# Persistence of Cell Types in Monolayer Cultures of Dispersed Cells from the Pituitary Pars Distalis as Revealed by Immunohistochemistry [1]

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*ABSTRACT* The objective was to study the fate of specific secretory cell types of the rat hypophysis when grown in primary monolayer cultures for periods ranging up to 32 days. The cells were identified immunohistochemically using peroxidase-labeled antibody. Early in the culture period TSH-cells were scarce and by 12 days they could no longer be identified. In most cultures LH-cells were well stained and common for eight to 12 days, after which they underwent involution. Growth hormone cells were a prominent feature up to six days but by 12 days they were declining in number, size, and stainability; in contrast, prolactin cells proliferated and were large and intensely stained throughout the period of study, ultimately becoming the dominant secretory cell type. Corticotropic cells also continued throughout the period of study without regression. Thus drastic shifts occur with time in the relative proportions of cell types in monolayer cultures of rat pituitary cells.

Cultures containing dispersed or isolated cells of the pars distalis are being used with increasing frequency in the study of factors that regulate pituitary secretion. As observed by Vale et al. ('72), cultures of dispersed pituitary cells grown as monolayers have two major advantages over those containing slices or fragments of the gland. First, a homogeneous cell population is obtainable which facilitates the consideration of several variables in one experiment. Second, the dispersed cells are sufficiently responsive to stimulating agents so that the amount of hormone released can be related directly to the amount of stimulating agent used.

Both Vale et al. ('72) and Steinberger et al. ('73) have found that the capacity of monolayer cultures of pituitary cells to secrete certain hormones declines with time, this observation indicating that significant cytological alterations occur in the cell population. Thus far, histochemistry and electron microscopy have not permitted satisfactory analysis of the fate of specific secretory cell types in monolayer cultures. For example, Kobayashi et al. ('71) stained with Giemsa or the periodic acid-Schiff technique and concluded that almost all cells become chromophobes by the end of the first week. Also, Rappay et al. ('73) found that the ultrastructure of rat pituitary cells grown in monolayer cultures for 11 to 27 days does not allow the identification of specific cell types.

Since immunohistochemistry has helped to solve many problems of cell identification in the intact hypophysis, in this study the method was applied to monolayer pituitary cell cultures in order to demonstrate changes that occur in the cell population as the cultures age. Thereby, a better understanding may be obtained of the capacity of such preparations to respond to factors that stimulate or inhibit secretion. This represents the first attempt to analyze by means of immunohistochemistry the cellular composition of normal pituitary tissue grown in vitro.

## MATERIALS AND METHODS

Holtzman female rats, in the diestrous stage of the cycle and weighing 200 to

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250 gm, served as the source of pituitary glands. After the rats were killed with $CO_2$ the hypophysis was excised and the posterior hypophysis discarded. In some of the earlier preparations portions of the pars intermedia were retained with the pars distalis; however, in subsequent experiments only the lateral portions of the pars distalis were used in order to eliminate insofar as possible cells of the pars intermedia. Monolayer cultures of cells from the pars distalis were prepared by the method of Vale et al. ('72), which involves incubation of the tissue in a medium containing hyaluronidase and collagenase followed by a 15- to 30-minute treatment with Viokase. The rate of dispersion is increased by gently drawing the fragments in and out of a siliconized Pasteur pipette every ten minutes throughout the dispersion procedure. Exceptions to the Vale et al. procedure were the use of Hanks' calcium- and magnesium-free balanced salt solution (Grand Island Biological Co.) instead of HEPES buffer as the medium for cell dispersion. Since Hanks' balanced salt solution was employed as the buffer, cell dispersion was carried out in a humidified $CO_2$ incubator. In addition, penicillin G (100 units/ml) and streptomycin (100 $\mu$g/ml) were added to the growth medium. The cells were cultured in Flaskettes-Biological Growth Chambers (Lab-Tek Products Division, Miles Laboratories). In general the cells of 15 hypophyses were dispersed at a time, thus providing sufficient material to prepare approximately 45 Flaskettes.

Seventy-four cultures were maintained for 1, 2, 4, 6, 8, 12, 16, 20, 24, 30, or 32 days. After removal of the incubation medium each culture was stained immunohistochemically for one to three cell types by the method of Nakane and Pierce ('67). The cell types studied were: growth hormone cells, prolactin cells, corticotropic cells, melanotropic (MSH-) cells, thyrotropic (TSH-) cells, and luteinizing hormone (LH-) cells. Antiserums were prepared in our laboratory by the method of Midgley et al. ('71) to human growth hormone (NIH-GH-HS 1395),[2] $\beta^{1-24}$-corticotropin, and human $\beta$-melanotropin. Anti-human thyrotropin[3] (anti-hTSH) was provided by Dr. W. D. Odell; anti-oLH and anti-rat prolactin by Dr. A. R. Midgley, Jr.; and anti-bTSH-$\beta$ and anti-bLH-$\beta$ by Dr. J. G. Pierce, Jr. For most preparations, 3,3'-diaminobenzidine was used as the substrate for peroxidase. For double staining $\alpha$-naphthol was employed as substrate for staining of the second cell type.

Evidence for the specificity of these procedures when applied to cells of the intact rat pituitary gland has been published from this laboratory as follows: for growth hormone and prolactin cells (Baker et al., '69; Baker, '70), corticotropic and MSH-cells (Baker et al., '70; Baker and Drummond, '72); TSH-cells (Baker and Yu, '71a,b) and TSH- and LH-cells (Baker et al., '72). In addition, numerous controls were employed in connection with immunohistochemical staining of the cultured cells. No staining was obtained under the following conditions: omission of the hormone-specific antiserum, prior absorption of the hormone-specific antiserum with the hormone used as the antigen, and application to the slide of unconjugated sheep anti-rabbit-$\gamma$-globulin prior to application of the peroxidase-conjugated $\gamma$-globulin. Prolactin represented an exception to these generalizations because absorption of anti-rat prolactin with ovine prolactin greatly reduced staining intensity but did not totally eliminate it.

## RESULTS

As noted by Vale et al. ('72), the dispersed pituitary cells were spherical when first placed in culture and this was their shape at the end of one day in our preparations. By the second day some cells had become flattened and stellate. With prolongation of the culture time a greater proportion of the cells were flattened. The pituitary epithelioid cells tended to form colonies which enlarged up to about six days. Such cell groups were illustrated by Steinberger et al. ('73) in ten-day cultures. With advancing time fibroblasts became

[2] We thank the following sources for the hormones indicated: NIAMD Pituitary Hormone Distribution Program for human growth hormone and bovine luteinizing hormone; Organon, Inc., W. Orange, N.J., for $\beta^{1-24}$-corticotropin (Cosyntropin®); and Professor H. J. Bein and Doctor W. Rittel of Ciba-Geigy Ltd., Basle, Switzerland for $\beta^{17-39}$-corticotropin and $\beta$-melanotropin.

[3] In abbreviations for hormones "b" indicates bovine origin, "o" ovine, and "h" human. Antiserums are indicated by the prefix "anti-" added to the name of the hormone.

predominant in areas intervening between the colonies. However, immunohistochemical staining showed that secretory cells occurred in the fibroblastic and other areas also (fig. 10).

*Growth hormone cells.* Between two (fig. 2) and four days (fig. 3) growth hormone cells did not show much change but at six days they were a prominent feature of epithelioid cell colonies (fig. 5). However, by 12 days in most cultures growth hormone cells were clearly declining in relative number and staining intensity. By 30 days the remaining growth hormone cells were few, small, weakly stained and generally stellate (fig. 6). Most growth hormone cells maintained their typical ovoid shape for about 12 days in culture; later a greater proportion were stellate.

*Prolactin cells.* Probably the most impressive feature of hypophyseal monolayer cell cultures was the ready adaptability of prolactin cells to the in vitro condition. At four days many attached to the slide and appeared as voluminous, flat, polyhedral cells (fig. 4). Their large cytoplasmic granules and the negative image of the Golgi apparatus were clearly evident. By six to eight days prolactin cells were the dominant type in epithelioid cell colonies and at 12 days and later (fig. 8) they far outnumbered the declining growth hormone cells. Even at 30 to 32 days, when fibroblasts seemed to be overtaking the culture, prolactin cells were still abundant (figs. 9, 10).

Cultures were doubly stained for growth hormone and prolactin at four and 24 days. At both times growth hormone and prolactin cells were differentiated clearly from each other; hence, modulation leading to the production of both hormones by a single cell had not occurred. In contrast, clonal strains of rat pituitary tumor cells secrete both prolactin and growth hormone (Tashjian et al., '70).

*Corticotropic cells.* At four days the morphology of corticotropic cells resembled that in the intact gland (fig. 11) but after attaching to the glass corticotropic cells appeared larger. Distinctive of the corticotropic cells was their stability in culture and they appeared to increase somewhat in relative number and size (fig. 12) during the 32-day period of study.

Cells stained with anti-$\beta$-MSH showed the same characteristics as described above for corticotropic cells (fig. 13). Attempts to demonstrate two separate cell types by double-staining with anti-$\beta^{1-24}$-corticotropin and anti-$\beta$-hMSH were unsuccessful; thus it appears that in these cultures one cell type contained both corticotropin and melanotropin.

*TSH-cells.* TSH-cells were regularly demonstrable with both anti-hTSH and anti-bTSH-$\beta$ at one and two days and in one of three cultures at each of the following times: four (fig. 14), six and eight days. TSH-cells were undetectable in all other speciments at all other times. When observed they were rare, small, stellate, and their size was never equivalent to that of TSH-cells in the intact gland.

*LH-cells.* LH-cells were stained with both anti-oLH and anti-bLH-$\beta$. Although the two antiserums were not used in the double staining procedure, it seemed certain that the same cell type was delineated with both. Early in the culture period numerous LH-cells were ovoid but by four days many were flattened and polyhedral (fig. 15). LH-cells were numerous and intensely stained through 12 days (fig. 16). Subsequently they became small, poorly stained, and were difficult to find at 30 days (fig. 17).

Double staining of a four-day culture with anti-TSH-$\beta$ and anti-bLH-$\beta$ revealed that separate cells secreted the two hormones. Similar results were obtained with a 32-day culture stained with anti-$\beta^{1-24}$-corticotropin and anti-oLH (fig. 18).

## DISCUSSION

When viewing microscopic sections of the intact pituitary gland, one's assurance in the identification of cell types on the basis of their tinctorial properties is strengthened by the typical shape, size, and intraglandular distribution of each cell type. However, when dealing with cultured cells one is more dependent on staining properties, because the distributional feature is lost, ovoid cells usually become polyhedral when attached to the slide, and relative size differences become distorted. Indeed, the light microscopic differentiation of some pituitary secretory cells from fibroblasts is impossible without the assist-

ance of immunohistochemistry. Since we are concerned with the capacity of specific cell types to maintain their structural and functional integrity in culture, the irregularity in the rate of decline for certain cell types is important. Thus, after a certain period of culture the prominence of a specific cell type may vary considerably from one culture to another. The reason for this variation is not now apparent.

There is fairly close correlation between the capacity of pituitary cell types to survive in vitro as observed in our study and their capacity to secrete hormones, with and without stimulation (fig. 1) by hypothalamic releasing hormones. In dispersed cultures of Kobayashi et al. ('71) secretion of gonadotropin stopped in the second week. Vale et al. ('72) observed that LH was quantifiable after four hours at a low level of less than 1 $\mu$g in the medium of four-day monolayer cultures but it was undetectable by either bio- or radioimmunoassay at 9, 15, and 21 days. Likewise, the amount of LH in the cells declined rapidly from day 4 to day 21. Similarly, Steinberger et al. ('73) found high levels of LH in the culture medium at three days which was followed by a steady decline to an undetectable level at 16 to 41 days. In the latter two studies, addition of hypothalamic extract or synthetic LH-releasing hormone to the culture at four or seven days increased the output of LH, but responsivity of the pituitary cells declined with age of the culture. In many of our

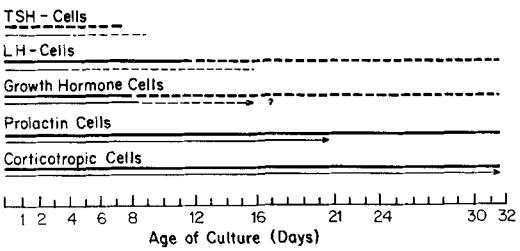


Fig. 1 A chart summarizing the duration of survival for specific cell types in monolayer cultures of the rat hypophysis. Also charted is the presence of various hormones in the medium of pituitary cultures based on published information as summarized in the text. Indicators: ———, presence of well-formed cells in all cultures; — — —, cells are involuting and/or irregularly present; ———, hormone is regularly present in the medium; — — —, hormone is irregularly present in the medium.

8- or 12-day preparations, a decline in the number of well stained LH-cells was evident. At later times this regression characterized all cultures.

A similar correlation exists between the output of thyrotropin and presence of TSH-cells. Vale et al. ('72) found that four-day cultures secrete a small amount of thyrotropin but that this hormone is undetectable at nine or more days. The content of thyrotropin in the cells fell rapidly with time. Also the strong responsivity of four-day cultures to thyrotropin releasing hormone had declined progressively at 9 and 15 days and was absent at 21. In our cultures TSH-cells were maintained poorly, being found in only some cultures at four and eight days and being absent from those that were 12 or more days old.

Present evidence indicates that when corticotropic cells are grown in vitro, they continue to secrete actively for some time although Guillemin and Rosenberg ('55) reported that secretion of corticotropin disappears from tissue cultures of rat hypophysis after four days. Explants of rat hypophysis maintained in vitro for 12 days can still stimulate adrenocortical fragments when placed together in a single culture (Schaberg and de Groot, '58). A similar relationship has been demonstrated between human fetal hypophysis and adrenal cortex (Stark et al., '65). According to Gyévai et al. ('69) pituitary fragments secrete corticotropin for as long as five weeks. Fleischer and Rawls ('70) showed that in monolayer cultures of pituitary cells the amount of intra- and extracellular corticotropin increased sharply over a period of three to ten days in direct relation to the increase in the total number of cells, but that the concentration of corticotropin per cell appeared to remain constant. Our observations support the conclusion that corticotropic cells continue in good condition for some time since they were at least as large, as numerous, and as intensely stained at 32 days as at earlier times.

The apparent presence of both corticotropin and melanotropin in a common cell is of considerable interest because it suggests that corticotropic cells maintained in vitro acquire the capacity to secrete melanotropin, whereas in the intact gland cor-

ticotropic cells of the pars distalis do not contain melanotropin. Since cells of the pars intermedia do contain both hormones (Baker and Drummond, '72) their exclusion from the cultures was crucial. Although care was taken to do this our experiment is still not conclusive because in the normal gland a few cells containing melanotropin may be found in the pars distalis, especially along its caudal border. Nevertheless, had they been included in the culture we should have been able to differentiate corticotropic from MSH-cells by double immunohistochemical staining. This was not possible so in contrast to the other cell types, one may deduce that corticotropic cells underwent some modulation in culture and acquired the capacity to produce melanotropin.

With respect to the fate of growth hormone cells and prolactin cells in vitro, our observations with monolayer cultures resemble those obtained by Pasteels ('63) with organ cultures of rat and adult and fetal human hypophyses. He found that most cell types were present in the peripheral portion of the fragment for several days; however, they underwent atrophy and no growth hormone cells were identifiable in rat cultures after nine days. Proliferating around the fragment was a veil of prolactin cells which according to Pasteels is the only cell type that continues active secretion of hormones for some time after initiation of the culture. Furthermore, Pasteels demonstrated that the amount of prolactin secreted increased with age of the culture for about three weeks. Concurrently, the secretion of growth hormone declined rapidly by nine days in cultures of human fetal hypophyses. The most rapid increase in prolactin secretion occurred in the cultures with the most intense mitotic proliferation.

In summary, in monolayer cultures of dispersed pituitary cells TSH-cells are extremely short-lived; growth hormone cells and LH-cells decline with time while prolactin cells come to dominate the culture. In these respects our results agree with Pasteels' observations with organ cultures. In addition, corticotropic cells also persist but in smaller numbers than the prolactin cells.

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## PLATE 1

### EXPLANATION OF FIGURES

The magnification of all photographs is × 400.

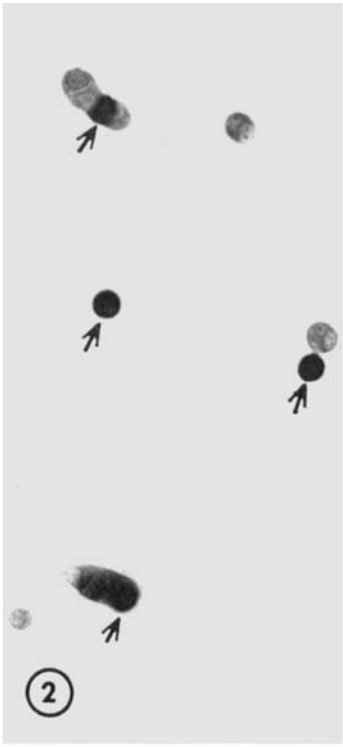
2 A one-day culture illustrating growth hormone cells (arrows). At this time practically all cells are spherical.

3 A four-day culture illustrating growth hormone cells (arrows). Many cells are flat on the slide and have become stellate.

4 A four-day culture illustrating prolactin cells (arrows). Prolactin cells are polyhedral, contain large peripheral granules, and are much larger than growth hormone cells (fig. 3). The large Golgi area, almost devoid of large granules, may be identified in several cells.

5 A six-day culture showing a large colony of epithelioid cells stained for growth hormone. The growth hormone cells tend to retain their natural ovoid shape. Inconspicuous fibroblasts appear at the periphery of the colony.

6 A 30-day culture depicting a colony of epithelioid cells stained for growth hormone. Growth hormone cells (arrow) are few, small, and poorly stained.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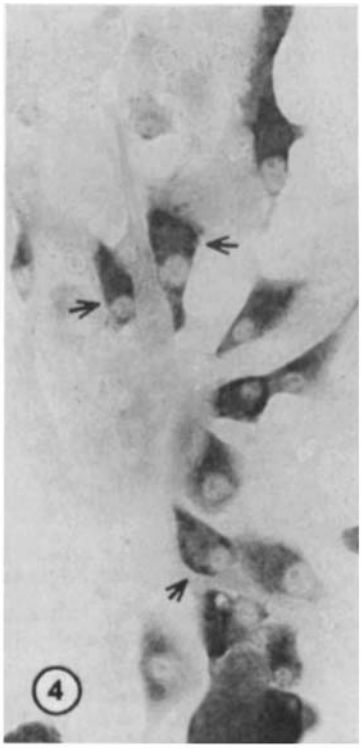
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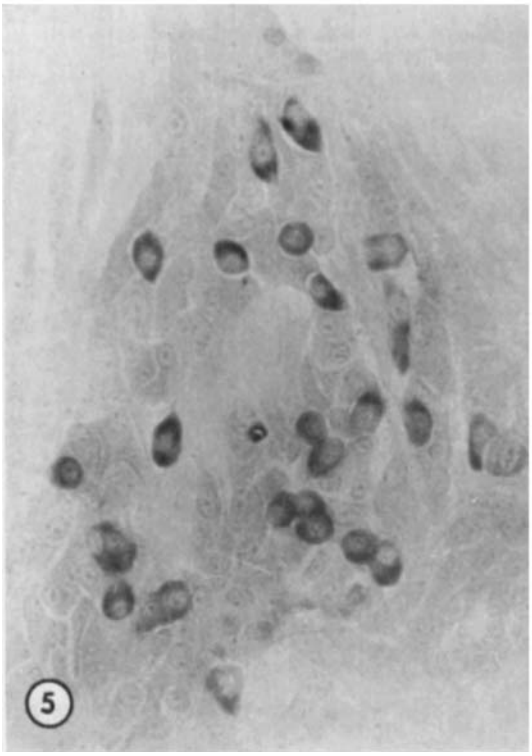
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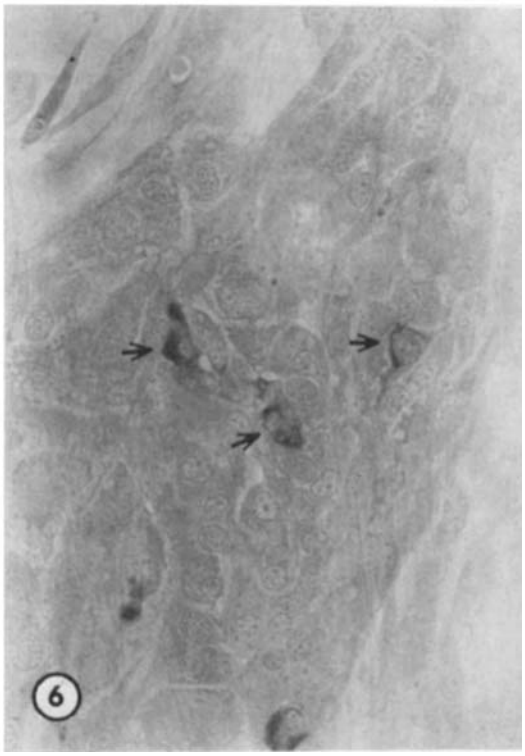
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## PLATE 2

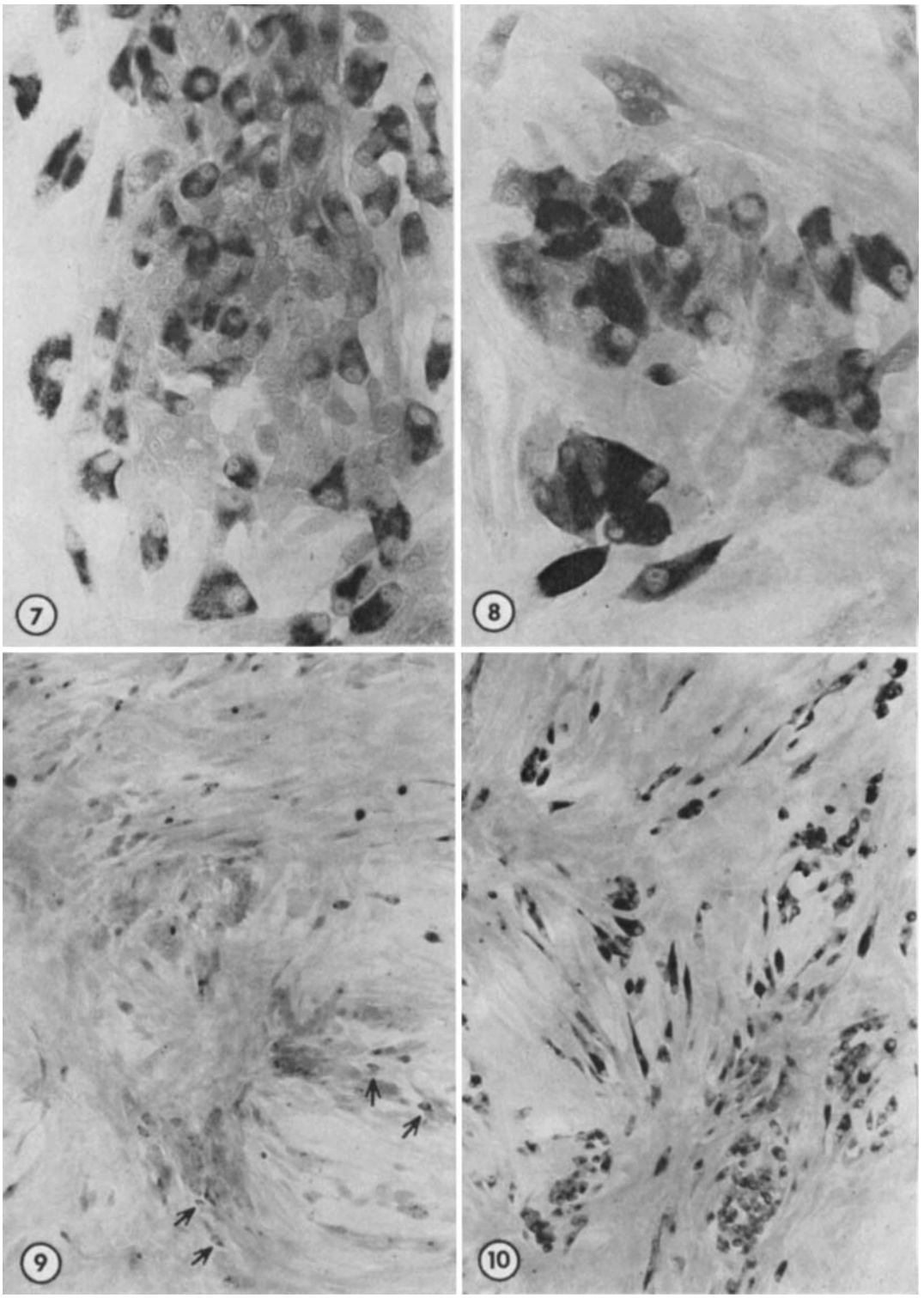
### EXPLANATION OF FIGURES

7 A 16-day culture illustrating a colony of epithelioid cells stained for prolactin. Prolactin cells are the dominant cell type. × 400.

8 A 30-day culture showing a small colony with large, well stained prolactin cells. × 400.

9 A 32-day culture stained for growth hormone. A few small epithelioid cell colonies are visible but most of the field is composed of fibroblasts. Growth hormone cells (arrows) are few and poorly stained. × 100.

10 Another area of the 32-day culture illustrated in figure 9 but stained for prolactin. Prolactin cells predominate in the small colonies and also are scattered through the fibroblastic areas. × 100.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## PLATE 3

### EXPLANATION OF FIGURES

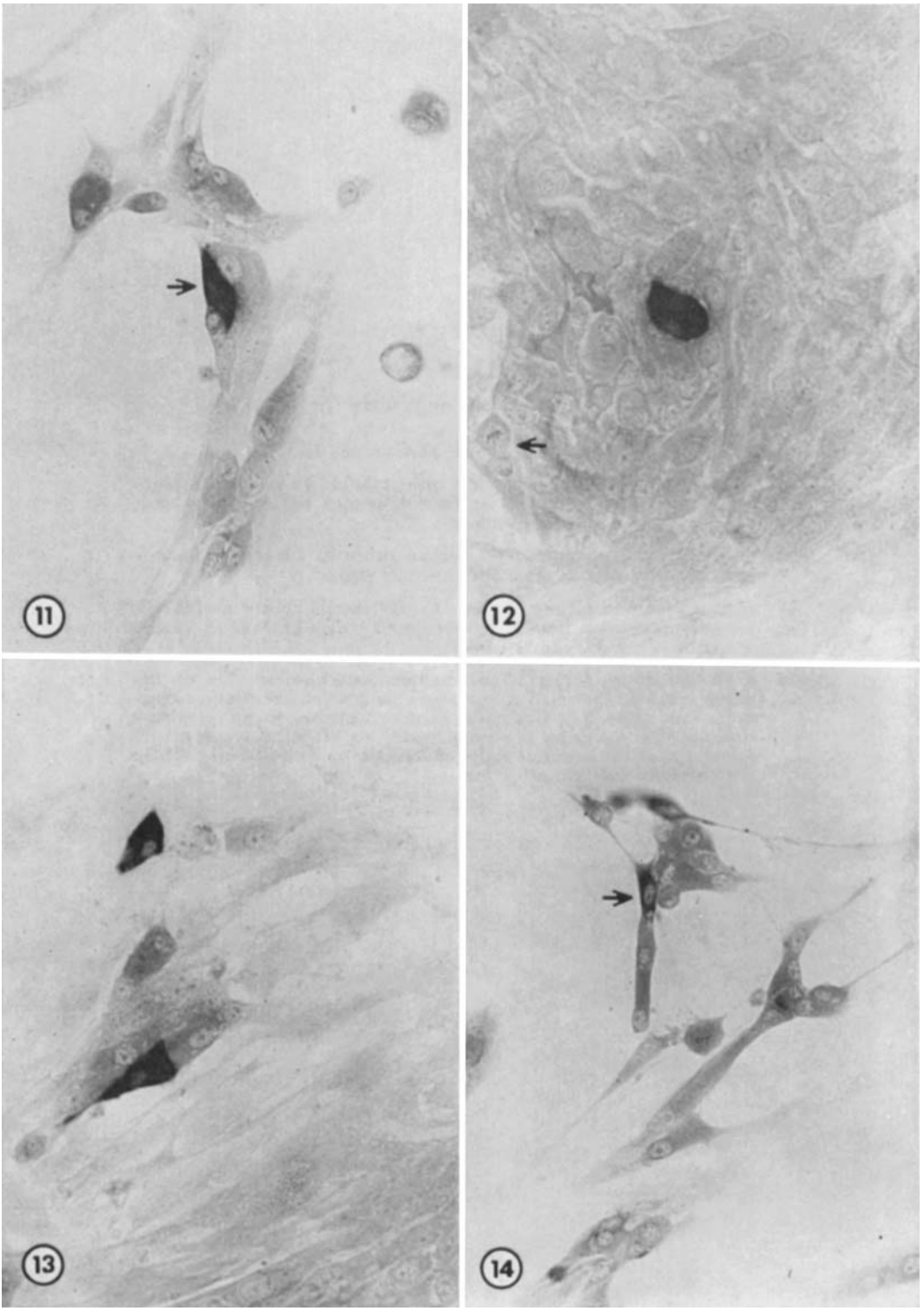
The magnification of all photographs is × 400.

11 A four-day culture stained for corticotropin. An intensely stained corticotropic cell (arrow) is shown.

12 A 32-day culture illustrating a large colony of epithelioid cells including one intensely stained corticotropic cell. The arrow indicates a mitotic figure.

13 A 30-day culture stained for β-MSH. Two intensely stained cells containing β-MSH are shown. Note the similarity of these cells in size, staining capacity, and morphology to the corticotropic cells of figures 11 and 12.

14 A four-day culture stained for thyrotropin with anti-bTSH-β. Only one TSH-cell (arrow) is shown. As compared with the other cell types illustrated previously, it is small and contains little hormone.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## PLATE 4

### EXPLANATION OF FIGURES

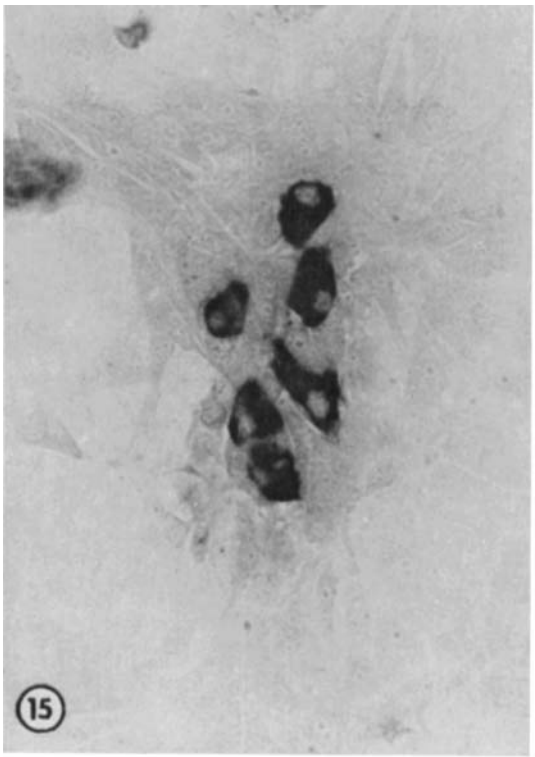
The magnification of all photographs is × 400.

15 A four-day culture stained for LH with anti-oLH. In this small colony six LH-cells are illustrated. At four days they are generally large, polyhedral, and intensely stained.

16 A 12-day culture prepared for demonstration of LH-cells with anti-oLH. LH-cells are still large and intensely stained.

17 A 30-day culture stained for LH. In this sizable colony no LH-cells are present. At this time in other cultures a few LH-cells in various stages of regression can be identified.

18 A 24-day culture doubly stained immunohistochemically for corticotropin and LH. The small colony above contains at least three corticotropic cells (gold, following use of diaminobenzidine as the peroxidase substrate). At the arrow is a small regressing LH-cell (lavender, with α-naphthol as the substrate followed by staining with pyronin). These are distinctly different cell types.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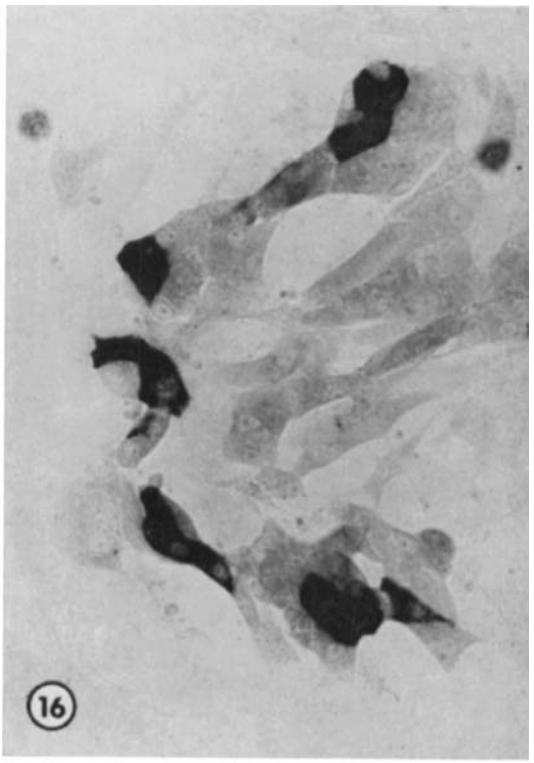
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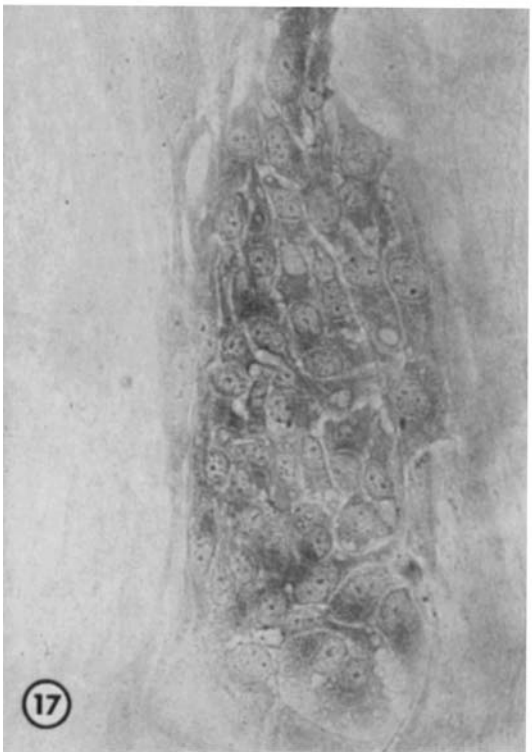
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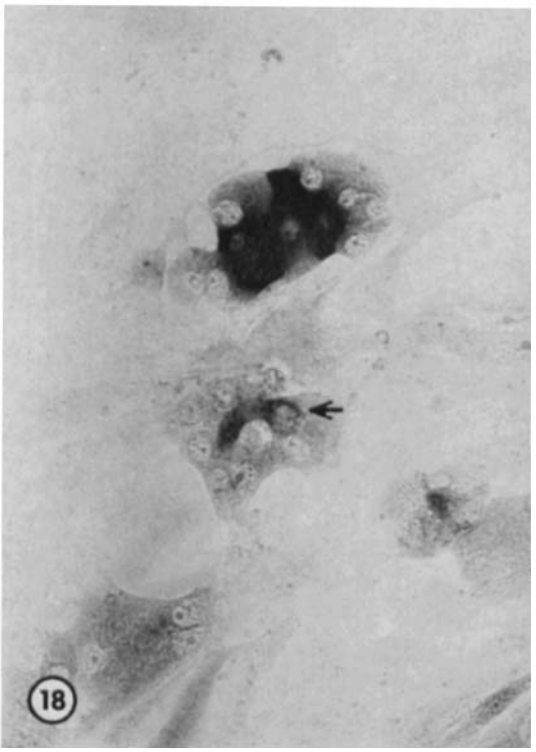
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