ISOLATION OF RAT PITUITARY GRANULES AND THE STUDY OF THEIR BIOCHEMICAL PROPERTIES AND HORMONAL ACTIVITIES

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ABSTRACT

A simple and rapid method is described for the isolation of the acidophilic and basophilic granules from the anterior pituitary gland of the rat. The method involves chromatography of pituitary particulates on columns of No. 545 Celite equilibrated and developed with 0.25 M sucrose. Mitochondria are retained quantitatively on the column. The granules and microsomes which are not retained on the Celite are further fractionated on a discontinuous density sucrose gradient and by differential centrifugation. Essentially homogeneous populations of acidophilic and basophilic granules were obtained as indicated by 1) extensive electron microscopic studies, 2) enzymatic determinations, and 3) fatty acid and RNA analyses on the granule pellets. Microfiltration studies indicated that the acidophilic granules were smaller than 450 mμ, but greater than 300 mμ in diameter. They were found, unlike the basophilic granules, to be partially stable to extraction with water, but were unstable on incubation in 1 mM EDTA at 37°. Magnesium ions were not detected in the granules. The acidophilic and basophilic granules contained, respectively, 5 and 8 per cent of the protein present in the whole homogenate. Extensive hormone studies showed that growth and lactogenic hormones were associated with the acidophilic granules, while thyroid-stimulating hormone and gonadotropin were associated with the basophilic granules. ACTH was not present in significant amounts in either of the granule fractions, but was localized in a particulate fraction which contained microsomes and small granules. The association of the pituitary hormones with specific granules and cell types is discussed.

INTRODUCTION

The assumption that there are specific pituitary cell types involved in the production, storage, and release of the tropic hormones from the anterior pituitary gland of the rat has been a basic tenet for many years (15, 24). Recently, electron microscopic studies of anterior pituitary tissue taken from a variety of experimentally treated rats not only have advanced the understanding of the fine structural details of the individual pituitary cells, but also have provided a detailed scheme for the production, storage, and release of hormones from these specific cell types (9).

Studies concerned with the separation of pituitary gland homogenates into particulate fractions by use of differential centrifugation were done during the past decade (17, 21). The results from these studies indicate, in general, that the tropic hormones are, in part, associated with particulates, but since the fractions were impure it was

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not possible to associate a specific tropic hormone with a specific cytoplasmic particulate.

The best evidence for the association of a hormone with a specific population of isolated secretory granules was reported by Hartley *et al.* (16). An essentially homogeneous sample of basophilic granules was isolated by means of differential centrifugation, isopycnic gradient centrifugation, and microfiltration. The granules were found to contain high levels of gonadotropin, indicating that this hormone is associated with the 200 mμ maximal diameter granules found in certain basophils of the anterior pituitary gland of the rat.

The acidophilic granules are similar in size and density to the mitochondria, and attempts to isolate the 350 mμ maximal diameter acidophilic granules by differential centrifugation, density gradient centrifugation, and microfiltration proved unsuccessful in our hands. On the basis of the report of Riley *et al.* (26) in which Celite was used for the purification of melanin granules, an attempt was made to use columns of Celite for the isolation of pituitary granules. A preliminary report on the partial purification of the acidophilic and basophilic granules by the use of Celite has already appeared (19). This report will describe in detail the further development of the method of isolation, and the results of studies on the biochemical and hormonal properties of the isolated granules.

MATERIALS AND METHODS

Preparation of Homogenates

Adult male rats[1] of the Holtzman strain served as the source of the pituitary glands. The animals were killed by cervical dislocation, and the pituitary glands were removed immediately, separated from the posterior lobes, and placed in an ice-cooled moist chamber. Glands from 8 to 10 animals were required for an experiment. A 5 per cent homogenate was made in a medium consisting of 0.25 M sucrose and 7.3 per cent polyvinylpyrrolidone (PVP)[2] adjusted to pH 7.4. The homogenization was done in a cold, sharp-pointed homogenizing tube and mashed rather than ground with the pestle to avoid undue solubilization of the hormones.

Differential Centrifugation and Microfiltration

The whole homogenate was centrifuged in the multispeed head of a Model PR-2 International refrigerated centrifuge at 275 *g* for 10 minutes to obtain a pellet of red blood cells, nuclei, and unbroken cells. The supernatant S1 was filtered through a Millipore filter with 5.0 μ diameter pores in a Swinny adapter fitted on a 5 ml syringe. This filtered S1 (FS1) was the fraction which served as the starting material for further fractionation procedures involving chromatography on columns of Celite and centrifugation on discontinuous density gradients.

Preparation of Celite Columns

A 10 per cent (W/V) slurry of Celite No. 545 (Johns-Manville Company, Chicago) in 0.25 M sucrose was allowed to settle and the fines were decanted. This procedure was repeated four times over a period of 2 hours. In a few experiments Celite No. 535 and No. 503 were used. A 1.5 x 20 cm column with a 3 cm length of 1 mm diameter capillary tubing at the bottom of the tube was used. A small disc of silk mesh cloth was placed at the bottom of the column as support for a 1 cm layer of No. 203 glass microbeads (Microbeads, Inc., Jackson, Mississippi). A disc of coarse filter paper (Reeve Angel No. 202) cut to the inside diameter of the column was placed over the glass beads. The Celite slurry was then pipetted into the column until a level of 12.8 cm was reached. A reservoir containing 0.25 M sucrose was then attached to the column, and this solution was passed through the column for 1 hour. The pH of the eluted sucrose was close to neutrality.

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[1] These rats were supplied by the Endocrine Laboratories, Madison, and the Biochemistry Department, University of Wisconsin, Madison, Wisconsin.

[2] Abbreviations and terminology used in this report: The terms acidophilic and basophilic granules refer, respectively, to those granules isolated from the acidophilic and basophilic cells of the anterior pituitary gland and not to the staining properties of these granules. Fractions in Fig. 1: WH, whole homogenate; NF, nuclear fraction; S1, supernatant from nuclear fraction; FS1, filtered S1; G3LSP, acidophilic granule fraction; G3HSP, basophilic granule fraction; G1, G2, and G3, the consecutive layers from top to bottom of the discontinuous density sucrose gradient, Grad. Super, the top part of the gradient to the first interface after centrifugation; WCP, whole column pellet; WCS, whole column supernatant. Hormones: STH, growth hormone; LTH, lactogenic hormone; ACTH, adrenocorticotropic hormone; TSH, thyroid-stimulating hormone; Gonadotropin, follicle-stimulating and luteinizing hormones. PVP, polyvinylpyrrolidone; EDTA, ethylenediaminetetraacetic acid; mμ, millimicrons; mg eq FT, milligrams equivalent fresh tissue.

In some experiments the Celite column was washed and developed in:

1) 0.25 M sucrose containing 7.3 per cent PVP and 0.9 per cent saline.

2) 0.25 M sucrose containing 7.3 per cent PVP.

3) 0.25 M sucrose in 0.01 M, pH 7.0 phosphate buffer.

In another series of experiments Celite No. 545 was washed with either acid or base. Twenty gm of Celite was stirred with 1 liter of 1 M HCl for several hours. The suspension was allowed to settle, the acidic fines were decanted, and the Celite was then washed several times with distilled water until the pH of the slurry was close to neutrality. The column was then packed with this acid-washed Celite and developed in the usual manner with 0.25 M sucrose. In other experiments the Celite was washed in a similar manner with 1 M NaOH.

Operation of the Column

One ml of FS1 was carefully layered on the top of the Celite column. The time required for the FS1 to pass into the column was 4 to 5 minutes. Twenty-five to 30 minutes after the addition of the FS1 to the column cloudy material was collected. This cloudy suspension when pooled gave a volume of 5.0 ± 0.2 ml. This suspension was divided into 3 aliquots (1.6 ml each) and each aliquot was layered over a discontinuous density gradient which had been prepared by successively layering the following solutions: 0.5 ml 80 per cent (W/V) sucrose, 0.9 ml 65 per cent sucrose, 0.9 ml 50 per cent sucrose, and 0.9 ml 35 per cent sucrose. The three gradients were placed in the buckets of the SW-39L rotor and centrifuged at 100,000 *g* for 1 hour in a Spinco Model L centrifuge. Three bands were present at different levels in the gradient. A dense white band was easily identified at the 50 to 65 per cent sucrose interface. A lighter more diffuse band was present at the 35 to 50 per cent sucrose interface, and a faint band (seen only with difficulty) was present at the layering to 35 per cent sucrose interface. These bands were termed, from the top to bottom, G1, G2, and G3.

The layers were removed from the gradient tubes with a long bent-tipped needle fitted to a 1 ml syringe. This syringe was placed in a special holder (15) which made it possible to lower the tip of the needle into the desired layer and quantitatively withdraw a single band into the syringe. The bottom bands (G3) from the 3 gradients were drawn off, pooled, diluted to 5 ml with 0.88 M sucrose, and centrifuged in the swinging bucket rotor at 20,000 RPM (40,000 *g*) for 1 hour. A small white pellet, termed G3LSP (low speed pellet) was present in the bottom of the centrifuge tube. The supernatant from the G3LSP was carefully decanted into another centrifuge tube and centrifuged at 35,000 RPM (100,000 *g*) for 1 hour. A small white pellet was present in the bottom of the centrifuge tube and this pellet was designated G3HSP (high speed pellet).

The G2 layer was also drawn off from the three gradient tubes, combined, diluted with 0.88 M sucrose, and centrifuged at 100,000 *g* for 1 hour. A small white pellet G2P was present in the bottom of the centrifuge tube. A pellet did not form when the top layer G1 was removed from the gradient and centrifuged at 100,000 *g* for 3 hours. In some experiments the G2 and G1 layers were combined and centrifuged to recover the particulates for hormone assays. A summary of the method is outlined in Fig. 1.

The above mentioned pellets were: *a*) fixed for electron microscopy, *b*) prepared for various bioassays, and *c*) used for biochemical studies.

Preparation of Material for Electron Microscopy

The pellets mentioned above were fixed in 1 per cent OsO_4 in 0.25 M sucrose and kept in the cold overnight. Rapid dehydration was done in a series of methyl alcohol solutions of increasing concentrations. The pellets were embedded in a 5:1 *N*-butyl and methyl methacrylate (2 per cent benzoyl peroxide) and incubated at 50°C for 24 to 36 hours. Sections of the pellets were studied with a Philips EM-75B electron microscope.

Bioassay Procedures Used for Determination of the Hormones in the Particulate Fractions

Growth hormone (STH) was assayed by the tibia test of Greenspan *et al.* (13). Hypophysectomized female rats 26 days of age, obtained from the Hormone Assay Laboratories, Inc., Chicago, were used.

Lactogenic hormone (LTH) was assayed by the pigeon crop sac method of Lyons (20) as modified by Cherms *et al.* (5), using 6-week-old White King pigeons obtained from Fred Manske, Lake Mills, Wisconsin.

Adrenocorticotropic hormone (ACTH) was assayed by the adrenal ascorbic acid depletion method reported by Sayers *et al.* (27) as modified by Munson *et al.* (22). Hypophysectomized male rats ranging in weight from 120 to 160 gm were obtained from the Hormone Assay Laboratories, Inc., and used for this assay.

The gonadotropic hormone was assayed using P^{32} uptake by the chick testis as reported by Florsheim *et al.* (11).

Thyroid-stimulating hormone (TSH) was assayed using the uptake of P^{32} by the chick thyroid as reported by Greenspan *et al.* (14).

Hormone preparations used as standard: Growth hormone (STH), lactogenic hormone (LTH), follicle-

stimulating hormone (FSH), and luteinizing hormone (LH) were supplied by the Endocrinology Study Section of the National Institutes of Health. A U.S.P. reference standard TSH preparation was used for the thyrotropin assays, and Upjohn corticotropin for the ACTH assays.

Biochemical Determinations

PROTEIN: The protein content of the fractions was determined by the biuret method of Gornall *et al.* (12) modified by the inclusion of 0.03 per cent sodium desoxycholate in the biuret reagent. Beef serum albumin was used as the standard. The volumes of the reagents were reduced so that 0.025 ml of the fractions could be analyzed in a total volume of 1.0 ml. The results are expressed as mg of protein per ml of whole homogenate (23).

PHOSPHATASE: The acid and alkaline phosphatases were measured by the method of Dryer *et al.* (7) as modified by Perdue *et al.* (23).

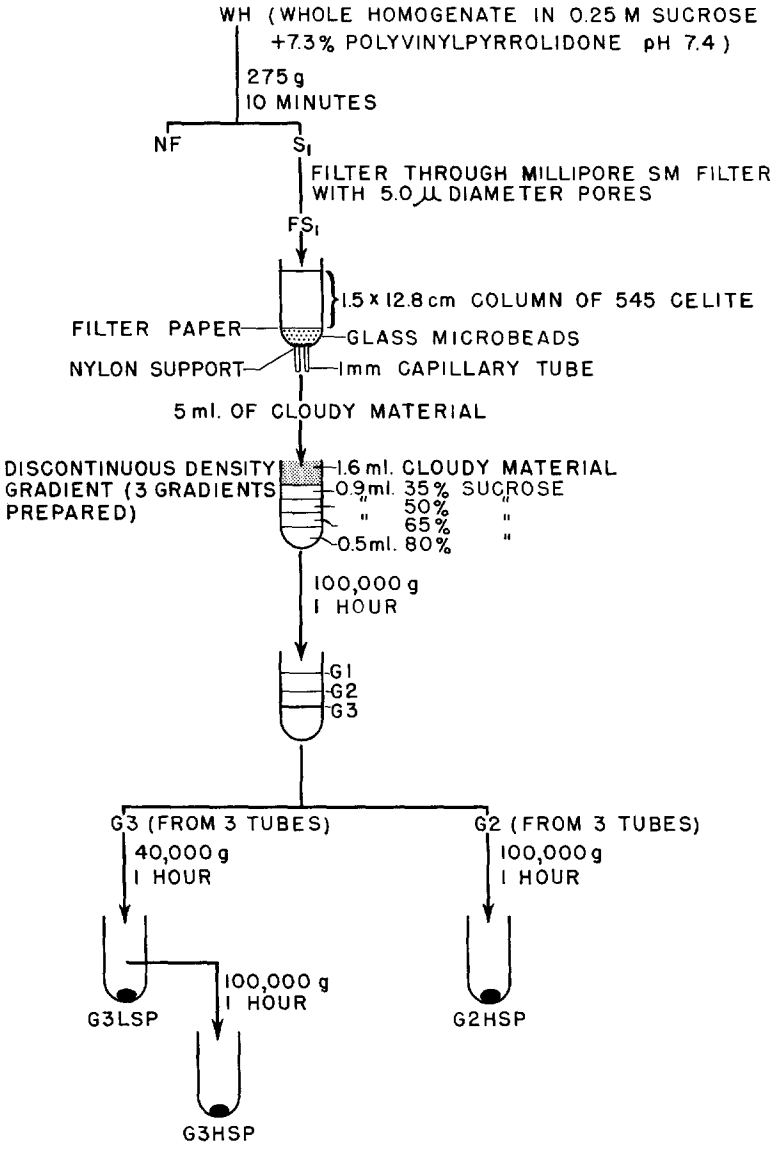


FIGURE 1

Procedure for isolation of pituitary secretory granules.

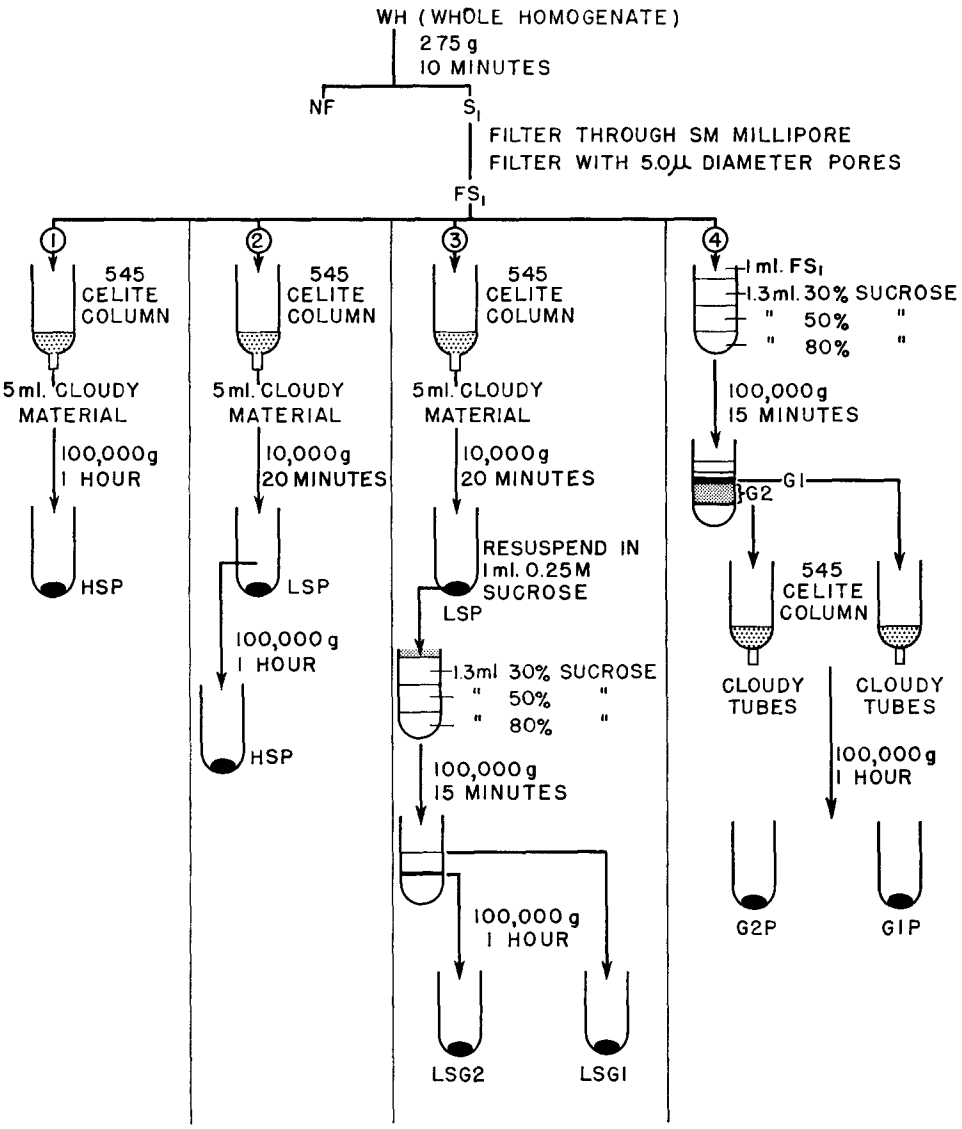


FIGURE 2

Preliminary procedures used in the development of the method for the isolation of pituitary secretory granules.

SUCCINIC DEHYDROGENASE: The microindophenol method of Ziegler (31) was used to determine the succinic dehydrogenase activity of the pituitary fractions. The results are expressed as μmoles of succinate oxidized per minute per ml of homogenate.

CYTOCHROME OXIDASE: This enzyme was assayed by the microspectrophotometric method reported by Cooperstein *et al.* (6). The rate of enzymatic oxidation of reduced cytochrome *c* was measured, and the results are expressed as Δ Log (Ferrocytochrome *c*) per minute per ml of pituitary homogenate.

FREE SULFHYDRYL (—SH) groups were determined by the method of Boyer (3) and the results are expressed in terms of microequivalents —SH per ml of homogenate.

MAGNESIUM: The method of Schacter (28) was used for the determination of magnesium.

FATTY ACIDS: The fatty acid composition of the FS1, G3LSP, and G3HSP fractions was deter-

mined with a model 10 Barber-Colman gas chromatograph.

RNA: Ceriotti's (4) modification of Barrenscheen and Peham's (1) adaptation of the Bial orcinol reaction was used as a measure of RNA ribose.

RESULTS

Electron Microscopy of the Rat Anterior Pituitary Gland

Electron microscopic work on the anterior pituitary gland has led to definitions of the cell types and the hormones which are probably present in them. A system for the classification of the various cell types is based primarily on the maximal diameter of the secretory granules present in the cytoplasm of the pituitary cells (9, 15). A summary of this classification is as follows:

Hormone	Cell type		Diameter of granules* mμ
	General	Specific	
STH	Acidophil	Somatotrope	350
LTH	Acidophil	Mammotrope	600
TSH	Basophil	Thyrotrope	140
FSH & LH	Basophil	Gonadotrope	200
ACTH	?	Corticotrope or Follicular cell	?

* Maximum diameter of the granules as determined from sectioned material.

An electron micrograph of a thin section taken from the anterior pituitary gland of a young adult male rat is shown in Fig. 3. The difference in the sizes of the granules present in the basophils and acidophils is shown in this micrograph.

Isolation of the Acidophilic and Basophilic Granules

The method of isolation which was developed for the purification of the acidophilic and basophilic granules is given in Fig. 1. Several isolation schemes were tried in the development of the final method. These preliminary procedures are given in Fig. 2 and will be discussed later in order to preserve the continuity of this report.

Electron micrographs of thin sections through the FS1 pellet, the particulate fraction which was chromatographed on the column of Celite, are shown in Figs. 4 and 5. The micrographs show that acidophilic and basophilic granules, mitochondria, and microsomal material were present in this fraction.

Electron micrographs of thin sections taken from different levels in the low speed pellet G3LSP (Fig. 1) are shown in Figs. 6 and 7. Examination of these micrographs revealed a population of densely packed granules in this pellet. The major part of these granules have a maximum diameter of 350 mμ and, on the basis of their size, are identified as those which are found in the cytoplasm of certain acidophilic cells of the pituitary gland. They are designated as the acidophilic granule fraction. The pellet of these granules was finally sectioned throughout and, on examination of the sections with the electron microscope, it was found to consist essentially of pure granules.

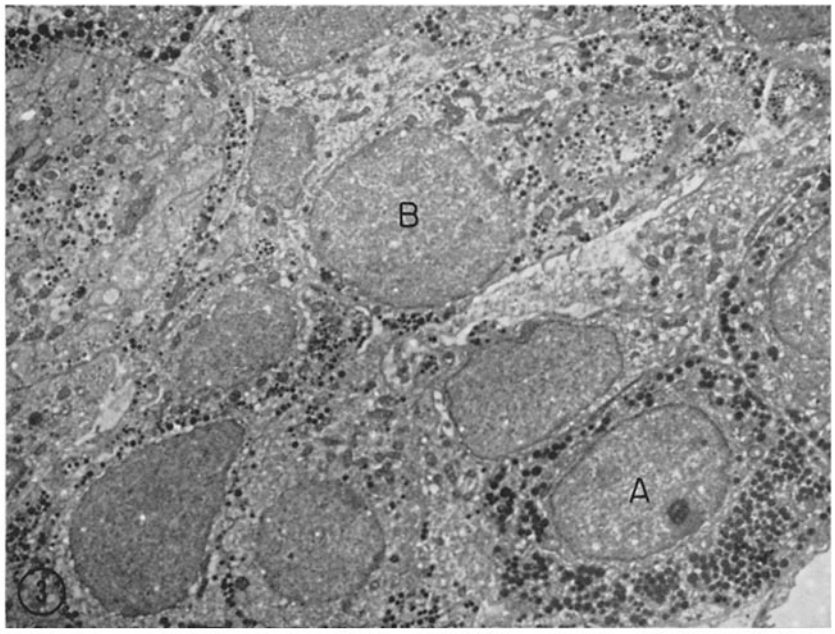
Electron micrographs of the high speed pellet are shown in Figs. 8 and 9. These micrographs show that many granules are present in this pellet. They have a maximum diameter of 200 mμ and are identified as the granules which come from the cytoplasm of the basophils of the pituitary gland.

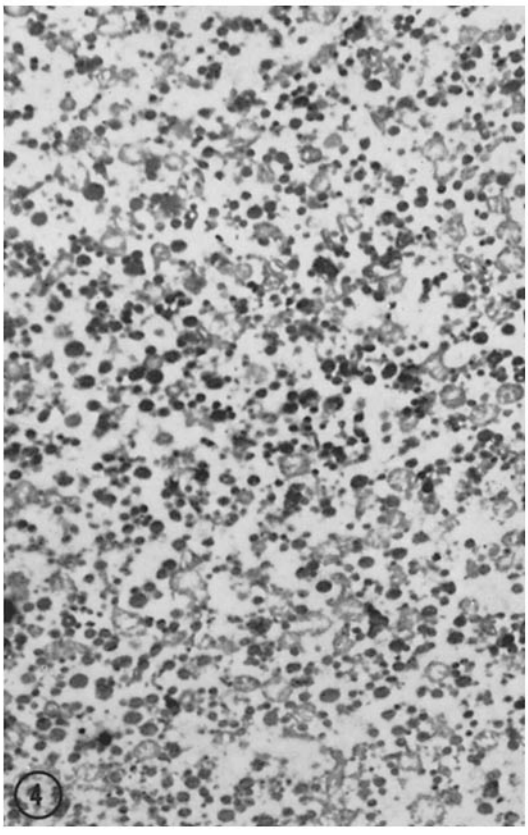
FIGURE 3

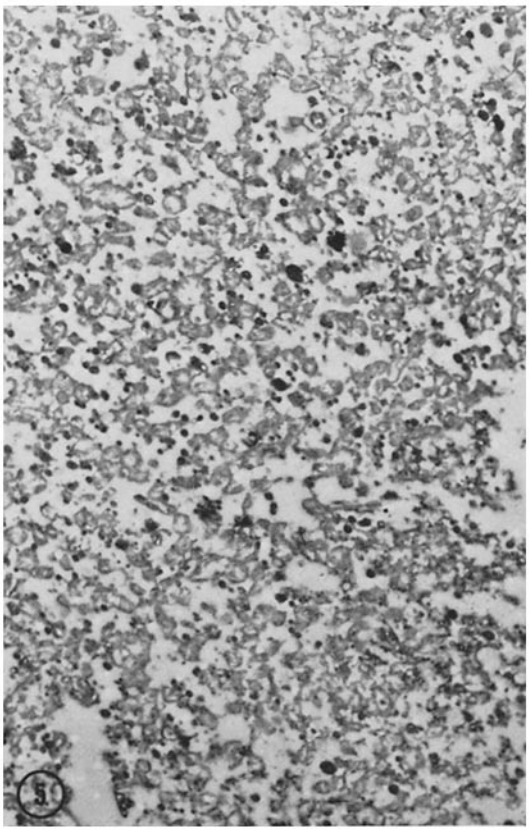
An electron micrograph of a thin section taken from the anterior pituitary gland of an adult male rat. Several acidophils (*A*) and basophils (*B*) are present. × 6,500.

FIGURES 4 AND 5

Electron micrographs of thin sections through the FS1 pellet. Stratification of the material in this pellet is evident. This fraction consists of mitochondria, acidophilic and basophilic granules, and microsomes. This is the fraction which was chromatographed on the Celite columns. Fig. 4, × 8,500. Fig. 5, × 5,000.


B
A





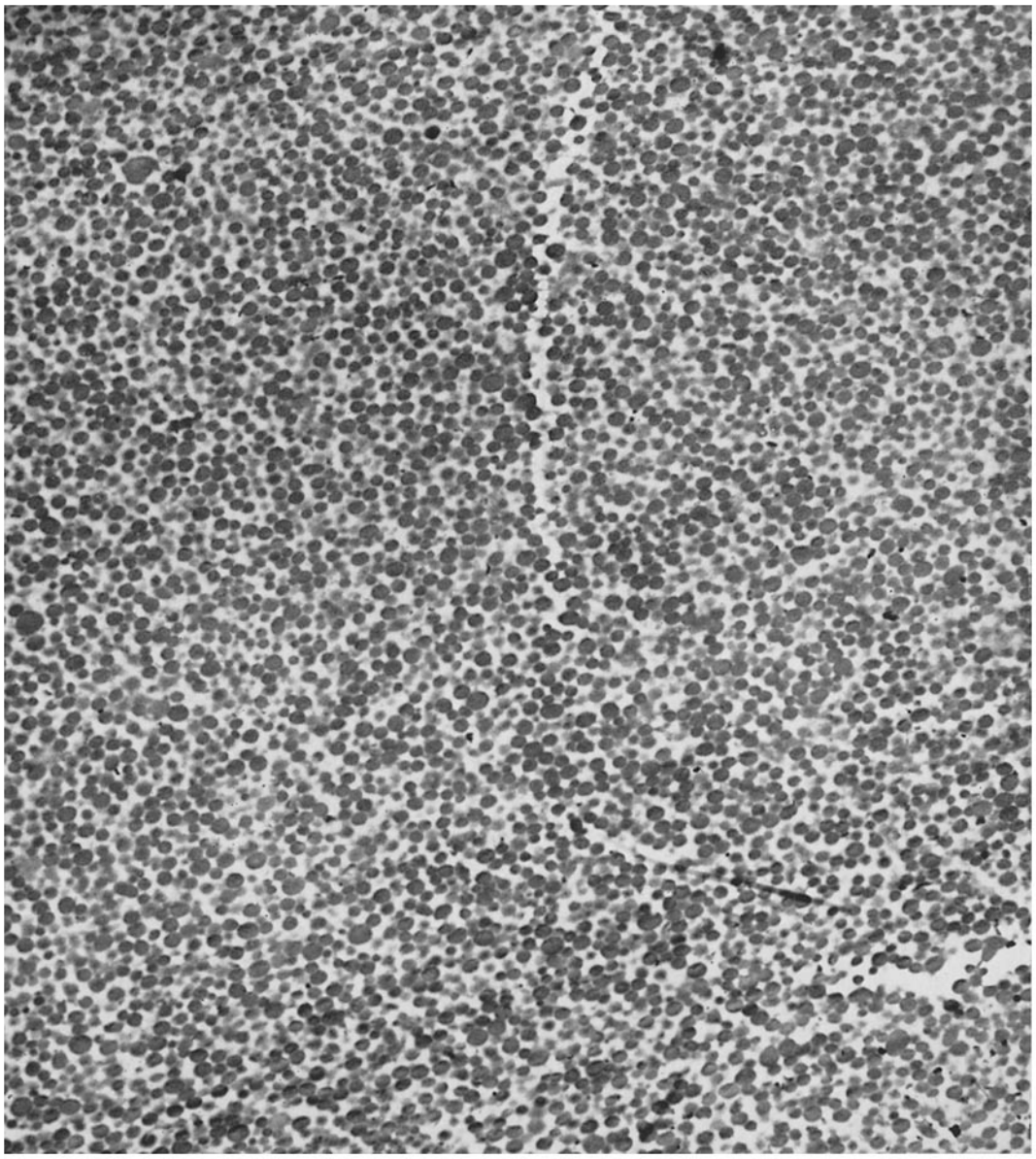


FIGURE 6

An electron micrograph of a thin section through the G3LSP pellet. The majority of the granules in this pellet have a maximal diameter of 350 μ, and there are a few granules which have a diameter larger than 350 mμ. This is the purified acidophilic granule fraction. × 9,000.

Chemical Properties of the Granule Fractions

The morphological homogeneity of the acidophilic and basophilic granule fractions discussed above was further confirmed by enzymatic and chemical studies. Cytochrome oxidase and succinic dehydrogenase activities were not detected in either the G3LSP or G3HSP fraction (Table I). These findings support the electron microscopic observations that these granules were not contaminated with mitochondria. The evidence for

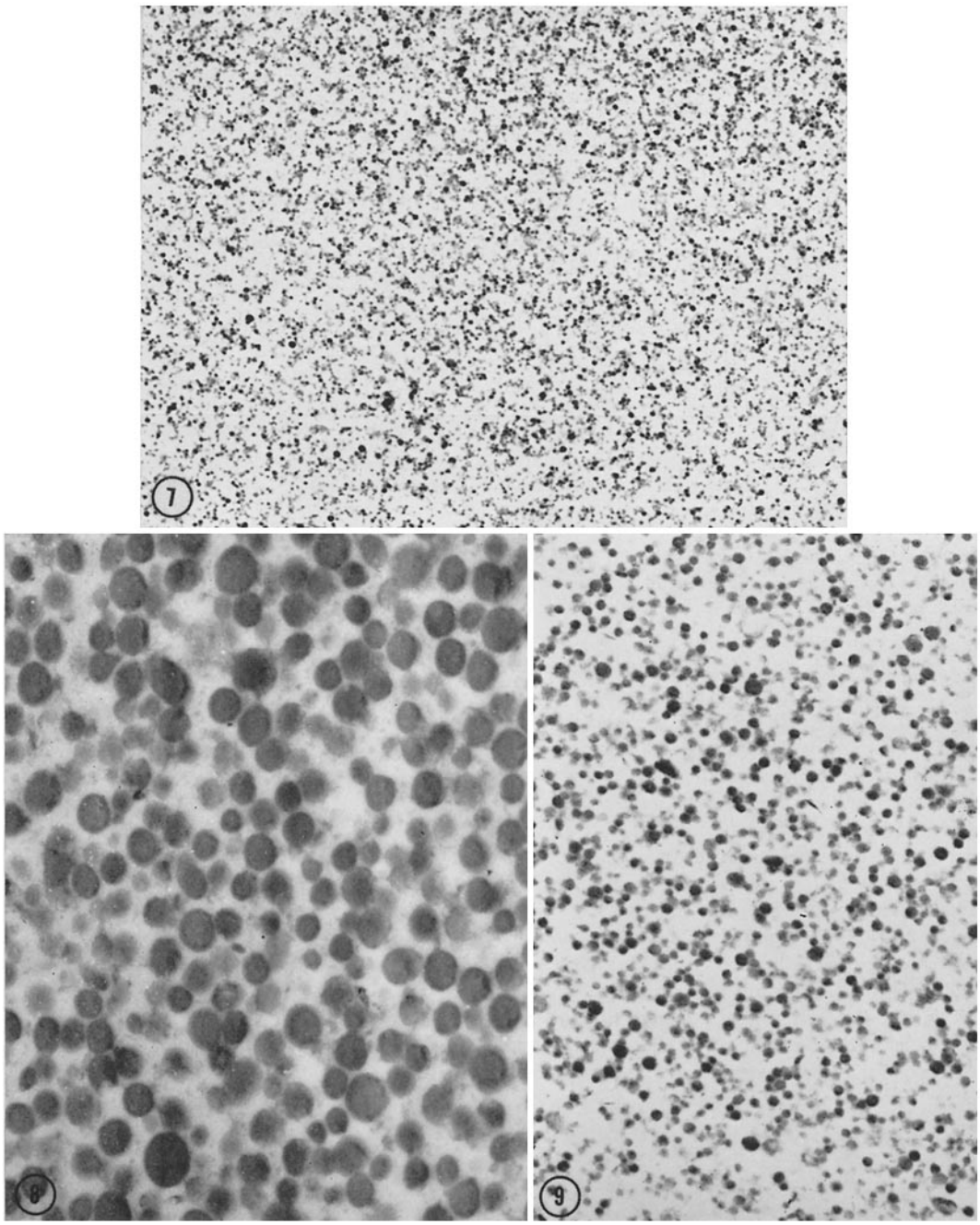


FIGURE 7

An electron micrograph of a thin section through the G3HSP pellet. The granules in this micrograph have a maximal diameter of 200 mμ. This is the basophilic granule fraction. × 4,500.

FIGURE 8

An electron micrograph of a thin section through the acidophilic granule pellet (G3LSP). × 25,000.

FIGURE 9

An electron micrograph of a section through the basophilic granule pellet (G3HSP). × 12,000.

TABLE I

Biochemical Properties of the Particulate Fractions (Fig. 1)

Fraction	Protein *mg/ml**	Succinic dehydrogenase‡	Cytochrome oxidase§	RNA *μg/ml*	Phosphatase *μg P/min/ml* Acid	Phosphatase *μg P/min/ml* Alkaline	Microequiv. *—SH/ml*
WH	7.6	—	2.4 ± 0.01	—	83	27	3.6 ± 0.06
FS1	4.3	0.118 ± 0.011	1.8 ± 0.008	236 ± 1.5	45	14	1.2 ± 0.10
G3LSP	0.4	0.0¶	—‖	0.0	0.0	0.0	0.04 ± 0.0
G3HSP	0.6	0.0	—	0.0	0.0	0.0	0.04 ± 0.0
G1 + G2	1.2	—	—	108 ± 1.8**	0.9	0.4	0.1 ± 0.0
Grad. Super	—	—	—	—	2.2	1.2	0.15 ± 0.0
WCP	1.4	0.0	0.0	—	3.5	5.1	0.58 ± 0.07
WCS	2.8	0.004 ± 0.0	0.08 ± 0.002	—	—	—	0.15 ± 0.01
Wash from Celite column‡‡	—	—	0.12 ± 0.002	—	—	—	—

* Mg protein/ml of pituitary homogenate.

‡ Expressed as μmoles succinate oxidized/minute/ml of pituitary homogenate.

§ Expressed as Δ Log (Ferrocytochrome *c*)/minute/ml of pituitary homogenate.

¶ Indicates that activity was not present in the fraction.

‖ Indicates that fraction was not assayed.

** This value was obtained from the G2 fraction only. The RNA in the G1 fraction was not determined.

‡‡ After the column was developed, the top 3 cm of Celite was removed, suspended in 0.25 M sucrose, and centrifuged to obtain the supernatant which was assayed for cytochrome oxidase.

the lack of mitochondria was further extended by the results of the fatty acid analysis given in Table II. Arachidonic acid, a 20 carbon acid with 4 double bonds, which is found in mitochondria (25), was not present in either of the granule fractions. On the other hand, arachidonic acid was found in the FS1 fraction which is consistent with the electron microscopic observations and cytochrome oxidase and succinic dehydrogenase determinations showing that mitochondria were present in this fraction. Since the mitochondrial enzymes were not detected in the whole column pellet (WCP), a fraction which contained all the particulates which passed through the column, it is concluded that columns of Celite are effective in the quantitative separation of the mitochondria from the acidophilic and basophilic granules, and the microsomal material that passed through the column. Acid and alkaline phosphatases and RNA were not detected in the granule fractions (Table 1), indicating that other contaminating elements such as microsomal, lysosomal, and cell membranous materials were also not present.

Electron micrographs of thin sections taken from the pellet obtained by centrifugation of the middle layer G2 from the gradient (Fig. 1) are shown in Figs. 10 and 11. These micrographs

TABLE II

Fatty Acids Found in Certain Pituitary Particulate Fractions (Fig. 1)

Fraction	Fatty acids identified	Relative* amounts
FS1‡	16 methyl heptadecanoic	1
	13 methyl tetradecanoic	2
	15 methyl hexadecanoic	3
	arachidonic	4
G3LSP	n-hexadecanoic	1
	14 methyl pentadecanoic	2
	12 methyl tridecanoic	3
	13 methyl tetradecanoic	4
	15 methyl hexadecanoic	5
G3HSP	n-hexadecanoic	1
	14 methyl pentadecanoic	2
	11 methyl dodecanoic	3
	12 methyl tridecanoic	4
	13 methyl tetradecanoic	5
	15 methyl hexadecanoic	6

* Fatty acids increase in amount with increasing numbers.

‡ The FS1 fraction was run at a lower sensitivity than the two granule fractions. As a result, 5 additional fatty acids were detected in the FS1, but these were not identified.

reveal that this fraction was heterogeneous. A high percentage of 140 mμ maximal diameter granules, microsomal material, and a few 200 mμ maximal diameter granules were present in this fraction. It was not possible to sediment the particulates in the G1 layer of the gradient (Fig. 1). This material is undoubtedly very fine in character since it did not sediment into the gradient. The G1 and G2 layers were not contaminated with mitochondria since, as mentioned above, mitochondria were not present in the WCP. It is of interest in this connection, however, that approximately one-half of the RNA which was associated with particulates in the FS1 was present in the G2 fraction (Table I). This result confirms the electron microscopic evidence that microsomes were present in this fraction. Low levels of acid and alkaline phosphatases were also detected in the upper layers of the gradient, suggesting that other elements such as lysosomes and cell membrane fragments may be present in this fraction.

The protein contents of the acidophilic and basophilic granule fractions were, respectively, 5 and 8 per cent of the protein present in the whole pituitary homogenate (Table I). Analysis of the acidophilic and basophilic granules for free —SH groups showed that only low levels were present in these granules which is in agreement with the histochemical studies of Barrnett *et al.* (2), showing that very few sulfhydryl groups are present in pituitary cells.

As was mentioned above, mitochondrial fatty acids such as arachidonic acid were not present in the granule fractions. Several branched-chain methyl fatty acids, however, were found in the acidophilic and basophilic granules (Table II). This finding is of particular interest in that, as far as could be determined, branched chain fatty acids are not usually found in animal tissues.

Physical Properties of the Isolated Acidophilic Granules

Experiments involving microfiltration were done to verify further, on a morphological basis, the homogeneity of the acidophilic granules (G3LSP). Two acidophilic granule fractions were prepared, and each was carefully resuspended in 1 ml of 0.25 M sucrose. One suspension was filtered on a HA Type Millipore filter which had 0.45 μ diameter pores, while the other suspension was filtered on a PH Type Millipore filter which had 0.3 μ diameter pores. It was shown by electron microscopy that the granules passed through the paper with the 0.45 μ diameter pores, but they were held by the fibers of the filter paper with the 0.3 μ diameter pores. These results show that 1) the acidophilic granules have a diameter less than 450 mμ but greater than 300 mμ, and 2) the acidophilic granules were not contaminated by small basophilic granules, as these would have passed through the filter with 0.3 μ diameter pores had they been present in the acidophilic granule fraction.

The stability of the acidophilic granules was studied by extracting them twice in 5 ml of distilled water and centrifugation each time at 100,000 *g* for 2 hours. Electron micrographs of the extracted pellet (Fig. 12) show that these granules were partially stable to this treatment, although many of them appear to be in various stages of fusion and disruption. In relation to these results, it is of interest that the gonadotropin associated with the small granule fraction obtained by differential centrifugation is readily dissolved when this fraction is treated with water (21), and the purified basophilic granules are easily disrupted by treatment with 0.9 per cent saline with resulting solution of the gonadotropin (16).

Ribosomes have been shown by Siekevitz *et al.* (29) to swell in the presence of EDTA. On the basis of this finding the acidophilic granules were incubated in 1 mM EDTA at 37° for ½ hour. Electron micrographs (Fig. 13) showed that the acidophilic granules were not stable to this treatment, suggesting the possibility that a divalent cation such as Mg may be present in the granules. When the granules were analyzed for Mg by the fluorimetric method of Schacter (28), this element was not detected in either the acidophilic or basophilic granules. It would be of interest, however, to examine the granules further for the presence of other divalent cations such as Ca, Mn, and Zn.

Solutions Other than 0.25 M Sucrose Used for Washing and Developing the Celite Columns

In a series of experiments, Celite No. 545 was washed with either 0.25 M sucrose containing 7.3 per cent PVP, or with 0.25 M sucrose containing 7.3 per cent PVP and 0.9 per cent saline. The particulates did not pass through the column after these treatments. The large quantities of PVP apparently caused the adsorption of the particulates to the Celite in the column, or prevented their passage through the interstices in the column.

In another series of experiments it was found that certain pH changes occurred during the elution of the particulates from the column. The pH was 7.0 after the FS1 was layered on the column, 6.1 when the cloudy material first emerged from the column, 7.1 after the last of the cloudy material was collected, 7.6 after 5 additional ml of 0.25 M sucrose was collected, and 7.0 shortly thereafter. In the light of these pH changes, an experiment was done in which chromatography of the particulates was attempted on a column of Celite which was previously washed and then developed with 0.25 M sucrose containing 0.01 M pH 7.0 phosphate buffer. This solution was effective in maintaining a constant pH, but the particulates were held on the column. Attempts to elute the particulates from the column with 0.25 M sucrose buffered at pH 6.0 and at 8.0 were not successful.

The effect of washing the Celite with 1 M HCl or 1 M NaOH on the chromatography of the particulates was also studied. An electron micrograph of the G3LSP fraction obtained from an acid-washed Celite column is given in Fig. 16 and one of the WCP from a base-washed column is shown in Fig. 17. It is obvious from these micrographs that mitochondria as well as granules and microsomes passed through these columns. These results show that either acid or base changed the properties of the Celite so that it did not retain or adsorb the mitochondria.

Although the results obtained when the columns were developed with these various solutions do not clarify the mechanism by which the Celite in the column effects the separation of the granules from the mitochondria, they suggest that adsorption of the mitochondria (as well as other particulates) by the Celite is involved. This mechanism presupposes that the Celite and particulates have charges on their surfaces. Filtration or sieving processes may also play a role since the best separations were obtained when Celite No. 545, which consists of particles with large pore diameters, was used.

Preliminary Methods for the Isolation of Pituitary Secretory Granules

The preliminary methods, mentioned earlier and given in Fig. 2, led directly to the development of the final method given in Fig. 1. The results obtained from these preliminary methods are outlined briefly below.

1. Fig. 2-①. This method was used for obtaining the whole column pellet WCP. Electron microscopy showed that this fraction consisted of acidophilic and basophilic granules, and microsomal material.
2. Fig. 2-②. This method involved the fractionation of the material which passed through the column into a low speed pellet and a high speed pellet by differential centrifugation. Electron microscopy revealed that the low speed pellet consisted mainly of acidophilic granules while the high speed pellet contained basophilic granules and microsomal material.
3. Fig. 2-③. This procedure consisted of the fractionation of the particulates in the low speed pellet of Figure 2-② by discontinuous density gradient centrifugation. Two bands

FIGURES 10 AND 11

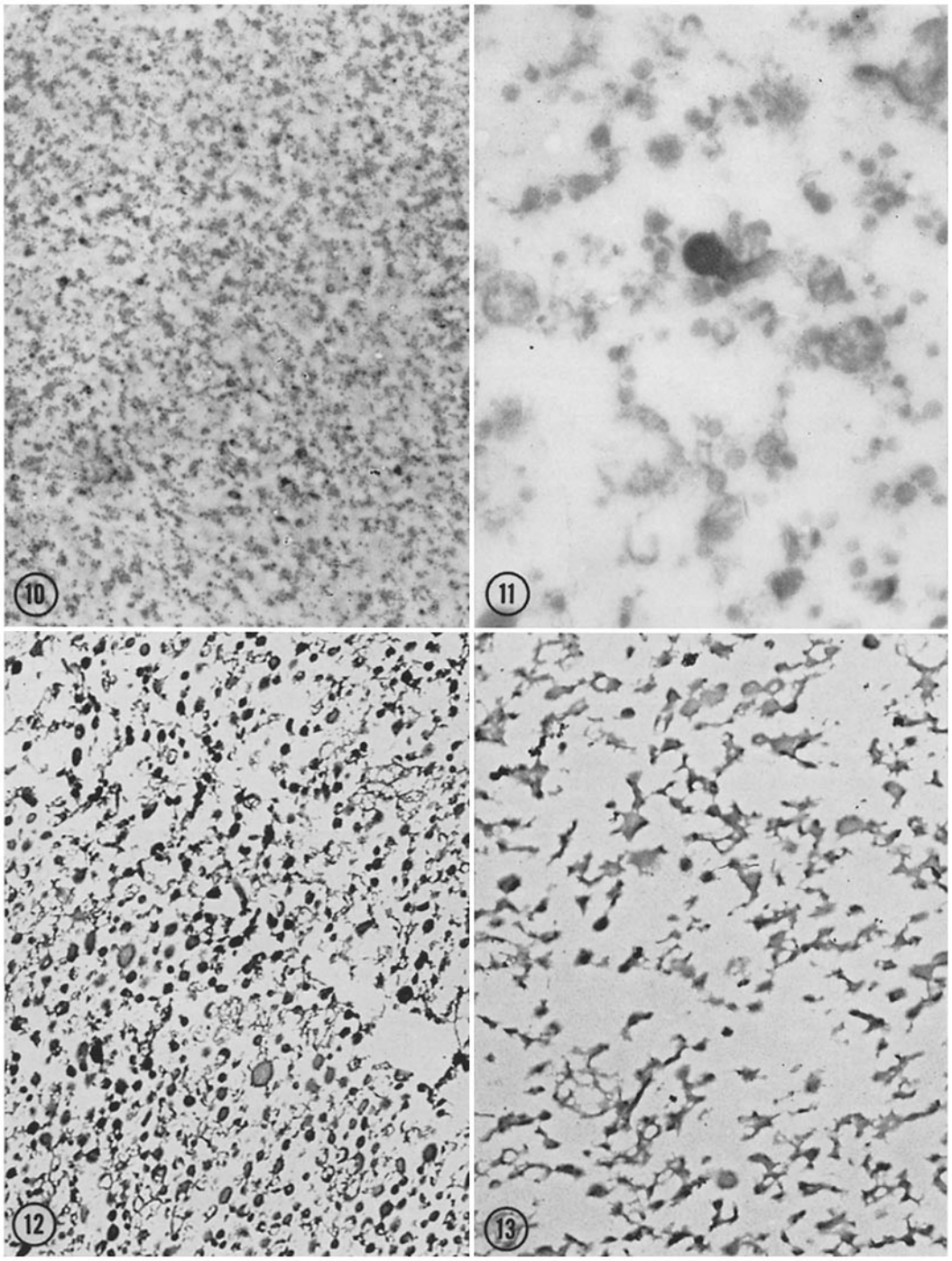
Electron micrographs of sections from the G2P pellet (Fig. 1). The majority of the granules in this fraction have a maximal diameter of 140 mμ or less. Microsomes are also present in this fraction. Fig. 10, × 4,500. Fig. 11, × 29,000.

FIGURE 12

An electron micrograph of a thin section through the pellet obtained when the G3LSP was extracted twice with distilled water. The granules are partially stable to this treatment. × 14,000.

FIGURE 13

An electron micrograph of a thin section of the pellet obtained when the G3LSP was incubated at 37°C for 30 minutes in 1 mM EDTA. The granules were unstable in this medium. × 18,000.


10
11
12
13

formed in the gradient. The pellet from the lower band (LSG2) consisted of highly purified acidophilic granules, while the material in the upper band (LSG1) consisted of basophilic granules and microsomal material. Since this method involved resuspension of the LSP, a process which often results in partial loss of hormone activity from the granules, the method was not used further.

4. Fig. 2-④. The FS1 was fractionated on a discontinuous sucrose gradient followed by chromatography of the two lower layers from the gradient on columns of Celite. Electron micrographs of the G2P and G1P fractions (Figs. 14 and 15) showed excellent preparations of acidophilic and basophilic granules. When these granules were tested for gonadotropic hormone activity, none was detected in either of the granule fractions. On the other hand, when the columns were washed with 60 per cent sucrose (the approximate concentration in the gradient where the granules were located) rather than the usual 0.25 M sucrose, activity was recovered in the cloudy material from the column. These granules could not be sedimented into a pellet due to the high concentration of sucrose in which they were suspended and, because of this, the method was not used further. The results gained from this method are of interest, however, since they tend to indicate at least that some hormones such as gonadotropin can be liberated from the granules while leaving the granular structure relatively intact.

Hormones Associated with the Various Particulate Fractions Isolated by the Method of Fig. 1.

The specific hormones associated with the particulates isolated by the method outlined in Fig. 1 were studied rather extensively. The results serve to clarify, confirm and extend certain of the early results obtained from studies using differential centrifugation, and they also confirm and extend the postulates of Farquhar (9) which are based on electron microscopic studies of rat pituitary glands. It is already clear from the work of Hartley *et al.* (16) and Perdue and McShan (23) that at least a large part of the gonadotropic and thyrotropic hormone activities are associated, respectively, with 200 mμ and 140 mμ maximal diameter secretory granules.

The results given in Table III show that a major part of the growth hormone is associated with the 350 mμ maximal diameter granules present in certain acidophilic cell types of the anterior pituitary gland of the rat. Essentially all of the growth hormone which passed through the Celite column in particulate form (WCP) was localized in the acidophilic granules (G3LSP). Since an epiphyseal plate width up to 185 μ is considered as a non-specific response (8), it is clear that STH was not associated with the small granule fraction (G3HSP) although a slight amount of activity was found in the G1 + G2 fraction.

The results obtained from the assays on the

FIGURE 14

An electron micrograph of a thin section through the G2P pellet (Fig. 2-④). This pellet contains densely packed acidophilic granules. × 7,500.

FIGURE 15

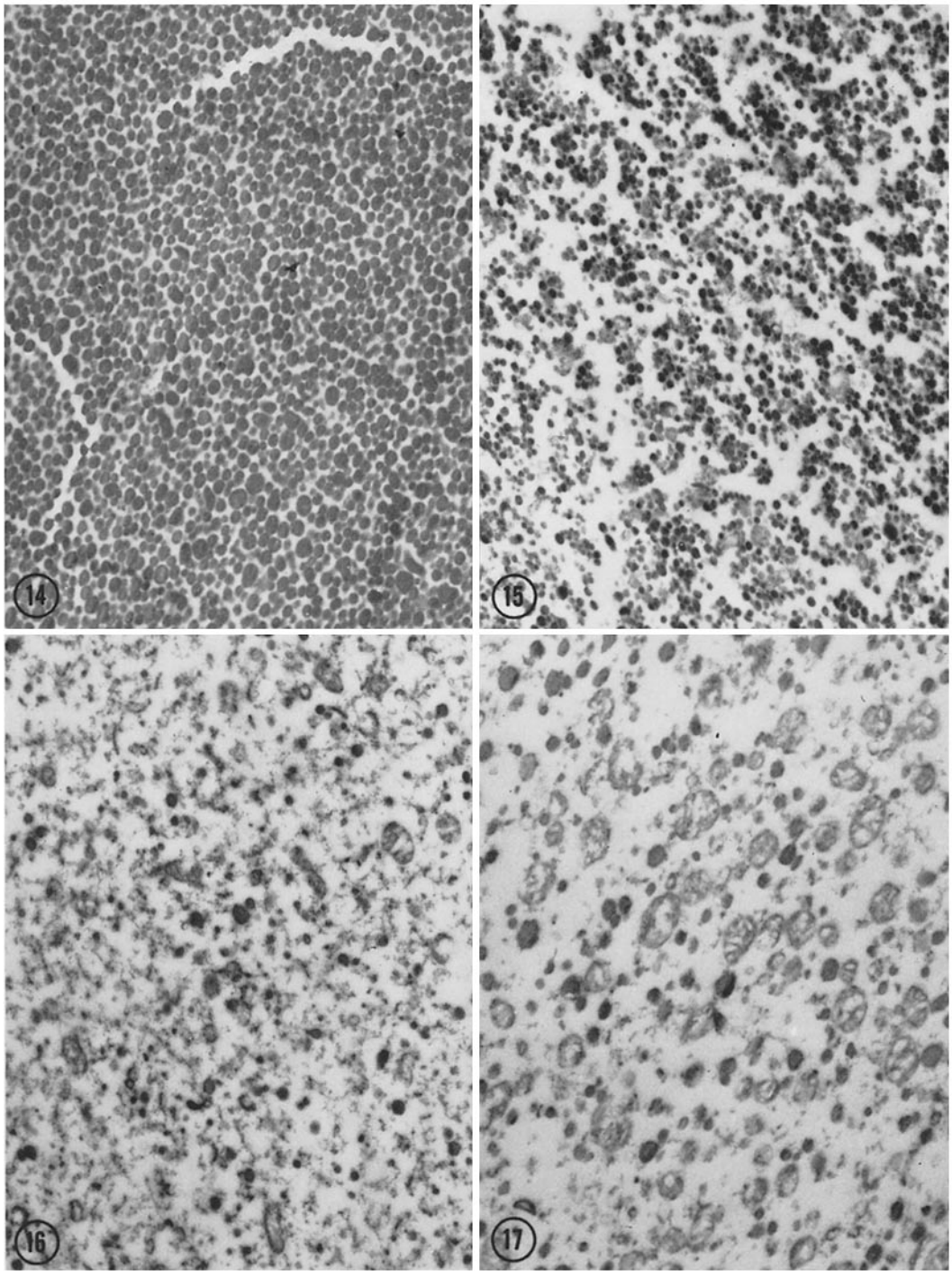
An electron micrograph of a thin section through the G1P pellet (Figs. 2-④). This pellet contains basophilic granules and microsomal material. × 7,500.

FIGURE 16

An electron micrograph of a thin section through the G3LSP fraction which was isolated on a column of No. 545 Celite previously washed with 1 M HCl. The material in this fraction is heterogeneous. Several mitochondria are present. × 10,000.

FIGURE 17

An electron micrograph of a thin section of the whole column pellet (WCP) which was isolated on a column of No. 545 Celite previously washed with 1 M NaOH. This fraction is heterogeneous and contains mitochondria. × 14,000.


14
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17

TABLE III

Growth and Lactogenic Hormone Activities of the Pituitary Fractions (Fig. 1)

Growth hormone				Lactogenic hormone				
							Mean response stim. area	
Fraction	Dose *mg eq FT**	No. of animals	Width tibia μ	Fraction	Dose *mg eq FT**	No. of animals	Units‖	mg
Saline	—	4	128 ± 7.0‡	FS1	4	4	1.0	98
FS1§	10	4	346 ± 19.0	FS1	8	7	1.25	164
G3LSP	10	4	234 ± 1.4	G3LSP	30	1	1.0	144
G3HSP	10	4	161 ± 7.1	G3LSP	40	1	1.0	131
G3HSS	10	4	168 ± 15.4	G3HSP	30	1	0.5	51
G1 + G2	10	4	194 ± 20.4	G3HSP	40	1	0.5	88
Grad. Super	10	4	186 ± 33.5	G3HSS	8	2	0.0	0
WCP	10	4	256 ± 11.3	G1 + G2	40	2	0.0	0
WCS	10	4	141 ± 7.7	Grad. Super	8	2	0.0	0
Standard¶	40 μg	4	222 ± 12.1	Standard**	25 μg	2	1.0	102
Standard	200 μg	4	285 ± 10.2	Standard	50 μg	2	1.75	170

* Indicates mg equivalents of fresh pituitary tissue in this and subsequent tables.

‡ Standard error of mean in this and other tables.

§ See list of abbreviations.

¶ Bovine growth hormone supplied by the Endocrinology Study Section, Nat'l. Inst. of Health.

‖ Reece-Turner Units (*Research Bull. Mo. Agr. Exp. Sta.*, 1937, 266).

** Ovine prolactin supplied by the Endocrinology Study Section, Nat'l. Inst. of Health.

TABLE IV

TSH and Gonadotropic Hormone Activities of Pituitary Particulate Fractions (Fig. 1)

Thyroid-stimulating hormone				Gonadotropin			
Fraction	Dose *mg eq FT*	No. of chicks	% increase in P^{32} uptake over control thyroids	Fraction	Dose *mg eq FT*	No. of chicks	% increase in P^{32} uptake over control testes
FS1	0.5	15	121 ± 31	FS1	0.5	15	197 ± 24
FS1	1.0	14	140 ± 19	FS1	1.0	15	318 ± 25
FS1	2.0	13	163 ± 6.5	FS1	2.0	15	310 ± 26
G3LSP	2.0	11	15 ± 6.4	G3LSP	2.0	14	26 ± 5.1
G3HSP	2.0	15	59 ± 21	G3HSP	2.0	15	94 ± 15
G3HSS	2.0	8	−26 ± 12	G3HSS	2.0	9	110 ± 36
G1 + G2	2.0	12	51 ± 11	G1 + G2	2.0	14	71 ± 12
Grad. Super	2.0	5	−47 ± 37	Grad. Super	2.0	5	55 ± 16
WCP	2.0	14	87 ± 19	WCP	2.0	15	200 ± 37
WCS	2.0	9	21 ± 15	WCS	2.0	14	84 ± 15
Standard*	3.0	7	31 ± 11	Standard FSH‡	25 μg	11	88 ± 11
"	6.0	5	100 ± 39	"	50 μg	15	172 ± 19
"	18.0	5	216 ± 47	Standard LH‡	1 μg	14	61 ± 9.8
"	36.0	5	227 ± 33	"	3 μg	15	173 ± 11.0

* U.S.P. Reference TSH expressed in milliunits.

‡ FSH and LH supplied by the Endocrinology Study Section, National Institutes of Health.

distribution of LTH were somewhat disappointing. Only 25 per cent of the activity in the starting material FS1 was recovered in the acidophilic granule fraction G3LSP (Table III). These results may be explained by the relative lack of the large 400 to 600 mμ diameter granules, since lactogenic hormone is supposedly associated with these larger granules (10, 18). It is possible that these granules, due to their large size, did not pass through the column or that a large part of the LTH activity was adsorbed on the column. It should be pointed out, however, that the pigeons used for the assays on the final granule fractions were relatively unresponsive to LTH as compared to other assays run on previous years. It is likely that the relative lack of LTH activity may be explained on this basis. The presence of approximately 25 per cent of the LTH in the acidophilic granules, however, still supports the concept that LTH is produced by the acidophilic cells of the anterior pituitary gland of the rat.

The results of the assays of the pituitary particulates for thyroid-stimulating hormone indicate that this hormone activity was divided between the basophilic granule fraction (G3HSP) and the particulates in the upper layers (G1 + G2) of the gradient (Table IV). These results confirm the observation of others (23) that TSH is associated with the basophilic granules. A high percentage of the granules in the G2 fraction ranged from 120 to 140 mμ in diameter, indicating that TSH is probably associated with these granules.

The gonadotropin assays (Table IV) indicate that this hormone was associated with particulate

TABLE V

ACTH Activities of the Particulate Fractions (Fig. 1)

Fraction	Experiment	Dose *mg eq FT*	Individual values *mg%**	Mean ±SE
Control	1	—	425, 443	434
WH	1	10	247, 210	228
Control	2	—	451	451
FS1	2	10	242, 269	255
WCP	2	10	297, 212	255
Control	3	—	394, 474, 579, 553, 519	504 ± 33
FS1	3	10	392, 248, 278	285 ± 24
G3LSP	3	20	423, 427, 401	417 ± 8.0
G3HSP	3	20	496, 479	487
G1 + G2	3	20	361, 249, 422	344 ± 50
WCP	3	20	306, 225, 355, 381	317 ± 34
Control	4	—	330, 371, 379, 361	360 ± 10
FS1	4	10	137, 96, 180, 194	151 ± 22
G3LSP	4	20	327, 363, 340, 213	311 ± 33
G3HSP	4	20	277, 352, 338, 325	323 ± 16
G1 + G2	4	20	359, 205, 247, 236	261 ± 33
WCP	4	20	388, 140, 376, 211	278 ± 61
Standard‡	4	3 mμ	256, 255, 271, 217	250 ± 11
Standard	4	12 mμ	209, 167, 100, 272	187 ± 36

Duncan-Bonnor§ significance test of the data of experiments 3 and 4 combined

Fraction	
FS1	**¶
G3LSP	NS¶
G3HSP	NS
G1 + G2	**
WCP	**

* Mg per cent ascorbic acid per 100 gm adrenal.

‡ Upjohn reference Corticotropin Standard.

§ Biometrics, March, 1955, **2**, No. 1, 1.

¶** indicates the fraction was significantly different from the control at the 0.01 level.

NS indicates fraction was not different from the control at the 0.05 level.

material localized in the basophilic granules, and the upper layers of the gradient. A significant part of the gonadotropin was solubilized from the basophilic granules by the 0.25 M sucrose used in this method of fractionation. This solubilized gonadotropin was either retained on the column of Celite, found in the gradient supernatant fraction, or in the supernatant from the basophilic granules. These assays in general agree with those reported by others (16, 23) that gonadotropin is associated specifically with 200 mμ maximum diameter granules.

The cell type which is concerned with the production and secretion of ACTH has been a matter of controversy for several years. It was of particular interest, therefore, to test the fractions for ACTH activity. The results of 4 assays are given in Table V. The individual values of the ascorbic acid concentration in the adrenal glands varied within an experiment as well as between experiments. This variability was expected in the light of results reported previously by other workers (27). The results given in Table V show that ACTH was associated with a particulate which passed through the Celite column and which was recovered in a pellet (WCP) by centrifugation. The assays of the particulates isolated by the method given in Fig. 1 show that a major part of the ACTH activity (significant at the 0.01 level) was present in the particulates recovered by centrifugation of the combined G1 and G2 layers from the gradient. Statistical analysis of the data also showed that ACTH was not present in significant amounts in the basophilic and acidophilic granules. The data suggest, however, that these granules were slightly contaminated with ACTH, probably as a result of the adsorption of the hormone on these granules during their preparation.

Electron micrographs of thin sections of the particulates from the G2 layer of the gradient (Figs. 10 and 11) show the presence of microsomal material, a few 200 mμ maximal diameter basophilic granules, and many small basophilic granules which measure 140 mμ in maximal diameter. The gonadotropic and thyrotropic activities found in these particulates correlate, respectively, with the concentrations of the 200 and 140 mμ diameter granules. These results and those discussed above are interpreted as indicating that ACTH is not associated with the 140, 200, 350, and 600 mμ diameter granules but is associated either with granules which have a maximal diameter less than 140 mμ or with other particulates such as microsomal material which are present in the G1 + G2 fraction.

It would be of interest to determine whether three populations of granules, one with a diameter of 200 mμ, one with a diameter of 140 mμ, and one with a diameter less than 140 mμ are present in the particulates of the G1 + G2 fraction from the gradient. If this could be shown, these populations of granules might then correlate with gonadotropin, TSH, and ACTH activities, respectively. In this connection electron micrographs of the cell type which is believed, but not proven, to produce ACTH (10) show a relative lack of cytoplasmic development and few, if any, granules in in the cytoplasm. This apparent lack of granules may be misleading, however, as granules 100 mμ or less in diameter would not likely be found in great numbers in sections of their cell type. It is of interest that Siperstein (30) suggested recently from the results of radioautographic studies that ACTH is produced by a chromophobic type cell. On the basis of the present results, the association of ACTH with granules should be made with caution, although the alternative that it is associated with microsomal material in the G1 + G2 fraction appears to be less likely. The microsomal elements in the G1 + G2 fraction are derived from the different pituitary cells which should make it impossible to assign the ACTH to a specific cell type should it be associated with the microsomal elements.

DISCUSSION

The results of the experiments presented above are significant from several standpoints. Thus, a simple and highly repeatable method for the isolation of the acidophilic and basophilic granules from the anterior pituitary gland of the rat has been developed. These granule fractions are obtained within 3 hours after sacrifice of the animals. Briefly, the method consists of the passage of acidophilic and basophilic granules and microsomes through a column of No. 545 Celite with retention of the mitochondria on the column. Essentially homogeneous acidophilic and basophilic granules were then obtained by centrifugation of the material from the column on a discontinuous density sucrose gradient and differential centrifugation of the G3 fraction from the gradient (Fig. 1). The homogeneity of the two kinds of granules was

demonstrated by 1) electron microscopy, 2) enzymic studies, and 3) fatty acid and RNA analyses.

The results given in this report show that under favorable conditions relatively large particulates can be chromatographed. The mechanism by which the mitochondria are separated from the pituitary granules is not entirely clear. Charges on the Celite and/or the particulates are almost certainly involved and the separation may also depend in part on sieving action by the Celite.

The presence of branched-chain fatty acids in the granules is of interest from the biochemical standpoint in that these acids are rarely found even at low levels in animal tissues. The significance of the presence of branched-chain fatty acids in the granules is not clear from the present results and further studies on this aspect of the problem are underway. The fact that acidophilic and basophilic granules have different physical properties as indicated by the stability studies suggests that they are also different chemically. If the hormones are loosely bound to the inner electron opaque portion of the granule, the smooth surfaced membrane probably prevents the dissolution of the electron opaque material and the hormone during the preparation of the granules. There is also the possibility that the hormone is adsorbed to the outer surface of the smooth surfaced membrane.

It is suggested that the cell type which produces ACTH has small granules less than 140 mμ in diameter, and it is more likely that the ACTH is associated with these granules than with the microsomal material present in the particulate fraction with which ACTH was found to be associated in this study. Additional experiments are necessary to further substantiate this hypothesis.

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