In Cow Anterior Pituitary, Growth Hormone and Prolactin Can Be Packed in Separate Granules of the Same Cell

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ABSTRACT The ultrastructural localization of growth hormone and prolactin in cow anterior pituitary was studied by double immunocytochemical labeling using specific antibodies and protein A-gold particles of different sizes.

The two hormones were found in specific somatotrophs and mammotrophs as well as in somatomammotropic cells which were multinucleated and predominantly arranged in clusters in the central area of the lobules. In these mixed cells the two hormones were packaged (*a*) in different granules of the same cell, (*b*) in the same granules where they were segregated in different portions of the granule content, or (*c*) in the same granules but evenly intermixed. The relative proportion of these three types of granules varied in somatomammotrophs of different animals.

A single large Golgi complex was generally present in somatomammotrophs. Small, immature granules containing either growth hormone or prolactin or both hormones were found randomly distributed along Golgi stacks. This suggests that in these cells the two hormones are processed in the same Golgi cisternae and that mechanism(s) exist(s) to sort out the two hormones from each other.

Acidophilic cells of the anterior pituitary have been among the favorite models for the study of intracellular transport, storage, and release of secretory proteins. So far, most of these studies have been carried out in the rat, in which two distinct types of cells are easily identified even by conventional thin section electron microscopy: somatotrophs and mammotrophs, which secrete growth hormone (GH)[1] and prolactin (PRL), respectively (1–3). In other mammalian species the identification of these cell types is not as straight forward as in the rat, but the existence of two distinct cell populations, one for each hormone, is almost generally accepted (4–10). Mixed cells, secreting both GH and PRL, have been proposed to exist in normal human pituitary gland (11) and in human and rat tumors, the latter including some clones of the well-known cultured cell line GH_3 (12–14); somatomammotrophs have also been described in human adenohypophysis in pregnancy and postpartum period (15) and in estrogen-treated rats (16).

During the last several years, our laboratory has been interested in the study of the mechanisms which underly the intracellular transport (in particular the packaging within the secretion granules) of GH and PRL. In the course of these studies, an immunocytochemical approach has been developed to reveal the localization of the hormones as well as that of other minor components of the granule content, which were suspected to play a role in the packaging process (17). An unexpected result was the observation that, in the cow anterior pituitary, GH and PRL have a dual localization: in specific somatotrophs and mammotrophs as well as in mixed, somatomammotropic cells, which have a different morphology and localization within the gland. The interest of this observation is that it provides some clues on the packaging of hormones in the acidophilic cells of the anterior pituitary.

MATERIALS AND METHODS

Antisera: Anti-bovine GH (NIH-GH-B18) antiserum was developed in rabbits and antibodies were purified by affinity chromatography as previously described (18). The specificity of eluted immunoglobulins was determined by two-dimensional PAGE of proteins immunoprecipitated from total anterior pituitary homogenate (18), and by immunoblotting after SDS PAGE of total anterior pituitary. No cross-reactivity with PRL was observed.

Antiserum against ovine PRL was the kind gift of Dr. C. H. Li (Hormone Research Laboratory, University of California, San Francisco, CA). No cross-reactivity with GH was observed by immunoblotting after SDS PAGE of total anterior pituitary proteins.

Immunoelectron Microscopy: Anterior pituitaries from seven 2–3-y-old Holstein Friesian nursing cows and from one virgin calf were collected immediately after slaughter and fixed for 1 h at 4°C with 2% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4); the tissues were then postfixed for 1 h at 4°C with 1% OsO_4 in the same buffer, dehydrated, and embedded in Epon.

[1] *Abbreviations used in this paper*: GH, growth hormone; PRL, prolactin.

Protein A-gold complexes of different sizes were prepared as previously described (19). Briefly, 3–5-nm gold particles were obtained by reduction of tetrachloroauric acid (Sigma Chemical Co., St. Louis, MO) with white phosphorous; for 16–18-nm gold particles, sodium citrate was used. After conjugation with the adequate amount of protein A (Sigma Chemical Co.) the complexes were centrifuged on a 10–30% linear gradient of glycerol to obtain particles of homogeneous size.

For double immunolabeling, thin sections were mounted on uncoated nickel grids and treated for 1 h with sodium-meta-perjodate (20); GH and PRL were immunolocalized, each on one side of the section, using protein A-gold particles of different sizes (21). The specificity of the immunostaining was tested by (*a*) substituting GH and/or PRL antisera with preimmune sera; (*b*) omitting one component of the reaction; or (*c*) using the same antiserum on both sides of the section.

RESULTS

In cow anterior pituitary, cells are organized in large lobules, separated by fibrillar connective tissue containing the vessels. The majority of the cells at the periphery of the lobules are mononucleated and heterogeneous in size and shape, as well as in the dimension and electron density of their secretion granules. The central area of lobules is occupied primarily by cells of larger size containing several nuclei usually arranged around a well-developed Golgi area; in their cytoplasm, dense secretory granules were clustered primarily toward the periphery, in between the nuclei and the plasma membrane (Fig. 1). Within the lobules the cells are closely apposed and interdigitated. No basal lamina is visible in between the cells.

By the use of specific antibodies and immunogold technique, the smaller cells located preferentially at the periphery of the lobules were found to be accounted for by the various pituitary hormone producing cells: corticotrophs, gonadotrophs, thyrotrophs (not shown), and, especially, mammotrophs and somatotrophs (Fig. 2, *A* and *B*). The large, multinucleated cells, on the other hand, were found to be mixed cells, containing in all cases GH as well as PRL (Figs. 2*C*–4). It was calculated that approximately half of the cells reactive for either one of the two hormones was reactive also for the other in cow anterior pituitary sections. Under our experimental conditions, GH and PRL immunoreactivities were detectable on secretory granules only. In all the seven pituitaries investigated, GH and PRL were found packaged in separate granules (Fig. 2, *C* and *D*). No major differences were found between GH– and PRL–containing granules: they were both spherical or ovoidal, and their size varied considerably (diameter of sectioned profiles between 200 and 900 nm). Within the cytoplasm, the granules of the two classes were intermingled, apparently at random.

Co-localization of GH and PRL within individual granules was also observed (Fig. 3), giving rise to two distinct patterns: granules with heterogeneous content (Fig. 3*A*) and granules in which the two hormones were evenly intermixed (Fig. 3*B*). The granules with heterogeneous content had parts composed of either GH or PRL directly opposed, with no intervening structures in between. The relative proportion of the two components varied from approximately 1:1 to 1:10 in the sectioned profile. Granules of this type were seen in very few cells, which also contained granules positive for either of the two hormones. The granules with the two hormones evenly intermixed in the content were found in a large proportion (~70%) of the somatomammotrophs of one cow. In some of these cells granules positive for either GH or PRL were also observed (Fig. 3*C*).

Secretion granules are known to be assembled in the Golgi complex. In the cow pituitary, the degree of condensation of GH and PRL which is achieved in the *trans* Golgi cisternae is less pronounced than in the rat, as indicated by the absence of dense granule cores within those cisternae. In addition, the immunocytochemical procedure that we used was not sensitive enough to reveal unambiguously the diluted concentrations of the hormones which exist within the Golgi cisternae. Although at these sites the labeling appeared higher than background, it was too low to permit conclusions as to the relative distribution of the two hormones. Indirect information about the situation in Golgi cisternae might be deduced from the study of the small granules in the Golgi area, which are believed to be newly assembled as a consequence of the fusion of Golgi-derived vesicles and the concomitant concentration of the content (22). In all cells investigated, these granules were found to have the same structure as the larger

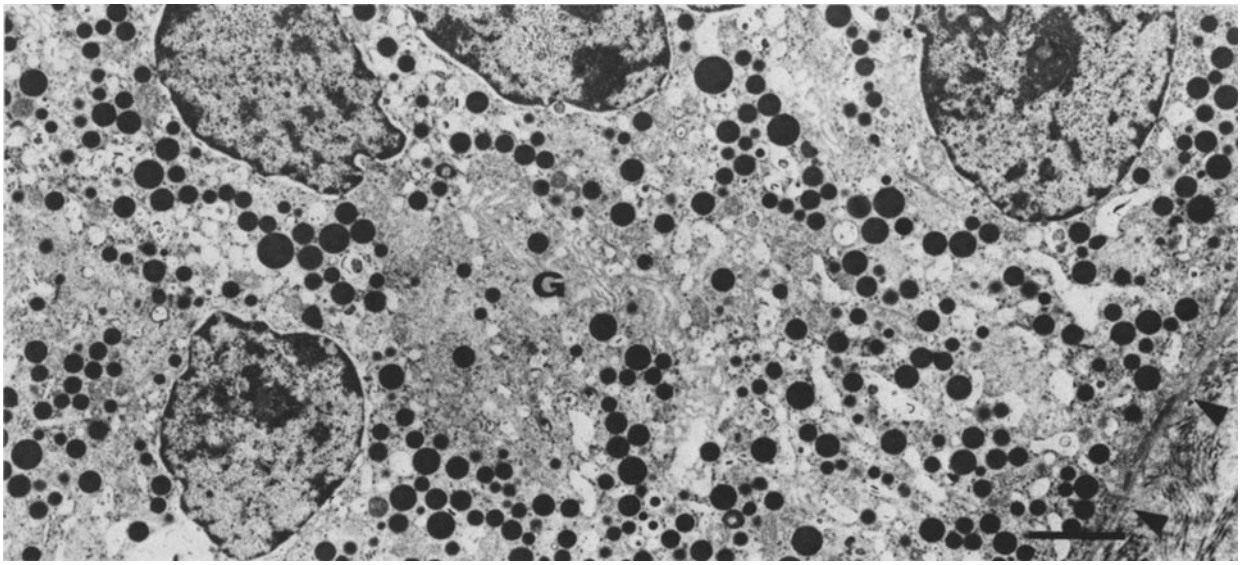

FIGURE 1 Low magnification micrograph of a large multinucleated cell of the cow anterior pituitary. A well-developed Golgi complex (G) is present in the center of the figure; granules are of heterogeneous size. Basal lamina and fibrillar connective tissue outline the periphery of the lobule (arrows). Bar, 2 μm. × 6,600.

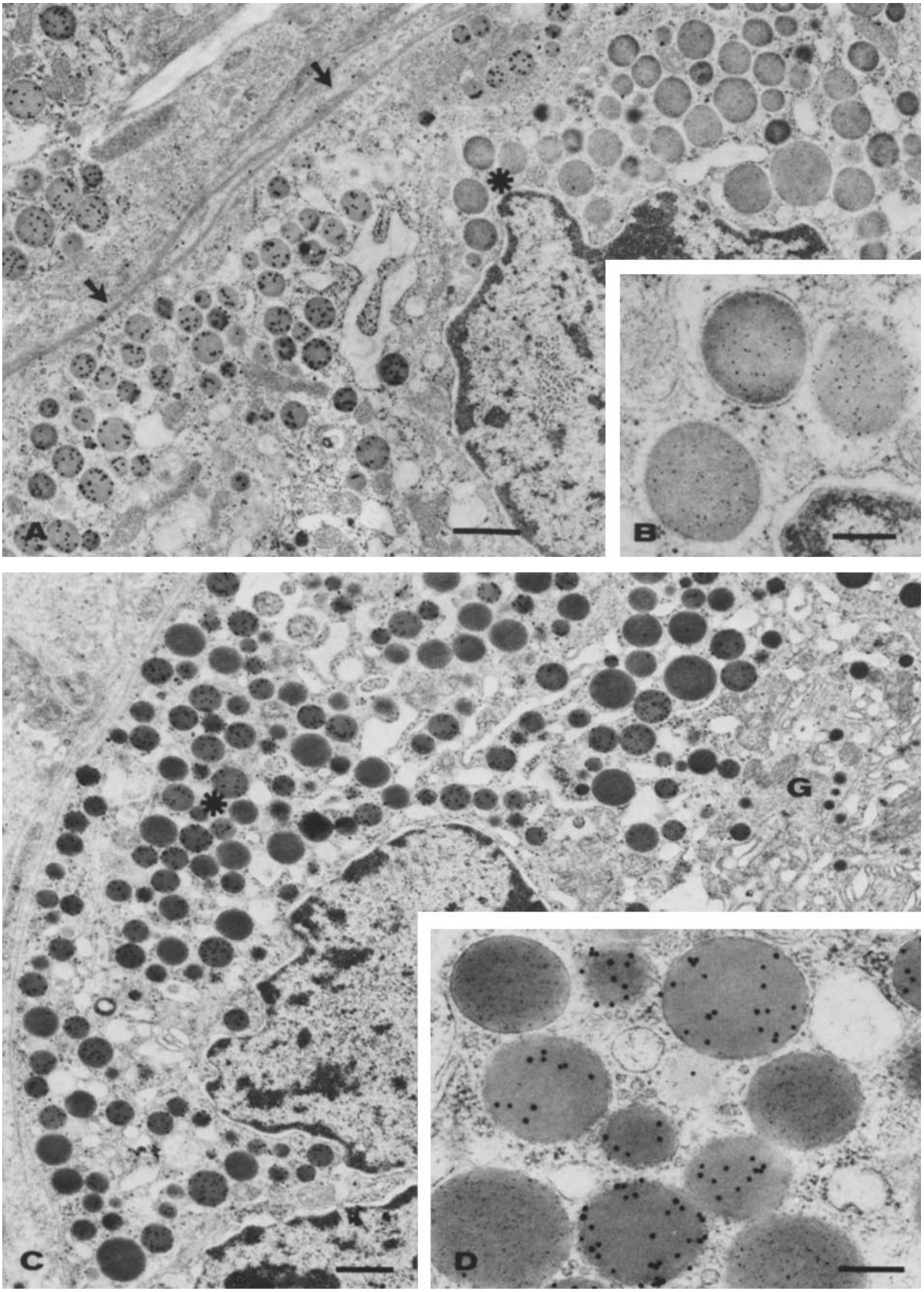

FIGURE 2 Immunocytochemical localization of GH (small gold particles) and PRL (large gold particles) in cow anterior pituitary. In *A*, two adjacent lobules are separated by the basal lamina (arrows). Two mammotrophs and one somatotroph are seen: each cell contains granules immunoreacting for one hormone only. The asterisk indicates the area enlarged in *B* which shows the immunolocalization of GH in the somatotroph cell. In *C*, a large, multinucleated somatomammotroph is shown. A large Golgi region is present. The asterisk indicates the area enlarged in *D*; note that each granule is immunoreacting for one of the two hormones only. (*A*) Bar, 1 μm; × 12,000. (*B*) Bar, 300 nm; × 38,000. (*C*) Bar, 1 μm; × 10,000. (*D*) Bar, 300 nm; × 39,000.

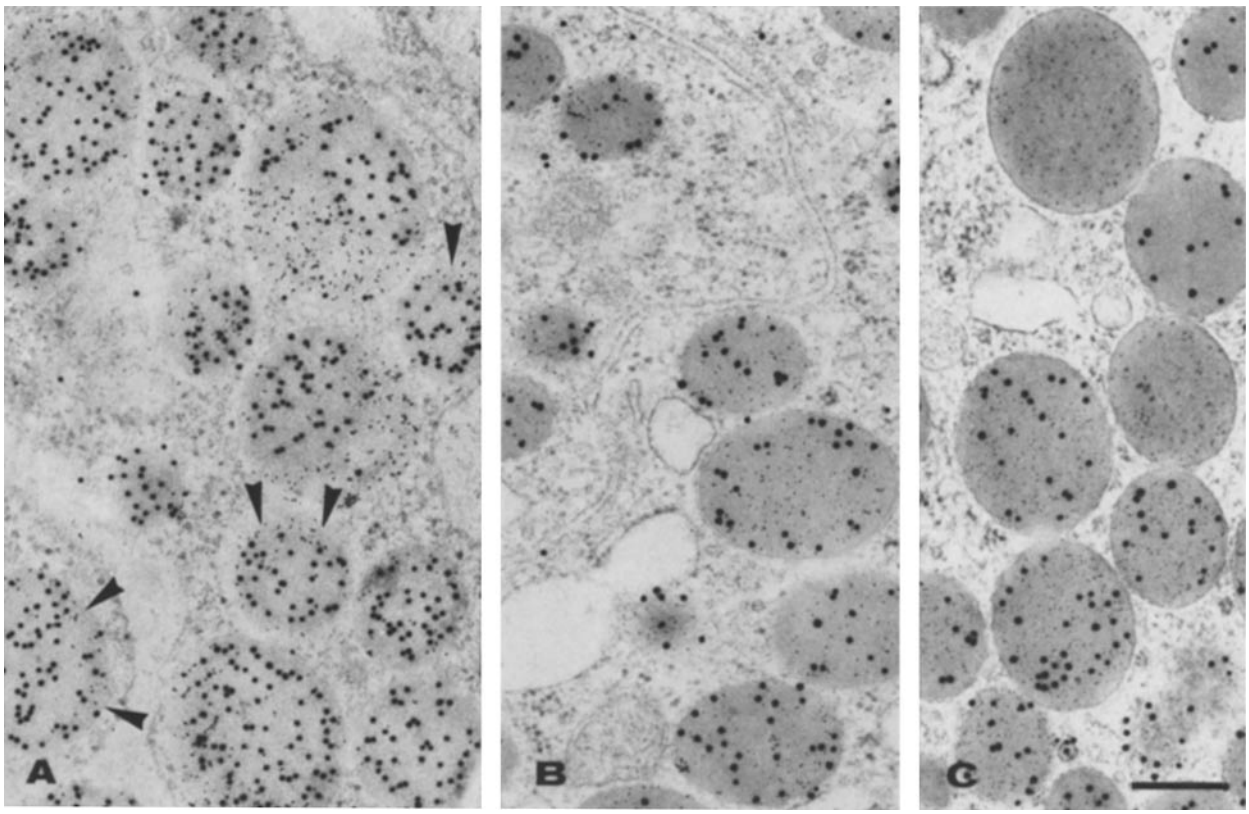

FIGURE 3 Immunocytochemical localization of GH (small gold particles) and PRL (large gold particles) in cow anterior pituitary. Some of the granules in *A* have heterogeneous content with parts composed of either GH or PRL. Note that PRL is always contained in a mass whereas GH is often restricted to a rim (arrowheads). In *B*, the two hormones are evenly intermixed in the granules of two adjacent cells. In *C*, granules immunoreacting for the two hormones are present together with granules positive for either GH or PRL. Bar, 300 nm. × 41,500.

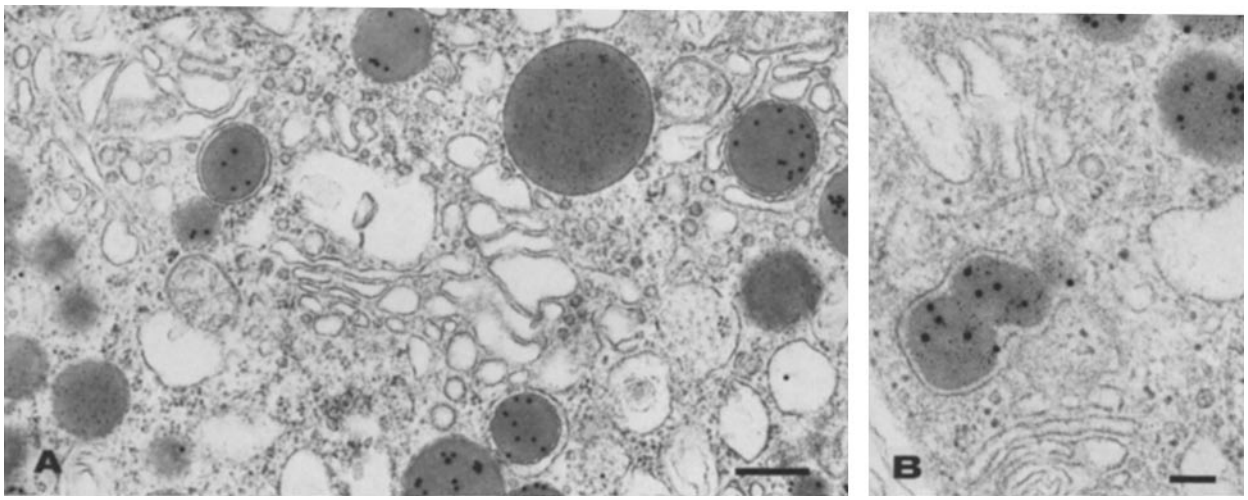

FIGURE 4 Immunocytochemical localization of GH (small gold particles) and PRL (large gold particles) in cow anterior pituitary. (*A*) Golgi stacks are surrounded by granules of different size containing either of the two hormones. Immunoreactivity is undetectable in the Golgi cisternae. (*B*) Small granules that contain evenly intermixed GH and PRL are close to Golgi profiles. Intermixing of the hormones also occurs in the immature, pleomorphic granule. Bar, 200 nm. (*A*) × 31,000; (*B*) × 50,000.

granules located more peripherally. Thus, small granules containing either GH or PRL were found randomly distributed in the Golgi area of somatomammotropic cells (Fig. 4 *A*), and small and occasionally pleomorphic (immature) granules containing intermixed GH and PRL were seen in the Golgi area of most of the somatomammotrophs of one cow (Fig. 4 *B*).

DISCUSSION

The hormone specificity of the cow pituitary cells was investigated previously by Dacheux and Dubois (4). As far as acidophilic cells are concerned, they commented about somatotrophs and mammotrophs being scattered and in clumps, respectively. They also mentioned the existence of

marked polymorphism of PRL-containing cells but apparently did not notice the presence of multinucleated somatomammotropic cells. Mixed somatomammotrophs were described in tumors (12–14). However, the subcellular localization of the hormones in concentrated form, whether in the same or in separate secretion granules, was not clearly established.

The origin of the large, multinucleated somatomammotropic cells of the cow anterior pituitary is not clear at present. Since the cows studied were lactating until shortly before death, we felt initially that these cells might have resulted in response to stimulation, i.e., that during lactation somatotropic cells were stimulated to express PRL as well. Recently, however, we have studied the pituitary of a virgin cow and found mixed somatomammotrophs as well, although in a lower number (data not shown). Thus, lactation is apparently not necessary for the appearance of these cells. Being multinucleated, somatomammotropic cells could derive by fusion of pre-existing cells (expressing either one of the two hormones) or by nuclear duplication without subsequent cytodieresis. At the moment we have no data to choose between these two alternatives.

Recent studies by Gumbiner and Kelly have indicated that at least two secretory pathways exist in a pituitary cell line, AtT-20 (23). Of these pathways, one appears to be regulated and devoted primarily to the transport of secretion products specific for that type of cell, the other to be constitutive and to be used primarily for membrane and extracellular matrix components. Other recent results (24) demonstrated that, when the gene for a typical secretory protein (insulin in the Gumbiner and Kelly experiments) is cloned into a heterologous secretory cell (AtT-20 cells which expresses ACTH), the products traveled intracellularly along the regulated pathway, together with the indigenous hormone ACTH. These observations appear in line with the fact that in glandular cells that produce many different secretory proteins (22 in the pancreatic acinar cells) all these are co-packaged together in one single type of granule (25). This rule, however, has exceptions. In granulocytes, the classical studies of Bainton and Farquhar (26) revealed that the two types of granules (one of which is however of lysosomal nature) are produced asynchronously at the opposite faces of the Golgi stack: first the azurophilic, and later, the specific granules. In the exocrine pancreas of various species mixed, exo-endocrine cells have been described (27). Granules were reported to be produced either in separate Golgi stacks, or at opposite sides of Golgi cisternae, and to migrate then to opposite poles of the cells: the apical pole for zymogen granules and the basal pole for B granules. Finally, in the thyroid gland, thyroglobulin-containing B granules seem to originate from Golgi cisternae, whereas peroxidase-containing A granules were reported to originate from GERL (28).

Segregation of GH and PRL in different secretory granules of the same cell could be a result of asynchronous synthesis of the two hormones. Although we cannot rule out this possibility, it appears to be unlikely because pituitary glands are long-lived and GH and PRL have fast turnover so that their synthesis and discharge, although quantitatively modulated with time, are continuously going on in vivo. In addition, this hypothesis does not explain the co-existence of somatomammotrophs containing granules with evenly intermixed hormones along with somatomammotrophs packaging GH and PRL in different granules within the same pituitary. Origin of granules containing either of the two hormones from separate Golgi complexes appears also unlikely; in fact, the mixed cells have usually only one single, although very large Golgi area. It is possible that the large Golgi complex is formed by the fusion of different Golgi complexes. Under our experimental conditions, immunoreactivity was detectable in secretory granules only: therefore, we cannot either prove or rule out that the large Golgi complex is compartmentalized in small units, each one processing either of the two hormones. However, the presence of a population of small granules positive for either of the two hormones randomly distributed close to individual Golgi stacks and, above all, the existence of intermixed GH-PRL-containing granules would not be explained by this model. At the moment we favor an alternative explanation, i.e., that granules are assembled in one and the same Golgi complex, where mechanism(s) exist(s) to sort out the two hormones from each other. The existence in some animals, together with granules containing either hormone, of other granules of the intermixed type suggests that the sorting process has limited efficacy and can be overwhelmed.

Newly synthesized PRL molecules are believed to be free in solution within the lumen of endoplasmic reticulum cisternae (22). Previous studies, on the other hand, demonstrated that within the granules, the molecules of the hormone are arranged in a solid-state structure (29), which is only marginally affected by the removal of the granule-limiting membrane (29, 30). Insolubilization of PRL, a process which appears to take place at the *trans* face of the Golgi complex (22), could account for the sorting of the hormone from GH. Although the mechanisms which regulate PRL insolubilization have not been identified with certainty, the changes of the physicochemical environment (pH, ionic environment, and ionic strength) occurring along the secretory pathway could be of importance. In addition, minority components of the granules, such as a "secretogranin," a tyrosine-sulfated protein recently characterized (17, 31), could play some role in the process. In fact, this protein is present in mammotrophs but absent from somatotrophs (17). Further studies to correlate the subcellular distribution of GH, PRL, and "secretogranin" in somatomammotrophs are now in progress.

Whatever the mechanism(s), the possibility that a preferential insolubilization of PRL is responsible for our data appears attractive because it would explain the different behavior of the pituitary PRL and GH with respect to other secretory proteins. In addition, in that the mechanism is based on the tendency of a protein to come out of solution, rather than on specific, high-affinity molecular recognition, it would be expected to be less stringently regulated and to allow some degree of spill (formation of mixed granules).

The mixed granules containing separate masses of the two hormones (Fig. 3*A*) could originate from granules containing GH and PRL evenly intermixed (as in Fig. 3*B*) where phase separation of the two proteins had occurred. However, phase separation did not occur in that pituitary where a large proportion of somatomammotrophs were packaging the two hormones evenly intermixed in the same granules. Therefore, a more likely explanation is that these granules originate from the fusion of preassembled granules containing either GH or PRL. This process occurs, at least in the rat, during maturation of PRL granules (32). In somatomammotropic cells it could involve fusion of heterologous (GH- and PRL-containing) granules as well. In this respect it should be mentioned

that in these mixed-segregated granules (Fig. 3 *A*), PRL (which we expect to be the more insolubilized component) was always contained in a mass, whereas GH was often restricted to a rim, as it had been squeezed around the PRL mass after granule fusion. The granules containing the two hormones intermixed together (Figs. 3, *B* and *C*, and 4 *B*), on the other hand, would be expected to occur in somatomammotropic cells whenever the conditions leading to PRL insolubilization would become inefficient and/or saturated.

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