Separation of the Two Different Sizes of Storage Granules with GH or ACTH Activity from the Pellets of Acidophils Isolated from Rat Anterior Pituitaries*

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Synopsis

It was preliminarily revealed by a histogram made from the electron micrographs of acidophils on the tissue sections of anterior pituitaries in normal rats that the two different sizes of storage granules were distributed in high population in many acidophils. These two granules, small and large in size, were isolated from the pellet of isolated acidophils and separated each other by filtrations through membrane filters with various pore sizes and by discontinuous sucrose density gradient centrifugations. The other histogram made from the electron micrographs of these separated granules coincided with the above histogram. ACTH, GH and prolactin activities were bioassayed on the free granules. GH activity was found only in the large granules with diameter between 300 and 450 $m\mu$. ACTH activity was detected only in the small granules with diameter between 100 and 200 $m\mu$, though the content of ACTH contained in the isolated granules represented only a few per cent of the total ACTH content of the homogenate of anterior pituitary. No prolactin activity was found in the both kinds of granules.

These findings strongly suggested that the two kinds of granules originated from the acidophils of the rat anterior pituitaries were involved in GH and ACTH storage and the acidophils were responsible for ACTH production. The findings were also discussed related to the mode of intracellular localization of the hormones.

Efforts to determine the secreting cells of various trophic hormones have been made by many investigators who bioassayed the secreting granules isolated by ultracentrifuging method from the whole anterior pituitary materials (Herlant, 1952; Ziegler and Melcjoir, 1956; Brown and Hess, 1957; Reid and Segaloff, 1958; Brown *et al.*, 1960; Perdue and McShan, 1962). The possible storing sites of ACTH have been enumerated as follows: Acidophilic granules with 350 $m\mu$ diameter (Brown and Hess, 1957), granules with 140–200 $m\mu$ diameter (Perdue and

McShan, 1962), and microsome fraction and granules whose diameter was less than 140 $m\mu$ (Hymer and McShan, 1963). Recently Costoff and McShan (1969) noted that the granules of ACTH and TSH which could not be distinguished from each other by their size (50–180 $m\mu$ in diameter) co-existed in the same fraction of granules obtained from the anterior lobe. The materials of the whole anterior pituitary seem unsuitable for the purpose of presenting direct evidence of the ACTH secreting cells, because the free granules with the same size can be originated from the different cells. The identification of the original cells of the granules has not been, therefore, impossible by the above procedures. Ishikawa *et al.* (1971) isolated the six kinds of anterior pituitary cells by their original

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method. Upon the bioassays of ACTH, GH, prolactin and α -MSH on these cells, these authors demonstrated that ACTH was localized predominantly both in the acidophils and "acidophilic intermediate cells" but not in all the basophilic cells. For this reason, the pellets of the isolated acidophils were used in this study as the materials for separating their intracellular granules. The two different sizes of storage granules were successfully separated from the pellets of acidophils by the filtration through membrane filters with

various pore sizes and by the centrifugation on the discontinuous sucrose density gradient. Major purpose of this study was to determine the relationship between the size of free granules originated from the isolated acidophils and their specific hormonal activities.

Materials and Methods

Acidophils were isolated by the method of Ishikawa (1969) from the anterior pituitaries of 200 male adult

Fig. 1 Fractionation procedure of large granules from a pellet of isolated acidophils.

rats of Wistar-Imamichi strain. A pellet of acidophils was used for bioassay and chemical identification of ACTH.

Qualitative chemical analysis of hormones from acidophils

Qualitative analysis of hormones was carried out by the two dimensional chromatographic and electrophoretic separation on paper sheet according to the method of Jacobowitz *et al.* (1963). Two mg of the pellet of the acidophils was homogenized in 2 ml of glacial acetic acid and stocked at 4°C. A lyophilized powder was made from the supernatant and used for the specimens after being dissolved in 0.5 ml of distilled water. The specimens were applied to the origin point of sheets of Whatman No. 3 chromatography paper, which were then subjected to electrophoresis at 1,000 volts for 1.5 hr, using a pH 3.5 buffer of pyridine: acetic acid: water (1: 10: 89). The papers were dried and subjected in the second dimension to ascending chromatography, for 20 hr, using a solvent consisting of butanol: acetic acid: water (4: 1: 5). ACTH(NIH-S₈), GH(NIH-S₁₂), alpha-MSH (Sigma), prolactin (NIH-S₆), oxytocin (Sigma) and lysin vasopressin (Sigma) were used as

the reference standard in the chromatographic and electrophoretic analysis.

Isolation of granules from the acidophils

The isolation of granules was performed according to the method of Costoff and McShan (1969) with some modifications. The homogenizing medium containing 0.33 M sucrose in 0.33 M Tris (tris hydroxymethyl aminoethane) buffer was adjusted to pH 7.3. About 300 mg of the pellets of acidophils were suspended in a small volume of the buffer, and homogenized at 1,000 rpm for 2 min in a Virtis homogenizer. The homogenate was diluted by the buffer to make a 5% homogenate and rehomogenized at 1,000 rpm for 2 min at 4°C in a motor driven Teflon Pestle homogenizer. The obtained homogenate was centrifuged twice at $900 \times g$ for 10 min by model RC-2B (Sorval Co.). The supernatant (PLSs) was used as the starting material for the preparation of granules (Fig. 1).

Separation of large granules (300 to 450 μ in diameter) from PLSs (Fig. 1)

As shown in Figure 1, PLSs diluted with 20 times volume of the homogenizing medium was centrifuged at $5,000 \times g$ for 20 min. The residue (PLp1) was

Fig. 2 Fractionation procedure of small granules from a pellet of isolated acidophils.

suspended with the medium to make a 5% homogenate which was sucked by negative pressure successively through the membrane filters (Gelman Co. Ltd.) with diminishing pore size, namely 5,000, 1,200, 800 and 500 $m\mu$. The filtrate (FPL) was centrifuged twice at $14,000 \times g$ for 20 min by a Hitachi centrifuge (18 PR-type), then the pellet (PLp2) was suspended in the small volume of medium and centrifuged at $75,000 \times g$ for 90 min on the discontinuous sucrose density gradient of 50%, 55%, 57% and 65%. The substantial amount of granules was accumulated only at the interphase between 57% and 65%, and the layer containing granules (LGF) was obtained by slicing the tube with a spinco tube slicer. The layer (LGF) diluted with an approximately equal volume of 1 *M* sucrose was then passed through the membrane filter of 450 $m\mu$ pore size, and the filtrate was then passed through the filter of 300 $m\mu$ pore size. The residue on the second filter were suspended in 1.2 *M* sucrose and centrifuged at $75,000 \times g$ for 90 min on the discontinuous sucrose density gradient of 55% and 75%, and the pellet recovered from the bottom of the centrifuge tube was designated as large granules with diameter 300–450 $m\mu$.

Separation of small granules (100 to 200 $m\mu$ in diameter) from PLSs (Fig. 2).

As shown in Figure 2, the supernatant (Ss) obtained by a centrifugation of PLSs were filtered successively through the membrane (millipore) filters of 1,200, 650, 450, and 300 $m\mu$ pore size in decreasing sequence. The final filtrate (FS) was centrifuged twice at $14,000 = g$ for 20 min. The sediment (Sp) was suspended in the homogenized medium to make a 5% suspension and then centrifuged at $75,000 = g$ for 180 min on the continuous sucrose density gradient ranging from 10 to 40% (the 17.5% diodrast solution was used to make the sucrose gradient). The third layer (SGF) located three-quarters of the distance from the top of the gradient (Fig. 2) was separated, diluted by 1 *M* sucrose, and passed through a membrane filter of 200 $m\mu$ pore size. The filtrate was passed again through a filter of 100 $m\mu$ pore size, and the residue on the filter was suspended in 1 *M* sucrose and centrifuged at $105,000 \times g$ for 80 min on the discontinuous sucrose density gradient of 50 and 55%. Pellet composed of only small granules (100–200 $m\mu$ in diameter) was sedimented at the bottom of the tube.

Electron Microscopy

The portions of the pellets of granules prepared by the above mentioned procedure were in a cacodylate-buffered 2% glutaraldehyde 1.2 *M* sucrose solution at 4°C for 12 to 15 hr. The pellets were embedded in Epon 812 through the routine procedure and sectioned with Porter-Blum ultratome. The sections stained with

uranyl acetate were observed with J.E.M. type-7 electron microscopy.

Bioassay method

1) *ACTH*

The whole anterior pituitaries were homogenized 1,000 rpm for 2 min in 0.33 *M* sucrose in 0.030 *M* Tris buffered at pH 7.3. The pellets of isolated acidophils were homogenized according to the same manner. The pellets of large or small granules were suspended in 1 ml of 199-medium. 0.1 ml of the above homogenates and suspension were bioassayed *in vitro*, using $1 \times 10^7/2$ ml clonic neoplastic cells propagated vegetatively from the spontaneous adrenal tumor cells in rats (Table 2). The detailed procedure of this *in vitro* bioassay should be referred to our preceding paper (Ohtsuka *et al.*, 1971).

2) *GH*

GH was determined by the modified method of Greenspan *et al.* (1949) which is based on an increase in width of tibial epiphyseal plates in hypophysectomized juvenile rats. Each pellet of large and small granules is suspended in 10 ml of the physiological saline. This suspension was given to the 30 assay rats hypophysectomized at the 26th day after birth. Daily injection of 0.1 ml of suspension was repeated for 10 days starting from the first day after the operation. Thus, the maintenance test of GH was carried out, and the width of epiphyseal cartilage plate of the tibia was measured either upon the $AgNO_3$ -treated tibia or its histological preparations sectioned perpendicularly and stained with hematoxyline-eosin.

3) *Prolaction*

Each pellet was suspended in 10 ml of the physiological saline. The suspensions were injected repeatedly (0.1 ml/day for 10 days) into the pigeons. Assay was performed according to the clop sack method of Kanematsu and Sawyer (1963).

Results

The hormones contained in the pellets of isolated acidophils were chemically identified by two dimensional chromatographic and electrophoretic separations on paper sheets. The results of this analysis were shown in Table 1. Figure 3 showed the two-dimensional separation on the chromatographic paper of the acid extract of the acidophils in comparison with that of the authentic samples.

The presence of ACTH, GH, and prolactin in the acid extract of the acidophils was proved

Table 1. Chromatographic mobility(rf) and electrophoretic migration(Em) for spot 1,2, and 3, and for various purified standards

	rf	Em
Spot 1	0	10
Spot 2	0	7.0
Spot 3	0	5.5
ACTH	0	10
GH	0	7.0
LH	0	8.5
Alpha-MSH	0.09	10
Prolactin	0	5.5
Oxytocin	0.22	8.0
Lysin vasopressin	0.06	9.8

Fig. 3 Tracing of the ninhydrin positive spots following electrophoretic and chromatographic separation of an extract of pellet of acidophils. The dotted spots indicate the standards.

by comparing the chromatographic and electrophoretic mobilities of spots 1, 2, and with those of the authentic samples. No other pituitary hormones than the above three hormones were detected through the analysis.

Before the isolation of acidophils, many

Fig. 4 Histogram of granules established on the electron Micrographs of acidophils *in situ*, showing the relationship between their number and diameter. The total number of measured granules was 50,000 for making a histogram. Anterior pituitary tissues for making the histogram were fixed in buffered 2% osmium tetroxide at 4°C for 2 hr. The tissues were embedded in Epon 812, sectioned, and stained with uranyl-acetate.

Fig. 5 Histogram of free small and large granules from the pellets of acidophils showing the relationship between their number and diameter.

acidophils on tissue sections were examined electron microscopically. The histogram as to the size of the granules on the acidophils was made by measuring the diameters of the granules. This histogram (Fig. 4) indicated the high population of two kinds of granules with diameter of 350 m μ and of 130 m μ in the acidophils. Consequently a possibility was presented that the two different sizes of granules co-existed in a single acidophil *in situ*.

This histogram was compared with that made from the electron micrographs of the

pellet of isolated granules (Fig. 5). The two histograms coincided with each other, showing the similar pattern of distribution as to the granular size (Figs. 4 and 5). The free granules from the pellets of acidophils were distributed highly at 350 and 130 m μ in diameter. The electron micrographs of the pellets of granules (Figs. 6 and 7) showed the good separation of the granules with the negligible contamination of the different sizes granules. The results of hormone bioassays on the free granules were shown in Table 2:

Table 2. ACTH, GH, and Prolactin contents of the homogenates of whole anterior pituitaries and isolated acidophils, and of the free granules from the isolated acidophils

	diameter of granules (m μ)	wet wt. (mg)	ACTH (μ g)	GH (μ g)	Prolactin (μ g)
Homogenate of whole anterior pituitaries		10	24.6 $\pm$ 3.5*	59.4 $\pm$ 5.2*	0.31 $\pm$ 0.05*
Homogenate of isolated acidophils		2	0.49 $\pm$ 0.02	10.2 $\pm$ 0.9	0.06 $\pm$ 0.007
Free granules**	100-200		0.25 $\pm$ 0.04	N.D.	N.D.
	300-450		N.D.	8.4 $\pm$ 0.9	N.D.

* mean $\pm$ S.E.

** Segregated from 2 mg of a pellet of acidophils isolated from 10 mg of whole anterior pituitaries.

N.D. not detectable

Fig. 6 An electron micrograph of the isolated large granules, about 350 m μ in diameter, from a pellet of acidophils. Growth hormone activity was localized in these free large granules $\times$ 20,000.

Fig. 7 An electron micrograph of the isolated small granules about $130\text{ m}\mu$ in diameter, from a pellet of acidophils. ACTH activity was localized in these free small granules $\times 27,000$.

The ACTH content collected from the homogenate of 10 mg of whole anterior pituitary was about $24.6\text{ }\mu\text{g}$, while that from 2 mg of pellets of acidophils obtained was remarkably reduced to about $0.49\text{ }\mu\text{g}$. ACTH content of small granules isolated from the same volume of pellets of acidophils was about $0.25\text{ }\mu\text{g}$. Thus, during isolation and centrifugation, a large part of ACTH activity was lost. On the other hand, GH activity did not diminish and was stable during the treatments. There was evidence that the large storage granules $300\text{--}450\text{ m}\mu$ in diameter were regarded as the stable intracellular carrier of GH. Prolactin activity was not, however, detected in the both sizes of granules, notwithstanding the conspicuous activity of prolactin in the acidophils or in the whole anterior pituitaries before separation of granules (Table 2).

Discussion

It has been generally known that the acidophils of the normal rat pituitary contain abundant dense granules with the correspond-

ing diameter approximately $350\text{ m}\mu$. Most acidophils have been thought to belong to the "somatotrophs" rich in large granules, but Ishikawa (1969) distinguished the acidophilic cells of rat anterior pituitary into the "acidophilic intermediate cells" (immature acidophils), the typical (mature) acidophils and so-called prolactin secreting acidophils. The first was characterized by the occurrence of small granules with a diameter of $150\text{--}200\text{ m}\mu$: The second was identical with the somatotrophs: The third contained a number of huge, irregularly shaped granules with a maximal diameter over $700\text{ m}\mu$. However, the granules in the somatotrophs are not always of the same dimension. Kurosuni (1968) found such atypical somatotrophs as containing both the large granules $350\text{ m}\mu$ in diameter and small ones below $200\text{ m}\mu$ in diameter in the normal rat anterior pituitary. Ishikawa (1969) examined electron microscopically the pellets of acidophils isolated from the rat anterior pituitaries, and found only a few number of the contaminated acidophilic intermediate cells in the pellets of acidophils. The materials used for isolating the intracellular granules

in this study seemed, therefore, to consist of both the typical acidophils (so-called somatotrophs) and prolactin secreting acidophils. The simultaneous existence of the small and large granules in the same somatotrophs was suggested, because the first histogram as to the granular size made *in situ* coincided with the second one made on the free granules originated from the isolated acidophils.

In isolating the storage granules from a pellet of acidophils, the present authors modified the method of Costoff and McShan (1969) in the following respects: Membrane filter was used twice before and after the concentration on the sucrose density gradient. The collected residues of large and small granules were observed electron microscopically to determine if they were intermingled with each other. Since the contamination was negligible, the present technique of isolation seemed to be appreciated. The hormonal activity of the middle-size of granules between 200 and 300 $m\mu$ in diameter were not chanced to be examined, because they were passed away through the mesh.

The present bioassay data revealed that the small granules 100–200 $m\mu$ contained only ACTH, while large ones 300–400 $m\mu$ only GH. Despite the high prolactin activity of the homogenate of isolated acidophils, the free small and large granules had no prolactin activity. Big granules irregularly shaped with the maximal diameter beyond 400 $m\mu$ which were identical with so-called “prolactin granules” (Nakane, 1970) were excluded by the membrane filter (pore size was 400 $m\mu$). There was the remarkable difference of ACTH activity among the free granules themselves, the homogenates of the isolated acidophils and those of the whole pituitaries (Table 2). Anyhow, the present results revealed that GH and ACTH might be localized independently in the two kinds of storage granules. In other word, the two kinds of granules surely correspond to the carriers of the two hormones. In consideration of the previous data (Ishikawa *et al.*, 1971) that

ACTH activity was present only in the isolated acidophils and acidophilic intermediate cells, it was assumed that a large part of intracellular ACTH might be discharged freely from the acidophils through the cytomembrane during isolation while small storage granules with ACTH activity still remained in the cells. On the other hand, GH activity might be stable and not markedly reduced during isolation, because there was evidence that a large part of GH might be tightly bound with the matrix of large storage granules. However, it has not still given a reliable conclusion concerning whether both the small and large granules are simultaneously originated from a single acidophil or independently from the different acidophils, but the possible co-existence of both sizes of granules in the same acidophil was suggested. The results of bioassays of ACTH, GH and prolactin on the separated two kinds of storage granules from the isolated acidophils thus strongly supported by our previous theory that the cells along the acidophilic axis was responsible for ACTH production (Totsuka 1967; Ishikawa *et al.* 1969; Ishikawa and Yoshimura, 1969; Yoshimura *et al.*, 1969; Ohtsuka *et al.*, 1971; Ishikawa *et al.*, 1971)

Reference

- Brown, J. H. U. and M. Hess (1957). *Am. J. Physiol.* **188**, 25.
Brown, J. H. U., F. S. Labella, and F. Ulvedal (1960). *Endocrinology* **66**, 1.
Costoff, A. and W. H. McShan (1969). *J. Cell Biol.* **43**, 564.
Greenspan, F. S., C. H. Li, M. E. Simpson and H. M. Evans (1949). *Endocrinology* **45**, 455.
Herlant, M. (1952). *Ann. Endocrinol.* **13**, 611.
Hymer, W. C., and W. H. McShan (1963). *J. Cell Biol.* **17**, 67.
Ishikawa, H. (1969). *Endocrinol. Japon.* **16**, 517.
Ishikawa, H., F. Soyama, and F. Yoshimura

- (1969). *Ibid.* **16**, 331.
- Ishikawa, H. and F. Yoshimura (1969). *Gumma Symposia Endocri.* **6**, 267.
- Ishikawa, H., T. Watanabe and F. Yoshimura (1971). *Endocrinol. Japon.* **18**, 223.
- Jacobowitz, D., B. H. Marks and J. Vernikos-Danellis (1963). *Endocrinology* **72**, 592.
- Kanematsu, S. and C. H. Sawyer (1963). *Ibid.* **72**, 243.
- Kurosumi, K. (1968). *Arch. Hist. Japon.* **29**, 329.
- Nakane, P. K. (1970). *J. Histochem. Cytochem.* **18**, 1.
- Ohtsuka, Y., H. Ishikawa, T. Omoto, Y. Takasaki, and F. Yoshimura (1970). *Endocrinol. Japon.* **18**, 133.
- Perdue, J. F., and W. H. McShan (1962). *J. Cell Biol.* **15**, 159.
- Reid, E. and A. Segaloff (1958). *Proc. Soc. Exp. Biol. Med.* **97**, 187.
- Totsuka, S. (1967). *Endocrinol. Japon.* **14**, 295.
- Yoshimura, F., K. Harumiya, H. Ishikawa, and Y. Ohtsuka (1969). *Ibid.* **16**, 531.
- Ziegler, D. M., and J. B. Melchior (1956). *J. Biol. Chem.* **222**, 721.