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Morphometric analyses of adrenal gland growth in fetal and neonatal sheep. I. The adrenal cortex

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INTRODUCTION

Studies of endocrine gland development have demonstrated species-specific patterns of timing in prenatal growth and differentiation, and have provided explanations of the association between glandular differentiation and concomitant changes in particular target tissues. Mammalian adrenal epigenesis follows a general pattern in which coelomic mesothelial proliferations lying in anteromesial relation to the metanephric kidneys become right and left adrenocortical masses which are then invaded medially by sympathochromaffin cells migrating from nearby sympathetic ganglia. The chromaffin cells pass between the cortical cells and finally, as the medulla, occupy the centre of the gland. Further growth and differentiation of the glands then depend on trophic influences exerted principally by the anterior pituitary gland (Jost, 1975) and a range of peptide growth factors (Hornsby & Gill, 1977).

The patterns of fetal adrenal growth, however, differ between species. Human fetal adrenal gland weights increase linearly during the second half of gestation (Neville & O'Hare, 1982), whereas in fetal sheep and pigs, adrenal gland weights increase abruptly near term (Comline & Silver, 1961; Lohse & First, 1981). By contrast, fetal rat adrenal growth rates decrease as term approaches (Cohen, 1963). Such growth patterns generally correlate well with fetal plasma corticosteroid levels which appear to serve as regulatory mechanisms for initiating specific developmental events (Ballard, 1979). Murphy (1982) reported a mid-gestational fall and a late gestational rise in human fetal serum cortisol, the former coinciding with a rapid drop in relative adrenal weight. In both fetal sheep and pigs, increased fetal adrenocortical secretory activity in the later stages of gestation has been described (Alexander *et al.* 1968; Wintour *et al.* 1975; Lohse & First, 1981). Fetal rat corticosterone concentrations, although high at about Day 19, have fallen precipitately by Day 20 and remain low until just after birth (Holt & Oliver, 1968).

Although some simple mensuration has been undertaken, prior analyses of adrenocortical growth and differentiation have been predominantly qualitative (Comline & Silver, 1961; Durand, Bosc & Nicolle, 1978; Robinson, Rowe & Wintour, 1979; Yamauchi, 1979; Boshier, Holloway & Liggins, 1980). In his comprehensive review of the fetal adrenal cortex, Nussdorfer (1986) did not record one morphometric study of adrenal gland epigenesis.

We therefore consider it of value to report this quantitative study of the development of the ovine adrenal gland over the period from 53 days gestation to two days *postpartum*. The study considers the fetal adrenocortical inner zone when it is actively steroidogenic (53 days), quiescent (100 days), becoming increasingly

responsive to ACTH (130 days), actively steroidogenic (144 days, and 2 days postnatal), as described by Wintour *et al.* (1975) and Glickman & Challis (1980).

MATERIALS AND METHODS

Experimental animals and tissue preparation

Adrenal tissues were obtained from fetal or neonatal New Zealand Romney sheep whose dams were pasture-fed and of known mating dates (gestation length = 147 days). The donor ewes were anaesthetised (sodium pentobarbitone, i.v.), the fetuses exteriorised, and then perfused through the thoracic dorsal aorta with cold (4 °C) Karnowsky's electron microscope (EM) fixative diluted 1:1 with 0·1 M phosphate buffer at a pressure of 70 cm water for 10 minutes, at which time the adrenal glands were pale and firm. The ewe was killed by an overdose of anaesthetic as the embryonic perfusion began. Postnatal lambs were killed by exsanguination before aortic perfusion with EM fixative.

The paired adrenal glands were then dissected clear of perirenal connective tissue and, except for the 53 days specimens, the longest glandular dimension of each was measured using engineer's callipers. The adrenals were then placed for 3 hours in fresh fixative (53 days, 144 days, +2 days), or fresh fixative with 1·5% potassium dichromate in 0·1 M phosphate buffer (100 days, 130 days) to facilitate histological differentiation of the medullary region. The fixed glands (except 53 days) were then blotted dry, weighed, and remeasured in three dimensions (length, breadth and thickness). All glands were stored at 4 °C up to 7 days in EM fixative diluted 1:2 with phosphate buffer before being dehydrated through a series of ethanols and propylene oxide and embedded in Epox 812 resin (Ernest F. Fulham).

The glandular weights obtained after weighing (mean of 5 weighings per gland), and calculated from gland dimensions, where

$$W = V \times S.G. \quad \text{where } S.G. = 1{\cdot}039 \text{ (Malendowicz, 1986)}$$

and

$$V = \frac{4\pi}{3}\frac{LBT}{8} \text{ (Aherne \& Dunnill, 1982)}$$

were compared in the 100 days (n = 10) and the 130 days (n = 10) groups using paired *t* tests and were found not to differ significantly at $P < 0{\cdot}05$. Consequently, the mean dry gland weights were considered representative of all age groups and were used for calculating the gland volumes in mm^3, where

$$V = \frac{W_{mg}}{S.G.}.$$

Before dehydration and embedding, the lengths of 5 glands in each of the 100 days and 130 days groups were remeasured and compared with their post-infusion lengths using paired *t* tests. No significant shrinkage occurred during the storage period. However, a 5% allowance for shrinkage during fixation and the embedding procedures (Hayat, 1981) has been incorporated into the volume calculations (Table 4).

Microscopy

The randomly selected right or left gland of each pair was kept for ultrastructural studies and the other was sectioned at 1 μm thickness on a Sorval JB4 microtome. Thirty sections, the second of which always contained zona fasciculata, equally spaced along its whole length were collected from each gland, thereby giving an

accurate record of the histological organisation of the gland. The 53 days sections were stained, firstly using the Masson–Hamperl method for argentaffin cells (Stevens, 1977) and then, as for all other sections, with 1:1 toluidine blue (1% aqueous) and Azure II (1% aqueous).

In efforts to determine the extent of functional zonal differentiation in the fetal and neonatal adrenal cortex, 10 μm frozen sections of fresh adrenal glands (133 days, n = 4; 140 days, n = 4; +2 days, n = 2) were stained for 3β-hydroxysteroid dehydrogenase (Chayen, Bitensky, Butcher & Poulter, 1969; Hornsby, 1985) and then examined using bright-field and dark-ground illumination in a Leitz Orthoplan microscope.

Morphometric analyses

Standard point-count methods for biological morphometry as described by Weibel (1979) were used in all analyses. Preliminary studies indicated that the volume density (Vv) of the adrenal zona fasciculata nuclei, the smallest structures of significance in this study, was approximately 0·07. Allowing for a relative probable error of 5% of mean Vv indicated that approximately 5000 points had to be counted in each age group (Weibel, 1979; p. 114). Such point-count data were collected using a stepping-motor driven mechanical stage on a Leitz Orthoplan microscope controlled by a BBC B+ microcomputer. The computer programme allowed selection of a range of test line units (d) from 100 μm to 1 mm in length arranged in a coherent multipurpose system, and for the storage of the point counts designated as specific anatomical features. Each section was examined at ×100 and 'd' selected, knowing the least diameter of the gland, so as to give the appropriate number of points per animal.

To allow determination of the nuclear and steroidogenic cell volumes in the various age groups, the longer nuclear axis was measured using an eyepiece micrometer in approximately 500 nuclei selected randomly throughout the inner cortical zone (excluding zona glomerulosa) of each gland. The shorter nuclear axis was also measured in 100 nuclei of each of the 130 days and 144 days groups and the profile axial ratio (PAR) calculated as mean ± SE (PAR 130 days = 1·21 ± 0·02; PAR 144 days = 1·22 ± 0·02). With these PAR values the nuclei may reasonably be considered to be spherical (Weibel, 1979). Following the approach of Williams (1977) and using the Abercrombie correction, the mean nuclear diameter $\bar{D}$, was calculated for each age group from the profile diameter data and then used to calculate the nuclear volume and ultimately the average steroidogenic cell volume.

Statistical analyses

For comparisons within and between the five age groups, non-parametric χ^2 tests, as four-fold or 2×2 contingency tables (Brand & Snedecor's formula), *t* tests for determining differences between two proportions, or Kolmogorov–Smirnov tests were employed.

RESULTS

Functional zonation

53 *days*

The 53 days old glands were composed of a series of relatively loose cords of cortical cells separated by wide anastomosing sinusoids leading to the central region of the gland (Fig. 1). The cortical cells contained a large round nucleus with up to three prominent nucleoli. All sections showed evidence of development of the zona glomerulosa, for although some cell cords appeared homogeneous from the capsule to the centre of the gland, others had smaller, more palely staining cells with rounded

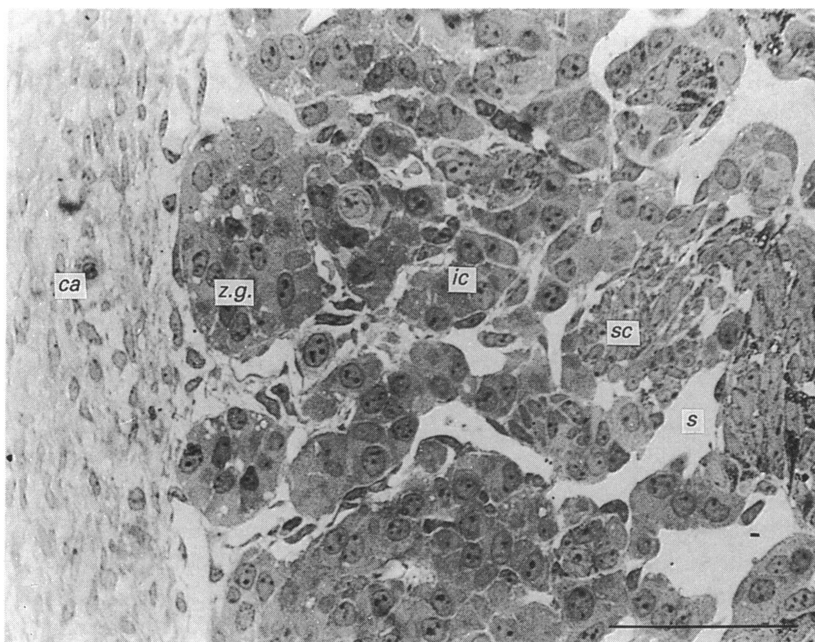

Fig. 1. Section of 53 days fetal adrenal gland illustrating the degree of development of the zona glomerulosa (*z.g.*) and inner cortex (*ic*). Migratory sympathochromaffin cells (*sc*) are also shown. The cortical sinusoids (*s*) have their origins in thin-walled vessels in the capsule (*ca*). The scale bar represents 50 μm.

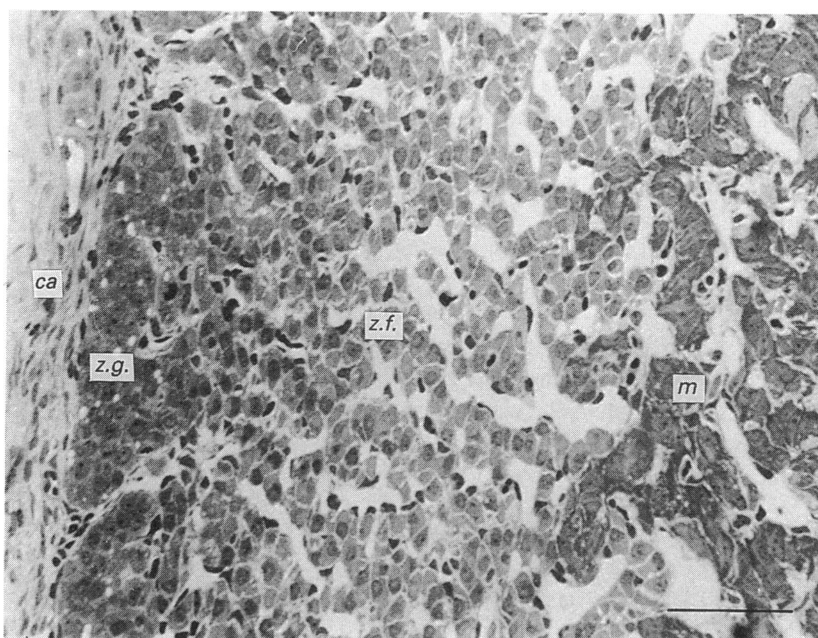

Fig. 2. Section of 100 days fetal adrenal gland showing the completed zonation into zona glomerulosa (*z.g.*), zona fasciculata (*z.f.*) and medulla (*m*). The scale bar represents 50 μm.

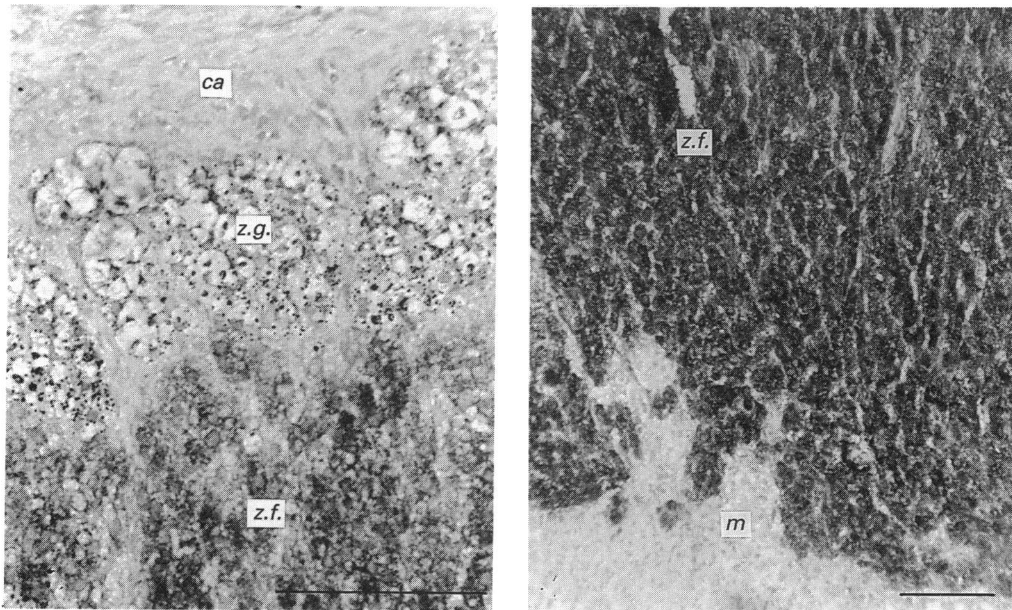

Fig. 3. Photomicrograph of histochemical preparation staining 144 days fetal cortical tissue for 3β-hydroxysteroid dehydrogenase. Positive staining is absent in the zona glomerulosa (*z.g.*), but present in the zona fasciculata (*z.f.*). Both Figs. 3 and 4 are also representative of the 130 days fetus and the newborn lamb. The scale bar represents 100 μm.

Fig. 4. A similar preparation of the corticomedullary region (*m* = medulla) showing how the zona fasciculata cells are positively stained for 3β-hydroxysteroid dehydrogenase right to the medullary junction and therefore that there is no differentiation of the innermost cortex into a zona reticularis. The scale bar represents 100 μm.

nuclei containing numerous small nucleoli arranged in a rounded cap-like manner over the centripetally directed cortical cells. Other areas showed the cap region to be discrete and organised within its own connective tissue framework in a typically glomerulosa-like fashion. Clumps of elongated, migratory sympathochromaffin cells were frequently located among the cortical cells or between the sinusoids.

100 *days*

By 100 days gestation, differentiation of the z. glomerulosa and z. fasciculata was well established (Fig. 2). All cortical cells in the subcapsular region were organised as rounded, cap-like aggregations associated with inwardly directed cells, or as discrete balls of cells surrounded by the subcapsular loose connective tissue. This interglomus connective tissue marked the site of entry of the cortical blood vessels, some of which ramified among the z. glomerulosa cells; however the majority of them rapidly branched within the z. fasciculata into anastomosing sinusoids which were narrower than those present at 53 days. At the junction with the well-defined medullary region, the sinusoids recombined into larger vessels separated by wide cords of elongated chromaffin cells which had their long axes normal to the blood vessels. There was no specific differentiation apparent in the juxtamedullary cortical cells.

130 *days*, 144 *days*, 2 *days postnatal*

The basic pattern of the fetal ovine adrenal cortex was established by 100 days and later development was primarily growth-related. There was no histological or histochemical evidence of the development of the z. reticularis by 2 days *postpartum*.

Table 1. *Volume density of major regions within fetal and neonatal sheep adrenal glands**

Volume density	Gestational age (days) 53 (n = 5)†	100 (n = 6)	130 (n = 6)	144 (n = 6)	Postnatal age (days) 2 (n = 3)
Capsule	0·21±0·03	0·14±0·02	0·15±0·02	0·12±0·02	0·12±0·06
Zona glomerulosa	0·15±0·03	0·14±0·02	0·13±0·01	0·10±0·02	0·12±0·04
Zona fasciculata	0·48±0·06	0·35±0·06	0·36±0·06	0·41±0·06	0·50±0·07
Medulla	0·16±0·06	0·37±0·06	0·36±0·07	0·37±0·05	0·26±0·06
Zona fasciculata (z.f.) nuclei	0·08±0·04	0·08±0·03	0·07±0·02	0·06±0·01	0·08±0·04
z.f. secretory cell cytoplasm	0·16±0·09	0·14±0·02	0·15±0·02	0·19±0·07	0·26±0·07
z.f. blood vessels	0·18±0·04	0·07±0·01	0·07±0·02	0·08±0·03	0·06±0·04
z.f. connective tissue and intercellular space	0·06±0·01	0·06±0·02	0·07±0·01	0·08±0·03	0·10±0·04

* Mean±95% C.I.
† n refers to the number of glands studied.

Table 2. *Volume density of tissue components within the adrenocortical zona fasciculata of fetal and neonatal sheep*

Volume density	Gestational age (days) 53 (n = 5)†	100 (n = 6)	130 (n = 5)	144 (n = 6)	Postnatal age (days) 2 (n = 3)
Secretory cells	0·49±0·08	0·64±0·07	0·53±0·16	0·63±0·09	0·61±0·15
Secretory cell nuclei	0·15±0·06	0·24±0·06	0·18±0·06	0·15±0·01	0·13±0·06
Secretory cell cytoplasm	0·34±0·04	0·40±0·02	0·35±0·17	0·48±0·08	0·48±0·14
Blood vessels	0·38±0·08	0·19±0·03	0·20±0·07	0·19±0·08	0·21±0·08
Connective tissue and intercellular space	0·13±0·01	0·17±0·06	0·27±0·14	0·18±0·06	0·18±0·04

* Mean±95% C.I.
† n refers to the number of glands studied.

Table 3. *Proportionate comparisons of secretory cells with other zona fasciculata components at different developmental stages of fetal and neonatal sheep*

	Age groups comparisons 53/100 days	100/130 days	130/144 days	144/+2 days
Secretory cells/zona fasciculata*	$P < 0·05$	$P < 0·05$	$P < 0·05$	N.S.
Proportional value†	0·49 0·64	0·64 0·53	0·53 0·63	0·63 0·61
Nucleus/cytoplasm	$P < 0·05$	N.S.	$P < 0·05$	N.S.
Proportional value	0·46 0·56	0·56 0·49	0·49 0·28	0·28 0·27
Blood vs/secretory cells	$P < 0·05$	N.S.	$P < 0·05$	N.S.
Proportional value	0·78 0·30	0·30 038	0·38 0·30	0·30 0·34
Connective tissue and intercellular space/ secretory cells	N.S.	$P < 0·05$	$P < 0·05$	N.S.
Proportional value	0·24 0·27	0·27 0·51	0·51 0·29	0·29 0·30

* After using *t* tests for determining differences between proportions.
† The proportional values are the volume ratios of the first component of the contrasted pair to the second at the development age specified, using the volume data presented in Table 4.

Table 4. *Morphometric characteristics of the adrenocortical zona fasciculata in fetal and neonatal sheep*

	Gestational age (days)				Postnatal age (days)
	53	100	130	144	2
Mean total adrenal wt. (mg)*	22·1 ± 1·6	121·5 ± 7·8	290·0 ± 13·8	560·1 ± 54·5	914·1 ± 71·6
Mean total adrenal vol. (mm³)*, †	22·26 ± 1·6	122·64 ± 7·9	293·01 ± 13·9	566·06 ± 55·1	923·79 ± 72·4
Vv_a zona fasciculata‡	0·48 ± 0·05	0·35 ± 0·05	0·36 ± 0·05	0·41 ± 0·06	0·50 ± 0·11
Volume of z.f. (mm³)§	10·68	42·92	105·48	232·08	461·90
Vol. connective tissue and i/cell. space‖	1·39	7·30	28·48	41·77	83·14
Vol. blood vessels (mm³)‖	4·06	8·15	21·10	44·10	97·00
Vol. secretory cells (mm³)‖	5·23	27·47	55·90	146·21	281·76
Vv nucleus of secretory cells	0·31	0·36	0·33	0·22	0·21
Mean nuclear diameter (μm)	6·69	5·99	6·42	7·27	7·68
Mean nuclear volume (μm³)	156·8	112·5	138·5	201·2	237·2
Mean secretory cell vol. (μm³)	505·8	312·6	419·7	914·5	1129·5
Number of secretory cells (× 10⁶)	10·34	87·88	133·19	159·88	249·46
No. of secr. cells per mm³ (× 10⁶)	1·98	3·20	2·38	1·09	0·89

* Mean ± S.E., per fetus, post-fixation weight.
† Corrected for shrinkage (+5%; Hayat, 1981) and specific gravity (÷1·039; Malendowicz, 1986). Volume therefore approximates pre-fixation volume.
‡ Vv_a Volume density within whole adrenal gland, mean ± 95% C.I.
§ Mean values used, as Vv zona fasciculata was not determined in all animals supplying weight and volume data.
‖ Within zona fasciculata, calculated by using Vv in Table 2.

Staining of 133 days, 140 days and 2 days *postpartum* adrenocortical tissues for 3β-hydroxysteroid dehydrogenase showed the enzyme to be lacking in the z. glomerulosa but present at uniformly moderate levels in the z. fasciculata from the sub-glomerulosa region to its junction with the medulla (Figs 3, 4).

Morphometry

Volume densities within developing adrenal gland

The volume densities found for the capsule, z. glomerulosa, z. fasciculata and medulla from 53 days gestation to 2 days *postpartum*, expressed as means ± 95% confidence intervals, are presented in Table 1. The total Vv of the z. fasciculata is further divided into its components, viz. secretory cell nuclei, secretory cell cytoplasm, blood vessels, and intercellular space plus general connective tissue.

Over the study period, the fetal glands increased in weight from 22 mg to 914 mg and in volume from 22 mm^3 to 924 mm^3 in an essentially logarithmic fashion (Table 4; Fig. 5). However, this pattern is the sum of a number of growth events, for consideration of the interrelationships of specific zones within the glands showed marked changes in their proportionate contribution to gland weight and volume through the developmental period (Tables 1, 2, 3). Between 53 and 100 days the significant change in regional proportions within the gland was due principally to an increase in the medullary component ($\chi^2 = 11·79$, D.F. = 3, $P < 0·001$). Thence until 2 days *postpartum*, no significant changes in the general corticomedullary organisation of the glands were apparent.

Within the cortex, the z. glomerulosa Vv declined between 130 and 144 days ($\chi^2 = 58·16$, D.F. = 1, $P < 0·001$). The z. fasciculata Vv also decreased between 53 and 100

Fig. 5. Graph of mean total adrenal gland weight of the five age groups studied, illustrating that gland growth is essentially logarithmic during the latter two thirds of development and immediately postnatally (term = 147 days). The regression line has the form $Y = 0{\cdot}4875 + 0{\cdot}159X$, $r = 0{\cdot}99$.

days ($\chi^2 = 91{\cdot}60$, D.F. = 1, $P < 0{\cdot}001$), then increased continually to 2 days *postpartum* ($\chi^2 = 48{\cdot}77$, D.F. = 1, $P < 0{\cdot}001$). Within the z. fasciculata cell, the nuclear Vv remained relatively constant through the study period, but the cytoplasmic Vv increased by over 50 %. On the other hand the Vv of the z. fasciculata blood vessels fell between 53 and 100 days ($\chi^2 = 5{\cdot}53$, D.F. = 1, $P < 0{\cdot}02$); the apparent increases in the intercellular space plus connective tissue were not statistically significant.

Volume densities of the zona fasciculata components

The serial developmental changes in Vv of the components of the z. fasciculata are shown in Table 2 and their statistical analyses are summarised in Table 3. Over the study period, the volume of the z. fasciculata increased markedly from 10·7 mm^3 at 53 days to 462 mm^3 two days postnatally (Table 4).

Within the z. fasciculata, the Vv of steroidogenic cells changed as development proceeded, increasing from 0·49 at 53 days to 0·64 at 100 days, decreasing to 0·53 at 130 days, increasing again to 0·63 at 144 days and then remaining stable (Tables 2, 3). Within the secretory cells, the nucleus:cytoplasm ratio also varied, increasing from 0·45 to 0·56 between 53 and 100 days, and then decreasing from 0·49 at 130 days to 0·28 by 144 days and 0·27 at 2 days *postpartum* (Tables 3, 4). The converse of these data shows that there was a decrease in the amount of cytoplasm per steroidogenic cell by 100 days and relative cytoplasmic growth thereafter to the *postpartum* stage.

As the Vv of the z. fasciculata blood vessels halved between 53 and 100 days of fetal development, the volume of the z. fasciculata blood vessels relative to the volume of the secretory cells also decreased over this period, falling from the fraction 0·78 at 53 days to 0·30 at 100 days. After 100 days, associated with the cytoplasmic growth in the steroidogenic cells alluded to above, the proportion of the blood vessels rose from 0·30 to 0·38 at 130 days, which is suggestive of an increase ($\chi^2 = 3{\cdot}55$, D.F. = 1, $P < 0{\cdot}1 > 0{\cdot}05$), then fell to 0·29 by 144 days, thereafter showing no further detectable change.

The volume relationships between the secretory cells and the intercellular spaces and connective tissue components within the z. fasciculata also varied during the period of development, increasing significantly from 0·27 at 100 days to 0·51 at 130 days, decreasing by a similar amount to 144 days and then remaining stable over the next five days.

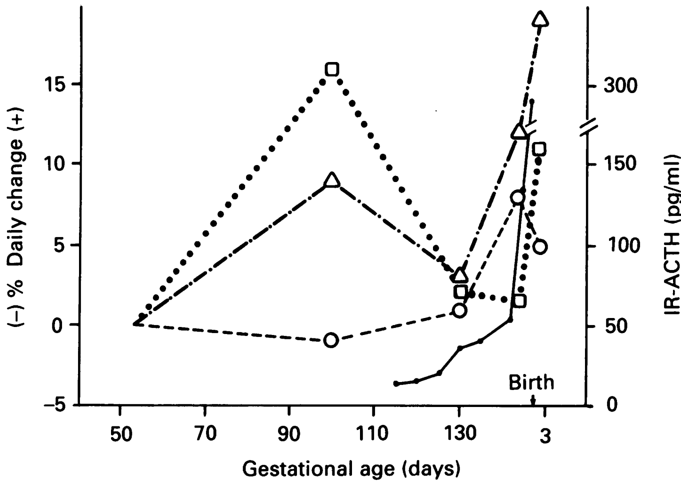

Fig. 6. Velocity diagrams of relative changes in total steroidogenic cell volumes (△), total cell numbers (□), and individual cell volumes (○) during growth of the zona fasciculata from 53 days gestation to two days *postpartum*, where:

$$\text{Daily change \%} = \frac{\dfrac{\text{Datum}_n - \text{Datum}_{n-1}}{\text{Growth period (days)}}}{\text{Datum}_{n-1}} \times 100.$$

Lines joining the data points are indicative of the changes occurring, for our data do not allow determination of the true inflections. Immunoreactive ACTH levels (——) from MacIsaac *et al.* (1985) correlate well with the increases in total cell numbers and total steroidogenic cell volume.

Growth velocities within the adrenal gland and its zona fasciculata

Growth patterns within the adrenal gland may be deduced from the parameters recorded in Table 4 as they were derived from the mensuration and Vv data, and compared, as represented in Fig. 6.

The mean nuclear diameter fell from 6·7 μm at 53 days to 6·0 μm at 100 days and then it increased with accelerating rapidity to 7·3 μm at 144 days and 7·7 μm in the neonatal gland. Application of the Kolmogorov–Smirnov test to these data showed that these diameters all differed significantly at $P < 0{\cdot}05$. The mean volume of the individual secretory cells also decreased by 100 days, then increased throughout the remainder of the study period. The number of secretory cells per mm^3 of z. fasciculata, reflecting this, was highest at 100 days and then fell throughout the rest of the developmental period as the cells became larger. Between 100 and 130 days of development, the rate of increase in the total steroidogenic cell volume decreased markedly. However, the lower rate of cellular proliferation also shown over this period was linked with the first signs of cellular hypertrophy, for the mean secretory cell volume increased by one third over those 30 days.

Application of the Vv figures to the mensuration data allows calculation of the real-time changes occurring within the glands (Table 4; Fig. 6). The adrenal gland weights and volumes and the velocity diagrams of changes in steroidogenic cell volumes and numbers show that there were two major periods of growth in the z. fasciculata (Fig. 6). The first growth period in mid-pregnancy was essentially one of secretory cell proliferation with a slow but significant decrease in cell volume, both events being associated with a profound decrease in the blood vascular compartment. The second growth period showed two phases: the first, one of cellular hypertrophy during the

period from 130 to 144 days, passed in its latter stages into the second, one of hyperplasia associated with a lesser rate of cell volume increase during the perinatal period.

DISCUSSION

This, the first linear quantitative study of adrenocortical growth in a fetal mammal, has confirmed and offered explanations for the early observations that the rapid rise in the weight of the ovine fetal adrenal gland occurred mainly during the last week of gestation (Comline & Silver, 1961; Alexander *et al.* 1968). Neither of these groups of workers distinguished between the cortical and the medullary contributions to the overall increase in size, except to suggest that the cortical was the greater. Boshier *et al.* (1980) later showed a very close correlation between the weight of the gland and cortical thickness, indicating that the increased size of the gland from 136 to 147 days resulted from differential growth of the inner cortical zone, for neither the z. glomerulosa nor medulla grew much during that period. Durand *et al.* (1978) demonstrated that this rapid increase in adrenal size resulted from two growth phases: the first, of gradual onset, from 123 days to 137 days; the second, a period of cellular hypertrophy, from 143 days to term. Our data suggest that cellular hypertrophy had begun before 143 days.

Growth studies of the early to mid-gestation adrenal gland are difficult because of its small size and highly vascular state. We found the 100 days and all older glands to be amenable to mensuration and undertook the analyses of shrinkage effects to validate our further use of the mensuration data in determining real-time growth phenomena. Mathieu, Claassen & Weibel (1978) and Hayat (1981) have confirmed the effects of various EM solutions in influencing later tissue and organ dimensions. As the degree of shrinkage or swelling they described is within the limits of our measuring techniques and we found no changes in organ dimensions between fixation and embedding, we are confident that our quantitative data, the use made of them and the conclusions drawn from them are valid.

Our data suggest that the development of the fetal sheep adrenal cortex between 53 days of gestation and 2 days postnatally occurred during three phases: 53 days–100 days, the establishment of functional zonation; 100 days–130 days, a period of quiescence and then reactivation; 130 days–2 days postnatal, the attainment of structural and functional maturation. These phases reflect the interactions of the cellular kinetic phenomena of hyperplasia and hypertrophy.

The establishment of functional zonation: 53 *days*–100 *days*

This, the first growth phase of the fetal adrenal gland, was characterised by the completion of sympathochromaffin cell migration and the separation of the cortical and medullary components. The presence of a well-defined z. glomerulosa at 53 days correlates well with the ability of the fetal sheep adrenal gland to secrete aldosterone at 40 days gestation (Wintour *et al.* 1975).

Within the inner cortical zone, two main events had occurred by 100 days of gestation; the volume of steroidogenic tissue in the z. fasciculata had increased at a rate second only to that present during the last week of gestation, and the cortical blood vasculature and associated connective tissue had become reorganised so that the volume density of the vascular sinusoids was halved. Following their qualitative analyses of adrenocortical histogenesis, Robinson *et al.* (1979) concluded that the fetal adrenal gland grew in size from 40 days to 60 days, but did not appear to grow from 60 days to 100 days. Our quantitative data indicate that growth continued beyond 60

days, but was much reduced by 100 days of fetal life. The increased volume of steroidogenic cells over this period was due principally to cellular hyperplasia, for individual cell volumes had decreased by 100 days.

The reorganisation of the blood vasculature and inner zone connective tissue is probably intimately linked with the development of the definitive zonation, for Hornsby (1985) has emphasised the role of the blood vasculature in the supply of materials and growth factors to growing and differentiating adrenocortical cells. By 100 days, although the glandular growth rate and steroidogenic activity had declined (Glickman & Challis, 1980), the centripetally-directed vascular network of the cortex was well defined and the structural and functional zonation was established.

Quiescence and reactivation: 100 *days*–130 *days*

Whilst the fetal adrenal gland at 100 days of gestation reflects prior hyperplasia rather than hypertrophy, the following period of 30 days encompassed the transition from a period of the lowest rate of organ growth and steroidogenic cell multiplication to one of increased glandular activity at all levels. It was the only period in which the rate of the increase in total steroidogenic tissues slowed, although there was some increase in individual cell volumes, probably predominantly towards the end of the period.

Functional reactivation also occurs, for by 120 to 125 days, the z. fasciculata cells can respond to ACTH stimulation by the production of increased levels of cortisol (Bousquet, Lye & Challis, 1984). Norman, Lye, Wlodek & Challis (1985) consider the adrenal maturation sequence to be expressed in the fetal hypothalamo-hypophysial axis by 110 to 115 days, as plasma ACTH levels are then measurable and increase from 110 days. During the 120 to 140 days period, fetal ACTH secretion becomes pulsatile, down-regulation of the pituitary does not occur, and the numbers of ACTH receptors on the z. fasciculata cells increases from 123 days (Challis, Mitchell & Lye, 1984; Durand, 1979). There is little doubt that the cytodifferentiation of the fetal adrenal gland and probably its hormonal secretion are controlled by the hypothalamo-hypophysial axis via ACTH (Nussdorfer, 1986).

Structural and functional maturation: 130 *days*–2 *days postpartum*

The gradual and then profound increases in fetal plasma ACTH levels over this period (MacIsaac *et al.* 1985; Norman *et al.* 1985) are closely associated with the final phase of growth and maturation in the z. fasciculata (Madill & Bassett, 1973). We found that the rates of cell growth and cell division increased, the rate of increase in the volume of steroidogenic cells becoming greater than at any other stage. Cellular hypertrophy preceded cellular hyperplasia, for the rate of individual cell cytoplasmic growth was greatest between 130 and 144 days. The rate of cortical cell division also increased between 130 days and 144 days but attained its maximum only after 144 days gestation. This confirms the observations of Dallman (1984/85) that ACTH stimulates hypertrophy rather than hyperplasia, and of Hornsby (1985) that there is a time lag of some days before there is stimulation of hyperplasia by ACTH.

Growth of the z. fasciculata, its cytodifferentiation and increasing responsiveness to ACTH (Madill & Bassett, 1973) all contribute to the first evidence of its functional maturity, viz. the rapid rise in fetal plasma corticosteroids which starts several days before birth (Alexander *et al.* 1968; Bassett & Thorburn, 1969), and which ultimately initiates parturition (Liggins, 1968). Fetal cortisol itself has direct autocrine effects on the inner cortical cells, facilitating their maturation (Boshier, Holloway & Liggins, 1981).

The control of fetal adrenocortical growth and differentiation

In vivo, adrenal gland growth is under complex and multifactorial control which, on the whole, is poorly understood (Dallman, 1984/85). However, Hornsby (1985) considered that ACTH is clearly the major hormonal regulator of adrenocortical growth *in vivo* and probably is an indirect mitogen, acting to increase the delivery of peptide growth factors to the adrenocortical tissue. Whether $ACTH_{1-39}$ or other proopiomelanocortin (POMC)-derived substances is the primary trophic stimulus is not yet resolved.

Non-corticotroph-derived growth factors effective in stimulating adrenocortical cell growth *in vivo* are an adrenal growth factor (Samsoondar & Kudlow, 1987) and a placental-derived adrenocortical mitogenic factor (Simonian, Capp, Templeman & Chang, 1987). The possible roles of peptide growth factors in adrenocortical growth became apparent when fibroblast growth factor (FGF) – but not epidermal growth factor (EGF) – was shown to be mitogenic for bovine adrenal cells in culture (Gospodarowicz, Ill, Hornsby & Gill, 1977). A second group of peptides with potent growth-promoting abilities is the insulin-like growth factors (IGF-I, IGF-II) and evidence is accumulating that IGFs have distinct effects on cells of mesodermal origin. IGF-I stimulated DNA synthesis in cultured bovine z. glomerulosa cells (Horiba *et al.* 1987), and the gene coding for IGF-II, which is an embryonal mitogen, is expressed relatively abundantly in human fetal adrenal tissue (Scott *et al.* 1985).

It is probable, therefore, that the role of ACTH is to stimulate adrenocortical connective tissue cells to produce IGF-II (Han, d'Ercole & Lund, 1987) and the cortical cells to produce cortisol which will then act in concert with other growth factors. Growth of the fetal adrenal cortex may follow other patterns of fetal growth in being controlled by autocrine and paracrine influences. Growth factors termed competence factors, e.g. platelet-derived growth factor (PDGF) and FGF, probably derived from the cortical blood vessels and connective tissue cells, could make the adrenocortical cells in G_1 capable of responding to a second set of growth factors, the progression factors, e.g. IGF-II. The newly programmed cells then progress to the S-phase and undergo mitosis, or differentiate and become hypertrophied (Stiles *et al.* 1979; D'Ercole, 1987). The epigenesis of the fetal sheep adrenal gland which occurs during the two growth phases, as we have shown, therefore reflects the interactions of these cellular kinetic phenomena of hyperplasia and hypertrophy.

SUMMARY

This, the first linear morphometric analysis of the epigenesis of the fetal mammalian adrenal cortex, has shown that in the fetal sheep during the latter two thirds of gestation and in the newborn lamb, there are two periods of rapid growth separated by a period of much reduced growth. The fetal ages studied were 53 days (0·36 gestation), a period when the fetal adrenal cortex is actively steroidogenic; 100 days (0·68 gestation), a period of adrenocortical quiescence; 130 days (0·88 gestation), the period of increasing responsiveness to ACTH and cortisol production; 144 days (0·98 gestation), the period of maximal adrenocortical steroidogenesis; and 2 days *postpartum*, when cortisol production is normally maintained. The first adrenocortical growth period extends to mid-gestation, then growth slows to 0·85 gestation when the second growth period begins.

The changes between the first growth period (0·36 gestation) and the period of quiescence (0·68 gestation) are characterised by the attainment of normal adreno-

cortical zonation and the separation of the medulla. The rate of adrenocortical cell division slows and the zona fasciculata cells become smaller in size. The volume density of the adrenocortical blood sinusoids decreases significantly.

The onset of the second growth phase is associated with the previously reported increased levels of fetal plasma ACTH at 0·85 gestation and is expressed initially as a hypertrophic response. Cellular hypertrophy increases from 0·88 gestation to 0·98 gestation and then declines over the birth period. The rate of adrenocortical cell division increases from 0·88 gestation and maintains a maximal rate from 0·98 gestation to 2 days *postpartum*. These interactions of cellular hypertrophy and hyperplasia, which result in adrenocortical growth, may be explained as a response to fetal ACTH, which has the ability to stimulate the production of peptide growth and differentiation factors, e.g. IGF-II, and cortisol, which then control adrenocortical development in an autocrine and paracrine fashion.

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