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A Simple Method for the Isolation of Adrenal Chromaffin Granules on a Large Scale

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Highly purified chromaffin granules can be obtained from homogenates of either ox, pig, horse or rat adrenal medullae by ultracentrifugation of the large-granule fraction layered on 1·6M-sucrose solution, by using angle-head rotors. The chromaffin granules are obtained as a pink sediment that is only slightly contaminated by mitochondria and lysosomes.

The catecholamines of the adrenal medulla are stored in a specific cell particle, the chromaffin granule. Methods for the isolation of chromaffin granules have involved the use of sucrose density gradients in the swing-out head of the ultracentrifuge. By this means it has been possible to obtain chromaffin granules almost entirely free from mitochondria (Blaschko, Hagen & Hagen, 1957) and from lysosomes (Smith & Winkler, 1966).

In the present paper a method is described that allows the isolation of chromaffin granules on a large scale with the angle-head rotor of the ultracentrifuge; the procedure does not involve the preparation of sucrose density gradients. The method is based on the observations by Blaschko, Born, D'Iorio & Eade (1956) and by Eade (1958) that chromaffin granules could be sedimented through a hyperosmotic solution of sucrose of uniform density. The isolation procedure described in the present paper has been used in studies of the soluble proteins (Smith & Winkler, 1967) and lipids (Blaschko, Firemark, Smith & Winkler, 1966) of bovine chromaffin granules.

METHODS

Preparation of the large-granule fraction. Adrenal glands of ox, horse and pig were obtained from the slaughterhouse, where they had been placed on ice within 15min. of death. Adrenal glands from the rat were obtained within 2min. of death. The cortex was removed as quickly as possible and the medullae were stored in ice-cold 0·3M-sucrose for up to 1hr. before homogenization. The adrenal medullae were finely chopped and homogenized in a Potter–Elvehjem homogenizer (Kontes Glass Co., Vineland, N.J., U.S.A.) having a glass mortar and Teflon pestle (clearance 0·08mm.) to give a 1:5 (w/v) homogenate in ice-cold 0·3M-sucrose. It was necessary to dilute the suspension of rat adrenal medullae to a convenient volume for homogenization. Homogenization was usually complete after the pestle had been passed up and down four times.

In the centrifugal data given below, all the values for g were calculated by using the radius from the centre of rotation to the bottom of the tube. The centrifugal force is given as g-min.

The homogenate was centrifuged at low speed ($12 \times 10^3 g$-min.) to remove unbroken cells and cell nuclei. The low-speed supernatant was centrifuged at $2{\cdot}42 \times 10^5 g$-min. in the A30 rotor of the Spinco model L ultracentrifuge at 2°. The 'fluffy layer', which lay loosely on top of the sediment, was decanted along with the supernatant. The sediment was resuspended in ice-cold 0·3M-sucrose, but care was taken to exclude some erythrocytes that were present at the bottom of the sediment. A 1ml. sample of the resuspended sediment (large-granule fraction) corresponded to 1–2g. of original tissue.

Isolation of chromaffin granules. The polyallomer centrifuge tubes of the A40 rotor of the Spinco ultracentrifuge were used for this stage. The tubes contained 7·5ml. of ice-cold 1·6M-sucrose solution, on which was layered no more than 3ml. of the resuspended large-granule fraction. After centrifugation for $66 \times 10^5 g$-min., several different layers could be distinguished within the tube: these are numbered 1–5 in Fig. 1. The total supernatant (layers 1–3) was decanted and the inside of the tube above the sediment was cleaned with paper tissues. Then 1ml. of 1·6M-sucrose was added to the tube and the loosely adhering layer (4) was resuspended by slightly shaking the tube: the material was then decanted. This washing procedure was repeated once more. A pink sediment (5) of chromaffin granules was left in the tube.

Isolation and analysis of fractions. The different fractions obtained after centrifugation of the large-granule fraction over 1·6M-sucrose (see Fig. 1) were separated for analysis as follows: fractions 1 and 2 were each removed with polythene Pasteur pipettes; fraction 3 was removed by decantation; fraction 2′ was then sucked from the side with a Pasteur pipette and was combined with fraction 2; the sediment in each tube was washed twice with 1·6M-sucrose, as described above, and the washings (fraction 4) were combined with fraction 3. Fraction 5 was resuspended in 0·3M-sucrose.

The catecholamines were measured by the method of Euler & Hamberg (1949), but with citrate–phosphate buffers (McIlvaine, 1921) instead of acetate buffers. In the experiments with rat adrenal medulla, the fluorimetric

method of Euler & Lishajko (1961) was used for the determination of the catecholamines. Total acid-insoluble N was determined, after precipitation with trichloroacetic acid (final concn. 5%, w/v), by the micro-Kjeldahl method. ATP was measured by the firefly method of Strehler & Totter (1954). For enzyme assays the fractions were diluted with tris–sodium succinate buffer, pH 5·9 and I 0·015, to lyse the cytoplasmic particles. The diluted fractions were dialysed against the same buffer for 15 hr. at 3° to decrease the otherwise high enzyme blanks. Fumarase activity was measured by the spectrophotometric method of Racker (1950). Estimation of deoxyribonuclease activity was made by a modification (Smith & Winkler, 1966) of the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955).

RESULTS

Separation of particulate constituents of the large-granule fraction from ox adrenal medulla. The large-granule fraction contained 63% of the catecholamines present in the homogenate. A drawing of the centrifuge tube, after centrifugation of the large-granule fraction in 1·6 M-sucrose, is given in Fig. 1. Five distinct regions were seen and these are briefly described in Fig. 1. The material labelled 2′ was dark brown and was clearly derived from the material of region 2 that had become attached to the tube during centrifugation in the angle-head rotor.

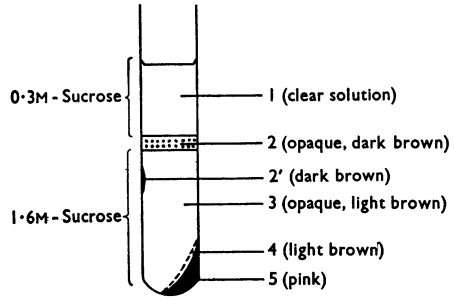

Fig. 1. Appearance of centrifuge tube after centrifugation of the large-granule fraction in 1·6 M-sucrose. A 3 ml. sample of the large-granule fraction, suspended in 0·3 M-sucrose, was layered on 7·5 ml. of 1·6 M-sucrose. The tube was spun at $66 \times 10^5 g$-min. in the A 40 angle-head rotor of the Spinco ultracentrifuge.

The different fractions were each analysed for total acid-insoluble N (largely protein), catecholamines (a marker for chromaffin granules), fumarase (a marker for mitochondria) and acid deoxyribonuclease (a marker for lysosomes). The results are given in Table 1 as the percentage of the constituent recovered in each fraction, and show that each of the markers has a different distribution. Thus fumarase activity was concentrated in fractions 2 and 2′, whereas nearly three-quarters of the acid-deoxyribonuclease activity was recovered in fractions 3 and 4. The pink sediment (fraction 5) contained three-quarters of the catecholamines but only 37% of the protein and a trace of fumarase or deoxyribonuclease activities.

The pink sediment contained $15{\cdot}7 \pm 2{\cdot}3$ ($n = 7$) μmoles of catecholamines/mg. of acid-insoluble N and was also rich in ATP, a characteristic constituent of chromaffin granules (Blaschko *et al.* 1956). The molar ratio of catecholamines to ATP was 4·85, which is close to the ratio found by Banks (1965) for granules prepared by sucrose-density-gradient centrifugation.

The cytochrome spectrum of fraction 2 and of fraction 5 was measured after reduction with dithionite as described by Banks (1965). The spectrum of the sediment (fraction 5) had a marked peak at 559 mμ and a shoulder at 526 mμ, whereas that of fraction 2 had a peak at 552 mμ and another at 600 mμ.

Isolation of chromaffin granules from the adrenals of other species. The large-granule fractions from the adrenal medullae of pigs, horses and rats contained 57, 53 and 78% respectively of the catecholamines present in the original homogenate. The large granules were further fractionated exactly as described for the bovine material. After centrifugation, the appearance of the tubes was very similar to that found with the large-granule fraction from ox adrenal medulla. The different fractions were analysed for catecholamines and the

Table 1. *Distribution of protein, enzymes and catecholamines after ultracentrifugation of the bovine large-granule fraction in 1·6 M-sucrose*

The results are expressed as percentages of the total constituent present in the centrifuge tube. Details of the centrifugation and the numbering of the fractions are given in Fig. 1. The values are the means ± S.D. of six experiments, except for the values for acid-insoluble N, which are the means of two experiments. The recoveries of the constituents of the large-granule fraction after centrifugation were 96–100%.

Fraction	Acid-insoluble N	Fumarase	Acid deoxyribonuclease	Catecholamines
1	1·5	< 1·0	0·8 ± 0·3	4·5 ± 1·2
2 + 2′	42·2	91·1 ± 3·4	19·6 ± 1·1	4·8 ± 0·8
3 + 4	18·8	6·9 ± 1·3	72·8 ± 1·9	14·6 ± 3·1
5	37·3	2·1 ± 0·9	6·5 ± 0·7	76·6 ± 4·3

Table 2. *Distribution of catecholamines after ultracentrifugation of the large-granule fraction from the adrenal medullae of three species*

The results are expressed as percentages of the total catecholamines present in each tube. The values are the means of those from two experiments. The fractions are numbered according to Fig. 1 and the centrifugation conditions are given in the legend to Fig. 1.

Fraction	Horse	Pig	Rat
1	3·4	5·0	1·75
2+2′	5·3	9·3	6·3
3+4	6·2	6·7	16·8
5	84·9	79·0	75·2

results are given in Table 2. In each case the sediment contained a large proportion of the catecholamines. A very similar distribution of catecholamines was obtained from a homogenate of a human phaeochromocytoma (A. D. Smith & H. Winkler, unpublished work). The number of μmoles of catecholamines/mg. of protein N was $7·3 \pm 1·0$ ($n=3$) in the sediment of the equine granules and $6·8 \pm 1·38$ ($n=3$) in the sediment of porcine granules. These values are about half that obtained with bovine granules.

DISCUSSION

The distribution of the constituents of the large-granule fraction after ultracentrifugation in 1·6M-sucrose agrees with what has been found previously by means of sucrose-density-gradient centrifugation (see Blaschko *et al.* 1957; Smith & Winkler, 1966). The bulk of the chromaffin granules were sedimented through 1·6M-sucrose and were separated from the mitochondria and the lysosomes.

Evidence that the pink sediment was composed mainly of chromaffin granules was its high content of catecholamines, its cytochrome spectrum and its low content of enzymes. The number of μmoles of catecholamines/mg. of protein N was as high as that found by other workers in granules prepared by means of a sucrose density gradient (Blaschko *et al.* 1957; Banks, 1965). The cytochrome spectrum of the sediment had the same characteristic peak at 559mμ as the cytochrome present in chromaffin granules (Banks, 1965; Ichikawa & Yamano, 1965), but did not have the peaks at 552 and 600mμ characteristic of mitochondrial cytochromes. A more sensitive indication of the presence of other cell particles is the use of enzymes as markers. The very low content of fumarase (2·1%) and acid deoxyribonuclease (6·5%) in the sediment indicates that it is only slightly contaminated by mitochondria and by lysosomes respectively. It is possible that the light-brown layer that could be readily washed off the pink sediment was composed of lysosomes. That the contamination of the chromaffin-granule sediment with other cell particles is low may in part be due to the fact that its upper surface can be easily washed, whereas in a sucrose density gradient the chromaffin granules remain suspended in solution.

The present method of isolating chromaffin granules has several technical advantages over methods that have been used in the past. First, the method does not involve making up sucrose density gradients. Secondly, it is no longer necessary to use the small capacity tubes from the swing-out head of the Spinco ultracentrifuge: the use of the angle-head rotor (A 40) allows the isolation of chromaffin granules from a homogenate of a large number (up to 100) of bovine adrenal medullae in 1 day. Finally, the chromaffin granules are obtained in a highly concentrated form, as a pellet in a centrifuge tube. The method can also be used to isolate a mitochondrial fraction that is not significantly contaminated by chromaffin granules, but that is still contaminated by lysosomes. A lysosomal fraction can be obtained that is contaminated slightly by mitochondria and chromaffin granules.

The results given in Table 2 show that the chromaffin granules from the adrenals of horse, pig and rat are, like those from the ox adrenal, largely sedimented from 1·6M-sucrose. It is concluded that the present method can be used to isolate purified chromaffin granules from the adrenals of several species.

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