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ONTOGENY AND SUBCELLULAR LOCALIZATION OF 3β-HYDROXYSTEROID DEHYDROGENASE (3β-HSD) IN THE HUMAN AND RAT ADRENAL, OVARY AND TESTIS

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Summary—Primates are unique in having adrenals that secrete large amounts of the precursor sex steroids (PSS) dehydroepiandrosterone (DHEA) and especially DHEA-sulfate The adrenal PSS require the action of 3β-hydroxysteroid dehydrogenase/5-ene-4 ene isomerase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α-reductase and/or aromatase to form the androgen dihydrotestosterone (DHT) or the estrogens 17β-estradiol and androst-5-ene-diol Knowing the crucial role of 3β-HSD and 17β-HSD in sex steroid biosynthesis both in classical as well as in peripheral steroidogenic tissues, we have concentrated our efforts on the elucidation of the molecular structure of these enzyme families We have thus characterized two types of human 3β-HSD cDNA clones and their corresponding genes which encode deduced proteins of 371 and 372 amino acids and share 93 5% homology Human type I 3β-HSD is the almost exclusive mRNA species expressed in the placenta and skin, while human type II is the predominant mRNA species in the adrenals, ovaries and testes We have also recently elucidated the structure of three types of rat 3β-HSD cDNAs which all encode a 372 amino acid protein The predicted rat type I and II 3β-HSD proteins expressed adrenals, gonads and adipose tissue share 94% homology while they share 80% similarity with the liver-specific type III 3β-HSD Transient expression of human type I and II as well as rat type I and II 3β-HSD cDNAs in HeLa human cervical carcinoma cells reveals that 3β-ol dehydrogenase and 5-ene-4-ene isomerase activities reside within a single protein and that these cDNAs encode functional 3β-HSD proteins The expressed rat type III protein possesses a unique property catalyzing selectively the reduction of 3β-androstane 5α-steroids such as DHT Furthermore, we have also demonstrated by site-directed mutagenesis that the lower activity of expressed rat type II compared to rat type I 3β-HSD protein is due to a change of four amino acid residues potentially involved in a membrane-spanning domain In parallel, we have characterized the complete nucleotide sequence of human 17β-HSD cDNA clones encoding a 327 amino acid protein as well as two in tandem 17β-HSD genes Two major 17β-HSD mRNA species have been detected in several tissues due to a tissue-specific alternative site of initiation of transcription It is quite clear that our attention should be focused on intracrinology, in order to better understand the physiological mechanisms controlling local steroid formation

INTRODUCTION

A discovery of major importance in the physiology and pathology of sex steroids is that men and women are unique among animal species in having adrenals that secrete large amounts of the inactive precursor steroids dehydroepiandrosterone (DHEA), its sulfate (DHEA-S) and androstenedione which are converted into potent androgens and estrogens in peripheral tissues (Fig 1) Adrenal secretion of DHEA and DHEA-S increases during the adrenarche in children at the age of 6 to 8 years and elevated values of circulating DHEA-S are maintained throughout adulthood [1–5] In fact, plasma DHEA-S levels in adult men are 100 to 500 times higher than those of testosterone [5, 6], thus providing the high level of substrate required for conversion into active sex steroids in peripheral tissues

This situation of a high secretion rate of precursor adrenal androgens and estrogens in men and women is thus completely different from most animal models used in the laboratory, e g rats, mice, guinea pigs, or others (except monkeys), in which the secretion of sex

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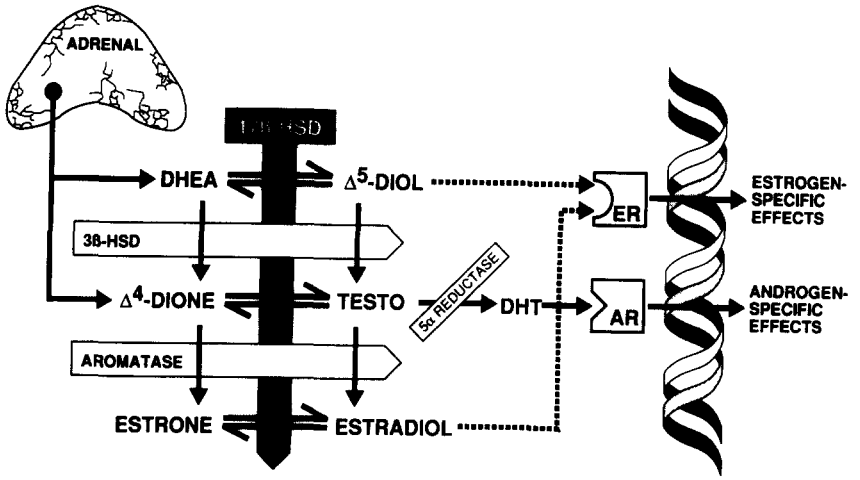

Fig 1 Biosynthetic steps involved in the formation in peripheral target tissues of the estrogens Δ^5-diol (androst-5-ene-3β,17β-diol) and estradiol, and of the androgens testosterone (testo) and DHT (5α-dihydrotestosterone) Δ^4-dione, androstenedione, 17β-HSD, 17β-hydroxysteroid dehydrogenase, ER, estrogen receptor, and AR, androgen receptor

steroids is exclusively from the gonads [5, 7, and refs therein] In these animals, no significant amounts of androgens and estrogens are left after castration [5, 7] Such findings open a new field of endocrinology, namely that of "intracrine" secretion or intracrinology [8] Through intracrine activity, locally produced androgens and/or estrogens exert their action inside the same cells where synthesis took place Since local formation of androgens and estrogens is likely to play a major role in both normal and tumoral hormone-sensitive tissues, an important proportion of our research program has recently been devoted to this exciting and therapeutically promising area

A key enzyme in steroidogenesis is 3β-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase (3β-HSD), the enzyme required for the biosynthesis of all classes of hormonal steroids, namely progesterone, glucocorticoids, mineralocorticoids, androgens and estrogens The 3β-HSD enzymatic system is present in the adrenals, testes, ovaries, and placenta as well as in many peripheral tissues including the prostate, breast, liver and skin [9–11] Congenital deficiency of 3β-HSD activity causes severe depletion of steroid formation by the adrenals and gonads and can be lethal in early life [12]

Since the structure of 3β-HSD was not known, we have cloned cDNAs encoding human, rat, macaque and bovine 3β-HSD, and we have deduced the amino acid sequences of the corresponding proteins [13–19] We have also elucidated the structure of two human 3β-HSD genes [20, 21] localized to the p13 band of chromosome 1 [22]

Since 3β-HSD plays such a crucial role in steroidogenesis, we have studied the localization and ontogeny of 3β-HSD in the human adrenal, testis, ovary and placenta as well as in the same rodent tissues [23–28]

LOCALIZATION OF 3β-HSD IN RAT ADRENAL AND GONADS

Compartmentalization of enzymes plays an essential role in the precise modulation of steroid biosynthesis in the gonads and adrenals Using immunocytochemistry, we have recently reported that 3β-HSD immunoreactivity could be observed in the guinea pig adrenal cortex as well as theca interna and corpus luteum cells in the ovary and interstitial cells of the testis [27] Since the rat is the most widely used model to study the control of steroidogenesis, and the precise distribution of 3β-HSD at the microscopic level has not yet been reported in this species, we have localized this enzyme in the gonads and adrenals of the adult rat using light microscopic immunocytochemistry and *in situ* hybridization [26]

In the adrenal glands, immunostaining was observed exclusively in the three layers of the cortex, the medulla was devoid of any reaction Moreover, the intensity of the staining was similar in the zona reticularis, fasciculata, and glomerulosa At the cellular level, labeling was exclusively observed in the cytoplasm When preimmunized serum or primary antiserum

previously absorbed with purified 3β-HSD was used, no staining could be detected, thus indicating the specificity of the reaction observed [26]

In the testis, immunostaining was exclusively seen in the interstitial cells; the seminiferous tubular elements, including the Sertoli cells, were unlabeled [Fig 2(a)] Staining of the inter-

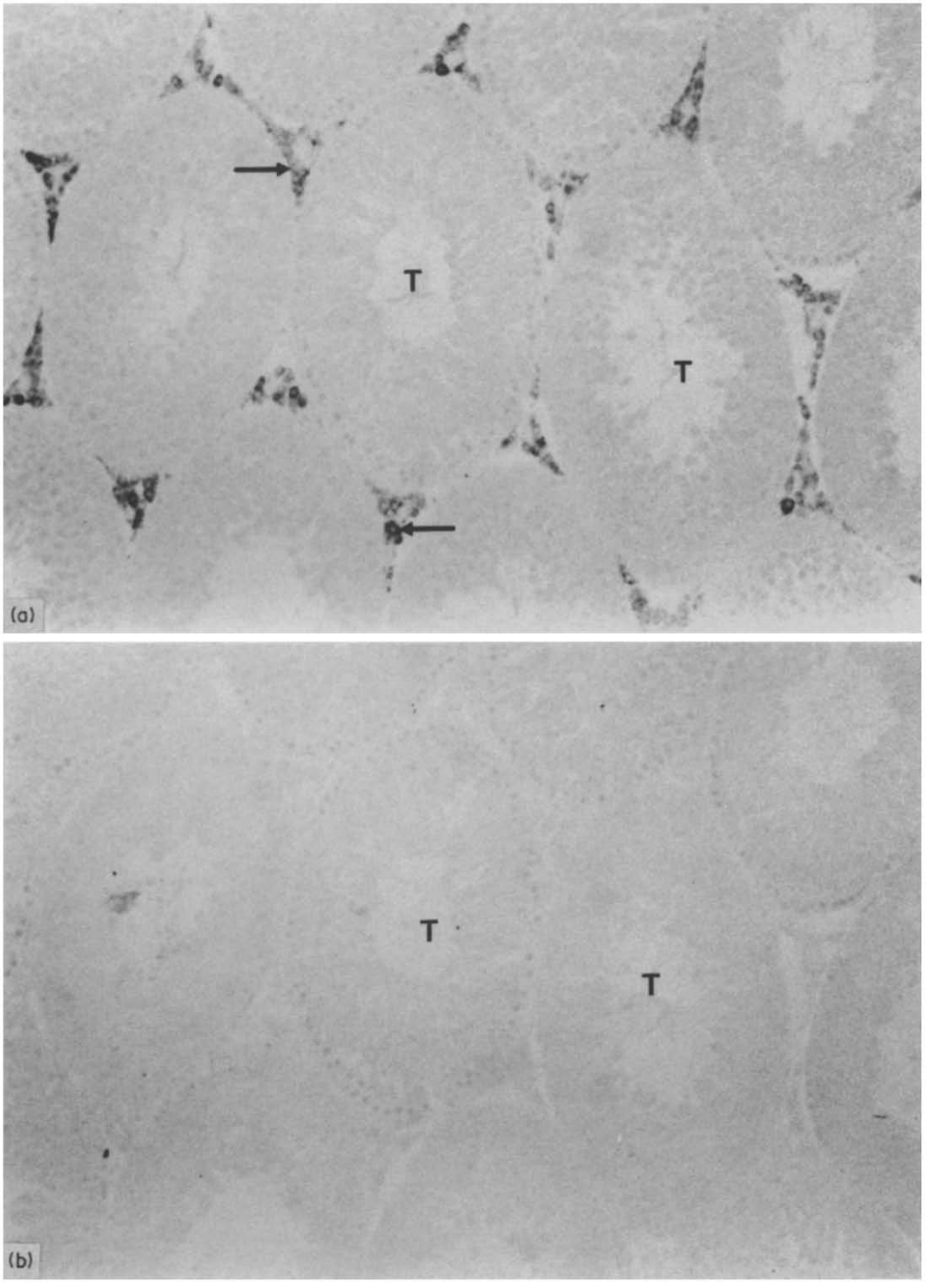

Fig 2 (a) Section through a rat testis Immunostaining is only found in interstitial cells (→) The seminiferous tubules (T) show no positive reaction Magnification, ×200 (b) Section through a rat testis Immunoabsorption of the antiserum with 3-HSD completely prevented staining Seminiferous tubules Magnification, ×200 [26]

stitial cells was completely abolished when antiserum was immunoabsorbed with purified 3β-HSD [Fig 2(b)] [26]

Sections from ovaries obtained at each stage of the estrous cycle were examined Immunolabeling was detected in the cytoplasm of corpus

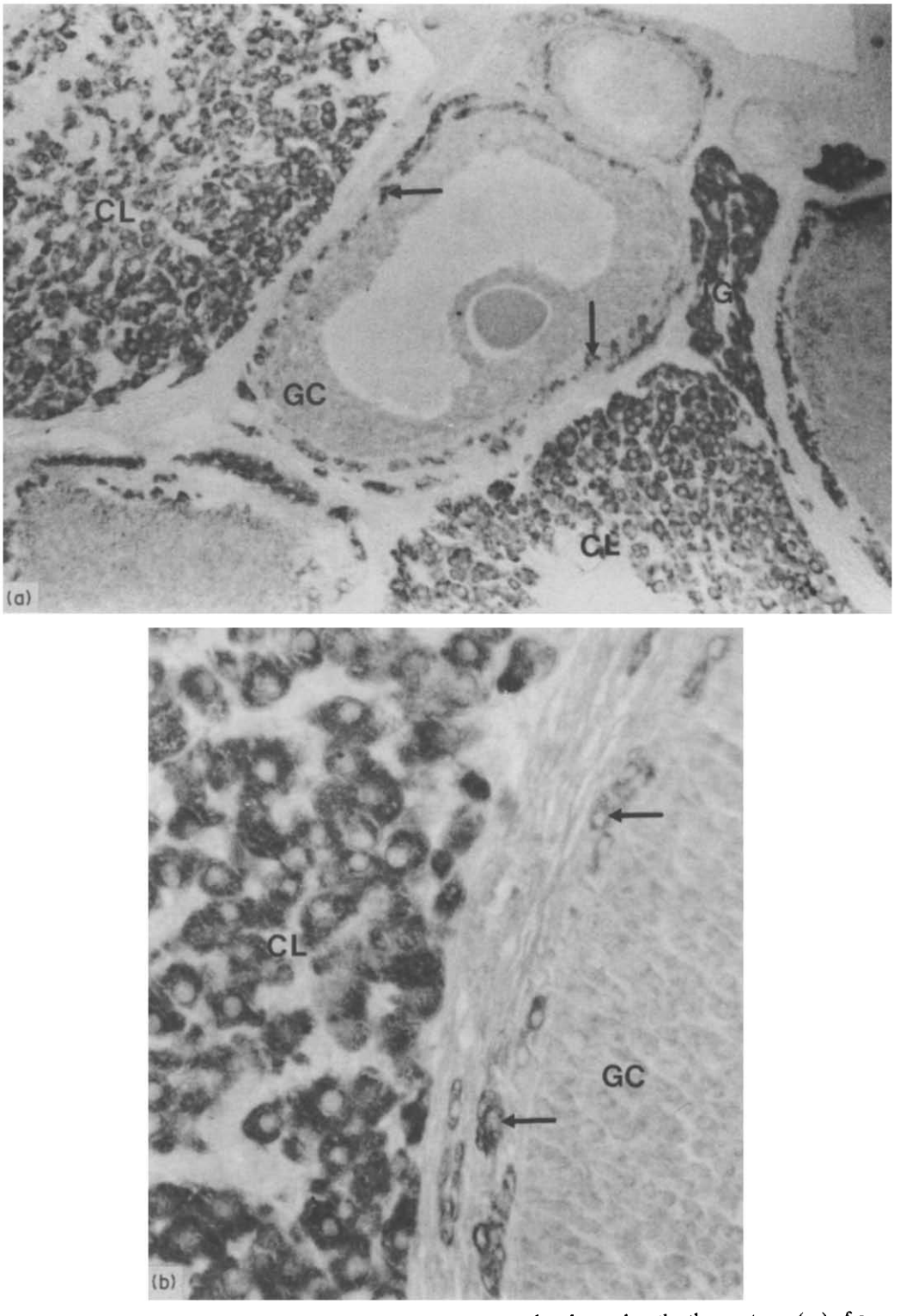

Fig 3 (a) Section through a rat ovary Immunostaining can be observed in the theca interna (→) of a mature follicle, corpus luteum (CL), and interstitial gland cells (IG) Granulosa cells (GC) are unlabeled Magnification, ×200 (b) Higher magnification of a follicle and corpus luteum The reaction product appears exclusively located in the cytoplasm of the corpus luteum (CL) cells and theca interna cells (→) The granulosa cells (GC) are devoid of any reaction Magnification, ×500 [26]

luteum cells, interstitial cells, and theca interna cells (Fig 3) Labeling of the theca interna was present in growing and mature follicles, but not in primordial follicles No staining could be detected in the granulosa cells at any stage of follicular development

In the adrenal glands, *in situ* hybridization yielded results similar to those obtained by immunocytochemistry A homogenous autoradiographic reaction was detected in the cortex, the medulla was devoid of reaction Pretreatment with RNase completely prevented the autoradiographic reaction over the cortex [26]

In the testis, X-ray film autoradiographs revealed that the hybridization signal had a peritubular location Light microscopic observations clearly demonstrate an accumulation of silver grains over the interstitial cells, the tubular elements were very weakly labeled In RNase-pretreated sections, only a few uniformly distributed silver grains were observed [26]

As observed in X-ray films, strong labeling was detected over the corpus luteum and at the periphery of follicles at all stages of the estrous cycle (Fig 4) At higher resolution, it was clearly observed that the autoradiographic reaction was found in corpus luteum cells, theca interna cells of growing and mature follicles, and interstitial cells In the follicles, the results were similar to those obtained with immunocytochemistry, no granulosa cell was labeled at any stage of follicular development [26] The present data clearly establish for the first time the precise localization of 3β-HSD in rat gonads and adrenals at the light microscopic level, using a combination of immunocytochemistry and *in situ* hybridization techniques In the ovary, the positive immunostaining and the hybridization signal found in the theca interna of the follicle are in good agreement with previous data suggesting that biosynthesis of the C_{19}, steroids androstenedione and testosterone takes place in the theca cells and that C_{19} steroids are subsequently aromatized into estrogens by the granulosa cells [10, 29] This 3β-HSD was also found in cells of the interstitial glands, which are known to be involved in steroid biosynthesis [29–31], although their precise role remains to be established

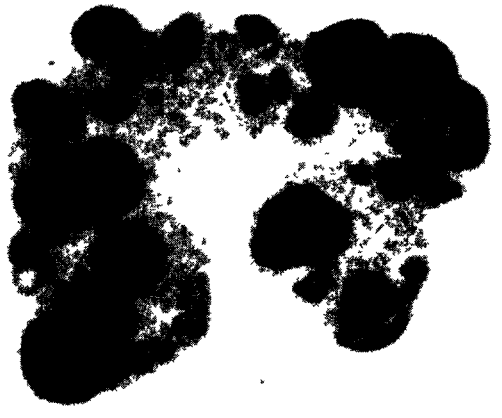

Fig 4 X-ray autoradiograph showing the hybridization signal in a rat ovarian section Strong labeling of the corpus luteum (CL) can be observed Magnification, × 15 [26]

ONTOGENY OF 3β-HSD IN RAT ADRENAL AND TESTIS

Adrenal

The essential role of the adrenal cortex during the last part of fetal life is clearly indicated by the requirement for glucocorticoids for the development and maturation of a series of fetal organ systems [32] Since the enzyme 3β-HSD catalyses an essential step for the biosynthesis of all biologically active steroid hormones, namely glucocorticoids, mineralocorticoids, progesterone, estrogens and androgens [13, 17, 19, 20, 29, 33], data on adrenal gland ontogeny of 3β-HSD should provide information useful for our understanding of development and control of steroidogenesis in the adrenal cortex The zones of the adrenal cortex contain distinct subpopulations of cells which share a common mesodermal origin and are differentiated into the zona glomerulosa, which secrete mineralocorticoids, zona fasciculata and reticularis which secrete mainly glucocorticoids Distribution of 3β-HSD during development of the rat adrenal cortex has been previously investigated using histochemistry [34], giving rise to the controversial suggestion of the existence in the fetal capsule of a 3β-HSD type which transforms more specifically 5β-androstan-3β-ol-17-one than DHEA, the latter being metabolized preferentially in the middle cortex In the adult rat adrenal, it is currently thought that 3β-HSD activity is restricted to the cortical parenchyma while the capsule is immunocytochemically devoid of steroidogenic activity [26] The availability of antiserum against human 3β-HSD [13] and a rat cDNA probe [17], offers us the possibility of

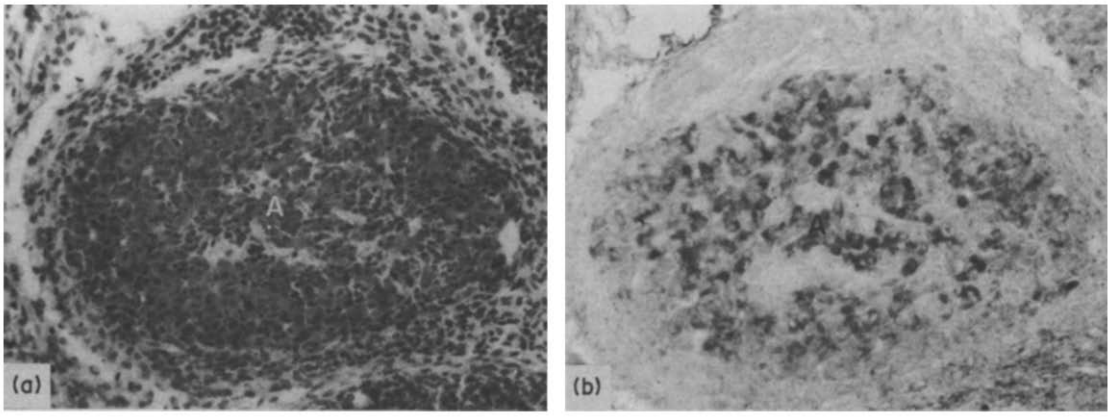

Fig 5 (a) Section showing an adrenal (A) from a 17-day-old fetus stained with eosin-hematoxylin Magnification ×200 (b) Section adjacent to that shown in (a) Heterogeneous immunostaining can be observed exclusively in cells of the adrenal gland (A) Magnification, ×200 [28]

localizing 3β-HSD during development of the rat adrenal using immunocytochemistry and *in situ* hybridization at the light microscopic level [28]

In the fetal adrenal glands, 3β-HSD expression was first detected by *in situ* hybridization on day 16, the silver grains being located over the cortical cells On the 17th day of gestation, however, heterogenous immunostaining could first be detected [28, Fig 5] Between days 18 to 21, immunostaining as well as silver grain became detectable in the cytoplasm of the adrenocortical cells, the capsule being negative by both techniques [28] However, two distinct zones could be distinguished on the basis of the intensity of immunostaining and hybridization signal, namely the highly labeled reticular and fascicular zones and the less active glomerular zone [28]

After birth and until day 15, strong immunolabeling was observed in the cytoplasm of cortical cells, the glomerular zone still being labeled to a lower degree than the remaining cortex (Fig 6) The same distribution of

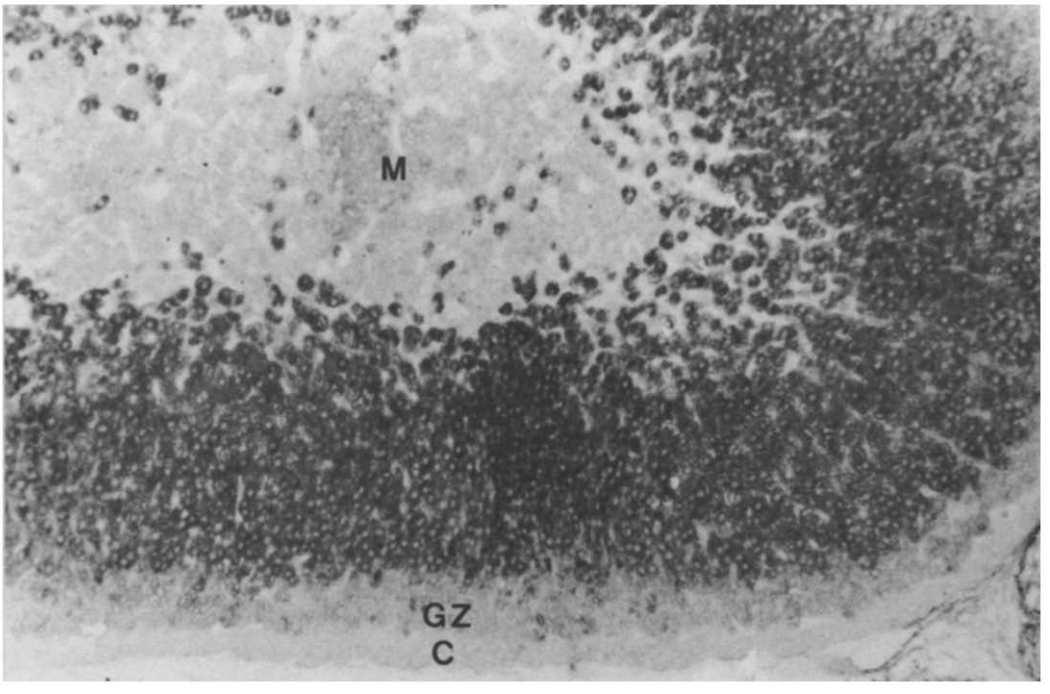

Fig 6 Section through the adrenal of a 5-day-old animal Immunostaining can be observed in the three layers of the cortex although to a lower degree in the zona glomerulosa (GZ) Note the marked heterogeneity in the labeling in this layer The medulla (M) and capsule (C) are completely devoid of reaction Magnification, ×200 [28]

3β-HSD mRNA was obtained by *in situ* hybridization [28] At the light microscopic level, accumulation of silver grains was seen over cells of zona fasciculata and reticularis and to a lesser extent over glomerulosa cells while only very weak and nonspecific labeling could be detected over chromaffin cells of the medulla [28]

From day 25 after birth, the results obtained were identical to those seen in adult animals In fact, immunostaining was then observed exclusively in the three layers of the cortex while the medulla was devoid of any reaction Moreover, the intensity of staining was similar in the zona reticularis, fasciculata and glomerulosa *In situ* hybridization yielded results superimposable to those obtained by immunocytochemistry [28]

The present results on the expression of 3β-HSD during development of the rat adrenal, as evaluated by *in situ* hybridization and immunocytochemistry are in agreement with previous data obtained by histochemistry [34] In addition, the present data clearly demonstrate that the expression of the 3β-HSD gene into mRNA and its transcription into the 3β-HSD immunoreactive protein are closely related events during development of the rat adrenal gland In fact, the first appearance of 3β-HSD mRNA and immunoreactivity was observed at 16 and 17 days postconception, respectively On day 18, we could see two distinct zones of 3β-HSD signal intensity, namely the highly labeled reticular and fascicular zones and the less active glomerular zone This observation coincides with the onset of fetal ACTH secretion on day 17–18 and with accelerated adrenocortical growth and differentiation [35–37]

The present data also clearly show that 3β-HSD is expressed earlier in the reticular and fascicular zones compared to the glomerulosa zone Since the glomerula zone is under the positive regulation of angiotensin II, it might be postulated that the delay in 3β-HSD expression in this zone is related to the low plasma levels of angiotensin II observed during postnatal development in the rat [38] The marked modulation of 3β-HSD mRNA and protein levels during development of the adrenal cortex strongly suggest that regulation of the 3β-HSD gene could be an important site of control of steroidogenesis in the adrenal cortex [28]

Testis

In mammalian species, Leydig cells of the fetal testis have been identified as the source of testosterone which is required for masculinization of the accessory organs of reproduction, the secondary sex structures, and the neural substrate which, in the adult, will mediate sexually dimorphic functions of the central nervous system [39, 40] In most mammals, including rats, Leydig cells undergo two distinct periods of development, the first one occurring during fetal life and the second one taking place at puberty [41–43], thus leading to two populations of Leydig cells On one hand, the fetal and neonatal Leydig cell population, showing 3β-HSD activity from 17 days onwards is localized largely in the interstitial space between the seminiferous tubules while, on the other hand, the postnatal Leydig cell population showing 3β-HSD activity from the 16 postnatal day onwards, surrounds the individual seminiferous tubules [42]

So far, the immunocytochemical localization of 3β-HSD during development of the testis has not been studied In order to obtain more information about the age-specific expression of 3β-HSD in testis, we have localized this enzyme by immunocytochemistry at the light microscopic level during the fetal and postnatal development of the rat testis

No immunostaining for the 3β-HSD enzyme was detected at 15 and 16 days of fetal life while on the 17th day of gestation, only a few weakly stained cells could be observed On day 18, several cells were labeled Some positive cells appeared without any specific orientation while others were located around poorly defined seminiferous tubules From 19 to 21 days of fetal life, strongly immunopositive cells are seen in large clusters occupying the space between the seminiferous tubules (Fig. 7)

Shortly after birth, immunopositive Leydig cells are forming clusters in the large intertubular spaces However, during the days following birth, the number and size of positive cells rapidly decreased In fact, at 5 and 10 days after birth, the stained cells appeared isolated or forming small complexes

Fifteen days after birth, groupings of positive Leydig cells appeared more numerous At that time, some cells had an elongated shape and were situated alongside the seminiferous tubules These cells probably correspond to the already described peritubular Leydig cells [39, 41–44] Other positive cells having a round shape were arranged in small clusters in the large peritubular spaces At the onset of puberty or between 20 to 30 days of

Fig 7 Section through the testis of a fetus rat at 21 days of gestation Numerous Leydig cells (→) are strongly labeled The seminiferous tubules (T) are devoid of any reaction Magnification ×180

age, the seminiferous tubules have grown and most of the Leydig cells which are much more abundant appear strongly labeled and located in both the peritubular and intertubular spaces (Fig 8) At 40 days of age and adulthood, both strongly and weakly immunolabeled

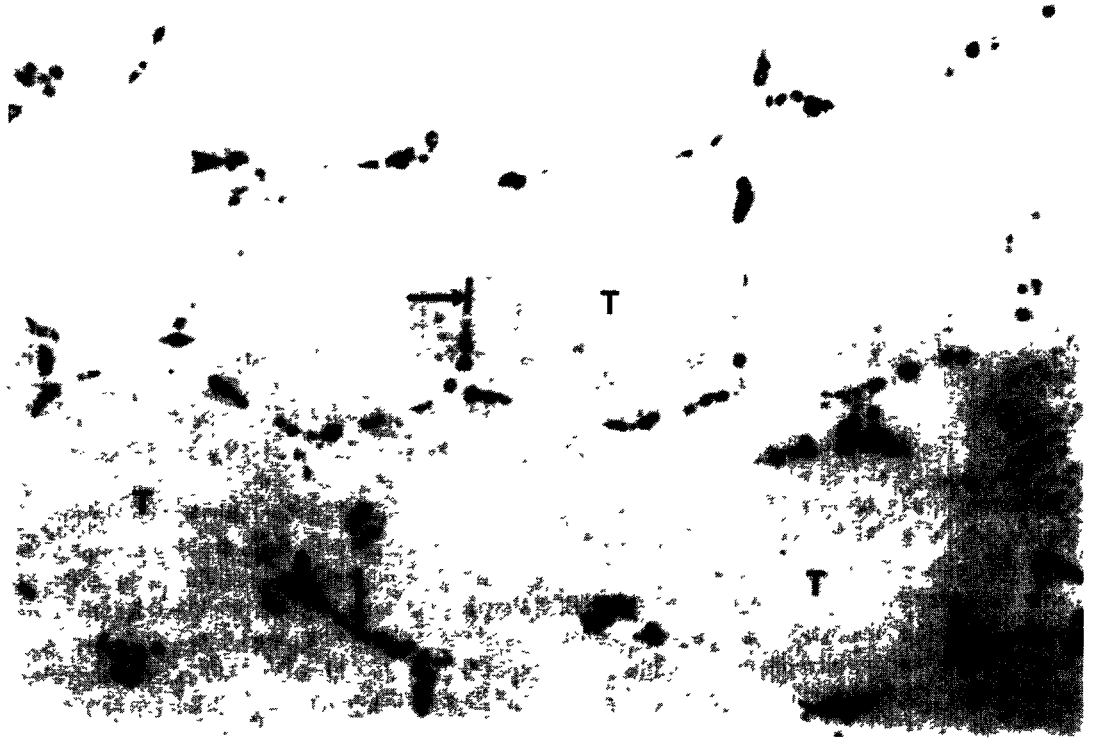

Fig 8 Twenty-five days after birth The peritubular (→) and intertubular (◗) Leydig cells are strongly stained Seminiferous tubules Magnification, ×180

Leydig cells are observed, mostly located in the intertubular spaces The tubular elements always remain unreactive at any stage of development

These findings clearly indicate that marked changes occur in 3β-HSD immunoreactivity during pre- and postnatal development in Leydig cells In fact, immunostaining could be first detected on the 17th day of gestation while a plateau was reached between days 19 and 21 of fetal life At the end of gestation, the Leydig cells appeared well developed and strongly stained Soon after birth, there was a rapid decrease in immunoreactivity which reached the lowest levels during the first 10 days of postnatal life During the days preceding the onset of puberty and at the onset of puberty, there was a moderate increase in the number of 3β-HSD containing cells which were located in both peritubular and intertubular spaces These positive cells continued to increase in number and size during and after puberty to become mostly concentrated in the intertubular spaces during adulthood

Using histochemical techniques, Ziegler *et al* [45] have shown that during fetal development maximal 3β-HSD enzyme activity was seen on day 19 of fetal life In the present study, we have observed that 3β-HSD immunoreactivity remained maximal from day 19 until birth There was no detectable decrease in the number and staining of Leydig cells on the 20th and 21st days of gestation

The present data support the previous reports on changes in plasma testosterone levels during fetal life [40] which were low on day 17 and increased during the following days of gestation to reach maximal values before birth There thus seems to exist a correlation between plasma testosterone levels and the amount of 3β-HSD present in Leydig cells during fetal life

The progressive atrophy of Leydig which takes place during the days following birth is possibly related to the very low levels of circulating gonadotropins observed during the neonatal period [46] Such changes in plasma gonadotropins and the present data on the level of immunoreactive 3β-HSD during the neonatal period can provide an explanation for the low levels of plasma testosterone observed on days 3 and 15 after birth [40] The increase of Leydig cells especially those of the peritubular type, from day 10 after birth is probably related to the progressive increase in gonadotropin levels which takes place after the first week of postnatal life [46–48]

LOCALIZATION AND ONTOGENY OF 3β-HSD IN HUMAN OVARY

Since there has been so far no report about the age-specific expression of 3β-HSD in the human ovary, we have thus localized this enzyme by immunocytochemistry at the light microscopic level during fetal and postnatal periods of development in the human ovary as well as during adult pre- and postmenopausal ages [25]

No immunostaining could be detected in ovarian tissue prior to 28 weeks of gestation In ovaries of 28-week-old fetuses, immunostaining could be observed exclusively in the cytoplasm of thecal cells surrounding small primary follicles (Fig 9) Small primordial follicles were devoid of any staining At 34 weeks of fetal life, staining was observed not only in thecal cells of growing follicles but also in interstitial cells

After parturition until puberty, no immunostaining could be detected in any ovarian structure During this period, only primordial and small primary follicles were observed From puberty until menopause, immunostaining was seen in theca interna cells in large primary as well as secondary and mature follicles Atretic follicles often exhibited immunostaining of the theca interna Primordial and small primary follicles were devoid of any reaction Theca externa did not show any labeling during folliculogenesis Granulosa cells did not express 3β-HSD immunoreactivity in primary and secondary follicles, while in large antral and mature follicles, these cells exhibited labeling which was always less intense than that observed in thecal cells Interestingly, one to several layers of the theca interna cells which were lying directly beneath the basement membrane did not show any immunoreactivity In the corpus luteum, immunostaining was detected in both luteinized theca and granulosa cells, with equal intensity in both cell types Occasionally, layers of luteinized theca cells in proximity to granulosa cells did not show any immunoreactivity [25]

In the menopausal and postmenopausal ovaries, immunostaining could only be detected in the cytoplasm of a few dispersed interstitial cells No staining could be detected in any ovarian structure when the antiserum to

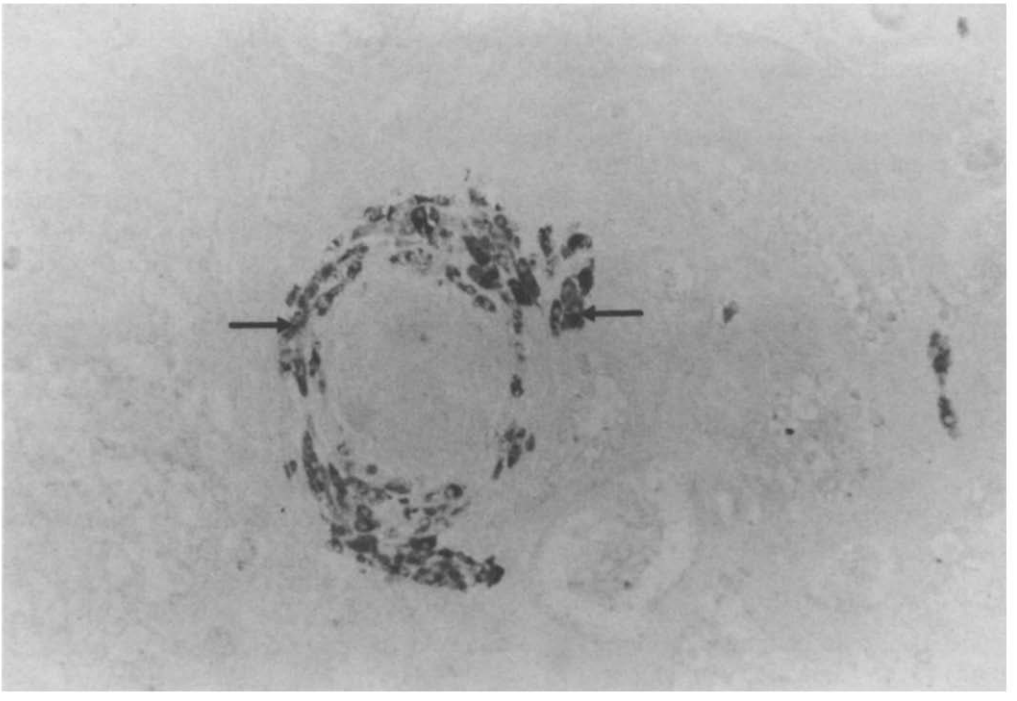

Fig 9 Immunoreactivity of 3β-HSD in a ovary at 28 weeks of fetal life Immunostaining is observed exclusively in the cytoplasm of thecal cells (→) surrounding primary follicles Magnification, ×170 [25]

3β-HSD previously immunoabsorbed with purified 3β-HSD or preimmune antiserum was used [25]

The present data clearly demonstrate that during human fetal life, immunolabeling for 3β-HSD can be detected in theca interna cells surrounding primary follicles and also in interstitial cells Immunostaining was first observed at 28 weeks of gestation and persisted throughout the third trimester of gestation Such results suggest that human fetal ovaries may be involved in sex steroid synthesis during the last weeks of gestation provided other steroid-synthesizing enzymes such as aromatase are also expressed in fetal ovaries This finding was rather unexpected since previous reports have clearly indicated that only minimal amounts of estrogens can be synthesized by fetal ovaries in several mammalian species, including man [49, 50] The high levels of estrogens found in the fetal circulation are in fact thought to originate from placenta [51] The development of primordial follicles to the stage of primary follicles as well as the expression of 3β-HSD observed from the 28th week of gestation could be related to the increase in fetal plasma FSH and LH levels which occurs during the end of the second trimester of gestation [51]. hCG which has been shown to increase 3β-HSD mRNA levels in rat ovaries [52] might also be responsible for induction of the expression of the enzyme during fetal ovarian development

From birth to the prepubertal period, no 3β-HSD immunostaining could be detected This finding is in agreement with the low levels of sex steroids observed during this period of life [54] During active folliculogenesis, immunostaining for 3β-HSD was observed in theca interna cells but not in the theca externa of growing and mature follicles These results are consistent with previous histochemical reports [54, 55] and immunohistochemical data [56]

In menopausal and postmenopausal women, 3β-HSD immunoreactivity was found in dispersed interstitial cells, thus indicating that active steroidogenesis could occur after menopause These results are in agreement with the findings of Aiman *et al* [57] who reported the presence of 17β-estradiol in ovarian venous blood in postmenopausal women However, the main steroid secreted by the human ovary after menopause is androstenedione In fact, in postmenopausal women, about 30% of circulating androstenedione is of ovarian origin, the remaining being of adrenal sources [58, 59]

LOCALIZATION AND ONTOGENY OF 3β-HSD IN HUMAN TESTIS

In the human male fetus, Leydig cells secrete testosterone, which in turn promote male differentiation of the Wolffian ducts, urogenital sinus and external genitalia The production of testosterone by the testis and the concentration of plasma testosterone in the fetus start to rise at the end of the second month of gestation and shortly thereafter attain high values that are maintained until late gestation when they decrease [60]

At the time of birth, plasma testosterone levels are very low and shortly afterwards, they begin to rise and remain elevated for approx 3 months falling to low levels by the age 1 year [61–63] The concentration remains low until the onset of puberty when the concentration again increases to reach adult levels by the age of 17 [64, 65]

In order to correlate possible changes in 3β-HSD with the well known variations in testosterone production during development, we have localized the 3β-HSD by immunocytochemistry during different fetal and postnatal periods of development in the human testis

In testis of 22-week-old fetı, immunostaining was observed exclusively in the cytoplasm of interstitial cells whereas the seminiferous tubular elements remained completely unreactive [Fig 10(a)] [24]

At 28 weeks of fetal life, strong immunolabeling could be detected in the cytoplasm of interstitial cells [Fig 10(b)] The seminiferous tubules were similar to those of 22-week-old fetı Interestingly, the interstitial cells appeared much larger than those observed at 22 weeks of gestation

In fetı of 31 weeks [Fig 10(c)], immunostaining was also observed in the cytoplasm of interstitial cells It was of interest to note heterogeneity in labeling, approximately half the interstitial cells being weakly labeled In 8-month-old infants and during the childhood, no immunostaining for 3β-HSD could be observed in the testis With puberty, dramatic changes were observed in the testis histology As observed in sections through a testis of a 15-year-old boy, the seminiferous tubules appeared well developed and the interstitial cells contain immunoreactive material The same intensity of immunostaining was also observed in adult testes [24] No staining could be obtained when preimmunized rabbit serum or the primary antiserum immunoabsorbed with excess of purified 3β-HSD was used [Fig 10(d)]

The present data clearly shows that 3β-HSD could be detected in Leydig cells in the fetus and that immunolabeling was absent 8 months and 12 years after birth to become detectable at the puberty time These immunocytochemical results agree well with previous data from Baillie *et al* [66] who used enzyme histochemistry to detect 3β-HSD activity in testes of human fetı from 8 to 22 weeks of gestation Our results which indicate that 3β-HSD is present in fetal testis during the second and third trimester of gestation (22, 28 and 31 weeks) agree well with previous results demonstrating that in human embryo plasma testosterone levels are high during the second and third trimester of gestation and decrease during the last weeks of gestation [60]

In summary, we have demonstrated variation in the testicular content of 3β-HSD during pre- and postnatal development in the human Since the pattern observed in 3β-HSD content is similar to that observed for androgen production, it might be suggested that activation of 3β-HSD by trophic hormones plays an important role in androgen production during fetal life and postnatal development

LOCALIZATION AND ONTOGENY OF 3β-HSD IN HUMAN ADRENAL

The human fetal adrenal cortex is morphologically and functionally differentiated into two zones, namely the neocortex and the fetal zone While the fetal zone primarily secretes 5-ene-steroid 3-sulfates [67, 68], the neocortex secretes higher amounts of 3-keto-4-ene-steroids and is the primary site of cortisol production on the fetus [67] Circulating levels of free and conjugated 3β-hydroxy-5-ene-steroids such as DHEA, pregnenolone and 17-hydroxypregnenolone undergo dramatic changes with a rapid decline after birth, a gradual rise during late childhood and puberty, and a smaller decline in old age [4, 69, 70] Analyses of intraadrenal [69], circulating and urinary [70] 4-ene- to 5-ene-steroids ratios during development have suggested that these phenomena reflect, at least in part, changes in the activity of the adrenal microsomal 3β-HSD complex In order to obtain more information about the age-specific expression of 3β-HSD in the human adrenal, we have localized this enzyme by immunocytochemistry at the light microscopic level during

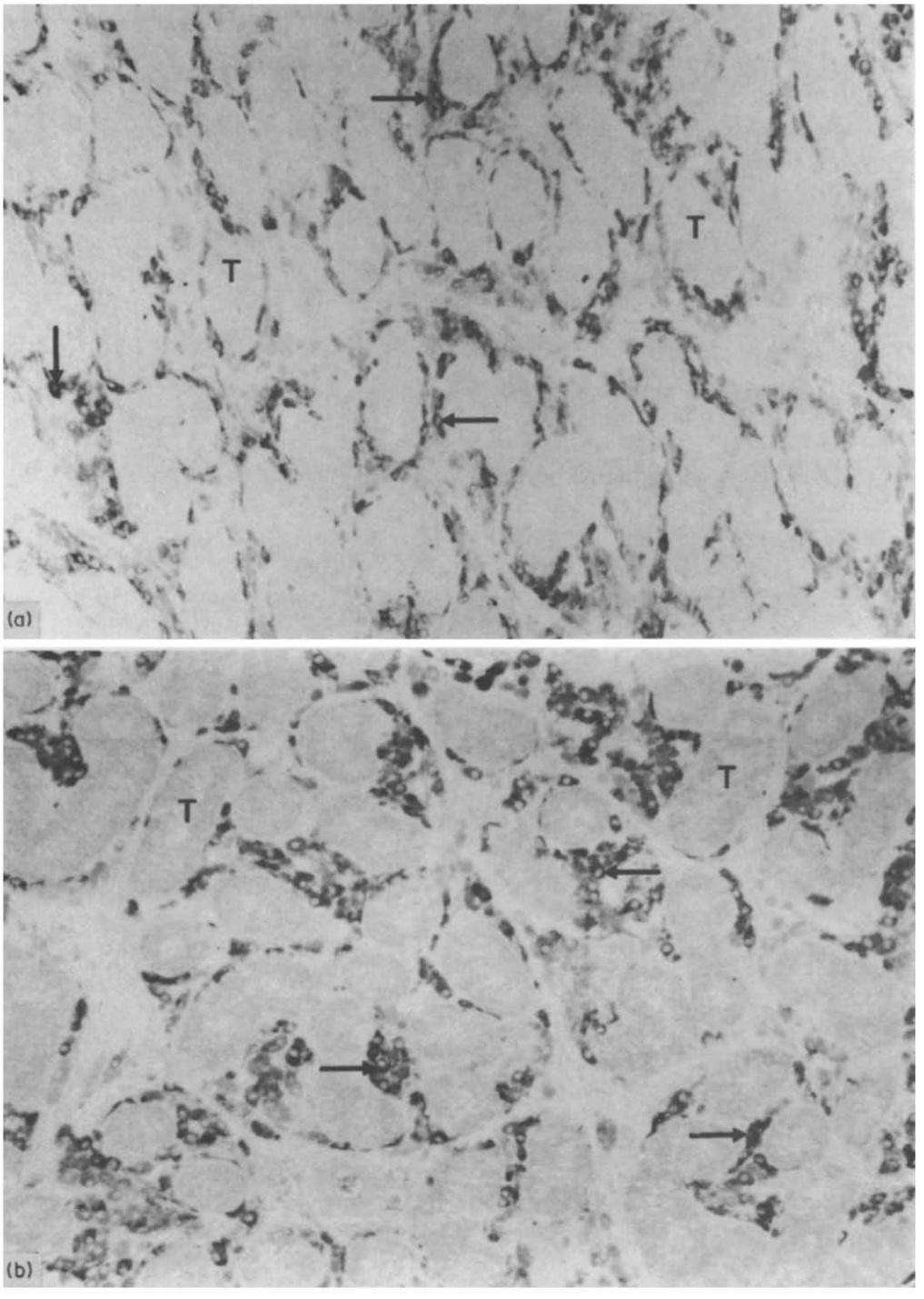

Fig 10(a, b)—*legend opposite*

fetal and postnatal periods of development in the human adrenal [23]

In the fetal adrenal from mid-gestation (22 weeks), immunostaining was observed exclusively in the cytoplasm of cells of the outer cortex [Fig 11(a)] Six weeks later, at the beginning of the third trimester (28 weeks), [Fig 11(b)] immunolabeling was more widely distributed and extended to all cells of the neocortex In term fetal adrenals, on the other hand, strong staining was observed in the presumptive zona glomerulosa [Fig 11(c)] [23]

In 2-month-old infant adrenals, immunostaining was seen in both the zona glomerulosa and fasciculata [Fig 12(a)] No staining could be detected in the zona reticularis which could

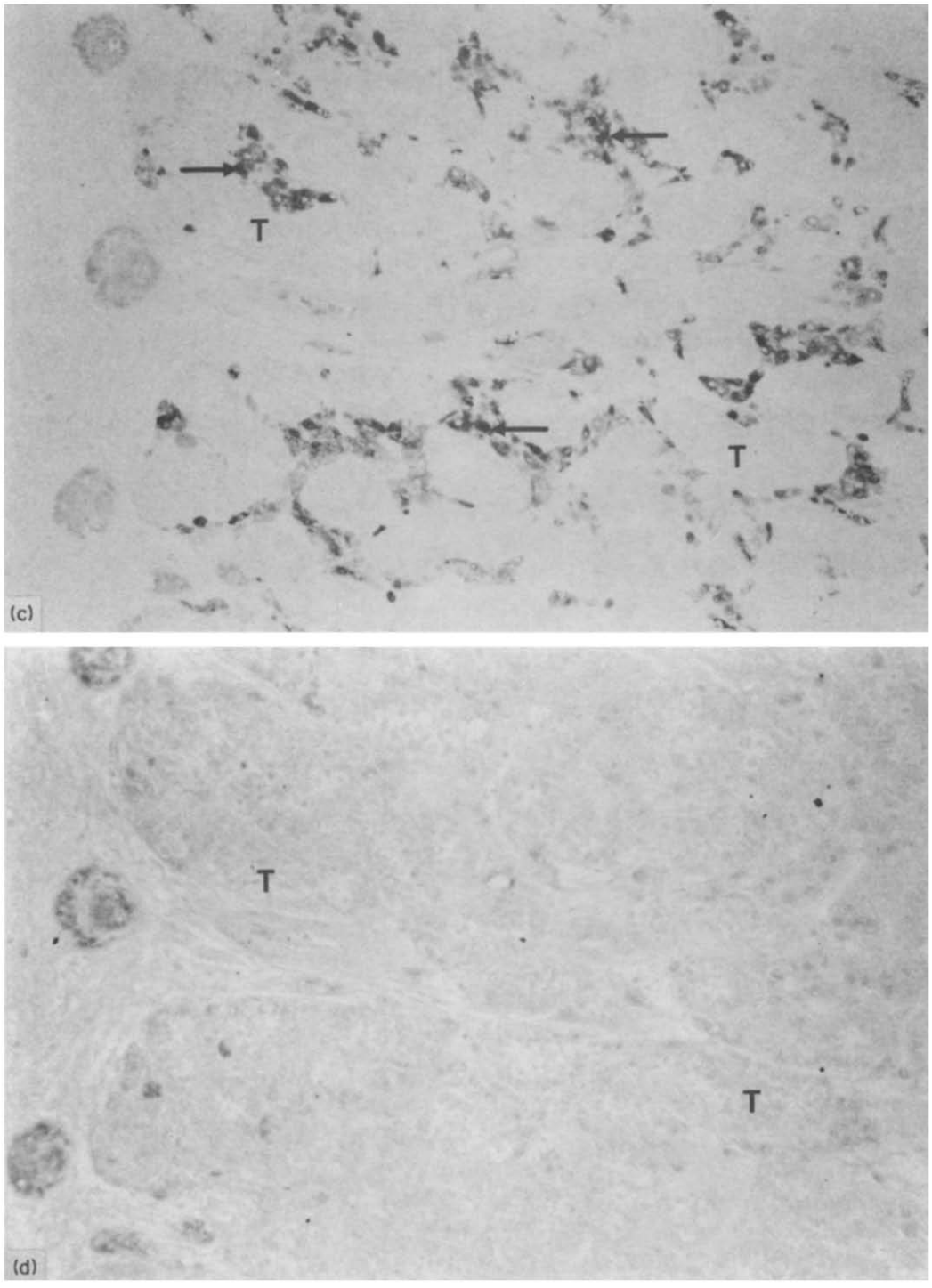

Fig 10(c,d)

Fig 10 Sections through human fetal testes immunostained for 3β-HSD Magnification, ×200 (a) 22 weeks of gestation Immunostaining can be observed in the cytoplasm of interstitial cells (→) The tubules (T) are unstained (b) 28 weeks of gestation Strong immunostaining is present in interstitial cells (→) which appeared more prominent than those observed at 22 weeks of testation [see (a)] T tubules (c) 31 weeks of gestation Heterogeneous intensity of immunostaining of interstitial cells (→) can be observed T tubules (d) Control section adjacent to that shown in (c) Immunoabsorption with excess of human 3β-HSD (10^{-6} M) has completely prevented labeling T tubules [24]

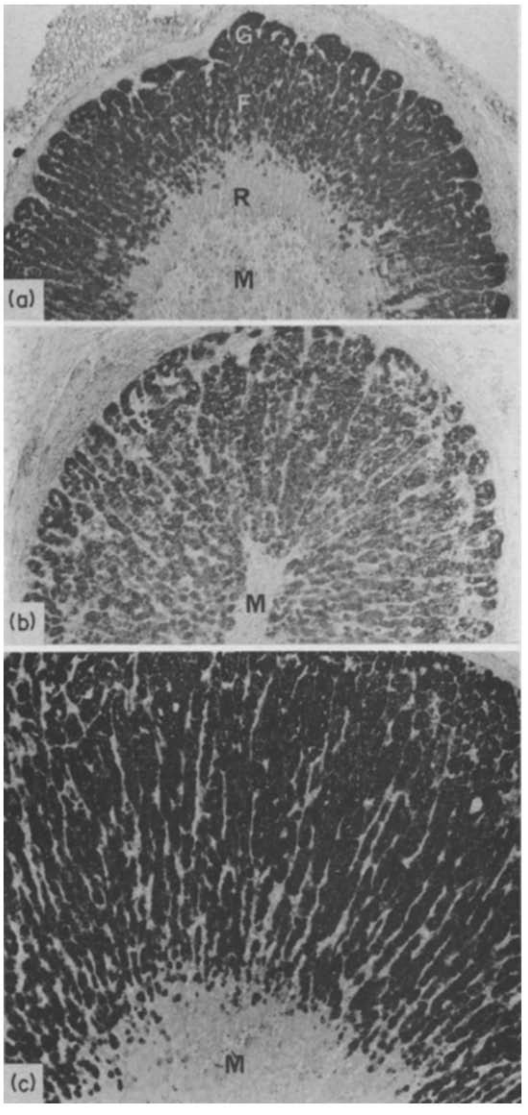

Fig 11 Immunostaining of sections through human fetal adrenal glands (a) The reaction product is observed in the cytoplasm of the secretory cells in the neocortex (N) of adrenal tissue from a 22-week-old fetus (b) In the second trimester of gestation (28 weeks), staining extends to all cells of the neocortex (c) In a mature fetus (35 weeks), immunostaining appears exclusively localized in the cytoplasm of glomerulosa cells (GC) and in some cells differentiated into fasciculata cells Magnification, ×75 [23]

be easily identified Similar 3β-HSD localization was seen in the 8-month adrenals In 2-year-old infants and adults [Fig 12(b and c)], on the other hand, immunostaining was observed in the three layers of the cortex, the medulla being devoid of any reaction Moreover, the intensity of staining was similar in the zona reticularis, fasciculata and glomerulosa At the subcellular level, the labeling was exclusively observed in the cytoplasm [23]

The fetal adrenal gland derives from the dorsal coelomic epithelium At 8 weeks of gestation, the human adrenal comprises chromaffin cells which will later form the adrenal medulla as well as the cortex which is divided into two zones, namely the outer or definitive zone and the inner of fetal zone The outer zone further differentiates into zona glomerulosa, fasciculata and reticularis during the period of infancy and early childhood, while the inner zone which occupies approx 80% of the adrenal volume during fetal life regresses following birth

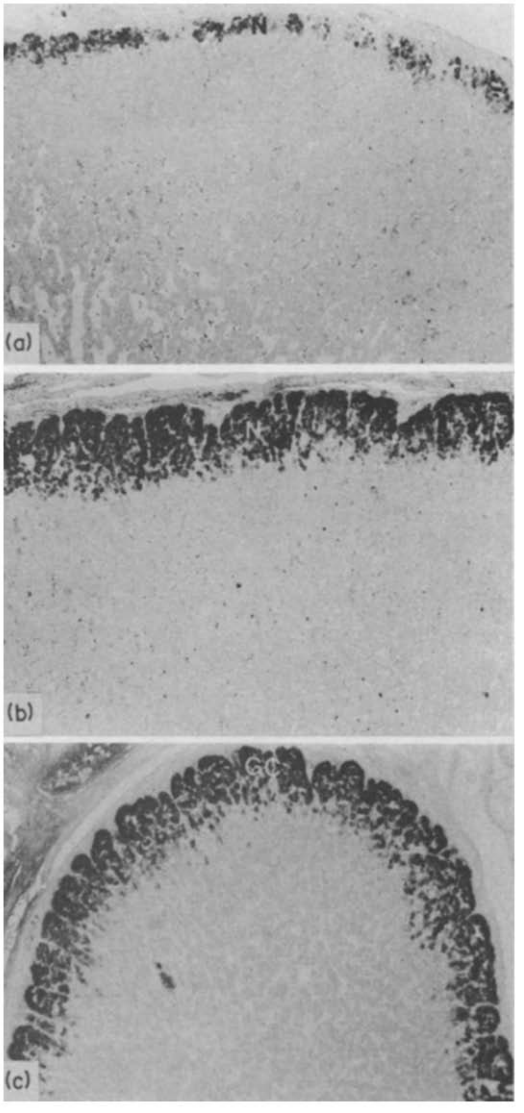

Fig 12 Immunostaining of sections through human adrenal glands (a) 2-month-old infant Immunostaining can be found in the cytoplasm of the secretory cells in the zona glomerulosa (G) and fasciculata (F) No staining is present in the zona reticularis (R) (b) 2-year-old infant Uniform labeling can be observed through the three layers of the adrenal cortex while the medulla is devoid of any reaction (c) Adult adrenal Labeling can be detected in the three cortical zones which are more developed than in the first years of life M, medulla Magnification, ×75 [23]

Contrary to the observation of Murakoshi *et al* [71] who localized 3β-HSD in the inner zone of the human 20-week fetal adrenal by histochemistry, our data clearly identifies 3β-HSD exclusively in the outer zone at this period of fetal life During early to mid-gestation, it is generally thought that 3β-HSD activity in fetal adrenal tissue is minimal [71] and must use placental progesterone as a precursor for the synthesis of cortisol At mid-gestation and approaching term, the present data indicate an increase in 3β-HSD levels [Fig 12(b and c)], a finding which could well explain the increase in circulating cortisol at mid-gestation [72] Thus, the increase in 3β-HSD level in the outer zone of the adrenal cortex could provide the basis for increased cortisol secretion by the fetal adrenal independently from placental transfer of cortisol [73, 74] Such data also suggest that the fetal adrenal is actively involved in progesterone synthesis contrary to the generally accepted view that progesterone is synthesized from precursor pregnenolone by the placental 3β-HSD In fact, 3β-HSD activity is only 3-fold higher in the outer adult-type definitive adrenal tissue compared with that in the fetal cortex [75] Moreover, removal of the fetus in baboons resulted in a 80% decline in serum estradiol concentration and a 70% decrease in serum progesterone [76] The decrease in circulating estrogen presumably reflects removal of estrogen precursors secreted by the fetus while the loss in circulating progesterone might be due, at least in part, to the removal of fetal 3β-HSD or to factors of fetal origin which regulate placental progesterone production [77].

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