ISOLATION AND BIOLOGICAL PROPERTIES OF SECRETORY GRANULES FROM RAT ANTERIOR PITUITARY GLANDS

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ABSTRACT

A method is described for the isolation of secretory granules from rat anterior pituitary glands. The method consists of differential and isopycnic gradient centrifugations, followed by filtration of the zones containing granules on Nuclepore filters to remove mitochondria. Highly purified granules were obtained as indicated by electron microscopy. Major parts of the thyrotropin (TSH) and adrenocorticotropin (ACTH) were recovered in a single fraction of granules as were follicle-stimulating (FSH) and luteinizing (LH) hormones. The somatotropin (STH) and prolactin (LTH) were recovered in separate granule fractions. The major parts of the six different hormones were associated with their respective granule fractions as shown by bioassays specific for each of the hormones. The diameters of granules in sections of intact rat pituitary glands and in isolated pellets were measured, and the means and ranges were in close agreement. These results contribute to the identification of the cell types which produce the different pituitary hormones.

INTRODUCTION

The fractionation of the secretory granules and other particles from rat, sheep, cow, and hog pituitary glands by differential centrifugation has been summarized by McShan and Hartley (1). Highly purified granules were not obtained by differential centrifugation. Hartley et al. (2) used isopycnic gradient and differential centrifugations to obtain fractions of granules with which gonadotropic activity was associated. Perdue and McShan (3, 4) obtained a fraction of granules which showed TSH, ACTH, FSH, and LH activities. Chromatography on Celite (Johns Manville, Chicago) followed by discontinuous gradient centrifugation was used by Hymer and McShan (5) to obtain purified large and small granules. These granules were assayed for the six tropic hormones, and in some cases there was considerable overlap in their biological activities. The biochemical properties of the granules were also studied. Recently Tesar et al. (6, 7) prepared and studied purified granules from the anterior pituitary gland of the cow.

This report describes a method for the isolation of secretory granules from rat pituitary glands by isopycnic gradient centrifugation, microfiltration, and differential centrifugation. The granules from the different cell types were shown by electron microscopy to be free of microsomal and mitochondrial contamination. The biological assays showed that a major part of the hormones was associated with individual granule fractions.

METHODS AND MATERIALS

Preparation of Homogenates

Pituitary glands from 3-month-old male rats weighing 300–350 g were obtained from the Holtzman Company, Madison, Wisconsin. The animals were

killed by cervical dislocation; the pituitary glands were removed immediately, and the anterior lobes were separated and placed in an ice-cooled, moist chamber. A 5% homogenate was made in a medium consisting of 0.25 M sucrose and 7.3% polyvinylpyrrolidone (PVP) adjusted to pH 7.4. The homogenization was performed in a cold, sharp-pointed glass homogenizing tube and mashed rather than ground with a pestle so as to minimize breaking the granules and solubilizing the hormones.

Differential Centrifugation and Microfiltration

The whole homogenate was fractionated at 4°C in the multispeed head of a refrigerated Model PR-2 International Centrifuge. The nuclear pellet (NP) was obtained by centrifugation at 275 *g* for 10 min. The supernatant (S1) was filtered through a SS Millipore filter with 3.0 μ diameter pores in a Swinny adapter fastened to a 5-ml Luer-Lok syringe. This filtered supernatant (FS1) was layered on to the continuous density gradient.

Preparation of Continuous Density Gradients

The gradients were prepared by a modification of the method of Hartley et al. (2). Diodrast, the diethanolamine salt of 3,5-diiodo-4-pyridone-*N*-acetic acid, which is available (Winthrop Laboratories, Evanston, Ill.) as a 35% (w/v) aqueous solu-

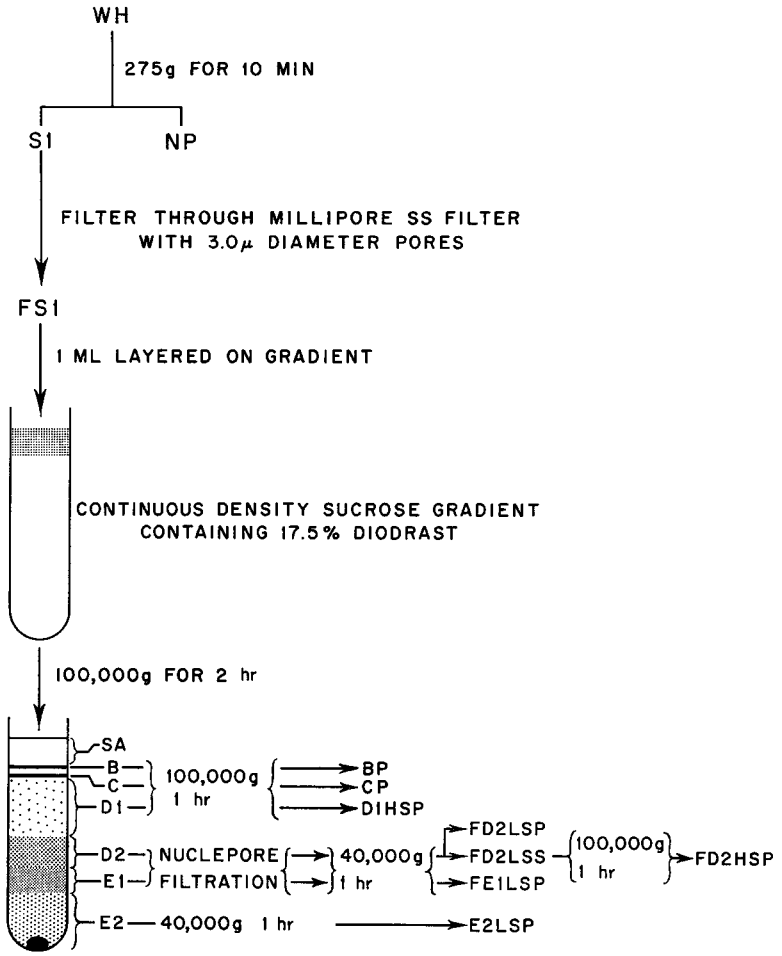

FIGURE 1 Procedure for isolation of rat pituitary secretory granules. Designation of fractions: *WH*, Whole homogenate (50 mg FT per ml); *NP*, Nuclear pellet, *S1*, Supernatant from nuclear pellet; *FS1*, Filtered supernatant; *SA* to *E2*, Zones obtained on the continuous density gradient; *SA*, Soluble A zone; *BP*, Ribosomal pellet, *CP*, Microsomal pellet, *D1HSP*, D1 high-speed pellet; *FD2LSP*, Filtered D2 low-speed pellet; *FD2LSS*, Filtered D2 low-speed supernatant; *FD2HSP*, Filtered D2 high-speed pellet; *FE1LSP*, Filtered E1 low-speed pellet, *E2LSP*, E2 low-speed pellet.

tion, was mixed with sucrose for making the gradients (8). The solutions used were 6 and 45% (g per 100 ml) sucrose containing 17.5% (g per 100 ml) Diodrast and 5 × 10⁻⁴ M Versene. The pH of these solutions was adjusted to 7.2 with potassium hydroxide. In preparing the gradients, the machine (9) was calibrated to deliver a total of 4.4 ml into each tube. The density ranged from 1.11 at the top to 1.26 at the bottom of the gradient.

Isopycnic Gradient Centrifugation

1 ml of the FS1 fraction was layered onto the surface of the gradient with a 2-ml syringe. The tubes were placed in a SW-39 swinging bucket head and centrifuged in a Beckman Spinco Model L centrifuge for 2 hr at 100,000 *g*. The zones of the gradient were individually removed with a long needle attached to a 2-ml syringe placed in a holder that enabled careful removal of the desired layer.

Recovery of Granules

The zones were removed starting from the top of the gradient and were identified as shown in Fig. 1. The SA layer containing hormones in solution was removed and used for assays. The other zones were diluted with 0.88 M sucrose prior to centrifugation for recovery of the particles.

Zones B, C, and D1 were individually removed, diluted, and centrifuged at 33,000 rpm (100,000 *g*) for 1 hr to obtain the respective high-speed pellets. Zones D2 and E1 were removed and slowly filtered through moistened Nuclepore filters (General Electric Co., Pleasanton, Cal.) with 0.5-μ diameter pores mounted in a Swinny adapter attached to a 2.0-ml Luer-Lok syringe. These filtered solutions, FD2 and FE1, were diluted and centrifuged at 17,000 rpm (40,000 *g*). The resulting filtered low-speed pellets were designated as FD2LSP and FE1LSP. When the supernatant (FD2LSS) from the FD2LSP pellet was centrifuged for 1 hr at 100,000 *g*, a small pellet, FD2HSP, was obtained. The lowest zone, E2, which usually included a diffuse pellet at the bottom of the tube, was removed, diluted, and centrifuged at 40,000 *g* for 1 hr for obtaining a pellet of large particulates. The above-mentioned pellets were recovered and used for electron microscopy or were diluted with saline for bioassays.

Fixing and Embedding of Whole Tissue and Pellets for Electron Microscopy

The whole tissue was fixed in Caulfield fixative (10) for 1 hr at 4°C and then dehydrated in a series of increasing concentrations of ethyl alcohol. The glands were embedded in Epon-Araldite according to Mollenhauer (11) and stained with uranyl acetate and lead citrate (12).

The pellets obtained during the fractionation were fixed overnight at 4°C in 1% OsO_4 containing 0.25 M sucrose according to the method of Palade and Siekevitz (13). The pellets were rapidly dehydrated through a series of methyl alcohol solutions of increasing concentrations and then embedded in a mixture of methyl and *N*-butyl methacrylates containing 2% benzoyl peroxide according to the method of Palade (14) or Epon-Araldite (11).

The pellets were sectioned at different levels for determining whether stratification had occurred. The sections were viewed by use of a Hitachi HS-7S electron microscope equipped with a high-voltage stabilizer and double condenser. An accelerating potential of 50 kv and a 50-μ objective aperture were used. Magnification was calibrated with a carbon replica of a diffraction grating with 54,864 lines per inch (Fullam Co., Schenectady, N. Y.).

Diameters of Sections of Granules

The diameters of sections of isolated granules and of granules in whole tissue for each of the cell types were measured with a 7× magnifier with 0.1-mm scale divisions. The longest axes of the granules were measured to the outside of the membrane on micrographs usually enlarged × 30,000.

Biological Assays

The zones from the gradient were diluted and centrifuged so as to obtain the pellets which were suspended in 0.9% saline for bioassay. The assay data were analyzed by the parallel line method of Bliss (15). The fractions from two to four gradients were required for assay of each of the hormones.

THYROTROPIN: The pituitary preparations were assayed for TSH by a modification of the ^{32}P uptake assays of Lamberg (16) and of Greenspan et al. (17). 20 μc of ^{32}P diluted with saline were administered to 1-day-old chicks kept in the dark at 24°C in order to increase the sensitivity of the assays.

ADRENOCORTICOTROPIN: This hormone was determined by using male rats weighing 140–190 g according to the Saffran and Schally in vitro method (18).

FOLLICLE-STIMULATING HORMONE: The preparations were assayed according to the method of Steelman and Pohley (19).

LUTEINIZING HORMONE: The ovarian ascorbic acid depletion method of Parlow (20) was used for assaying the fractions for LH. The determination of total ascorbic acid was performed according to the modification reported by Schaffert and Kingsley (21). The slope of the regression of ascorbic acid content on ovarian weight was calculated by analysis of covariance (22). Estimates of potency of LH preparations were calculated by the parallel line method (15).

SOMATOTROPIN: The somatotropin content of

the fractions was determined by a modification of the procedure of Greenspan et al. (23). Female rats, hypophysectomized at 25 days of age, were obtained from the Hormone Assay Laboratories, Chicago. Beginning at 30 days of age, these animals were injected with the granule preparations twice daily for 3 days, and autopsies were performed 25 hr after the last injection.

PROLACTIN: The prolactin in the fractions was determined by a modification (24) of the in vitro method developed by Mishkinsky et al. (25). Rat inguinal mammary gland explants were cultured in disposable sterile 35 × 10-mm Petri dishes containing supplemented M-199 medium. The Petri dishes were placed in a closed, moist incubator at 38°C, flushed with 95% O_2–5% Co_2, and removed after 48 hr. A positive alveolar response of the hematoxylin-stained explant is the basis for this assay.

RESULTS

Electron Microscopy

The method developed for the isolation of secretory granules from rat anterior pituitary glands is outlined in Fig. 1. An electron micrograph of a section from a male rat anterior pituitary gland shows STH and TSH cells (Fig. 2).

The 5% pituitary homogenate when centrifuged for 10 min at 275 *g* gave a nuclear pellet (NP) which consisted mainly of nuclei and red blood cells. The supernatant from this pellet was filtered to give fraction FS1 which was layered on the gradient and centrifuged at 100,000 *g* for 2 hr.

The top zone of the gradient, SA, contained low levels of soluble hormones. The BP and CP pellets obtained after high-speed centrifugation consisted of fibrous material, ribosomes, and microsomes. Electron microscopy of the D1HSP pellets (Fig. 3) showed that they consisted of homogeneous granules and were essentially free of microsomal and ribosomal contamination. The mean diameter of sections of these granules was 90 mμ (Table III). The FD2LSP (Fig. 4) obtained after Nuclepore filtration and low-speed centrifugation revealed granules with a mean diameter of 210 mμ, and they were free of mitochondrial contamination. The FD2HSP pellet (Fig. 5) obtained from the supernatant of the above FD2LSP consisted of granules with a mean diameter of 150 mμ which were not contaminated by other organelles. The FE1LSP pellet (Fig. 6) when sectioned showed granules that were 235 mμ in mean diameter and were free of mitochondria. Electron microscopy of the drop of material left on the Nuclepore filter showed that it consisted almost exclusively of mitochondria. The unfiltered E2LSP pellets (Fig. 7) were stratified and consisted of granules 220–800 mμ in diameter. A few mitochondria were present.

Biological Activities of the Gradient Fractions

Biological activities in terms of relative potencies and confidence limits are indicated in Table I. The ranges of the indices of precision and number of assays for each hormone fraction are also given in this table.

The percentages of hormones recovered from each fraction are presented in Table II. The maximum amounts of the individual hormones recovered in a single granule fraction ranged from 51.4% of the LH in fraction FD2HSP to 89.3% of the LTH in fraction E2LSP. A significant amount of each of the hormones was present in the SA soluble fraction. Essentially none of the hormones was found in the nuclear pellet (NP) and the ribosomal plus microsomal fractions (BP and CP). Little or none of the hormones was found in fraction FD2LSP. The STH and LTH were mainly present, respectively, in granule fractions FE1LSP and E2LSP.

Diameters of Sections of Granules

The ranges and the mean diameters of sections of granules from the isolated pellets and from the whole pituitary tissue were determined and are given in Table III. 600 measurements were made from electron micrographs for each of the granule fractions and the cell types. These granule measurements indicate that most of the TSH and ACTH granules were associated with the D1HSP pellets, while granules from LH and FSH cells were found in the FD2HSP fraction. The diameters of sections of granules in the FE1LSP pellets coincide with those in STH cells, and the E2LSP pellets consist of granules similar in size to those from LTH cells.

DISCUSSION

A method was developed for preparing secretory granules from rat anterior pituitary glands. Essential features of this reproducible method are isopycnic gradient centrifugation followed by filtration on Nuclepore filters and differential centrifugation (Fig. 1). This method has been

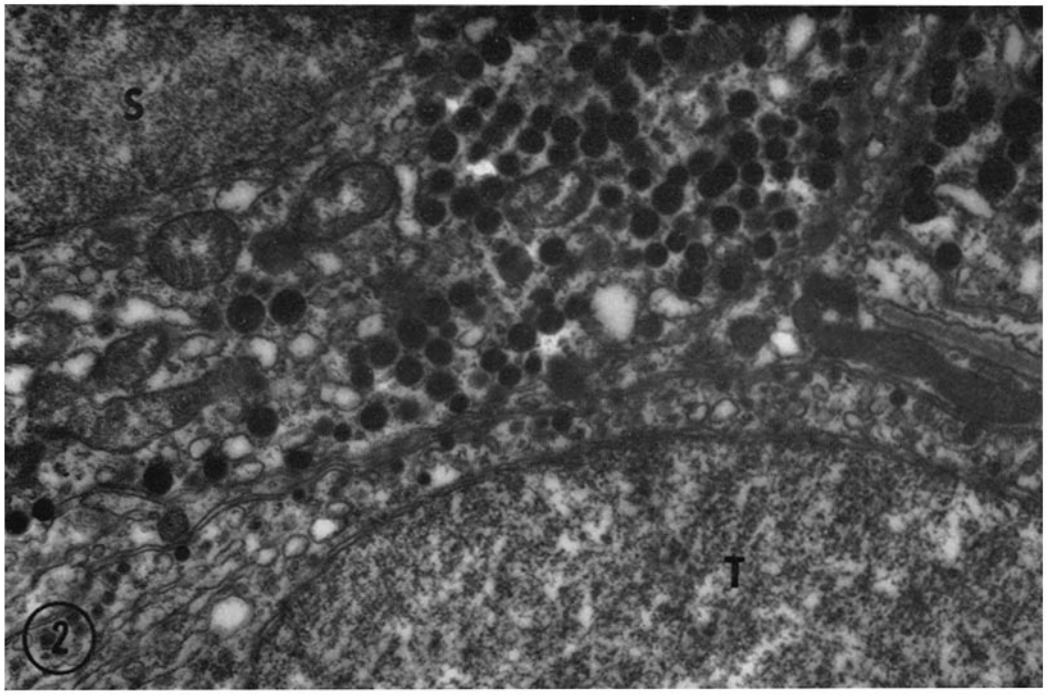

FIGURE 2 An electron micrograph of the anterior pituitary gland of an adult male rat. A somatotrope (*S*) and a thyrotrope (*T*) are shown. × 15,000.

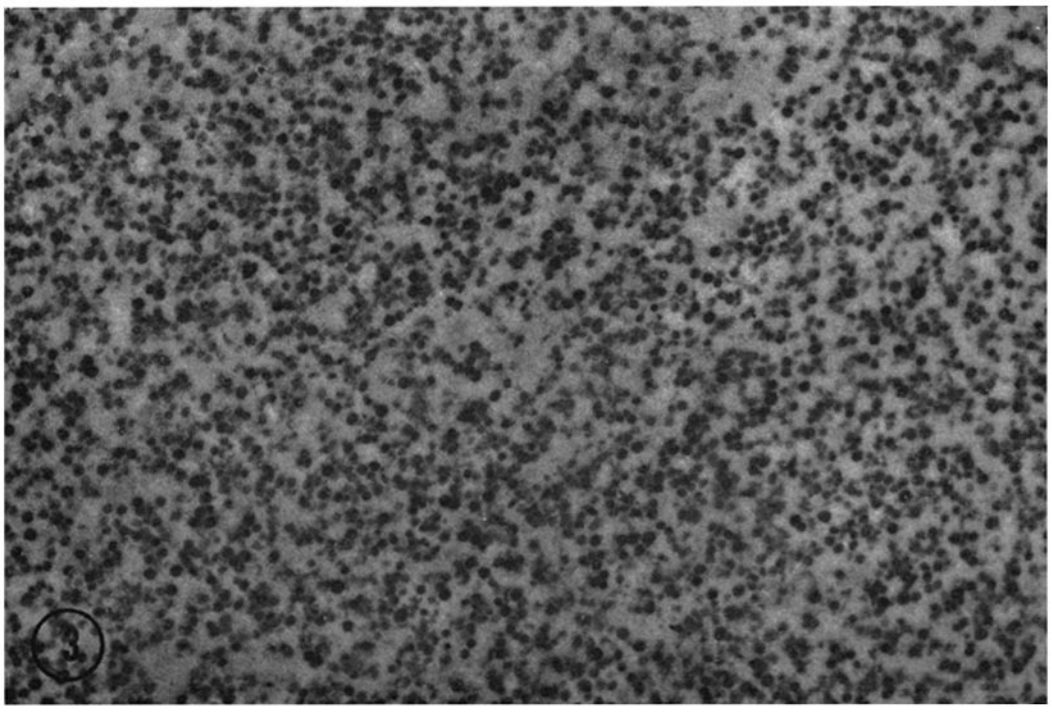

FIGURE 3 The D1HSP secretory granule fraction with which TSH and ACTH are associated. × 15,000.

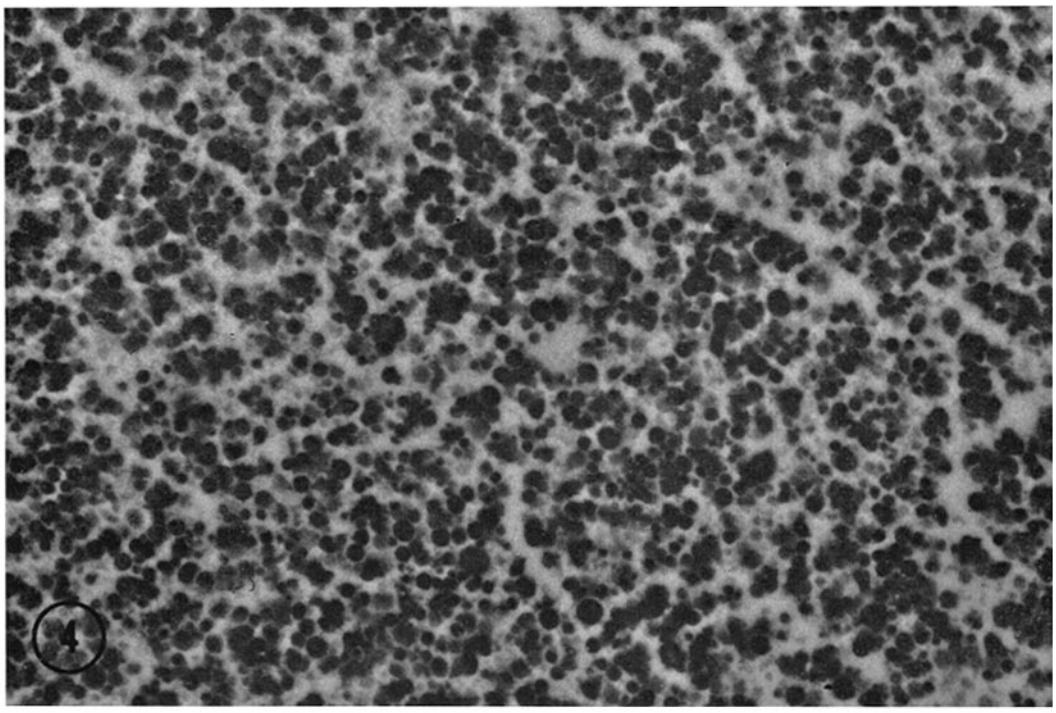

FIGURE 4 An electron micrograph of a section through the granules of the FD2LSP pellet. × 15,000.

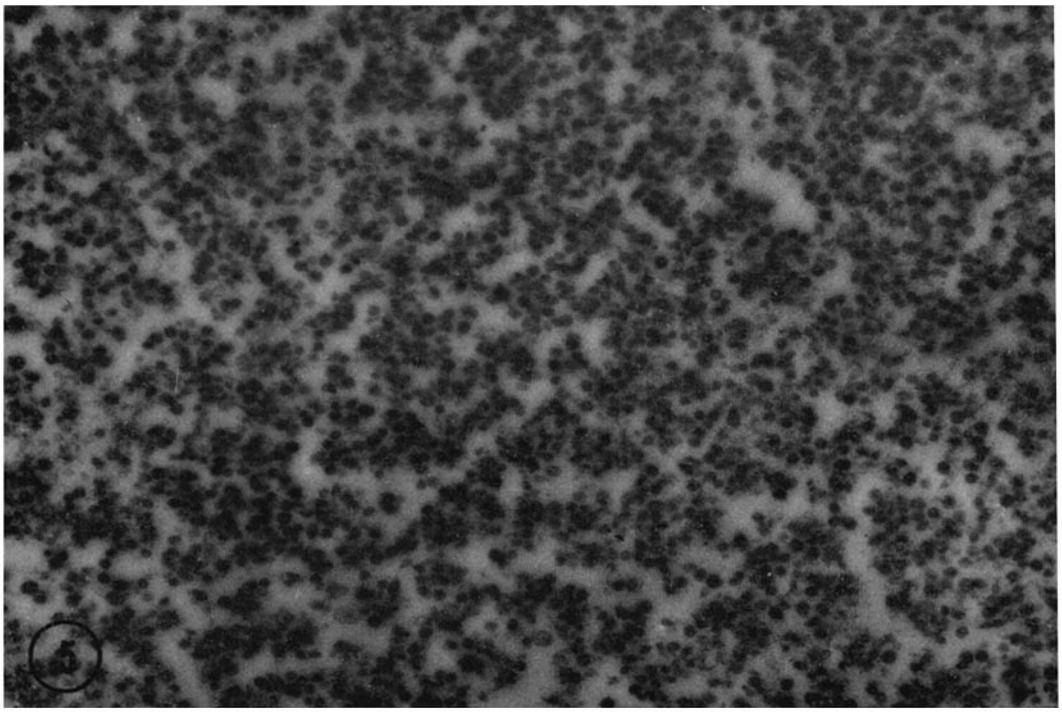

FIGURE 5 A section from the FD2HSP pellet with which the gonadotropins are associated. × 15,000.

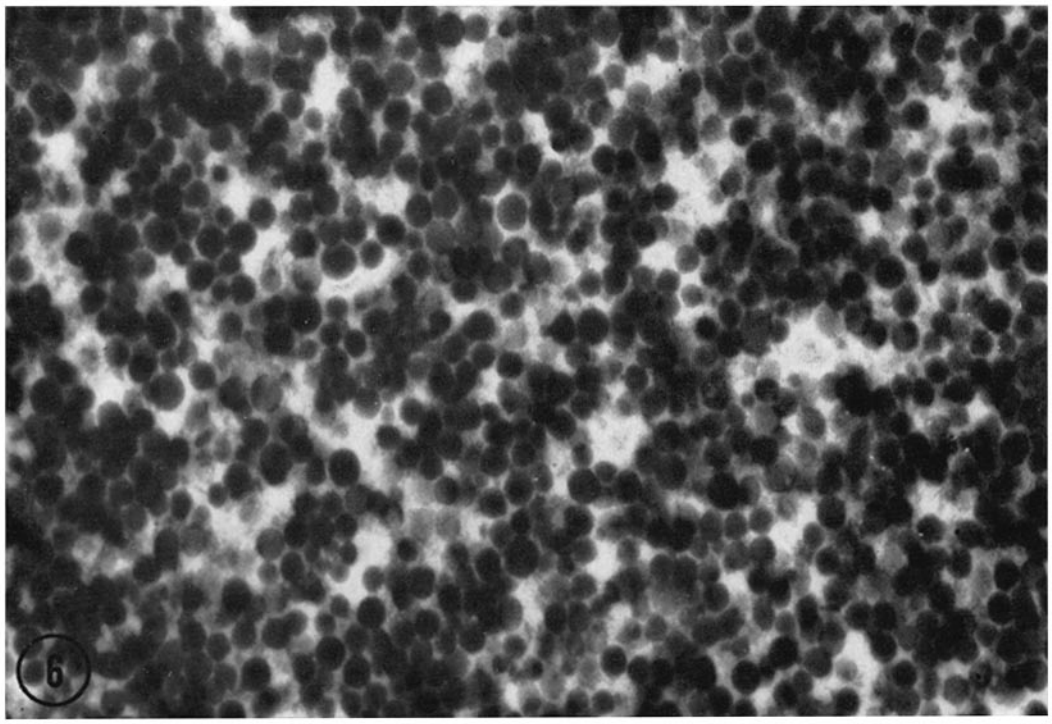

FIGURE 6 An electron micrograph of the FE1LSP secretory granule pellet containing growth hormone activity. × 15,000.

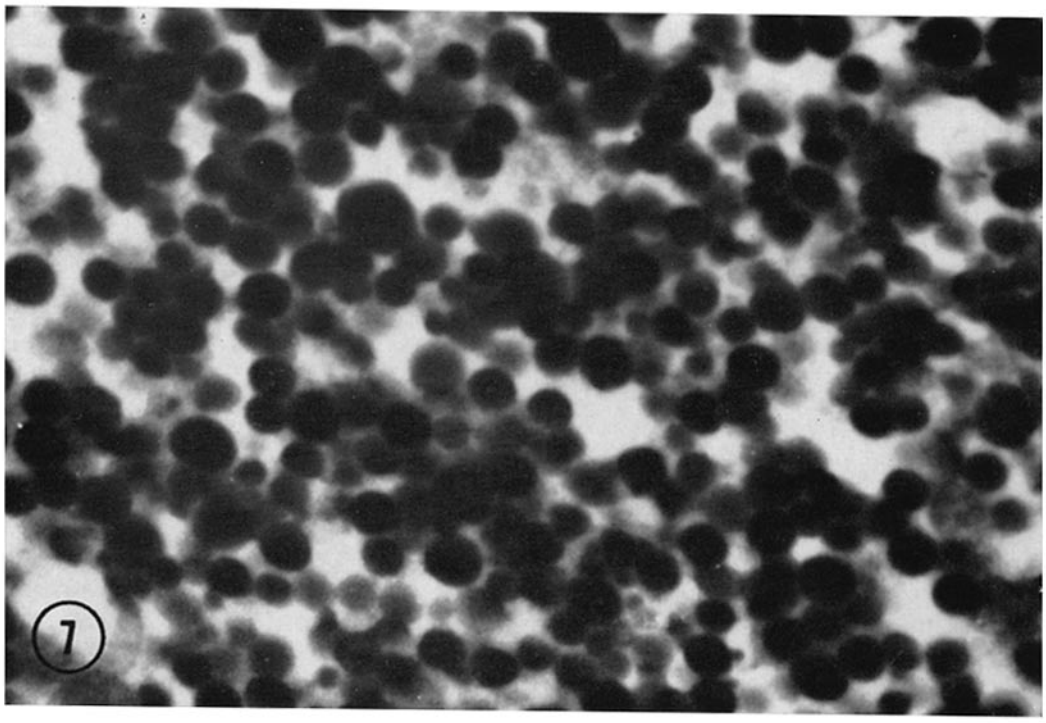

FIGURE 7 The E2LSP fraction of the large acidophilic LTH granules. × 15,000.

TABLE I

Relative Potencies of the Hormones in the Anterior Pituitary Fractions

Fractions	TSH	ACTH	FSH	LH	STH	LTH
	*Milliunits per milligram equivalent of fresh tissue**					
FS1	9.31‡ (5.82–14.85)§	42.50 (39.10–46.62)	35.90 (29.21–39.52)	2.08 (1.13–3.84)	69.00 (44.10–108.64)	12.20 (9.70–15.00)
NP	0.00	0.50 (0.40–0.72)	0.36 (0.29–0.40)	0.01	0.28 (0.21–0.43)	0.00
SA	0.37 (0.18–0.76)	8.10 (7.38–9.15)	4.84 (4.03–5.29)	0.48 (0.24–0.82)	13.33 (8.16–21.62)	1.05 (0.85–1.28)
BP+CP	0.02	0.00	0.00	0.00	0.00	0.00
D1HSP	7.62 (4.75–12.10)	22.70 (21.30–24.68)	1.11 (0.71–1.43)	0.02	0.00	0.00
FD2LSP	0.00	1.00 (0.80–1.32)	3.74 (2.82–4.31)	0.16 (0.07–0.38)	4.14 (3.02–5.27)	0.00
FD2HSP	0.14 (0.07–0.21)	4.60 (4.10–5.83)	20.35 (15.85–22.13)	1.07 (0.59–1.91)	0.00	0.00
FE1LSP	0.00	0.00	0.00	0.01	45.92 (28.34–77.57)	0.12 (0.08–0.15)
E2LSP	0.00	0.00	0.00	0.00	1.68 (1.12–2.58)	10.90 (8.92–13.41)
Indices of precision (Ranges)	0.34–0.36	0.08–0.10	0.08–0.11	0.19–0.25	0.30–0.35	0.10–0.15
No. of Assays	3	4	4	3	2	2

* Relative potencies given as follows: TSH—adjusted to NIH-S1 Standard, expressed as USP mu/mg eq FT (equivalent of fresh tissue). ACTH—adjusted to Third International Standard, mu/mg eq FT. FSH, LH, STH, and LTH—adjusted to NIH-S1 Standards, mu/mg eq FT.

‡ Mean relative potencies calculated as arithmetic means.

§ The 95% confidence limits of the potency determinations.

repeated 20 or more times. Because the separation is based on the particles moving through the gradient to their level of density equilibrium, turnover effects were minimized and essentially homogeneous granule preparations were obtained. Electron microscopy of the granule pellets obtained by low- and high-speed centrifugations showed them to be free of mitochondrial, ribosomal, and microsomal contamination (Figs. 3–7). The fine contaminants were recovered in the B and C zones at the top of the density gradient. Mitochondria were removed from the D2 and E1 zones by filtration on Nuclepore filters which have pores of uniform diameter. Since mitochondria are flaccid, precaution is necessary so as to prevent them from passing through the pores of the filter. It is important to apply slight pressure to the syringe plunger and to leave a film of liquid on the filter. The mitochondria are found in this film.

From 4 to 23% of the hormonal activities were found in the SA soluble fraction. It is suggested that most of the hormones recovered in this fraction were solubilized from the granules and

TABLE II

Percentages of Hormonal Activities Recovered from the Gradient Fractions

Fractions	Percentages*					
	TSH	ACTH	FSH	LH	STH	LTH
NP	0.0	1.0	0.0	0.5	0.0	0.0
SA	4.0	19.1	13.5	23.1	19.3	8.6
BC+CP	0.2	0.0	0.0	0.0	0.0	0.0
D1HSP	82.0‡	53.4	3.1	1.5	0.0	0.0
FD2LSP	0.0	2.4	10.4	7.7	6.0	0.0
FD2HSP	1.5	10.8	55.7	51.4	0.0	0.0
FE1LSP	0.0	0.0	0.0	0.0	66.6	1.0
E2LSP	0.0	0.0	0.0	0.0	2.4	89.3
Total recoveries	87.7	86.7	82.7	84.2	94.3	98.9

* Based on the amounts of the FS1 fraction added to the gradient, taken as 100% as given in Table I line 1.

‡ The maximum percentage recoveries for each of the hormones are underlined.

TABLE III

Diameters of Sections of Granules from Rat Anterior Pituitary Glands

Diameters from sections of	Cell types					
	TSH	ACTH	FSH	LH	STH	LTH
	mμ	mμ	mμ	mμ	mμ	mμ
Intact cells						
Mean	85 ± 9*	106 ± 10	133 ± 12	145 ± 13	240 ± 17	318 ± 22
Range	40–145	50–180	75–200	75–235	100–390	250–870
	Fractions from gradient‡					
	D1HSP	D1HSP	FD2HSP	FD2HSP	FE1LSP	E2LSP
	mμ	mμ	mμ	mμ	mμ	mμ
Isolated granule pellets						
Mean	89 ± 9*	89 ± 9	150 ± 15	150 ± 15	242 ± 16	361 ± 22
Range	35–150	35–150	75–218	75–218	180–380	220–880

* Standard error of the mean.

‡ Fractions (Fig. 1) from which maximum amounts of the hormones were recovered.

released from the cisternae of the endoplasmic reticulum during homogenization. The slopes for the dose-responses of the hormones in this fraction were less than those for the granule fractions. The reason for this is not known, but it may be due to compounds or complexes present in the SA fraction such as hydrolytic enzymes from broken lysosomes and alkaline protease released from granules. The latter enzyme has been shown to be associated with small granules from anterior pituitary glands of the rat (3) and of the cow (7).

82% of the TSH and over 53% of the ACTH activities were found in the D1HSP fraction. Over 50% of the FSH and LH activities were associated with the FD2HSP pellet. The FE1LSP fraction contained 66.6% of the STH activity, and 89.3% of the LTH was associated with the E2LSP granule fraction. Furthermore, the latter two fractions were essentially free of other hormones, which is the first time STH and LTH have been prepared in this degree of purity from granules. The relative potencies of each of these hormones are given in Table I and the total percentage recoveries in Table II.

The earliest fractionation studies, as reviewed by McShan and Hartley (1), employed only differ-

ential centrifugation which gave incomplete separation of the granules and other organelles, resulting in their cross-contamination. The present results indicate that, in most cases, greater yields of hormones were recovered than previously reported, and the small granules were essentially free of fine contamination as compared with discontinuous gradient studies (3, 5).

The FD2LSP pellet contained a significant amount of granules, but low levels of the pituitary hormones were found in this fraction (Table II). It is suggested that the larger granules were from MSH cells of the pars intermedia which remained with the anterior pituitary when they were dissected from the posterior lobes. These granules were 300 mμ in maximum diameter, which is in agreement with Kobayashi (26) who reported that granules of MSH are 200–300 mμ in maximum diameter.

The diameters of granules in sections of isolated pellets were compared with those in the different cell types, and their ranges and means were in close agreement (Table III). The biological assays substantiate the above findings. For example, 82% of the TSH was associated with the D1HSP pellet having granules with a mean diameter of 89 mμ as compared with 85 mμ for those in thyrotropes. Similar findings hold true for the other hormones (Tables II and III). The diameters of the small granules are in accord with the measurements reported by Perdue and McShan (3). The maximum diameters of granule sections for each cell type are probably close to the actual diameters which vary to some extent for each cell type.

Because the ACTH and TSH, and FSH and LH granules are of similar size and density, it does not appear likely that they will be further separated by present methods. However, granules prepared by this method were solubilized and the solution was subjected to analytical electrophoresis on polyacrylamide gel. In this experiment, FSH was separated from LH activity as reported by Hodges et al (27).

An improved method is presented for the isolation of secretory granules, and, in addition, the bioassays and granule measurements support the ultrastructural identification of the rat pituitary cell types as reported by other workers (24, 28–35).

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