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ISOLATION AND CHARACTERIZATION OF NORADRENALIN STORAGE GRANULES OF BOVINE ADRENAL MEDULLA

OLE TERLAND, TORGEIR FLATMARK and HARALD KRYVI

Department of Biochemistry and Institute of Anatomy, University of Bergen, Årstadveien 19, N-500 Bergen (Norway)

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Summary

A method is described for the preparation of (1) the heavy population of bovine adrenal chromaffin granules ($\overline{S}_H$ (average sedimentation coefficient) = 12 400 S in 0.25 M sucrose) essentially free from contamination with mitochondria and other organelles, and (2) a subpopulation of this heavy population which is highly enriched in noradrenalin ($\gtrsim$95% of the total catecholamine is noradrenalin). The method is based on isopycnic gradient centrifugation using a self-generating gradient of polyvinylpyrrolidone-coated colloidal silica particles (Percoll) in 0.5 M sucrose medium.

The isolated population of noradrenalin granules appeared highly electron dense in transmission electron microscopy and revealed a rather narrow size distribution. The specific content of amine and adenine nucleotides (with reference to total granule protein) was markedly higher than for the total population of heavy chromaffin granules. The molar ratio of amines to adenine nucleotides was, however, lower in the noradrenalin granules, i.e. 4.8 vs. 11.9.

Introduction

The idea that adrenalin and noradrenalin are synthesized by different cells in the adrenal medulla arose from light-microscopic [1—3] as well as electron-microscopic [4] studies of the intact tissue. In the latter case [4] two types of cells were identified in the bovine medulla, i.e. one type with small, electron-dense granules and one with large, electron-light granules. Experimental evidence was presented that the two types of granules contained noradrenalin and

Abbreviation: HPLC, high-performance liquid chromatography.

adrenalin, respectively [4], but this conclusion has not been verified by biochemical analyses of highly purified subpopulations of the storage granules having the described morphology. So far, only populations enriched in noradrenalin have been obtained by density gradient centrifugation in sucrose media [5,6,7].

By analytical differential centrifugation we have recently found that the chromaffin granules of bovine adrenal medulla homogenates can be resolved into two main populations of storage granules whether adrenalin or noradrenalin is used as the specific marker [8]. The heavy population ($\overline{S}_H$ (average sedimentation coefficient) = 12 440 S in 0.25 M sucrose), which appeared to be the most homogeneous one, has now been studied in more details by isopycnic gradient centrifugation using a self-generating gradient of polyvinylpyrrolidone-coated colloidal silica particles (Percoll), a gradient medium recently introduced by Pertoft and Laurent for separation of cells and cell organelles [9].

Materials and Methods

Preparation of homogenate, large granule fraction and heavy chromaffin granules. A homogenate of bovine adrenal medulla and its large granule fraction were prepared as previously described [8]. The heavy population of chromaffin granules ($\overline{S}_H$ = 12 440 S in 0.25 M sucrose) was isolated from the homogenate by differential centrifugation using the HB-4 rotor of the Sorvall RC 5 centrifuge (Du Pont Instruments) at 4°C and a centrifugal effect [10,11] of $103 \cdot 10^7$ min^{-1} (R_{max} = 14.4 cm and R_{min} = 6.2 cm). The pellet, containing the total 12 440 S population, was washed [12] five times in 0.25 M sucrose in order to remove contaminating light granules; a centrifugal effect of $103 \cdot 10^7$ min^{-1} was used for the sedimentation of the 12 440 S particles. By this procedure more than 99% of the light granules in the homogenate was removed (calculated as in Ref. 12). The final pellet, representing a crude preparation of heavy granules, was carefully resuspended in a minimum amount of 0.25 M sucrose medium.

Rate zonal centrifugation. The rate zonal centrifugation experiments were carried out slightly modified from the procedure described by Winkler [7] using a Ti 50 fixed angle rotor in the Spinco L-50 ultracentrifuge (Beckman Instr., U.S.A.). 2 ml of the large granule fraction, resuspended in ice-cold 0.25 M sucrose, containing 1.0 mM EDTA, pH 6.5, were layered on the top of the centrifuge tubes filled with 7 ml of sucrose medium of molarities ranging from 1.40 to 2.40 M (0.1 M intervals). The pellets were treated as described in Ref. 8.

Isopycnic density gradient centrifugation in Percoll/sucrose. In order to separate the total population of chromaffin granules from other contaminating subcellular particles (notably mitochondria), 0.2 ml of the crude heavy population (see above) was resuspended in 8.0 ml of a medium containing 0.5 M sucrose and 50% (v/v) Percoll, pH adjusted to 7.2 by 1 N HCl. After centrifugation (Beckman L2-65 B, 40 Rotor, 4°C) at an optimal centrifugal effect ($4 \cdot 10^{10}$ min^{-1} or 38 000 rev./min for 20 min, plus the contribution from the acceleration and deceleration of the rotor) the gradient was fractionated. Light

scattering was continuously recorded at λ = 510 nm on a Zeiss Spectrophotometer supplied with a 80 μl flow-through cell, and 0.43 ml fractions were collected for further analyses.

Assay of catecholamines, nucleotides and ascorbate by high-performance liquid chromatography. A constant volume high-performance liquid chromatographic (HPLC) pump (Model Constametric IIG from Laboratory Data Control, FL, U.S.A.) supplied with a 20 μl Rheodyne valve loop injector (Berkely, U.S.A.) was used. The chromatographic separation of the catecholamines was achieved at 20°C on a strong cation exchanger (Zipax SCX, prepacked from Du Pont Instruments in a 60 cm × 2.1 mm inner diameter stainless steel column). The mobile phase, consisting of 30 mM acetate buffer, pH 5.2, was pumped at a flow rate of 2 ml · min^{-1}. The catecholamines were detected using a spectrofluorometer (Model SFM 22 from Kontron, Switzerland) essentially as described previously [13], λ_{ex} = 283 nm and λ_{em} = 313 nm.

The chromatographic separation of the nucleotides was achieved at 20°C on a strong anion exchanger (Partisil 10-SAX, prepacked from Whatman in a 25 cm × 4.6 mm inner diameter stainless steel column). The mobile phase, consisting of 0.45 M potassium phosphate, pH 3.93 (ADP and ATP) [14] or 0.01 M potassium phosphate, pH 3.90 (AMP) was pumped at a flow rate of 2 ml · min^{-1} (1200 lb/$inch^2$), giving the following retention times: t_R(AMP) = 6.2 min, t_R(ADP) = 3.0 min and t_R(ATP) = 12.2 min.

In all chromatographic systems a short pre-column (40 mm × 2 mm inner diameter) of pellicular silica (HC Pellosil from Whatman) was used to protect the ion-exchange columns.

To each fraction of the collected density gradient was added an equal volume of absolute ethanol containing 2% (v/v) thiodiglycol and 60 mM acetic acid [8]. The precipitated protein was removed by centrifugation and the clear supernatant analyzed for catecholamines and nucleotides without further purification; the content of the individual components was determined from standard curves. In the assay of adenine nucleotides aliquots were alternatively analyzed following extraction, and precipitation of protein, by 0.4 M perchloric acid [15].

Electron microscopy. Isolated fractions of chromaffin granules were fixed in 3% (v/v) glutaraldehyde in a medium containing 0.5 M sucrose and 25 mM potassium phosphate buffer, pH 6.5, for 6 h at 2°C. Subsequently, the material was rinsed in this medium, post-fixed in 1% osmium tetroxide and dehydrated in ethanol. The preparations were embedded in Epon, and ultrathin sections contrasted with uranyl acetate and lead citrate, were studied in a Siemens Elmiskop 1A. From micrographs at 25 000 × the diameter of the granules were measured by a Kontron MOP AM 03 Morphometric Analyzer (Kontron, Switzerland).

Other analytical methods. The density of each fraction collected from the Percoll gradient was determined at 20°C by weighing a defined volume of the fraction and using water as a reference.

Malate dehydrogenase activity in the isolated fractions was assayed spectrophotometrically by measuring the rate of oxidation of NADH [16]. The reaction mixture contained in a volume of 1 ml : 50 μM NADH, 0.9 mM oxalo-

acetate, 50 mM potassium phosphate buffer, pH 6.5, and 0.2% (v/v) Triton X-100. When a maximum of 0.05 ml of each fraction from the density gradient was used in the assay, the catecholamines present did not interfere with the reaction.

Protein was determined by the Coomassie brilliant blue method of Bradford [17] as the catecholamines present do not interfere with the assay of protein by this method [18]. There was no interference from Percoll on the color development, but when Percoll was present the color was read at exactly 2 min to avoid interference from the slowly developing light scattering.

Results

Rate zonal centrifugation of the total population of chromaffin granules in hypertonic sucrose media

Using the simplified technique of Winkler [7] it is seen from Fig. 1 that the pellets are considerably enriched in noradrenalin when the dense sucrose medium contains a high concentration of the solute. However, the cross-contamination of adrenalin was in all experiments found to be >25%, and only 5—10% of the catecholamines were recovered (figure not shown).

Purification of the heavy population of chromaffin granules by density gradient centrifugation

The heavy population of chromaffin granules obtained by differential centrifugation, which is contaminated by mitochondria, lysosomes, microbodies and some microsomes, was further purified by isopycnic density gradient centrifugation on a self-generating gradient of colloidal silica particles (Percoll). Starting with 50% (v/v) of Percoll in 0.5 M sucrose, pH 7.2 (standard procedure), two main bands, each with a small satellite band (see below), were observed by the naked eye and the light-scattering profile (λ = 510 mm) of the collected gradient confirmed this pattern (Fig. 2A). Based on measurements of catecholamines and malate dehydrogenase activity in fractions collected from

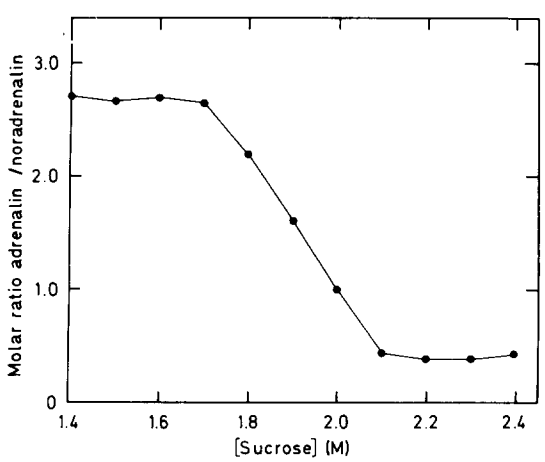

Fig. 1. Effect of sucrose concentration on the molar ratio of adrenalin to noradrenalin in chromaffin granules recovered as a pellet on rate zonal centrifugation. For experimental details, see text.

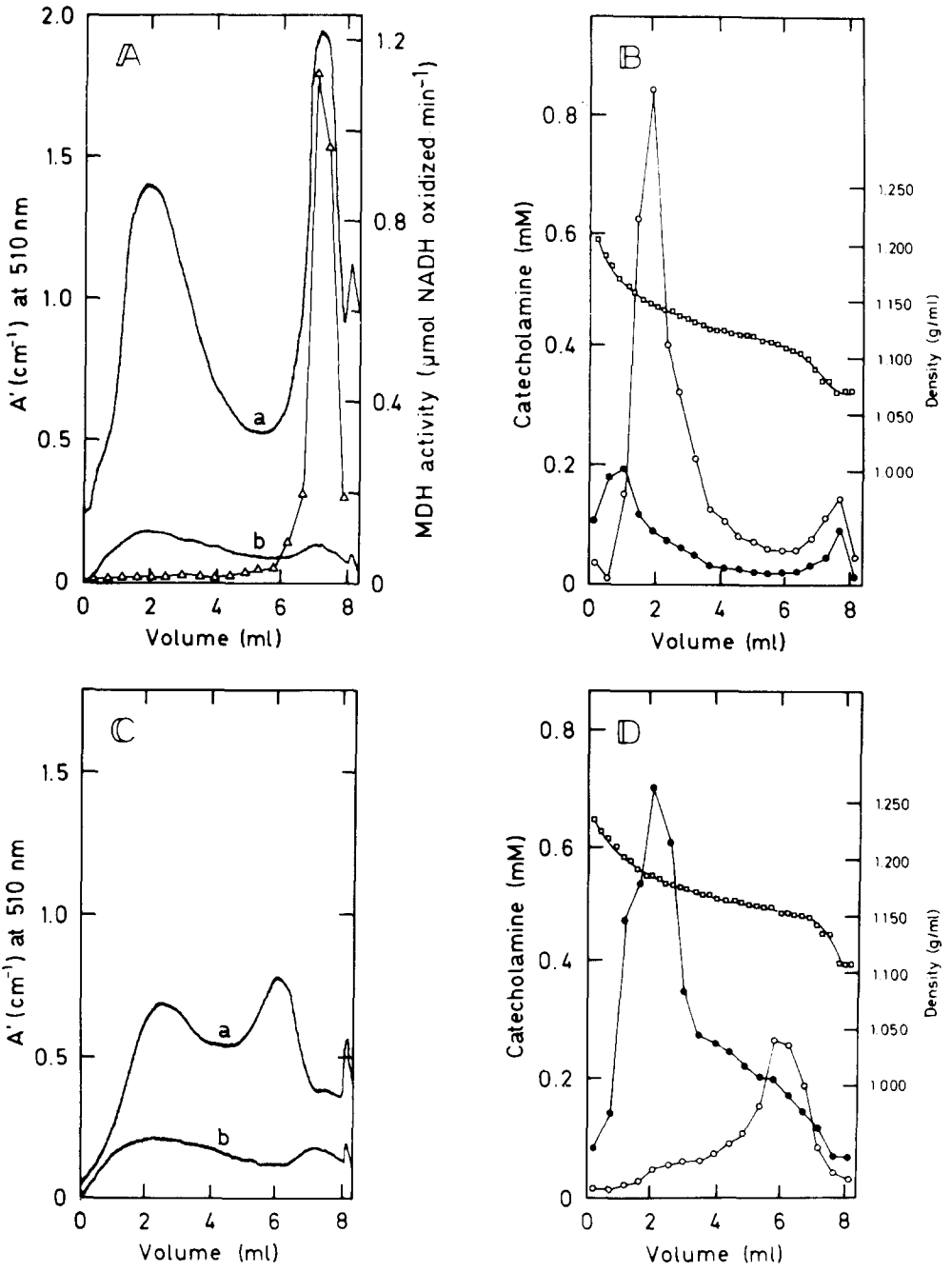

Fig. 2. Distribution of biochemical markers after density gradient fractionation of a crude population of heavy chromaffin granules (A and B) and of a noradrenalin-enriched population of heavy chromaffin granules (C and D). For experimental details, see text. ———, light scattering of the subcellular particles (a in A and C) and of the colloidal silica particles alone (b in A and C); △———△, malate dehydrogenase activity (A); ●———●, noradrenalin (B and D); ○———○, adrenalin (B and D); □———□, density (B and D).

the gradient (Fig. 2A) it is seen that the major fraction of chromaffin granules ($\overline{\rho}$ = 1.145 g · ml^{-1}) is completely separated from mitochondria ($\overline{\rho}$ = 1.079 g · ml^{-1}). Thus, less than 3% of the total malate dehydrogenase activity in the gradient was recovered in the lower 3/4 of the gradient and the activity was negligible in the peak catecholamine fraction. The small amount of microsomes in the preparation was found in the satellite band just above the mitochondrial band (data not shown). The profiles presented in Fig. 1 were found very reproducible from one preparation to the other.

From Fig. 2B it is also seen that noradrenalin bands at a slightly higher density (observed as a satellite band by the naked eye) than adrenalin (main band).

Isolation and characterization of noradrenalin granules

In order to improve the separation of granules containing noradrenalin and adrenalin, the Percoll concentration of the standard procedure was slightly increased to give a smoother density gradient profile in the critical region ($1.150 < \overline{\rho} < 1.200$). The first 0.85 ml of seven identical gradients (as in Fig. 1A and B) of the standard procedure, i.e. a total of 5.95 ml gradient material containing 33% of the noradrenalin and 4% of the adrenalin, was mixed with 50% (v/v) Percoll and 0.5 M sucrose, pH 7.2, to give a final volume of 8.2 ml, and centrifuged as described above. From Fig. 2C and D it is seen that the chromaffin granules were now separated into two bands. The lower band ($\overline{\rho}$ = 1.185 g · ml^{-1}) contained almost exclusively (approx. 95%) noradrenalin (Fig. 3B) whereas the upper band ($\overline{\rho}$ = 1.155 g · ml^{-1}) contained both adrenalin

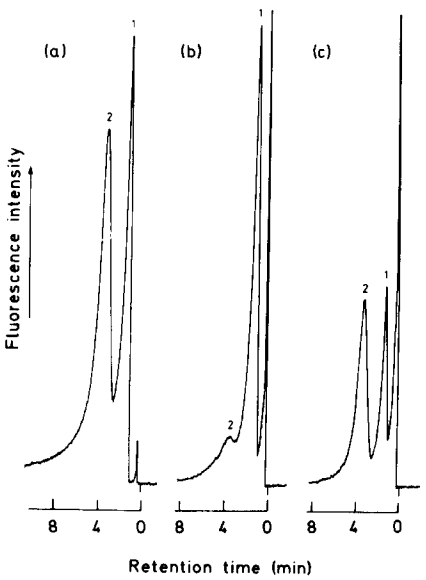

Fig. 3. HPLC fluorescence elution pattern of noradrenalin (peak 1, t_R = 1.0 min) and adrenalin (peak 2, t_R = 3.3 min) from a standard solution of the amines (a), from a diluted extract of either isolated noradrenalin granules (b) or isolated adrenalin-enriched granules (c). 20 µl of the diluted (16 times) extract of chromaffin granules were injected into the liquid chromatograph; λ_{ex} = 283 nm and λ_{em} = 313 nm. For experimental details, see text.

TABLE I

THE CONTENT OF CATECHOLAMINES AND ADENINE NUCLEOTIDES IN NORADRENALIN GRANULES AND IN THE TOTAL POPULATION OF HEAVY CHROMAFFIN GRANULES

	μmol · mg protein^{-1}			Molar ratio [(Catecholamines)/ (Adenine nucleotides)]
	Noradrenalin	Adrenalin	Adenine nucleotides *	
Noradrenalin granules	6.74	0.42	1.54	4.7
	7.98	0.35	1.66	5.0
	6.25	0.04	1.38	4.6
Heavy granules	1.15	2.40	0.28	12.7
	0.88	2.34	0.30	10.7
(total population) **	1.19	2.50	0.30	12.3

* ATP + ADP.

** The amount of catecholamines recovered represents approx. 75% of that present in the crude preparation of heavy chromaffin granules.

and noradrenalin (Fig. 3C). In a series of preparations of noradrenalin granules (Fig. 2 and Table I) the noradrenalin content was found to vary from 94 to 99% ($\bar{x}$ = 96%, n = 4), and the recovery in this step was approx. 50%.

Electron microscopy of the noradrenalin granules revealed a very pure population of particles with no detectable contamination by mitochondria, lysosomes, peroxysomes and fragments of endoplasmic reticulum (Fig. 4). The granules were all highly electron dense and revealed a more narrow size distribution (Fig. 5) than the total population of chromaffin granules [19].

From Table I it is seen that the noradrenalin granules have a content of catecholamines and adenine nucleotides which is approx. two and five times, respectively, higher than in the total population of heavy chromaffin granules. Hence, the molar ratio of catecholamine to adenine nucleotides average 4.8 : 1 and 11.9 : 1 for the noradrenalin granules and the total population of heavy

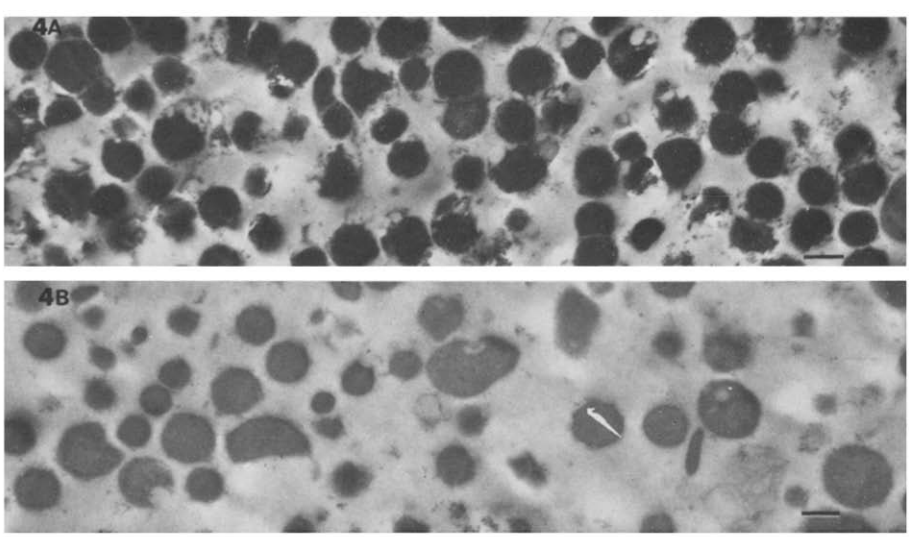

Fig. 4. Electron micrographs of isolated noradrenalin granules (A) and adrenalin-enriched granules (B). Scale bar represents 0.2 μm.

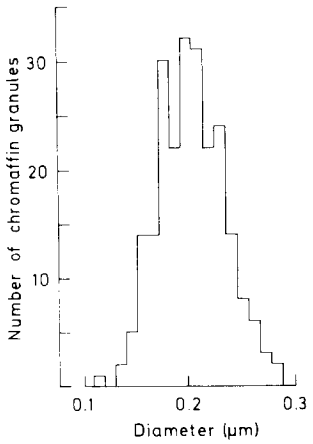

Fig. 5. Size distribution of isolated noradrenalin granules measured from transmission electron micrographs. The diameter was found to be 0.216 ± 0.142 μm (mean ± S.D; n = 229).

granules, respectively. Almost identical ratios were obtained when the nucleotides were assayed following extraction by perchloric acid, i.e. 4.9 : 1 and 14.1 : 1, respectively. It should be stressed that AMP contributed <4% to the content of adenine nucleotides in the total population of chromaffin granules and <1% in the population of noradrenalin granules.

Discussion

Although populations of chromaffin granules enriched in noradrenalin have been obtained by rate zonal centrifugation in sucrose density gradients [5—7], the poor yield and the significant cross-contamination by adrenalin-enriched granules when applied to bovine adrenal medulla (Fig. 1), suggest the need for development of an improved method for the isolation of the noradrenalin granules in a more highly purified form. In the present study, advantage has been taken of our recent finding [8] that the total population of chromaffin granules of the bovine adrenal medulla contain two major, rather distinct populations of storage granules with different $\overline{S}$ values, i.e. heavy population and a light population. The heavy population was the more homogeneous of the two [8], and this population was therefore selected for the isolation of noradrenalin granules. In the present study, this population has been further resolved by isopycnic density gradient centrifugation using a self-generating gradient of colloidal silica (Percoll). Two distinct bands of particles were obtained, one containing on average (n = 4) 96% noradrenalin and the other was highly enriched in adrenalin. The isolated population of noradrenalin granules appeared highly electron dense in transmission electron microscopy, in good agreement with the prediction made by Coupland and Hopwood [4] based on electron microscopy of intact cells. A more detailed comparative study of the two types of granules is now possible, but already at this stage we have found that the noradrenalin granules have a higher content of amine (by a factor of two) and adenine nucleotides (by a factor of five) on a mg protein basis than do the total population of heavy granules. Since this population

largely consists of adrenalin-enriched granules (see Fig. 2B), our data (Table I) point to a difference in the matrix complex of the two types of granules. The molar ratio of catecholamine to adenine nucleotides (ATP + ADP) found for the noradrenalin granules, i.e. 4.8 : 1, is close to the value of 4 : 1 originally reported by Hillarp [20] and others [21-23] for the total population of bovine adrenal chromaffin granules. On the other hand, for the total population of heavy chromaffin granules we have found a ratio as high as 11.9 : 1, which is closer to the value of 7.5 : 1 recently reported for the total population of chromaffin granules [15] and the ratio of 7.3 : 1 found for bovine splenic nerve vesicles [24]. Although it appears to be premature to discuss the significance of these findings in terms of physicochemical properties of the storage complexes in the matrix of different subpopulations of chromaffin granules, our findings are in good agreement with recent ^{1}H NMR studies [25]. Although part of the catecholamines are complexed with ATP inside the chromaffin granules, neither the binary nor the ternary complexes in the presence of metal ions can account for the storage of high concentrations of catecholamines [25].

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