THE NUMBER OF CATECHOLAMINE STORAGE GRANULES IN ADRENAL GLANDS*

W. R. Burack,† E. Avery, P. R. Draskóczy, and N. Weiner

Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

IT HAS been established that the catecholamine hormones of the adrenal medulla, epinephrine and norepinephrine, are stored within intracytoplasmic "heavy granules" which are distinct from mitochondria and which can be harvested in a homogeneous pellet by a combination of high speed differential and sucrose density gradient centrifugation. (Blaschko *et al.*, 1955, 1956). In speculations about the mechanism by which the catecholamines are stored, their molar concentration within individual heavy granules holds considerable importance. From observations which Carlsson and Hillarp (1958) have made of the catecholamine, and total and extragranular water contents of heavy granule pellets from bovine adrenal glands, the concentration in granule water appears to be greater than 0·5 M. The purpose of this communication is to present the results of investigations designed to determine content and molar concentration of catecholamines in single heavy granules, an estimation made possible by a technique for counting these subcellular particles. Adrenal glands of fowl (cross-bred Cornish and Light Rock strains) were used as the source of granules because they contain a greater proportion of modulla tissue than the adrenal glands of other small laboratory animals. (Elliott and Tuckett, 1906).

METHODS

1. *Preparation of Granules*

Adrenal glands, removed immediately after death by decapitation, were trimmed, weighed and homogenized in ice-cold, neutralized 0·3 M sucrose containing 0·01 M disodium-ethylenediamine tetraacetic acid (EDTA). An aliquot was taken for the colorimetric determination for catecholamines (Euler and Hamberg, 1950). After centrifugation for 10 minutes at 800 × *g* to remove cell debris, nuclei and unbroken cells,

the "large granule fraction" consisting of mitochondria and heavy granules (Blaschko *et al.*, 1956) was sedimented from the supernatant by centrifuging at 12,000 × g for 30 min. This sediment was gently rehomogenized in 0·3 M sucrose in a tapered glass homogenizer of 5 ml. capacity*, layered over 2·0 ml. 1·8 M sucrose in a 10 ml. tube and centrifuged at 27,000 × g overnight in a swinging bucket rotor.† The heavy granule pellet which was obtained was again gently rehomogenized in a measured volume (usually 4·0 ml) of 1·8 M sucrose. This material was used for microscopy after further dilution with 1·8 M sucrose (see below).

2. *Counting Technique*

Human red blood cells were washed several times with and then suspended in 0·9% sodium chloride solution. The number of red cells per milliliter of the suspension was ascertained by a standard microscopic technique using 125-fold magnification and a ruled hemocytometer with a well 100 μ deep. A volume of cell suspension was then diluted with many (usually 20) volumes of 1·8 M sucrose and an aliquot mixed with an equal volume of the resuspended heavy granules. The concentration of red cells in the final cell-granule mixture was approximately 2×10^8/ml. A very small droplet of the mixture was placed on a glass slide and covered with a 20 mm micro cover slide and the resulting thin film observed by phase contrast with a Carl Zeiss Standard GFL binocular microscope. The apertures of the 25 × oculars were reduced by the introduction of metal discs with 2·5 mm central circular apertures, one of which was fitted with cross hairs. Use of a 100 power oil immersion objective afforded 2500 fold magnification. The light source was a 6 V, 15 W bulb with a green filter. Immediately adjacent fields were observed in several randomly chosen places, granules and red cells being counted simultaneously until at least 25 red cells had been found. By counting one-quarter of the field at a time, individual heavy granules could be counted without confusion. (Some constant readjustment of the fine-focusing knob was necessary to avoid overlooking granules which would otherwise have been out of focus since the film did not, in fact, contain a monolayer of granules.) Granule counts were calculated by relating the number of observed granules to the number of observed red cells, the latter functioning as an internal standard. A correction was then made for the destruction of granules during the rehomogenization and resuspension procedures. The necessary correction factor was determined as follows: The resuspended granules from 17 pairs of adrenal glands were each divided into equal fractions. One fraction was again centrifuged overnight at 27,000 × g and the protein content of the

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* Kontes Glass Co., Vineland, N. J.

† Servall, RC-1, refrigerated centrifuge; HS rotor.

recovered granules was compared with the total protein content of the other fraction. Any difference was assumed to be due to the destruction of granules by the homogenization-resuspension procedure. Mean protein
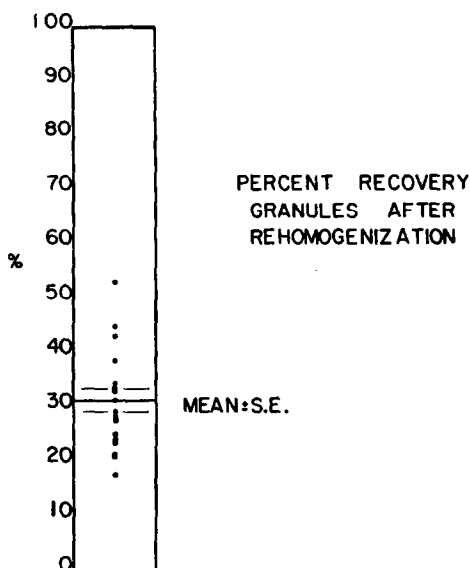

FIG. 1

content of the recovered pellets was $30 \cdot 8\% \pm 3 \cdot 3$ (S.E.) of that in the uncentrifuged fractions (Fig. 1). Therefore, heavy granule counts were divided by $0 \cdot 31$ to correct for this expected "breakage".

3. Chemical Determinations

Catecholamines were estimated in an aliquot of the whole gland homogenate and in an aliquot of heavy granule suspension after extraction with an equal volume of $0 \cdot 8$ N perchloric acid ($HClO_4$). They were adsorbed on and eluted from alumina columns, converted to trihydroxyindole derivatives by ferricyanide at pH $6 \cdot 0$ (Euler and Lishajko, 1960) and measured spectrophotofluorimetrically (Lund, 1950). Correction was introduced for recovery which, in the case of standards was greater than 75% for epinephrine and usually greater than 85% for norepinephrine.

Proteins were washed twice with $0 \cdot 2$ N perchloric acid, dissolved in 1 N sodium hydroxide and estimated colorimetrically (Lowry et al., 1951).

RESULTS

1. Microscopic Appearance of Heavy Granules

The heavy granules of normal glands appeared as identical, tiny black dots against a light background and exhibited much Brownian

motion. Rarely, larger particles with less dark centers, probably mito-
chondria, were seen. When strict attention was given to performing all
of the heavy granule harvesting techniques at a temperature near zero,
clumping of granules was minimal or absent.

The heavy granules of glands from fowl to which reserpine had
been administered previously were found to lack clear-cut outlines
("ghost granules") and were difficult to see. Therefore, it was not poss-
ible to perform reliable counts of the heavy granules from glands of
reserpine-pretreated animals.

2. *Heavy Granule Counts*

All but a few per cent the catecholamines of the adrenal gland is
contained within the heavy granules (Burack *et al.*, 1961). Division of
the number of heavy granules in each pellet by the fraction of gland
catecholamines which the pellet contains yields a figure for the number
of heavy granules in the intact pair of glands from which the pellet
was harvested. Granule counts for 11 pairs of glands ranged from $1 \cdot 6$
to $10 \cdot 6 \times 10^{12}/g$ wet weight. The mean was $5 \cdot 0 \pm 0 \cdot 8 \times 10^{12}/g$.

3. *Content of Catecholamines in Individual Heavy Granules*

In 11 instances the mole content of catecholamines in a pellet was
divided by its granule count to determine the amount in a single gran-
ule. The average catecholamine content of single granules ranged
from $0 \cdot 6$ to $2 \cdot 8 \times 10^{-17}$ mole. The mean was $1 \cdot 4 \pm 0 \cdot 2$ (S.E.) $\times 10^{-17}$
mole.

4. *Concentration of Catecholamines in Individual Heavy Granules*

The average volume of individual heavy granules was estimated
from electron microphotographs of hamster adrenal medulla cells pub-
lished by de Robertis and Sabatini (1960). The mean radius, r, of 87
granule sections was $0 \cdot 13\mu$. When $0 \cdot 13$ divided by a "radius-correction
factor" $(0 \cdot 785)$, the equatorial radius, r, is obtained, viz. $0 \cdot 17$ μ. Since
the heavy granules are always nearly round in cross section, they may
be considered to be spherical. The volume of a sphere with $r = 0 \cdot 17$ μ
is 19×10^{-8} l. (volume $= 4/4$ πr^3). The radius-correction factor is nec-
essary because random slices through any sphere will more often be
located on one side or other of its equator, i.e. the (equatorial) radius
of heavy granules is not the "mean" radius as directly measured from
electron microphotographs. This is illustrated by Fig. 2, which repre-
sents one half of an infinitely thin slice through the equator of a sphere.
The "mean" radius, r, as measured from electronmicrographs, is the
average height of the semicircle. Since the area of the semicircle can
be expressed either as π $r^2/2$ or as $(2$ $r)$ $(\bar{r})$, r can be stated in terms of $\bar{r}$.
r is found to be $\bar{r}/0 \cdot 785$.

Division of mole content of catecholamines per granule by granule volume gives a value for molarity. The mean calculated molarity of the
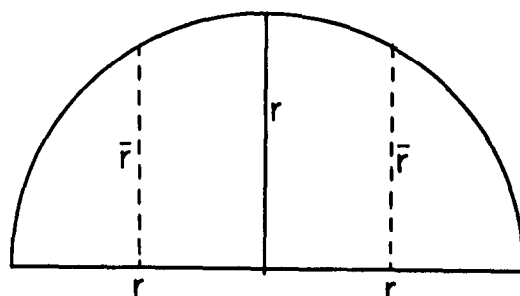
AREA $= \pi r^2/2 = (2r)(\bar{r})$

$\therefore \bar{r} = .785\,r$

FIG. 2

catecholamines in the heavy granules from 11 pairs of glands ranged from 0·3 to 1·1 and the mean was 0·7.

DISCUSSION

Since the adrenal glands of fowl contain approximately as much cortex as medulla (Elliott and Tuckett, 1906), the number of granules/g of medulla tissue may be assumed to be about 10^{13}, twice the counts/g of whole gland. It is not possible at this point to estimate the average number of granules contained in each medulla cell.

The amount of hormone ($1 \cdot 4 \times 10^{-17}$ mole) in a single heavy granule represents about 8 million molecules of catecholamines. de Robertis and Sabatini (1959) have published microphotographs of adrenal medulla cells from hamsters whose splanchnic nerves had been stimulated electrically in order to produce a heightened discharge of catecholamines. Heavy granules are aligned adjacent to the cell membrane apparently discharging their entire contents through a break where granule and cell membranes touch. If this is the way in which the granules are ordinarily emptied, 8 million molecules of hormone may be regarded as a quantum of adrenal medulla secretion.

Estimation of the molar concentration of catecholamines in a single granule requires a knowledge of granule volume. The dimensions of an average heavy granule were derived from the microphotographs of hamster adrenal medulla cells of de Robertis and Sabatini (1960). No similarly detailed pictures of as many fowl heavy granules are available but hamster and fowl granules are of similar size (Kleinschmidt and Schümann, 1961). Our calculations indicate that if a heavy granule had a volume of 1 l., it would contain about 0·7 mole catecholamines. How-

ever, it must be borne in mind that only about 70% of the weight of a heavy granule is water (Carlsson and Hillarp, 1958), since it also contains considerable amounts of adenine nucleotides and protein, so the molar concentration of catecholamines in granule water is even greater than 0·7. Carlsson and Hillarp's data similarly indicate that the catecholamine concentration within intragranular water exceeds half molar. It may be concluded that catecholamines are probably stored within the heavy granule in a bound state, for if they existed as a half molar solution of free ions, they would exert an osmotic pressure of several atmospheres and granule integrity in all likelihood could not be maintained. In fact, there is evidence both indirect* (Hillarp and Thieme, 1959; Burak *et al.*, 1961) and direct† (Weiner *et al.*, 1961), that catecholamines are indeed bound to polyvalent adenine nucleotides in a storage complex. Whether formation of this complex is of itself enough to lower the concentration of osmotically active particles within the granule to a degree compatible with their survival, or whether to this end the complex must in turn be bound to granule protein remains to be explored.

SUMMARY

(1) A method has been described for counting the catecholamine-containing heavy granules of adrenal glands. There are $5·0 \pm 0·8$ (S.E.) $\times 10^{12}$ granules/g wet weight of fowl adrenal gland.

(2) Individual heavy granules contain about 8 milion molecules of catecholamines ($1·4 \times 10^{-17}$ mole).

(3) Reference to published electron microphotographs of adrenal medulla cells allows estimation of the average volume of heavy granules and calculation of the intragranular concentration of catecholamines. The latter probably exceeds 0·5. Therefore, unless catecholamines are chemically bound to some polyvalent constituent within the heavy granules, their osmotic activity would be expected to be greater than is consistent with the maintenance of granule integrity.

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* Catecholamines and adenine nucleotides are present in chemically equivalent amounts in heavy granules.

† Nuclear magnetic resonance spectroscopy of solutions containing catecholamines and adenine nucleotides indicates that the two compounds interact by both ionic and hydrogen bonds.

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