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Immunohistochemical localization of 3β-hydroxysteroid/Δ^5-Δ^4-isomerase, tyrosine hydroxylase and phenylethanolamine *N*-methyl transferase in adrenal glands of sheep fetuses throughout gestation and in neonates

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Summary. Immunoreactive 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) was localized in adrenal glands of sheep fetuses in cortical-type cells, but not in medullary-type cells, from day 43 of gestation to term and in 2–4-day-old neonates. From day 54 of gestation, the formation of distinct zones within the adrenal cortex was apparent and immunoreactive 3β-HSD was found in cortical cells in the zona fasciculata and in groups and cords of cortical cells within the developing medulla, with weak positive staining in the zona glomerulosa. At this stage, most medullary cells were positive for immunoreactive tyrosine hydroxylase, and some of these cells with a juxta-cortical distribution also stained positively for immunoreactive phenylethanolamine *N*-methyl transferase (PNMT). Between days 65 and 130, the adrenal medulla increased in size with little change in the width of the cortex. Organization and zonation of immunoreactive 3β-HSD staining cells were evident in the zona fasciculata and in groups of cells in the medulla. Between day 130 and term, uniform immunoreactive 3β-HSD staining was found throughout the zona fasciculata, and there was also staining in single cells and small clusters of cells throughout the medulla. At this stage, immunoreactive tyrosine hydroxylase was distributed in most cells throughout the medulla, but in two distinct patterns: cells staining intensely for immunoreactive tyrosine hydroxylase in the central region of the medulla, and cells exhibiting weaker staining for immunoreactive tyrosine hydroxylase localized in a juxta-cortical position. These juxta-cortical cells were also positive for immunoreactive PNMT. Similar patterns of immunoreactive 3β-HSD, tyrosine hydroxylase and PNMT staining were found in 2–4-day-old neonates. We conclude that immunoreactive 3β-HSD is present in adrenal glands of sheep fetuses throughout gestation and in neonates, that progressive organization of cells containing 3β-HSD to form the fetal adrenal cortex occurs during gestation, and that immunoreactive 3β-HSD-positive cells may be closely associated with catecholamine biosynthesizing medullary cells during fetal life.

Keywords: adrenal gland; 3β-hydroxysteroid isomerase; fetus; sheep

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Introduction

The output of cortisol by the adrenal gland of sheep fetuses is important for fetal development, including lung maturation and catecholamine biosynthesis in the fetal adrenal medulla, and for the onset of parturition (Liggins, 1976; Challis & Olson, 1988). Formation of Δ^4-3-ketosteroids such as cortisol from Δ^5-3β-hydroxysteroids depends on the activities of the enzyme complex that contains 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145; 3β-HSD) and steroid Δ^5-Δ^4-isomerase (EC 5.3.3.1). Both activities reside in the same protein, but use different catalytic sites (Lachance *et al.*, 1990; Luu-The *et al.*, 1991). 3β-HSD has been purified from the human placenta; antibodies have been raised to it; and its cDNA cloned and sequenced (Luu The *et al.*, 1989, 1990).

Several studies have described in detail the anatomical development of the adrenal gland in ovine fetuses (Robinson *et al.*, 1979; Boshier *et al.*, 1980; Webb, 1980; Boshier & Holloway, 1989; Boshier *et al.*, 1989). The fetal adrenal gland can be recognized as early as day 28 of gestation, and develops as a series of cords of cortical and medullary cells. The zonation of the gland has been described from about day 60 of gestation into an outer zona glomerulosa and an inner zona fasciculata in the cortex, and an inner medulla. From biochemical studies it is known that the adrenal of sheep fetuses can use Δ^5 steroids as precursors for steroid synthesis from as early as day 50 of gestation. Adrenal steroid output decreases in mid-gestation, but increases again after days 120–125 (Wintour *et al.*, 1975; Glickman & Challis, 1980). This is associated with alterations in the activity of 3β-HSD, as well as that of $P450_{c17}$ and with changes in the abundance of $P450_{c17}$ mRNA (Tangalakis *et al.*, 1989). However, there is little information available concerning the localization of these enzymes within the different zones of the fetal adrenal gland, or on the anatomical relationship of steroid producing cells to those cells that will form the fetal adrenal medulla, particularly in early gestation when the gland is becoming organized or at term when fetal cortisol is thought to influence the activities of medullary catecholamine synthesizing enzymes.

We have therefore used immunohistochemical techniques to determine the distribution and localization of 3β-HSD, and to define its anatomical relationship to the catecholamine biosynthetic enzymes tyrosine hydroxylase and phenylethanolamine *N*-methyl transferase (PNMT), in adrenal glands of sheep fetuses throughout gestation and from newborn lambs.

Materials and Methods

Tissues

Pregnant ewes (n = 21) were killed with an overdose of Nembutal (Euthanyl, Abbott Laboratories, Montreal, Canada), at the following stages of gestation: day 43 (n = 2 animals); day 54 (2); days 60–65 (2); days 75–90 (3); days 100–120 (3); days 125–135 (4); days 140–term (term between days 145–147; 3). Tissue was also obtained from two lambs at 2–4 days of age, after spontaneous delivery at term. Fetal adrenal glands were removed immediately, cut transversely into 3–4 mm thick slices and fixed in Bouin's fluid for 24 h. Tissues were washed in 70% ethanol, and embedded in paraffin.

Immunohistochemical protocol

Adrenal tissue was cut into 5 μm sections which were placed on glass slides coated with 3-aminopropyltriethoxysilane (Sigma Chemical Co, St Louis, MO, USA). Sections were deparaffinized, rehydrated and washed in phosphate-buffered saline (PBS; 0·01 mol l^{-1}; pH 7·5). Endogenous peroxidase activity was quenched by incubating sections with hydrogen peroxide (3% in H_2O) for 30 min. Sections were washed in PBS and incubated with 10% normal goat serum to eliminate nonspecific staining. Excess goat serum was blotted off, and a range of dilutions of the primary antibody was applied to the sections to ensure optimum immunostaining and also to confirm weak immunoreactivity by incubating with a low antibody dilution. The following primary antibodies were used: a polyclonal antibody (used at a dilution of 1:500–1:2000) raised in rabbits against purified human placental 3β-HSD (the specificity of this antibody was confirmed previously by western immunoblotting (Luu-The *et al.*, 1989)); two polyclonal antibodies raised in rabbits against tyrosine hydroxylase (used at 1:500) and PNMT (used at 1:1000), both purified from bovine

adrenal medulla (Eugene Tech International Inc., Allendale, NJ, USA). All antibodies were diluted in PBS containing bovine serum albumin (10 g l^{-1}) and NaN_3 (1 g l^{-1}). After overnight incubation at 4°C in a humid chamber, sections were washed and the primary antibody–antigen complex was localized using the avidin–biotin staining procedure (Dako Corp, Santa Barbara, CA, USA). Sections were incubated with biotinylated goat anti-rabbit IgG second antibody (1:500 dilution) for 1 h at room temperature. Sections were washed in PBS and Vectastain ABC solution was applied for 2 h. After a further wash, specific immunoreactivity was visualized by incubating with the chromagen 3,3′-diaminobenzidine in Tris buffer (0·05 mol l^{-1}) with 0·002% H_2O_2 for 5 min. Sections were counterstained with Carazzi's haematoxylin, dehydrated and mounted using Permount (Fisher Scientific Co., Fair Lawn, NJ, USA). Sections were examined and photographed using a Leitz Aristoplan light microscope.

Controls

As negative controls, sections of adrenal glands of sheep fetuses obtained at day 54 and day 130 of gestation and at term were treated as above except that the primary antibody was substituted by (i) antibody dilution buffer; (ii) nonimmunized rabbit serum (1:1000 dilution), or (iii) 3β-HSD antibody (1:1000 dilution) that had been preabsorbed overnight with purified human placental 3β-HSD 2·5 μmol l^{-1} (Luu-The *et al.*, 1989). As a positive control, a section of term human placenta was stained for immunoreactive 3β-HSD with each batch of slides that was processed.

Results

Days 43–65 of gestation

At day 43 of gestation, zonation of the adrenal gland of sheep fetuses was already apparent. Cells with large rounded nuclei and abundant cytoplasm were arranged in a layer 5–8 cells thick beneath the capsule surrounding the gland and these formed the outer zona glomerulosa. These cells stained weakly for immunoreactive 3β-HSD (data not shown). Cells arranged in cords 3–5 cells thick extended throughout the inner part of the gland and stained more strongly for immunoreactive 3β-HSD. Medullary cells, identified by their smaller size, less cytoplasm and smaller densely staining oval nuclei were also arranged in the central region in cords of cells between the cortical cells, and were immunonegative for 3β-HSD. Medullary cells were not present in the zona glomerulosa.

With increasing gestational age, the development of the zona fasciculata became more apparent, and cells in this layer stained strongly positive for immunoreactive 3β-HSD (Fig. 1a). By day 54, cords of medullary-type cells which were immunopositive for tyrosine hydroxylase were present in the central region of the gland (Fig. 1c). Immunoreactive PNMT was also present in some cords of medullary cells immediately underlying the zona fasciculata, and also in some centrally located cells (Fig. 1e).

By day 65, the zona fasciculata, consisting of a layer of approximately 10–15 cells, stained uniformly and positive for immunoreactive 3β-HSD, and there were few medullary-type cells in this layer (data not shown). At this stage, cords of cortical cells were still present in the central portion of the gland containing the medullary cells. Immunoreactive PNMT was found in medullary-type cells in a layer several cells thick principally underlying the zona fasciculata on the newly forming corticomedullary interface, although groups of immunoreactive PNMT cells were also found in the central portion of the medulla.

Days 78–125 of gestation

By day 78, the adrenal gland had developed into a distinct cortex and medulla. At this stage, the zona glomerulosa was 6–10 cells thick, and within the same section stained weakly for immunoreactive 3β-HSD, relative to the zona fasciculata which was approximately 20 cells thick and stained strongly and uniformly positive for immunoreactive 3β-HSD (Fig. 2a). There were very few medullary-type cells in the cortex. Some cortical-type cells that stained strongly positive for 3β-HSD were present within the medulla, both in cords towards the corticomedullary boundary and as single or small groups of 3–5 cells deeper within the medulla.

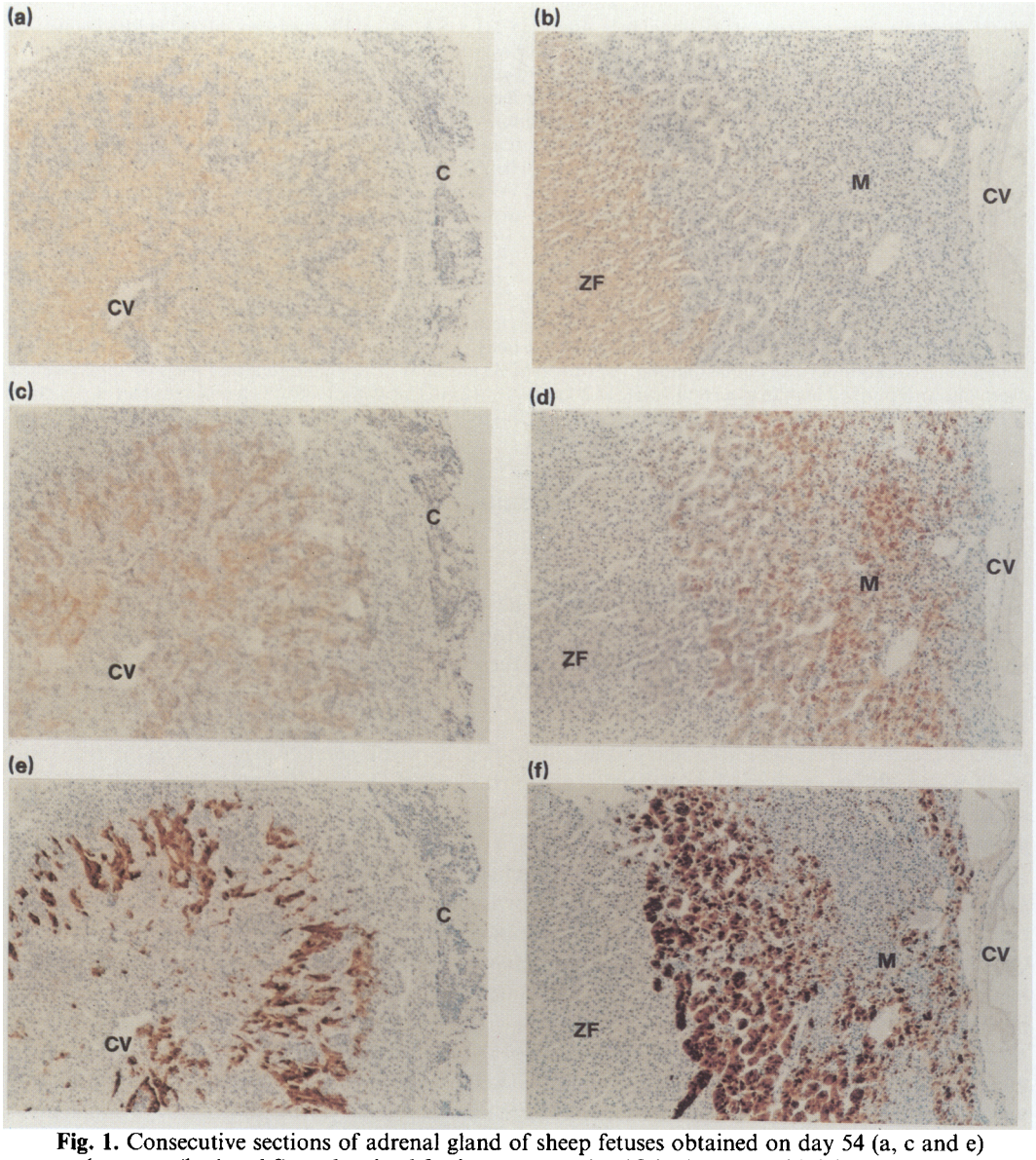

Fig. 1. Consecutive sections of adrenal gland of sheep fetuses obtained on day 54 (a, c and e) and at term (b, d and f), and stained for immunoreactive 3β-hydroxysteroid dehydrogenase (a and b), immunoreactive tyrosine hydroxylase (c and d), and immunoreactive phenylethanolamine *N*-methyl transferase (e and f); all ×200. C: capsule; CV: central vein; M: medulla; ZF: zona fasciculata.

Day 130 to term

At day 130 of gestation, the zona glomerulosa was approximately 10–15 cells thick, and stained weakly for immunoreactive 3β-HSD (Fig. 2b). The zona fasciculata was approximately 30 cells thick, and within the same section consistently stained strongly and uniformly for immunoreactive 3β-HSD (Fig. 2b). The thickness of both the zona glomerulosa and zona fasciculata further increased after day 130, so that, at term, they were 20–30 and 60–100 cells thick, respectively. Both layers stained uniformly for immunoreactive 3β-HSD, although the intensity of staining was stronger in the zona fasciculata (Fig. 2c). Immunoreactive tyrosine hydroxylase was present in most

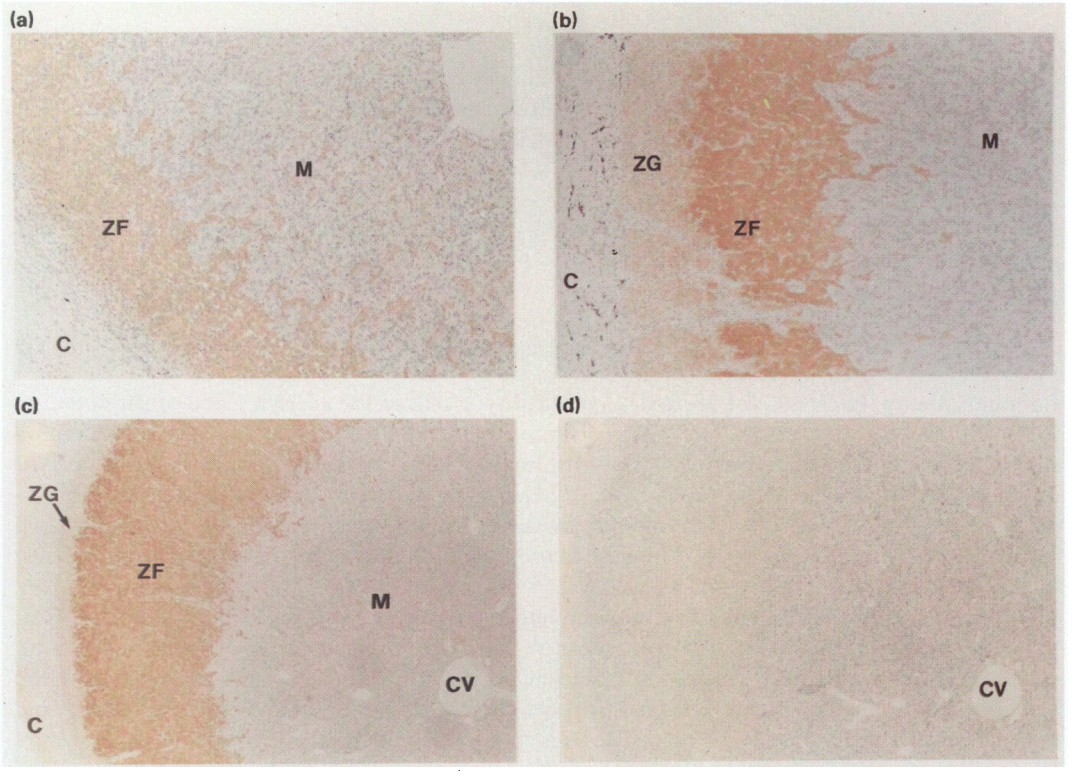

Fig. 2. Sections of adrenal glands of sheep fetuses at (a) day 78 of gestation and stained for immunoreactive 3β-hydroxysteroid dehydrogenase (3β-HSD; × 185), (b) day 133 of gestation demonstrating positive staining for immunoreactive 3β-HSD (× 185), (c) at term and stained for immunoreactive 3β-HSD (× 50), and (d) at term and incubated with 3β-HSD antibody that was preabsorbed with purified human placental 3β-HSD (× 50). C: capsule; CV: central vein; M: medulla; ZF: zona fasciculata; ZG: zona glomerulosa.

cells in the medulla, but within two distinctly different staining subpopulations (Fig. 1d). Intense immunoreactive tyrosine hydroxylase staining was found in a population of cells in the centre of the medulla, with a second population that exhibited weaker staining for immunoreactive tyrosine hydroxylase localized at the juxta-cortical interface. A few groups of weakly staining cells were also centrally located. From consecutive sections, we established that immunoreactive PNMT staining was localized in the cells staining weakly for immunoreactive tyrosine hydroxylase, principally in juxta-cortical cells with a few cells in the central region (Fig. 1f). Within any one section, the most intense immunoreactive PNMT was distributed in cells immediately adjacent to the cortex–medulla border. Tracts of nerve fibres descending through the cortex into the medulla were negative for immunoreactive 3β-HSD (Fig. 2b), tyrosine hydroxylase and PNMT.

Neonatal lambs, 2–4 days old

Weak positive immunoreactive 3β-HSD staining was localized in the outer layer of the adrenal cortex in contrast to the strong positive 3β-HSD staining that was found uniformly throughout the zona fasciculata in the same section. Further, an intensely staining layer of several cells which lay immediately underlying the zona glomerulosa was consistently found. Cortical-type cells staining positive for immunoreactive 3β-HSD in groups or as single cells were still located within the medulla. Immunoreactive tyrosine hydroxylase and PNMT staining was observed in a similar distribution to that in adrenal tissue of fetuses at term (data not shown).

Controls

When the 3β-HSD antibody was substituted by 3β-HSD antibody that had been preabsorbed with purified human placental 3β-HSD, positive immunoreactive 3β-HSD was abolished in sections of fetal adrenal gland obtained at day 54, day 130 and at term (compare Fig. 2c to 2d). No specific immunostaining was found in sections where the primary antibody had been substituted by either nonimmunized rabbit serum or antibody dilution buffer. In all of the positive control sections of human placenta, immunoreactive 3β-HSD was localized in syncytiotrophoblast and intermediate trophoblast cells.

Discussion

Immunostaining was found for 3β-HSD in cortical-type cells in the adrenal gland of sheep fetuses from day 43 of gestation. By day 54 of gestation, positive immunoreactive 3β-HSD immunostaining was found strongly and uniformly throughout the zona fasciculata, with weak positive immunoreactivity in the zona glomerulosa. Furthermore, groups of cells with cortical cell morphology that stained positively for immunoreactive 3β-HSD were found juxta-cortically, and also centrally within the medulla. This distribution of immunoreactive 3β-HSD was maintained throughout gestation and in neonates.

Cortical cells in the adrenal gland of sheep fetuses stained positively for 3β-HSD by day 43 of gestation, and by day 54 there was strong positive staining. This is in agreement with biochemical studies *in vitro* showing that on day 50 of gestation, adrenal glands of sheep fetuses possess 3β-HSD activity as determined by the conversion of pregnenolone to progesterone and also by corticosteroid biosynthesis; the basal output of corticosteroids by fetal adrenal tissue *in vitro* is greater at days 50–55 than at any other time of gestation (Wintour *et al.*, 1975; Glickman & Challis, 1980; Manchester & Challis, 1982). mRNA for $P450_{c21}$, $P450_{SCC}$ and $P450_{c17}$ is also found in cells of the fetal adrenal gland at this stage of gestation (Tangalakis *et al.*, 1989). Cortical cells in the layer immediately underlying the capsule, which corresponded to the zona glomerulosa, were weakly positive for 3β-HSD from days 43 to 60 of gestation. These cells also contained the mRNAs for $P450_{c17}$, $P450_{SCC}$ and $P450_{c21}$ determined by *in situ* hybridization analysis (Tangalakis *et al.*, 1989). Thus, within the fetal adrenal cortex early in gestation, genes for key steroidogenic enzymes, including immunoreactive 3β-HSD, are expressed and show some differentiation between zones.

Positive 3β-HSD immunostaining was localized in the zona fasciculata from days 78 to 120, a period that from biochemical studies is characterized by attenuated fetal adrenal steroidogenic activity (Glickman & Challis, 1980). Using *in situ* hybridization and northern blot analysis, Tangalakis *et al.* (1989) reported that $P450_{SCC}$ and $P450_{c21}$ mRNA were found in both the zona fasciculata and zona glomerulosa during this time. The relative abundance of mRNA for $P450_{c17}$ decreased between days 90 and 120, suggesting that it was a major rate-limiting enzyme to fetal adrenal steroid output in mid-pregnancy. The present results indicate that 3β-HSD is also expressed at mid-pregnancy. Enzymatic activity of 3β-HSD has been demonstrated at day 120 of gestation (Durand *et al.*, 1982), and is unlikely to constitute the major lesion in fetal adrenal steroidogenesis in mid-gestation.

After day 125, immunoreactive 3β-HSD was distributed principally in the zona fasciculata with weak staining in the zona glomerulosa. These findings agree in large part with a previous study using histochemical analysis that demonstrated 3β-HSD activity in the zona fasciculata but not in the zona glomerulosa of adrenal glands of sheep fetuses between day 133 and term (Boshier & Holloway, 1989). This is similar to the pattern and distribution of $P450_{SCC}$ and $P450_{c21}$ mRNA in the zona fasciculata. However, over this period $P450_{c17}$ mRNA increased as assessed by *in situ* hybridization and northern blot analysis (Tangalakis *et al.*, 1989), supporting the view that before this $P450_{c17}$ is a major rate-limiting block to cortisol production in fetal adrenal glands. We found that immunoreactive 3β-HSD was distributed uniformly throughout the zona fasciculata. This

correlates with morphometric analyses demonstrating that maturation occurs in the centripetal direction as there are no changes in the volume of mitochondria and smooth endoplasmic reticulum throughout the zona fasciculata (Boshier & Holloway, 1991).

Positive immunoreactive tyrosine hydroxylase staining was present in most medullary-type cells by day 54 of gestation, and some of these cells, in particular those underlying the zona fasciculata, also stained positively for immunoreactive PNMT. This indicates that even at a very early stage of gestation, functional differentiation of the medulla into adrenaline (immunoreactive tyrosine hydroxylase and immunoreactive PNMT positive) and noradrenaline (immunoreactive tyrosine hydroxylase positive and immunoreactive PNMT negative) secreting cells occurs. Within the medulla there were two different populations of cells staining for immunoreactive tyrosine hydroxylase. One subpopulation was present as groups of weakly staining cells found primarily in the juxta-cortical medullary region, with some cell groups in the central region of the medulla. These cells also exhibited positive immunoreactive PNMT staining. A similar distribution of immunoreactive PNMT was demonstrated in fetal adrenal glands by McMillen *et al.* (1988) from day 80 to term. A second subpopulation of cells staining strongly positive for immunoreactive tyrosine hydroxylase and which were negative for immunoreactive PNMT were also localized in the central portion of the medulla. This distribution agrees with morphological studies describing cells with adrenaline containing vesicles in the juxta-cortical position, and cells with vesicles containing noradrenaline in the central region (Boshier *et al.*, 1989). Furthermore, immunolocalization of dopamine β-hydroxylase also revealed two distinct patterns of staining that were similar to the distribution of immunoreactive tyrosine hydroxylase found in the present studies (McMillen *et al.*, 1988). From our studies comparing staining between consecutive sections it was not possible to determine whether immunoreactive 3β-HSD and catecholamine biosynthetic enzymes were co-localized in a particular population of cells in the adrenal medulla. However, catecholamine synthesizing cells are clearly in close proximity to steroidogenic cortical cells that may be involved in the paracrine control of the degree of interdigitation between cortex and medulla (Coulter *et al.*, 1989, 1991).

We conclude that immunoreactive 3β-HSD is present in cortical cells of the adrenal gland of sheep fetuses from day 43 of gestation and is present, primarily in the zona fasciculata, throughout the remainder of gestation. The close association between steroidogenic cells that were positive for immunoreactive 3β-HSD and catecholamine biosynthetic cells may underlie paracrine interactions between these cell types.

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