# HORMONE STORAGE GRANULES IN THE BEEF ANTERIOR PITUITARY

## I. Isolation, Ultrastructure, and Some Biochemical Properties

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ABSTRACT

A method is described for isolation of relatively large quantities of large and small hormone storage granules from the beef adenohypophysis. The hormone storage granules are highly purified, as indicated by ultrastructural and biochemical criteria. The average size of large granules is 400 mμ and of small granules is 220 mμ. The large granules contain growth hormone and prolactin; the small granules contain high concentrations of follicle-stimulating, luteinizing, and thyroid-stimulating hormones. An alkaline protease with a pH optimum of 8.3 is associated with the small granule fraction.

Evidence accumulated in the last twenty years indicates that the six known hormones of the adenohypophysis are contained in separate and distinct cell types (1–4). The acidophilic cells in the rat and mouse pituitary synthesize and store somatotropin and prolactin (5–8). The basophilic cells produce thyrotropin and two gonadotropins (9–12). The host cell responsible for production and storage of corticotropin is still uncertain. Ultrastructurally these cell types differ mainly in the size of their storage granules. In the rat adenohypophysis the acidophilic cells contain large storage granules with a diameter of 350–600 mμ (7), whereas the granules of the basophils are much smaller, their diameter ranging from 140–200 mμ (4, 9). Several attempts have been made to isolate the storage granules of the adenohypophysis (15–18). However, only McShan and his group have succeeded in preparing from the rat adenohypophysis a large granule fraction and a small granule fraction relatively free of contamination by other subcellular structures (13, 14).

The study reported here is part of a continuing investigation of the structural components in various storage granules. Since the yield of purified granules from the rat pituitary is necessarily limited, we have attempted to isolate the small and large storage granules from bovine adenohypophysis. Initially we used the method developed by McShan et al. (13, 14). However, it soon became apparent that this procedure was not suited to beef pituitary without extensive modification. In this communication we describe fractionation procedures suitable for the isolation of a large and a small granule fraction from the bovine adenohypophysis. The electron microscopic, biochemical, and biological studies indicate that these fractions are highly purified. The lipid and protein composition and the histochemical properties of these granules will be reported

separately. Some of these results have appeared in abstract form (19).

## FRACTIONATION PROCEDURE

### *Preparation of Homogenate*

Bovine pituitary glands were obtained in a local stockyard. Glands were removed and placed on ice within 30 min after slaughtering, and brought to the laboratory for processing. All procedures were performed at 0–4°C. The homogenizing medium contained 0.33 M sucrose in 0.030 M Tris (tris hydroxymethyl amino ethane) buffered at pH 7.3. The adenohypophysis was dissected away from the neurohypophysis and pars intermedia, and stripped of its connective tissue capsule. Approximately 15–20 g of anterior pituitary tissue were used for the large granule fraction and 25–30 g for the small granule fraction. The tissue was homogenized in a Virtis homogenizer (Virtis Co. Inc., Gardiner, N.Y.) in a small volume of the medium for approximately 2 min at 1,000 rpm. The resulting mince was then diluted to a final volume containing 5% and 10% homogenate for the large and small granule fractions, respectively, and further homogenized for 2–4 min in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle at 1,000 rpm. The homogenate (WH) was centrifuged at 900 *g* for 10 min. The supernatant was recentrifuged at the same speed for an additional 10 min and designated $LS_1$.

### *Isolation of Large Granules*

The procedure is outlined in Fig. 1. The supernatant, $LS_1$, prepared from a 5% homogenate was filtered with negative pressure through a series of Millipore filters (Millipore Filter Corp., Bedford, Mass.) of diminishing pore size, namely 5, 1.2, and 0.8 $\mu$. The filtered supernatant, designated $FLS_1$,

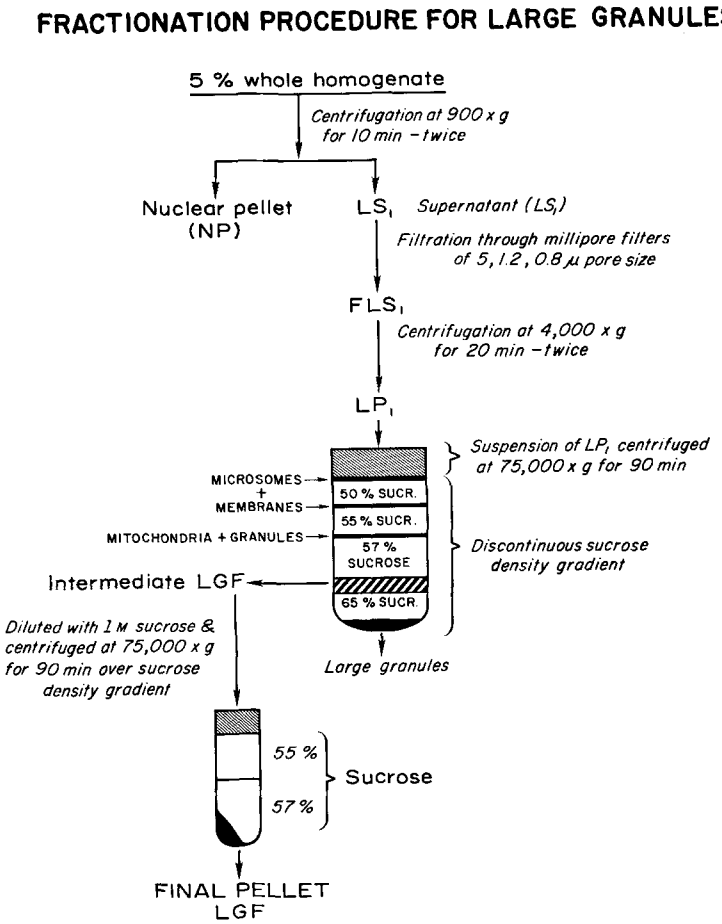


FIGURE 1 Isolation procedure for large hormone storage granules from bovine anterior pituitaries.

was centrifuged at 4,000 *g* for 20 min in a Sorvall centrifuge, Model RC 2-B. (Sorvall, Ivan, Inc., Norwalk, Conn.) The resulting pellet contained the bulk of large granules and mitochondria. The surface of the pellet was rinsed with a stream of the homogenizing medium delivered by a hypodermic syringe through a small-size needle so as to dislodge the superficial fluffy layer which could then be removed by suction. The remaining pellet was suspended in the homogenizing medium and re-centrifuged at the same speed. This procedure removed most of the microsomal contamination and some mitochondria. This pellet, designated $LP_1$, was resuspended in 8–10 ml of homogenizing medium, and placed on the top of a discontinuous sucrose gradient. The gradient system consisted of 50, 55, 57, and 65% sucrose in 0.02 M Tris buffer (pH 7.3) in 56-ml centrifuge tubes designed for the Spinco SW 25.2 rotor (Spinco Div., Beckman Instruments, Inc., Fullerton, Calif.). The volume of each layer was 8 ml, except for 20 ml in the 57% sucrose layer. The gradient was centrifuged at 75,000 *g* for 90 min in a Beckman $L_2$ preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The layer forming at the interface of 57 and 65% sucrose was removed either by sectioning the tube with a Spinco tube slicer or by aspiration of the layer with a syringe and a needle that had pierced the plastic tube slightly below the layer. This fraction containing most of the large granules was pooled and diluted with 34% buffered sucrose to a final concentration of about 45%. The granule suspension was placed on a discontinuous gradient of 55 and 57% buffered sucrose and again centrifuged in the SW 25.2 rotor at 75,000 *g* for 90 min. The resulting pellet contained the purified large granule fraction, LGF. Most of the pellet material was stored at −20°C for biochemical study. A small portion of the pellet remaining in the tubes was fixed overnight. Fixation of the pellet was performed with 2% glutaraldehyde in 1.2 M sucrose and 0.1 M cacodylate buffer of pH 7.3 at 4°C. About 1 mg of LGF protein was obtained from each gram of adenohypophyseal tissue.

### *Isolation of Small Granules*

The procedure is outlined in Fig. 2. The supernatant, $SS_1$, prepared from the 10% homogenate was centrifuged at 5,000 *g* for 20 min in a Sorvall Model RC 2-13 refrigerated centrifuge. The resulting supernatant, containing most of the small granules, was filtered (with negative pressure) through Millipore filters of graded pore size in the following order: 1.2 μ, 0.65 μ, 0.45 μ, and 0.3 μ. The last filtration step was repeated. The filtered fraction, $FSS_2$, was centrifuged at 14,000 *g* for 20 min to deposit a crude small granule fraction. The surface of the pellet was rinsed with a stream of homogenizing medium delivered by a hypodermic syringe through a small-size needle and the dislodged superficial layer was removed by suction. The remaining pellet was resuspended, centrifuged again at 14,000 *g* for 20 min, and the surface was cleansed in the same manner. This treatment removed most of the microsomal contamination. The pellet was resuspended in about 5 ml of homogenizing medium and placed on the top of a continuous linear gradient consisting of 10–40% sucrose in 17.5% Diodrast (diethanolamine salt of 3-diiodo-4-pyridone-*N*-acetic acid). Centrifugation was performed in the Spinco SW 25.2 rotor at 75,000 *g* for 180 min. Three distinct layers were formed at equilibrium. The upper two layers located near the top of the gradient contained microsomal material, some mitochondria, and granules. A third layer located three-quarters of the distance from the top of the gradient contained mainly small granules. This layer was removed with a syringe and needle or by sectioning the tubes with a Spinco tube slicer. The fraction was diluted with an approximately equal volume of 1 M sucrose, placed over a discontinuous gradient consisting of 50 and 55% buffered sucrose in 12-ml tubes and centrifuged in a No. 40 Spinco rotor at 105,000 *g* for 80 min. The final pellet contained the purified small granule fraction (SGF). A small portion of this fraction was fixed for electron microscopy and the remainder kept at −20°C for biochemical studies. The yield of the SGF was 0.1 mg per g of adenohypophysis.

### *Electron Microscopy*

Freshly excised adenohypophysis was cut into 1–2 mm pieces and immediately fixed in buffered 2% osmium tetroxide at 4°C for 2 hr. Fractions were fixed overnight at 4°C in cacodylate-buffered 2% glutaraldehyde with 1.2 M sucrose and were post-fixed in buffered 2% osmium tetroxide for 1 hr at 4°C. The pellets were embedded in Epon 612 or Araldite, sectioned, and stained with uranyl acetate. The stained sections were examined in a RCA EMU 3F electron microscope.

### *Biochemical Assays*

Protein was determined by the method of Lowry et al. (20). Beef serum albumin was used as a standard. Acid phosphatase activity was measured by the method of Andersch et al. (21), with the use of *p*-nitro-phenylphosphate as a substrate at pH 4.6. Succinic dehydrogenase activity was determined by the method of Slater and Bonner (22), and glucose-6-phosphatase by that of Swanson (23). Alkaline protease activity was measured by the method of Anson (24), with use of a 2% solution of denatured hemoglobin at pH 8.3 as a substrate. Alkaline protease results are expressed as relative

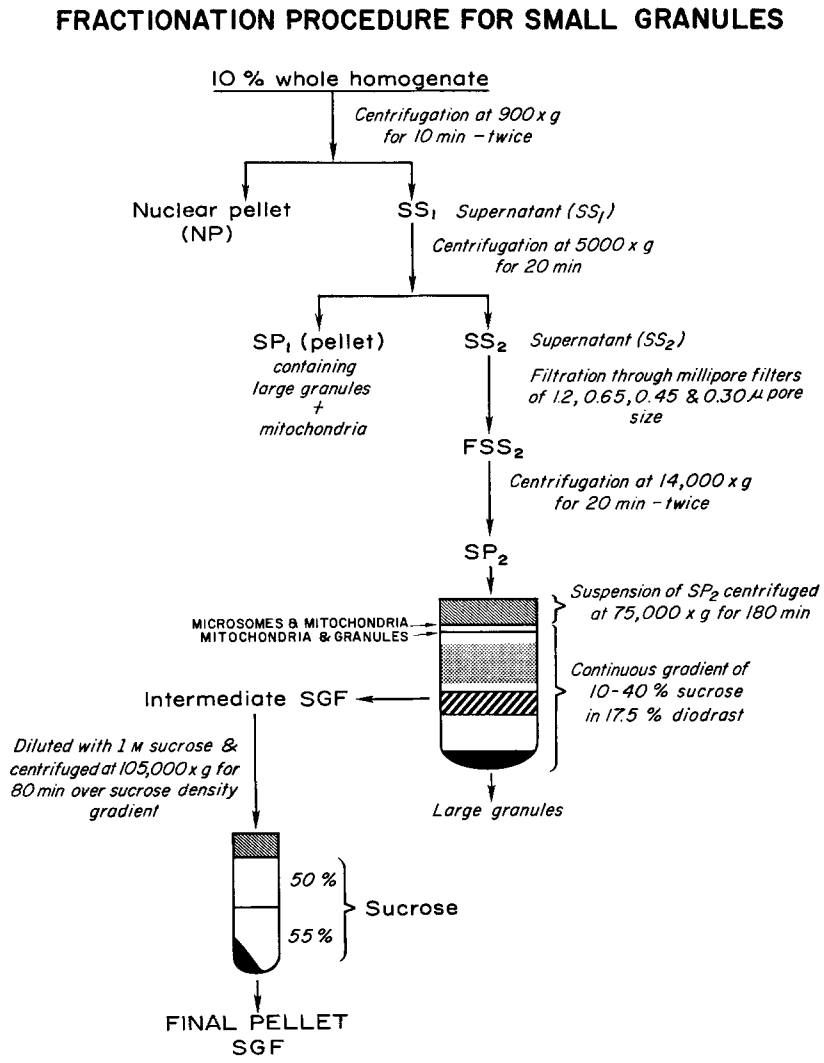


FIGURE 2 Isolation procedure for small hormone storage granules from bovine anterior pituitaries.

specific activities. The proteolytic activity at pH 8.3 of whole homogenate containing 1 mg of protein was arbitrarily chosen as an enzyme unit. All other enzyme activities are expressed as specific activities, i.e. units of enzyme per mg of protein. One unit of enzyme is defined as that amount which catalyzes the transformation of 1 mmole of substrate per minute.

## *Biological Assays*

Hormonal activities were measured by bioassay procedures. Somatotropin was determined by the method of Greenspan et al. (25) which is based on an increase in width of tibial epiphyseal plates in hypophysectomized juvenile rats. Prolactin was assayed by the method of Bates et al. (26) which employs a weight increase in the crop sac of adult pigeons as an end point. Thyrotropin assays were performed by the method of McKenzie et al. (27) which is based on an increase of blood radioactive $^{131}I$ released from the thyroid of mice. Luteinizing hormone assays were performed by the method of Greep et al. (28) which measures the weight increase in the anterior prostate of immature hypophysectomized rats. Follicle-stimulating hormone was assayed by the method of Steelman and Pohley (29) which is based on the weight increase of the ovaries. Immature 21-day-old female rats pretreated with injection of 20 μ of human chorionic gonadotropin were used.

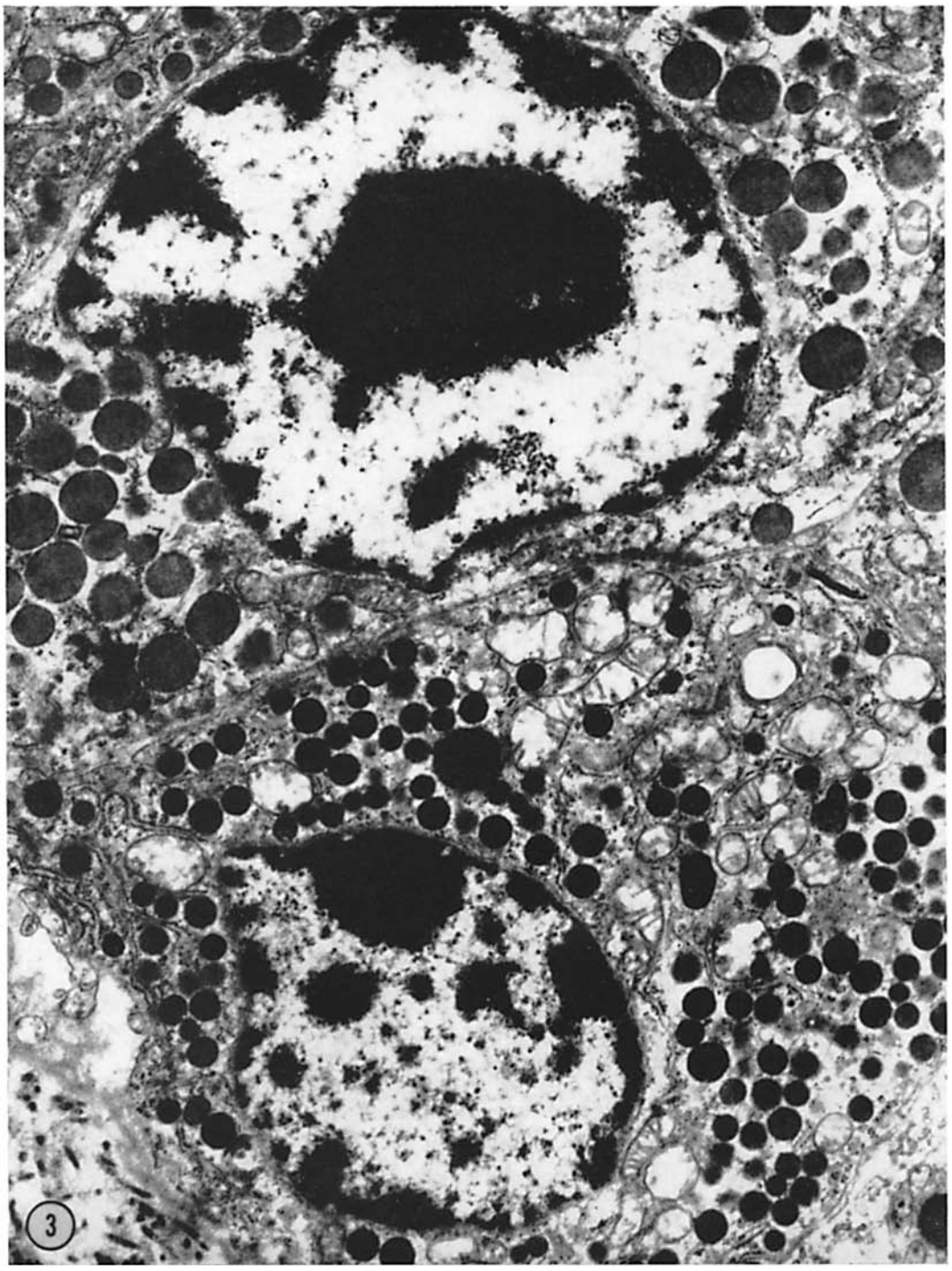


FIGURE 3 A section of a bovine adenohypophysis showing the ultrastructure of cells with large and small cytoplasmic granules. The small granules are more electron-opaque than the large granules (× 17,600).

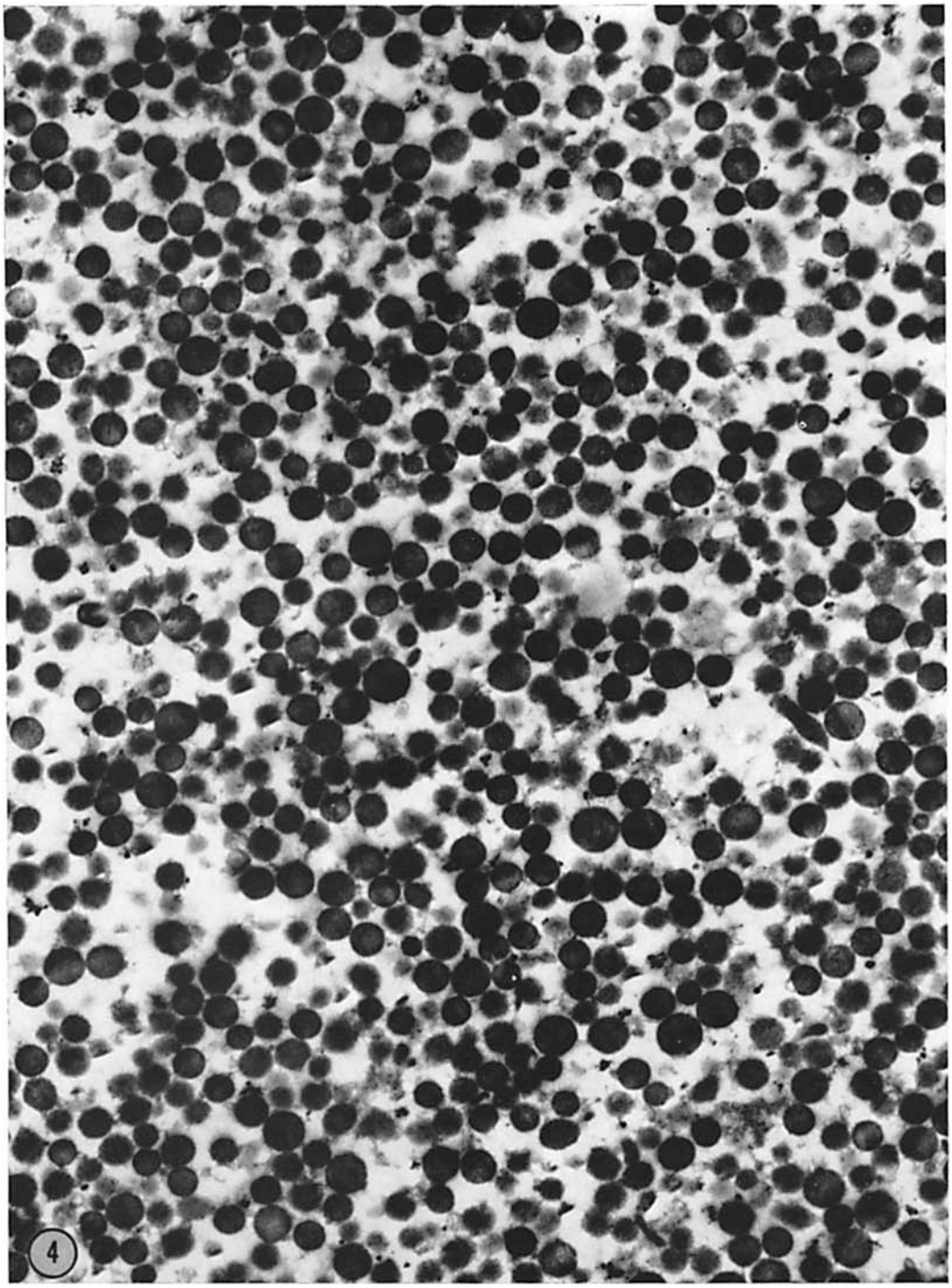


FIGURE 4 Electronmicrograph of a purified small granule fraction (× 17,600).

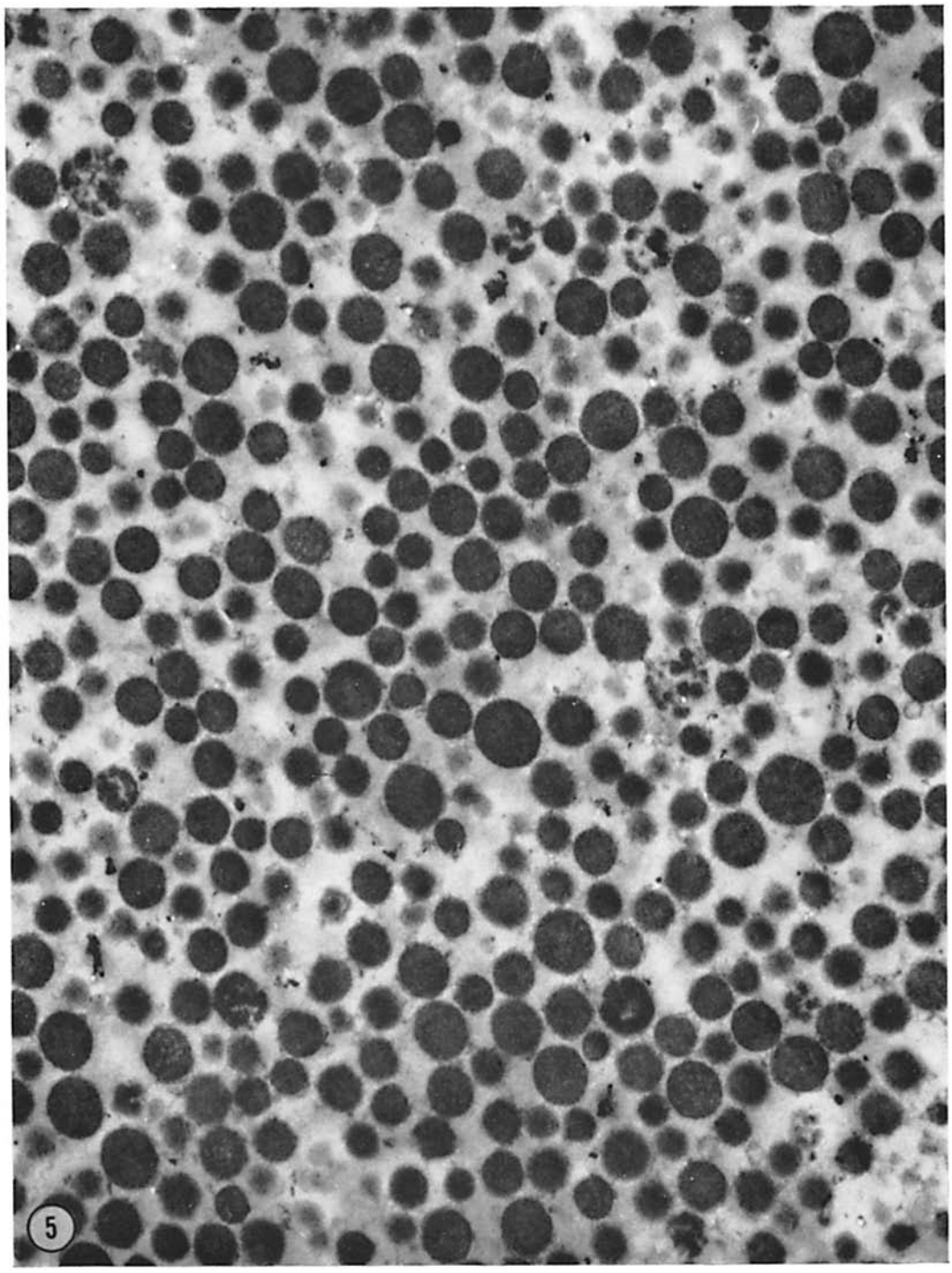


FIGURE 5 Electronmicrograph of a purified large granule fraction (× 17,600).

The pituitary hormones in the whole homogenate (WH), LGF and SGF were solubilized by homogenization and several cycles of freezing and thawing and were kept on ice until injection. Standard dose-response curves were constructed by administration of National Institutes of Health pituitary hormones of known hormone content. Preliminary experiments were done to determine the concentration of WH, LGF, and SGF required to give responses falling on the standard curve. Each definitive hormone assay was performed on two or more concentrations of tissue. Further details are given with the results.

compact granular masses, probably matrix of disrupted hormone storage granules, were the principal contaminants. The frequency distribution of the size of the granules in the SGF and LGF was in good agreement with that of the granules *in situ* (Fig. 6).

### *Biochemical Assays*

Further evidence of the purity of fractions was obtained by assays for enzyme markers associated with individual organelles (Table I). The degree

TABLE I

*Enzyme Activities in Whole Homogenate and Purified Granule Fractions of Bovine Adenohypophysis*

| Enzyme | Specific activity | | |
|---|---|---|---|
| | Whole homogenate | Large granule fraction | Small granule fraction |
| Succinic dehydrogenase | 60 ± 4.5 (5) | 3.2 ± 0.16 (5) | 3.2 ± 0.14 (5) |
| Acid phosphatase | 25 ± 2.3 (5) | 0.8 ± 0.2 (5) | 0 (5) |
| Glucose-6-phosphatase | 10 ± 0.7 (5) | 0 (5) | 0 (5) |
| Alkaline protease* | 1 ± 0.1 (4) | | 15.0 ± 1.5 (4) |

Specific activity is reported in milliunits per mg protein. Results are given as the mean ± standard error of the mean. The number of determinations is in parenthesis.

* The optical density increment (Δ O.D.) produced by incubation of 1 mg protein of WH with the substrate (according to the method of Anson) was taken as one unit of activity.

## RESULTS

### *Electron Microscopy*

Electron microscopy of intact beef adenohypophysis revealed the following cell types: (*a*) cells containing small cytoplasmic granules with an average diameter of 150–220 mμ, (*b*) cells with large granules ranging from 400 to 600 mμ in diameter, (*c*) cells with very few or no cytoplasmic granules (Fig. 3). These last cells may correspond to the chromophobe cells of light microscopy, or may represent cells which have discharged their storage granules. All granules consist of a compact, finely granular, electron-opaque material which is delimited by a single membrane. The granules isolated in the SGF (Fig. 4) and the LGF (Fig. 5) are ultrastructurally identical to those present *in situ*. Contamination of these purified fractions by other subcellular structures was minimal as determined by electron microscopy done at random on isolated fractions. Rare mitochondria, sparse membrane fragments of uncertain origin, and of mitochondrial contamination of the SGF and LGF, as measured by the succinic dehydrogenase activity, was minimal, the specific activity of these fractions being less than 6% the specific activity of the WH. Lysosomal and microsomal impurities are negligible, judging from the absence of acid phosphatase and glucose-6-phosphatase activity, respectively, in the SGF and the LGF.

An alkaline protease was found to be associated with the SGF but not with the LGF. The specific activity of the alkaline protease in the SGF was about 15 times greater than in WH or LGF. Other subcellular fractions were not tested for this enzyme.

### *Biological Assays*

The WH, LGF, and SGF were tested for activities of five pituitary hormones (Table II). It is evident that the LGF contains 4–6 times higher concentrations of somatotropin and prolactin than the WH, while the SGF contains 10–20 times higher concentrations of thyrotropin

TABLE II

*Hormone Activities in Pituitary Fractions*

| Hormone | Specific activity | | |
|---|---|---|---|
| | Whole homogenate | Large granule fraction | Small granule fraction |
| Somatotropin | 35 ± 5 (5) | 250 ± 24.2 (5) | 24 ± 3.5 (5) |
| Prolactin | 364 ± 84 (4) | 1560 ± 325 (4) | — |
| Thyrotropin | 5 ± 1.8 (6) | 5.1 ± 1.2 (3) | 100 ± 28 (6) |
| Follicle stimulating hormone | 7.1 ± 1.5 (5) | 5 ± 1.4 (5) | 70 ± 14.4 (5) |
| Luteinizing hormone | 7 ± 1.8 (4) | — | 72 ± 16 (4) |

The specific activity is expressed in milliunits per mg of protein. The results are given as the mean ± standard error of the mean. The number of determinations is in parenthesis. Each hormone in a given fraction was assayed in at least two concentrations. Standard curves were constructed by injection of NIH-pituitary hormone standards in 4–5 concentrations.

*Definition of units*: One mg of a standard preparation of somatotropin (NIH-GH-$B_{10}$), FSH (NIH-FSH-$S_3$), and LH (NIH-LH-$B_3$) is equal to one unit of the given hormone activity. Prolactin is expressed in international milliunits. One mg of the standard prolactin preparation (NIH-P-$B_1$) is equal to 13 IU. One mg of TSH standard (NIH-TSH-$B_3$) is equal to 2.73 units.

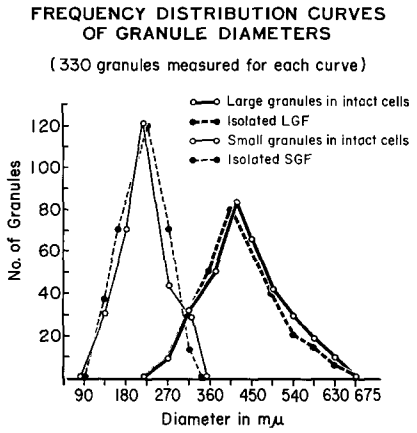


FIGURE 6 Comparison of frequency distribution of granule sizes *in situ* with that of granule sizes in the purified granule fractions. Measurements were made directly on electronmicrographs at magnification of × 24,600. Granules were selected at random.

and gonadotropins. Cross-contamination between the purified granule fractions is minimal since the LGF contains less gonadotropin and the SGF contains less somatotropin than the WH.

## DISCUSSION

Secretory proteins in various exocrine and endocrine glands are temporarily stored within special storage granules pending their discharge into the glandular lumina or blood stream. The essential features of the secretory cycle have now been established for several tissues, including the exocrine pancreas (30, 31), mammary gland (32), thyroid gland (33), fibroblasts (34), myelocytes (35), and eosinophils (36). Hormones, enzymes, or other proteins to be secreted are synthesized on ribosomes attached to the limiting membrane of the rough endoplasmic reticulum (RER). These proteins enter the cisternae of the RER, are thence transferred to the Golgi complex, apparently via small vesicles located at the periphery of the complex (31), and are subsequently packaged into storage granules within condensing vacuoles of the Golgi complex.

The present investigation was undertaken to isolate and characterize the end product of this process in the beef adenohypophysis, the hormone storage granule.

We have here described a method for preparing the storage granules of beef adenohypophysis in two fractions according to size, a large (LGF), and a small (SGF) granule fraction. Electron microscopic, biochemical, and biological studies have shown that these fractions are essentially devoid of contamination. The isolated granules are morphologically indistinguishable from granules fixed *in situ* within the intact cells. Removal of

mitochondria, whose sedimentation properties closely resemble those of the storage granules, was achieved by separating the "fluffy" layer from the crude granular pellet, followed by repeated isopycnic ultracentrifugations through sucrose density gradients. Filtration through celite columns, applied successfully by McShan and associates (13, 14) for removal of mitochondria in homogenates of rat adenohypophysis, proved unsatisfactory for processing bovine pituitary tissue. The yield of purified granules from beef adenohypophysis, 0.1 mg and 1.0 mg of SGF and LGF protein, respectively, per gram of tissue, has been adequate for biochemical studies that would have been exceedingly difficult to perform on rat material.

It is significant that the localization of hormones in the LGF and SGF from bovine adenohypophysis is identical to that of rat (McShan et al. 4, 13, 14). Thus the thyroid-stimulating hormone and the two gonadotropic hormones are concentrated within the SGF, while the growth hormone and prolactin occur in the LGF in both species. Recently Kwa et al. (37) also reported that growth hormone and prolactin are localized in a large granule fraction of beef adenohypophysis.

Earlier fractionation studies employing only differential centrifugation gave contradictory results due to incomplete purification and cross-contamination (reviewed in McShan, 4). In accord with McShan et al. (4), we also found an alkaline protease concentrated within the SGF. This enzyme was first reported in pituitary extracts by Adams and Smith (39), and subsequently confirmed by Brown and LaBella (17b) and Meyer and Clifton (38). The function of this alkaline protease is unknown. Possibly it is involved in the secretion of some pituitary hormones, as has been suggested (McShan et al. 4).

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