timed pregnancy (i.e., day of mating considered Day 0 of pregnancy) were housed in individual cages in a constant temperature room (21.5°) with illumination from 0600 to 1800 hr. Purina Rabbit Chow and tap water were allowed *ad libitum* with the exception of maternal dehydration experiment, in which the pregnant animals were not allowed access to water for 48 hr prior to sacrifice on Day 30. In the restraint experiment, pregnant animals (Day 30) were placed in restraining cages for 2 hr immediately prior to autopsy. For the developmental study, pregnant rabbits were sacrificed on Days 21, 24, 27, and 30 of gestation. All pregnant rabbits were rapidly sacrificed by an intravenous injection of 5 ml sodium pentobarbital (50 mg/ml) between 0600 and 0830 hr. Following laparotomy and exposure of the bicornuate uterus, the live fetuses were delivered, weighed (Table I), and rapidly decapitated. Neonates were similarly sacrificed within 12 hr of birth (Day 34).

The fetal and neonatal kidneys were excised, weighed on a Mettler balance (sensitivity = 0.1 mg) (Table II) and fixed in Zenker solution for 12 hr. They were subsequently rinsed under tap water for 24 hr and stored in 70% ethyl alcohol until sectioned (10 μm) and stained for renin granules according to the method of Wilson (11). Using light microscopy (100×), the juxtaglomerular index was determined by

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TABLE I. BODY WEIGHTS OF FETUSES DURING THE LATER STAGES OF DEVELOPMENT AND UNDER STRESS CONDITIONS

Gestation (days)	Condition	No. of fetuses	Body wt (g)
21	Nonstressed	24	3.9 ± 0.3[a]
24	Nonstressed	23	15.0 ± 0.6
27	Nonstressed	22	27.0 ± 0.8
30	Nonstressed	15	42.0 ± 1.8
30	Dehydration	15	34.1 ± 1.8[b]
30	Restraint	12	41.8 ± 1.7
34 (neonate)	Nonstressed	12	72.5 ± 3.3

[a] Mean ± SEM.
[b] Significantly ($P < 0.05$) lower than Day 30 nonstressed group.

ascertaining the number of renin granules per juxtaglomerular apparatus. This index was qualitatively graded from 0, indicating the absence of either the complete development of the juxtaglomerular apparatus or granulation, to +++ indicating the presence of the apparatus and the degree of granulation.

The adrenal glands were rapidly excised, placed in cold 0.9% saline, cleaned of extraneous tissue, and weighed on a torsion microbalance (Table II). Two to four fetal adrenal glands, depending upon the gestational age, were quartered and placed in 10-ml Teflon beakers containing 2.0 ml Krebs–Ringer bicarbonate (KRB) buffer with 11 m*M* glucose, pH 7.4, which had been gassed with 5% CO_2:95% O_2. Following a 30-min equilibration period in a shaking water (37°) bath, the buffer was discarded and representative glands were removed for the determination of preincubation concentrations of corticosteroids. To the remaining beakers, 1.5 ml fresh KRB buffer was added and the glands incubated for an additional 60 min at 37°. The glands were then placed in vials containing 2.5 ml of 0.9% saline–ethyl alcohol (4:1, v/v) solution and homogenized, while the KRB buffer was stored in separate vials. All samples were kept at −25° until analyzed for aldosterone, corticosterone, and cortisol.

The concentration of aldosterone in the adrenal homogenates and KRB buffer was determined by radioimmunoassay (kit obtained from New England Nuclear, North Billerica, Mass.). Briefly, the sample (50–100 μl) was extracted with 10 ml methylene dichloride and the extract successively washed with 1.0 ml each of 0.1 *N* NaOH, 0.1 *N* acetic acid, and distilled water. To each sample approximately 1300 cpm of (*N*)-*d*-[1,2-^{3}H]aldosterone (40–60 Ci/mmol) was added to ascertain individual sample percentage recovery. Aldosterone in the evaporated extract was resolved from the bulk of steroids with the potential for cross-reaction with the aldosterone antiserum (prepared against aldosterone-18, 21-dihemisuccinyl–bovine serum albumin) by passing the extract, dissolved in 0.2 ml CBM (cyclohexane:benzene:methanol, 60:40:10, v/v/v) through a minicolumn (1 cm I.D.; flow = 0.5 ml/min) containing 850 mg Sephadex LH-20 in 5.0 ml benzene–methanol (85:15, v/v). Three successive 6.0-ml CBM were passed through the column; the second and third 6.0-ml volumes contained the aldosterone and cortisol fractions, respectively. The dried aldosterone fraction was reconstituted in 1.0–2.0 ml phosphate buffer (pH 7.4) containing sodium azide (1 mg/ml) and bovine serum albumin (5 mg/ml). A portion of the buffer was added to a scintillation vial containing 15 ml of scintillation fluid (0.3 g POPOP, 5.0 g PPO, 200 ml Triton X-100, and 600 ml toluene) in order to ascertain individual sample recovery; to another portion (50–100 μl), labeled aldosterone (3500 cpm) and sufficient antiserum to yield 50% binding in the absence of unlabeled aldosterone were added. Following 3 hr incubation (4°), 0.8 ml cold dextran–charcoal was added, the sample centrifuged in the cold, and the supernatant decanted into a vial containing 15 ml scintillation fluid. Samples were counted for 10 min in a Packard TriCarb scintillation spectrometer (Model 3255). This procedure yielded a recovery of 85 ± 3% and had a sensitivity of <0.005 ng with intra- and interassay variations of 3.2 ± 0.3 and 5.1 ± 0.9%, respectively.

The concentration of corticosterone in the original sample or cortisol in the eluate fraction was determined from methylene dichloride extracts by the competitive protein binding method of Murphy (12), using

1.0 ml of a Tris buffer (pH 7.4) containing approximately 20,000 cpm of the tritiated steroid and sufficient pregnant monkey plasma to yield 60% binding in the absence of unlabeled steroids. After 5 min incubation at 45° and remaining for 15 min in an icebath, 80 mg Florisil was added to separate free and bound steroids. The latter fraction was decanted into a vial, mixed with 10 ml scintillation fluid, and counted as above. Ten nanograms cholesterol or pregnenolone exhibited no binding in this assay nor did they interfere with the binding of cortisol or corticosterone; moreover, as illustrated under Results, cortisol and aldosterone were present in very low concentrations. Thus it is assumed that competitive protein binding, as performed on original samples, is measuring primarily corticosterone, the predominant steroid of the rabbit adrenal gland. For corticosterone (sensitivity <0.1 ng) intra- and interassay variations were 1.0 ± 0.4 and 3.2 ± 0.6%, respectively, while for cortisol (sensitivity <0.1 ng) they were 1.3 ± 0.7 and 5.0 ± 0.9%, respectively. Protein (P) concentration was determined in all adrenal homogenates by the method of Lowry *et al.* (13).

The secretion rate was taken as the amount of steroid present in the KRB after 1 hr incubation, while the synthesis rate was calculated as follows: (posthomogenate concentration − prehomogenate concentration) + KRB buffer concentration. The data were analyzed, where applicable, using analysis of variance and nonpaired *t* tests.

Results. When the secretion data from the developmental study were examined on the basis of an individual adrenal gland (ng/adr/hr), it was apparent that aldosterone secretion increased essentially linearly as gestation progressed (Fig. 1). From a low rate of 0.08 ng/hr on Day 21, a single gland secreted 1.3 ng/hr by Day 30, while the glands of the neonates produced approximately 2.5-fold greater amounts of aldosterone than Day 30 fetal glands. These developmental changes in aldosterone secretion paralleled the increases observed in both kidney weight and juxtaglomerular index (Table II). Corticosterone production by a single gland on Day 21 exceeded by 20-fold its ability to secrete aldosterone. Thereafter, a linear increase in corticosterone secretion was apparent such that by Day 30, the gland was secreting at a 3- to 3.5-fold greater rate ($P < 0.05$) than those observed on Days 21 and 24. On the other hand, cortisol was secreted at a rate of 0.2 ng/hr on Day 21 and then gradually increased to 0.4 ng/hr by Day 30. The secretion rates of both cortisol and corticosterone were relatively unchanged between Day 30 and 12 hr after birth.

Throughout gestation, the synthesis pattern observed for each of the three corticosteroids paralleled that of secretion when reported as ng/mg P/hr (Fig. 1). From Days 21 to 27, the rates of aldosterone synthesis and secretion increased significantly ($P < 0.05$) and then exhibited essentially no change between Days 27 and 30. The protein concentrations (Table II) of the fetal adrenal glands followed a course which essentially paralleled that of aldosterone production. The glands of the neonate synthesized (28.8 ± 1.8 ng/mg P/hr) and secreted (27.1 ± 2.0 ng/mg P/hr) aldosterone at significantly ($P < 0.05$) greater rates than those (9.9 ± 0.5 and 9.6 ± 0.7 ng/mg P/hr, respectively) achieved by Day 30 prenatal glands. As gestation progressed, the production of corticosterone declined such that synthesis and secretion, which were 72.9 ± 6.6 and 65.1 ± 3.5 ng/mg P/hr, respectively, on Day 21 of gestation, had significantly ($P < 0.05$) decreased to 38.0 ± 6.5 and 35.6 ± 6.2 ng/mg P/hr, respectively, on Day 27. Thereafter (Day 27 to birth), corticosterone production remained essentially unchanged. The adrenal glands of developing rabbits produced rather meager amounts of cortisol; however, as with corticosterone, cortisol synthesis was greatest on Day 21 of gestation, while from Day 24 to birth cortisol secretion varied, though not significantly ($P > 0.05$), from 6.3 ± 0.9 on Day 24 to 3.3 ± 0.7 ng/mg P/hr at birth.

The effects of maternal dehydration and restraint on the production of aldosterone and corticosterone by the adrenal glands of Day 30 fetal rabbits are presented in Table III. Maternal dehydration elicited signifi-

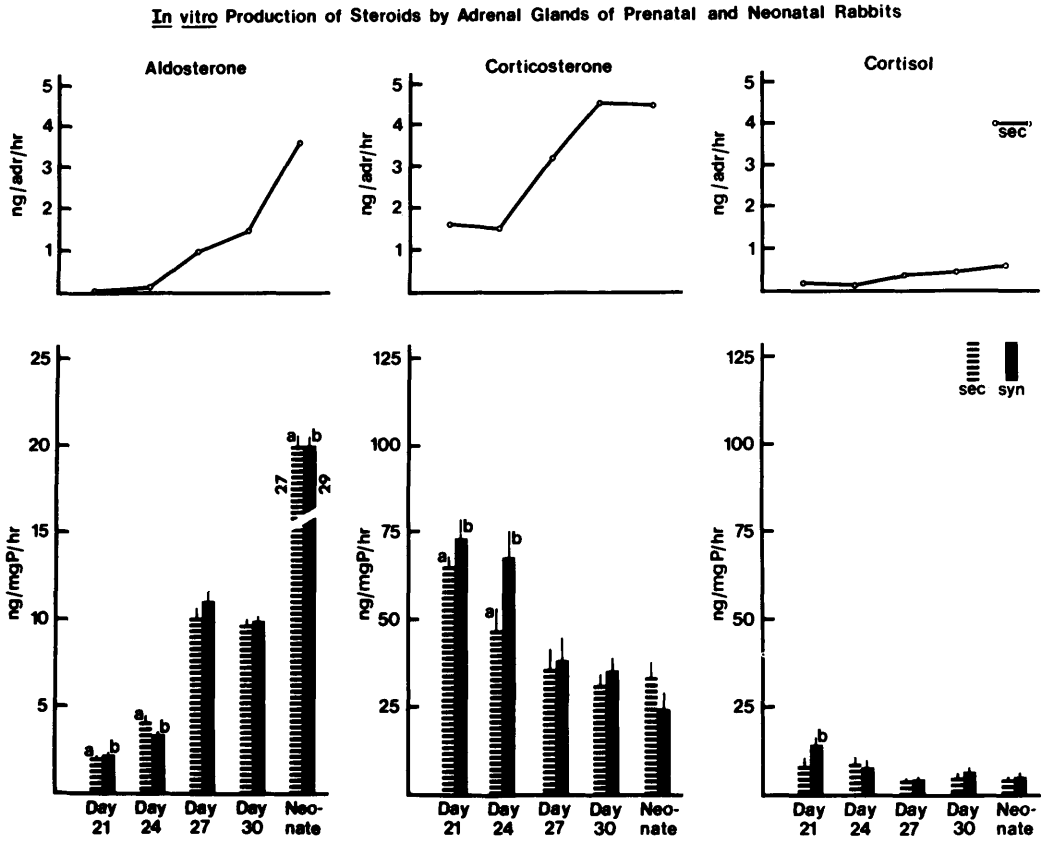

FIG. 1. Corticosteroid production by the adrenal glands of prenatal (Days 21–30) and neonatal (Day 34) rabbits. Upper panels: mean secretion of corticosteroids by single adrenal glands. Lower panels: bars represent mean with SEM above for the synthesis (syn) and secretion (sec), based upon adrenal protein (P) concentrations; a and b denote significance ($P < 0.05$) in sec and syn, respectively, from similar parameters observed in Day 30 fetuses.

cant ($P < 0.05$) increases in the fetal rates of aldosterone and corticosterone synthesis and secretion, while markedly decreasing fetal body weight (Table I). Aldosterone production increased approximately 3.5-fold over rates in the nonstressed fetuses, whereas corticosterone production increased approximately 2.5-fold. Restraint

TABLE II. ADRENAL AND RENAL PARAMETERS DURING THE LATER STAGES OF DEVELOPMENT

Gestation days	Adrenal Gland Wt (mg)	Adrenal Gland Protein (mg P/mg adr)	Kidney Wt (mg)	Kidney JGI[a]
21	0.75 ± 0.01[b]	0.032 ± 0.004	8 ± 2	0
24	1.25 ± 0.04	0.029 ± 0.002	23 ± 3	0
27	2.12 ± 0.02	0.040 ± 0.003	60 ± 5	+
30	2.67 ± 0.03	0.052 ± 0.004	102 ± 6	++
34 (neonate)	3.38 ± 0.16	0.050 ± 0.006	271 ± 8	+++

[a] Juxtaglomerular index.
[b] Mean ± SEM of single adrenal gland and kidney obtained from fetuses in Table I.

TABLE III. EFFECTS OF MATERNAL DEHYDRATION AND RESTRAINT ON THE *IN VITRO* SYNTHESIS (SYN) AND SECRETION (SEC) OF ALDOSTERONE AND CORTICOSTERONE BY THE ADRENAL GLANDS OF DAY 30 FETAL RABBITS

	Aldosterone ng/mg P/hr		Corticosterone ng/mg P/hr	
Condition	Syn	Sec	Syn	Sec
Nonstressed	6.1 ± 0.4[a]	6.5 ± 0.2	33.7 ± 2.1	33.2 ± 3.2
Dehydration	22.4 ± 0.8*	20.0 ± 1.4*	93.7 ± 3.5*	76.6 ± 8.5*
Restraint	3.4 ± 0.6*	3.4 ± 0.5*	29.0 ± 3.0	22.0 ± 3.2

[a] Mean ± SEM
* Significantly different ($p < 0.05$) from nonstressed fetuses for similar parameter.

of the pregnant animal for 2 hr (Table III) caused small decreases in fetal aldosterone and corticosterone synthesis and secretion; however, only the decreases in aldosterone production were significant ($P < 0.05$).

Discussion. The results of the present study provide the developmental pattern of corticosteroidogenesis in the fetal rabbit from Day 21 to birth (34 days). On the basis of a single adrenal gland, there were parallel increases in the production of aldosterone and corticosterone (ratio 1:10) and to a lesser extent in cortisol. Growth and differentiation of the adrenal gland in the fetal rabbit have been shown to proceed from Day 18 of gestation (14), and the appearance of enzymatic activities (e.g., 3β-hydroxysteroid dehydrogenase, 21-hydroxylase, and 11β-hydroxylase) necessary for autonomous synthesis have been demonstrated in the glands by Day 23 (5). Recently, Devaskar *et al.* (8) reported a near linear increase in the basal production of corticosteroids by dispersed fetal rabbit adrenal glands on Days 22 through 29 of gestation. Chouraqui and Weniger (2, 3) in qualitative studies have reported that fetal rabbit adrenal glands on Days 18, 22, and 26 of gestation are capable of synthesizing corticosteroids from [^{14}C]acetate and that the ^{14}C label was found in quantities such that aldosterone was synthesized to a greater extent than cortisol, and the synthesis of the latter steroid exceeded that of corticosterone. If one assumes that protein concentration, rather than adrenal weight or cell number, is a more accurate index of the true growth of the gland, the data presented herein indicate that those enzymes related to glucocorticoid steroidogenesis are present prior to those related to aldosterone synthesis; i.e., the enzymes expressing activity early (e.g., 21-hydroxylase, 11β-hydroxylase) in the synthesis of corticosteroids are manufactured prior to those involved in later steps (e.g., 18-hydroxylase, 18-dehydrogenase) in the biosynthetic pathway. In the absence of evidence demonstrating access of maternal plasma corticosteroidogenic factors (e.g., ACTH, angiotensin II, etc.) to the fetus and the questionable role of placental factors (15–17), it can be assumed that the fetal adrenal gland is to a great extent independent of these maternal influences. Moreover, given the level of development of the fetal hypothalamo–pituitary (HP) axis (18, 19), it is likely that the developmental pattern of corticosteroidogenesis described herein is under the control of fetal ACTH.

Upon examination of the corticosteroid pattern of the newborn rabbit, it is obvious that the production of aldosterone, but not glucocorticoids, markedly exceeds the observed prenatal rates, a finding similar to that noted in newborn guinea pigs (10). This would argue against a rise in fetal plasma ACTH; however, since the juxtaglomerular indices presented herein suggest that the renin–angiotensin system (RAS) is at least partially functional, and further since it is known that plasma K^+ is elevated in the neonate (20), it is postulated that a synergism of these two steroidogenic agents was likely responsible for the observed effect. However, further delineation of the precise origin of this neonatal elevation in aldosterone is required since the autonomy of

function demanded of the neonate could implicate not only these factors but others, such as arginine vasopressin, vasotocin, etc. (21, 22).

The present study demonstrated that throughout gestation, the synthesis and secretion of corticosterone exceeded not only aldosterone but also cortisol, and remained the predominant adrenal glucocorticoid of the 12 hr neonate. The latter finding is contrary to the results obtained by Hirose (23) who reported that in the neonatal rabbit the adrenal content of cortisol exceeded that of corticosterone for the first 20 hr postnatally, after which time corticosterone concentration was twice that of cortisol. This discrepancy may involve both methods of sacrifice and glucocorticoid determination. In the study by Hirose (23), the neonates were bled by carotid section under ether anesthesia, both of which would activate the hypothalamo–pituitary–adrenocortical (HPA) system (24–26) and/or the RAS (27); moreover, the steroids were determined fluorometrically. In the present study, the neonates were rapidly sacrificed by decapitation with a minimum of handling and the glucocorticoids were measured by competitive protein binding, a method with greater sensitivity than fluorometry. Thus, it is postulated that the more stressful method of sacrifice and the lesser sensitivity of the method employed for steroid determination may account for the discrepancy between these two studies.

Restraint is known to cause an increase in plasma corticosterone levels in adult rabbits (28) and is usually considered a neuropsychological stressor (29, 30). As such, it would be expected to activate the HP axis solely through higher centers in the pregnant rabbit. This is evident from the preliminary finding that *in vitro* corticosterone secretion in the pregnant animal increased significantly from 23.9 ± 1.3 ng/mg P/hr (control) to 110.2 ± 8.2 ng/mg P/hr during restraint. Obviously, restraint should not be considered a psychological stressor to the fetus, who *in utero* is essentially in a state of sensory deprivation. Thus, since fetal ACTH release would not be expected and further since maternal ACTH does not cross the placenta (15, 16), no increase in fetal corticosteroidogenesis was noted. In fact, the fetal production of both aldosterone and corticosterone decreased slightly. It is likely that some of the maternal unbound corticosterone, which was markedly elevated by restraint, crossed the placenta to an extent which exerted a negative feedback; i.e., it inhibited the fetal HPA system. This finding implicates fetal ACTH as a primary factor in the regulation of fetal corticosteroidogenesis.

Maternal dehydration resulted not only in significant 3.5-fold and 1.5-fold increases (preliminary findings) in maternal aldosterone and corticosterone secretion, respectively, but also in fetal dehydration as evidenced by the low fetal weights. This was associated with increases in the fetal production of both aldosterone and corticosterone. One of the factors involved could include plasma K^+ levels, since dehydration markedly increases plasma K^+ levels (20). Angiotensin II is not likely involved due to both the low Day 30 juxtaglomerular index reported herein and the low plasma renin activity and adrenal angiotensin II receptor concentration in fetal rabbits observed by Pernollet *et al.* (31). ACTH presumably plays a major role. The baroreceptors in the heart and periphery, which are present anatomically and functional in the fetus (32–34), were likely activated in response to decreased intravascular volume (24, 25), resulting in activation of the HP axis and the release of ACTH. Although the fetal HPA system could have been inhibited by the passage of maternal unbound corticosterone which was elevated in response to dehydration, it is probable that the stress of dehydration in the fetus was sufficient to override this inhibition. Thus, it is suggested that an interaction of fetal ACTH and K^+ was responsible for the corticosteroid pattern observed in the fetus in response to maternal dehydration.

Further investigations are necessary to resolve the multivariant parameters involved in regulation of corticosteroid secretion and synthesis in fetal and pregnant animals.

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