Localization of a 16,000-dalton Fragment of the Common Precursor of Adrenocorticotropin and β-Lipotropin in the Rat and Human Pituitary Gland

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ABSTRACT To clearly identify cells and organelles containing the common precursor (31,000 dalton) for both adrenocorticotropin (ACTH) and β-lipotropin (β-LPH), an immunohistochemical localization of a fragment (16,000 dalton) of the precursor that is not common to β-LPH and ACTH was conducted in rat and human pituitary glands. With the help of specific antibodies that do not cross-react with β-LPH and ACTH, the 16,000-dalton fragment was localized in the cells that also produce ACTH and β-LPH in both the pars distalis and pars intermedia of the rat pituitary. At the electron microscope level, the secretory granules that contain ACTH were also stained for 16,000-dalton fragment. In the human pituitary, the 16,000-dalton fragment was also observed in all the secretory granules of lipocorticotrophs. These results suggest that, after enzymatic cleavage, fragment(s) of the common precursor and/or the whole common precursor remain packaged within the secretory granules with peptides of known activity.

It is now well established that in the AtT-20 mouse pituitary tumor and the rat pituitary gland (3, 7, 12, 13) both adrenocorticotropin (ACTH) and β-lipotropin (β-LPH) come from a common precursor of ~31,000 mol wt. This common precursor for ACTH and β-LPH has been shown to be a glycoprotein (2, 13). Although we have reported that ACTH and β-LPH are contained not only in the same cells but also in the same secretory granules in the pituitary gland of several species (11), the localization of fragments of the precursor other than those immunologically related to ACTH and β-LPH has not been reported. There is also no indication of the presence of a common precursor in the human pituitary gland. With the help of antibodies specific to a fragment (16,000-dalton) (3) of the common precursor that does not cross-react with ACTH and β-LPH, we localized this fragment and ACTH in the pituitary glands of both man and rat.

MATERIALS AND METHODS

Pituitaries from three adult male rats weighing 200–250 *g* each were fixed by perfusion with 500 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Three human pituitaries obtained at hypophysectomy for breast cancer or diabetic retinopathy were fixed by immersion in the same fixative for 4 h. After dehydration in ethanol, the pituitary tissue was embedded in Araldite.

The immunohistochemical technique used for both light and electron microscope studies involved the use of the peroxidase-antiperoxidase complex (PAP) as described by Sternberger (15). For light microscopy, semithin (1.5-μm) sections were mounted on glass slides. Before the reaction, Araldite was removed with a saturated solution of Na ethoxide (1). For electron microscope studies, ultrathin sections were obtained and mounted on nickel grids. In brief, the sections were successively exposed to (*a*) rabbit primary antiserum (12 h at 4°C), (*b*) goat anti-rabbit gammaglobulins diluted 1:20 (30 min, at 22°C), and (*c*) PAP complex diluted 1:100 (30 min at 22°C). The peroxidase was visualized after incubation in a medium containing 3,3'-diaminobenzidine and H_2O_2 (4). The reaction was observed with a Siemens Elmiskop 102 electron microscope.

For localization of the 16,000-dalton fragment, we used the Georgie antiserum against mouse 16,000-dalton fragment supplied by Drs. R. E. Mains and B. A. Eipper. This antiserum has been shown to be specific for 16,000-dalton fragment with very low cross-reactivity with ACTH–β-LPH related peptides (3). ACTH was also localized with an antiporcine $ACTH^{1-39}$ (11) that does not cross-react with β-LPH and α-melanocyte-stimulating hormone (α-MSH).

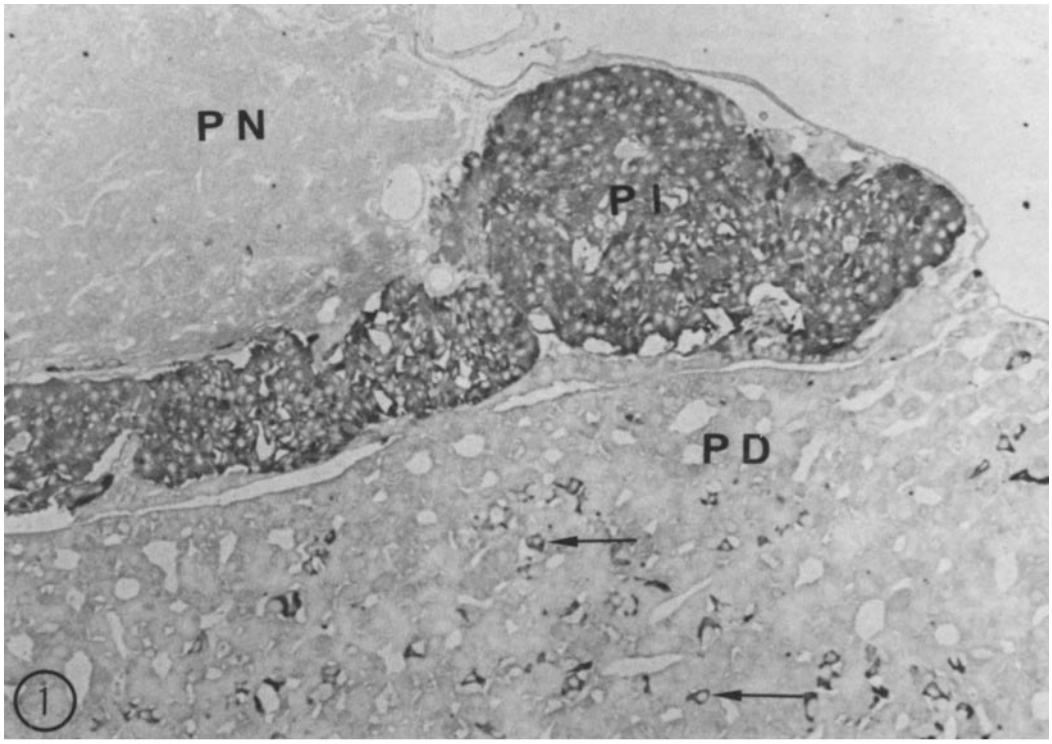
These primary antisera were used at a dilution ranging from 1:200 to 1:500 for light microscopy and 1:1000 to 1:8000 for electron microscopy. The specificity of the immunostaining was determined by absorption of each antiserum (diluted at 1:500 and 1:1000) with the following antigens at a final concentration of 10^{-6} M: purified porcine $ACTH^{1-39}$ (Sigma Chemical Co., St. Louis, Mo.), purified human β-LPH (6) (supplied by Dr. C. H. Li), purified ovine β-LPH (5) (supplied by Dr. M. Chrétien), purified 16,000-dalton fragment (3) (supplied by Drs. R. E. Mains and B. A. Eipper), synthetic human β-endorphin (supplied by Dr. D. H. Coy), synthetic human $ACTH^{1-39}$ (supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases, National Pituitary Agency), and synthetic α-MSH (Ciba-Geigy Corp., Summit, N. J.). Additional controls included omission of the primary antiserum or PAP.

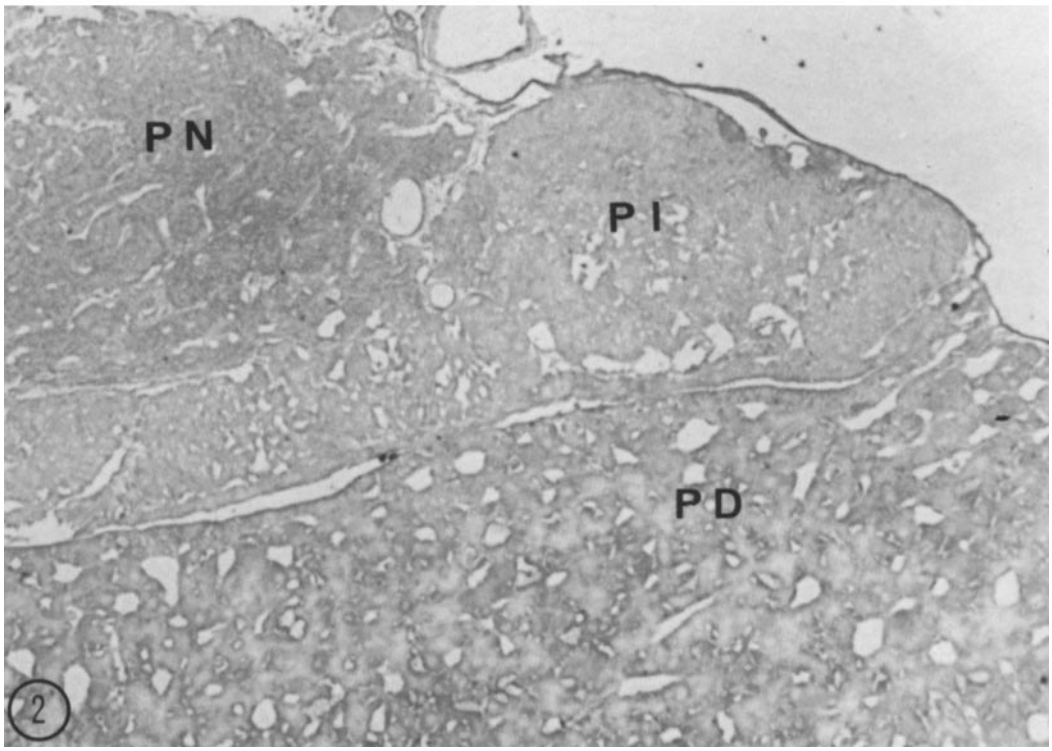
RESULTS

Rat Pituitary Gland

As revealed by immunostaining of semithin sections, the 16,000-dalton fragment appeared to be localized in all the secretory cells of the pars intermedia (Fig. 1). In the pars distalis, discrete stellate cells were also labeled.

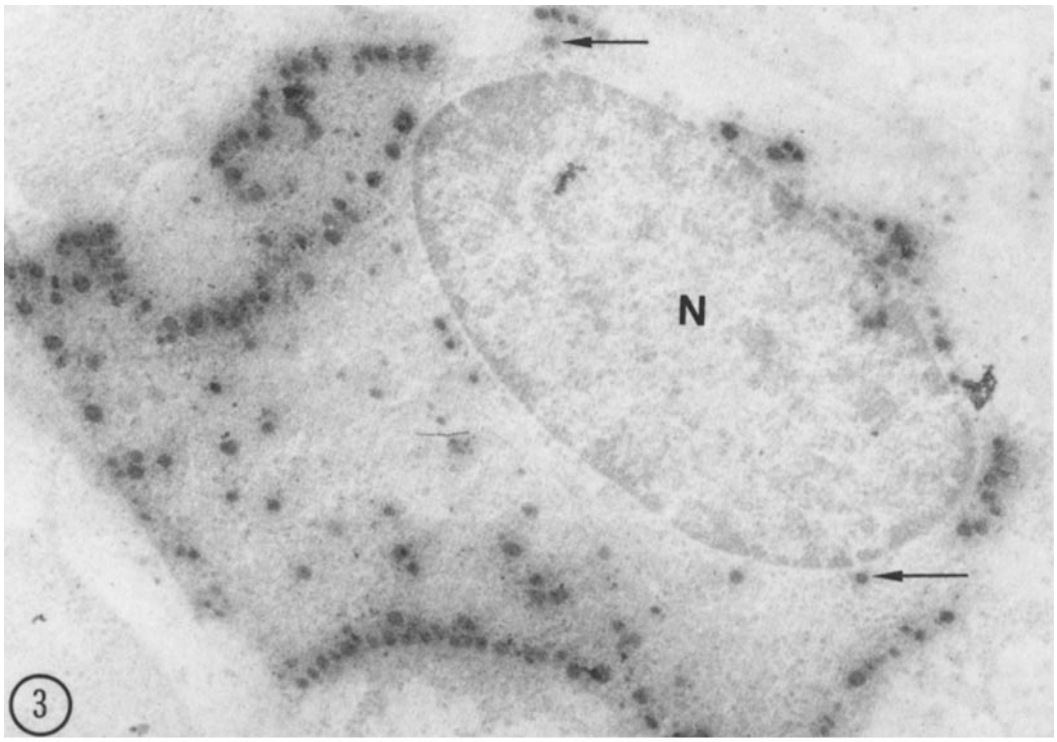
At the electron microscope level, when serial sections through the pars distalis were alternatively stained with anti-16,000-dalton fragment and anti-ACTH, it was observed that

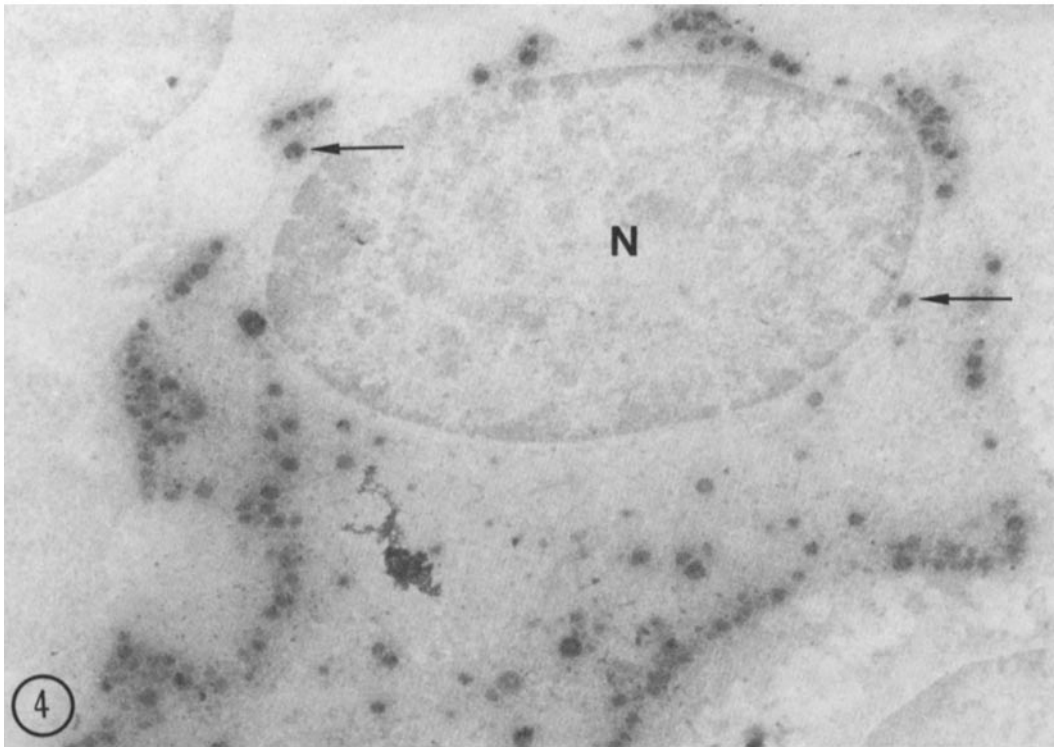




FIGURES 1 and 2 Adjacent semithin (1.5-μm) sections through rat pituitary gland. In Fig. 1, the section incubated with the anti-16,000-dalton fragment adsorbed with $ACTH^{1-39}$ and ovine β-LPH. The reaction product is found in all the cells of the pars intermedia (*PI*) and in scattered cells (→) in the pars distalis (*PD*). The pars nervosa (*PN*) is free of reaction. In Fig. 2, control section incubated with anti-16,000-dalton fragment absorbed with 16,000-dalton fragment. The staining has been completely prevented. × 240.

all the cells staining for 16,000-dalton fragment were also labeled with anti-ACTH (Figs. 3 and 4). Conversely, all the ACTH cells were positive for 16,000-dalton fragment. These cells were identified as typical corticotrophs with a stellate shape and containing secretory granules ~180–250 nm in diameter. With both antisera, the reaction product was mostly concentrated over the secretory granules. Generally, all the secretory granules were labeled in corticotrophs. Although sectioning 180–250-nm granules serially is difficult, it was occasionally possible to follow the same granules on two sec-





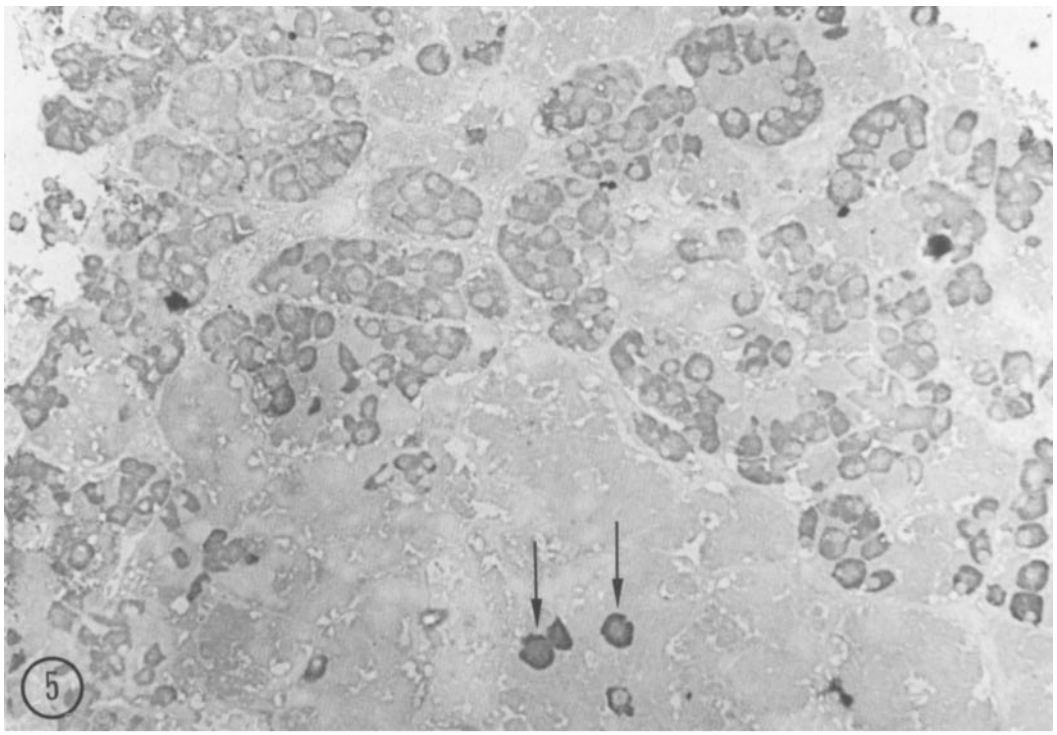
FIGURES 3 and 4 Adjacent ultrathin sections through the pars distalis of the rat pituitary. (Fig. 3) Typical ACTH cell, the secretory granules of which have been stained with the anti-ACTH previously absorbed with ovine β-LPH and 16,000-dalton fragment. (Fig. 4) Similar staining with the anti-16,000-dalton fragment. Arrows point to the positive granules that can be recognized in the two sections. *N*, nucleus. × 12,900.

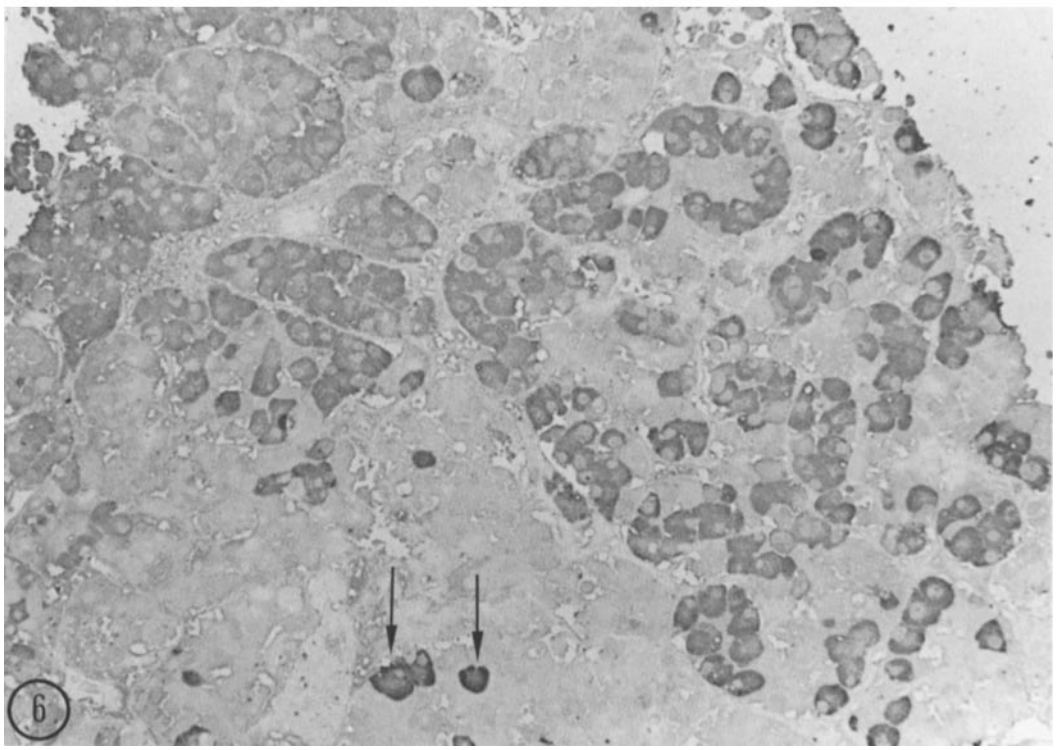
tions. In this case, 16,000-dalton fragment staining also was found in granules that also contain ACTH. In the secretory cells of the pars intermedia, as previously reported for ACTH and β-LPH (11), all the granules were generally labeled with the anti-16,000-dalton fragment.

Human Pituitary Gland

In the human pituitary, which lacks the pars intermedia, immunostaining of semithin sections through the pars distalis revealed that a positive reaction for 16,000-dalton fragment was only detected in cells labeled for ACTH (Figs. 5 and 6). Similarly, all the cells staining for ACTH were also positive for 16,000-dalton fragment. No staining could be detected in the neutral elements of the pars nervosa.

At the electron microscope level, the use of serial sections also demonstrated that both 16,000-dalton fragmentlike mate-

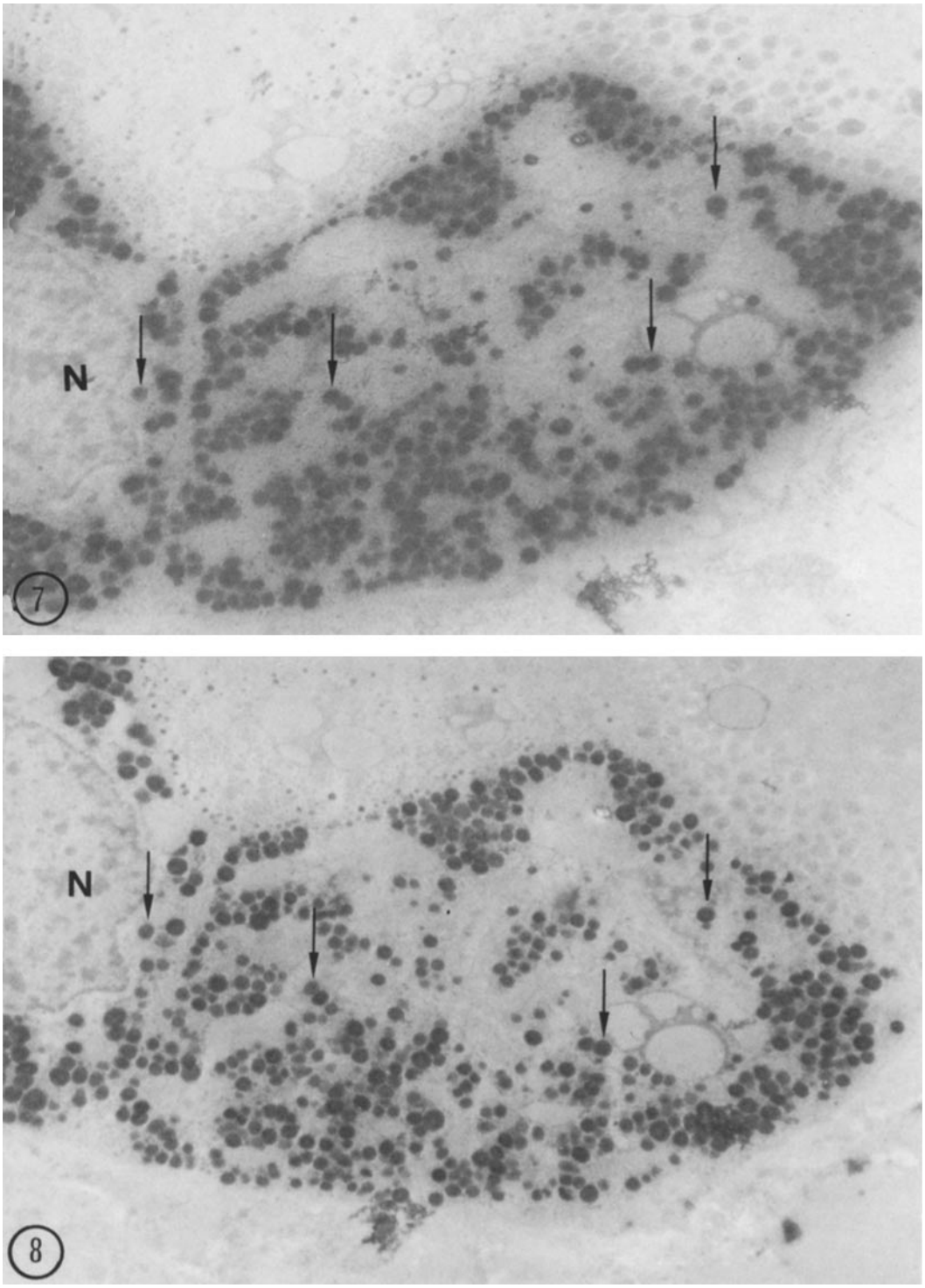




FIGURES 5 and 6 Adjacent semithin (1.5-μm) sections through the pars distalis of the human pituitary. Immunostaining for ACTH (Fig. 5) and 16,000-dalton fragment (Fig. 6) indicate that the same cells (→) contain the two peptides. × 250.

rial and ACTH could be found in the same cells (Figs. 7 and 8). These cells are characterized by the presence of numerous secretory granules ~300–600 nm in diameter (10) of which all were usually labeled with both antisera. Because these granules are larger than the corticotrophic granules in the rat, it was possible to identify several granules in two consecutive sections. Immunostaining for 16,000-dalton fragment and ACTH was then observed in the same secretory granules.

In both rat and human pituitaries, immunostaining was completely prevented when the antiserum to 16,000-dalton fragment was previously absorbed with purified 16,000-dalton fragment (Fig. 2) but was maintained when immunoabsorption was performed with ACTH (porcine and human) and β-LPH (ovine and human). β-Endorphin and α-MSH were also ineffective in blocking reaction. Similarly, the addition of ACTH, but not β-LPH, β-endorphin, α-MSH, and 16,000-dalton frag-



FIGURES 7 and 8 Adjacent ultrathin sections through the human pars distalis. (Fig. 7) Two portions of ACTH cells, the secretory granules (→) of which are stained for ACTH. (Fig. 8) Section stained for 16,000-dalton fragment. The same secretory granules (→) contain reaction product. *N*, nucleus. × 8,800.

ment to the ACTH antiserum abolished staining. These control experiments clearly confirm the absence of cross-reactivity between the antisera used in the experiment. Omission of the primary antiserum or PAP resulted in a complete absence of staining.

DISCUSSION

These results obtained with immunoperoxidase labeling strongly suggest that a fragment of the common 31,000-dalton precursor of ACTH and β-LPH is contained in the same pituitary cells (corticolipotrophs) that contain ACTH and β-LPH (10, 11). Because the 16,000-dalton fragment has not been found in cells other than those producing ACTH and β-LPH, this is a morphological indication that ACTH and β-LPH come from the 31,000-dalton precursor in the pituitary gland of rat and man. In both pars distalis and intermedia of the rat pituitary, the presence of a common precursor for both ACTH and β-LPH was recently reported by Eipper and Mains and by Roberts et al. (3, 13). In man, this immunocytochemical study also indicates the existence of a common precursor for ACTH and β-LPH that is immunologically related to the mouse 31,000-dalton common precursor. This strongly suggests that, as in the rat and mouse pituitary, ACTH and β-LPH come from a common precursor in the human pituitary. In both species studied in this report, immunoelectron microscope data clearly indicate that all the secretory granules, or at least a very high percentage of them, contain not only ACTH but also 16,000-dalton fragment. Because the corticotrophic granules have also been reported to contain both ACTH and β-LPH (11), it appears likely that, after enzymatic cleavage, fragment(s) of the common precursor remains packaged with fragment of known activity (ACTH, β-LPH, and endorphins). This localization of fragments of the 31,000-dalton fragment, which is a glycoprotein, can easily explain our previous findings, indicating the presence of glycoproteins in all the secretory granules of rat ACTH cells (9). From these immunohistochemical results, it cannot be excluded that antibodies to ACTH and 16,000-dalton fragment are also reacting with complete molecules of the 31,000-dalton precursor or even with some unknown peptides. On the other hand, because ACTH, β-LPH, and 16,000-dalton fragment are released on an equimolar basis (8), it is very likely that the three peptides exist as individual molecules within the secretory granules. This is a situation analogous to that found in β-cells of the endocrine pancreas where the C-peptide and insulin are stored in the same secretory granules and concomitantly released during exocytosis (14).

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REFERENCES

1. Baskin, P. G., S. J. Erlandsen, and J. A. Parsons. 1979. Influence of hydrogen peroxide on alcoholic sodium hydroxide on the immunocytochemical detection of growth hormone and prolactin after osmium fixation. *J. Histochem. Cytochem.* 27:1290–1292.
2. Eipper, B. A., and R. E. Mains. 1977. Peptide analysis of a glycoprotein form of adrenocorticotrophic hormone. *J. Biol. Chem.* 252:8821–8825.
3. Eipper, B. A., and R. E. Mains. 1978. Existence of a common precursor to ACTH and endorphin in the anterior and intermediate lobe of the rat pituitary. *J. Supramol. Struct.* 8:247–262.
4. Graham, R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291–302.
5. Li, C. H., L. Barnafi, M. Cretien, and P. Chung. 1965. Isolation and structure of beta-LPH from sheep pituitary glands. *Nature (Lond.).* 208:1093–1094.
6. Li, C. H., and D. Chung. 1976. Primary structure of human beta-lipopropin. *Nature (Lond.).* 260:622–624.
7. Mains, R. E., B. A. Eipper, and N. Ling. 1977. Common precursor to corticotropins and endorphins. *Proc. Natl. Acad. Sci. U. S. A.* 74:3014–3018.
8. Mains, R. E., and B. A. Eipper. 1978. Coordinate synthesis of corticotropins and endorphins by mouse pituitary tumor cells. *J. Biol. Chem.* 253:651–655.
9. Pelletier, G. 1971. Détection des glycoprotéines dans les cellules corticotropes de l'hypophyse du rat. *J. Microsc. (Paris).* 11:307–330.
10. Pelletier, G., F. Robert, and J. Hardy. 1978. Identification of human anterior pituitary cells by immunoelectron microscopy. *J. Clin. Endocrinol. Metab.* 46:534–542.
11. Pelletier, G., R. Leclerc, F. Labrie, M. Chrétien, and M. Lis. 1977. Immunohistochemical localization of β-lipotropic hormone in the pituitary gland. *Endocrinology.* 100:770–776.
12. Roberts, J. L., and E. Herbert. 1977. Characterization of a common precursor to corticotropin and β-lipotropin: identification of β-lipotropin peptides and their arrangements relative to corticotropin in the precursor synthesized in a cell free system. *Proc. Natl. Acad. Sci. U. S. A.* 74:5300–5304.
13. Roberts, J. L., M. Phillips, P. A. Rosa, and E. Herbert. 1978. Steps involved in the processing of common precursor forms of adrenocorticotropin and endorphin in cultures of mouse pituitary cells. *Biochemistry.* 17:3609–3618.
14. Steiner, D. F., W. Kemmler, H. S. Tager, and A. H. Rubenstein. 1974. Molecular events taking place during intracellular transport of exportable proteins. The conversion of peptide hormone precursors. *In* Advances in Cytopharmacology. Vol. 2. C. Ceccarelli, F. Clementi, and J. Meldolesi, editors. Raven Press, New York, 195–205.
15. Sternberger, L. A. 1974. The unlabeled antibody enzyme method. *In* Immunocytochemistry. Prentice-Hall, Inc. Englewood Cliffs, New Jersey, 129–171.