Cell Fractionation of Anterior Pituitary Glands from Beef and Pig*,‡

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PLATE 2

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ABSTRACT

Fresh anterior pituitary glands from beef and pig were separated by differential centrifugation into subcellular fractions. Nuclei and debris were obtained at 700 *g* for 15 minutes, secretory granules at 7000 *g* for 20 minutes, mitochondria at 34,000 *g* for 15 minutes, and microsomes at 78,000 *g* for 3 hours. Electron micrographs were taken of the individual fractions. Each fraction was analyzed for nitrogen, pentosenucleic acid (PNA), and phospholipide. Beef and pig anterior lobes were quite similar in their intracellular composition as seen in the subcellular fractions. Succinic dehydrogenase was localized in mitochondria, while alkaline phosphatase was concentrated in the microsomes. A proteinase with pH optimum at 8.2 was exclusively localized in microsomal and supernatant fractions. Acid phosphatase, acid ribonuclease, and acid proteinase were distributed among the subcellular fractions in another pattern, indicating the presence of a particle type distinct from mitochondria and microsomes. The distribution of cytoplasmic PNA paralleled that of alkaline phosphatase.

Cell fractionation of the anterior pituitary gland by differential centrifugation has been undertaken in this laboratory for the primary purpose of correlating hormonal activities with specific subcellular particles. The results of hormonal distribution will be presented elsewhere. This report deals with the morphological, chemical, and enzymatic criteria which served as a basis for separation of subcellular elements. In the few earlier studies concerned with this particular problem, cell components were arbitrarily separated into sedimentation classes with no regard to morphology (17, 30), on one hand, while others (4), who attempted to separate the components into morphologically homogeneous groups, were generally unsuccessful. Recently, Reid and Segaloff (21) have reported on the fractionation of rat pituitary, but give no data concerning their criteria for separation.

In the present investigation, preparations of secretory granules, mitochondria, and microsomes were obtained. Their homogeneity was checked by electron microscopy and their chemical and enzymatic properties studied by appropriate procedures.

Materials and Methods

Obtaining Glands.—Pituitaries were removed from the animals within 20 minutes after sacrifice in the case of pig glands[1] and within 40 minutes in the case of beef.[2] The glands were placed in glass vessels contained in ice, but at no time were the pituitaries allowed to freeze or to come in contact with the ice. After removing the connective tissue capsule, the anterior portion was freed of the other lobes.

Preparation of Cell Fractions.—The entire procedure was carried out at 2 to 4°C. All equipment and solutions were precooled to that temperature. Homogenization

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[1] Pig pituitaries were obtained from the White Provision Co., Atlanta, Georgia.

[2] Beef pituitaries were obtained from Armour and Co., Atlanta, Georgia.

of the tissue was performed within an hour and a half after the first gland was obtained. The tissue grinder used consisted of a glass grinding chamber and a fitted, motor-driven Teflon pestle. A 10 per cent (*w/v*) homogenate was prepared in 0.25 M sucrose containing 0.02 per cent heparin (Upjohn). Ten to 20 grams of anterior lobe were processed at one time.

1. Nuclei and Debris.—The homogenate was placed in 50 ml. plastic tubes and centrifuged at 700 *g* for 15 minutes in an International centrifuge, model QV-110. The final pellet, which was red or reddish-blue in color, was suspended in sucrose and stored in the cold. In all experiments, this first pellet occurred as a gel in which many cell components other than nuclei were trapped. Repeated homogenizations and centrifugations were necessary to obtain clear washings. Other media are being examined for their ability to prevent gel formation. Dounce (10) also obtained nuclear gels in the slightly acid sucrose medium, and he associated gel formation with intact mitochondria and suggested that ruptured mitochondria released an enzyme which prevents gel formation. Our findings disagree with this theory, since gel formation occurred regardless of the duration of homogenization. One centrifugation was performed with the following additives to the sucrose medium: 0.02 per cent heparin, 0.001 M ethylene diamine tetra-acetic acid, and 0.0004 M adenosinetriphosphate. Although a non-gel nuclear pellet resulted, the lighter cytoplasmic residue unexplainably could not be resolved into its components.

2. Secretory Granules.—The supernatant from fraction 1 was centrifuged at 7000 *g* for 20 minutes in a Spinco model L ultracentrifuge using plastic tubes to fit the No. 30 head. The material that collected at the bottom of the tubes consisted of a firm white pellet and an overlying tan colored, "fluffy" layer. The pellet and fluff were each suspended and centrifuged at 8000 *g* for 15 minutes; the supernatant was also centrifuged at 8000 *g*. From the recentrifuged white layer some fluffy material was separated, while from the recentrifuged fluffy layer a small, firm, white pellet was obtained. The pellets were pooled, as were the fluffs, and each suspended and recentrifuged at 10,000 *g* for 15 minutes. The milk-white pellets were pooled, resuspended, and stored in the cold.

3. Mitochondria.—The resuspended fluffy layer and the supernatant were each centrifuged at 34,000 *g* for 15 minutes, resulting in a firm, tan-colored pellet with an overlying fluffy layer. The firm and the fluffy material were suspended separately and each was again centrifuged at 34,000 *g* for 15 minutes. The firm pellet had a small overlying fluff layer and the tubes containing fluffy material from the previous run showed a small central pellet. In the present investigation the fluff and pellet were combined to form the mitochondrial fraction. Since the fluff contains high concentrations of microsomal elements, as indicated by pentosenucleic acid determinations, purer preparations of mitochondria could be obtained in the firm pellet if the fluff were kept separate. We have reported elsewhere (16) on the biochemical differences between the *heavy* and *light mitochondria*, as we have designated the pellet and fluff, respectively.

4. Microsomes.—The supernatant from fraction 3 was centrifuged at 78,000 *g* for 3 hours. The pellet produced by this centrifugation was a translucent red gel. Contaminating mitochondria, if present, tended to form a central zone which could easily be removed and added to the main mitochondrial fraction.

5. Supernatant.—The final supernatant was a perfectly clear, reddish solution.

In order to maintain a "balance sheet" of total enzyme activities and of total substances analyzed in the various fractions, all washings from a given pellet were added to the main supernatant. At the end of the separation procedure, the particulate fractions and the supernatant represented essentially 100 per cent of the starting material.

The visual appearance of the pellets obtained from anterior pituitary by centrifugation has been very useful in separating the various fractions. The white secretory granules sediment between the nuclei and the mitochondria; they tend to form a central pellet in the adjacent fractions and can readily be separated from them. The mitochondria occur as a dark-tan pellet and can be easily distinguished from the contaminating secretory granule layer. Similarly, the mitochondrial contamination in the microsome fraction is apparent, since the former occur as a dull, opaque region in the otherwise, clear, translucent pellet. By employing visual appearance alone, the composition of the various fractions can be consistently duplicated even if the centrifugal forces and times of centrifugation are varied.

Morphological Examination of Cell Fractions.—A few drops of the suspended material of each particulate fraction were mixed in 5 ml. of the buffered 1 per cent osmium tetroxide solution containing sucrose described by Caulfield (6) and fixed for 1 hour at 2–4°C. The amount of suspension taken for fixation was adjusted so that the pellet formed upon centrifugation was a very thin film. Several sections were cut from different loci on the pellet, and the appearance of the greatest number of similar sections was assumed to be most representative of the whole pellet. Increasing pellet thicknesses required disproportionately more sections in order to determine the typical particle population in a given fraction. The tube containing the fixed suspension was then filled with the sucrose medium and centrifuged in a Spinco No. 40 head at 110,000 *g* for 15 minutes. The fixative solution was poured off; the black film was freed from the bottom of the tube with a spatula, dehydrated successively with 50, 70, 95, and 100 per cent alcohols in the tube, and embedded in *n*-butyl methacrylate. Sections were taken from several

parts of the thin pellet for electron microscopical observation with a Philips instrument, model EM-100 A.

Chemical Analyses.—Aliquots of the suspension of cell fractions were separated into acid-soluble components, lipide-soluble fraction, deoxyribose nucleic acid (DNA), and pentose nucleic acid (PNA) according to the method of Schneider (23). Phosphorus was determined on the lipide extract according to Dryer *et al.* (11). Values for phospholipide were obtained by applying a factor of 26 to the values obtained for lipide phosphorus. PNA was determined by the orcinol reaction for pentose (3); ribose values were taken as 38 per cent of the weight of PNA. DNA determinations (9) were not run routinely, since a few analyses showed this material to be present exclusively in the nuclei and debris fraction. Nitrogen was determined by nesslerization (15). All chemical determinations were performed in triplicate.

Enzyme Assays.—It has been observed for most of the enzymes examined in the present investigation that maximum activity could not be obtained with fresh, untreated subcellular preparations contained in isotonic sucrose. Freezing and thawing or aging releases latent enzymic activity of anterior pituitary fractions, as reported for cell fractions from liver (8). Cell fractions from pituitary were routinely frozen before assaying for enzymatic activities.

1. Succinic dehydrogenase was assayed by the manometric method of Schneider and Potter (26) except that adenosinemonophosphate (0.012 M) and adenosinetriphosphate (0.012 M) were added to the reaction medium.

2. Alkaline phosphatase. The incubation medium contained 1.0 ml. of 0.1 M boric acid-KCl-NaOH buffer at pH 9.8, 0.5 ml, of 0.2 M beta-glycerophosphate (99.9 per cent minimum, Eastman), and tissue suspension and water to 2.0 ml. The reaction mixture was placed in a 12 × 125 mm. test tube and incubated at 37°C. for 10 minutes with occasional shaking. The reaction was stopped by the addition of 2.0 ml. of 10 per cent trichloroacetic acid (TCA). The tube was centrifuged and inorganic phosphorus was determined on 2.0 ml. of the supernatant by the method of Dryer *et al.* (11). Glycerophosphate inhibited color development in the determination of phosphorus. This could be prevented by decreasing the concentration of the substrate in the reaction medium or by determining inorganic phosphorus by the addition of 1 ml. of 5 N sulfuric acid and 1 ml. of 0.025 M ammonium molybdate to the TCA extract. The addition of these reagents, which are employed by Dryer to determine total phosphorus in digested samples, prevents the glycerophosphate inhibitory effect on color development.

3. Acid phosphatase assay differed from that of alkaline phosphatase in that the buffer consisted of 0.1 M acetic acid–sodium acetate at pH 4.5.

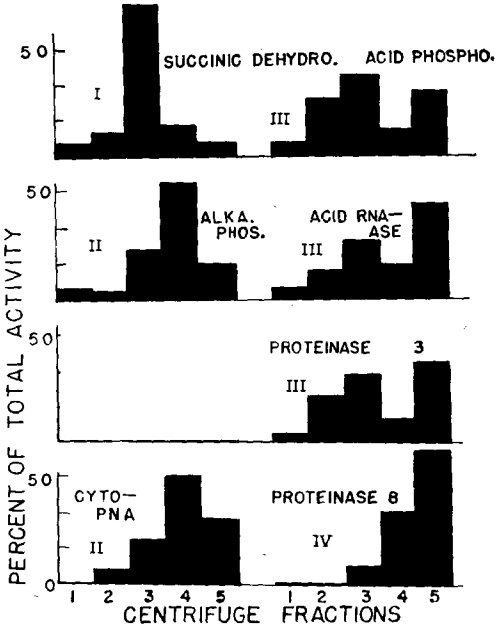
4. Ribonuclease was determined as described by de Duve *et al.* (8).

5. Proteinases were determined by the method of Adams and Smith (1).

For all fractions the aliquots used in the enzyme assays contained approximately the same amount of nitrogen. Appropriate blanks were run in each case and determinations were performed in triplicate.

RESULTS

Morphological Examination.—In Figs. 1 to 5 electron micrographs are presented of secretory granule, mitochondrial, and microsomal fractions from pig pituitary. The fractions from beef glands showed no noticeable morphological differences in electron micrographs. No micrographs of the nuclear fraction were obtained, since the sections prepared from the fixed pellet shattered consistently. The morphological integrity of the mitochondria, in particular, is considerably altered, and it is possible that these changes are due both to separation and fixation procedures. We have not examined sections of intact pig pituitary in the electron microscope, but micrographs of beef



TEXT-FIG. 1. The distribution of several enzymes and PNA among five subcellular fractions from pig anterior pituitary. The graphs are plotted from the averages obtained from 4 to 7 determinations. Numbers 1 to 5 along the abscissa represent nuclei and debris, secretory granules, mitochondria, microsomes, and supernatant, respectively. The Roman numerals refer to the specific type of distribution pattern among the various fractions.

TABLE I

Distribution of Nitrogen, PNA, and Phospholipide among Subcellular Fractions

	Nitrogen		PNA		Phospholipide	
	Beef	Pig	Beef	Pig	Beef	Pig
	per cent of total					
Nuclei and debris	11.6 ± 2.9	8.3 ± 2.2	15.8 ± 4.1	11.2 ± 2.4	4.7 ± 2.7	4.9 ± 1.7
Secretory granules	20.6 ± 3.2	13.1 ± 2.4	4.5 ± 2.2	3.8 ± 1.2	20.4 ± 3.8	13.8 ± 3.6
Mitochondria	13.0 ± 3.6	11.1 ± 3.6	15.6 ± 2.2	16.7 ± 6.7	28.1 ± 3.9	24.7 ± 7.8
Microsomes	11.2 ± 1.8	9.7 ± 1.2	38.9 ± 3.5	36.7 ± 5.4	30.8 ± 6.1	32.5 ± 6.1
Supernatant	46.5 ± 3.0	51.5 ± 1.5	24.2 ± 2.2	28.2 ± 4.9	14.6 ± 4.3	9.0 ± 4.5

Averages and standard deviations represent 4 to 7 determinations.

glands from earlier work by one of us (Brown, 1957) depict morphological characteristics which are very similar to those presented by Rinehart and Farquhar (22) for rat anterior pituitary gland. The oval, electron-dense elements in the secretory granule fraction appear to be almost exclusively in the size range of the acidophilic granules seen in tissue sections. There are a few granules in this fraction, and occasionally in others, which may be suspected of originating from basophiles. The rarity with which secretory granules in the size range generally encountered in basophile cells appear in the subcellular fractions has led us to believe that the basophilic granules dissolve when the gland is homogenized.

Enzyme Distribution.—The several enzymes studied in anterior pituitary demonstrate four patterns of distribution among the subcellular fractions (Text-fig. 1). Succinic dehydrogenase is localized in the mitochondria while alkaline phosphatase is associated with the microsomal fraction, these enzymes occupying similar loci in liver cells (14, 18). Acid phosphatase, acid ribonuclease, and acid proteinase (proteinase 3) are distributed in a pattern which is distinct from either of the two previously mentioned patterns and are spread over several fractions. Alkaline proteinase (proteinase 8) represents still another distribution pattern and is found almost exclusively in microsomal and supernatant fractions.

With the exception of succinic dehydrogenase, all the enzymes show high activity (usually 25 to 35 per cent of the total) in the supernatant. The activity in the soluble fraction is, no doubt, derived in part fron lysis of some of the particles with subsequent loss of contents. The small constant amount of each enzyme in the nuclear fraction may be a direct reflection of the number of unbroken cells which are present.

TABLE II

Absolute Values for Nitrogen, PNA, and Phospholipide in Subcellular Fractions

	Nitrogen		PNA		Phospholipide	
	Mg.	*per cent*	*Mg.*	*per cent*	*Mg.*	*per cent*
Homogenate	133		30.8		239	
Nuclei and debris	9.4	7.1	3.06	11.3	12.6	5.6
Secretory granules	18.1	13.8	1.16	4.3	33.3	14.7
Mitochondria	21.5	16.4	5.64	21.0	78.0	34.3
Microsomes	14.5	11.1	10.0	37.4	67.6	29.8
Supernatant	67.7	51.6	6.90	25.8	35.4	15.6
	131.2		26.76		226.9	
Recovery per cent	98.4		87.2		95.0	

Absolute values are presented for a single experiment in which 12.8 grams (wet weight) of fresh, pig anterior pituitary gland were employed.

The percentage distribution was calculated on the basis of the total recoverable nitrogen, PNA, or phospholipide, respectively.

Chemical Composition of Fractions.—The distribution of nitrogen, PNA, and phospholipide among the various cell fractions is presented in Tables I, II, and III. Beef and pig glands appear to be quite similar with the exception that the secretory granules make up a smaller proportion of the cell contents in the latter species, as seen in the low values for this fraction.

Cytoplasmic PNA is graphically depicted in Text-fig. 1 along with the distribution pattern of several enzymes in order to point out that the concentration of PNA in the various fractions parallels alkaline phosphatase activity. Since alkaline phosphatase is in highest concentration in the microsomal fraction and has been shown to be a microsomal constituent in liver (18), it seems likely that the presence of PNA in mitochondria and

TABLE III

The Concentration of PNA and Phospholipide in Subcellular Fractions

	PNA		Phospholipide	
	Beef	Pig	Beef	Pig
	μgm./mg. N			
Nuclei and debris	204 (200–210)	348 (239–497)	729 (510–1020)	951 (735–1340)
Secretory granules	42 (14–72)	51 (43–64)	1590 (1100–1990)	1435 (974–1840)
Mitochondria	243 (220–256)	214 (168–264)	3030 (2350–3660)	3420 (2600–4330)
Microsomes	630 (566–680)	704 (597–850)	4133 (3490–4670)	4900 (4370–5550)
Supernatant	106 (75–144)	94 (67–114)	510 (324–733)	281 (143–522)

Averages and ranges given represent 4 to 7 determinations.

secretory granules is due to microsomal contamination. Nuclear PNA is probably contaminated with a small amount of that substance from cytoplasmic elements present in unbroken cells which sediment along with the nuclei.

The microsomes are richest in PNA and phospholipide, and the phospholipide concentration increases from secretory granule fraction to mitochondria to microsomes. The nuclear fraction is very poor in phospholipide. The per cent lipide of the dry weight of each of several fractions was determined on one separation, and the results showed approximately 75 per cent of the secretory granules to be lipide, 50 per cent of the mitochondrial dry weight was lipide, and approximately 35 per cent of the microsomes. Since the secretory granules are relatively poor in phospholipide, the foregoing values indicate that these cellular constituents are very rich in non-phosphorus-containing lipides.

DISCUSSION

The present investigation has come closer to resolving the anterior pituitary gland into homogeneous subcellular fractions than any of the previous work. Most of the earlier studies were concerned simply with identifying hormonal activities with granular components of the anterior pituitary cell (5, 17, 30). Ziegler and Melchior (30) and Meyer and McShan (17) subdivided the cytoplasmic elements into large and small granules. Using the light microscope to examine his preparations, Herlant (12) attempted to isolate morphologically homogeneous fractions; he concluded erroneously that mitochondria of anterior pituitary were negligible in amount. The data of Brown and Hess (4), who also worked with beef and pig pituitaries, must be reevaluated on the basis of the findings of the present investigation, since the order in which the cell components were said to have been collected seems quite improbable. They reported that cellular fractions were obtained in the following order: nuclei, mitochondria, acidophilic granules, and basophilic granules. Pentose nucleic acid determination was the only other criterion employed by them (4) for identifying subcellular fractions. The earlier centrifugation scheme was ignored in the present study.

The centrifugal forces employed in the present investigation in segregating the subcellular elements of the anterior pituitary are considerably higher than those generally reported for other tissues. This point has been discussed in an earlier report (16).

The morphological and biochemical features of the subcellular particles obtained from anterior pituitary resemble those from other tissues. Palade and Siekevitz (19) observed in pancreas that the heavier microsomal fraction consisted mainly of circular membranous profiles with dense particles on the free surfaces of the outer membrane. The lighter microsomes, "post-microsomal" fraction, were clusters or chains of the dense granules. Similar features have been observed in fractions of anterior pituitary in the present investigation (Figs. 3 and 5). The microsomal fraction in liver was reported to contain 12 per cent of the total nitrogen, 33 per cent of the total PNA, and 22 per cent of the total phospholipide (19). These values are quite similar to those reported above for

anterior pituitary, except that the values are slightly higher in the latter tissue for both PNA and phospholipide. The composition of cellular fractions from pituitary is remarkably similar to that reported for liver by numerous workers (7, 13, 14, 24, 25).

Acid phosphatase, ribonuclease, and proteinases have been shown to be associated with the "light mitochondrial" fraction in liver (8, 18), and the existence of a distinct particle type, having sedimentation characteristics intermediate to those of mitochondria and microsomes, has been postulated on the basis of enzyme distribution studies. The name "lysosomes" has been given them because of their complement of hydrolytic enzymes (2, 8). In the present study, acid phosphatase, acid ribonuclease, and acid proteinase were found to have a distribution distinct from that of typical mitochondrial and microsomal enzymes; they are spread over several fractions, as opposed to the sharper localization of succinic dehydrogenase in mitochondria and alkaline phosphatase in microsomes. Adsorption of the individual hydrolytic enzymes by the other cellular elements must be ruled out, since it is highly unlikely that several different enzymes would be adsorbed in identical fashion. Therefore, it would appear that the hydrolases of anterior pituitary are localized in a distinct particle type which shows wide variations in size. In another report (16) we have shown that the specific activity of these "lysosomal" enzymes was greatest in the "heavy mitochondrial" fraction. Watson and Siekevitz (29) reported that succinic dehydrogenase was an insoluble enzyme associated with the membranous component of liver mitochondria. On the basis of this finding, one would not expect lysis of the mitochondria to result in a significant increase of activity of this enzyme in the soluble fraction. The relatively high percentage of the hydrolases in the supernatant suggests that the intracellular particle containing these enzymes has lysed during the procedures of homogenization and centrifugation or that the enzymes are diffusible and soluble. Both factors may be operative.

Proteinase 8 appears to be localized exclusively in microsomal and supernatant fractions. Assuming that at least part of the proteinase activity in the supernatant is due to lysis of microsomal vesicles, the percentage of activity found in the soluble fraction is much higher than observed for all the other enzymes studied. There appear to be two possibilities for the observed distribution. The proteinase may be a definite constituent of both the cell "sap" and the microsomes, or it may be associated with the lighter components of the endoplasmic reticulum, some of which may not have sedimented during centrifugation. Rademaker and Soons (20) studied the intracellular distribution of peptidases and proteinases in liver and found that certain enzymes were located only in the supernatant, others were absent from the supernatant, and still others were found in both particulate components and supernatant. In the present study acid proteinase is assumed to be localized only in particulate elements (lysosomes?). The activity of this enzyme in the supernatant is probably artifactual. Proteinase 8 appears to be a component of both particulate and soluble fractions of the cell.

The secretory granules possess properties quite similar to those seen in other intracellular granules. Siekevitz and Palade (27) found that the zymogen granules from pancreas were also obtained as a fraction between nuclei and mitochondria. It has been reported that the secretory droplets of the kidney, like the secretory granules described in the present study, contain less phospholipide than mitochondria and microsomes, are low in PNA, and demonstrate high acid phosphatase activity (28).

The fact that the basophilic granules were not detected by us following homogenization suggests that they may be soluble in sucrose solutions or that they depend upon the maintenance of intracellular integrity for their granular form. The use of other homogenizing media should answer this question. Hogeboom *et al.* (14) have also observed that secretory granules of the liver dissolved upon homogenization.

The electron microscopy in the present study was carried out by Dr. W. Duane Belt, Department of Anatomy, Emory University.

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Explanation of Plate 2

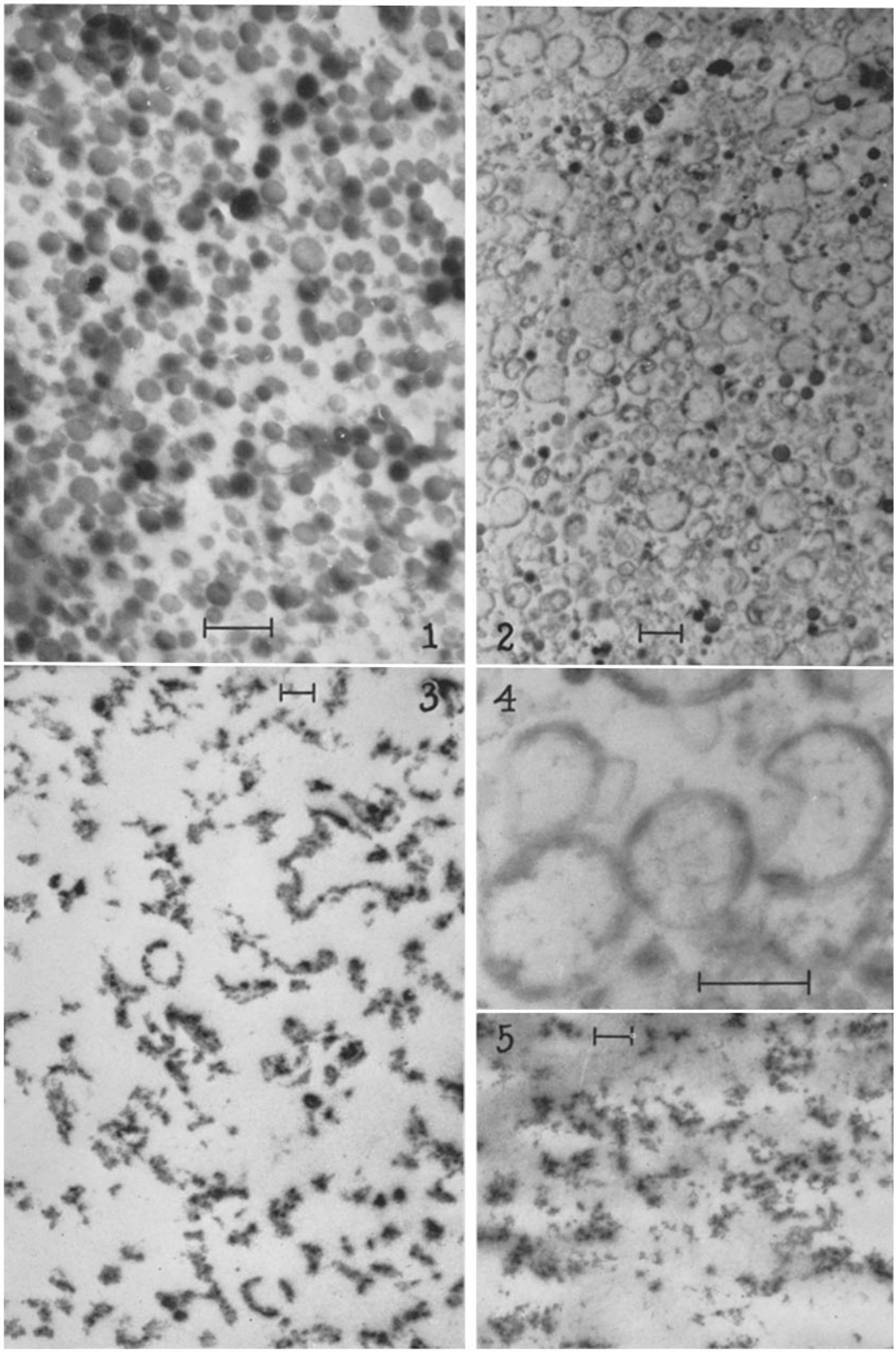
Fig. 1. Section from a pellet of secretory granule fraction. A few mitochondria are visible as well as more diffuse material. It is doubtful that the finer contaminant represents microsomes, since the PNA content of this fraction is extremely low. (Pig).

Fig. 2. Section from a pellet of the mitochondrial fraction. A few secretory granules are visible. The less distinct components of this fraction, upon closer examination, appear to be remnants of fragmented or ruptured mitochondria. (Pig).

Fig. 3. Section through a pellet of the microsomal fraction. A few circular profiles with adhering dense particles are visible. (Pig).

Fig. 4. Higher magnification of section from mitochondrial pellet shown in Fig. 2. The mitochondria are poorly preserved but do contain some dense material and show fairly distinct double membranes. (Pig).

Fig. 5. Section through a pellet of the microsomal fraction at a higher level in the pellet. The lighter PNA-containing elements are entirely in the form of clusters or chains of dense particles. (Pig).



(LaBella and Brown: Pituitary cell fractionation)