A ROUTINE AND IMMUNOCYTOCHEMICAL ELECTRON MICROSCOPIC STUDY OF THE GONADOTROPES IN THE HUMAN FETAL ANTERIOR PITUITARY

by

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ABSTRACT

Anterior pituitaries from 17 human fetuses were obtained immediately after hysterotomies in order to study the morphological development of gonadotropes as a function of age and sex. None of the specimens studied exhibited gross abnormalities nor signs of maceration. Specimens were divided into 3 groups: 2 females and 3 males, 9.5 to 10 weeks gestation; 3 females and 3 males, 11.5 to 13.5 weeks gestation; and 3 females and 3 males, 14.5 to 16.5 weeks gestation. In the youngest specimens, routine electron microscopy revealed numerous undifferentiated cells and relatively few granulated cells, exhibiting moderate numbers of organelles and granules of similar size, density and distribution. The intermediate group of specimens revealed a reduced number of undifferentiated cells and more granulated cells. Moreover, the latter cells contained well developed organelles and granules that displayed some variation in size, density, and distribution. In contrast to the younger groups, the oldest specimens revealed few undifferentiated cells but numerous well developed granulated cells with marked heterogeneity of granule size, density and distribution.

It became apparent that routine electron microscopy alone was inadequate in determining which granulated cells produced the gonadotropins. This was due to the similarities in appearance that all granulated cells had to one another in the youngest specimens as well as to the continuing differentiation these cells displayed during the gestational period studied. Therefore, immunocytochemical electron

microscopy, a more sensitive and specific method, was employed for the identification of gonadotropes following the protocol of Moriarty (1973). These cells contain LH and/or FSH, which have structural similarities, not only with one another, but also with the other glycoprotein hormones.

Accordingly, the LHβ and FSHβ antisera were each investigated using dilution and absorption tests to determine the degree of cross-reactivity with LHβ, LH, FSHβ, FSH, TSHβ, CGβ, and CGα antigens. It was shown that the LHβ and FSHβ antiserum specifically stained and totally cross-reacted with intact LH and FSH, respectively.

Cells containing LH and FSH were identified immunocytochemically in the pituitaries of all the fetuses examined. Sex and age differences were compared by qualitative assessment. In the youngest specimens (9.5 to 10 weeks gestation), the LHβ antiserum stained more cells and more granules per cell than did the FSHβ antiserum. As the age of the specimens increased to 16.5 weeks gestation, the number of cells and granules per cell, which were stained with either LHβ or FSHβ antiserum, also increased. In addition, the staining intensity of each antisera increased as a function of fetal age. A sex difference was also evident, in that pituitaries of older female specimens revealed more granules per cell and more cells stained with either LHβ or FSHβ antisera than those of males of comparable ages. Serially sectioned cells were found to contain granules immunostained with both LHβ and FSHβ antisera. This revealed the presence of both gonadotropins in the same cell.

The present study also clearly demonstrated the need for careful control experiments dealing with the cross-reactivity of antisera against intact LH and FSH with both the alpha and beta subunits of these

hormones. Although earlier studies using antisera against intact LH or FSH have detected the presence of gonadotropes as early as eight weeks gestation, the possibility of cross-reactivity with the alpha subunit was not excluded. Moreover, in these studies, which were all performed at the light microscopic level, when beta antisera were used, gonadotropes were not stained until 16 weeks gestation. In marked contrast to the above, the present investigation not only used both beta antisera which were shown not to cross-react with the alpha subunit, but also demonstrated the presence of gonadotropes as early as 9.5 weeks gestation by using the higher resolution of electron microscopy which is necessary to detect low levels of hormone during the early developmental stages of the human fetal pituitary.

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INTRODUCTION

Information concerning routine ultrastructural morphology of the adult mammalian anterior pituitary is rather extensive (see reviews by Rinehart and Farquhar, 1953; Herlant, 1964; Tixier-Vidal and Farquhar, 1975). There is also considerable information pertaining to the routine ultrastructural morphology of the human fetal pituitary (Dubois and Dumont, 1965, 1966; Dumont and Dubois, 1967; Dubois, 1968; Anderson et al., 1970, 1971; Satow et al., 1972), and that of the fetuses of a number of other mammals (Sano and Sasaki, 1969; Fink and Smith, 1971; Daikoku et al., 1973, 1976; Svalander, 1974).

Numerous studies have utilized either light microscopic immunohistochemistry or electron microscopic immunocytochemistry for identification of specific hormone producing cells in the anterior pituitary of adult (see reviews by Moriarty, 1973, 1976; Tougard et al., 1980), and fetal laboratory animals (Sétáló and Nakane, 1976; Thompson and Trimble, 1976; Chatelain et al., 1979; Gross and Baker, 1979; Watanabe and Daikoku, 1979). In addition, immunohistochemical light microscopic investigations have yielded abundant information in regard to the initial production and localization of pituitary hormones in the developing human (Dubois et al., 1973, 1975, 1978; Bugnon et al., 1974, 1976a,b, 1977; Dubois and Dubois, 1974; Baker and Jaffe, 1975). However, the only immunocytochemical studies at the ultrastructural level of the

human fetal pituitary reported are those of Li et al., (1977, 1979b).

Other studies have measured by radioimmunoassay, hormone content and concentration in the human fetal pituitary (Reyes et al., 1974; Clements et al., 1976; Kaplan et al., 1976) and have detected the presence of gonadotropins earlier in development than that reported in the aforementioned immunohistochemical light microscopic studies.

The objectives of this investigation were, therefore:

1. To describe the early development of the gonadotropes in the human fetal pituitary, using routine and immunocytochemical electron microscopy, and

2. To correlate the above mentioned morphological and immunocytochemical findings with other reported data on the endocrinological development of the human fetal hypothalamo-pituitary-gonadal axis.

LITERATURE REVIEW

In the human embryo, the anlage of the pituitary first becomes recognizable during the middle of the third week of gestation. A diverticulum referred to as Rathke's pouch, arises from the roof of the stomodeum and grows towards the diencephalon (Moore, 1977). By the fifth week, Rathke's pouch has elongated, and made contact with the infundibulum, i.e., the developing posterior lobe of the pituitary, and becomes constricted at its attachment to the stomodeum (Falin, 1961).

During the sixth week, Rathke's pouch becomes separated from the oral cavity (Moore, 1977). Subsequently, cells of the anterior wall of Rathke's pouch give rise to the pars distalis, or anterior lobe of the pituitary. Later a small extension of the anterior lobe forms the pars tuberalis around the infundibular stem. The posterior wall of Rathke's pouch gives rise to the ill-defined pars intermedia (Patten, 1968; Hamilton, Boyd and Mossman, 1972). By the eighteenth week of development, the anterior pituitary consists of a large number of small groups of glandular cells evenly dispersed throughout its extent. At this time, the anterior pituitary resembles the adult organ in its structure and arrangement of cells (Falin, 1961).

Early investigations of the human fetal pituitary were primarily concerned with developmental morphology (Atwell, 1926; Covell, 1926-7; Tilney, 1936). However, as interest in the cellular differentiation of the human fetal pituitary developed, three cell types, chromophobes,

acidophils, and basophils, were distinguished (Halpern, 1938). Acidophilic cells have been observed between eight and 12 weeks gestational age by various investigators (Pearse, 1953; Daikoku, 1958; Falin, 1961; Ellis et al., 1966; Conklin, 1968; Pavlova et al., 1968). On the other hand, basophils (PAS-positive cells) have been detected as early as seven weeks (Conklin, 1968); however, others have reported their initial occurrence at either eight weeks (Falin, 1961; Mitskevich and Levina, 1965; Pavlova et al., 1968) or 12 weeks gestation (Pearse, 1953). These discrepancies in the first appearance of acidophils and/or basophils were probably due to the technique used and identifying criteria of cell types.

Using tinctorial histochemistry, Mitskevich and Levina (1965) reported the detection of anterior pituitary cells containing LH in female specimens of 18 to 28 weeks gestational age but not in male specimens. Conklin (1968) was able to detect the first appearance of seven cell types and make assessments as to the hormone secreted by each cell type as follows: type III (ACTH) at seven weeks, type I (STH) at 11 weeks, type IV (TSH) at 12 weeks, type II (LTH) at 19 weeks, type V (LH) at 22 weeks, type VI (FSH) at 28 weeks, and type IX (?) at 32 weeks gestational age. Pavlova et al. (1968) were able to tentatively identify cells containing ACTH and STH, each first appearing at eight and 12 weeks of age respectively, while assayable amounts of ACTH and STH were detectable at nine weeks of age.

Using routine electron microscopy, Dubois and Dumont (1965) examined the anterior pituitaries of five human fetuses between 8.5 and

9.5 cm crown-rump length, and described four cell types, of which two were considered to be producing the same hormone (STH); one type in the synthesis phase, and the other in the storage phase.

Other ultrastructural studies revealed changes in the distribution of cell types as a function of age (Dubois and Dumont, 1966; Dumont and Dubois, 1967; Dubois, 1968). These studies described the anterior pituitaries of 7.5 and nine week old specimens as having many undifferentiated cells, and few granulated cells which were identified as STH cells. A 19 week specimen, in the above mentioned studied, was reported to have few undifferentiated cells and many granulated cells of several distinct types. However, the hormones produced by these differentiated cell types could not be determined with any certainty. Later studies by Dubois (1971a,b, 1972) of the human fetal pituitary described glycogen deposits in undifferentiatd cells, development of the Golgi apparatus in differentiating cells, and the maturation of capillaries.

Studies of Anderson et al. (1970, 1971) described the ultrastructural and histochemical observations of cell types in the anterior pituitaries of early to mid-gestation human fetuses. Although several granulated cell types were noted, these authors declined to identify them because of the changing morphology in the fetal pituitary during development and differences in identifying criteria between species.

Satow et al. (1972) identified cell types in the pituitaries of five normal human fetuses, using criteria, i.e., disposition of organelles and inclusions, established for human adult and other species, namely the rat and cow. By electron microscopy the pituitary of a three month female fetus revealed 41% STH cells, 10% TSH cells, 11% gonadotropes, 1% ACTH cells and 37% as unclassified cells. Light microscopic

observations of the total cell population revealed that 15-20% of the cells contained granules. STH cells were described as having large dense granules, 200-300 nm in diameter, distributed throughout the cytoplasm, and well developed rough endoplasmic reticulum. TSH cells were tentatively identified as having small polygonal-shaped cell bodies and containing few granules measuring 100-150 nm in diameter. Gonadotropes were classified as having uniformly-shaped dense granules, 100-150 nm in diameter throughout the cytoplasm and well developed rough endoplasmic reticulum. ACTH cells were identified as having cored vesicles or granules 100-150 nm in diamter, and small vesicular endoplasmic reticulum.

Even though the above mentioned investigations described and attempted to identify specific cell types and their hormone(s) produced, positive identification of the cell types of the human fetal pituitary by morphological comparison with the human adult as well as fetal and adult pituitaries of other species is at best only tentative. A more precise method used to resolve these discrepancies has been found with the introduction of immunohistochemical techniques for localization of tissue antigens. Fluorescent dyes chemically conjugated to specific antibodies were first used to detect tissue antigens (Coons, 1958). Subsequently, improved techniques utilized enzymes, such as horseradish peroxidase, conjugated to immunoglobulins for more sensitive localization of tissue antigens at both light and electron microscopic levels (Nakane and Pierce, 1966; 1967).

Immunofluorescent staining of the human fetal pituitary was first reported by Ellis et al. (1966) and subsequently by Porteous et al. (1968). Cells containing growth hormone were identified in specimens 12

weeks of age to birth, but not in specimens between eight and 11 weeks fetal age. Somatotropes have been initially identified at various ages, from nine to 14 weeks using light microscopic immunohistochemistry by Baker and Jaffe (1975), Bugnon et al. (1976b), Osamura (1977) and Cho et al. (1978).

In the human fetus, mammotropes, or prolactin containing cells, have been detected immunohistochemically as early as 10 to 16 weeks gestational age by Bugnon et al. (1974), Osamura (1977) and Cho et al. (1978).

Positive staining with 1-24-ACTH antiserum was reported in pituitaries of human fetal specimens ranging from eight to 10.5 weeks gestation (Bugnon et al. 1974; 1976b; Baker and Jaffe, 1975). On the other hand, 17-39 ACTH antiserum was reported to stain pituitaries somewhat sooner, at seven to eight weeks (Dubois et al., 1973; Baker and Jaffe, 1975; Bugnon et al., 1976a,b; Begeot et al., 1978). The earliest appearance of immunostained ACTH in the human fetal anterior pituitary was reported to be at five weeks fetal age (Osamura, 1977). MSH was detected in the anterior pituitary of a 14 week human fetus (Baker and Jaffe, 1975), while β MSH was detected at eight weeks (Bugnon et al., 1976b) and 13 weeks fetal age (Osamura, 1977). In addition, corticotropin and β MSH like-material have been reported to be in the same cells in pituitaries of older human fetal specimens (Dubois et al., 1973).

β LPH was reported to be immunostained in anterior pituitaries of eight to 10 week old human fetuses (Bugnon et al., 1974; Begeot et al., 1978). In addition, α and β endorphin were detected immunohistochemically in anterior pituitaries of human fetuses, eight weeks of gestational age by Begeot et al. (1978). Morever, these authors reported the presence of corticotropin, β LPH and α and β endorphin binding in the in the same cells of the pituitary of an 18 week specimen.

In contrast to the above mentioned cell types which appeared early in gestation, thyrotropes were reported not to be present until either 13 weeks (Baker and Jaffe, 1975; Bugnon et al., 1976a,b, 1977; Osamura, 1977), 14 weeks (Dubois and Dubois, 1974) or 15 weeks of gestational age (Dubois et al., 1978).

Dubois and Dubois (1974) and Dubois et al. (1975, 1978) used the indirect antibody method at the light microscopic level to investigate gonadotropes in the human fetal pituitary. As early as eight weeks of fetal age, hCG antiserum and ovine LH antiserum revealed positive reactions; however, neither porcine LHβ antiserum nor ovine LHβ antiserum produced a staining reaction before 16 weeks in female specimens and 20 weeks in male specimens. The stain observed after 16 and 20 weeks was interpreted as being due to the presence of LHβ subunits, while between eight and 16 weeks fetal age, the stain was due to the presence of only α subunits. The α subunit was detected, from the eighth week onwards without sex differences, whereas intact LH was detected during the third month, and sex differences were observed as early as the fourth and fifth months of gestation. Cells immunoreactive with hFSHβ antiserum were detected in female fetuses at the beginning of the fourth month and at 18 weeks were more numerous in female than male specimens (Dubois et al., 1975, 1978). Baker and Jaffe (1975) also reported the differential appearances of gonadotropes (LH cells) in the human fetal pituitary, depending upon the antiserum used. hCG antiserum immunostained gonadotropes first at 10.5 weeks, while bovine LHβ antiserum stained gonadotropes initially at 23 weeks gestation.

Bugnon et al. (1976a,b, 1977) reported the detection of α subunits initially at eight weeks gestation, and LHβ and FSHβ at 15 weeks gestation. Immunostainable gonadotropes have also been reported in pituitaries of human fetuses 13 weeks (Osamura, 1977) and 12 weeks gestational age (Cho et al., 1978). In addition to the above findings, Dubois et al. (1975, 1978) and Bugnon et al. (1976a,b, 1977), noted that both LH and FSH were contained in the same cell.

In regards to distribution of gonadotropes in the human fetal pituitary, Baker and Jaffe (1975) noted these cells throughout the anterior lobe, with an apparent concentration at the posterolateral border and in the anteromedian zone. On the other hand, other investigators described the distribution of these cells to be in the posteromedian zone and on the superior portion of the anterior lobe of the pituitary (Cho et al., 1978).

The apparent differences in the initial appearance of the various cell types noted in the foregoing immunohistochemical studies probably resulted from variations in technical procedures employed, such as 1) the highly sensitive peroxidase labeled antibodies versus fluorescence labeled antibodies, 2) the indirect labeled antibody method versus the less sensitive direct labeled antibody method, 3) mode of abortion and hence the integrity of the pituitary from the specimens, 4) fixation and subsequent dehydration and embedding of the tissue, 5) use of antibodies against intact glycoprotein hormone or β subunit, and 6) use of antibodies against glycoprotein hormone of species other than human.

In contrast to the aforementioned studies which were confined to the light microscopic level, there have been remarkably few studies utilizing immunocytochemical electron microscopy for identifying specific cell types in the human fetal pituitary. Li et al. (1977) reported the presence of somatotropes in fetuses ranging in age from eight to 19 weeks gestation. The cytoplasm of these cells contained well developed organelles and inclusions. Localization of growth hormone was confined to the secretory granules, which had a mean diameter of 300 nm. These authors also stated that during early development somatotropes were less numerous but by 12 weeks the average diameter of the granules was the same as at 19 weeks gestation. In a subsequent study, Li et al. (1979b) reported cells immunoreactive with α (17-39)ACTH antiserum, β (1-24)ACTH antiserum, β LPH antiserum, α endorphin antiserum, and β endorphin antiserum. However, all the aforementioned antisera immunostained one definitive cell type.

These studies of Li et al. (1977; 1979b) on the human fetal pituitary clearly demonstrated the advantage of immunocytochemistry at the ultrastructural level over that of light microscopic techniques to localize hormones to specific sub-cellular structures. In light of this fact, the present study was undertaken: 1) to clarify the discrepancies eluded to previously regarding the onset of hormone production, in particular the gonadotropins as studied by light microscopic immunohistochemistry, and 2) to correlate the findings with radioimmunoassay data regarding onset of as well as sex differences in LH and FSH production.

MATERIALS AND METHODS

SPECIMENS

Human fetuses were obtained immediately after hysterotomies which were performed for gynecosocial indications on otherwise healthy women[1]. A total of 17 fetuses ranging from 44 to 144 mm crown-rump (C.R.) length were used in this study and in no instance was the presence of gross abnormalities or maceration detected. Gestational (fertilization) age was determined from the C.R. length, using the plot of Patten (1968), Figure VII-3. Neither cord pulsation nor palpable cardiac activity, were detectable prior to the removal of the pituitary and gonads.

LIGHT MICROSCOPY

The gonads were fixed for several days in 10% neutral buffered formalin, dehydrated, embedded in paraffin, sectioned at 6 μm and subsequently stained with hematoxylin and eosin. The gonads were examined under a light microscope to determine the sex of each specimen.

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[1]Approval for the use of specimens for this study was obtained from the Faculty of Medicine Committee on the Use of Human Subjects in Research, University of Manitoba.

ELECTRON MICROSCOPY

Fixation

Each pituitary was divided sagittally into equal halves, and fixed for 2 hours at 4°C in 1% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4). Tissues were rinsed and stored overnight in 5% sucrose in 0.1 M Sorensen's phosphate buffer (pH 7.4). For routine electron microscopy, half of each pituitary was post-fixed for 2 hours at 4°C in 1% osmium tetroxide (OsO_4) in 0.1 M Sorensen's phosphate buffer (pH 7.4). For immunocytochemical electron microscopy, the remaining half of each pituitary was left in 5% sucrose in 0.1 M Sorensen's phosphate buffer wash (pH 7.4) and not post-fixed with osmium tetroxide.

Dehydration and Embedding

For both routine and immunocytochemical electron microscopy, tissues were rapidly dehydrated through ascending concentrations of ethanol. Following dehydration, the tissues were embedded in Epon 812 according to the method of Luft (1961).

Sectioning

Ultra-thin sections were cut on a Sorval Porter-Blum ultra-microtome, using a DuPont diamond knife. Sections of approximately 90-100 nm were lifted onto 300 mesh copper grids for routine electron microscopy or nickel grids for immunocytochemical electron microscopy.

Staining

Routine Electron Microscopy

For routine electron microscopy, tissue sections were stained first with a saturated aqueous solution of uranyl acetate for 30 minutes (Luft, 1961) and then with lead citrate for 3 minutes (Reynolds, 1963).

Immunocytochemical Electron Microscopy

Antisera, Antigens, and Reagents

The following antisera, prepared in rabbit, and antigens used in this study were obtained from the National Pituitary Agency, National Institute of Arthritis, Metabolism and Digestive Diseases: antiserum to the beta subunit of human LH (Batch #1), antiserum to the beta subunit of human FSH (Batch #1), beta subunit of human FSH (Batch #2), beta subunit of human TSH (N-785-B); all the aforementioned were prepared by A.F. Parlow; beta subunit of human LH (LER 1793B); beta subunit of hCG CR-115, alpha subunit of hCG (Center for Population Research; and human LH (LER 960). In addition, human FSH (S1191B) was obtained from M.R. Sairam, Clinical Research Institute of Montreal.

Goat antirabbit IgG and the "PAP" (peroxidase-rabbit antiperoxidase complex) were obtained from Cappel Laboratories and Sternberger-Meyer Immunocytochemical Inc., respectively. The "DAB" (3,3' diaminobenzidine tetrahydrochloride) was obtained from Polysciences Inc.

Antisera, antigens, and other staining reagents were stored in small aliquots (20 to 50 µl) in polyethylene microcentrifuge tubes (400 µl capacity) at -70°C.

Immunocytochemical Staining Procedure

For immunocytochemical electron microscopy, sections were stained according to the protocol of Moriarty (1973) with minor modifications. See Appendix II for the preparation of wash, and diluent buffers and the diaminobenzidine tetrahydrochloride-hydrogen peroxide (DAB-H_2O_2) solution used in the following staining procedure.

1. Alcoholic sodium hydroxide, 1%, 1 minute.
2. H_2O wash.
3. Normal goat serum 1/100 dilution, 3 minutes.
4. Primary antiserum, 48 hours at $4^{o}C$ in Beem capsules.
5. 0.05 M phosphate buffer wash.
6. Store (until ready) on 0.05 M phosphate buffer drops.
7. Normal goat serum, 1/100 dilution, 3 minutes.
8. Goat antirabbit IgG, 1/50 dilution, 3 minutes.
9. 0.05 M phosphate buffer wash.
10. Normal goat serum, 1/100 dilution, 3 minutes.
11. "PAP" peroxidase-antiperoxidase, 1/40 dilution, 3 minutes.
12. 0.05 M phosphate buffer wash.
13. Store (until ready) on 0.05 M phosphate buffer drops.
14. DAB-H_2O_2, 3 minutes.
15. H_2O wash, 3 minutes.
16. Osmium tetroxide (OsO_4) 4%, 10 minutes.
17. H_2O wash.
18. Dry on filter paper and store in grid box.

Figure 1 illustrates the sequential addition of the antibodies and reagents to the tissue section in the staining procedure.

Controls

For method and specificity controls, thin sections were cut from the pituitary obtained from a 144 mm (C.R. length) female specimen.

Method Controls--Dilution of Antisera. Various dilutions of each antiserum were tested to determine the working dilution. For the primary antisera, LHβ and FSHβ antisera, and normal rabbit serum (Figure 1, step 1), the dilutions ranged from 1/500 to $1/10^6$. For the goat antirabbit IgG (Figure 1, step 2) and peroxidase-antiperoxidase (Figure 1, step 3), the dilutions ranged from 1/10 to 1/3000 and 1/4 to 1/1200, respectively.

Specificity Controls--Absorptions of the Primary Antisera. Prior to the primary staining step (Figure 1, step 1) the LHβ antiserum, at its working dilution, was absorbed for 48 hours at 4°C with varying amounts (10 pg/ml to 10 μg/ml) of its homologous antigen, LHβ; with heterologous antigens, FSHβ, TSHβ, CGβ and CGα, and also with intact LH and FSH.

Similar absorptions were performed for the FSHβ antiserum.

Substitution and Omission Control. In each immunocytochemical staining procedure, additional grids were always included, in which the primary antiserum (Figure 1, step 1) was either omitted or substituted with normal rabbit serum at the same concentration as the primary antiserum.

Photography

Grids were viewed and sections photographed using a Philips EM 201 electron microscope. For quantification of the immunocytochemical staining intensity, all exposures were taken at the same magnification (X1,325) and enlarged (X7.5) to obtain micrographs at a final magnification of X10,000. For routine and immunocytochemical electron microscopic examination of the specimens, exposures were taken, and micrographs printed at either X5,000, X10,000, or X25,000.

Densitometry

Quantification of staining intensity was achieved by comparing the density of the granules (specifically, the reaction product over the granules) with the adjacent cytoplasm, using a Macbeth TD502 densitometer. The normalized staining intensity (N.S.I.)(Moriarty et al., 1973) was determined by expressing the optical density of the granule (ODg) as a percentage increase from the optical density of the adjacent cytoplasm (ODc) by using the formula

$$\frac{ODg}{ODc} - 1 = N.S.I.$$

Statistical Analysis

Means and standard error of the means were determined for the normalized staining intensities of observed granules at each dilution of LHβ antiserum, FSHβ antiserum and normal rabbit serum.

Similarly, for each absorption test, the means and standard error of the means of the normalized staining intensities were determined for

stained granules at each concentration of antigen (0; 10 pg/ml to 10 μg/ml) present in the LHβ and FSHβ antisera.

In addition, for each absorption test where the normalized staining intensity appeared to decrease, the absorption curve was divided into an "initial" and "final" part. The "initial" portion of the curve was where no decrease in normalized staining intensity was apparent. The "final" portion was where the normalized staining intensity decreased. The absorption tests that appeared to have no decrease in the normalized staining intensity were given only an "initial" portion.

For each absorption test, the means of the normalized staining intensities were used for linear regression analysis in order to obtain the "initial" regression line, and in some absorptions, also the "final" regression line.

In the absorptions where there was an apparent decrease in normalized staining intensities, the "initial" and "final" regression lines were compared, using Welch's approximate t-test, to determine if their slopes were different.

Finally, using Welch's approximate t-test, the slope of the "final" regression line of each homologous absorption was compared to the slope of the "final" regression line of the other antigens which decreased the normalized staining intensities for each antiserum.

Assessment of Gonadotrope Development

Differences due to sex and age of the specimens and the primary antiserum used were determined by qualitative comparison of micrographs at fixed magnifications. The parameters studied were the number of stained granules per cell, the number of cells containing stained granules, and the staining intensity of the granules.

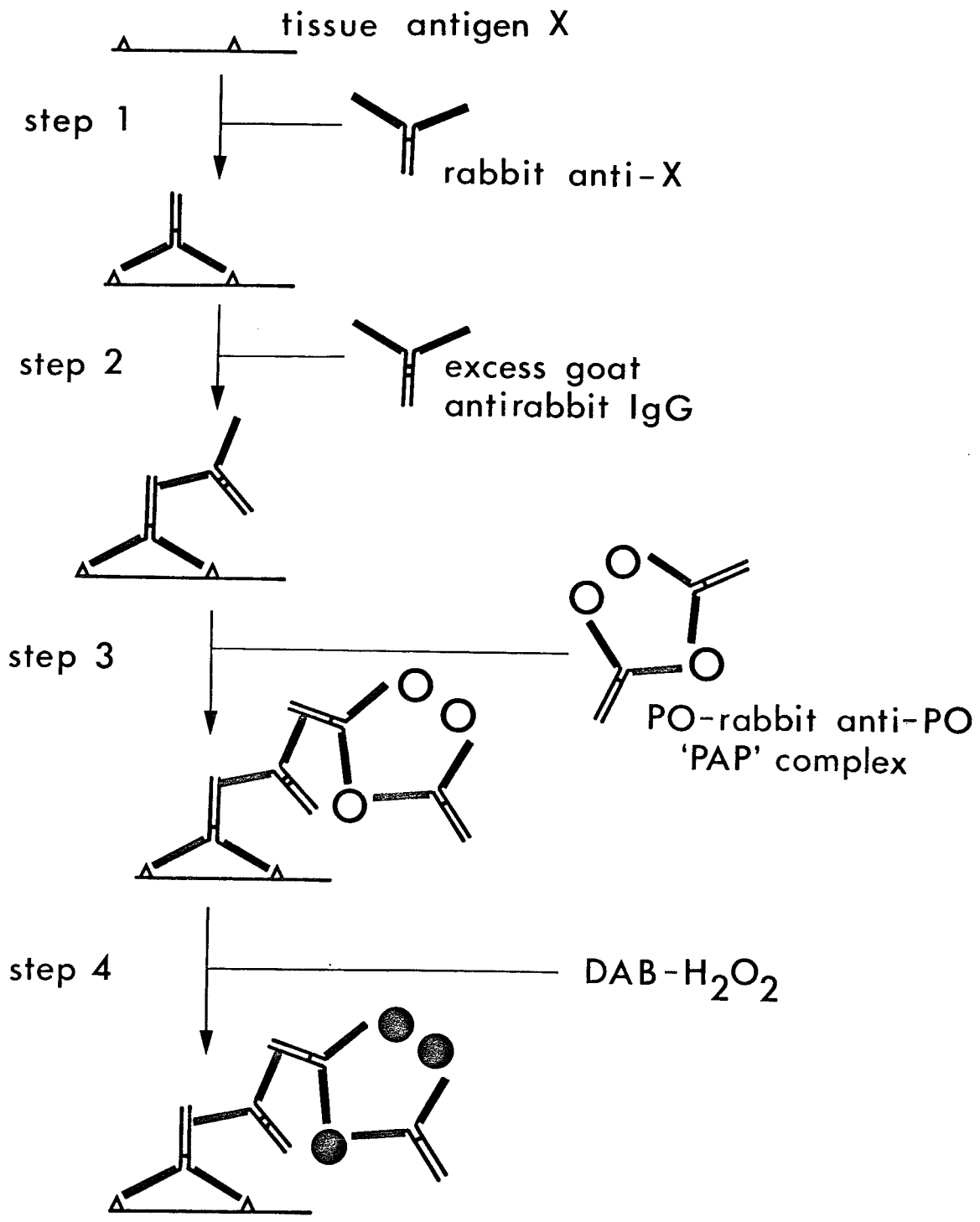
Figure 1

Schematic diagram of the immunocytochemical staining procedure. Tissue antigen X is exposed by etching with alcoholic NaOH. Rabbit anti-X (primary antiserum) binds to the exposed antigen X (step 1).

Excess goat antirabbit IgG binds to constant fragment (Fc) of the rabbit anti-X (step 2).

Three peroxidase enzymes (circles) conjugated with two rabbit anti-peroxidase are added as a "PAP" complex (PO-rabbit anti-PO; step 3). The goat antirabbit IgG (of step 2) binds the constant fragment (Fc) of the rabbit anti-peroxidase.

When diaminobenzidine tetrahydrochloride-hydrogen peroxide solution (DAB-H_2O_2) is added (step 4), a reaction product is formed over the peroxidase enzymes, and subsequently blacked with osmium tetroxide for electron microscopic visualization.


tissue antigen X
step 1
rabbit anti-X
step 2
excess goat
antirabbit IgG
step 3
PO-rabbit anti-PO
'PAP' complex
step 4
DAB-H_2O_2

RESULTS

SPECIMENS

The sex, determined by routine light microscopic evaluation of the gonads, crown-rump (C.R.) length, and estimated gestational age of each fetus used in this study are presented in Table 1.

Figure 2 illustrates the relationships of the pituitary and surrounding structures of a 70 mm C.R. length (11.5 weeks gestation) specimen.

ROUTINE ELECTRON MICROSCOPY

Human fetal anterior pituitaries examined were found to consist of granulated and non-granulated cells. The non-granulated cells were divided into stem or undifferentiated cells and supporting or follicular cells.

In anterior pituitaries of the youngest specimens (44 mm to 53 mm C.R. length), the stem cells and follicular cells (Figures 3a and 4a) were more abundant than the granulated cells (Figures 3b and 4b).

The stem cells were spherical in shape (Figures 3 and 4) and possessed a thin rim of cytoplasm containing few organelles. Follicular cells were stellate, small, with a dark cytoplasm containing few organelles and glycogen deposits (Figures 3 and 4). Long processes of follicular cells could be traced between the stem cells and granulated cells (Figures 3b and 4b). The granulated cells in the

pituitaries of the youngest specimens showed a homogeneity in size, shape and cytoplasmic structures. Most granulated cells were round to oval in shape, with abundant cytoplasm containing numerous mitochondria, stacks or single profiles of rough endoplasmic reticulum, Golgi apparatus and granules. Nuclei were round to oval in shape with a prominent nucleolus. Granules were usually observed along the cell membrane and were of a similar density and size (100 nm to 300 nm in diameter). The morphological characteristics of stem cells, follicular cells, and granulated cells did not appear to be altered according to the sex of the specimen.

Examination of anterior pituitaries from specimens ranging in C.R. length from 69 mm to 98 mm revealed more granulated cells than observed in the youngest specimens. In addition, the granulated cells were more variable in size, shape and content (Figures 5 and 6). Stem cells and follicular cells (Figures 5a and 6a) were morphologically similar to those observed in the youngest specimens studied; however, the stem cells did not appear as abundant as in the youngest specimens.

In anterior pituitaries of the oldest specimens studied (118 mm to 144 mm C.R. length), granulated cells were the most numerous (Figures 7 and 8). Stem cells and follicular cells were morphologically similar to those observed in younger specimens. With increased fetal age the processes of the follicular cells were more attenuated (Figures 7a and 8b) and stem cells appeared to decrease in number. Granulated cells displayed marked variability in shape, cytoplasmic content and

granule size and density. Distinct cell types were easily recognized. Some cells had small granules (100 to 300 nm in diameter) throughout the cytoplasm (Figures 7a and 8a), while other cells had larger granules (up to 750 nm in diameter) throughout the cytoplasm or located peripherally. Many granulated cells had an oval to oblong shaped nucleus with a prominent nucleolus, stacks of rough endoplasmic reticulum, occasionally dilated, and round or oblong mitochondria. No differences in stem cells or follicular cells were apparent due to the sex of the specimens; however, granulated cells of female specimens appeared to contain more dilated endoplasmic reticulum and more granules per cell than did the male specimens.

IMMUNOCYTOCHEMICAL ELECTRON MICROSCOPY

Controls

Method Controls--Dilution of Antisera. Serial dilutions of the primary antisera revealed a progressive decrease in the normalized staining intensity to undetectable levels (Figure 9). In addition, normal rabbit serum at high concentrations (1/500 - 1/1000) also showed a staining reaction over most granules, whereas at low concentrations (1/10K), staining was not evident.

The LHβ antiserum produced the most intense staining, detectable over numerous granules in most cells on the sections at a dilution of 1/500. At a lower concentration, i.e. 1/10K, most cells on the sections contained unstained granules, whereas a few cells contained

moderate to lightly stained granules. At a dilution of 1/30K, staining was not evident. Staining intensity of the FSHβ antiserum was similar to that noted for the LHβ antiserum; however, complete abolition of staining was not achieved until a dilution of 1/100K was used.

As a result of the above findings, a working dilution of 1/10,000 for both the LHβ and FSHβ antisera was chosen for subsequent testing.

Serial dilutions of goat anti-rabbit IgG and the peroxidase-antiperoxidase serum revealed that optimal staining was achieved using dilutions of 1/50 and 1/40, respectively. Higher dilutions of either serum resulted in a weak or undetectable stain.

Specificity Controls--Absorptions of the Primary Antisera. Comparison of the zero points, where homologous, heterologous and intact antigens were omitted, revealed some variations in the means and standard errors of the means of the normalized staining intensities, i.e., .23 to .32 for LHβ antiserum and .17 to .28 for the FSHβ antiserum (Figure 10 and 11, respectively).

Examination of the correlation coefficients of normalized staining intensities on antigen concentration for each of the "initial" regression lines revealed significances only for LHβ antiserum absorbed with CGα ($P<.05$, Figure 10g) and for the FSHβ antiserum absorbed with LH ($P<.001$, Figure 11d)(Table 2). In addition, the correlation coefficients for the "final" regression lines revealed significance for LHβ antiserum absorbed with LH ($P<.05$, Figure 10b), FSHβ ($P<.001$, Figure 10c) and TSHβ ($P<.05$, Figure 10e), and FSHβ antiserum absorbed with FSH ($P<.05$, Figure 11b) and LHβ ($P<.05$, Figure 11c)(Table 2).

Although there was no significant correlation ($P<.05$) with some of the data (Table 2), when the "initial" regression lines were compared to the "final" regression lines that best fit the data, significant decreases in the normalized staining intensities for some absorption tests were revealed. The following differences in the slopes of the regression lines in the LHβ antiserum absorptions were observed: (1) the "initial" and "final" regression lines of the LHβ antigen (Figure 10a) were different ($P<.01$); (2) the "initial" and "final" regression lines of the LH antigen (Figure 10b) were different ($P<.005$); (3) the "initial" and "final" regression lines of FSHβ antigen (Figure 10c) were different ($P<.001$); (4) the "initial" and "final" regression lines of CGβ antigen (Figure 10f) were different ($P<.005$)(Table 3). The following differences in the slopes of the regression lines in the FSHβ antiserum absorptions were revealed: (1) the "initial" and "final" regression lines of FSHβ antigen (Figure 11a) were different ($P<.05$); and (2) the "initial" and "final" regression lines of the FSH antigen (Figure 11b) were different ($P<.005$)(Table 3).

In addition, comparison of the slope of the "final" regression line of the homologous absorption test with the slope of the "final" regression line of the other absorptions of LHβ antiserum revealed that the following antigens had slopes different from LHβ antigen (Figure 10a): (1) FSH antigen (Figure 10d, $P<.05$); (2) TSHβ antigen (Figure 10e, $P<.005$); (3) CGβ antigen (Figure 10f, $P<.05$)(Table 4). The other "final" regression lines of the LHβ antiserum and those of the FSHβ antiserum were not significantly different from that of the homologous absorptions (Table 4).

The homologous absorptions (Figures 10a and 11a) and the absorption of the LHβ antiserum with intact LH (Figure 10b) required the least amount of antigen to bind to the antibodies and significantly decrease the

staining to an undetectable level, findings which are in contrast to those observed in the other absorptions. When FSH antigen was added to the FSHβ antiserum (Figure 11b), normalized staining intensities were also reduced to undetectable levels, but in order to achieve this approximately 10 times the concentration of the intact hormone over that of the homologous antigen was required. When FSHβ antigen was added to LHβ antiserum (Figure 10c) and LHβ antigen to FSHβ antiserum (Figure 11c), normalized staining intensities were reduced to undetectable levels. However, in order to achieve this, approximately 100 times the concentration of the heterologous antigens were required. In other words, the LHβ antiserum was approximately 100 times more sensitive to the LHβ and LH antigens and FSHβ antiserum was approximately 10 and 100 times more sensitive to FSH and FSHβ antigens, respectively, than to any of the other antigens tested.

There were insignificant decreases in normalized staining intensities in the absorptions of LHβ antiserum with FSH and TSHβ antigens (Figures 10d and e). However, CGβ antigen significantly decreased the normalized staining intensities of the LHβ antiserum (Figure 10f). A slight decrease in normalized staining intensity was evident in the absorption of LHβ antiserum by CGα antigen (Figure 10g) and FSHβ antiserum with LH antigen (Figure 11d), as revealed by the significant correlation coefficients (Table 2). No decrease in normalized staining intensity was evident in the absorptions of FSHβ antiserum with TSHβ, or CGβ antigens (Figures 11e and f). In one instance, when FSHβ antiserum was absorbed with CGα antigen, the results were inconclusive (Figure 11g).

Substitution and Omission Controls. Omission or substitution of the primary antiserum with normal rabbit serum (at a dilution of 1/10K) routinely resulted in undetectable staining of pituitary sections of all specimens (Figure 12).

Ultrastructural Localization of LHβ and FSHβ Antisera

Immunocytochemical staining with LHβ antiserum was evident in the anterior pituitary of all specimens studied. Anterior pituitaries of the youngest specimens (44 mm to 53 mm C.R. length) were observed to have moderate numbers of cells containing numerous lightly stained granules, distributed throughout the cytoplasm as well as along the cell membrane (Figures 13 and 14). Cells containing stained granules were usually found adjacent to one another. Female and male specimens of this group showed no noticeable variation of the LHβ antiserum stain.

Examination of anterior pituitaries of 69 mm to 98 mm C.R. length specimens stained with LHβ antiserum revealed numerous cells with many lightly stained granules (Figures 15 and 16). As was seen in the youngest specimens, the granules were found throughout the cytoplasm and subjacent to the cell membrane. However, the female specimens appeared to have more cells containing stained granules, and in addition, usually more stained granules per cell than that observed in the male specimens.

Anterior pituitary sections of the oldest specimens (118 mm to 144 mm C.R. length), stained with LHβ antiserum revealed cells containing many heavily stained granules, and other cells with moderately stained granules (Figures 17 and 18). This observation was more apparent in the female specimens (Figure 17a). As in the younger specimens,

the distribution of the granules was noted throughout the cytoplasm as well as along the cell membrane. However, the oldest specimens, when compared to both groups of the aforementioned specimens, revealed granules more intensely stained, more cells containing stained granules and usually more stained granules per cell. Among the oldest specimens, a sex difference was apparent, in that the female specimens appeared to have more cells containing stained granules. Moreover, these cells usually contained more stained granules than those from male specimens.

Immunocytochemical staining with FSHβ antiserum was also evident in the anterior pituitary from each specimen studied. Anterior pituitaries of the youngest specimens (44 mm to 53 mm C.R. length) were observed to have few isolated cells containing small numbers of moderately stained granules in close proximity to one another (Figures 19 and 20). Female and male specimens of this group showed no noticeable difference in the FSHβ antiserum stain.

Examination of the anterior pituitaries of 69 mm to 98 mm C.R. length specimens stained with FSHβ antiserum revealed more cells containing stained granules, as well as more stained granules per cell (Figures 21 and 22). Granules were moderately stained, and distributed throughout the cytoplasm and along the cell membrane. Morphological differences were not apparent with respect to the sex of the specimens.

Anterior pituitary sections of the oldest fetuses (118 mm to 144 mm C.R. length) stained with FSHβ antiserum revealed cells containing many heavily stained granules, and other cells with moderately stained granules (Figures 23, 24 and 25). As was seen in the younger specimens (69 mm to 98 mm C.R. length) stained granules were observed

to be distributed throughout the cytoplasm and subjacent to the cell membrane. However, the oldest specimens, when compared with the younger groups, revealed granules more intensely stained and the presence of more cells containing stained granules. Moreover, a sex difference was apparent in the oldest group of specimens stained with FSHβ antiserum. Female specimens appeared to have more cells containing stained granules, and usually more stained granules per cell. In addition, the reproducibility of the immunocytochemical stain was observed, as illustrated by the fact that the same group of granules stained with a similar intensity on serial sections (Figure 25).

Staining by LHβ antiserum (Figure 26a) and FSHβ antiserum (Figure 26b) was noted repeatedly on serial sections from the anterior pituitary from the 144 mm C.R. length female specimen. Cells stained with both LHβ and FSHβ antisera, were observed to contain lightly or moderately stained granules. Figure 26a (LHβ antiserum stain) illustrates two cells, one with moderately stained granules, the other with lightly stained granules. Figure 26b (FSHβ antiserum stain) illustrates the same two cells; however, the staining intensity of the granules was opposite to that seen with the LHβ antiserum stain.

Comparison of the LHβ and FSHβ antisera stains in the youngest group of specimens (44 mm to 53 mm C.R. length) revealed different staining patterns. LHβ antiserum stained granules were lightly stained, numerous, and usually seen in several cells adjacent to one another (Figures 13 and 14). FSHβ antiserum stained granules were moderately stained, few in number, and seen in isolated cells (Figures 19 and 20).

Specimens of 69 to 98 mm C.R. length revealed both similarities and differences when staining properties of the two antisera were compared.

Both antisera stained numerous granules, often in adjacent cells (Figures 15, 16, 21 and 22). However, LHβ antiserum lightly stained the granules (Figures 15 and 16), whereas FSHβ antiserum often stained the granules with moderate intensity (Figures 21 and 22).

Comparison of the staining of LHβ and FSHβ antisera on sections from the oldest group of specimens (118 mm to 144 mm C.R. length) revealed several similarities. Many granules, heavily or moderately stained, and observed in different cells, were frequently detected in sections stained with either LHβ (Figures 16 and 17) or FSHβ (Figures 23, 24 and 25) antisera.

TABLE 1

CROWN-RUMP (C.R.) LENGTH AND ESTIMATED GESTATIONAL AGE (WEEKS) OF HUMAN FETUSES

FEMALE SPECIMENS		MALE SPECIMENS	
C.R.(mm)	WEEKS	C.R.(mm)	WEEKS
		45	9.5
44	9.5	48	9.5
53	10.0	51	10.0
69	11.5	69	11.5
78	12.0	75	11.5
98	13.5	98	13.5
121	15.0	118	14.5
135	16.0	118	14.5
144	16.5	138	16.0

TABLE 2

SIGNIFICANCE OF CORRELATION COEFFICIENTS OF THE REGRESSION ANALYSIS OF THE NORMALIZED STAINING INTENSITIES ON ANTIGEN CONCENTRATIONS

LHβ ANTISERUM			FSHβ ANTISERUM		
Antigen	"Initial" Regression Line	"Final" Regression Line	Antigen	"Initial" Regression Line	"Final" Regression Line
LHβ	0.1<P	.05<P<0.1	FSHβ	0.1<P	0.1<P
LH	0.1<P	P<.05	FSH	0.1<P	P<.05
FSHβ	0.1<P	P<.001	LHβ	0.1<P	P<.05
FSH	0.1<P	0.1<P	LH	P<.001	
TSHβ	0.1<P	P<.05	TSHβ	.05<P<0.1	
CGβ	.05<P<.01	.05<P<.1	CGβ	0.1<P	
CGα	P<.05		CGα	0.1<P	

.05<P = No Significant Correlation

TABLE 3

COMPARISON OF THE SLOPES OF THE "INITIAL" AND "FINAL" REGRESSION LINES IN EACH ABSORPTION TEST

LHβ ANTISERUM		FSHβ ANTISERUM	
Antigen	Difference	Antigen	Difference
LHβ	P<.01	FSHβ	P<.05
LH	P<.005	FSH	P<.005
FSHβ	P<.001	LHβ	N.S.
FSH	N.S.	LH	N.A.
TSHβ	N.S.	TSHβ	N.A.
CGβ	P<.005	CGβ	N.A.
CGα	N.A.	CGα	N.A.

N.S. = No Significant Difference (.05<P)

N.A. = No Absorption Apparent

TABLE 4

COMPARISON OF THE SLOPES OF THE "FINAL" REGRESSION LINES

LHβ ANTISERUM ABSORPTIONS	
Antigens Compared	Difference
LHβ + LH	N.S.
LHβ + FSHβ	N.S.
LHβ + FSH	P<.05
LHβ + TSHβ	P<.005
LHβ + CGβ	P<.05

FSHβ ANTISERUM ABSORPTIONS	
Antigens Compared	Difference
FSHβ + FSH	N.S.
FSHβ + LHβ	N.S.

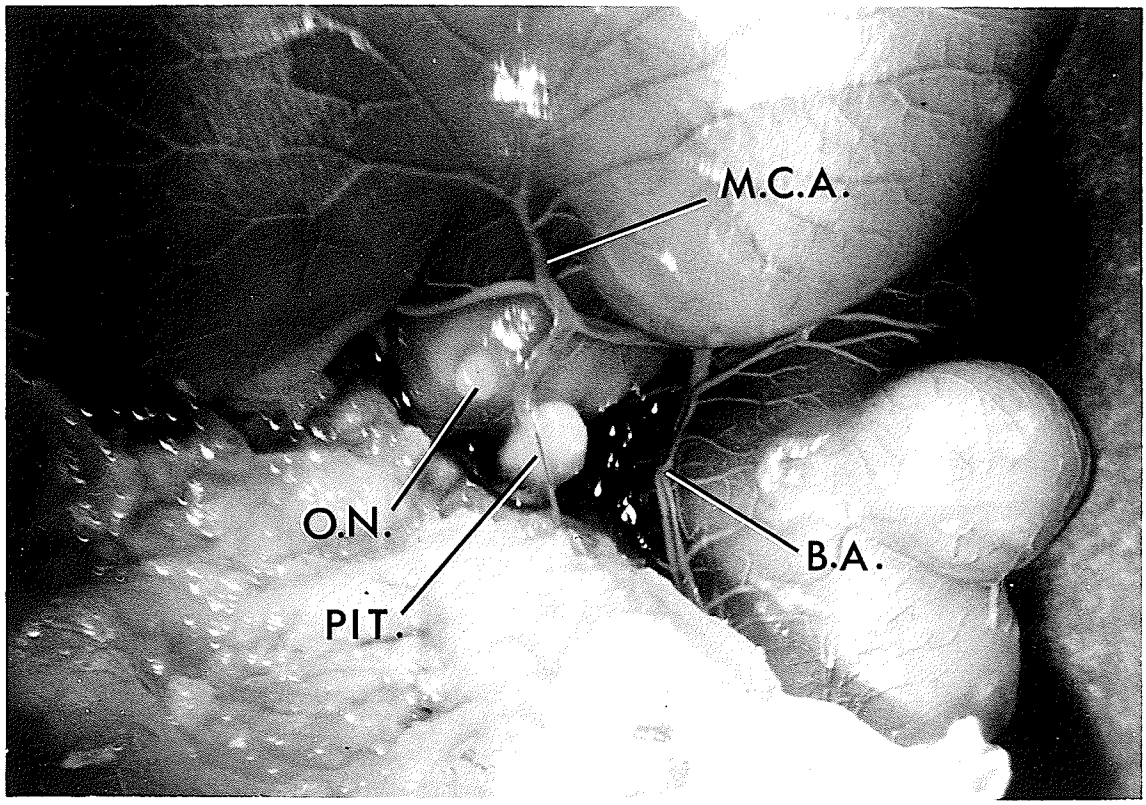
N.S. = No Significant Difference (.05<P)

Figure 2(a and b)

Photographs of the pituitary, hypothalamus and related structures from a 70 mm C.R. length specimen (11.5 weeks gestation).

2(a). Antero-lateral view of pituitary (PIT), optic nerve (O.N.), middle cerebral artery (M.C.A.) and basilar artery (B.A.). X4.5

2(b). Anterior view of pituitary (PIT), hypothalamus (HYPO) and anterior cerebral arteries (A.C.A.). X16


M.C.A.
O.N.
B.A.
PIT.

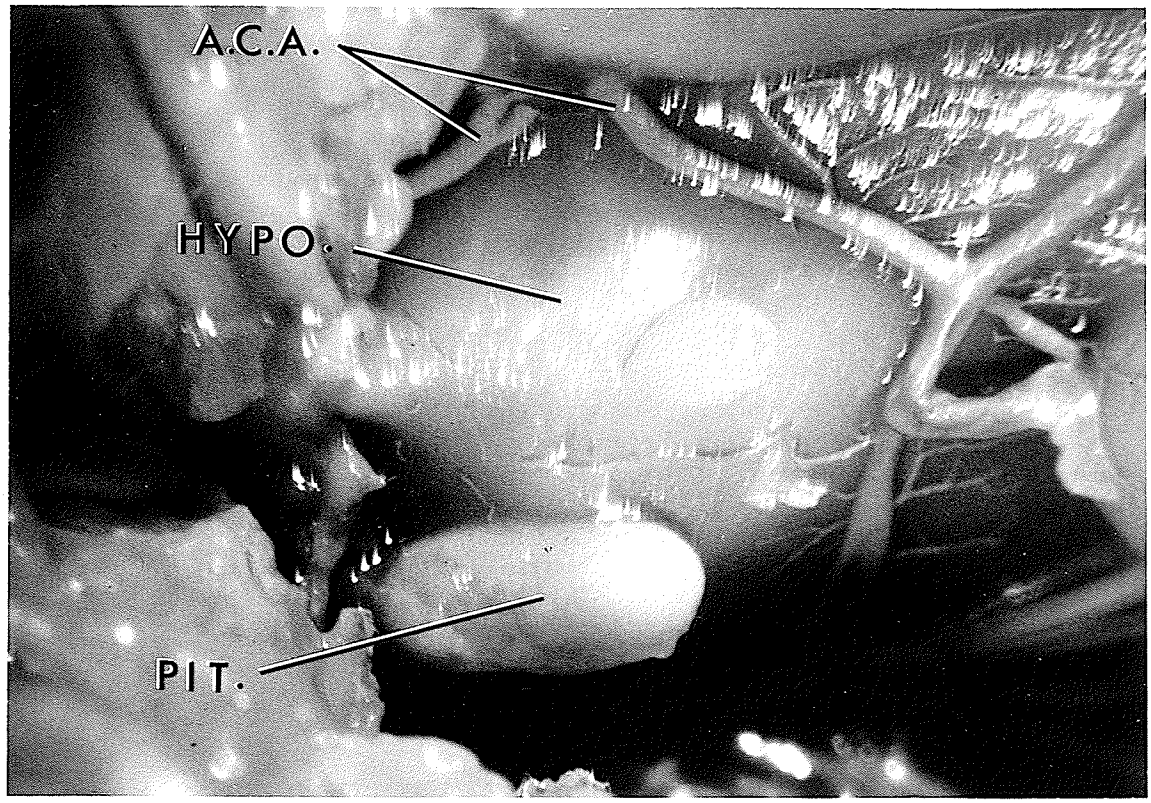
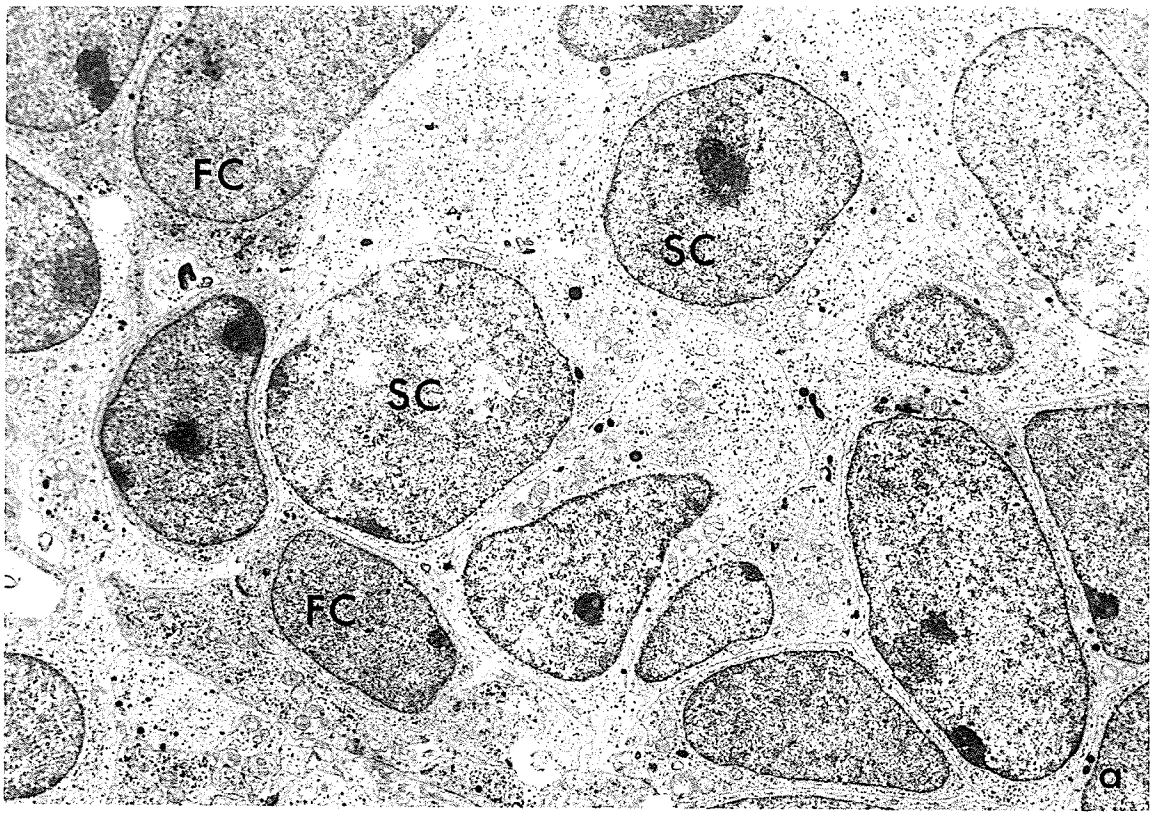

A.C.A.
HYPO.
PIT.

Figure 3(a and b)

Micrographs of the anterior pituitary from a 44 mm C.R. length female specimen (9.5 weeks gestation).

3(a). Area depicting sparsely granulated cells. Stem cells (SC), with a thin rim of cytoplasm, and follicular cells (FC) are evident. X5,000

3(b). Area depicting several granulated cells (GC). Granules (arrows) are of a relatively similar size and density. Also seen are follicular cells (FC), containing glycogen deposits (Gl), and exhibiting cytoplasmic processes (crossed arrow) between granulated cells. X5,000


FC
SC
SC
FC
a

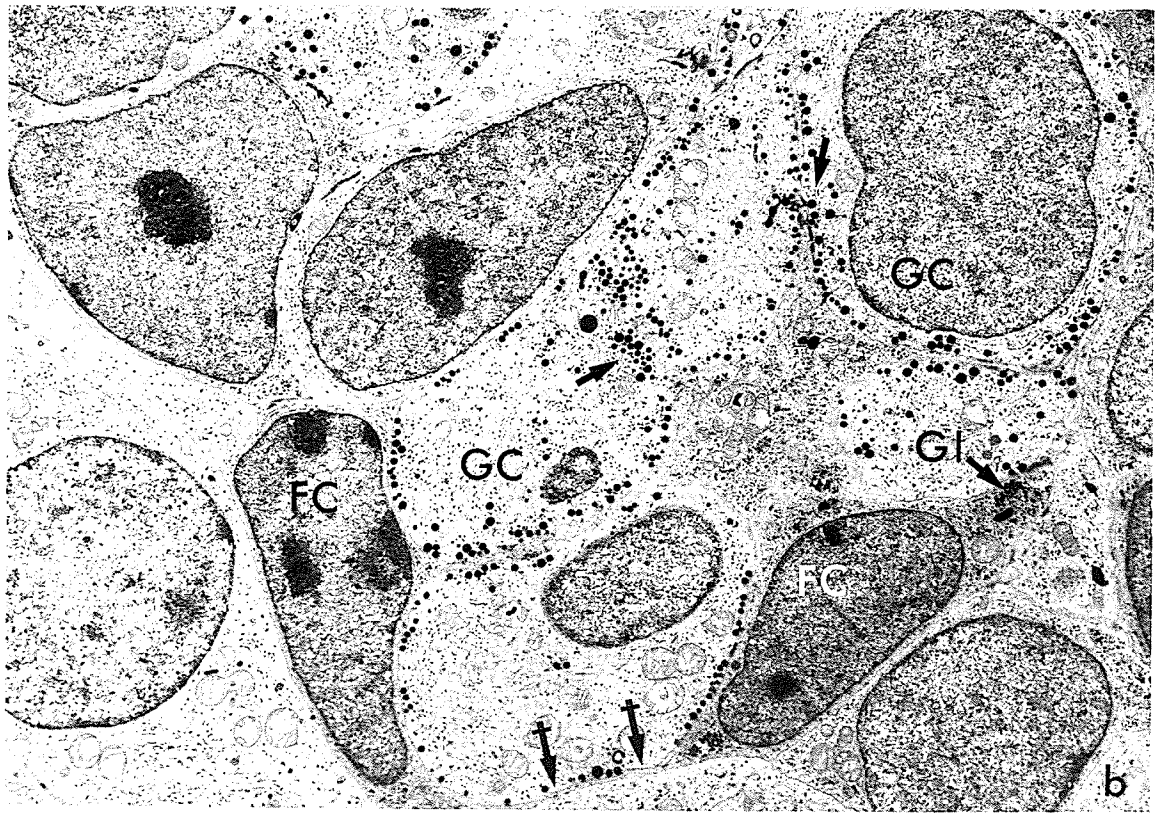

GC
GC
GI
FC
FC
b

Figure 4(a and b)

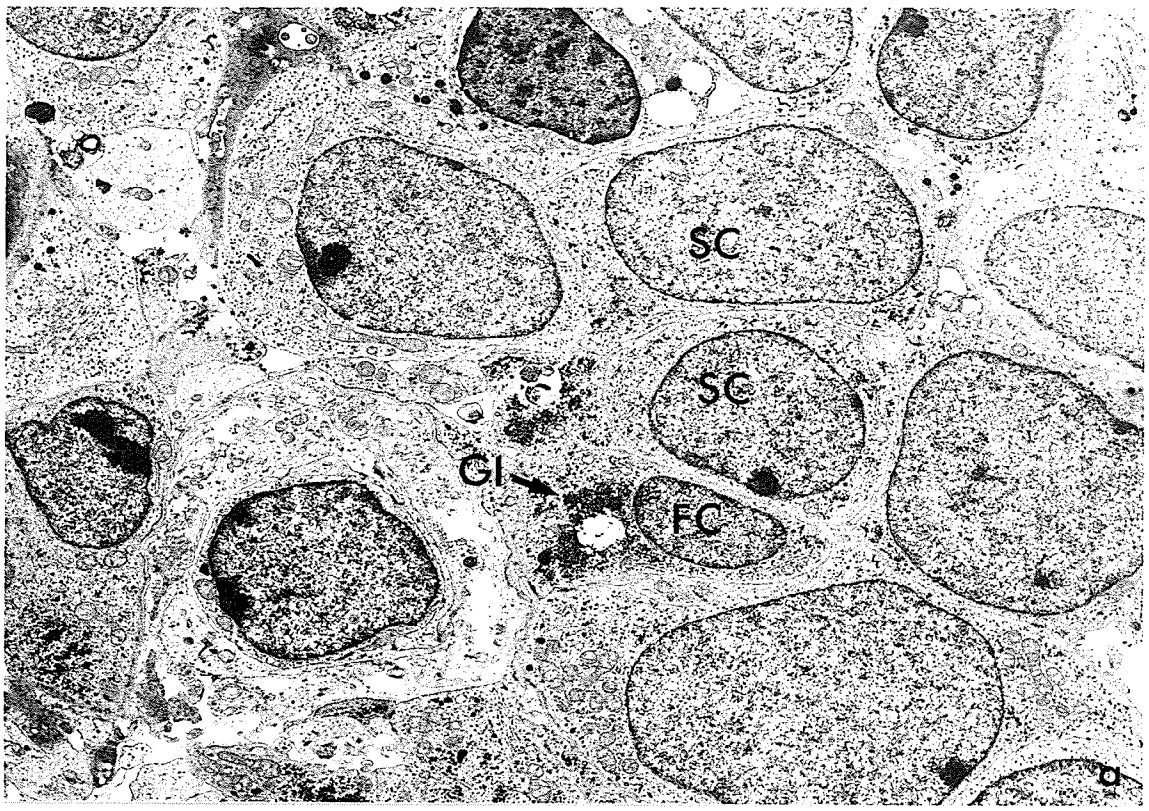
Micrographs of the anterior pituitary from a 45 mm C.R. length male specimen (9.5 weeks gestation).

4(a). Area depicting sparsely granulated cells. Stem cells (SC) and follicular cells (FC) such as those shown here are frequently observed in early fetal development. Glycogen (Gl).

X5,000

4(b). Area showing several granulated cells (GC). Granules (arrows) are of a relatively similar size and density. Follicular cells (FC), with long processes (crossed arrows) are frequently observed between stem cells (SC) and granulated cells.

X5,000


SC
SC
GI
FC
a

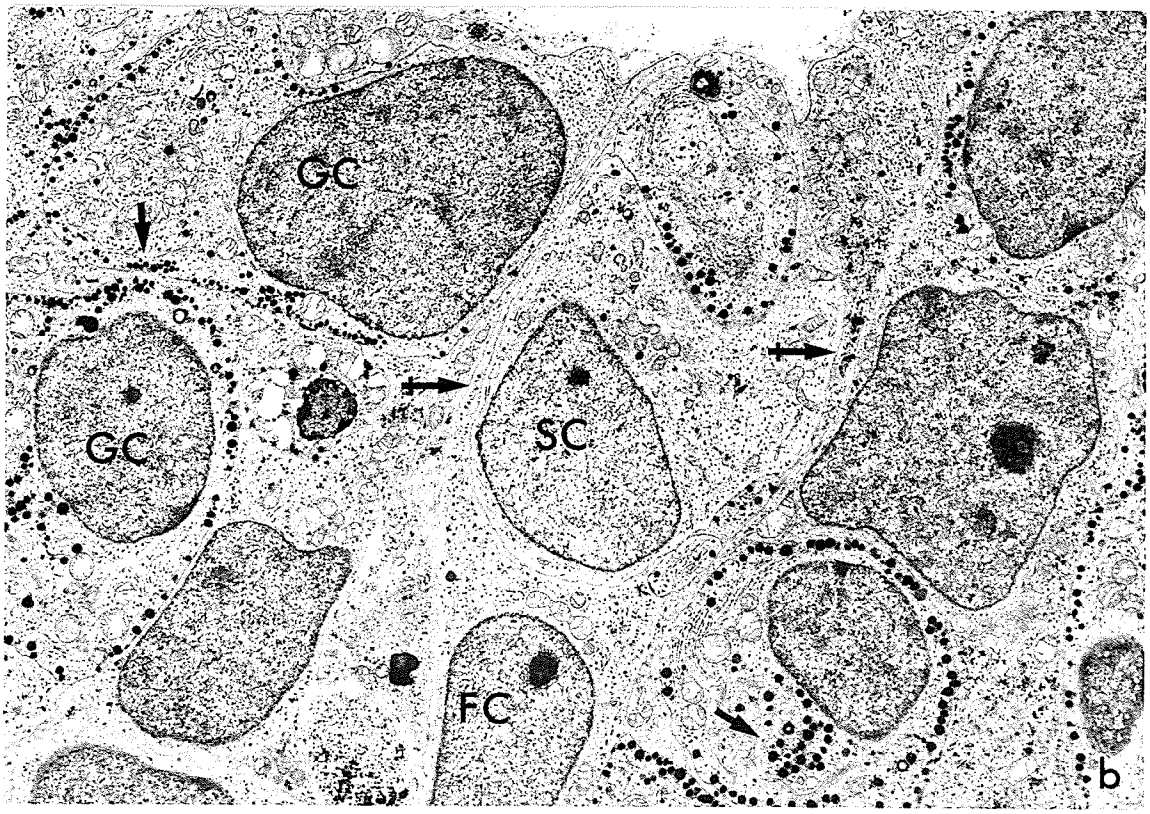
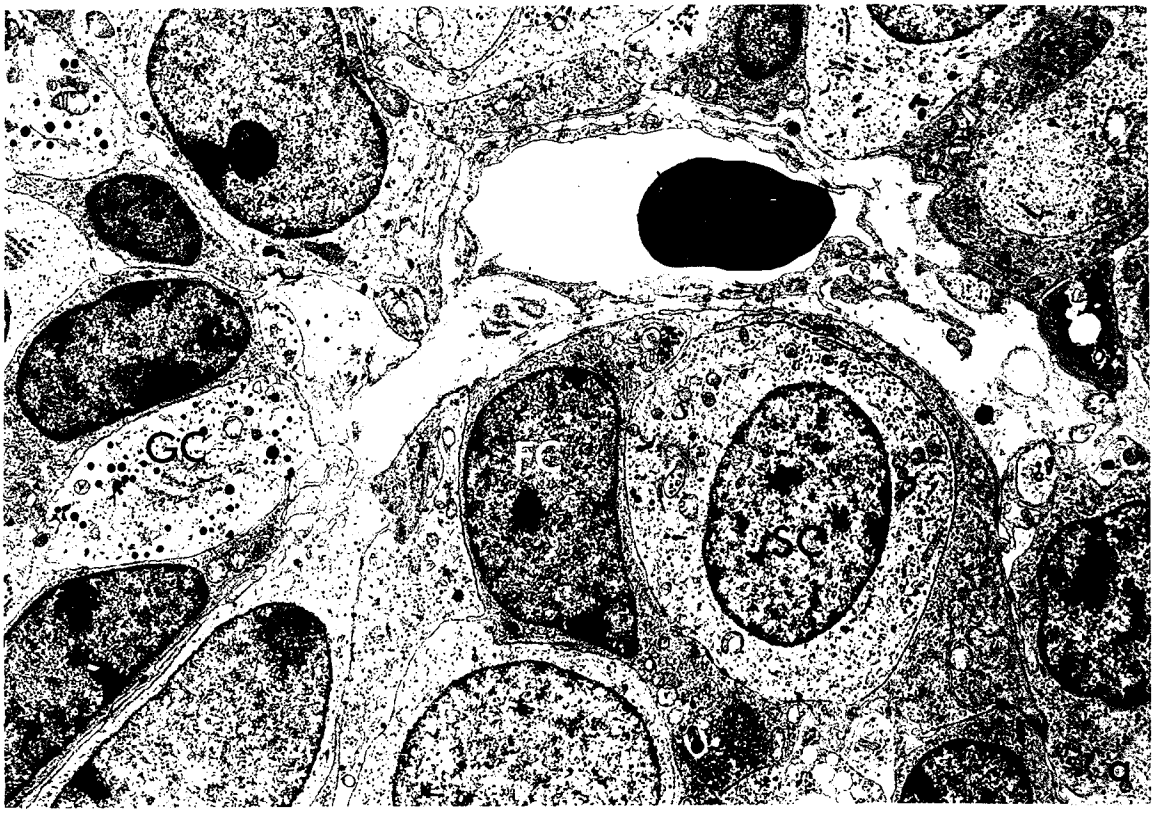

GC
GC
SC
FC
b

Figure 5(a and b)

Micrographs of the anterior pituitary from a 78 mm C.R. length female specimen (12 weeks gestation).

5(a). Area depicting a few lightly granulated cells (GC). Stem cells (SC) and follicular cells (FC) are also evident. X5,000

5(b). Area revealing several heavily granulated cells (GC). Granules (arrows) of different sizes are noted. X5,000


GC
FC
SC
a

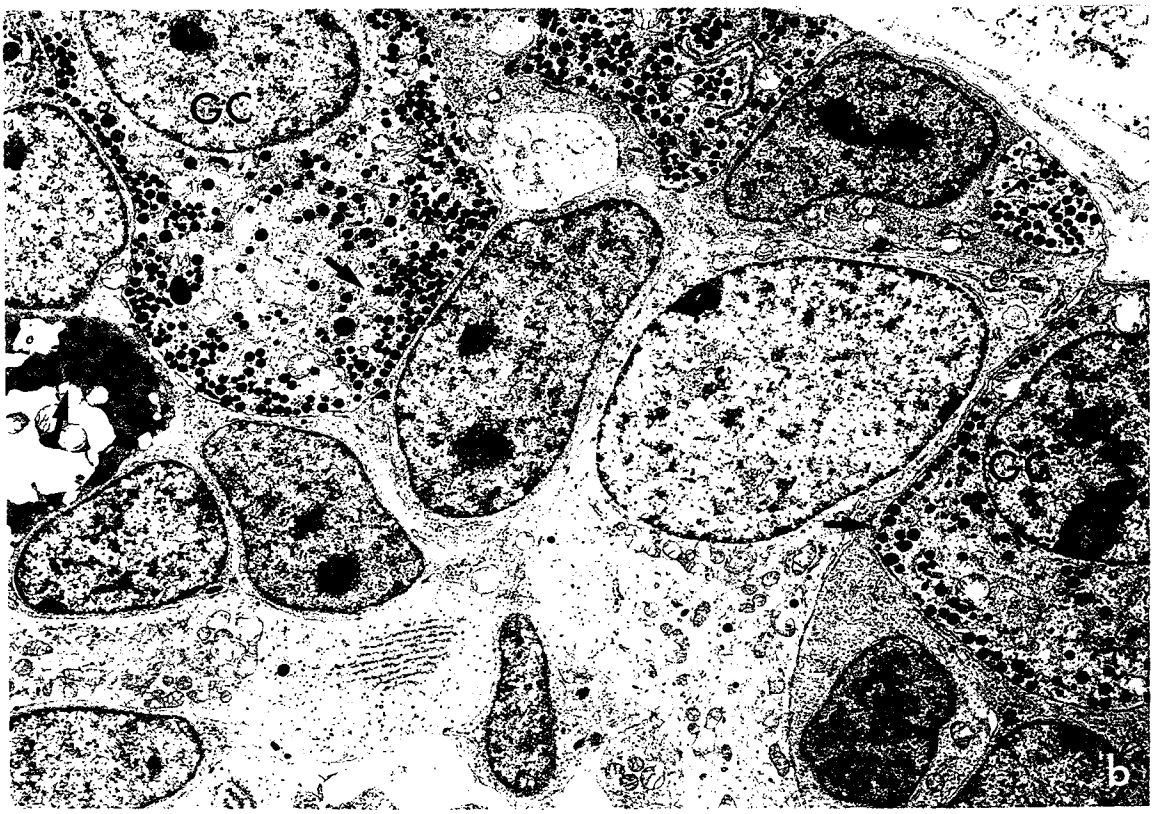
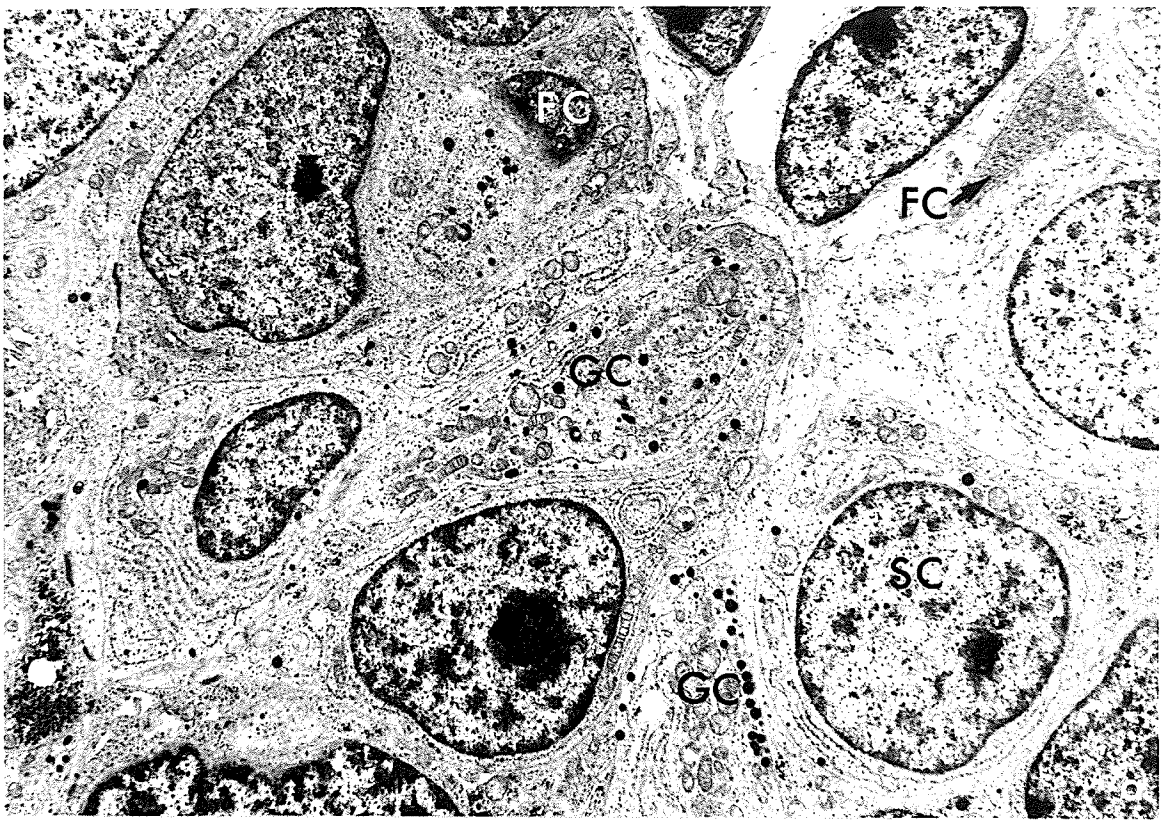

GC
GC
b

Figure 6(a and b)

Micrographs of the anterior pituitary from a 75 mm C.R. length male specimen (11.5 weeks gestation).

6(a). Area depicting sparsely granulated cells (GC), stem cells (SC) and follicular cells (FC). X5,000

6(b). Area depicting granulated cell (GC) with a moderate number of granules (arrows). X5,000


PC
FC
GC
SC
GC

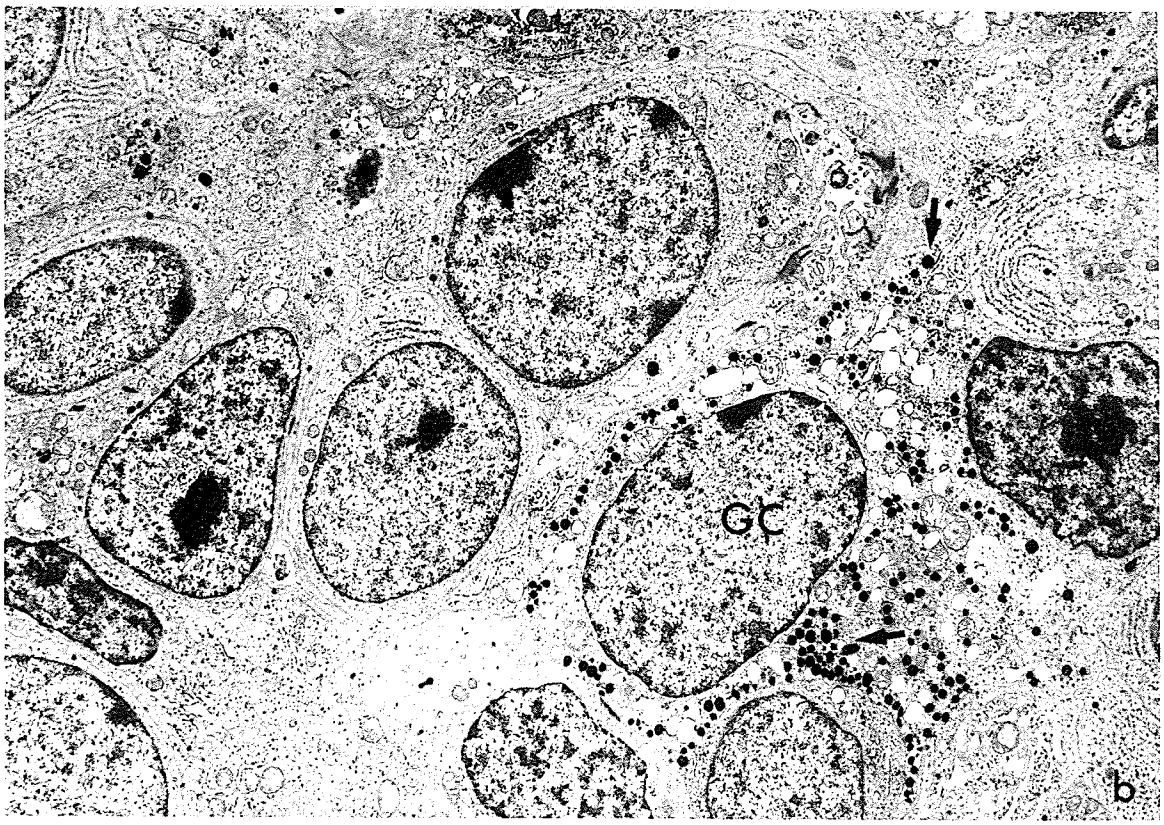
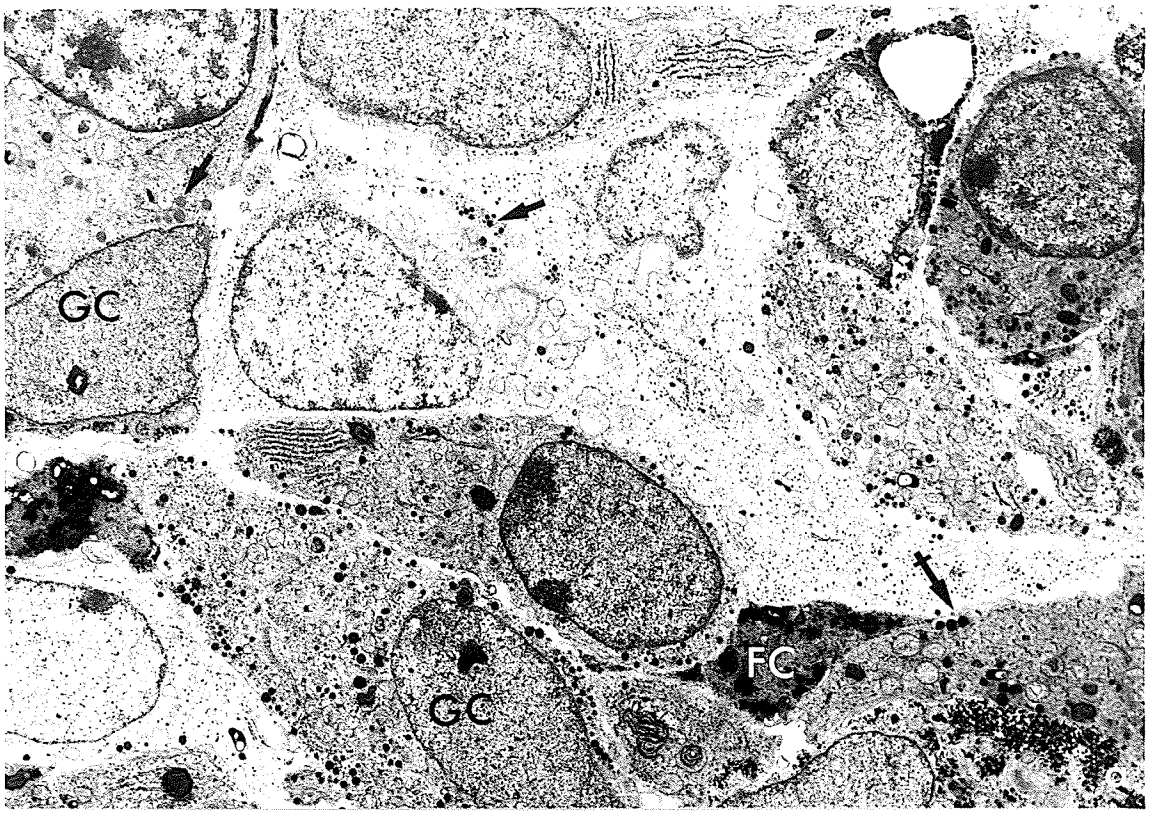

GC
b

Figure 7(a and b)

Micrographs of the anterior pituitary from a 144 mm C.R. length female specimen (16.5 weeks gestation).

7(a). Area with several lightly and moderately granulated cells (GC). Attenuated processes (crossed arrow) of a follicular cell (FC) are noted. Note the variation in size, density, and distribution of granules present (arrows). X5,000

7(b). Area depicting several moderately and heavily granulated cells (GC). Cells with dilated endoplasmic reticulum (ER) are frequently observed at 16.5 weeks gestation. Note the variation in size, density, and distribution of granules present (arrows). X5,000


GC
GC
FC

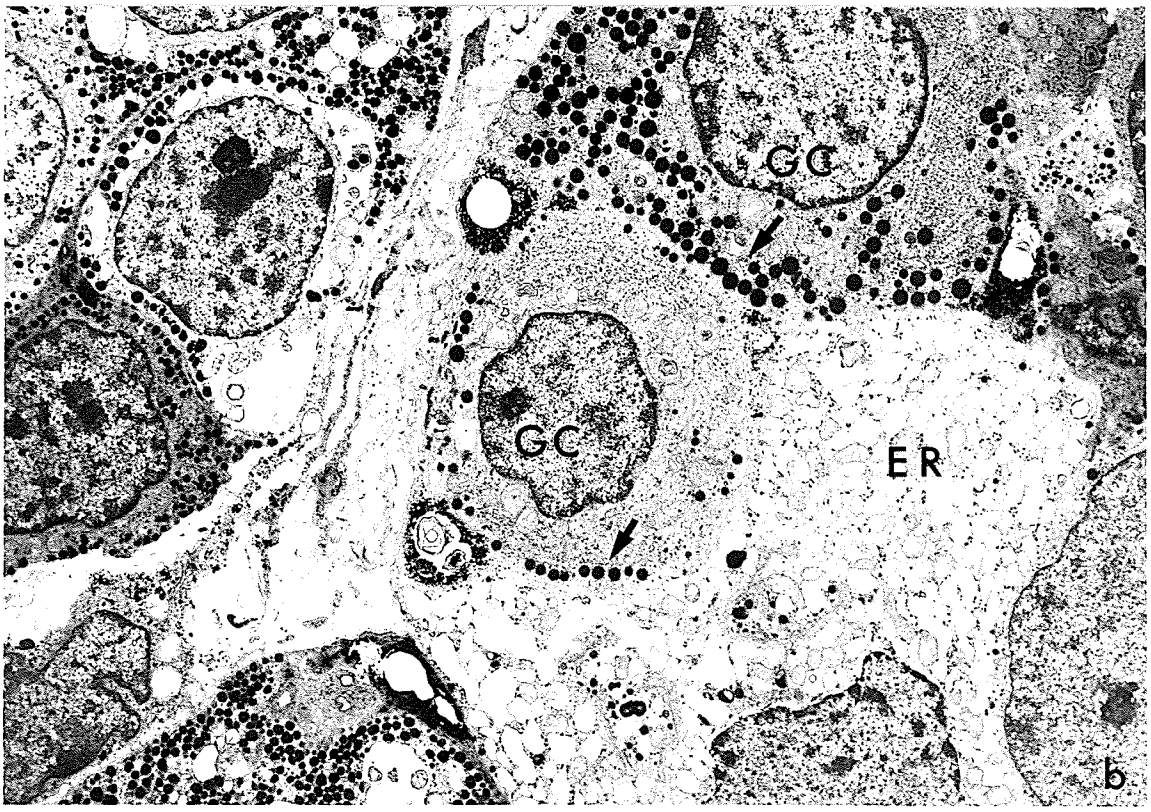
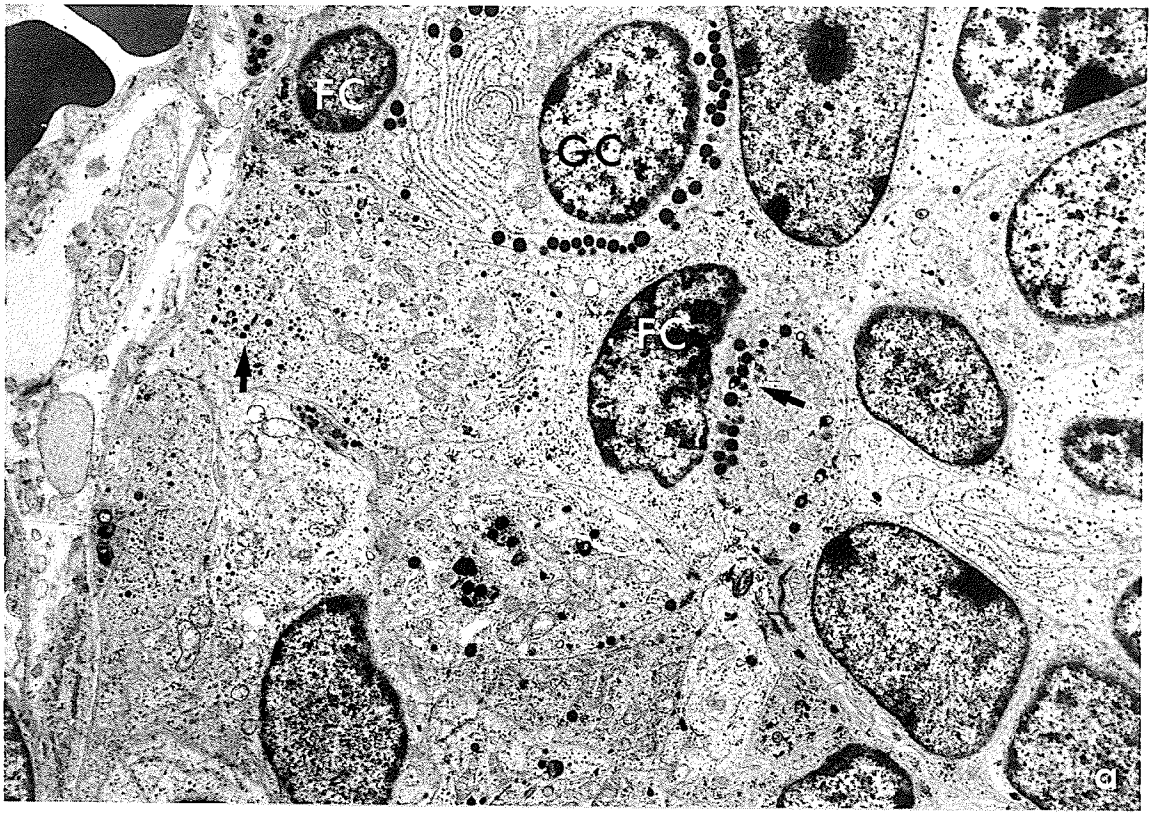

GC
GC
ER
b

Figure 8(a and b)

Micrographs of the anterior pituitary from a 138 mm C.R. length male specimen (16 weeks gestation).

8(a). Area depicting slightly and moderately granulated cells (GC). Note the variation in size, density and distribution of granules present (arrows). Follicular cells (FC). X5,000

8(b). Area of pituitary depicting several moderately and heavily granulated cells (GC). Size, density and distribution of granules vary from cell to cell (arrows). Attenuated processes (crossed arrows) of follicular cells are frequently observed. Cells with dilated endoplasmic reticulum (ER) are occasionally observed. X5,000


FC
GC
FC
a

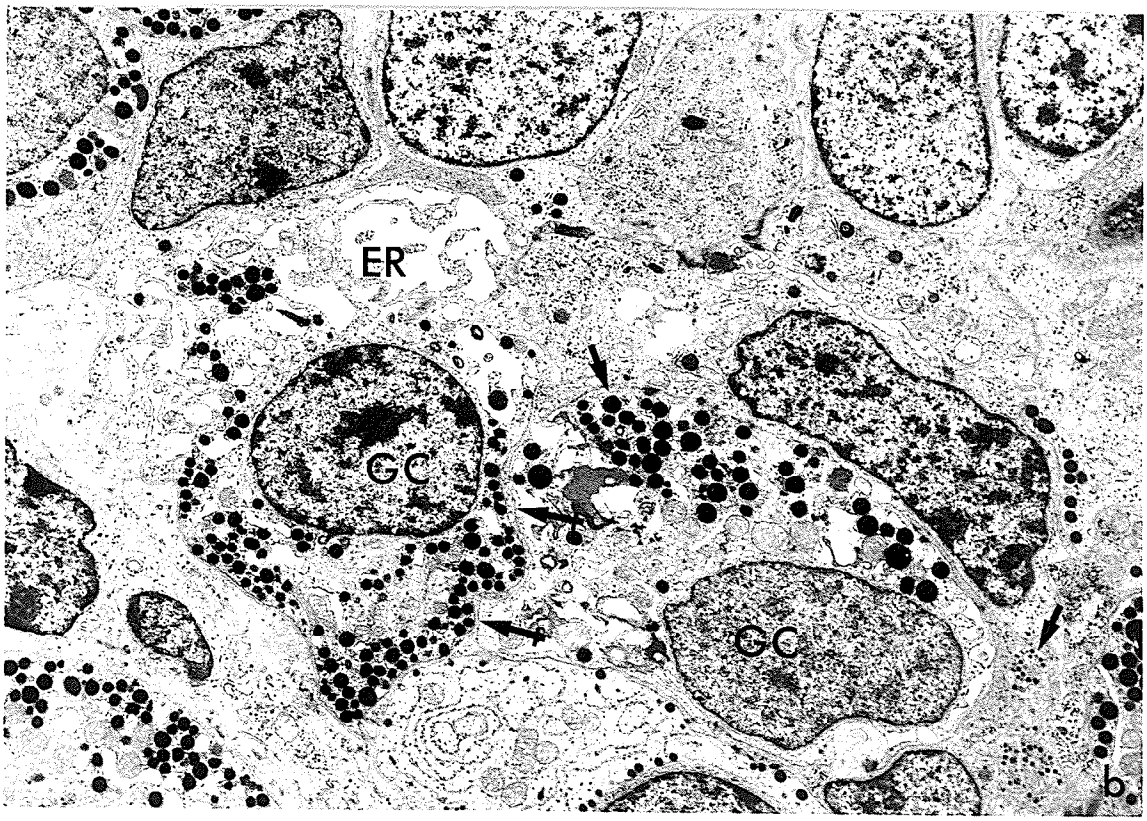

ER
GC
GC
b

Figure 9

Dilution curves of LHβ and FSHβ antisera and normal rabbit serum (NRS). Each data point represents measurements of 100 granules. $\bar{x}$ = mean ± S.E.M. K = x1000.

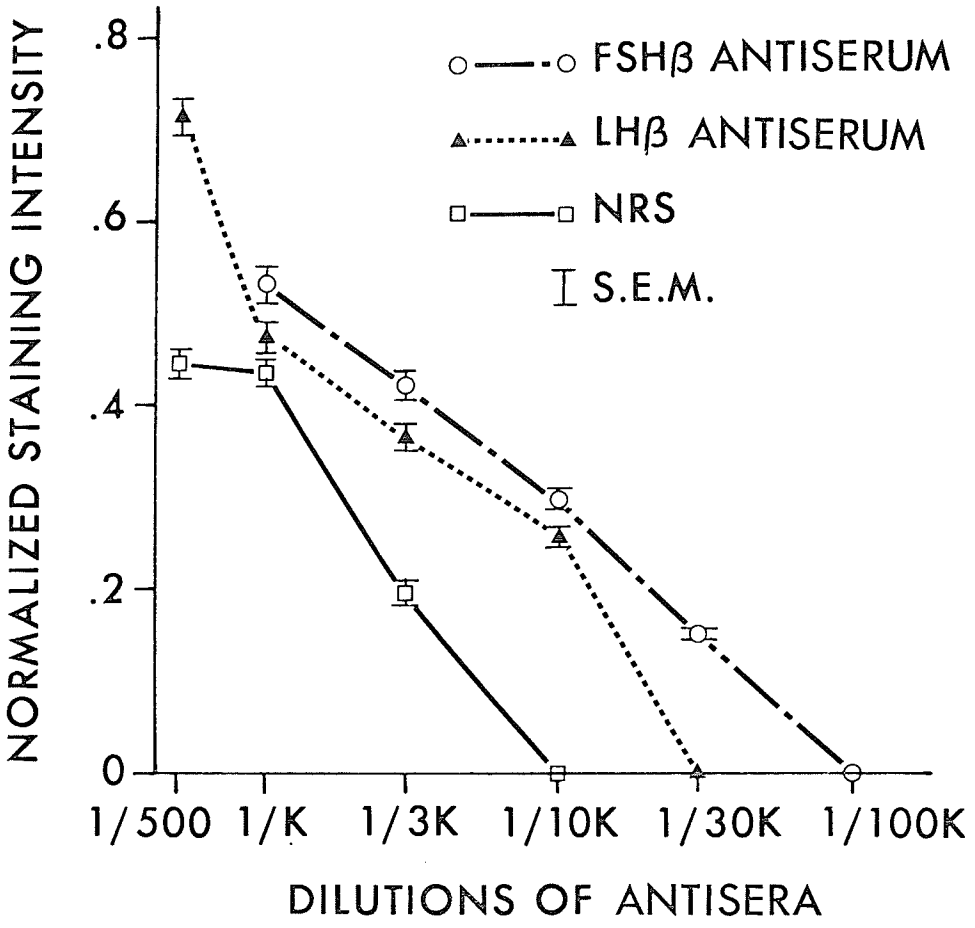

NORMALIZED STAINING INTENSITY
.8
.6
.4
.2
0
FSHβ ANTISERUM
LHβ ANTISERUM
NRS
S.E.M.
1/500
1/K
1/3K
1/10K
1/30K
1/100K
DILUTIONS OF ANTISERA

Figure 10(a-g)

Absorption of LHβ antiserum with selected antigens. Lines represent regression analyses of normalized staining intensity on antigen concentrations. Each data point represents measurements of 60 granules. $\bar{x}$ = mean ± S.E.M.

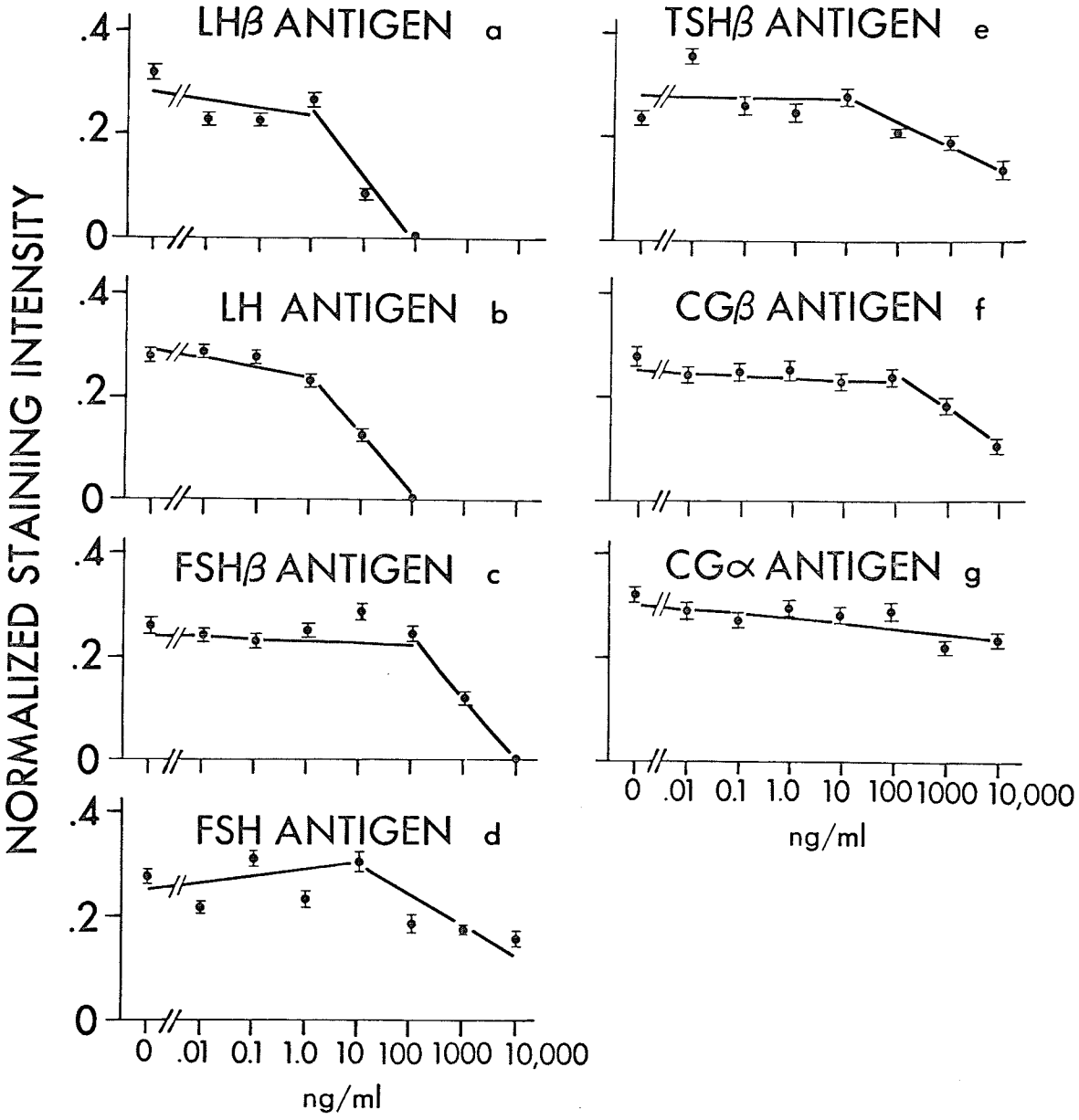

NORMALIZED STAINING INTENSITY
LHβ ANTIGEN a
LH ANTIGEN b
FSHβ ANTIGEN c
FSH ANTIGEN d
TSHβ ANTIGEN e
CGβ ANTIGEN f
CGα ANTIGEN g
.4
.2
0
0 .01 0.1 1.0 10 100 1000 10,000
ng/ml

Figure 11(a-g)

Absorptions of FSHβ antiserum with selected antigens. Lines represent regression analyses of normalized staining intensity on antigen concentrations. Each data point represents measurements of 60 granules. $\bar{x}$ = mean ± S.E.M.

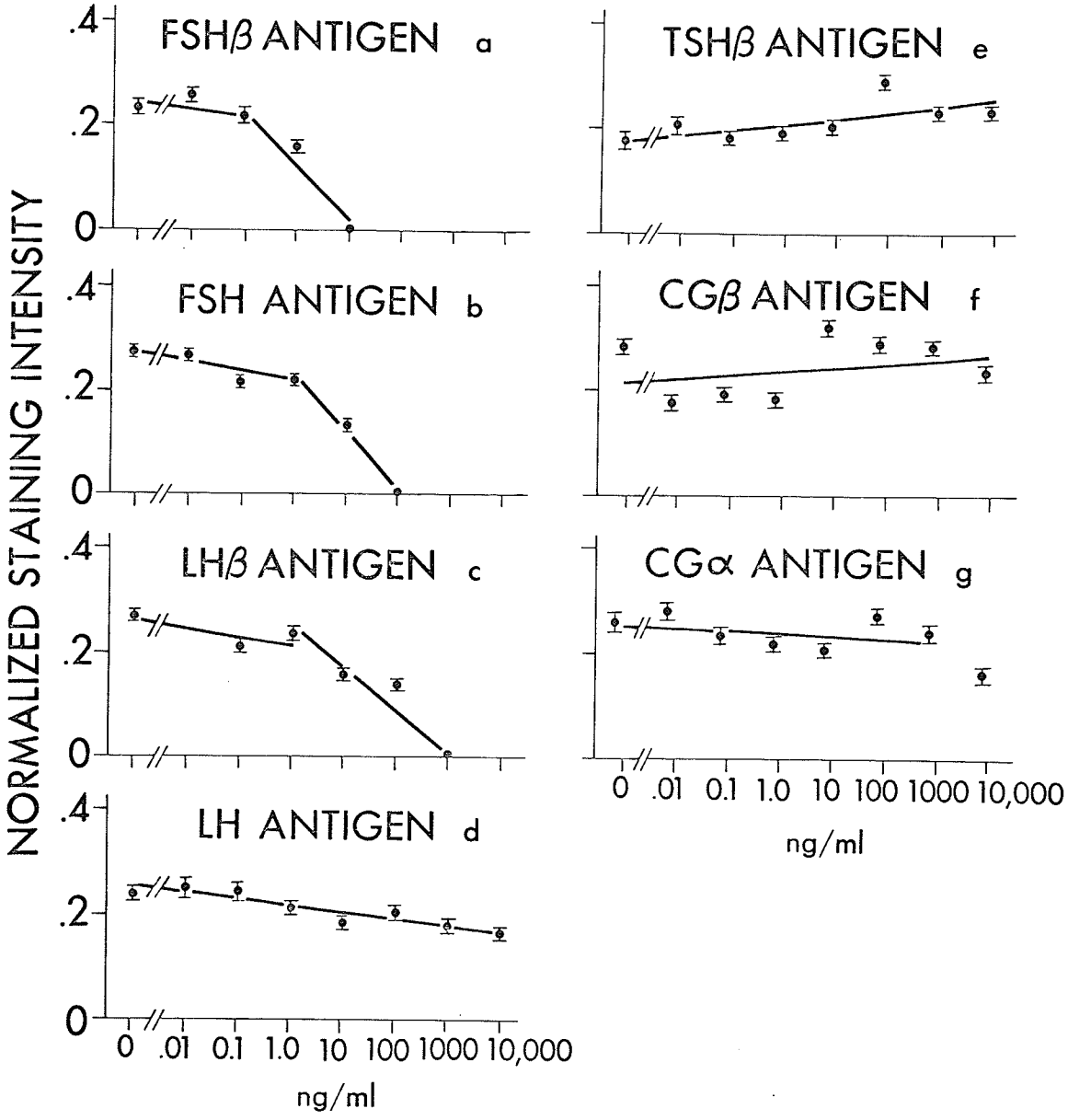

NORMALIZED STAINING INTENSITY
FSHβ ANTIGEN a
FSH ANTIGEN b
LHβ ANTIGEN c
LH ANTIGEN d
TSHβ ANTIGEN e
CGβ ANTIGEN f
CGα ANTIGEN g
.4
.2
0
0 .01 0.1 1.0 10 100 1000 10,000
ng/ml

Figure 12(a-c)

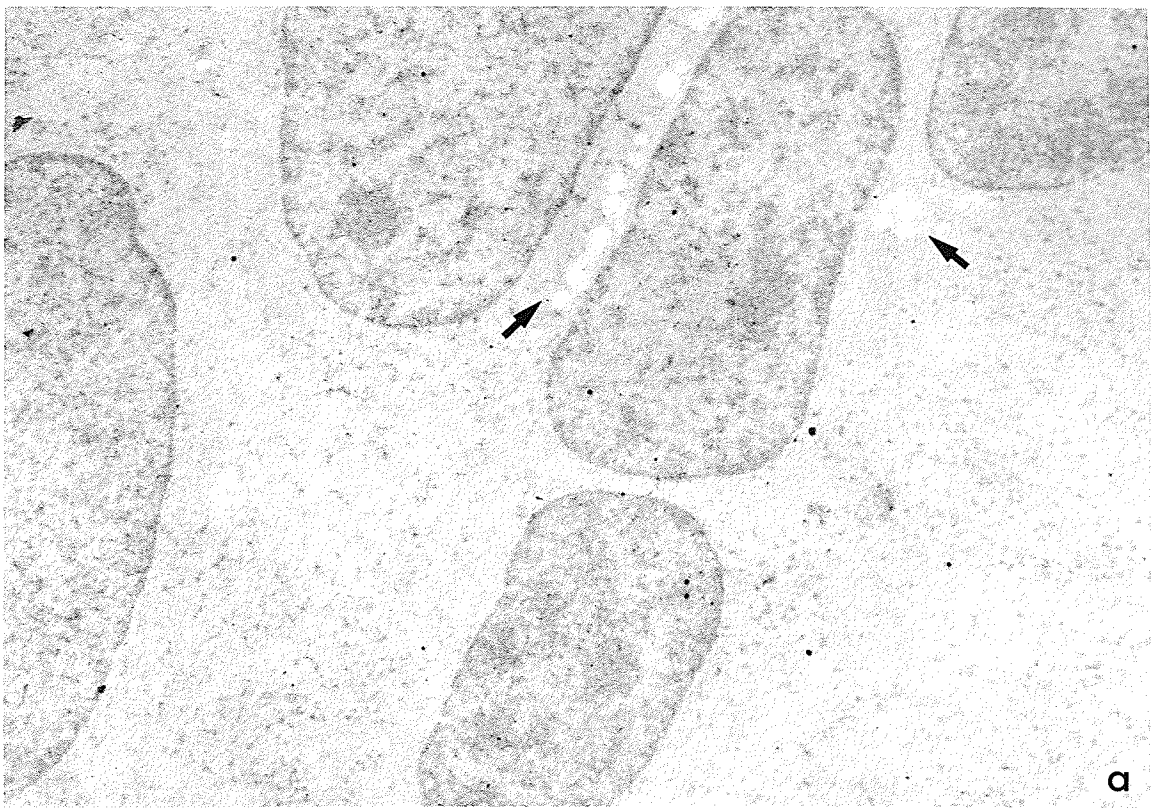
Micrographs of the anterior pituitary from a 144 mm C.R. length female specimen (16.5 weeks gestation), illustrating granules (arrows) devoid of the immunocytochemical stain.

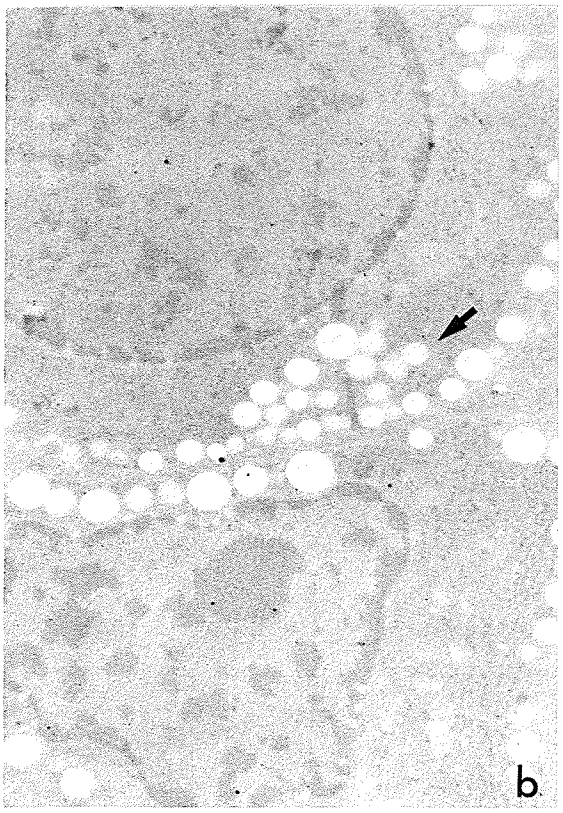
12(a,b). Pituitary section on which normal rabbit serum (1/10,000 dilution) was substituted for the primary antiserum.

X10,000

12(c). Pituitary section on which the primary antiserum was omitted.

X10,000


a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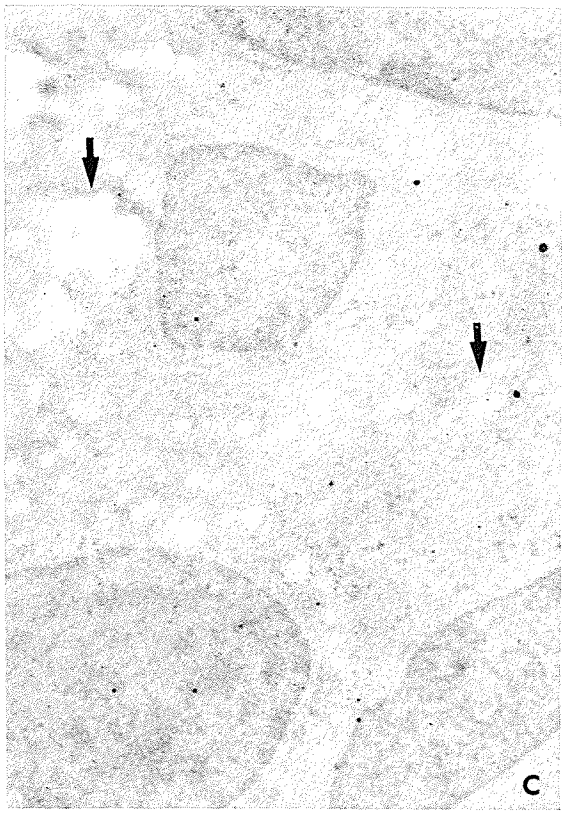

c

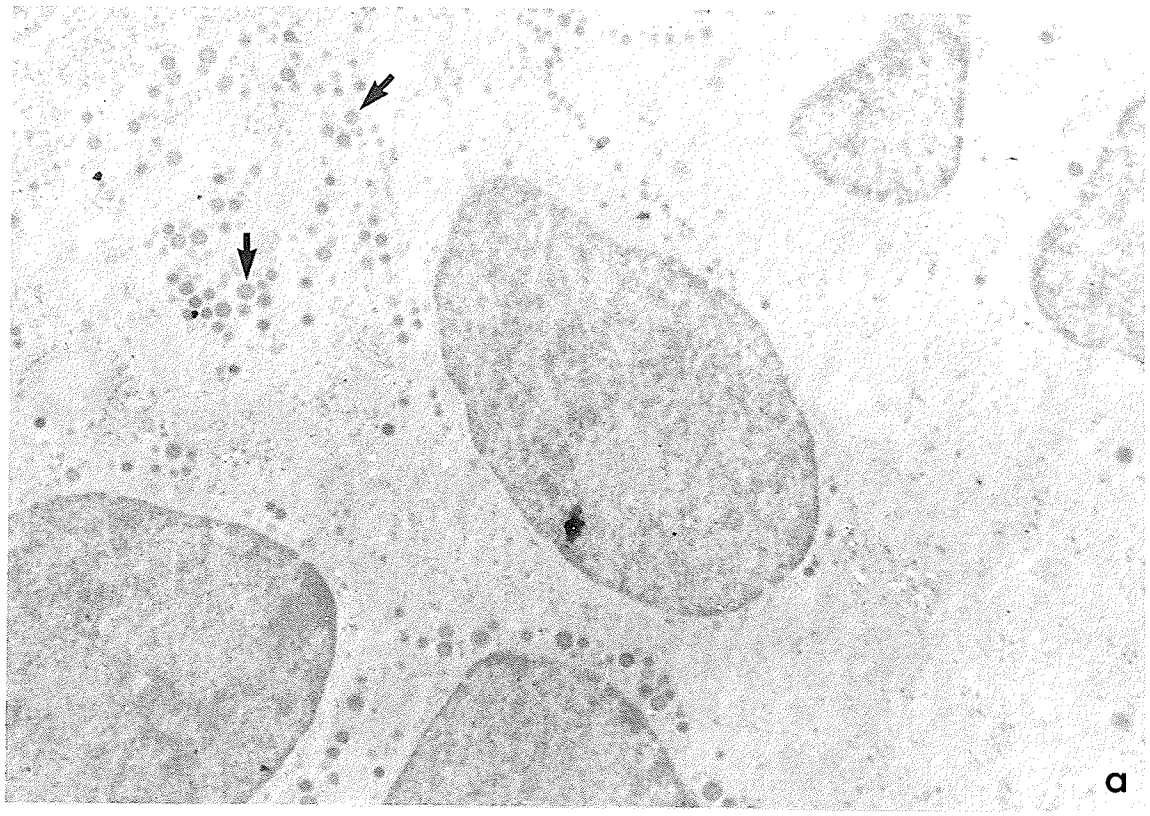
Figure 13(a-c)

Micrographs of the anterior pituitary from a 44 mm C.R. length female specimen (9.5 weeks gestation), stained immunocytochemically with LHβ antiserum at a 1/10,000 dilution.

13(a). Numerous lightly stained granules (arrows) are observed in several cells. X10,000

13(b). Higher magnification of granules (arrows) depicted in Figure 13(a). A light reaction product can be observed over the "PAP" complexes on the granules. X25,000

13(c). Cells illustrating granules (arrows) of similar size, with a more intense stain than that observed in Figure 13(b). X25,000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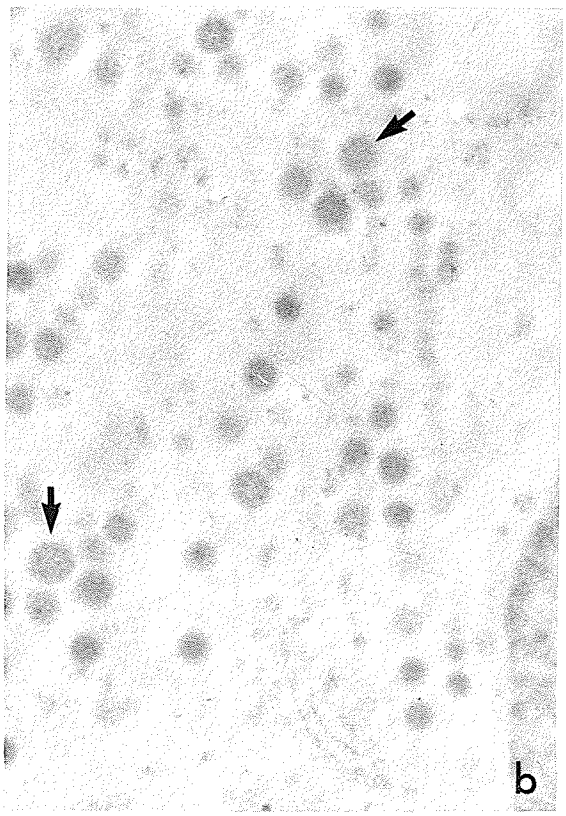
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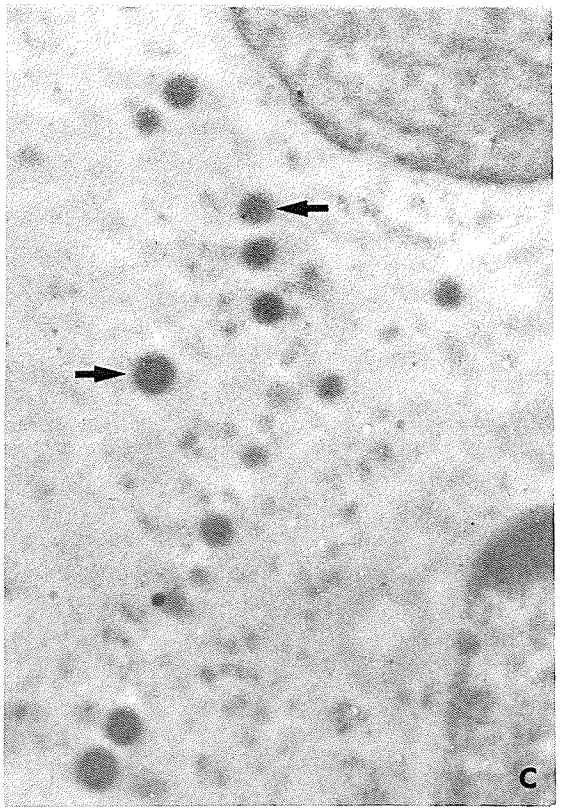

c

Figure 14(a-c)

Micrographs of the anterior pituitary from a 45 mm C.R. length male specimen (9.5 weeks gestation), stained immunocytochemically with LHβ antiserum at a 1/10,000 dilution.

14(a). Numerous lightly stained granules (arrows) are observed in two adjacent cells. X10,000

14(b). Higher magnification of granules (arrow) seen from the cell in the center of Figure 13(a). A light reaction product can be easily observed over the "PAP" complexes on the granules. X25,000

14(c). Cells from an area adjacent to those in Figure 14(a) illustrating stained granules (arrows) of similar size and staining intensity. X25,000

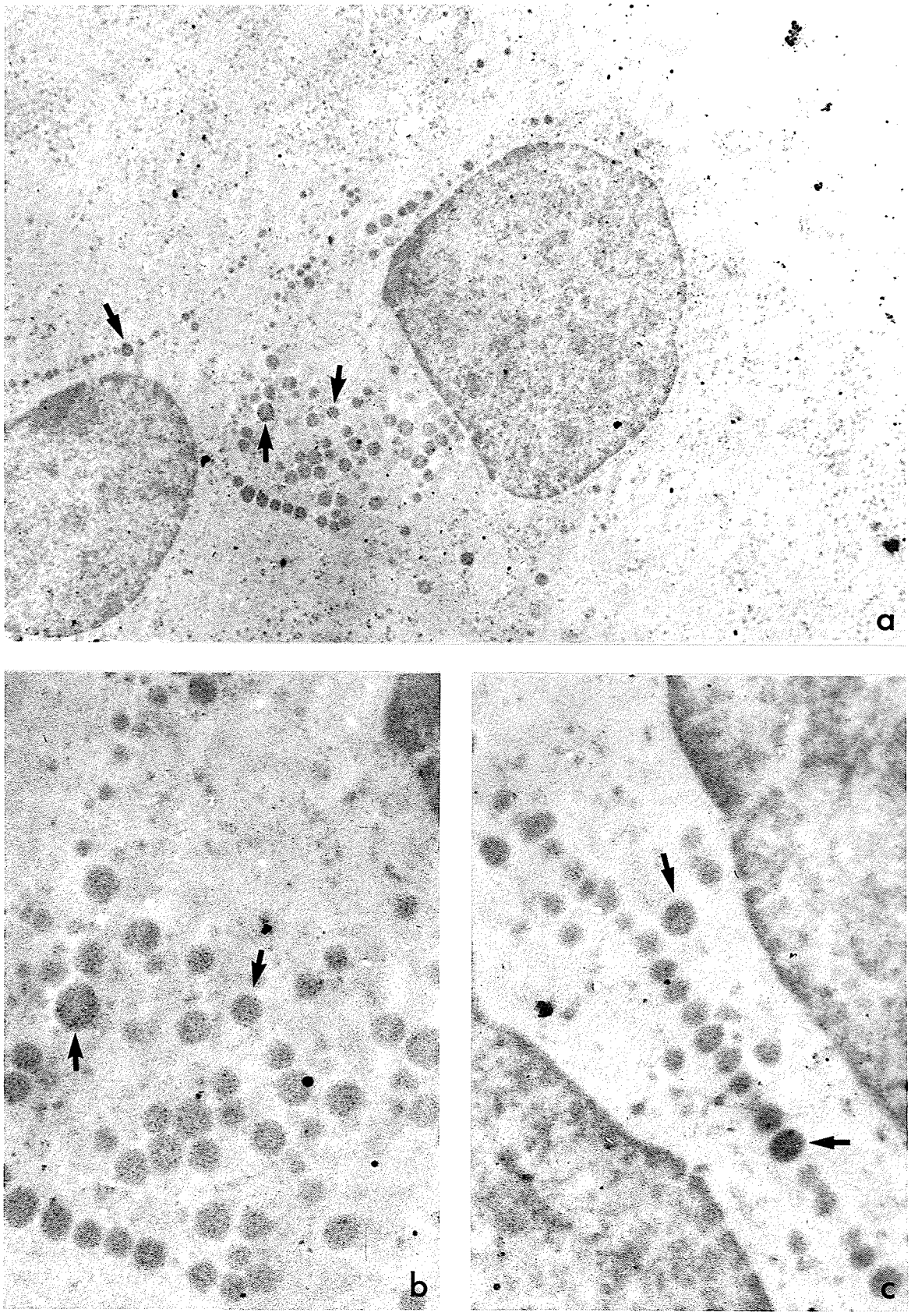

a
b
c

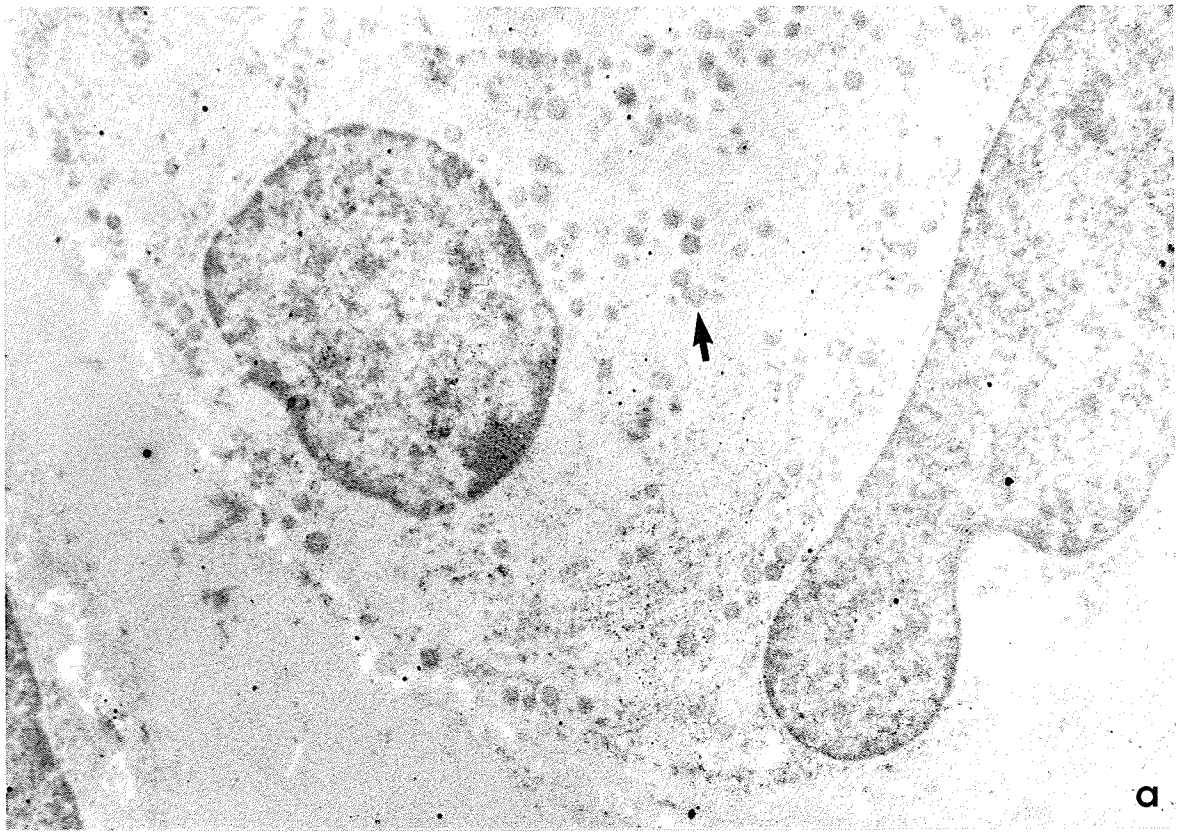
Figure 15(a-c)

Micrographs of the anterior pituitary from a 78 mm C.R. length female specimen (12 week gestational age), stained immunocytochemically with LHβ antiserum at a 1/10,000 dilution.

15(a). Numerous lightly stained granules (arrow) are seen distributed along the cell membrane and throughout the cytoplasm of a cell. X10,000

15(b). Higher magnification of granules (arrow) depicted in Figure 15(a). A light reaction product can be easily observed over the "PAP" complexes on the granules. X25,000

15(c). Stained (arrow) and unstained (crossed arrow) granules in close proximity are observed in two adjacent cells. X10,000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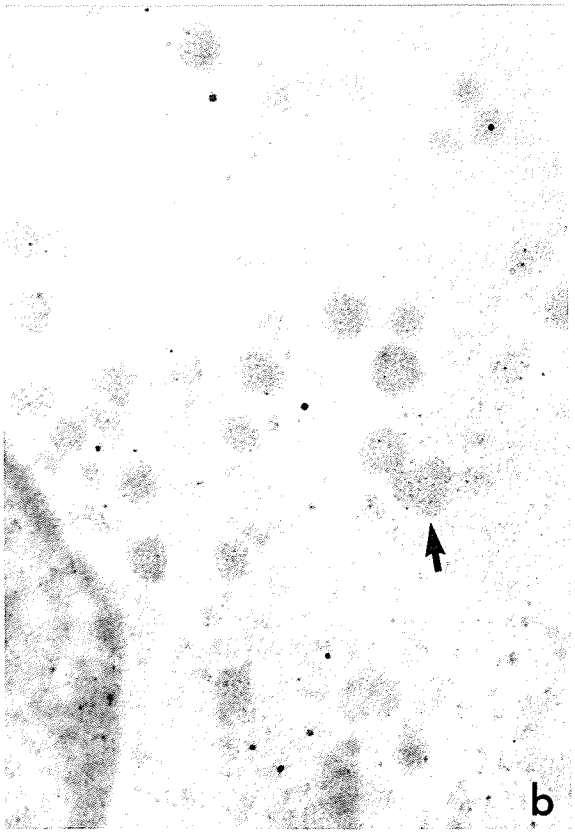
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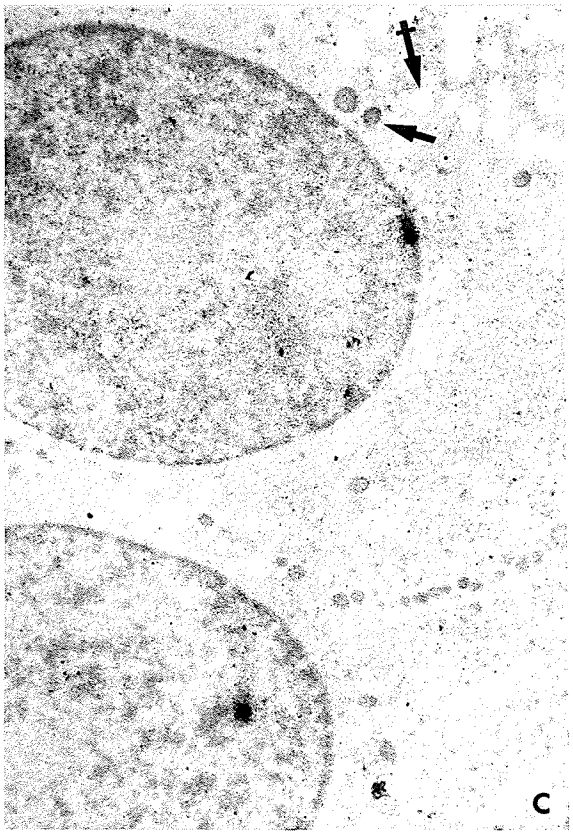

c

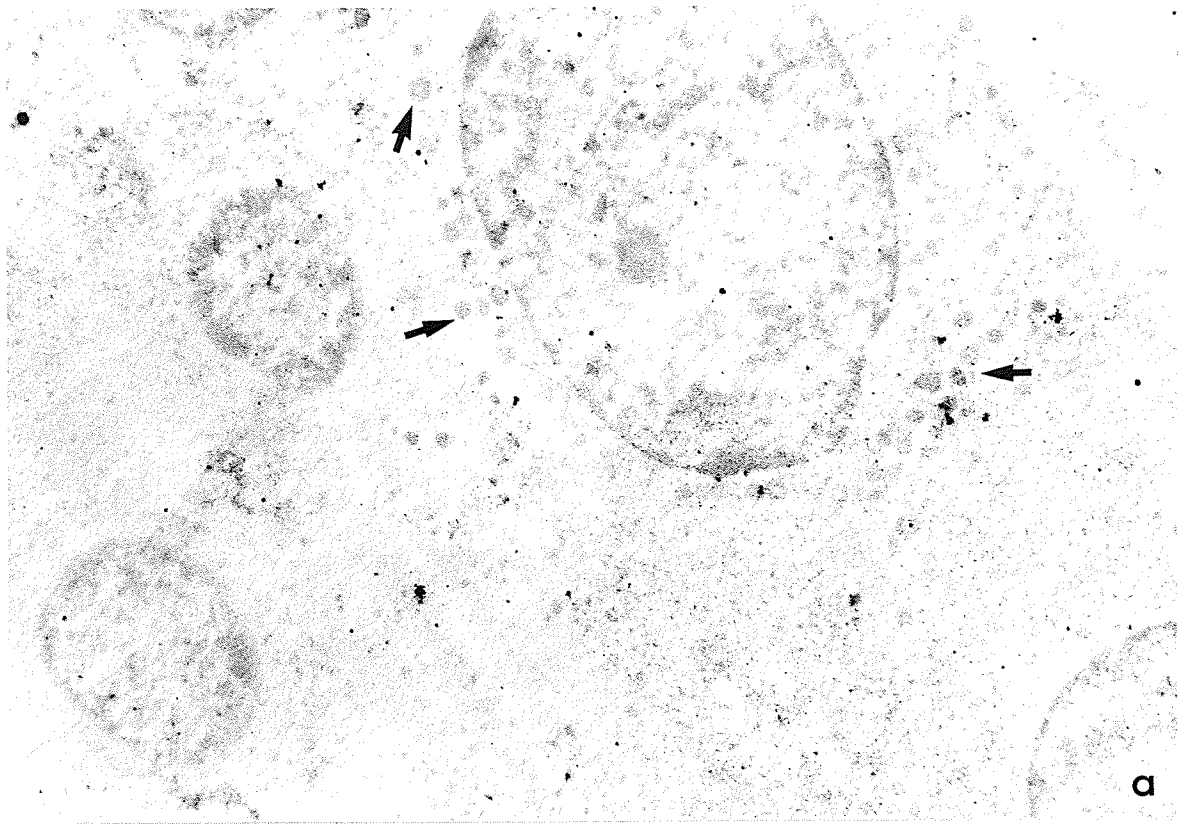
Figure 16(a-c)

Micrographs of the anterior pituitary from a 75 mm C.R. length male specimen (11.5 weeks gestation), stained immunocytochemically with LHβ antiserum diluted at 1/10,000.

16(a). Numerous lightly stained granules (arrows) are observed throughout the cytoplasm of a cell. X10,000

16(b). Higher magnification of the granules (arrows) depicted in Figure 16(a) showing a light reaction product (arrows). X25,000

16(c). A cell from an area adjacent to those in Figure 16(a) showing several moderately stained granules (arrows). X25,000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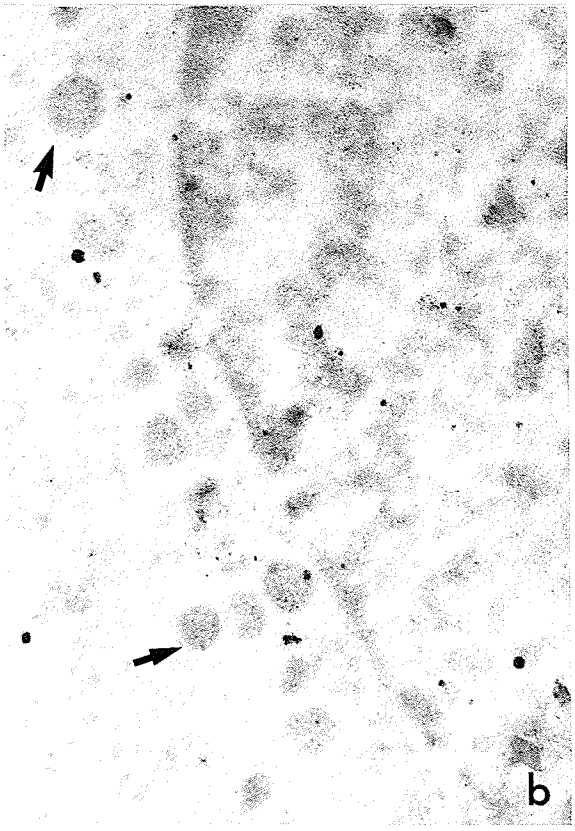
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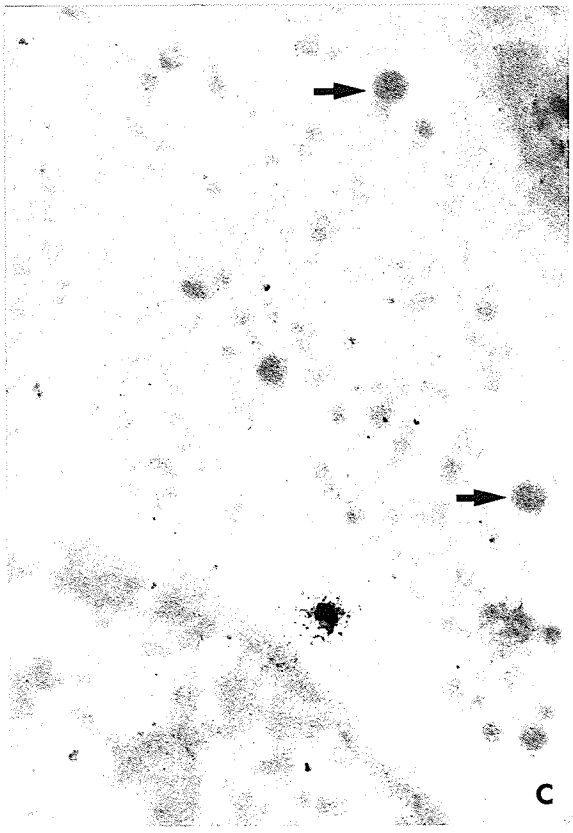

c

Figure 17(a-c)

Micrographs of the anterior pituitary from a 144 mm C.R. length female specimen (16.5 weeks gestation), stained immunocytochemically with LHβ antiserum diluted 1/10,000.

17(a). Numerous moderately stained (crossed arrow) and heavily stained (arrow) granules are observed in different cells. X10,000

17(b). Higher magnification of heavily stained granules (arrow) observed in Figure 17(a). X25,000

17(c). Higher magnification of moderately stained granules (arrow). X25,000

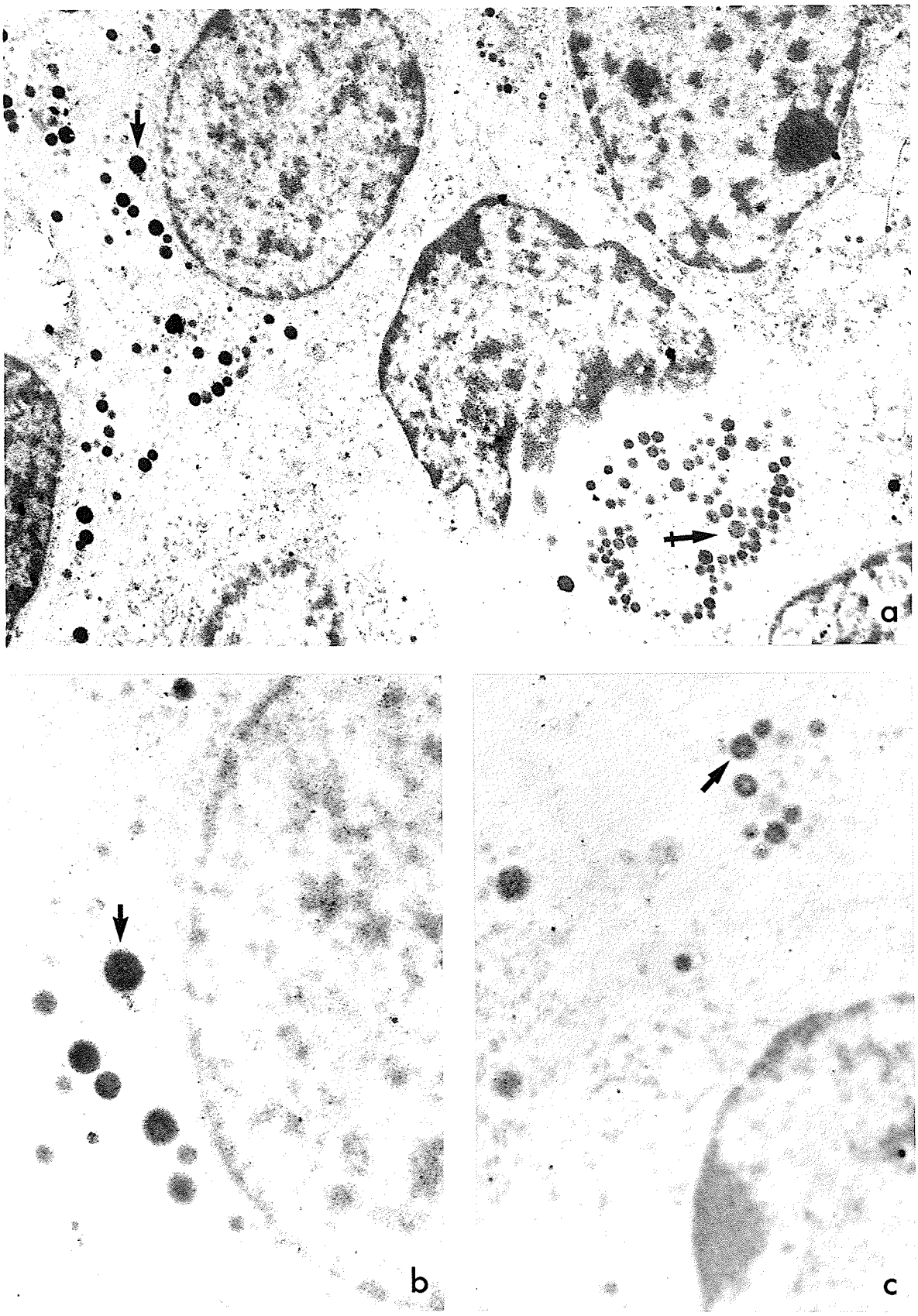

a
b
c

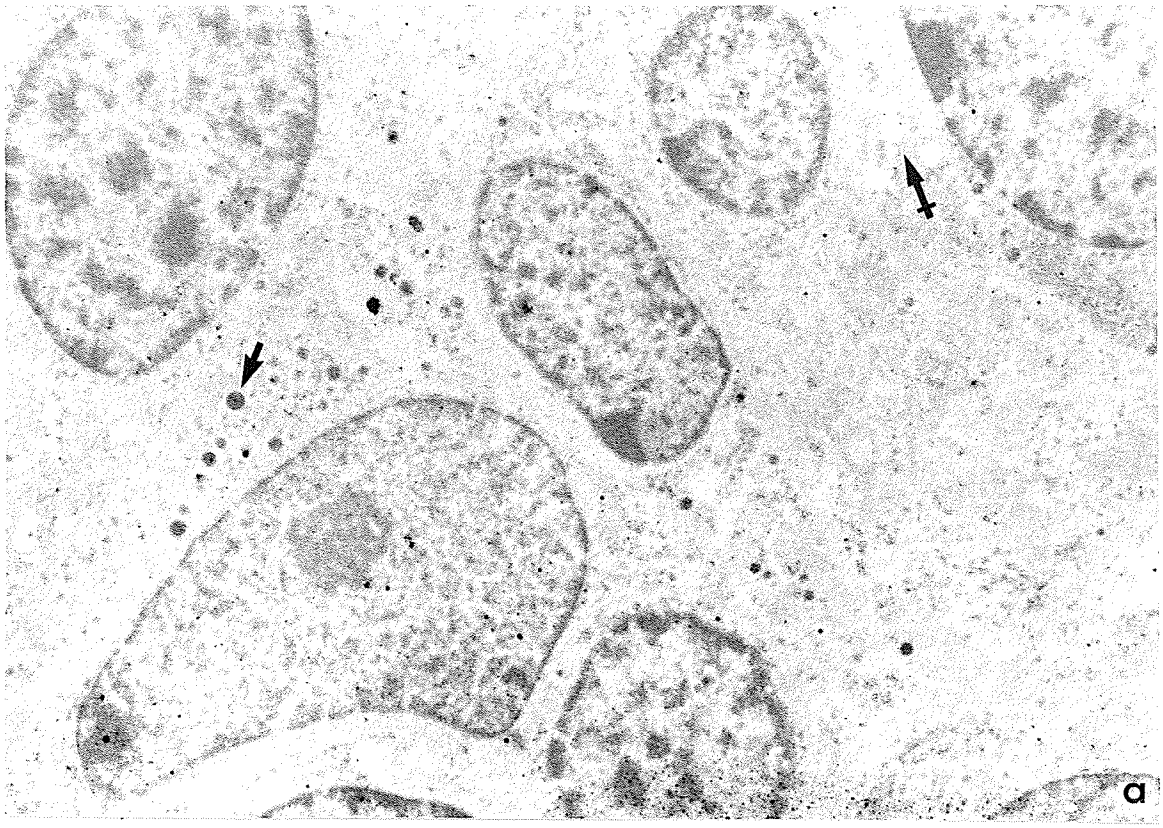
Figure 18(a-c)

Micrographs of the anterior pituitary of a 138 mm C.R. length male specimen (16 weeks gestation), stained immunocytochemically with LHβ antiserum at a 1/10,000 dilution.

18(a). Unstained (crossed arrow) and moderately stained (arrows) granules are observed in different cells. X10,000

18(b). Higher magnification of moderately stained granules (arrow) depicted in Figure 18(a). X25,000

18(c). A cell adjacent to the area depicted in Figure 18(a), revealing several moderately stained granules (arrow), with the reaction product clearly visible over the "PAP" complexes. X25,000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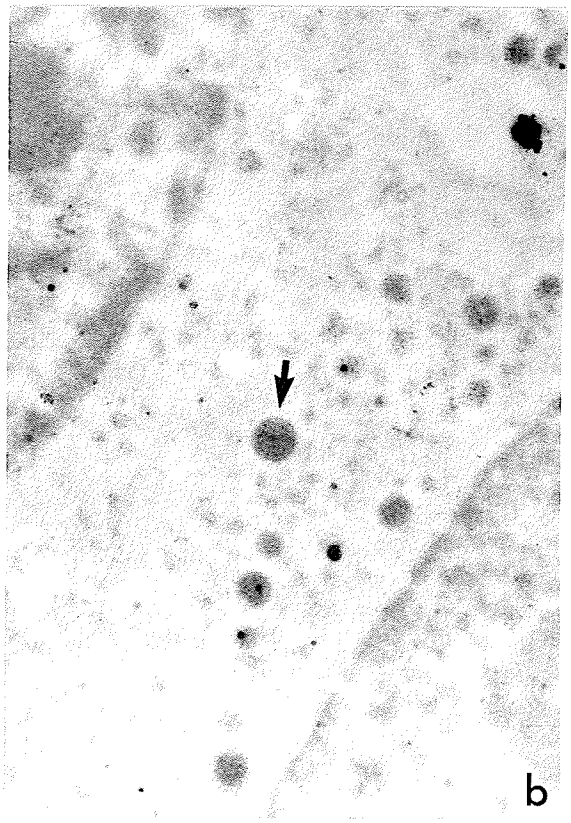
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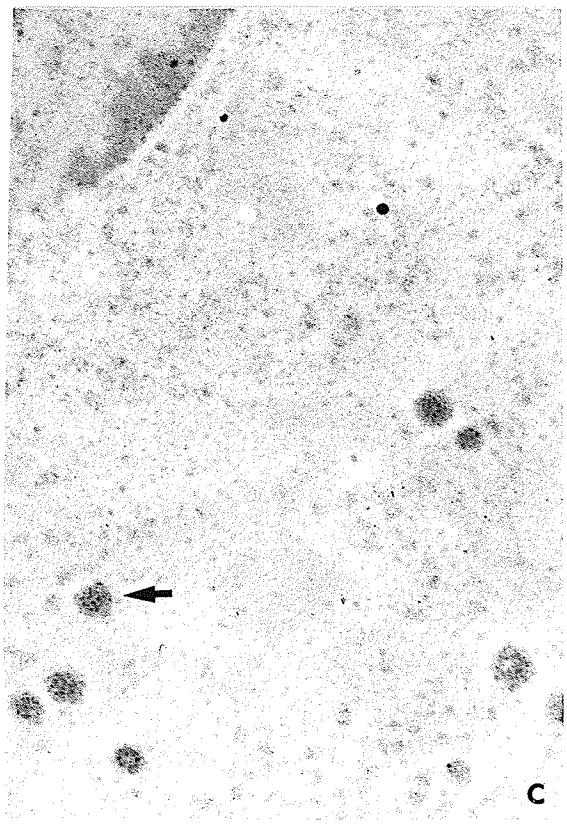

c

Figure 19(a-c)

Micrographs of the anterior pituitary of a 44 mm C.R. length female specimen (9.5 weeks gestation), stained immunocytochemically with FSHβ antiserum at a 1/10,000 dilution.

19(a). Several moderately stained granules (arrow) adjacent to one another are observed. X10,000

19(b). Higher magnification of the granules (arrow) depicted in Figure 19(a) showing reaction product clearly visible over the "PAP" complexes on the granules. X25,000

19(c). Two small groups of moderately stained granules (arrows) can be observed on the upper right and lower left of the micrograph. X10,000

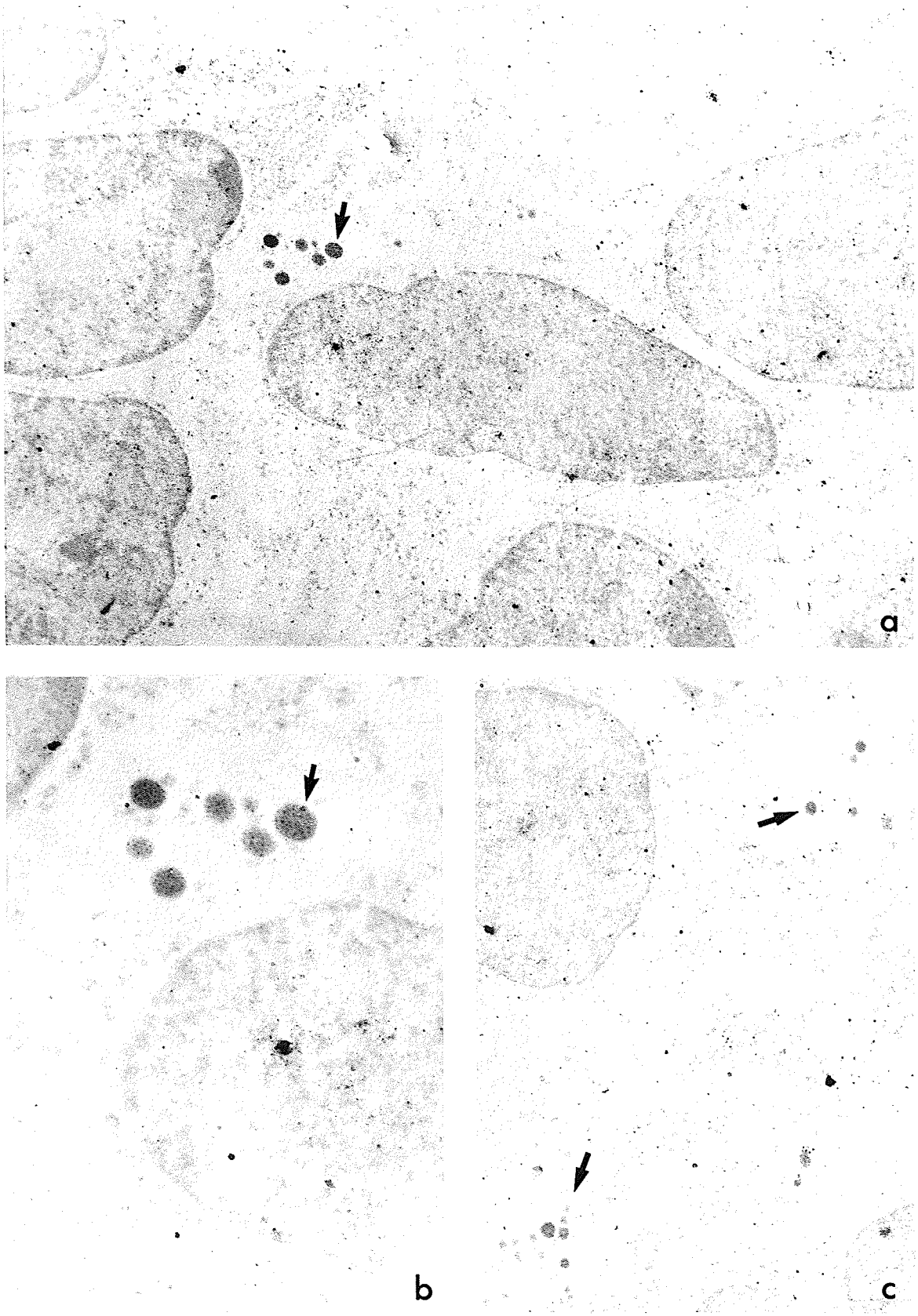

a
b
c

Figure 20(a-c)

Micrographs of the anterior pituitary of a 45 mm C.R. length male specimen (9.5 weeks gestation), stained immunocytochemically with FSHβ antiserum diluted 1/10,000.

20(a). A small group of several moderately stained granules (arrow) are observed. X10,000

20(b). Higher magnification of granules (arrow) depicted in Figure 20(a) show reaction product over the "PAP" complexes. X25,000

20(c). Three moderately stained granules (arrow) are observed close to several unstained granules (crossed arrow). X25,000

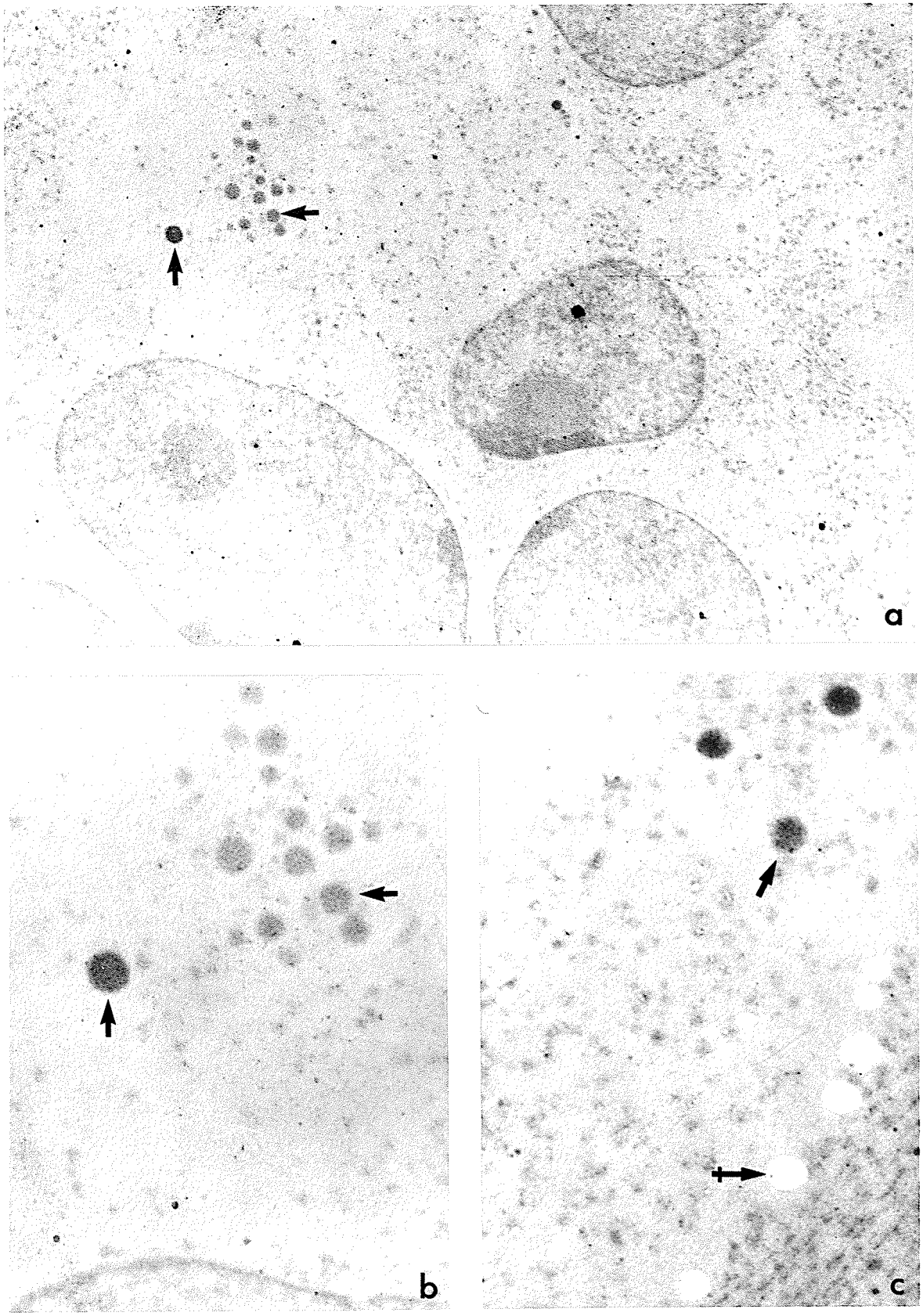

a
b
c

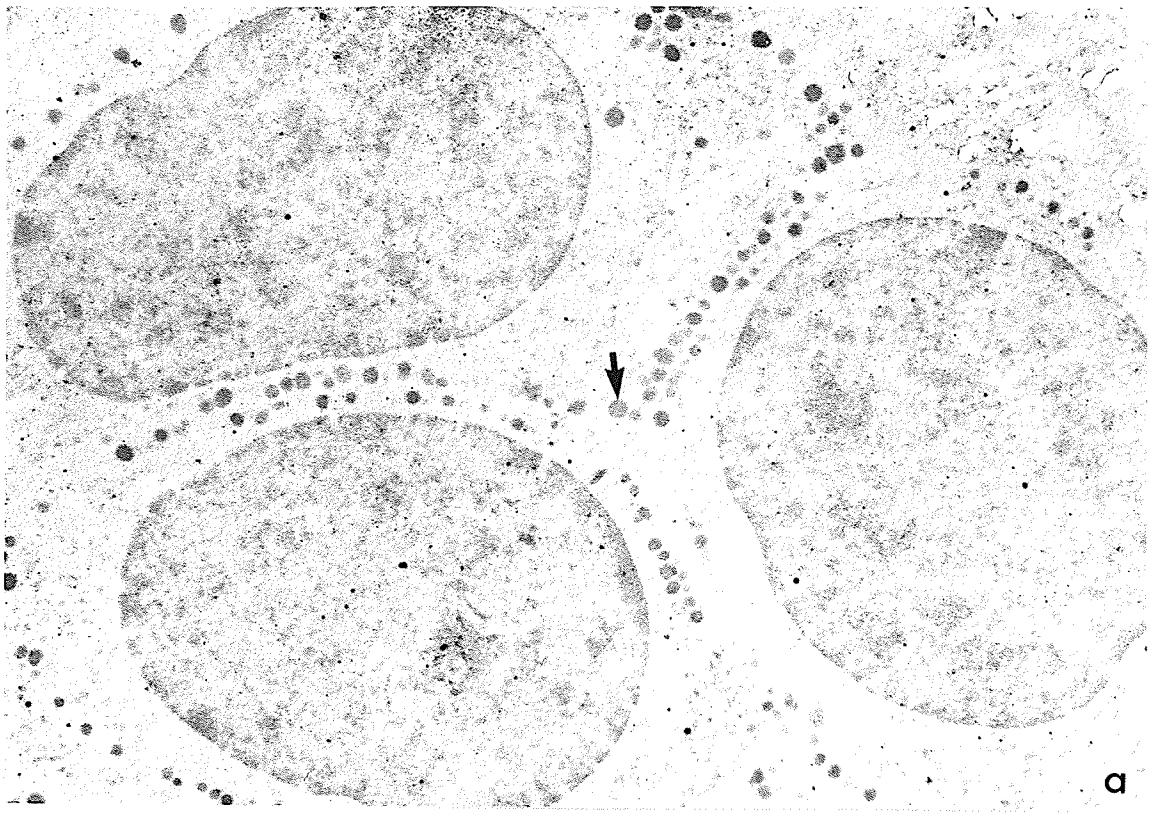
Figure 21(a-c)

Micrographs of the anterior pituitary of a 78 mm C.R. length female specimen (12 weeks gestation), stained immunocytochemically with FSHβ antiserum at a 1/10,000 dilution.

21(a). Numerous, moderately stained granules (arrow) are observed in adjacent cells. X10,000

21(b). Higher magnification of granules (arrow) depicted in Figure 21(a). X25,000

21(c). An area of the pituitary adjacent to the one depicted in Figure 21(a) exhibiting lightly stained granules (arrow) throughout the cytoplasm of a cell. X25,000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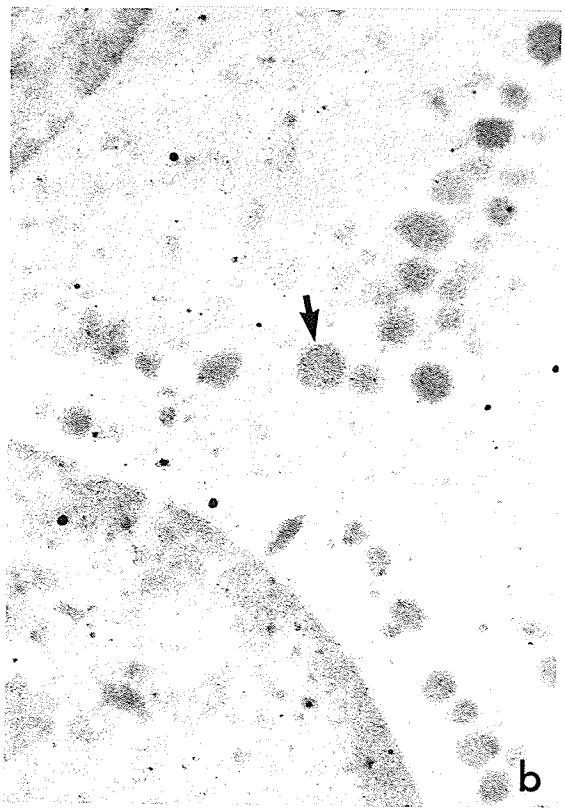
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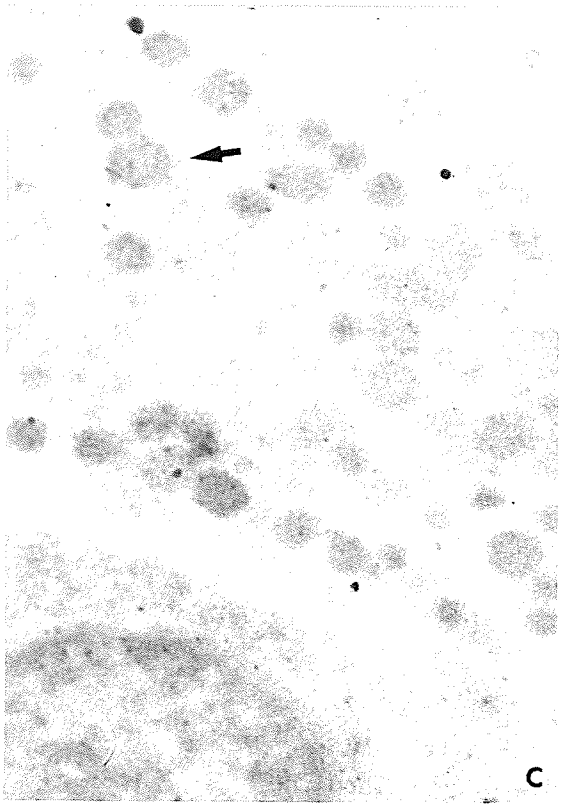
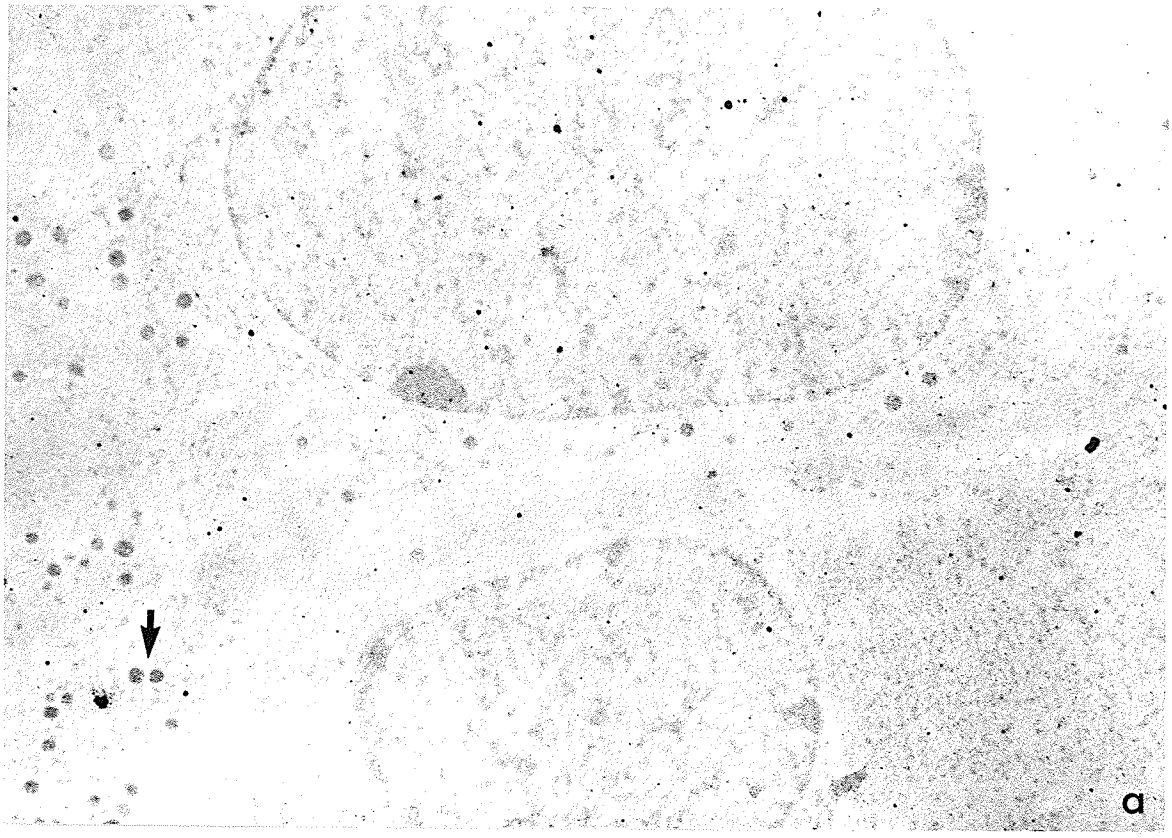

c

Figure 22(a and b)

Micrographs of the anterior pituitary of a 75 mm C.R. length male specimen (11.5 weeks gestation), stained immunocytochemically with FSHβ antiserum diluted 1/10,000.

22(a). Numerous lightly and moderately stained granules (arrow) appear distributed throughout the cytoplasm and along the periphery of the cell. X10,000

22(b). Higher magnification of granules (arrow) depicted in Figure 22(a) illustrating the reaction product over the granules. X25,000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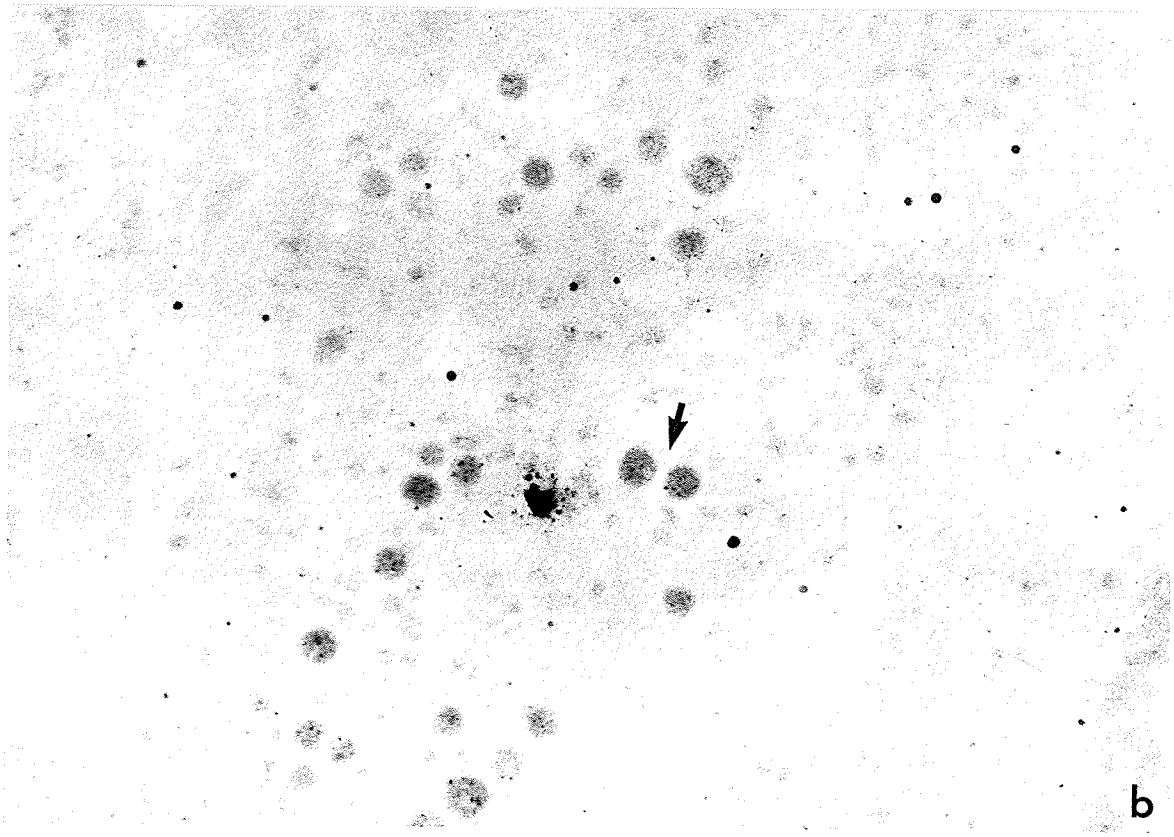

b

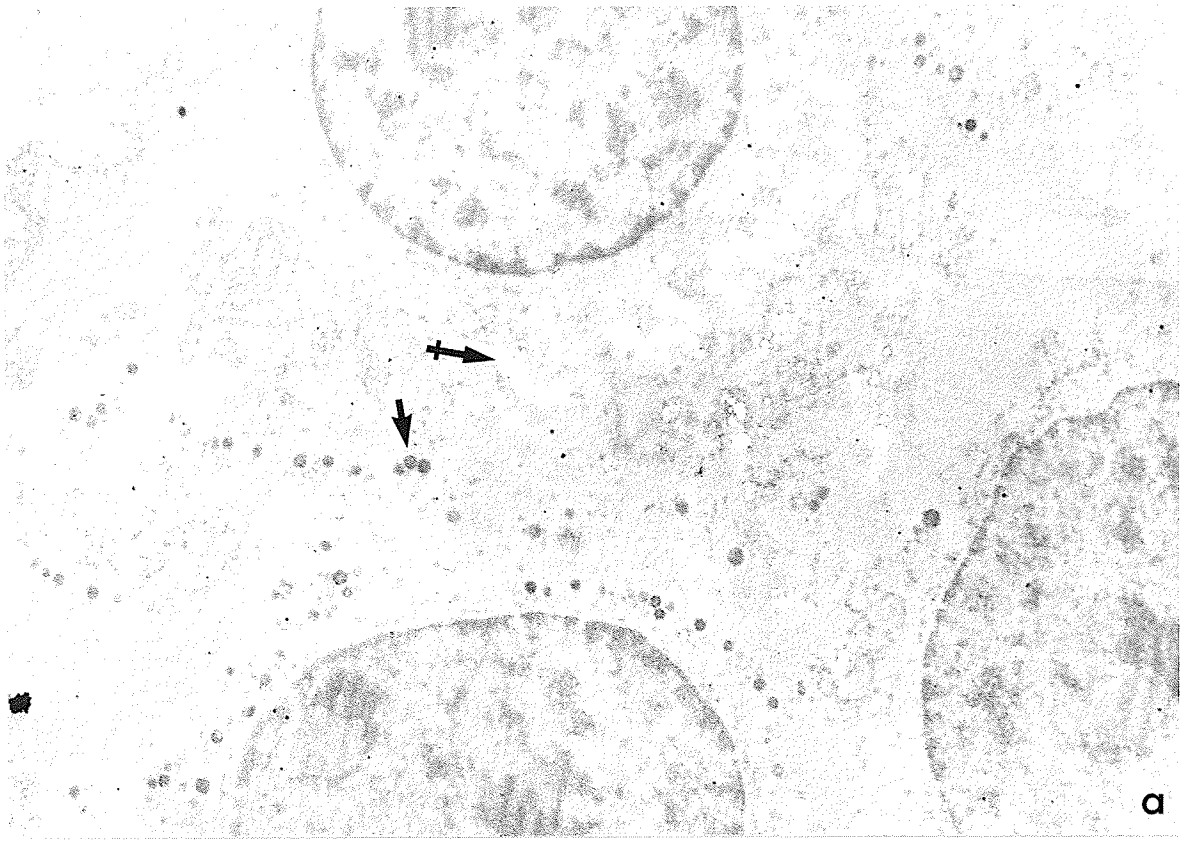
Figure 23(a-c)

Micrographs of the anterior pituitary of a 144 mm C.R. length female specimen (16.5 weeks gestation), stained immunocytochemically with FSHβ antiserum diluted 1/10,000.

23(a). Cell containing numerous lightly stained granules (arrow) and a cell containing numerous unstained granules (crossed arrow) are observed adjacent to each other. X10,000

23(b). Higher magnification of unstained (crossed arrow) and stained (arrow) granules depicted in the cells from Figure 23(a). X25,000

23(c). Numerous, heavily stained granules (arrow) dispersed throughout the cytoplasm are observed. Reaction product over the "PAP" complexes on the granules can be seen. X25,000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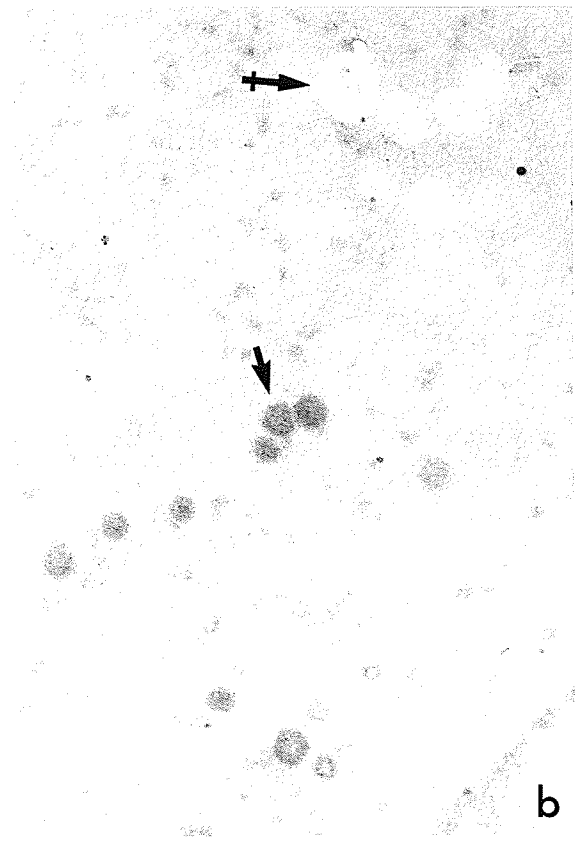
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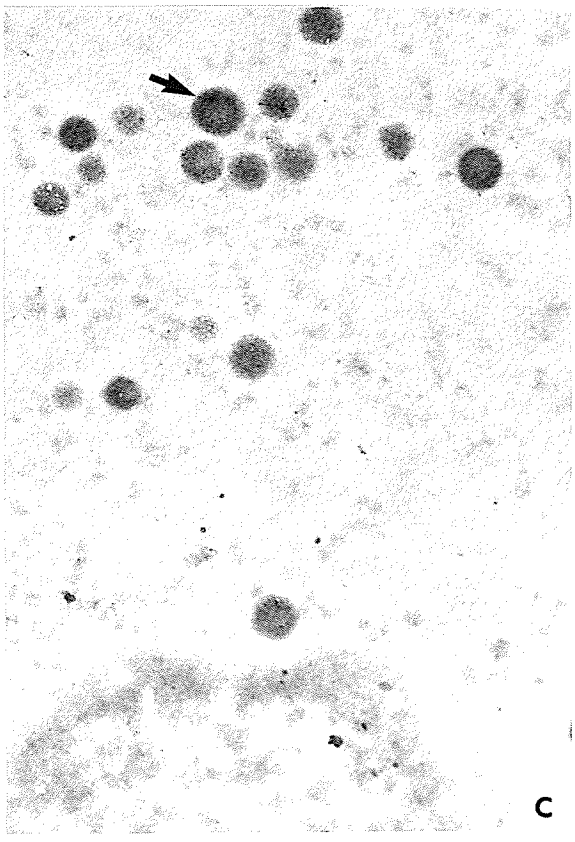

c

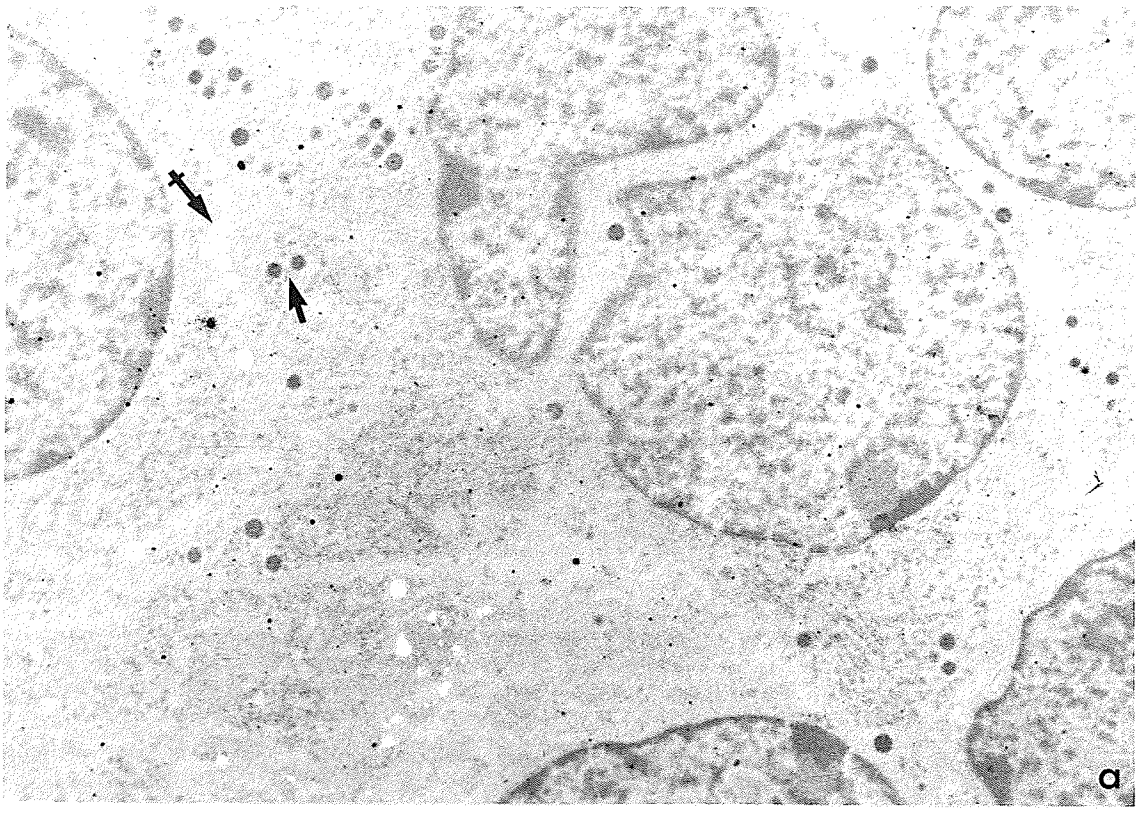
Figure 24(a-c)

Micrographs of the anterior pituitary of a 138 mm C.R. length male specimen (16 weeks gestation), stained immunocytochemically with FSHβ antiserum at a 1/10,000 dilution.

24(a). A number of moderately stained granules (arrow) and unstained granules (crossed arrow) appear in different cells. X10,000

24(b). Higher magnification of stained granules (arrow) and unstained granules (crossed arrow) depicted in Figure 24(a) are illustrated. X25,000

24(c). Numerous moderately stained granules (arrow) throughout the cytoplasm are seen. X10,000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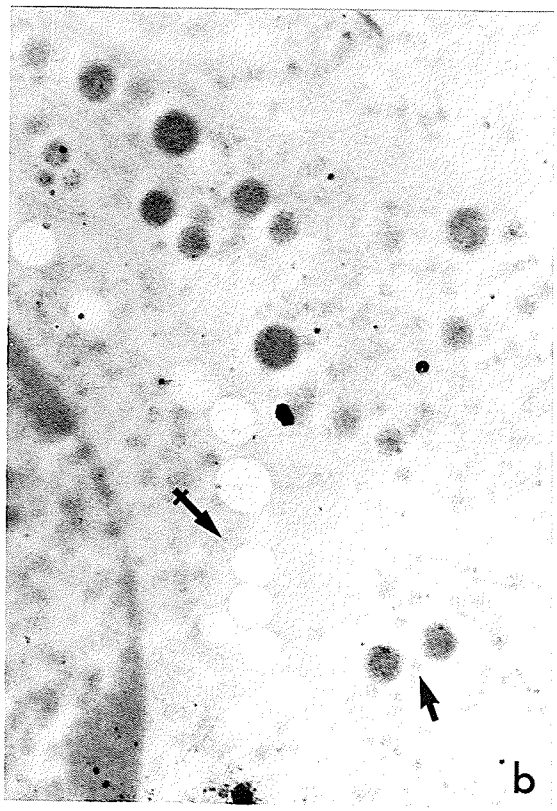
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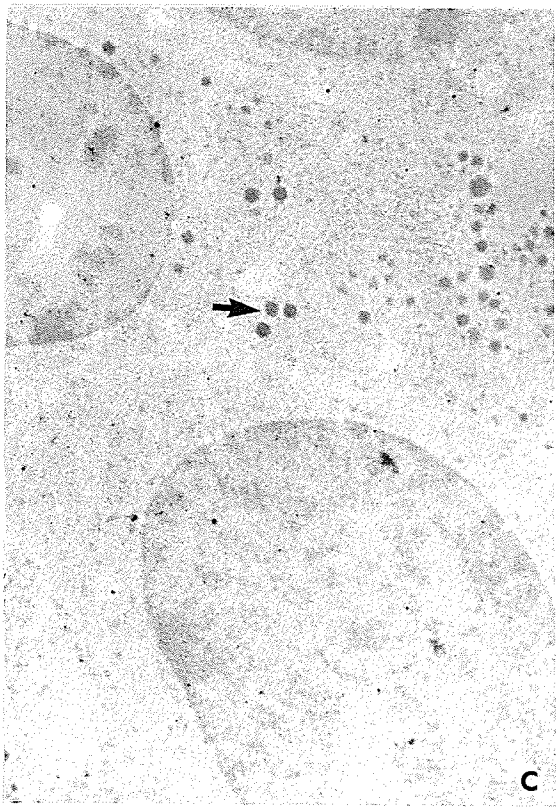

c

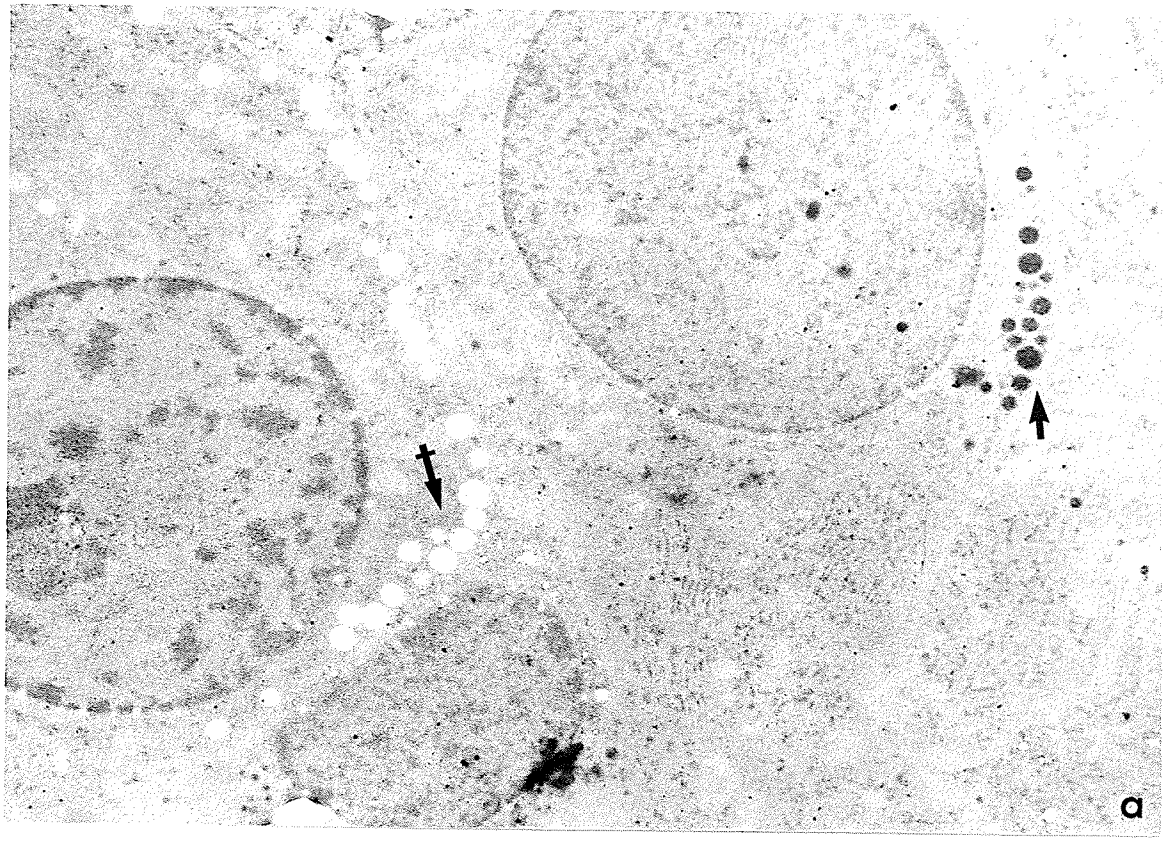
Figure 25(a-c)

Micrographs of the anterior pituitary of a 138 mm C.R. length male specimen (16 weeks gestation), stained immunocytochemically with FSHβ antiserum at 1/10,000 dilution.

25(a). Heavily stained granules (arrow) and unstained granules (crossed arrow) are observed in cells adjacent to one another. X10,000

25(b). Serial section of the immunocytochemically stained cell shown in Figure 25(a). Note the similarity in the staining intensity of the granules (arrow) in both sections. X10,000

25(c). Higher magnification of granules (arrow) depicted in Figure 25(b) clearly illustrating reaction product over "PAP" complexes. X25,000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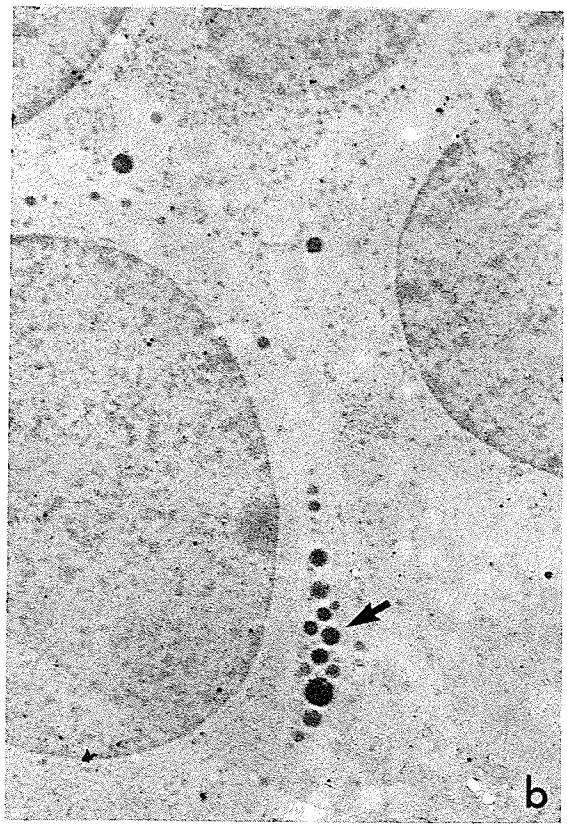
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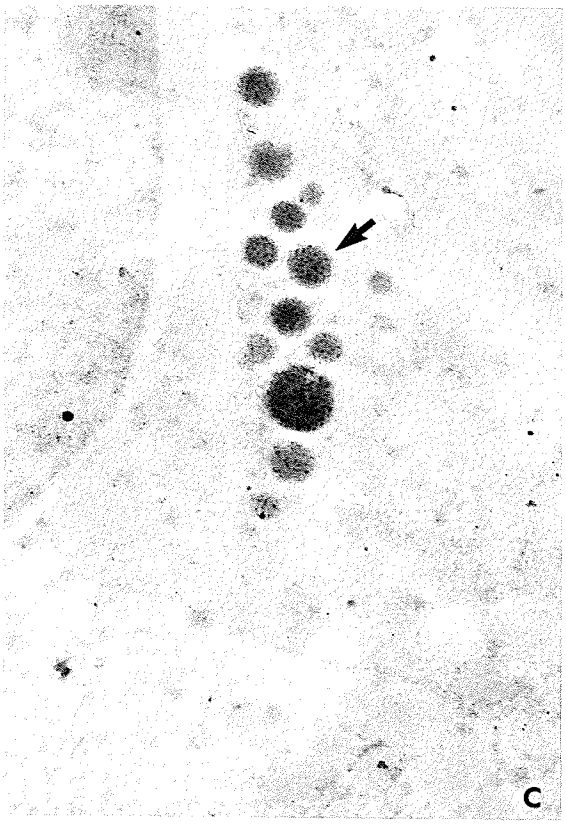
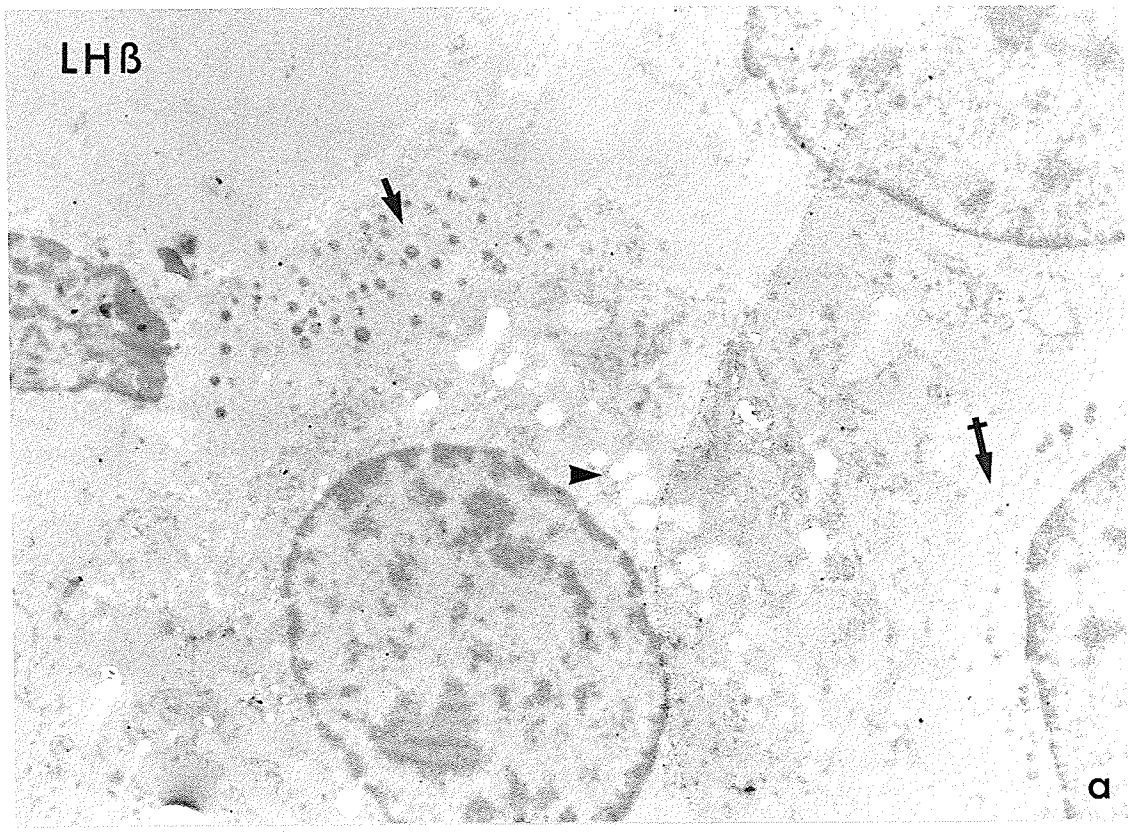

c

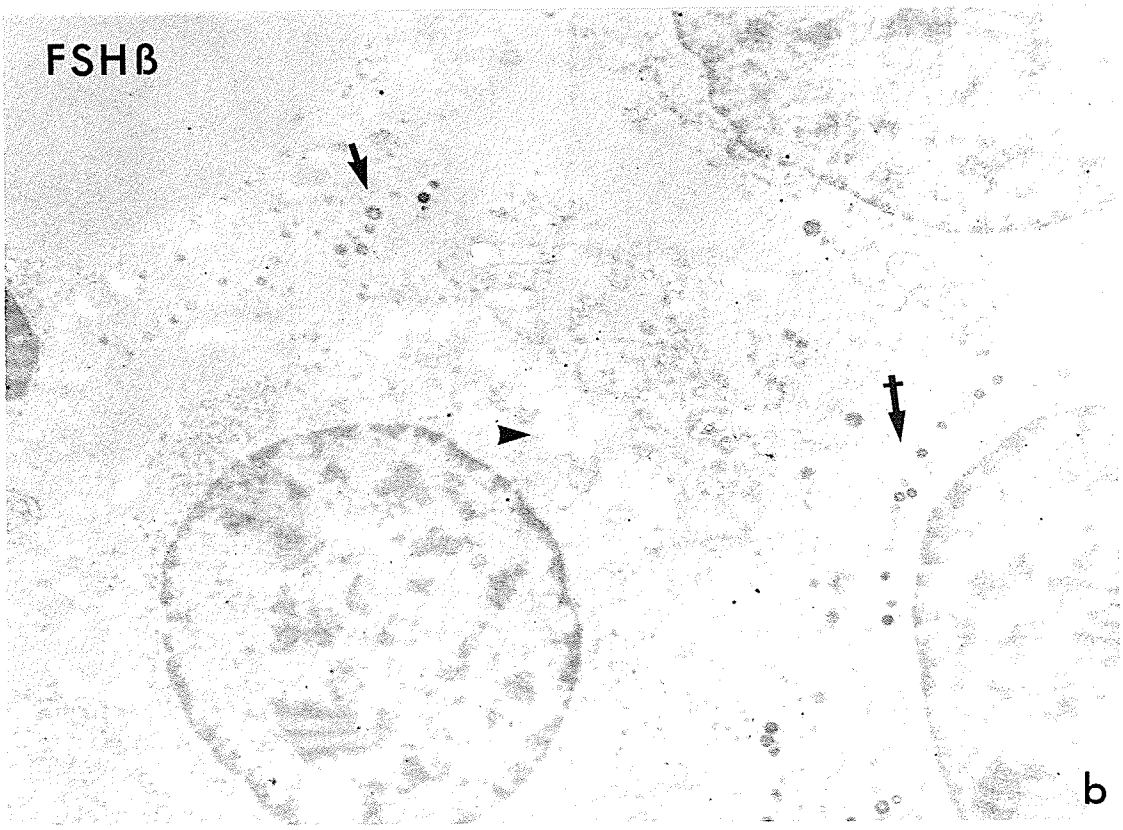
Figure 26(a and b)

Micrographs of the anterior pituitary of a 144 mm C.R. length female specimen (16.5 weeks gestation). Serial sections of the same cells are shown.

26(a). Staining with LHβ antiserum, at a 1/10,000 dilution, reveals moderately stained granules (arrow), lightly stained granules (crossed arrow) and unstained granules (arrowhead) in different cells. X10,000

26(b). Staining with FSHβ antiserum, diluted 1/10,000, also reveals lightly stained granules (arrow), moderately stained granules (crossed arrow) and unstained granules (arrowhead) in the same cells depicted in Figure 26(a). Note that the moderately stained granules, Figure 26(a)(arrow) and 26(b) (crossed arrow), are in different cells. X10,000


LHß
a


FSHß
b

DISCUSSION

ROUTINE ELECTRON MICROSCOPY

In the present investigation after careful assessment of the material studied, it became apparent that routine electron microscopy alone was inadequate in determining which granulated cells produced FSH and LH. This was due to the similar appearance all granulated cells had to one another in the youngest specimens as well as to the continuing differentiation these cells exhibited during the gestational period studied. Granulated cells were present in the anterior pituitaries of the youngest specimens (9.5 to 10 weeks gestation); however, undifferentiated cells or stem cells were predominant. At 9.5 weeks gestational age, cell types could not be distinguished among the granulated cells because of the homogeneity of granule size, density, distribution, and disposition of other cytoplasmic organelles. In older specimens (14.5 to 16.5 weeks gestation) granulated cells were predominant in the anterior pituitary and several distinct cell types, based on heterogeneity of organelles and inclusions, could be identified. The observations noted above confirm the difficulty experienced by others (Dubois and Dumont, 1965, 1966; Dumont and Dubois, 1967; Dubois, 1968, Anderson et al., 1970, 1971) in identifying not only gonadotropes but any cell type with respect to the specific hormone(s) produced. The changing proportion of granulated cells and nongranulated cells of the anterior pituitary through early human fetal development observed by others (Dubois and Dumont, 1966; Dumont and Dubois, 1967; Dubois, 1968) was also noted in the present investigation. In the aforementioned ultrastructural studies no reference was made to morphological differences due to the sex of the

specimen. This is in contrast to the results of the current investigation which suggested that the older female specimens (15 to 16.5 weeks gestation) were more active in producing hormones than male specimens of a comparable age. This was illustrated by the fact that cells in the female pituitary were repeatedly seen containing dilated rough endoplasmic reticulum and more granules per cell, both phenomena being indicative of increased cellular activity (Herlant, 1964). Other investigators using radioimmunoassay (Clements et al., 1976) have also reported a sex difference in that higher concentrations of LH and FSH were observed in the pituitaries and sera of female fetuses than in male specimens ranging from 12 to 20 weeks gestational age. It can be postulated, therefore, that the ultrastructural differences observed between the sexes in the present study was in part due to the higher production and storage of gonadotropins in the female specimens; however, identification of the hormones produced by these cells can only be answered with immunocytochemical electron microscopy.

IMMUNOCYTOCHEMICAL ELECTRON MICROSCOPY

Method and Specificity Controls

For reasons previously discussed, identification of gonadotropes in the human fetal pituitary using morphological criteria established by routine electron microscopy was shown to be unreliable. Therefore, immunocytochemical staining of the anterior pituitary was done using LHβ and FSHβ antisera to identify gonadotropes.

The validity of the electron microscopic immunocytochemical results presented here depends upon the specificity and cross-reactivity of the antisera used in the current investigation.

For the dilution and absorption controls, one pituitary was used, from a 144 mm C.R. length (16.5 weeks gestation), female specimen. This was chosen for the following reasons:

1. As previously reviewed, others have localized detectable levels of LH and FSH in the pituitary of 16 week human fetuses using light microscopic immunohistochemistry (Dubois and Dubois, 1974; Dubois et al., 1975, 1978; Bugnon et al., 1976a,b, 1977).

2. Higher levels of LH and FSH have been reported to be present at 16 weeks than earlier in gestation, determined by radioimmunoassay (Clements et al., 1976).

3. Pituitaries of female specimens were reported to have higher concentrations of LH and FSH than those of male specimens, of a comparable age (Clements et al., 1976; Kaplan et al., 1976).

For these reasons, it was thought that the 16.5 week female specimen would afford the heaviest stain, and that more gonadotropes would be present at this stage than in younger specimens and this would make the stain more easily detectable.

Serial dilutions of the primary antisera or normal rabbit serum revealed a progressive decrease in the normalized staining intensity to undetectable levels. This observation indicated that the immunocytochemical stain was due to the primary antiserum, and not to some other unknown factor. The dilution curves indicated that the specificity of both antisera was dependent upon their dilution (Van Leeuwen and Swaab, 1977). At dilutions of 1/500 and 1/1000, numerous granules in most cells were stained by both LHβ and FSHβ antisera, as well as by normal rabbit serum. As concentrations were decreased, i.e., at 1/10,000

dilution, staining was observed in far fewer cells, and detected only when using the two primary antisera. At the low dilutions of 1/500 and 1/1000 the antisera must have cross-reacted with several different antigenic sites to stain so many granules. The immunocytochemical stain observed when normal rabbit serum, at higher concentrations, was substituted for the primary antiserum was probably due to an adsorption phenomenon, i.e., the binding of rabbit immunoglobulins to the tissue sections, and the subsequent binding of the goat antirabbit IgG and rabbit peroxidase antiperoxidase complex.

As a result of the findings in the dilution controls, a working dilution (Moriarty et al., 1973), of 1/10,000 was chosen, for the absorption tests and staining of all the other specimens studied. At a 1/10,000 dilution it was noted that both LHβ antiserum and FSHβ antiserum stained granules of a similar size with similar intensity. Moreover, as one recalls the results from the routine electron microscopy, the anterior pituitaries of the youngest specimens had cells which contained granules of a similar size, density and distribution. Therefore from these two observations, it became clear that positive identification of gonadotropes regardless of the age of the fetus, would depend on the specificity of the primary antiserum. Furthermore, specificity of the antiserum becomes even more critical when one considers the likelihood of the same cell producing two or more different hormones as has been demonstrated for LH and FSH by light microscopic immunohistochemistry in both adult (Phifer et al., 1973; Pelletier et al., 1976) and fetal human anterior pituitaries (Bugnon et al., 1976a,b, 1977; Dubois et al., 1978). Similar observations have been noted using

immunocytochemistry at the ultrastructural level in the pituitaries of the adult rat (Moriarty, 1976) and human (Pelletier et al., 1978). For these reasons, an extensive series of absorption controls were done for each antiserum to determine if they cross-reacted with any other suspected antigens.

Swaab et al. (1977) and Hutson et al. (1979) questioned the usefulness of absorption tests alone to prove the specificity of an antiserum. These authors speculated that homologous absorptions prove no more than that all antibodies were bound to the added antigen when staining decreased to undetectable levels. Swaab et al. (1977) stated that homologous absorptions do not exclude the possibility of staining from two sources, namely unexpected or unwanted antibodies raised because of impurities in the injected antigen which then might be used in the absorption test. Most antigens have to be purified from biological material and will thus never be pure (Reichert, 1975; Stockell-Hartree, 1975). Secondly, cross-reactivity of antibodies with heterologous antigens is not excluded. Heterologous absorptions, although not completely excluding staining from unwanted or unexpected antibodies, or from cross-reactivity of the antibodies, can yield valuable information about the specificity of the antiserum. In the present study, the antisera were each absorbed with heterologous antigens and intact hormone with which they might cross-react.

The structural similarities of the glycoprotein hormones and their subunits (reviewed by Guidice and Pierce, 1978) are the basis for any cross-reactivity. The glycoprotein hormones LH, FSH, TSH and CG

each consist of an α and β subunit. The amino acid sequence is very similar or identical for all the α subunits within a species. Although the β subunits also have some similarities in amino acid sequence, distinct differences give the hormone its own characteristics, including biological activity. However, similarities in the β subunits can lead to cross-reactivity of β antisera with heterologous antigens. In addition, the α subunit can lead to cross-reactivity of antisera to intact hormone with other glycoprotein hormones (reviewed by Vaitukaitis, 1978).

In the current investigation, LHβ antiserum was equally sensitive to its homologous antigen, LHβ, and the intact hormone, LH. In addition, the "final" regression lines of the LHβ and LH absorptions of LHβ antiserum were parallel and indicated 100% cross-reactivity of intact LH with LHβ antiserum. Also, the "final" regression lines of the LHβ and FSHβ absorptions of LHβ antiserum were parallel, again indicating 100% cross-reactivity. However, a 100-fold increase in the concentration of FSHβ over that of LHβ was required to produce this cross-reactivity. Intact FSH, as well as TSHβ and CGβ subunits, each partially cross-reacted with LHβ antiserum. This was revealed by the "final" regression lines of these antigens not being parallel to that of the homologous absorption. In addition, much higher concentrations of these antigens were required to produce the partial cross-reactivity observed. The CGα antigen did not appear to cross-react with the LHβ antiserum, even though the regression line had a significant correlation coefficient, and a slight negative slope. From the foregoing

discussion, the LHβ antiserum appeared to be 100 times more sensitive to either LHβ or intact LH, than any of the other antigens tested.

FSHβ antiserum was also more sensitive to its homologous antigen than any of the other antigens tested. The "final" regression lines of the FSH and LHβ antigen absorption of FSHβ antiserum were parallel to the "final" regression line of the homologous absorption. This also indicated 100% cross-reactivity between the FSHβ antiserum and FSH and LHβ; however in both instances a 10 and 100 fold increase respectively, in the concentration of the antigen was required over that of the homologous absorption, to completely abolish the immunocytochemical stain. The intact LH appeared not to cross-react; however, the regression line did have a significant correlation coefficient and a slightly negative slope.

As shown by the absorption tests, FSHβ antiserum did not cross-react with the TSHβ or CGβ subunits. Absorption of the FSHβ antiserum with CGα subunit revealed no apparent cross-reactivity; however, at the highest concentrations of antigen tested, the result was inconclusive. From the absorption of FSHβ antiserum, it was apparent that this antiserum was also about 10 times and 100 times more sensitive to its homologous antigen than the intact FSH and any of the other antigens, respectively, tested.

In this study, the LHβ antiserum did not selectively distinguish between LHβ and LH. The FSHβ antiserum was more sensitive to FSHβ than FSH, however the serum was even less sensitive to LHβ or any of the other antigens tested. Kaplan et al. (1976) reported the presence of immunoreactive intact LH and FSH in a 10 week human fetal pituitary. However, these investigators were unable to detect the presence of free β subunits.

Although in the present study, both β antisera produced a positive stain in the pituitaries of all specimens studied, there is little supportive evidence for the presence of either free LHβ subunit or free FSHβ subunit. Therefore, due to the evidence just presented, and that of the absorption tests, it was thought that the stain observed was due to the cross-reactivity of LHβ and FSHβ antisera with intact LH and FSH, respectively.

Additional observations in this study also indicated that each antiserum bound separate and specific antigens. In the youngest specimen studied, LHβ and FSHβ antisera, each stained a distinctly different population of granules. Similarly, the immunocytochemical data supports the physiological changes reported during pituitary development and the differences noted between sexes (Clements et al., 1976).

Ultrastructural Localization of LH and FSH

Both LHβ and FSHβ antisera stained granules throughout the developmental period studied (9.5 to 16.5 weeks gestation). The earliest appearance of gonadotropes in the human fetal pituitary has been reported to be at eight weeks for the detection of α subunits, but not until 15 to 23 weeks gestation for the detection of β subunits (Dubois and Dubois, 1974; Baker and Jaffe, 1975; Dubois et al., 1975, 1978; Bugnon et al., 1976a,b, 1977). Considering the previously mentioned report of Kaplan et al. (1976), it seems likely that the above mentioned authors observed the presence of the β subunit in combination with the α subunit, i.e., as intact hormone. In the present investigation, neither LHβ nor FSHβ antiserum recognized the α subunit; and, as previously discussed, the positive stain seen at 9.5 weeks gestation was due to the

presence of β subunits in combination with α subunits, i.e., intact hormone. The current findings, therefore, are in contrast to the above mentioned observations, regarding immunohistochemical detection of β subunit or intact hormone at 15-23 week gestational age. The apparent discrepancies between these studies and the present investigation were most likely due to a number of factors. Firstly, the differences in the resolution of the microscopes used, i.e., the light stain observed by electron microscopy in the youngest specimens in all likelihood represents low hormone concentrations which were probably not detectable at the light microscopic level. Secondly, the mode of abortion, i.e., hysterotomy versus saline, prostaglandins, or spontaneous, in procurement of the material for study is critical for optimal cellular preservation and hence localization of tissue antigens. Thirdly, antibodies against LHβ and FSHβ of different species, i.e., ovine, porcine and human, do not all bind with the same avidity to human LH or FSH as observed by Baker and Jaffe (1975). Support for the current observations can be found in the radioimmunoassay studies of Kaplan et al. (1976) and Clements et al. (1976), in which detectable levels of intact LH and FSH were present as early as 10 weeks gestation.

In the youngest specimens examined in the current study, LHβ antiserum and FSHβ antiserum revealed a different staining pattern. LHβ antiserum stained numerous granules lightly, often in several adjacent cells, whereas FSHβ antiserum moderately stained few granules, in isolated cells. If the relative number of granules observed is indicative of the hormone content of the fetal pituitary, it can be postulated from these results that the pituitaries of the youngest specimens examined might contain more LH than FSH. This contention is supported by the work of Clements et al. (1976) and Kaplan et al. (1976) who have reported higher levels of LH than FSH in the human fetal pituitary during early gestation.

In the present study, with increasing fetal age the numbers of cells containing immunostained granules increased markedly. In addition, the staining intensity of some granules was very heavy in the older specimens. These observations are in agreement with those of Cho et al. (1978), who reported similar findings using light microscopic immunohistochemistry. Hence, it can be postulated that the aforementioned observations were indicative of increasing levels of LH and FSH in the anterior pituitary as a function of age. Support for this contention is found in the reports of several investigators, who noted an increase in assayable levels of pituitary gonadotropins from 12 to 18 weeks gestation (Reyes et al., 1974, 1976; Clements et al., 1976; Kaplan et al., 1976).

In the current investigation, the immunocytochemical electron microscopic observations revealed an apparent sex difference. The older female specimens, 15 to 16.5 weeks gestation, appeared to have more cells containing stained granules, and often more stained granules per cell than that observed in male specimens of comparable age. Further morphological evidence for a sex difference in the human fetal pituitary can be found in the studies by Dubois and Dubois (1974) and Dubois et al. (1975, 1978). These investigators, using light microscopic immunohistochemistry, detected the presence of LHβ in the pituitaries of only female human fetuses at 16 weeks gestation, and did not observe LHβ in male fetuses until 20 weeks gestation. In addition, Reyes et al. (1974, 1976), Clements et al. (1976), Kaplan et al. (1976), and Winter et al. (1977) also reported a sex difference in that

LH and FSH levels in the pituitary and serum were higher in female than male specimens between 12 and 20 weeks gestational age.

Another observation of this investigation was the staining of granules within the same cell by both LHβ and FSHβ antisera. This phenomenon has been previously reported in the human fetal pituitary after 15 weeks gestation, using light microscopic immunohistochemistry (Bugnon et al., 1976a,b, 1977; Dubois et al., 1978), as well as in the pituitary of the adult rat (Moriarty, 1976b) and human (Pelletier, 1978) at the ultrastructural level. In the present study this feature was observed repeatedly on serial sections of the 16.5 week female specimen, which was used for the dilution and absorption tests. The same cells containing granules stained with LHβ and FSHβ antisera, exhibited only lightly or moderately stained granules. In addition, heavily stained granules were observed, with both LHβ and FSHβ antisera, but not in the same cell on serial sections. Hence, it can be speculated that by 16.5 weeks gestation, at least in the female fetus, the pituitary might contain three types of gonadotropes; one containing LH, a second containing FSH, and a third containing both LH and FSH. The cells revealing heavily stained granules probably contained only LH or FSH. Moreover, the possibility exists that both LH and FSH are present in a number of the same granules. A possible reason for the light or moderate stain observed in cells with granules stained with both LHβ and FSHβ antisera on serial sections, might be due to a lower concentration of the hormone. If the granules contained a mixture of LH and FSH, their staining intensities would be less than that of granules containing only LH or FSH. However, before any definitive conclusions can be drawn from the aforementioned

observations, further studies on serially sectioned cells would have to be performed.

DEVELOPMENT OF THE HUMAN FETAL HYPOTHALAMO-PITUITARY-GONADAL AXIS: CORRELATION OF STRUCTURE AND FUNCTION

The stimulus for the initial production and increase in levels of LH and FSH observed in the current study of the human fetal pituitary is probably GnRH from the hypothalamus. GnRH has been localized immunohistochemically in cell bodies in the hypothalamus, and in their axonal terminals within the median eminence, between nine and 11 weeks gestation (Paulin et al., 1977; Bloch, 1978). In addition, immunoreactive GnRH has been detected in hypothalami of eight week old human fetuses (Winters, et al., 1974; Clements et al., 1980). Pituitaries from human fetuses, as young as 10 to 13 weeks gestation, have been shown to be responsive to GnRH in culture, by secretion of increased levels of LH and FSH (Goodyer et al., 1977; Li et al., 1979a).

The route by which hypothalamic GnRH reaches the anterior pituitary for initiation and/or increase of gonadotropin production early in gestation, is at present uncertain. Early studies of the hypothalamo-hypophyseal portal system reported that the vascular connections serving to transport hypothalamic releasing hormones to the pituitary were not established before 18-20 weeks gestation (D'Espinasse, 1933; Nimineva, 1949). However, a recent report (Thliveris and Currie, 1980) suggested that a direct vascular connection exists between the median eminence and pars tuberalis of the human fetus at least by 11.5 weeks gestation (the youngest specimens studied). Until further studies are performed on yet

younger specimens, i.e., prior to 11.5 weeks gestation, one can only speculate on the route by which GnRH reaches the pituitary. A possible alternate mode of transport early in gestation could be by simple diffusion or some specific active transport mechanism, since the hypothalamus is in direct contact with elements of the anterior pituitary by the fifth week in gestation (Falin, 1961). Although the route by which GnRH reaches the pituitary prior to 11.5 weeks gestation remains uncertain, detection of LH and FSH at 9.5 weeks gestation, and the subsequent increase in the levels of these hormones observed in the current study, implies that GnRH of fetal hypothalamic origin influences pituitary activity. Support for this contention can be inferred from the results of Clements et al. (1980) in that pituitary gonadotropin levels parallel the increasing levels of hypothalamic GnRH, between eight and 20 weeks gestation.

The target organs for the observed increase in LH and FSH noted in the present study, are the fetal gonads. However, neither LH nor FSH appear to be responsible for the onset of testosterone secretion, and hence genital differentiation (Clements et al., 1976). High levels of CG during the critical stage of sexual differentiation (eight to 10 weeks gestation) appears to be responsible for the stimulation of Leydig cells and subsequent production of high levels of testosterone (Clements et al., 1976). On the other hand, LH and FSH appear to be important in differentiation of the germinal elements in both the male and female gonads. Reyes et al. (1976) noted that transformation of primordial germ cells into spermatogonia and differentiation of Sertoli cells coincided with the initiation of fetal pituitary gonadotropin secretion. In addition, differentiation of primordial cells into oocytes and granulosa cells, and

proliferation of these elements to form follicles corresponded to the rising levels of LH and FSH. Absence of these hormones, on the other hand, in anencephalic and apituitary human fetuses and in hypophysectomized fetal monkeys, resulted in reduced gonadal weight in both sexes, as well as reduced numbers, luminal diameters, and coiling, of the seminiferous tubules in the male, and a paucity and arrest of follicular development in the female (Zondek and Zondek, 1965; Mroueh, 1970; Ross, G.T.: cited by Reyes et al., 1976).

In the present investigation, LH and FSH staining intensity and hence hormone concentration was higher in the female than male fetuses between 11.5 and 16.5 weeks gestation. This finding suggests a difference in feedback control sensitivity between the sexes. Support for this contention is provided by Clements et al. (1976) in that LH and FSH levels in the anterior pituitary as measured by radioimmunoassay were significantly higher in the female between 12 and 20 weeks gestation. In addition, whereas estradiol-17β levels are similar in both sexes between 11 and 17 weeks gestation, serum testosterone is much higher in the male fetus (Reyes et al., 1974). Finally, increasing levels of testosterone appear to have a direct feedback effect on the pituitary of male fetuses since GnRH levels are similar in both sexes (Reyes et al., 1976; Winter et al., 1977). In addition, as the male pituitary becomes sensitive to the increasing levels of testosterone, the amount of LH and FSH produced and secreted is reduced between 12 and 20 weeks gestation. The fall in GnRH levels and hence LH and FSH levels after 20 weeks gestation in both sexes (Reyes et al., 1976; Winter et al., 1977) probably represents the development of estrogen receptors in the hypothalamus and pituitary (Davies et al., 1975).

Other questions yet to be dealt with are those centered around the degree of interaction between the hypothalamus, pituitary, gonad and placenta during sexual differentiation and development. A clearer understanding of multiple endocrine interaction may be brought to light by studying localization of not only the principle hormones secreted by the various endocrine glands, but also determination of the presence and location of receptor sites for hormones of one gland in another during differentiation. Providing clues as to the likelihood of sex differences in neural, pituitary, gonadal, and placental structure and/or function during fetal life, could have important implications regarding postnatal pubertal development, adult reproductive function, and possibly sexual behaviour.

APPENDIX I

ABBREVIATIONS

ACTH - adrenocorticotropic hormone, adenocorticotropin.

CG - chorionic gonadotropin.

FSH - follicle stimulating hormone.

GnRH - gonadotropin releasing hormone.

h - human

IgG - immunoglobulin G

K - X1000

LH - luteinizing hormone.

LPH - lipotropic hormone, lipotropin.

LTH - luteotropic hormone, prolactin.

MSH - melanocyte stimulating hormone, melanotropin.

STH - somatotropic hormone, somatotropin, growth hormone.

TSH - thyroid stimulating hormone, thyrotropin.

APPENDIX II

0.05 M Phosphate Buffer Wash

The 0.05 M phosphate buffer wash consisted of a 1:4 combination of 0.2 M sodium phosphate, monobasic ($NaH_2PO_4.H_2O$), and 0.2 M sodium phosphate, dibasic ($Na_2HPO_4.7H_2O$), adjusted to pH 7.4 and diluted with distilled H_2O.

Diluent 0.05 M Phosphate Buffer

The 0.05 M phosphate buffer (pH 7.4)(above) with the addition of 2.5 mg/ml of human serum albumin was used for the dilution of the primary antisera, goat antirabbit IgG, peroxidase antiperoxidase, normal goat serum, and normal rabbit serum.

DAB-H_2O_2 Solution

25 mg of DAB (3,3'diaminobenzidine-tetrahydrochloride) was added to 175 ml of 0.05 M Tris(hydroxymethyl)aminomethane (Tris buffer) (pH7.6) to which 1.5 ml of 0.3% hydrogen peroxide (H_2O_2) had been added.

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