ISOLATION AND BIOCHEMICAL STUDY OF SECRETORY GRANULES FROM RAT PITUITARY GLANDS

J. F. PERDUE and W. H. McSHAN, Ph.D.

From the Department of Zoology, University of Wisconsin, Madison, Wisconsin

ABSTRACT

Secretory granules from anterior pituitary glands of young adult male castrate rats were isolated by differential centrifugation, microfiltration, and discontinuous density gradient centrifugation. The granules were obtained as pellets, sectioned, and studied with the electron microscope. A major part of the gonadotropin and a substantial amount of the TSH were associated with the isolated granules. Negligible amounts of growth hormone and prolactin were present as contaminants. Succinic dehydrogenase, glucose-6-phosphatase, acid protease, and acid and alkaline phosphatases were not found in the granules. Alkaline protease was the only enzyme found to be associated with the granules, and it is suggested, in the light of these results, that the alkaline protease may be involved in the release of the hormones.

INTRODUCTION

Electron microscope studies of anterior pituitary glands from rats by Farquhar and co-workers (1–5) have shown that the two representative cell types, acidophils and basophils, contain secretory granules of a specific maximum diameter. Certain of the acidophilic cells contain 350-mμ and others 600-mμ secretory granules reported to be associated, respectively, with growth and lactogenic hormones, while the two basophilic cell types have granules 140 mμ (thyrotropin) and 200 mμ (gonadotropins) in diameter (6).

The separation of pituitary cytoplasmic particulates into various fractions and the determination of the hormones associated with them have been the objective of several workers employing differential centrifugation procedures similar to those used by Hogeboom *et al.* (7) for the preparation of liver particulates. These pituitary studies (8–20) were done with glands from different species, using different homogenizing media, and different times and speeds of centrifugation. The results of these studies are of interest in that they provided information concerning the hormonal activities of the fractions. The degree of purity of these fractions was not established and, as a consequence, generalizations cannot be made as to the specific particulates with which the hormonal activities are associated. In a recent study, however, Hartley *et al.* (6, 21), using differential centrifugation, microfiltration, and isopycnic gradient centrifugation succeeded in isolating highly purified granules, 200 mμ in diameter, from anterior pituitary glands of castrate rats. A substantial amount of the original homogenate's gonadotropic hormone activity was associated with these granules.

The present report is concerned with the extension of this work, in that a shorter procedure was developed for obtaining the granules, young

adult male rats instead of discard breeders were used after castration as the source of the pituitary glands, and the hormonal and enzymatic activities of the granule and other particulate fractions were studied.

MATERIALS AND METHODS

Preparation of Homogenates[1]

A homogenate containing 50 mg of fresh tissue per ml of 0.25 M sucrose and 7.3 per cent polyvinylpyrrolidone (PVP) was prepared, as described previously, from anterior pituitary glands of male castrate rats (21).

Differential Centrifugation and Microfiltration

The homogenates were fractionated in the multi-speed head of a refrigerated International Centrifuge (IC) at 4°C by a modification of the method reported by McShan and Meyer (10). The whole homogenate (WH) was centrifuged at 275 *g* for 10 minutes. The pellet (NF) containing nuclei, red blood cells, and whole and broken cells was saved for assay. The resulting supernatant (S_1) was filtered through a series of Millipore filters having pore diameters of 5.0, 1.2, 0.8 and 0.65 μ.

The filter with 5.0 μ pores was placed in a "Swinny hypodermic adapter," washed with 0.88 M sucrose, and fastened to a 5.0 ml Luer-Lok syringe containing the S_1. Filtration was accomplished by putting light pressure on the plunger of the syringe and filtering into a centrifuge tube. This procedure was repeated but each time with a filter of decreasing pore diameter. The used filters were dissolved in a small amount of acetone, diluted with 0.9 per cent sodium chloride, and assayed.

The filtered S_1 (FS_1) was centrifuged at 4,400 *g* for 10 minutes. The supernatant (S_2) was decanted and the large granule (LG) pellet obtained was fixed for electron microscopy or resuspended in the homogenizing medium for biological and enzyme assays.

The S_2 was filtered through dry Millipore filters with pore diameters of 0.45 and 0.3 μ. The filtrate (FS_2) was centrifuged in the swinging bucket rotor (SW-39L) in the Spinco Model L Ultracentrifuge at 100,000 *g* for 60 minutes. The pellet (FS_2SP) was fixed for electron microscopic observation or resuspended and assayed.

[1] The young adult male rats used as the source of pituitary tissue were supplied by the Endocrine Laboratories, Madison, Wisconsin, and by the Department of Biochemistry of the University of Wisconsin. We thank the Enzyme Institute, Madison, Wisconsin, and Merck & Co., Rahway, New Jersey, for providing the coenzyme Q_2.

Discontinuous Gradient Centrifugation

The choice of a discontinuous gradient was made on the basis of results obtained by Hymer (22) in which a gradient of this kind was used to prepare acidophilic granules from anterior pituitary glands of normal male rats. The gradient used in this work consisted of 2.5 ml of 0.44 M sucrose containing 17.5 per cent Diodrast (the diethanolamine salt of 3,5-diiodo-4-pyridone-*N*-acetic acid) and 5×10^{-4} M Versene which was layered over 0.5 ml of 1.31 M sucrose containing the same amounts of Diodrast and Versene. These solutions were adjusted to pH 7.4. (In Fig. 1 the trade name Diodon is used in place of Diodrast.)

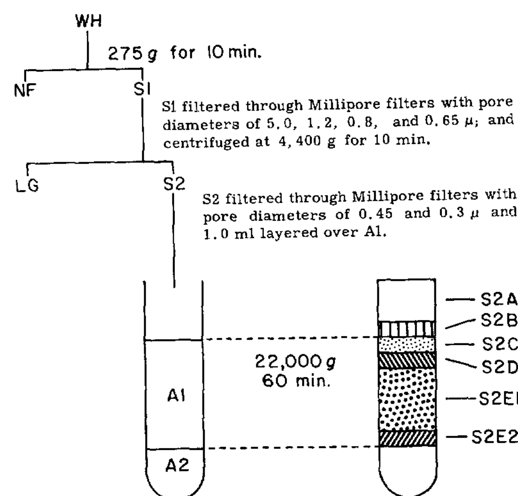


FIGURE 1

Procedure for the fractionation of homogenates from anterior pituitary glands of castrate rats. Designation of fractions: WH—Whole homogenate (50 mg FT per ml); NF—Nuclear fraction; LG—Large granule; S1—Supernatant 275 *g*; S2—Supernatant 4,400 *g*; A1—0.44 M Sucrose; A2—1.31 M Sucrose (both A1 and A2 contained 17.5 per cent Diodrast (Diodon) and 5×10^{-4} M Versene); S2A–S2E2 represent layers obtained on the discontinuous gradient by centrifugation.

One ml of the above filtered S_2 (FS_2) was layered on this gradient and centrifuged at 22,000 *g* for 60 minutes in the SW-39L rotor. The layers (Fig. 1) S_2A through S_2E_2 were removed from the gradient, assayed, or diluted with 0.88 M sucrose and centrifuged at 100,000 *g* for 1 hour to give a pellet (SP) and a supernatant (SS). The pellets were fixed for

electron microscopy or used for assays along with the supernatant solutions.

Biological Assays

GONADOTROPIN: The gonadotropic hormone activity of each fraction was determined in 21-day-old normal female rats of the Holtzman strain. The basis of the assay is the increase in the weight of the ovaries (10).

Hypophysectomized male and female rats were also used for the gonadotropic assay of certain of the fractions. These rats were obtained at 25 days of age, hypophysectomized at 27 days of age, and 0.5 ml injections of the saline-diluted fractions were made twice daily for 4.5 days beginning on the afternoon of day 29. The animals were killed on the morning after the last injection and the ovaries, testes, ventral prostates, seminal vesicles, and adrenals were removed and weighed. The animals were checked for completeness of hypophysectomy by visual examination of the sella turcica.

OTHER HORMONES: The tibiae from the above hypophysectomized rats were removed, fixed, stained, and used for the estimation of the growth hormone content of the fractions, essentially according to the method of Greenspan *et al.* (23). Because the control width of 217 μ is higher than that reported in the literature, possibly reflecting a change in procedure, the data are also expressed as per cent increase above that of the control. The method involving intradermal injections over the crop glands of pigeons reported by Lyons and Page (24), as modified by Breitenbach and Meyer (25), was used to assay the prolactin content of the fractions. The degree of gland stimulation was estimated on a rating scale of negative to four plus. The method of McKenzie (26), as modified by Schuetz *et al.* (27), was used for determining the thyrotropin (TSH) activity of the rat pituitary fractions. The basis of this method is the determination of the per cent increase of blood I^{131} in normal rats after an intravenous injection of the unknown fraction.

Biochemical Determinations

PROTEIN: The protein content of the fractions was determined by the biuret method of Gornall *et al.* (28), using beef serum albumen as a standard, modified by the inclusion of 0.03 per cent sodium desoxycholate. The volumes of the reagents were reduced so that 0.025 ml of fractions could be analyzed in a total volume of 1.0 ml. The results are expressed as mg of protein per ml of fractions diluted to their original volumes.

PROTEASE: The method of determining the protease activity of the fractions was a modification to a micro scale of the method originally reported by Anson (29), as modified by Meyer and Clifton (30). The incubation medium consisted of 0.72 per cent denatured hemoglobin buffered at pH 3.8 in 0.074 M citrate and at pH 8.3 in 0.015 M glycylglycine. The substrate (0.5 ml) was placed in a 1.0 ml Misco glass centrifuge tube with 0.05 ml of the pituitary fraction and incubated in a Dubnoff shaker for 60 minutes at 38°C. The reaction was stopped by the addition of 0.15 ml of 9.9 M perchloric acid (31). The coagulated protein was removed by centrifugation and the optical density of the supernatant fraction was determined at 278 mμ in a Beckman DU spectrophotometer. The control values were obtained by adding the acid to the substrate before its incubation with the pituitary fraction. The results are expressed as μg of tyrosine liberated per minute per ml of the fraction less the control value. Because of the variability in these and the other enzyme assays the determinations were made in quintuplet and repeated 3 to 5 times to obtain the final mean value.

PHOSPHATASE: The method of analysis for inorganic phosphorus with sodium beta-glycerophosphate as the substrate, reported by Dryer *et al.* (32), was used for the determination of the acid (pH 4.6) and the alkaline (pH 9.3) phosphatases of the pituitary fractions. The method was modified by decreasing the amount of each reagent so that incubation could be carried out in 1.0 ml Misco glass centrifuge tubes which were shaken for 30 minutes at 38°C. The results in these and the glucose-6-phosphatase assays are expressed in μg of phosphate liberated per minute per ml of pituitary fraction.

GLUCOSE-6-PHOSPHATASE: The procedure of Swanson (33) was used to determine glucose-6-phosphatase activity. The fractions, 0.025 ml, were incubated in Misco glass centrifuge tubes for 30 minutes at 38°C in 0.025 ml of 0.1 M glucose-6-phosphate and 0.075 ml of 0.1 M malic acid buffer of pH 6.5. The reaction was stopped by the addition of 0.5 ml of 15 per cent trichloroacetic acid, and the increase in the inorganic phosphate was determined.

SUCCINIC DEHYDROGENASE: The microindophenol method of Ziegler (34) 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 pituitary fraction.

Electron Microscope Procedures

The pellets obtained during the fractionation were fixed overnight in the homogenizing medium containing 1 per cent osmium tetroxide (35). Intact pituitary tissue was fixed for 2 hours at 0°C in Palade fixative at pH 7.4. The pellets and intact tissue were dehydrated in a series of methyl alcohol solutions of increasing concentrations and embedded

in either methacrylate (6) or Araldite (36). Thin sections were prepared on the Porter-Blum ultramicrotome, placed on carbon or formvar films and studied in the Philips EM-75B electron microscope. The sections in Araldite were stained with potassium permanganate (37) prior to examination in the microscope.

Distribution Frequency of Sectioned Secretory Granules

The presence of two distinct populations of secretory granules was determined by measuring the diameters of the sectioned granules in randomly selected areas. The measurements were made from electron micrographs of similar magnification and enlargement. The data were plotted as the per cent distribution of the diameters of the granules in the isolated pellet, and in intact thyrotropes and an intact gonadotrope.

TABLE I

Gonadotropic Hormone Activity of Pituitary Glands from Young Adult and Old Breeder Castrate Rats

WH	Ovarian weights	
	Young adult rats	Old breeder rats
(mg FT)*	*(mg)*	*(mg)*
1.0	31 ± 2.7‡ (3)§	19 ± 0.2 (3)
3.0	85 ± 7.2 (3)	44 ± 6.8 (3)
5.0	166 ± 27.8 (3)	73 ± 2.0 (3)
10.0	240 ± 23.5 (3)	211 ± 19.1 (3)

* Indicates fresh tissue in this and subsequent tables.
‡ Standard error of mean.
§ Figures in parentheses in this and subsequent tables indicate number of animals used or determinations made.

RESULTS AND DISCUSSION

Gonadotropic Hormone Activity of Young Adult and Old Breeder Castrate Rats

The results of preliminary studies using the fractionation procedures of Hartley *et al.* (6, 21) and young adult castrate male rats indicated that large amounts of the gonadotropic hormone were solubilized, and that considerable amounts were sedimented with the particulates of the LG fraction. In this respect the results differed from those reported by Hartley *et al.* (6, 21) using old breeder castrate animals. Because of this difference the pituitary glands from the two kinds of animals were assayed for gonadotropic hormone activity. The results (Table I) indicate that the glands from the young castrate rats at the lower dose levels were almost twice as active as those from the old breeder castrate males. This difference may account for the patterns obtained with the glands from the two groups of animals during fractionation. In view of this, the method was modified to obtain a highly purified granule fraction using pituitaries from the young adult castrate animals.

Development of a Method for Fractionation of Pituitary Homogenates

The procedures illustrated in Fig. 1 were developed and used consistently for the preparation of a highly purified sample of secretory granules from the pituitary glands of young adult castrate male rats. The distinguishing features of this method as compared to previous methods (6, 10, 21) are microfiltration of the S_1 and S_2 fractions prior to centrifugation and the use of the discontinuous gradient.

The filtration of the S_1 with Millipore filters of decreasing pore diameters removed cellular particulates not sedimented with the nuclear fraction (NF). The removal of these particulates permitted the centrifugation of the filtered S_1, to give the large granule (LG) and the S_2 fractions, to be carried out in 10 minutes rather than the 20 minutes used in earlier studies (6, 10, 21). The amount of gonadotropin remaining on the filters was negligible (Table II) and that in the LG fraction was found now to be less than with the previous procedures.

It was observed in the early stages of this work that filtering the S_2E_1 layer from the discontinuous gradient with filters having pores 0.45 and 0.3 μ in diameter resulted in the removal of a major part of the activity. This retention of activity on the filters may be due to the diffusion of Diodrast into the particulates and their concomitant swelling. For this reason the supernatant fraction (S_2) was filtered before it was placed on the discontinuous gradient which contained Diodrast. When this procedure was used, the amount of gonadotropic activity retained on the filters was negligible as shown by the assay data in Table II.

The discontinuous gradient made with two different concentrations of sucrose, each containing Diodrast and Versene, has consistently formed the layers S_2A through S_2E_2 (Fig. 1) when

TABLE II

Gonadotropic Hormone Activity of Rat Pituitary Fractions

Fractions*	Dose mg eq FT	Normal rats Ovaries	Hypophysectomized rats Ovaries	Hypophysectomized rats Testes	Hypophysectomized rats Ventral prostates	Hypophysectomized rats Seminal vesicles
		(mg)	*(mg)*	*(mg)*	*(mg)*	*(mg)*
Saline inj. control	0	17 ± 0.9 (6)	10 ± 0.3 (10)	248 ± 20.1 (5)	10 ± 0.4 (5)	5 ± 0.3 (5)
WH	2.5	35 ± 1.5 (5)	38 ± 2.3 (4)	546 ± 33.0 (6)	61 ± 9.7 (6)	24 ± 1.5 (6)
	5.0	138 ± 4.4 (4)	123 ± 8.9 (5)	518 ± 16.0 (5)	57 ± 3.9 (5)	27 ± 2.7 (5)
	10	219 ± 15.7 (5)	167 ± 6.9 (4)	595 ± 17.9 (3)	51 ± 7.5 (3)	30 ± 1.0 (3)
	20	227 ± 34.8 (4)	165 ± 10.3 (8)	706 ± 38.3 (4)	74 ± 5.2 (4)	34 ± 1.6 (4)
NF	20	44 ± 4.9 (18)	21 ± 1.3 (3)	594 (2)	68 (2)	27 (2)
	40	125 ± 12.0 (12)				
LG	20	47 ± 5.2 (10)	15 ± 0.3 (3)	678 ± 50.8 (3)	49 ± 9.4 (3)	26 ± 2.6 (3)
	40	121 ± 16.6 (5)				
Filter	20	29 ± 3.6 (6)		588 ± 57.3 (3)	44 ± 5.9 (3)	20 ± 2.5 (3)
5.0 to 0.65 μ	40	36 ± 4.8 (4)				
0.45 μ	20	17 ± 2.1 (5)				
	40	18 ± 4.2 (3)				
0.3 μ	20	32 ± 4.7 (11)				
0.45 to 0.3 μ	20	25 ± 2.8 (8)		507 (1)	38 (1)	17 (1)
FS_2SP	20	162 ± 7.4 (9)				
FS_2SS	20	81 ± 6.8 (14)				
S_2A	20	36 ± 9.5 (27)	21 ± 1.6 (3)	618 ± 18.6 (4)	55 ± 6.4 (4)	22 ± 2.8 (4)
	40	97 ± 12.4 (5)				
S_2B	20	21 ± 4.2 (3)				
S_2C	20	14 ± 1.5 (2)				
S_2D	20	23 ± 1.5 (5)	15 ± 1.7 (3)	624 ± 39.4 (3)	51 ± 5.8 (3)	16 ± 1.8 (3)
	40	29 ± 4.6 (3)				
S_2B–S_2D	20	44 ± 5.3 (4)				
S_2E_1SP	20	105 ± 9.5 (17)	60 ± 12.0 (5)	606 ± 44.8 (4)	45 ± 9.1 (4)	24 ± 2.6 (4)
	40	267 (1)				
S_2E_1SS	20	38 ± 2.3 (23)	14 ± 4.8 (3)	538 ± 9.4 (3)	47 ± 7.4 (3)	19 ± 1.8 (3)
S_2E_2SP	20	25 ± 1.9 (13)	14 (1)	566 ± 39.4 (4)	41 ± 4.9 (4)	17 ± 0.9 (4)
S_2E_2SS	20	33 ± 1.5 (11)	12.2 (1)	528 (2)	50 (2)	18 (2)

* The blank space in this and subsequent tables represents fractions not hormonally or enzymatically assayed.

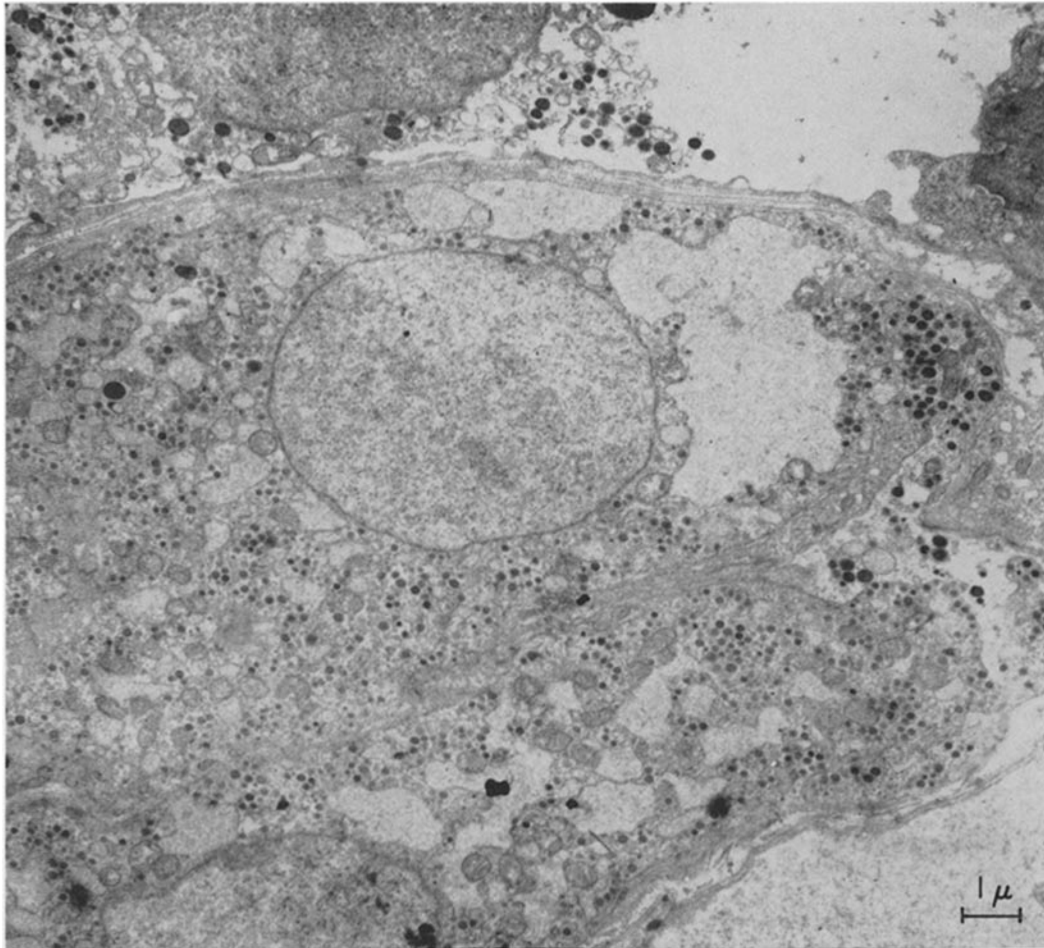


FIGURE 2

An electron micrograph of a thin section from the anterior pituitary gland of a young adult male rat castrated for 10 weeks. The basophilic gonadotrope present in this section shows mainly secretory granules less than 200 mμ in diameter, and a high degree of vesiculation. × 7,500.

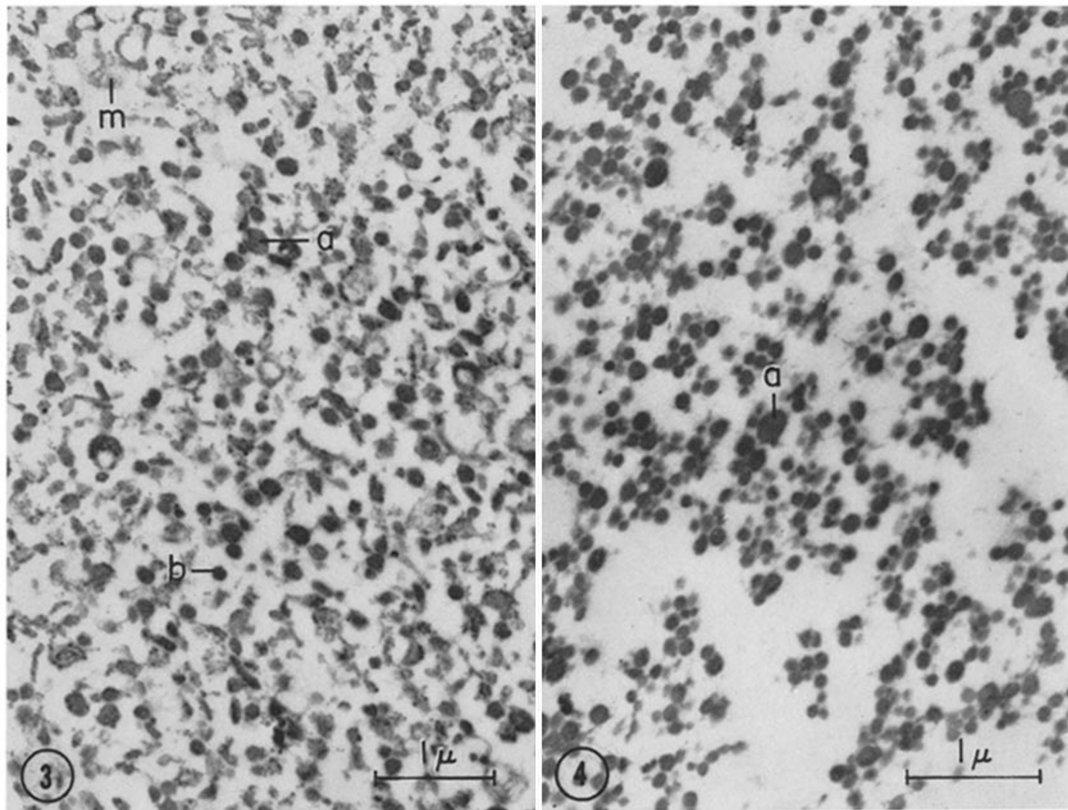
1 ml of the FS_2 (filtered S_2) was placed on it and centrifuged at 22,000 *g* for 60 minutes.

Electron Microscopy of Intact Cells and Fractions

The pituitary basophilic cell shown in Fig. 2 is from a young male rat castrated for 10 weeks. The gonadotropic hormone produced by this cell type is associated with 200-mμ (maximum diameter) secretory granules (2, 21). Vesiculation occurs to some degree within this cell and, with lengthened periods of castration, "signet-ring" cells are often formed (2).

The filtered S_2 pellet (FS_2SP) obtained by filtering the S_2 through Millipore filters with pore diameters of 0.45 and 0.3 μ and centrifuging at 100,000 *g* for 60 minutes contains secretory granules, mitochondria, and vesicular elements (Fig. 3). The majority of the granules (Fig. 4) were less than 200 mμ in diameter.

The particulates in the layers S_2C through S_2E_2 were recovered as pellets by high-speed centrifugation and studied with the electron microscope. The section of the S_2C pellet (Fig. 5) shows mainly small vesicles and a few secretory granules. Numerous granules, the largest having a diameter of 170 mμ, along with membranous



FIGURES 3 AND 4

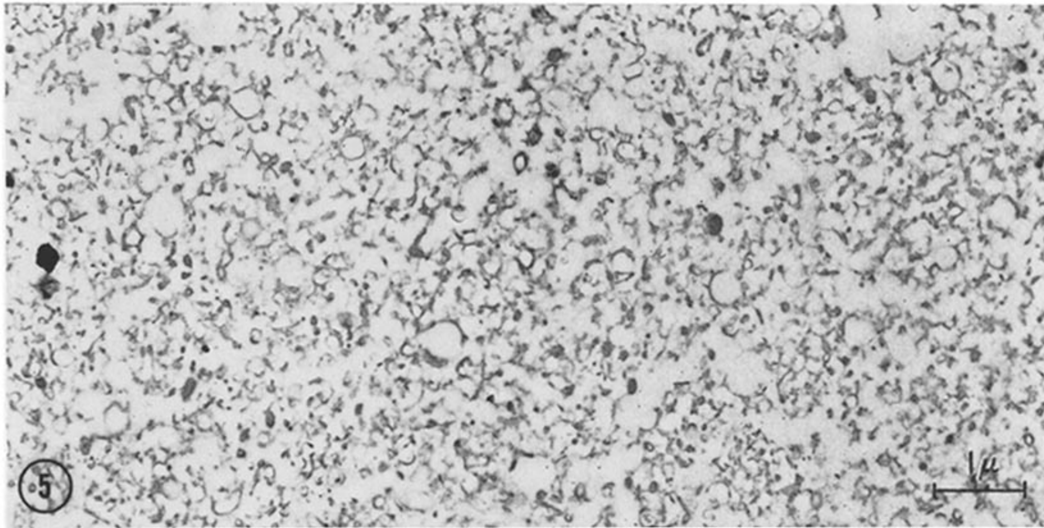
Electron micrographs of sections from different levels of the filtered S_2 pellet showing acidophilic (*a*) and basophilic (*b*) secretory granules, mitochondria (*m*), and vesicles (Fig. 3). Fig. 4 is an area in the pellet containing predominantly secretory granules. The acidophilic granules, of which the largest (*a*) has a diameter of 280 mμ, are easily distinguished from the more numerous basophilic granules. Fig. 3, × 16,000. Fig. 4, × 18,000.

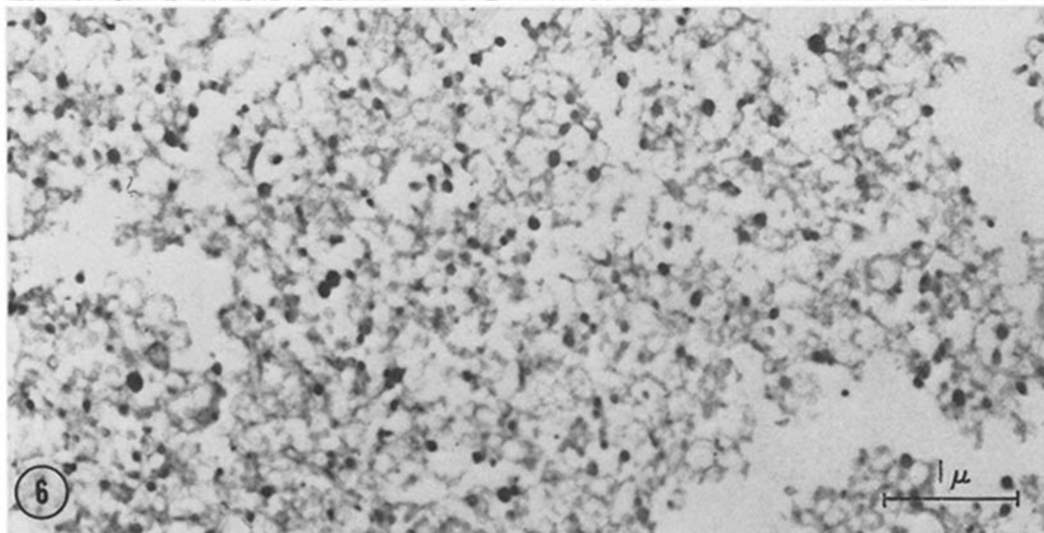
elements are present in the S_2D pellet (Fig. 6). Secretory granules and composite units tentatively identified as broken and swollen mitochondria are shown in the section of the S_2E_2 pellet (Fig. 7). The majority of these granules are probably from acidophils.

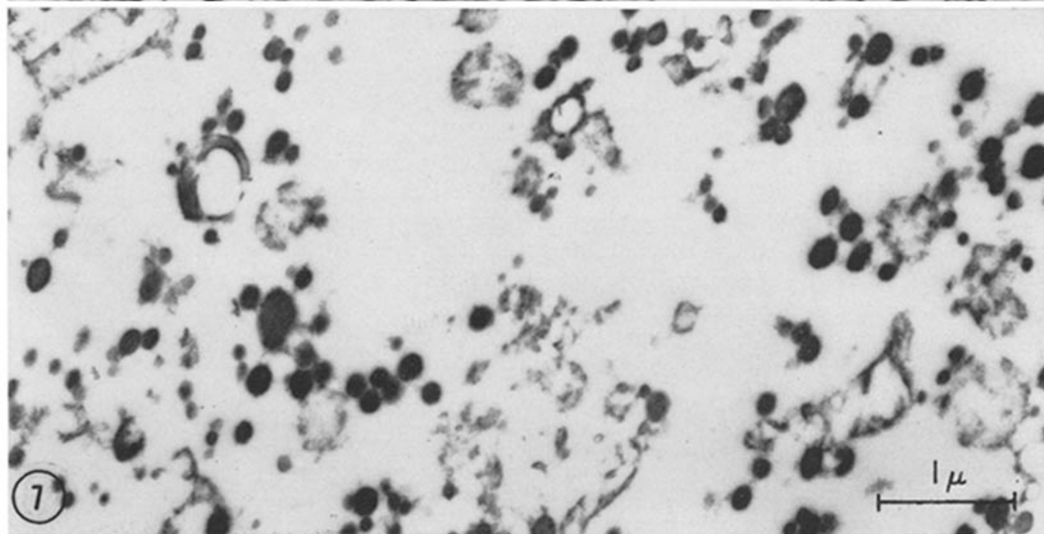
The pellet (S_2E_1SP, Figs. 8 and 9) consists of secretory granules with some fine contaminating membranous material. The percentage distributions of the diameters of sectioned secretory granules from the isolated pellet, the intact thyrotropes and gonadotrope are shown in Fig. 10. The diameters of the TSH granules are unimodally distributed over a range of 40 to 160 mμ with a maximum at 80 mμ. The diameters of gonadotropin granules also appear to be unimodally distributed, having a range of 40 to 180 mμ with a maximum at 120 mμ. The diameters of the granules in the pellet (S_2E_1SP) are bimodally distributed with maxima at 80 and 120 mμ and a range of 40 to 190 mμ. These results, which indicate that two distinct populations of secretory granules are present in the isolated fraction, agree with the assays for TSH and gonadotropin discussed in the next section.

Hormonal Activities of the Pituitary Fractions

GONADOTROPINS (FSH AND LH): Gonadotropic hormone assays in normal female rats indicated that small amounts of activity were associated with the nuclear and LG fractions, and


5
1μ


6
1μ


7
1μ

a negligible amount with the filters (Table II). The major portion of the gonadotropin of the whole homogenate (WH), about 70 per cent, was found in the filtered S_2. The gonadotropic activity was distributed among the layers S_2A through S_2E_2 after centrifugation of the filtered S_2 on the discontinuous gradient, but the greater part of it was found in the S_2E_1 layer. The fractions S_2A through S_2D and the S_2E_2 contained little activity. The hormone in the S_2A layer represents that solubilized during fractionation as well as that in a soluble form in the intact gland.

The gonadotropic activity of the above fractions was also determined in hypophysectomized male and female rats. The major part of the activity was found in the S_2E_1SP when assayed in the female (Table II). The assays carried out in the male, however, gave variable results. The increase in the weights of the testes, an indicator of FSH, showed activity not only in the S_2E_1SP but also in the LG, S_2A, and S_2D fractions. The increase in weight of the ventral prostate (VP) and the seminal vesicles (SV) indicated the presence of luteinizing hormone in the NF, LG, S_2A, S_2D, and the S_2E_1 fractions. It is likely that the LH activity found in these fractions reflects contamination with particulates from pituitary glands of animals with a high level of LH.

GROWTH HORMONE (GH): The fractions shown in Table III were assayed for growth hormone by the increase in the width of the epiphyseal plate. The active S_2E_1SP contained a small amount of growth hormone as did some of the other fractions, especially the S_2A. The presence of large amounts of this hormone in the soluble fractions reported previously by Ziegler and Melchior (13) and by Reid and Segaloff (15) may be due to the hormone's being in the soluble form in the intact gland and/or to its solubilization during fractionation.

LACTOGENIC HORMONE: Lactogenic hormone activity was found to be low in the S_2E_1SP (Table III) whereas in the S_2E_2SP it was substantial in amount.

THYROTROPIC HORMONE (TSH): The TSH activity, in milliunits, was determined in the WH, S_2D and the S_2E_1SP (Table III). The S_2E_1SP contained approximately one-third and the S_2D about one-fifth of the TSH activity found in the WH. Brown *et al.* (19) have reported that TSH is associated with the mitochondria. This observation is not supported since the active S_2E_1SP containing TSH has been assayed for the presence of succinic dehydrogenase and none was found. In view of this, it is more likely that TSH is associated with the secretory granules, an opinion which is supported by the observation that thyroidectomy in the rat causes hyperplasia of pituitary basophils containing secretory granules 140 mμ in diameter (3).

Biochemical Properties of the Pituitary Fractions

PROTEIN. The values for the protein content of the various fractions (Table IV) agree

FIGURE 5

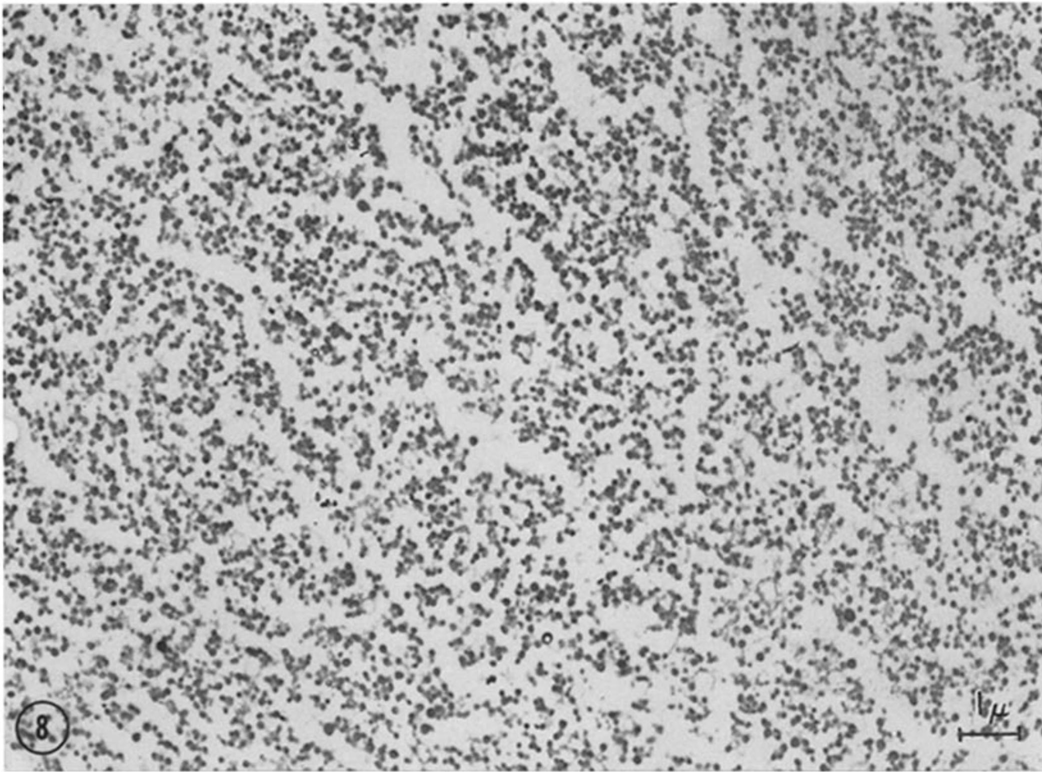
An electron micrograph of a section from the S_2C pellet obtained by removing the S_2C layer from the sucrose-Diodrast-versene discontinuous gradient and centrifuging it at 100,000 *g* for 60 minutes. This layer contains mainly membranes, tentatively identified as microsomes, and a few small granules. $\times$ 12,000.

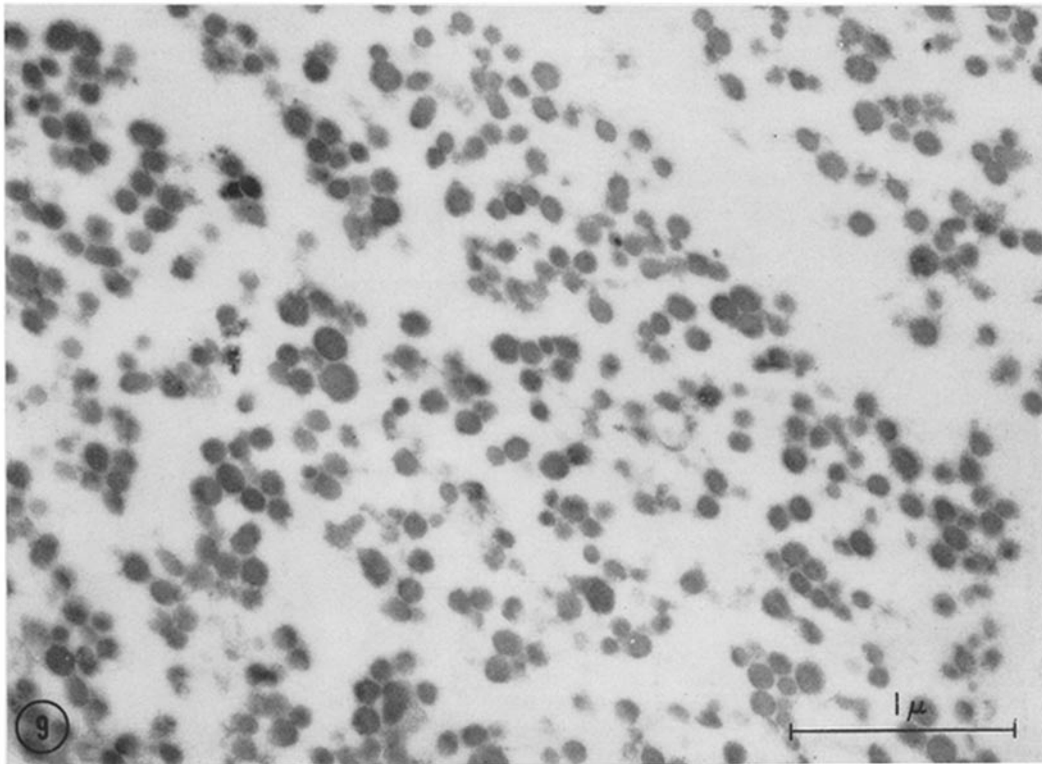
FIGURE 6

An electron micrograph of a section from the pellet obtained from the S_2D layer. Basophilic secretory granules, the largest having a diameter of 170 mμ, and membranous material are found in this fraction. $\times$ 18,000.

FIGURE 7

A section of the pellet (S_2E_2SP) from the S_2E_2 layer showing secretory granules, the largest an acidophil having a diameter of 460 mμ, and particulates suggestive of mitochondria. $\times$ 18,000.


8
1μ


9
1μ

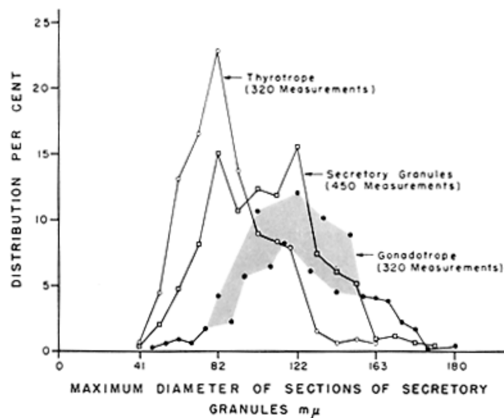


FIGURE 10

The diameter distribution of secretory granule sections from randomly selected areas of a thyrotrope, gonadotrope, and the isolated secretory granule pellet S_2E_1SP. The distribution curve for the sectioned gonadotrope granules lies within the stippled area with granules of 122 mμ in diameter being most frequently observed.

with results reported by other workers (11, 13). The protein value obtained for the active granule fraction (S_2E_1SP), although small, is a maximum one since this pellet contains Diodrast which reacts with the biuret reagent.

GLUCOSE-6-PHOSPHATASE: The activity of glucose-6-phosphatase, known to be associated exclusively with microsomal material (38), was determined on certain of the fractions (Table IV). The presence of a small amount of this enzyme in the active S_2E_1SP indicated slight contamination of this fraction with microsomal material.

SUCCINIC DEHYDROGENASE: The S_2E_1SP was free of enzyme activity (Table IV), indicating the purity of this fraction with respect to mitochondrial contamination since Hogeboom *et al.* (7) showed that succinic dehydrogenase is associated with the mitochondria. The effectiveness of microfiltration with Millipore filters as compared to differential centrifugation for the removal of mitochondria from fraction S_1 was demonstrated in this study.

PHOSPHATASE: The sum of the acid and alkaline phosphatase activities found in the pituitary fractions (Table V) was essentially the same as that found in the starting homogenate (WH). Alkaline phosphatase was not found in the S_2E_1SP but there was present a slight amount of acid phosphatase. The latter might have been caused by contamination with lysosome-like material since acid phosphatase has been shown by de Duve *et al.* (39) to be associated with this cytoplasmic particulate. Kuff and Dalton (40) also found this enzyme in the Golgi complex of the rat epididymes. Sobel (41) has shown by histochemical methods that there is an increase in acid phosphatase in stimulated rat pituitary thyrotropes. He concluded that this, too, appeared to be located in the Golgi complex. It is more likely, therefore, that the enzyme activity found in S_2E_1SP is not that of lysosomal contamination but rather that of the Golgi complex.

PROTEASES: The protease activities of the fractions were determined at pH 3.8 and 8.3 (Table V). The activity of the soluble S_2A could not be assayed because of the presence of substantial amounts of Diodrast which absorbs at 278 mμ. Luck (42) found that Diodrast inhibits liver transferase, but it did not influence the activity of the enzymes reported in this study. Acid protease was present in all the fractions except the S_2E_1SP. This lack of activity substantiates the opinion that the S_2E_1SP is not contaminated with lysosomes since de Duve *et al.* (39) found an acid pH-dependent protease with these cytoplasmic particulates.

Alkaline protease was present in the S_2E_1SP and is the only enzyme found in this fraction in appreciable amounts (Table V). This suggests that the alkaline protease is either associated with the secretory granules or is adsorbed on them during their preparation. If the former is the case, this protease may play a role in hormone

FIGURES 8 AND 9

Electron micrographs of sections through the pellet (S_2E_1SP) obtained from the discontinuous gradient. Present in this fraction are small secretory granules representing two distinct granule populations (TSH and gonadotropin) and some fine membranous contamination. Fig. 8, × 8,000. Fig. 9, × 30,000.

TABLE III

Growth, Lactogenic, and Thyrotropic Hormone Activities of Rat Pituitary Fractions

Fraction	Dose mg eq FT	Hormone: Growth Epiphyseal plate μ	% above control	Lactogenic Crop gland rating 0 to 4	Thyrotropic milliunits
Control	Saline	217 ± 10 (41)			0.2
WH	2.5	385 ± 9 (33)	77		23 ± 2.0
	5.0	420 ± 12 (25)	93		
	10.0	381 ± 11 (21)	76	1.3 (3)	
	20.0	398 ± 11 (33)	83	1.8 (4)	
NF	20.0	314 ± 11 (15)	45		
LG	20.0	311 ± 14 (24)	43		
S_2A	20.0	346 ± 12 (22)	59	0.2 (3)	
S_2C	20.0			0.7 (3)	
S_2D	2.5				4.5 ± 0.5
S_2E_1SP	2.5				7.2 ± 1.5
	20.0	284 ± 8 (34)	31	0.3 (3)	
S_2E_1SS	20.0	270 ± 13 (21)	24		
S_2E_2SP	20.0	310 ± 15 (14)	43	1.5 (2)	
S_2E_2SS	20.0	346 (3)	59		

TABLE IV

Protein Content and Glucose-6-Phosphatase and Succinic Dehydrogenase Activities of Rat Pituitary Fractions

Fraction	Protein mg/ml	Glucose-6-phosphatase μg P/min/ml	Succinic dehydrogenase μmole succinate oxidized/min/ml
WH	8.5 ± 0.3 (6)	2.4 ± 0.1 (3)	0.25 ± .01 (6)
NF	3.7 ± 0.2 (7)	0.2 ± 0.2 (3)	0.02 (4)
S_1	5.5 (1)		0.20 (2)
FS_1	5.0 (1)		0.14 (2)
LG	0.5 ± 0.2 (6)	0.2 ± 0.1 (3)	0.01 (3)
S_2	4.8 (1)		0.12 (2)
FS_2	3.9 ± 0.5 (5)	1.1 ± 0.1 (3)	0.12 (5)
S_2A	2.3 ± 0.3 (4)	0.1 ± 0.1 (3)	0.07 (3)
S_2E_1SP	0.2 ± 0.1 (3)	0.1 ± 0.0 (3)	0.0 (4)

release or secretion in a manner analogous to that reported by Litonjua (48) for thyroid hormones. He has recently confirmed the existence of a protease bound intimately to thyroglobin which can autolytically degrade this molecule to release smaller peptide fragments. These may then be acted on by peptidases to liberate the thyroid hormones.

The presence of proteases in pituitary tissue was first reported by Adams and Smith in 1951 (43). Later, Meyer and Clifton (30) found that alkaline protease activity increased 30 per cent in the pituitaries of rats treated with diethylstilbestrol which causes protein synthesis and hypersecretion of prolactin. The acid protease activity did not increase. Recently LaBella and Brown (18) and Brown *et al.* (19), using hog and cow pituitary glands, found that alkaline protease was confined principally to the microsomal and supernatant fractions. These fractions also contained significant amounts of FSH activity.

GENERAL CONCLUSIONS

The position of the secretory granules in the general plan of synthesis, storage, and secretion of the pituitary hormones was proposed by Far-

quhar and Wellings, and by Farquhar (4, 44, 45). The purpose of this investigation was to isolate those secretory granules with which gonadotropic hormone has been shown to be associated (2, 6, 21) and to study their biological and biochemical properties.

In previous isolations old breeder castrate rats were used as the source of pituitary tissue (6, 9, 10, 21). This study indicates that pituitaries from young adult males contain a higher level of gonadotropin following castration than do those of old breeder castrates. The preparation of the S_2 fraction from the pituitaries of the young males is shorter but does not differ markedly from the method used previously (6, 9, 10, 21). Microfiltration, however, was used more extensively than in the earlier studies (6, 21, 46), and this aids in the purification of the granules as indicated by the enzyme and protein results.

The procedure consisted of layering the filtered S_2 (FS_2) fraction on a discontinuous gradient and centrifuging to obtain the S_2E_1 layer. When this layer was centrifuged at 100,000 *g* a secretory granule fraction S_2E_1SP was obtained which contained predominantly gonadotropin and about one-third of the TSH activity of the original homogenate. Only negligible amounts of the other pituitary hormones were present. The electron micrographs and the results of the enzyme studies, particularly the absence of glucose-6-phosphatase, succinic dehydrogenase, and the phosphatases, support the conclusion that the active pellet of granules is of a high degree of purity.

The method for obtaining these granules is simple and takes a relatively short time. The critical part of the procedure is the length of time and speed of centrifugation necessary to obtain the proper separation of the particulates into the pattern shown in Fig. 1.

The presence of alkaline protease with the secretory granules suggests that it may be involved in the release or secretion of the hormones associated with these granules. This has also been suggested earlier by Adams and Smith (43), Meyer and Clifton (30) and Ellis (47).

TABLE V
Phosphatase and Protease Activities of Rat Pituitary Fractions

Fraction	Phosphatase μg P/min/ml*		Protease μg T/min/ml	
	pH 4.6	pH 9.3	pH 3.8	pH 8.3
WH	8.7 ± 0.4 (5)	4.7 ± 0.8 (3)	16.6 ± 1.6 (4)	7.4 ± 1.5 (6)
NF	2.2 ± 0.3 (5)	2.8 ± 0.9 (4)	7.7 ± 2.8 (3)	3.8 ± 0.2 (6)
LG	1.9 ± 0.3 (5)	0.0 (3)	1.9 ± 0.7 (4)	0.7 ± 0.2 (6)
FS_2	3.8 ± 0.2 (5)	1.4 ± 0.5 (3)	6.6 ± 2.4 (4)	3.6 ± 0.9 (5)
S_2A	1.4 (1)	0.6 ± 0.3 (4)		
S_2E_1SP	0.3 ± 0.1 (4)	0.0 (3)	0.0 (3)	1.6 ± 0.3 (6)

* μg/min/ml is based on a ml of the fraction resuspended to its original volume.

This investigation was supported by research grant G9895 from the National Science Foundation and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

Received for publication, June 6, 1962.

REFERENCES

1. Rinehart, J. F., and Farquhar, M. G., *J. Histochem. and Cytochem.*, 1953, **1**, 93.
2. Farquhar, M. G., and Rinehart, J. F., *Endocrinology*, 1954, **54**, 516.
3. Farquhar, M. G., and Rinehart, J. F., *Endocrinology*, 1954, **55**, 857.
4. Farquhar, M. G., and Wellings, S. R., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 319.
5. Hedinger, C. E., and Farquhar, M. G., *Schweiz. Z. allg. Path.*, 1957, **20**, 766.
6. Hartley, M. W., Ph.D. Thesis, University of Wisconsin, 1959.

7. Hogeboom, G. H., Schneider, W. C., and Palade, G. E., *J. Biol. Chem.*, 1948, **172**, 619.
8. Catchpole, H. R., *Fed. Proc.*, 1948, **7**, 19.
9. McShan, W. H., and Meyer, R. K., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 407.
10. McShan, W. H., and Meyer, R. K., *Endocrinology*, 1952, **50**, 294.
11. McShan, W. H., Rozich, R., and Meyer, R. K., *Endocrinology*, 1953, **52**, 215.
12. Herlant, M., *Ann. Endocrinol.*, 1952, **13**, 611.
13. Ziegler, D. M., and Melchior, J. B., *J. Biol. Chem.*, 1956, **222**, 721.
14. Ziegler, D. M., and Melchior, J. B., *J. Biol. Chem.*, 1956, **222**, 731.
15. Reid, E., and Segaloff, A., *Proc. Soc. Exp. Biol. and Med.*, 1958, **97**, 187.
16. Brown, J. H. U., and Hess, M., *Am. J. Physiol.*, 1957, **188**, 25.
17. LaBella, F. S., and Brown, J. H. U., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 833.
18. LaBella, F. S., and Brown, J. H. U., *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 17.
19. Brown, J. H. U., LaBella, F. S., and Ulvedal, F., *Endocrinology*, 1960, **66**, 1.
20. Brown, J. H. U., and Ulvedal, F., *Endocrinology*, 1960, **66**, 175.
21. Hartley, M. W., McShan, W. H., and Ris, H., *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 209.
22. Hymer, W. C., Master's Thesis, University of Wisconsin, 1959.
23. Greenspan, F. S., Li, C. H., Simpson, M. E., and Evans, H. M., *Endocrinology*, 1949, **45**, 455.
24. Lyons, W. R., and Page, E., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1049.
25. Breitenbach, R. P., and Meyer, R. K., *Proc. Soc. Exp. Biol. and Med.*, 1959, **101**, 16.
26. McKenzie, J. M., *Endocrinology*, 1958, **63**, 372.
27. Schuetz, A. W., Strauss, W. F., and Meyer, R. K., to be published.
28. Gornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.*, 1949, **177**, 751.
29. Anson, M. L., *J. Gen. Physiol.*, 1938, **22**, 79.
30. Meyer, R. K., and Clifton, K. H., *Arch. Biochem. and Biophysics*, 1956, **62**, 198.
31. Derechin, M., *Biochem. J.*, 1961, **78**, 443.
32. Dryer, R. L., Tammes, A. R., and Routh, J. I., *J. Biol. Chem.*, 1957, **225**, 177.
33. Swanson, M. A., *in* Methods in Enzymology, (S. P. Colowick and N. O. Kaplan, editors), New York, Academic Press, Inc., 1955, **2**, 541.
34. Ziegler, D. M., *Am. J. Clin. Nut.*, 1961, 9, Part II, 43.
35. Palade, G. E., and Siekevitz, P., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 171.
36. Luft, J. H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
37. Lawn, A. M., *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 197.
38. Hers, H. G., Berthet, J., Berthet, L., and de Duve, C., *Bull. Soc. chim. biol.*, 1951, **32**, 21.
39. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelman, F., *Biochem. J.*, 1955, **60**, 604.
40. Kuff, E. L., and Dalton, A. J., *in* Subcellular Particles, (T. Hayashi, editor), New York, The Ronald Press Co., 1959, 114.
41. Sobel, H. J., *Endocrinology*, 1961, **68**, 801.
42. Luck, D. J. L., *J. Biophysic. and Biochem. Cytol.*, 1961, **10**, 195.
43. Adams, E., and Smith, E. L., *J. Biol. Chem.*, 1951, **191**, 651.
44. Farquhar, M. G., *Angiology*, 1961, **12**, 270.
45. Farquhar, M. G., *Tr. New York Acad. Sc.*, 1961, series II, **23**, 346.
46. Jordon, W. K., and Darwin, J., *Experientia*, 1960, **16**, 167.
47. Ellis, S., *J. Biol. Chem.*, 1960, **235**, 1694.
48. Litonjua, A. D., *Endocrinology*, 1960, **67**, 829.