MOLECULAR ORGANIZATION OF RAT PROLACTIN GRANULES

I. In Vitro Stability of Intact and "Membraneless" Granules

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Studies carried out on a number of secretory cell systems suggest that the specific cytoplasmic granules in which the secretion products are stored before their release are complex organelles which can possess a distinct molecular organization. For instance, it has been reported that in some granules the segregated secretion products are organized into crystalline structures (1–3) or large intermolecular aggregates (4–8). It is likely that all phenomena of this type are favorable to the economy of the cell, in the sense that they reduce the energy required for storage of the secretion products.

The prolactin (LTH)[1] granules of the rat pituitary possess a number of morphological features which strongly suggest that the molecule(s) of their content might be arranged in a relatively stable structure. Thus, these granules are remarkably polymorphic in shape, and their membrane is usually separated from their content by a clear space. Furthermore, identifiable LTH granules devoid of their membrane are often seen in the pericapillary space, suggesting that upon discharge by exocytosis they are dissolved only slowly (9). However, no studies specifically concerned with the mechanisms of LTH storage have been reported so far.

In order to obtain some information on this question, we have studied the behavior of isolated granule fractions incubated in vitro under a variety of carefully controlled experimental conditions.[2]

MATERIALS AND METHODS

Anterior pituitary glands (obtained from female Sprague-Dawley rats, 200–250 g in weight) were homogenized and fractionated by centrifugation as described in reference 10. The following fractions were used in this study: (*a*) a 15,000-*g*, 30-min pellet obtained from a postnuclear supernate (crude granule fraction; P in reference 10); (*b*) a purified LTH granule fraction (fractions 3 + 4 of reference 10).

LTH was assayed by microdensitometry after separation by two different procedures of polyacrylamide gel electrophoresis: the nondissociating method of Ornstein and Davis (11, 12), modified as in reference 13, and the Na dodecylsulfate (SDS) method of Maizel (14, 15). Protein was measured according to Lowry et al. (16); phospholipids were extracted and purified according to Folch et al. (17) and assayed according to Ames (18).

For electron microscopy, the pellets were fixed overnight at 4°C in 1% OsO_4 in 0.1 M cacodylate buffer (pH = 7.2), then stained in block with 0.5% Mg uranyl acetate in 0.9% NaCl, and processed as described in reference 10. Purified LTH granules were also studied by negative staining with 2% phosphotungstic acid (PTA), pH = 6.9, containing 0.1% bovine serum albumin. The sources of the materials used are listed in reference 15.

RESULTS AND DISCUSSION

A partial characterization of the cell fractions used in this work has been reported previously (10, 19). The crude granule fraction contains the bulk of the LTH granules originally present in the pituitary homogenates but also includes most of the GH granules and many mitochondria and microsomes, whereas the purified LTH granule preparations contain very few contaminants. The morphology and the biochemical composition of the latter fraction were further investigated in the course of this work.

The isolated LTH granules appear as dense structures, 3,500–4,000 Å in diameter, round, oval, or polycyclic in shape, surrounded by a membrane usually separated from the granule core by a clear space 100–500 Å wide. At high magnification, both in sections and after negative staining, such a core reveals neither a crystalline nor a periodical organization (Fig. 1). By both the polyacrylamide gel electrophoresis procedures that we used, the

[1] *Abbreviations used in this paper:* LTH = prolactin; GH = growth hormone; SDS = sodium dodecylsulfate; DOC = sodium deoxycholate; EDTA = ethylenediaminetetraacetic acid; PLP = phospholipids; PTA = phosphotungstic acid.

[2] A part of this work was presented at the Advanced Study Institute on Cytopharmacology of Secretion, Venice and Milan, Italy, 1973.

THE JOURNAL OF CELL BIOLOGY · VOLUME 64, 1975 · pages 246–251

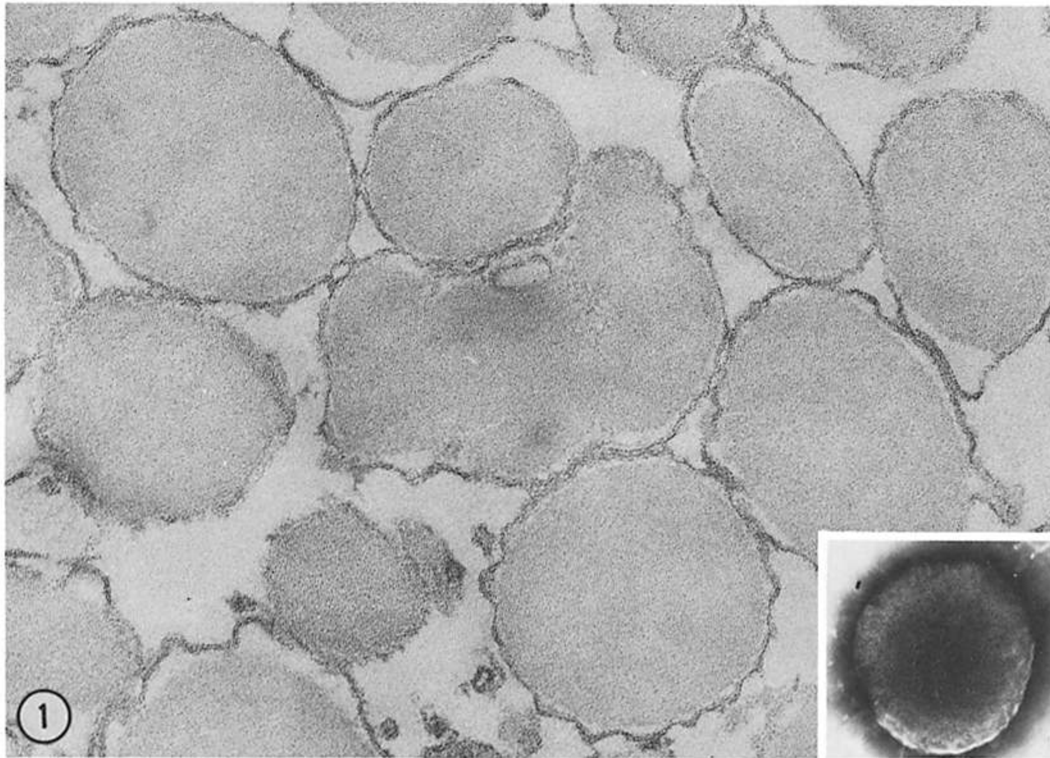


FIGURE 1 Purified LTH granule fraction. Several large, dense, irregularly shaped granules are shown in the field. They appear surrounded by a continuous limiting membrane which is usually separated from the granule core by a clear space. × 110,000. The *inset* shows one LTH granule after negative staining with PTA. × 81,600.

proteins of the purified granules were found to be mostly accounted for by the hormone (> 80% of the total)[3] (Fig. 2, B and D). A few other minor components (probably membrane proteins) were revealed by the SDS method.

In order to get some indirect information on the mechanisms of intragranular hormone storage, LTH granules were submitted to a number of treatments, most of which are known to severely damage isolated organelles (20–25). For convenience, many of these experiments were carried out with crude granule preparations; however, with purified granules similar results were obtained. The fractions were incubated in vitro under the conditions specified in Table I and in Fig. 3. After the incubation the preparations were centrifuged at 100,000 *g* for 45 min in a Spinco 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Pellets and supernates were assayed for total protein and LTH.

We found that LTH granules are apparently not affected by any of the experimental conditions listed in Table I, which include severe hypotonic shock and strong hypertonicity, treatment with different concentrations of nonelectrolytes, monovalent and divalent cations, and chelating agents. Furthermore, LTH granules are stable over a wide pH range: from 3.6 to 7.4. A considerable number of them is dissolved only under very acidic conditions (pH 2.6), while at pH 8.5 only a moderate solubilization occurs (Fig. 3). The pellets obtained by centrifuging the incubated granules were routinely inspected by electron microscopy: in all cases where the biochemistry failed to reveal a large solubilization of the hormone, numerous intact granules were observed. Thus, the absence of solubilization is most likely due to the preservation

[3] This value is higher than that reported previously (10) for the same fraction. However, the inconsistency is only due to the inaccuracy of the LTH calibration curve previously used.

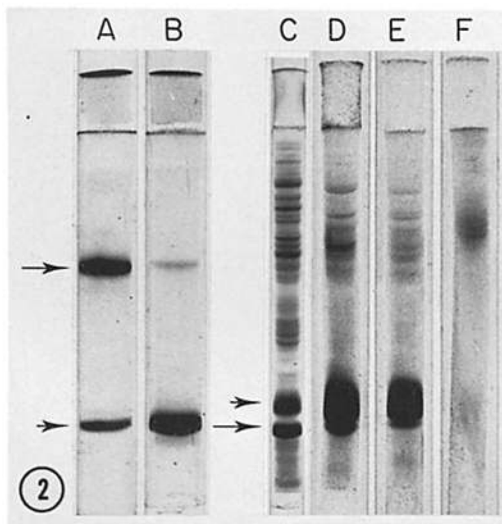


FIGURE 2 Polyacrylamide gel electrophoresis of LTH granules and granule subfractions. A and C = crude LTH granules; B and D = purified LTH granules; E = purified membraneless granules, obtained by treatment with Lubrol PX; F = membrane proteins, solubilized by the detergent. The gels A and B (7% acrylamide, 5 cm in length, 0.5 cm in diameter) were obtained by the method of Ornstein and Davis and stained with Amido Black; the gels C, D, E, and F (10% acrylamide, 5 cm in length, 0.3 cm in diameter) were obtained by the SDS method and stained with Coomassie brilliant blue. The long and short arrows label the bands containing GH and LTH, respectively.

TABLE I

In Vitro Treatments which do not Affect the Stability of the LTH Granules (Hormonal Release <5%)

Treatment	Temperature	Time
	°C	*min*
Water	4	60
Sucrose 0.08–1.0 M	4	60
Sucrose 0.32 M	4; 37	60
Sucrose 0.32 M	24	15–120
Sucrose 0.32 M plus EDTA 0.1–1.0 mM	4; 37	60
KCl 0.04–1.0 M	4	60
$CaCl_2$ 0.025–0.5 M	4	60
$MgCl_2$ 0.025–0.5 M	4	60

The pH of the incubation media was 6 ± 0.2. The experiments were repeated two to four times. Either crude or purified granule preparations were used in these experiments, with identical results. They were incubated in vitro under the conditions specified above and then centrifuged at high speed. The lack of LTH granule solubilization was established by polyacrylamide gel electrophoresis of both the pellets and supernates and by electron microscopy of the pellets.

of the LTH granules and not to the precipitation or adsorption of released hormone molecules.

The effect of detergents was also investigated. In agreement with our previous findings (15), we observed that high concentrations of SDS produced a complete solubilization. In contrast, milder detergents, such as Na deoxycholate (DOC) or Lubrol PX, had little or no hormone-releasing activity (Table II). However, when the granules treated with these detergents were examined by electron microscopy, it was found that they are almost completely devoid of their limiting membrane. Lubrol PX-treated granules maintain their typical shape and their dense texture (Fig. 4): hereafter they will be designated as "membraneless LTH granules." DOC-treated granules appear more damaged and exhibit a moth-eaten outline (not shown).

In agreement with these morphological observations, we found that the bulk of the phospholipids originally present in the granule preparations is solubilized by the treatment with the detergents (Table II). Furthermore, the proteins solubilized

FIGURE 4 Membraneless LTH granules. The field is occupied by numerous dense, polymorphic granules devoid of limiting membrane. The structure of the granules is well preserved. However, some thick, short filaments which are continuous with the texture of the granules appear to radiate from their surface. A fragment of detached membrane is indicated by the arrow. × 100,000. The appearance of the membraneless granules after negative staining is shown in the *inset*. × 81,600.

FIGURE 5 Membraneless LTH granules incubated for 60 min at pH 8.5 (see Fig. 3). Most of the material sedimented by centrifugation from these preparations is accounted for by a network of thick, twisted filaments which are often continuous with partially disarranged LTH granules (arrows). × 140,000.

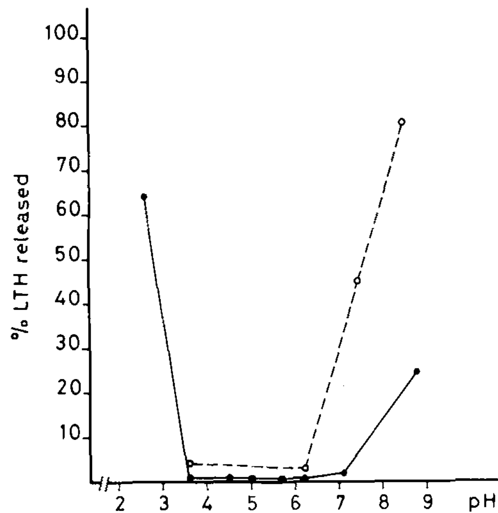


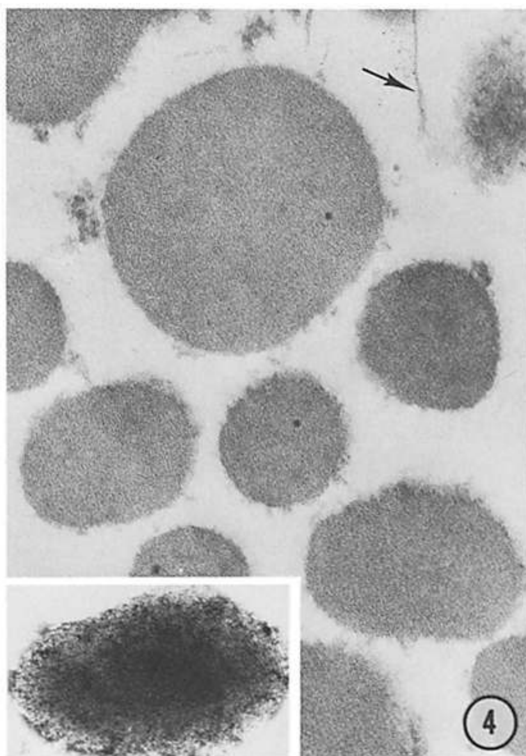
FIGURE 3 Effect of the pH on the stability of intact (continuous line) and membraneless (broken line) LTH granules. Isolated preparations were resuspended in 0.32 M sucrose, and the pH was adjusted to the specified values by the addition of phosphate buffer, 10 mM final concentration. The solubilization was estimated as described in the text and in Table I.

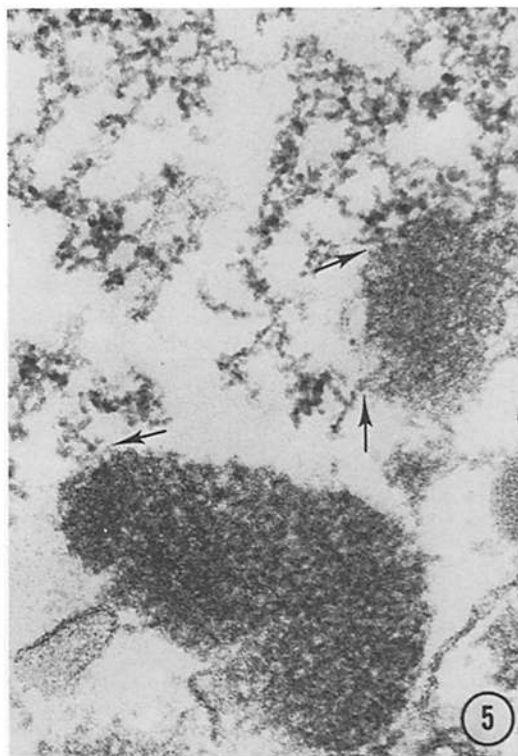
TABLE II

Effect of DOC and Lubrol PX on the Protein, LTH and PLP Release from Purified LTH Granules

Detergent	% Release*		
	Protein	LTH	PLP
%			
0.2 DOC	20.0	13	78.5
0.4 Lubrol PX	13.5	<5	95.0

* The values given are the averages of two consistent experiments. Purified LTH granules were resuspended in 0.32 M sucrose containing either 0.2% DOC or 0.4% Lubrol PX (detergent:protein ratio (wt/wt) ~6.5 and ~13.0, respectively). After 1 h of incubation at 4°C the preparations (0.5–0.7 ml) were layered on a cushion of 1.2 M sucrose and centrifuged at 30,000 *g* for 60 min in a Spinco SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div.). The percent of release of the molecular components originally present in the preparations was estimated by assays carried out on both the pellets (which contain the membraneless granules) and the load region of the gradients (which contains the solubilized material).





by Lubrol PX do not include LTH but only a few minor components (most likely, membrane components) (Fig. 2, E and F). Membraneless granules were tested as to their in vitro stability. Analogously with the situation described previously for intact granules, no hormone solubilization was obtained by incubation in 0.04–1.00 M KCl and 0.025–0.50 M $CaCl_2$. Membraneless granules are also stable at pH varying from 3.6 to 6.1, whereas at pH 7.4 and 8.5 their solubilization is much larger than that of the intact granules: over 45 and 80%, respectively (Fig. 3). Moreover, most of the granules recovered in the pellets obtained from the preparations incubated at pH 8.5 appear severely damaged or even completely disarranged into a network of twisted filamentous structures (Fig. 5).

In conclusion, these results clearly indicate that in the LTH granules the hormone is stored not in soluble form, but as a stable solid-state structure. In fact, (*a*) isolated LTH granules are apparently unaffected by a number of treatments which are known to solubilize secretory granules derived from different sources (19–25), and (*b*) such unusual resistance is primarily dependent on the organization of the granule content, since it is little changed after removal of the limiting membrane of the organelle.[4] Only in relation to the pH of the suspending medium did the behavior of membraneless and intact granules prove to be different: intact granules are stable upon incubation at pH 7.4 and only a small proportion of them is solubilized at pH 8.5, whereas membraneless granules are much more fragile. This phenomenon might have a physiological significance in relation to the mechanism by which the LTH granules released by exocytosis are solubilized in the slightly alkaline environment of the extracellular space.

Whether the stable organization of the LTH granules is simply due to the formation of polymeric aggregates of hormone molecules, or whether it requires the involvement of other molecules (for instance inorganic cations, organic polyanions, or nucleotides) is at present under investigation in our laboratory.

[4] The preservation of the structure of the granule content after removal of the membrane might not be a peculiarity of LTH granules only, since it has been observed with the specific granules of the eosinophilic leukocytes (Murer, H., and M. Baggiolini. Personal communication.) and with the granules of mast cells (25) as well. However, in these other cases the phenomenon was not studied in detail, and in particular, a comparison of the characteristics of the intact and membraneless granules was not made.

We are grateful to Dr. F. Clementi for helpful suggestions and to Miss E. Matera for technical assistance.

Received for publication 7 May 1974, and in revised form 22 August 1974.

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