Studies on the relationship between the catecholamine distribution in the atrium and the specific granules present in atrial muscle cells

1. Isolation of a purified specific granule subfraction

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AUTHORS’ SYNOPSIS Electron microscopy of mammalian atrial muscle cells reveals a population of specific granules suggesting that the atrial muscle cells may have a secretory function. As a preliminary step towards the investigation of the function of the specific granules, a procedure was designed to isolate these particles by differential and density gradient centrifugation of rat atrial homogenates. The specific granule preparation obtained showed a considerable degree of purification with respect to marker enzymes. The isolated granules retained their in situ fine structure and staining properties.

The unfailing presence in mammalian atrial muscle cells of a population of granules which morphologically resemble those granules present in secretory cells has attracted the attention of several investigators during the past 15 years. It is not clear at present whether these particles, presently referred to as specific granules, play an endocrine role or whether they constitute an organelle involved in the intracellular metabolism of cardiocytes (Poche, 1957; Bompiani, Rouiller, and Hatt, 1959; Bloom, Oslund, von Euler, Lishajko, Ritzen, and Adams-Ray, 1961; Palade, 1961; Jamieson and Palade, 1964; Kisch, 1965; Bencosme and Chang, 1968; Martínez-Palomo and Bencosme, 1966a, b; Trillo, Martínez-Palomo, and Bencosme, 1966; Chang and Bencosme, 1969; Ferrans, Hibbs, and Buja, 1969; Hibbs and Ferrans, 1969; Okamoto, 1969; Otsuka, Okamoto, and Tomisawa, 1969; Manasek 1969; Sosa-Lucero, de la Iglesia, Lumb, Berger and Bencosme, 1969; Tomisawa, 1969; Strosberg, Katzung, and Lee, 1970; Bencosme and Berger, 1971; Berger and Bencosme, 1971; Berger, Sosa-Lucero, de la Iglesia, Lumb, and Bencosme, 1972).

The inability to perform direct biochemical analysis on the specific granules has handicapped to a large extent further developments in the investigation of these particles. The present work, aimed to isolate the specific granules from rat atrial cardiocytes, was conceived as an essential step in the investigation of the composition and function of these particles.

A method for the isolation of a specific granule fraction has been reported using ultracentrifugation of atrial tissue homogenates over a continuous sucrose gradient (Sosa-Lucero et al., 1969). The use of this type of gradient to obtain isolated specific granules in a preparative scale, however, did not provide an adequate resolution of a specific granule fraction. It was found that a
combination of differential centrifugation and density gradient centrifugation using a discontinuous sucrose gradient provides not only better resolution but also a faster methodology to isolate a specific granule fraction.

A procedure for the isolation and purification of specific granules by preparative ultracentrifugation of rat atrial tissue homogenates is described below. This procedure is evaluated in the light of electron microscopic studies and biochemical determination of marker enzymes in the different fractions obtained during the isolation procedure.

**Methods**

**Biological material**

Tissue fractionation experiments were carried out on atria of male Sprague–Dawley rats (200–300 g). The animals had food and water *ad libitum* until 2 hr before beginning the experiment. The animals were killed by decapitation. Forty rats were used in each experiment. Immediately after death the thorax was quickly opened, the heart removed, washed in ice cold 0.25 M sucrose, and placed in a container with ice cold 0.25 M sucrose. At this time, the large vessels and fat were dissected away and the tissues were washed a second time in ice cold 0.25 M sucrose followed by dissection of the atria.

Homogenization of the tissue was carried out in a cold room at 1–3°C.

**Sucrose solutions**

Three concentrations of sucrose were used: 0.25 M, 1.60 M, and 2.00 M. All three solutions contained 0.25% glycogen and 1 mM EDTA adjusted to pH 7.0 with NaOH. The glycogen was prepared as a 20% solution (w/v) in water and was centrifuged at 30,000 g for 30 min in order to remove sedi-

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**FIG. 1** Outline of the procedure to isolate a purified specific granule subfraction by differential and density gradient centrifugation of rat atrial tissue homogenates.
mentable impurities (Maunsbach, 1966). The final concentration of glycogen in this solution was assessed by refractometry.

Fractionation procedure
Figure 1 represents an outline of the differential and density gradient centrifugation procedures.

Preparation of atrial tissue homogenates
The washed atrial tissues were quickly blotted on filter paper, weighed, and immediately chopped with a razor blade. The resulting pulp was washed into a glass homogenizer tube with 10 volumes of 0.25 M sucrose.

The resulting suspension was homogenized by 20 strokes of a loosely-fitting Teflon pestle driven at 1,000 rpm. The resulting homogenate will be referred to as initial homogenate.

Preparation of a crude granule fraction by differential centrifugation
The initial homogenate was filtered into a 50 ml. polyethylene tube through four layers of cheese cloth previously dampened with 0.25 M sucrose. This filtrate was centrifuged at 1,900 g_{max} for 10 min in the SS-34 head of a refrigerated RC2B Sorvall centrifuge. The supernatant, designated as the postnuclear fraction, was filtered and centrifuged again in the same fashion. The supernatant from this second centrifugation is referred to as the postmitochondrial fraction. The pellets obtained during this procedure were designated nuclear and mitochondrial fractions respectively. The postmitochondrial fraction was centrifuged at 32,000 g_{max} for 10 min in a Spinco 50 rotor in a Model L Beckman preparative ultracentrifuge. The supernatant obtained after this latter centrifugation was called 32,000 g_{max} supernatant, whereas the pellet was designated crude granule fraction. The crude granule fractions obtained from 20 animals were resuspended in 0.25 M sucrose, pooled, and centrifuged at 32,000 g_{max} for 10 min as described above. The supernatant from the latter centrifugation step was pooled with the 32,000 g_{max} supernatant. This supernatant was further centrifuged at 144,880 g_{max} for 90 min in the head 40 of a L2-65B Beckman preparative ultracentrifuge. The supernatant and the pellet obtained after this centrifugation were designated high speed supernatant and microsomal fraction respectively.

Purification of crude granule fraction by density gradient centrifugation
The tube containing the crude granule fraction was carefully drained and the pellet was partially resuspended by addition of approximately 4 ml of 1.6 M sucrose. Resuspension was further accomplished by placing the partially resuspended pellet in a glass homogenizer tube and homogenizing by two strokes of a Teflon pestle driven at approximately 100 rpm.

After resuspension, 4.0 ml of the resuspended crude granule fraction was pipetted on top of 0.5 ml of 2.0 M sucrose and topped with 1 ml of 0.25 M sucrose in a nitrocellulose tube fitting the SW 39L rotor of the Model L Beckman preparative ultracentrifuge. Centrifugation of the gradients thus formed was carried out immediately at 154,000 g_{max} for 60 min.

After centrifugation, five subfractions were obtained from the gradients by slicing the tube in a Beckman tube slicer (see Fig. 16a).

Chemistry
Cytochrome c oxidase was determined according to Cooperstein and Lazarow (1951). Acid phosphatase was determined at 37°C in a 2 ml incubation mixture containing 0.05 M sodium acetate/HCl buffer, pH 5.0, and 0.05 M sodium β-glycerophosphate adjusted to pH 5.0 with HCl. In order to solubilize the enzyme, all samples were frozen and thawed five times before incubation. The incubation time varied between 30 and 60 min. The reaction was stopped by addition of 2 ml 20% (w/v) of trichloroacetic acid.

The inorganic phosphate liberated in the incubation mixture was determined by the method of Fiske and Subbarow (1925). Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951).

Electron microscopy: processing of tissues and fractions
Fixation of heart tissues for routine electron microscopic examination was carried out on 1 mm thick blocks for 15 min at 4°C in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, with 0.4 mM CaCl₂ added. After fixation, the tissues were washed in three changes of the same buffer plus 10% sucrose (w/v). Postfixation was carried out in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 15 min at 4°C (Bencosme and Tsutsu, 1970).

For silver methenamine histochemistry the tissues were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 4 hr without postfixation (Chang and Bencosme, 1968).

Pellets obtained after differential centrifugation were fixed in situ with 3% glutaraldehyde in 0.1 phosphate buffer, pH 7.4, for 1 to 2 hours at 4°C. After fixation, the pellets were loosened from the walls of the centrifuge tube, cut in small blocks,
washed, and postfixed in 2% osmium tetroxide as for tissues.

For silver methenamine histochemistry the pellets were fixed as described for tissues.

After postfixation or fixation in 5% glutaraldehyde, the samples were washed in three changes of 0.1 M phosphate buffer, pH 7.4, plus 10% sucrose (w/v).

Tissues and pellets after differential centrifugation were dehydrated and embedded using a fast method (Bencosme et al., 1970).

Micromethod for processing subfractions

In order to avoid loss of material during processing, the subfractions obtained after density gradient centrifugation were processed in 400 µl plastic tubes fitting a Beckman microfuge (Beckman Ultramicroanalytical System) as follows: after fixation in suspension, for 15 min in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, the subfractions were transferred in aliquots to the microfuge tubes, each time centrifuging for 15 sec and discarding the supernatants until the subfraction was pelleted in the bottom of the microfuge tube. The resulting pellets were washed by shaking in a Beckman microfuge for 10 sec and sedimented by centrifuging for 10 sec. This procedure was repeated three times. Postfixation was carried out in the microfuge tube by adding 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, and shaking for 10 sec. After 15 min the samples were pelleted by centrifugation for 10 sec. The pellets were subsequently washed as described after fixation. Dehydration was carried out in successive changes of 70%, 80%, 90%, and 100% ethanols and propylene oxide, shaking and centrifuging for 10 sec in each step. The propylene oxide supernatant was discarded, a mixture of epoxy resin (Luft, 1961) and propylene oxide (3:1, v/v) added, and the tubes shaken for 5 min. The resulting suspension was

*FIG. 2* Section through a portion of the rat atrial wall. The central sarcoplasmic core from an atrial muscle cell shows numerous specific granules (SG), mitochondria (M), and Golgi complex profiles (G). The sarcoplasm between myofibrils also contain these organelles in variable amounts. Capillary (C), nerve fibre (N). ×7,000.
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![Image](https://example.com/image.png)

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sedimented by centrifugation for 10 sec, the supernatant was discarded, and undiluted resin was added to half the volume of the tube. Shaking was carried out for 5 min and the samples were sedimented by centrifugation for 5 min. The tubes were cut just above the level of the epoxy mixture and allowed to polymerize for 45 min in a 70°C oven and at 90°C for 45 min longer.

Sections were cut on a LKB ultratome. Thick sections (approximately 1 μ) were stained with toluidine blue for orientation purposes. Thin sections (approximately 400 to 800 Å) were contrasted with uranyl acetate (Stempak and Ward, 1964) and lead citrate (Venable and Coggeshall, 1965), and mounted on naked copper grids. The mounted sections were examined on a Hitachi HU-11C electron microscope.

**Silver methenamine histochemistry**

The silver methenamine stain of Chang and Bencosme (1968) was modified to diminish the size of the silver grain.

The silver methenamine solution was prepared by adding 5 ml. 5% solution of silver nitrate to 45 ml. 3% hexamine solution. To this mixture, 20 ml. M/20 borax were added.

Gold coloured sections of Epon-embedded material fixed in 5% glutaraldehyde in phosphate buffer were floated in a small container containing the silver methenamine solution. The container was transferred to a 60°C oven until the floating sections turned to brown (approximately 1-2 hr). The stained sections were mounted on naked copper grids, rinsed in three changes of distilled water, and examined under the electron microscope.
Results

Conventional electron microscopy and silver methenamine histochemistry of atrial tissue

Conventional electron microscopy of rat atrium confirmed the general organization of this tissue as described by others (Kisch, 1956; Bompiani et al., 1959; Hibbs et al., 1969; Forssmann and Girardier, 1970; Berger and Rona, 1971). Figure 2 illustrates some of the components of the rat atrial wall such as muscle cells, nerve fibres, and a capillary vessel. The specific atrial granules present within the atrial muscle cells appear as membrane-bound, electron-dense bodies with an average diameter of approximately 350 nm; and are more concentrated in the paranuclear zones. In agreement with the previous findings (Bencosme et al., 1968; Berger et al., 1971), sections of atrial tissue fixed in glutaraldehyde without postfixation and stained with silver methenamine, showed the core of the specific granules to be silver negative. The use of the modified silver methenamine technique with its finer deposition of the silver grain showed that the silver negative core of the specific granules is separated from the surrounding structures by a silver-positive halo (Fig. 3). This halo, approximately 50 nm thick, was formed by heavy aggregations of silver grains enclosing the core of the specific granules (Fig. 4).

Isolation of specific granules

Morphological studies of fractions obtained after differential centrifugation of atrial homogenates

The nuclear fraction was formed by a mixture of unbroken cells, nuclei, cellular debris, blood elements, endothelial, connective and nerve tissue components. In contrast, the remaining fractions obtained during differential centrifugation of atrial homogenates proved to be composed of a fairly well-defined group of particles. The mitochondrial fraction was mainly composed of a population of mitochondria showing different degrees of preservation. Mitochondrial matrices appeared with different electron opacities. In this and all the remaining fractions, some of the mitochondria disintegrated producing electron-opaque bodies referred to here as mitochondrial debris (Figs. 5 and 6). A few specific granules were occasionally found throughout this fraction. A small amount of cellular debris usually contaminated this pellet.

The crude granule fraction was formed by a mixture of mitochondria smaller than those found in the preceding pellet, specific granules, smooth and rough vesicles, and electron-opaque bodies probably representing mitochondrial debris. The specific granules were considerably more abundant in this pellet than in the mitochondrial pellet (Fig. 7).

The microsomal fraction was composed mostly of smooth vesicles (Fig. 8).

FIG. 5 Section through a mitochondrial fraction. The mitochondria show different matrix densities and some of them (heavy arrows) appear broken at different points producing mitochondrial debris (MD). × 6,000.

FIG. 6 Mitochondrial fraction. High power showing mitochondrial debris. × 30,000.
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Morphological studies of subfractions obtained after density gradient centrifugation of the crude granule fraction

Subfractions I and V after density gradient centrifugation of the crude granule fraction did not provide a pellet after fixation and centrifugation. Subfraction II was the largest subfraction obtained from the density gradient and proved to be essentially composed of mitochondria. A few membranous profiles and specific granules were also present in this subfraction together with particles resembling mitochondrial debris and very few smooth and rough vesicles (Fig. 9).

Subfraction III was formed by a mixture of mitochondria, a fairly large proportion of smooth and rough vesicles, specific granules and other granular structures (Fig. 10).

Subfraction IV contained the highest concentration of specific granules and it will be referred to here as the specific granule subfraction. The
FIG. 9 Subfraction II from the density gradient. This pellet is composed of mitochondria (M), membranous profiles (MP), mitochondrial debris (MD), smooth (SV), and rough (RV) vesicles and some specific granules (SG). × 6,000.

FIG. 10 Subfraction III from the density gradient. The pellet consists of mitochondria (M), many of which appear swollen, mitochondrial debris (MD), a few specific granules (SG), and smooth (SV) and rough (RV) vesicles. × 6,000.

Largest contributors to the contamination of the specific granule subfraction were membrane-bound granules, probably lysosomal in nature, some mitochondria and dense bodies probably representing mitochondrial debris (Fig. 11). The fine structure of the isolated specific granules was essentially the same as that observed in situ (Fig. 12). By conventional electron microscopy, the contaminants of the specific granule subfraction such as bodies resembling mito-
chondrial debris (Fig. 13) and other granular structures, including the so-called B granule described by others (Berger et al., 1971) (Fig. 14), can be differentiated in most instances from the specific granules. This distinction, however, can be best accomplished using the silver methenamine stain. With the latter, the core of the specific granules remained negative whereas most other particles presented different degrees of silver deposition. As shown for the specific granules in situ (Figs. 3 and 4), the isolated granules also appeared surrounded by a silver positive halo after silver methenamine treatment (Fig. 15).

**Distribution of protein, cytochrome c oxidase and acid phosphatase content of crude granule fraction after density gradient centrifugation**

The content of protein, cytochrome c oxidase, and acid phosphatase in the various subfractions

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**FIG. 11** Subfraction IV or purified specific granule subfraction from the density gradient. Most of the structures present in this subfraction are specific granules. Other granular bodies may represent lysosomes (Ly) and mitochondrial debris (MD). × 7,500.
after density gradient centrifugation of the crude granule fraction are given in Figs. 16b, c, and d respectively. An almost identical distribution throughout the gradient was obtained for protein and cytochrome c oxidase, while acid phosphatase showed a slight shift towards the denser region of the gradient as compared with protein or cytochrome c oxidase. The specific granule subfraction (subfraction IV) showed mean activities of 3% of the total cytochrome c oxidase and 12% of the total acid phosphatase activities present in the crude granule fraction.

**Discussion**

The effectiveness of a procedure to isolate subcellular particles of known activity is usually measured in terms of the increase of specific activity of the particle-containing fraction with respect to the starting material. Under appropriate experimental conditions, this ‘marker’ activity may also be used to assess the physical integrity of the isolated particles. Morphological studies are often valuable to establish the physical basis of such activity.
In the absence of a known marker for the specific granules, the isolation procedure described in the present work was largely evaluated in terms of electron microscopic data. This was used to estimate the concentration of particles in a given fraction as well as the structural integrity of the isolated specific granules. The structural integrity of the isolated granules was evaluated in terms of preservation of fine structure, affinity to contrasting agents and to silver methenamine stain. Furthermore, the degree of contamination by mitochondria and particles of lysosomal nature, was assessed by their marker enzymes; cytochrome c oxidase and acid phosphatase. As suggested by the electron microscopic pictures, the activity of these enzymes in the purified granule subfraction showed that a relatively large degree of purification was achieved with respect to the cytochrome c oxidase and acid phosphatase content of the crude granule fraction.

The electron microscopic appearance of the isolated specific granules closely corresponded to that found in situ after conventional contrasting and silver methenamine. It has been shown in the past, that the specific granules remain unstained after silver methenamine treatment, while most other structures within atrial muscle cells show various degrees of silver positivity (Bencosme et al., 1968; Berger et al., 1971). When applied to the specific granule subfraction, the silver methenamine technique proved most valuable for the identification of the isolated granules. The greater ultrastructural detail afforded by the modified silver methenamine technique used in this work allowed the in situ demonstration of a silver-positive halo surrounding the silver-negative
core of the specific granules. Attesting to the ultrastructural integrity of the isolated specific granules, this silver-positive halo was found after isolation. The evidence thus indicates that a substantial amount of specific granules in a reasonable degree of purity and with preservation of ultrastructural detail can be obtained with the preparative procedure described in the present work.

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References


