Biochimica et Biophysica Acta, 464 (1977) 53—64

BBA 77554

SOME PHYSICAL PROPERTIES OF ADRENAL MEDULLA CHROMAFFIN GRANULES ISOLATED BY A NEW CONTINUOUS ISO-OSMOTIC DENSITY GRADIENT METHOD

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(Received May 21st, 1976)

Summary

Centrifugation of crude preparations of chromaffin granules on iso-osmotic continuous gradients of sucrose and metrizamide produce granule fractions of high purity without pelleting.

These granules have a buoyant density of 1.123 at 300 mosM, which increases to 1.222 at 1800 mosM.

Exchange of water for ^2H_2O greatly increases the density of the granules. An apparent water space for the granules can be calculated from the difference in density in ^2H_2O and water. The water space of 0.63 at 300 mosM diminishes as the osmolality of the suspension medium is increased, suggesting that the granule membrane is a good osmometer and that the core contains a large osmotically active water space.

Other separation methods for chromaffin granules are discussed in terms of these results.

Introduction

Methods for the purification of adrenal medulla chromaffin granules have recently been reviewed by Winkler and Smith [1]. Although it is possible to partially purify granules by filtration [2,3], the purest fractions are obtained by the use of continuous or discontinuous sucrose gradients [4,5] which produce granules of high catecholamine and ATP content and low mitochondrial and lysosomal contamination. However, these granules are quite dehydrated by the high osmolality sucrose (1.6—2.0 M) in which they equi-

Abbreviations: HEPES, 2-(*N*-2-hydroxyethyl-piperazin-*N'*-yl)-ethanesulfonic acid; INT, 2-(*p*-iodophenyl)-3-*p*-nitrophenyl tetrazolium · HCl.

librate [6,7] and tend to lyse when the sucrose concentration is readjusted to isotonicity [8,9].

To reduce the damage caused by osmotic pertubations, Trifaró and Dworkind [3] developed a purification method based on a sucrose/Ficoll/^2H_2O step gradient which is iso-osmotic at 300 mosM. It has the disadvantage that the granules are pelleted at the bottom of the tube and are coated with a layer of mitochondria. The purity of these granules is therefore quite variable.

Iso-osmotic continuous gradients formed from glycogen [6] or Ludox silica gel [7] suspended in constant concentrations of sucrose have been employed to purify granules, but both gradients give poor separations from mitochondrial contaminants. In addition the high molecular weight of the gradient-forming material leads to rapid distortion of this type of gradient [10] thereby varying the position of the bands in the gradient.

The method reported here employs iso-osmotic gradients of sucrose and metrizamide. Metrizamide solutions have a much higher density and lower viscosity than the equivalent weight/volume solution of Ficoll. In addition metrizamide osmotic pressure is a linear function of concentration as opposed to Ficoll, which although it has a low osmolarity at low concentrations shows an exponential rise with increasing concentration [11].

Our results show that the new method can produce granules of greater purity than the Ficoll/^2H_2O method without pelleting the granules. Experiments with ^2H_2O as a solvent for these gradients show that ^2H_2O is rapidly exchanged for water in the granules and suggest an explanation for the basis of the Ficoll/^2H_2O and continuous sucrose gradient methods of separation.

Materials and Methods

Metrizamide is produced by Nyegaard, Oslo and was purchased from Molter, GmbH, Heidelberg. HEPES (2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)-ethanesulfonic acid)), Amido Schwarz and ^2H_2O were purchased from E. Merck, Darmstadt; [1-^{14}C] tyramine from Amersham Buchler, Braunschweig; phenolphthalein β-glucuronide, luciferin-luciferase and INT (2(*p*-iodophenyl)-3-*p*-nitrophenyl tetrazolium · HCl) from Sigma, Munich and sodium succinate from Boeringer, Mannheim. All other chemicals were reagent grade and were used without further purification.

The experimentally observed non-linearity between osmotic pressure and molality of sucrose solutions is due to the non-ideality of sucrose as a solute [12]. Conversion of molarity to osmolality for sucrose can be calculated from the equation: osmolality = $M/(1 - VM)\rho s$ [13] where M = molarity ρs = density of the solvent and $V = 0.3087$ rather than the sucrose specific volume = 0.215 (Schultens, H.A., unpublished). Metrazamide osmolality shows a linear dependence on concentration which varied about 5% over four batches. For this work the osmolalities of the sucrose and metrazamide solutions were measured directly on a Knauer semi-micro freezing point depression osmometer. For convenience solutions are identified in milliosmolality (mosM) but recipes are given in terms of w/v. Density and refractive index at 0—1°C (λ = 589 nm) are also listed.

Bovine adrenal glands were obtained from a local slaughter house and placed

on ice within 30 min of the death of the animals. Fractionation was started within 1.5 h of death. The medullae were minced and homogenized in 0.26 M sucrose, 10 mM HEPES, pH 7.2 (buffered sucrose), centrifuged at 1000 × *g* for 10 min at 2° C and the pellet (P_1) discarded. The resulting supernatant (S_1) was centrifuged 20 min at 27 000 × *g* at 2° C. The upper layer of the resulting pellet (mostly mitochondria) was washed away with buffered sucrose and the resulting pink pellet (P_2) was resuspended in buffered sucrose and recentrifuged. The resulting pellet was once again washed to remove mitochondria and the final (P_3) pellet resuspended in buffered sucrose and loaded onto the density gradients.

Linear iso-osmotic gradients of sucose and metrizamide were formed in "lusteroid" tubes for centrifugation in a Beckman SW41 Ti rotor. For the 300 mosM gradient described below, the gradients were generated from 300 mosM sucrose (8.95 g/100 ml, η_D = 1.3492, ρ = 1.036) and 300 mosM metrizamide (Batch No. 505069, 36 g/100 ml η_D = 1.3938, ρ = 1.208). Osmolarities were measured by freezing point depression using a Knauer Semi Micro Osmometer. Approx. 1.5 ml of the crude granule pellet resuspended in 300 mosM sucrose was layered atop the gradients and centrifuged for 90—120 min at 32 500 rev./min at 1—2° C (129 600 × g_{ave}). Preliminary experiments showed that the optical pattern and the distribution of markers did not change appreciably after 90 min of centrifugation. For comparative purposes granules were purified on sucrose step gradients and Ficoll/sucrose/^2H_2O step gradients [3]. The first of these gradients consisted of 2.0 ml of resuspended granules layered atop of a single step of 10 ml 1.6 M sucrose and centrifuged in the SW41 Ti rotor as above; the fractionation scheme is presented in Fig. 1B. The second type of

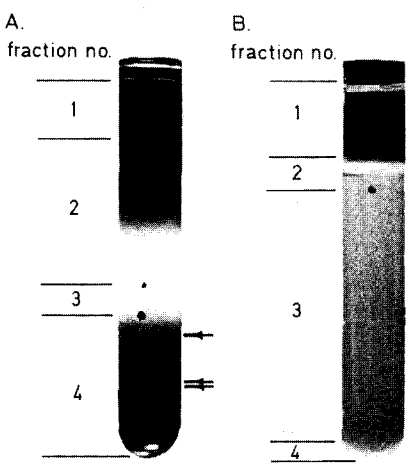

Fig. 1. Fractionation scheme for (A) 300 mosM continuous sucrose/metrizamide gradients and (B) discontinuous sucrose step gradients described in Materials and Methods. Tubes were marked by eye with a felt pen then cut in a tube slicer and the fractions collected as noted. The lines and shadows at the bottom of A are artifacts from the plexiglas holder used for photography. Fraction 1 of both gradients includes most of the original sample volume. Fractions 2 and 3 of A were divided arbitrarily but the lower boundary of fraction 3 was set above the faint band (marked with an arrow) which is probably the bulk of the lysosomes. The lowest band (double arrow) is the mitochondria. Fraction 2 of B is the material at the 0.26/1.6 M sucrose interface. Fraction 4 of B is the pellet, which was resuspended in 1.6 M sucrose.

gradient was prepared by dissolving 19.5 g Ficoll (Batch No. 6580) and 8.95 g sucrose in 100 ml of HEPES-buffered ^2H_2O (w/v). 6.5 ml of this mixture was placed in polycarbonate "Oakridge" type centrifuge tubes for the Beckman AH65 rotor. 1.5 ml of the resuspended crude granule pellet were layered atop the gradient and centrifuged for 60 min at 40 000 rev./min ($\approx 102\,000 \times g_{ave}$) at 1—2° C. Fractionation of this gradient followed the scheme of Trifaro and Dworkind [3].

Fractionation of the iso-osmotic sucrose/metrizamide gradients was performed in two ways: Fractionation method I consisted of piercing the bottom of the tube and collecting approx. 25 5-drop fractions. To control the flow, air was pumped into the gradient via a silicone rubber tube which was passed through an LKB Vario Perpex pump model 1200-2 (flow rate approx. 1 ml/min) connected to a rubber stopper which had been placed into the top of the gradient tube before the tube was pierced. For fractionation method II, the tubes were removed and marked by eye, as noted on Fig. 1A then fractionated with a tube slicer. Fraction No. 3, previously judged to be the ATP-rich succinate dehydrogenase, β-glucuronidase monoamine oxidase-poor region of the gradient can be identified as the area just above the sharp opaque line and below the diffuse lightly opalescent area (Fig. 1A).

Protein was assayed by the Campbell [14] modification of the Amido Schwarz method of Schaffner and Weissmann [15]. ATP, taken to be a positive marker for the granules, was measured by the luciferin-luciferase method as modified by Dowdall et al. [16].

Monoamine oxidase, a positive marker for the mitochondrial outer membrane and succinate dehydrogenase, marking the mitochondrial inner membrane were measured by the methods of Wurtman and Axelrod [17] and Pennington [18], respectively.

β-Glucuronidase, assayed by the method of Gianetto and de Duve [19], was used as a marker for lysosomes.

Ultra thin sections of a pellet of sucrose/metrizamide fraction 3 were prepared as described previously [20] and examined in a Siemens Ia electron microscope.

Results

Fig. 2 summarizes the results of the separation of mitochondria and chromaffin granules on iso-osmotic density gradients. As can be seen, the mitochondria band out below the granules at low osmolalities but become relatively less dense than the granules at osmolalities greater than approx. 800 mosM. The densities of the components noted on the figure are results of single representative gradients. Selected results of these experiments as well as data from other studies are presented in Table I.

The distributions of protein, ATP, monoamine oxidase, succinate dehydrogenase and β-glucuronidase in the 300 mosM gradient fractionated by method I are presented in Fig. 3. It is possible to select fractions of very high purity using this method. However, yields are small and there is the danger of selecting subpopulations of granules (c.f. below).

High yields of granules can be collected from the continuous gradient by

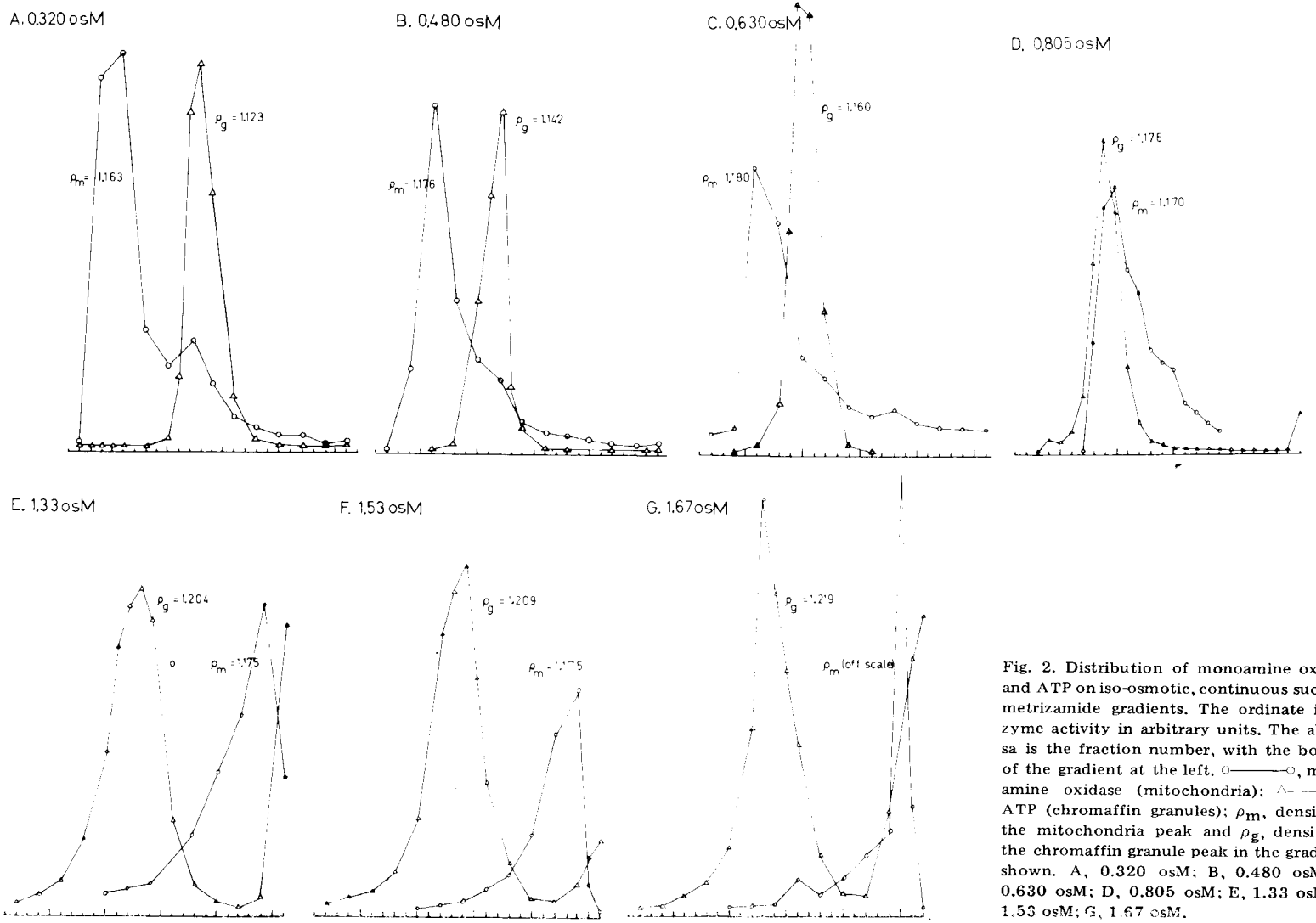

Fig. 2. Distribution of monoamine oxidase and ATP on iso-osmotic, continuous sucrose/metrizamide gradients. The ordinate is enzyme activity in arbitrary units. The abscissa is the fraction number, with the bottom of the gradient at the left. ○———○, monoamine oxidase (mitochondria); △———△, ATP (chromaffin granules); ρ_m, density of the mitochondria peak and ρ_g, density of the chromaffin granule peak in the gradients shown. A, 0.320 osM; B, 0.480 osM; C, 0.630 osM; D, 0.805 osM; E, 1.33 osM; F, 1.53 osM; G, 1.67 osM.

TABLE I

VARIATION OF CHROMAFFIN GRANULE DENSITY WITH OSMOTIC PRESSURE

Reference	Gradient		Solvent	Osmolality (mosM)	Mitochondria density (ρ)	Chromaffin granules	
	Type	Composition				Density (ρ)	Water space ($^{v}H_2O$)
6	continuous	sucrose/glycogen	water	300	1.079	1.120 [1]	0.67
						1.123 [2]	
7	continuous	sucrose/Ludox	water	350	~1.17	≈1.17	
	continuous	sucrose/metrizamide	water	280	1.163	1.119	
				300		1.123	
				530		1.156	
				790		1.175	
				1200		1.203	
				1800		1.222	
			water : $^{2}H_2O$ 38 : 62	280		1.158	0.63 [3]
				530		1.192	0.58 [3]
				790		1.214	0.56 [3]
3	step	sucrose/Ficoll	$^{2}H_2O$	300			
6	continuous	sucrose	water	≈2430 [4]	1.180	1.213	
			$^{2}H_2O$	≈1960 [4]	1.212	1.265	

[1] Catecholamine peak.
[2] Dopamine-β-hydroxylase peak.
[3] Calculated from Eqn. 4.
[4] Calculated from de Duve et al. [13] using the granule densities reported in ref. 6.

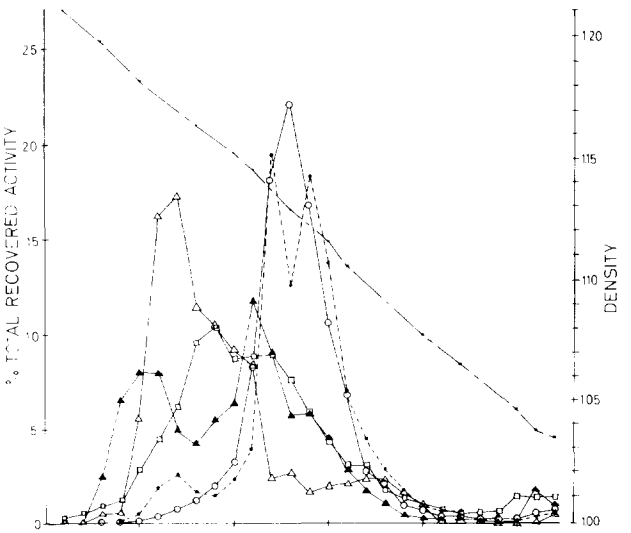

Fig. 3. Distribution of various enzymes, ATP and protein on the 0.300 osM iso-osmotic sucrose/metrizamide gradients. The abscissa is the fraction number, with the bottom of the gradient at the left. •— — —•, density; •- - - - - -•, protein; ○———○, ATP; □———□, β-glucuronidase; △———△, succinate dehydrogenase; ▲———▲, monoamine oxidase.

means of a tube slicer (method II). This can be done quite rapidly and reproducibly. Fig. 4 gives the results of this type of fractionation from all three sorts of gradients tested. Results are presented in terms of percentage recovery rather than specific activity since the latter is biased by the protein concentration; e.g. relatively high total contamination of the granule fractions would have relatively low specific activity due to the large amount of granule protein. We report standard deviations of the mean rather than the standard error of the mean (S.E. = S.D. $\cdot \sqrt{n}$) to give a better idea of the variability of the method.

Higher ratios of granules to contaminants may be obtained by raising the lower boundary of fraction 3. The boundaries for this study were deliberately chosen to be wide enough to include approx. 70% of the recovered ATP. This approaches the ATP recovery from the Ficoll/^2H_2O-purified granules and shows the significantly higher purity of the sucrose/metrizamide granules. As can be seen, collection of sucrose/metrizamide gradient fractions 2 + 3 together give granules of lower contamination than the fraction 5 Ficoll/^2H_2O gradient granules (Fig. 4).

The separation on the sucrose/metrizamide gradient might be improved somewhat by running a shallower gradient starting with e.g. a 300 mosM mixture of 65% sucrose/35% metrizamide ($\rho \approx 1.10$) and ending with 300 mosM metrizamide, thus spreading out the ATP peak and perhaps better resolving the distribution of contaminants. However, this precludes the use of gradients for studying the shift in the density of the granules as they release their stores of catecholamines, ATP and protein and become ghosts [20].

Although the mitochondrial and lysosomal markers are denser than the granules at 300 mosM, succinate dehydrogenase, β-glucuronidase and to a lesser extent monoamine oxidase all show second peaks in the lower region of the

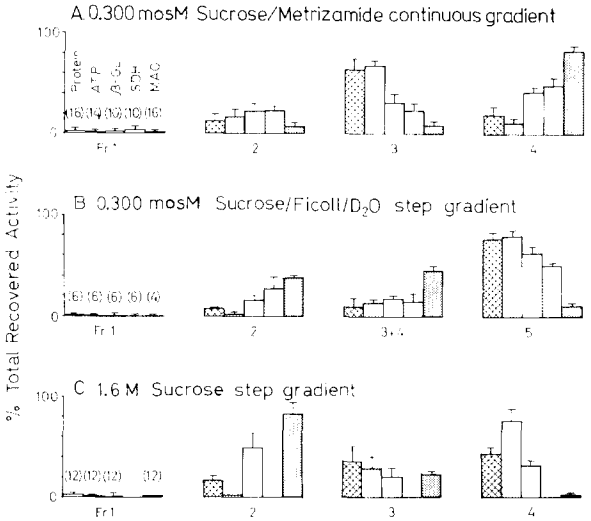

Fig. 4. Distribution of protein, ATP and various enzymes from (A) 0.300 osM sucrose/metrizamide gradients, (B) 0.300 osM discontinuous sucrose/Ficoll/^2H_2O gradients and (C) discontinuous sucrose step gradients fractionated as described in Fig. 1 and Materials and Methods. Activities of the P_3 crude granule starting material (mean ± S.D. (No. of experiments): protein, 3.16 ± 0.94 (8) mg/ml; ATP, 1.63 ± 0.38 (8) nmol/ml; monoamine oxidase, 1.11 ± 0.29 (8) μg tyramine converted/ml per h; succinate dehydrogenase, 335 ± 156 (3) μg neotetrazolium reduced/ml per h; β-glucuronidase, 264 ± 152 (5) μg phenolphthalein liberated/ml per h. Recoveries of activities compared to the starting material varied from 67 to 108%. These values were normalized to 100% to aid comparison of results. Error bars are ± S.D. The number of gradients analyzed is in brackets.

ATP peak which suggests that there is a fraction of both lysosomes and mitochondria which are bound to chromaffin granules (Fig. 3). This seems to be more pronounced for the mitochondrial inner membrane than for whole mitochondria. Similar results were found for the Smith and Winkler [1] type of sucrose step gradient which produced granules with the least monoamine oxidase contamination. However, about the same amount of contaminating β-glucuronidase as the sucrose/metrizamide gradient was found. Inclusion of 0.1 mM EDTA or 20 mM KCl in the gradient solutions gave distinctly poorer separations which implies that the contamination is due neither to aggregation by divalent ions [4] nor to non-specific absorption due to low ionicity.

A sample from the sucrose/metrizamide fraction 3 was fixed, centrifuged, stained and embedded for electron microscopy as described separately and the thin sections were cut so that the pellet could be scanned from top to bottom in the electron microscope [20]. Fig. 5 is a photomontage of six approximately equally spaced fields of this pellet. The granules appear closely packed and somewhat flattened due to the high *g*-force (150 000 × *g* for 1 h), otherwise they are similar in appearance to granules taken from continuous sucrose gradients [4]. Only three mitochondria profiles compared to approx. 5000 granule profiles were observed.

It may be noted that granules prepared by the sucrose/metrizamide method show the same morphologic changes when subjected to increased [Ca^{2+}] [21] as seen previously for granules isolated from continuous sucrose gradients [4]. Metrizamide does not affect the ability of the granule ghost to respond to

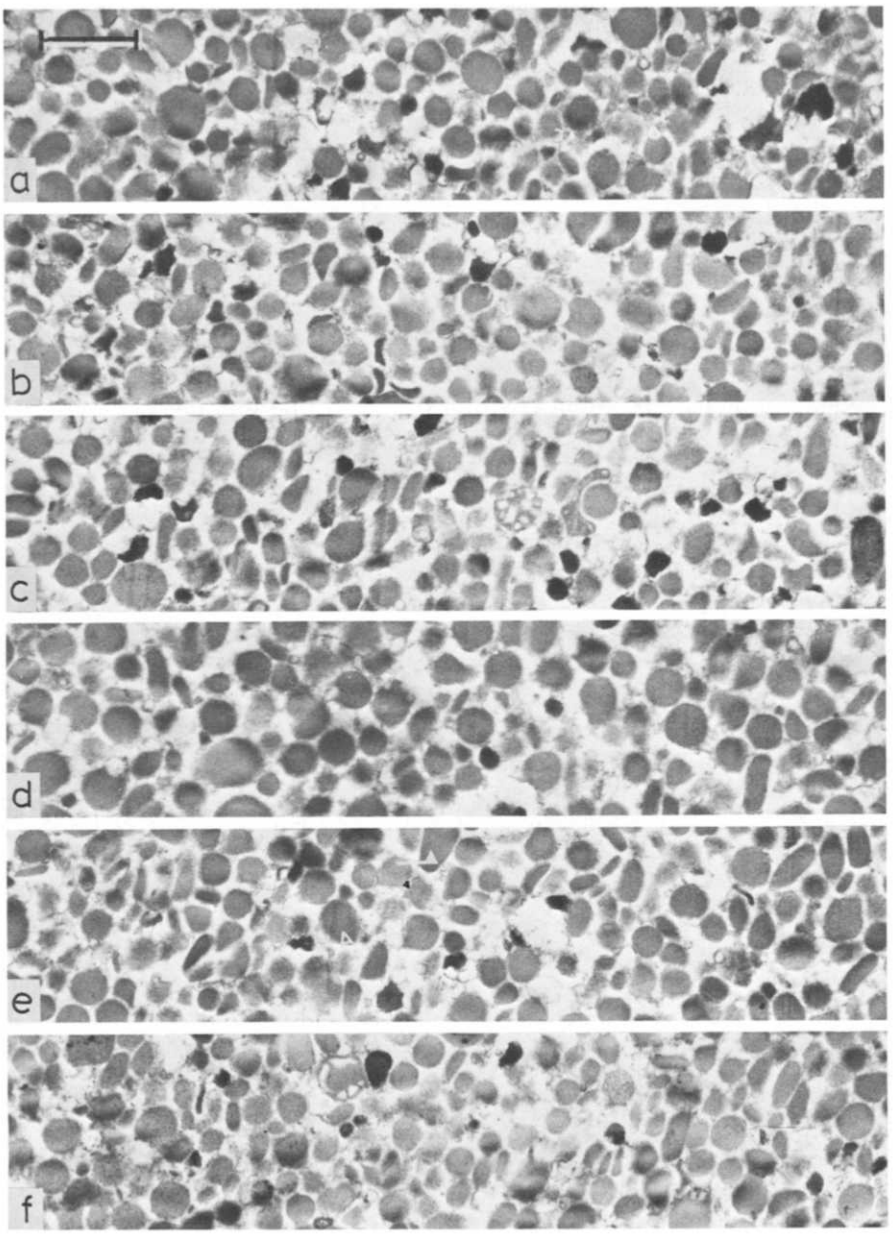

Fig. 5. Electron micrographs of sucrose/metrizamide fraction [3]. The photos represent fields varying from the top (a) to the bottom (f) of the pellet. See text for details. Bar = 1 μm.

changes in osmotic pressure [22] nor to bind calcium, magnesium or lanthanide ions (Morris, S.J. and Schober, R., unpublished). Systematic study of the effect of metrizamide on granule enzymes have not yet been done.

The effects of ^2H_2O on the density of granules and mitochondria were assessed by centrifugation of P_3 material on iso-osmotic gradients of sucrose/metrizamide using approx. 62% ^2H_2O as a solvent. The results which are quali-

tatively similar to those for H_2O gradients (Fig. 2) are presented in Fig. 6 and Table I. It was deemed from the distribution of ATP and monoamine oxidase that such gradients offered no advantage over their H_2O counterparts as a separation method.

It is possible to calculate the water space of the granules to a first approximation from the water and ^2H_2O gradient data. Assuming density to be an additive property [23],

$$\rho_{g_{H_2O}} = \rho_m v_m + \rho_c v_c + \rho_{H_2O} v_{H_2O} \quad (1)$$

and

$$v_{H_2O} + v_m + v_c = 1 \quad (2)$$

where ρ = density and v = volume fraction of the whole granule (g), external membrane (m), non-water core material (c), and core water (H_2O), respectively in H_2O. When H_2O is replaced by a mixture of H_2O and ^2H_2O:

$$\rho_{g_{mix}} = \rho_m v_m + \rho_c v_c + \rho_{mix} v_{H_2O} \quad (3)$$

Assuming that the presence of ^2H_2O causes no change in volume fractions or densities of non-water components and subtracting Eqn. 3 from Eqn. 1 yields:

$$v_{H_2O} = \frac{\rho_{g_{mix}} - \rho_{g_{H_2O}}}{\rho_{mix} - \rho_{H_2O}} \quad (4)$$

for these experiments $\rho_{mix} - \rho_{H_2O} = (1.062 - 1.000) = 0.062$. The accuracy of the v_{H_2O} determination is dependent upon the accuracy of the density determination of the constituents. Since the density measurements reported here are accurate to approx. 1%, whereas the shift in density is only 6%, this method

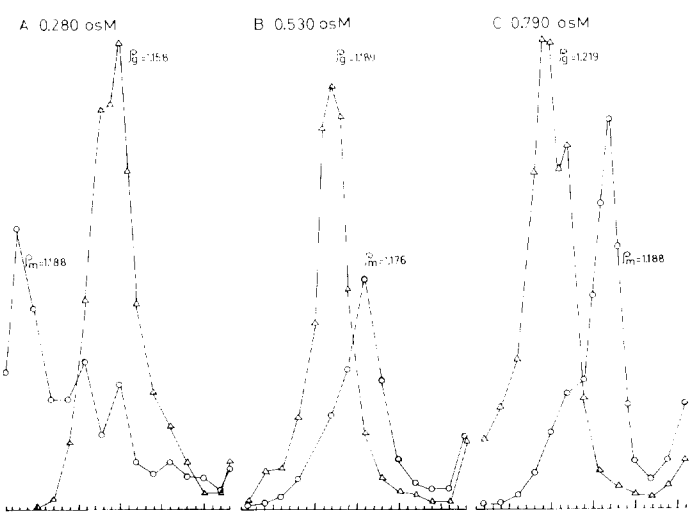

Fig. 6. Distribution of monoamine oxidase and ATP on iso-osmotic sucrose/metrizamide gradients using 38% water/62% ^2H_2O as a solvent. The ordinate is enzyme activity in arbitrary units. The abscissa is the fraction number, with the bottom of the gradient at the left. ○———○, monoamine oxidase; △———△, ATP; ρ_m, mitochondria peak density and ρ_g, chromaffin granule peak density for the gradients shown. A, 0.280 osM; B, 0.530 osM; C, 0.790 osM.

provides only a first approximation of the water space. However, since the denominator of Eqn. 4 remains constant the trend in the data (Table I) suggests that the granules have a large water space which diminishes as the particles dehydrate in response to increasing osmotic pressure.

Discussion

Although the use of continuous or step sucrose gradients produced granules with lower mitochondrial contamination, the iso-osmotic sucrose/metrizamide did as well or better than both sucrose and Ficoll/^2H_2O gradients in removing contaminating lysosomes, and was far superior to the Ficoll/^2H_2O gradient in removing both contaminants. In addition, the granules are not pelleted in the sucrose/metrizamide; therefore they need not suffer the mechanical abrasion required to resuspend them.

Granule fractions of high purity can be obtained from the continuous gradient by dropwise fractionation. Yields are very small, however, and there is the danger of selecting subpopulations of granules which are enriched in catecholamines compared to dopamine-β-hydroxylase [24,25] or adrenaline compared to noradrenaline [26,27]. However, such fractionation offers an excellent analytical tool for investigation of such effects, or establishing the presence in the granules of putative enzyme activities.

Bulk fractionation using a tube slicer provides large quantities of reasonably pure granules which, never having been subjected to hyper-osmotic dehydration, make excellent starting material for physical and biochemical studies.

As can be seen in Table I, increasing the osmotic pressure of the granule suspension medium also increases the granule density. At high osmolalities the granule density approaches that achieved in continuous sucrose density gradients. The results of this study supports the contention of Laduron [6] and Lagercrantz et al. [7] that this phenomenon is due to loss of water from the granules by dehydration. Thus the granules placed on a continuous sucrose gradient dehydrate as they move down the gravitational field. The particle stops its movement down the gradient at the point where further removal of water from the particle cannot compensate for increased density of the medium [13]. The difference between the densities of the granules on water and ^2H_2O continuous gradients is discussed by de Duve et al. [13].

Trifaró and Dworkind [3] suggest that the basis of the iso-osmotic Ficoll/sucrose/^2H_2O separation is that the Ficoll layer ($\rho \approx 1.205$) is less dense than the granules in a continuous sucrose gradient but slightly denser than the mitochondria (cf. Table I). However, as noted above the high density of granules isolated on sucrose gradients is due to dehydration; granules at 300 mosM are considerably less dense than the Ficoll/sucrose/^2H_2O mixture.

Extrapolating from the work of Laduron [6] and Lagercrantz et al. [7], Winkler and Smith [1] assume that the granules have a large water space and suggest that ^2H_2O/water exchange could account for the pelleting of the granules in the 300 mosM Ficoll/^2H_2O. Our experiments using 62% ^2H_2O solvent give a water space of approx. 0.63 for the granules at this osmolarity which is in good agreement with Hillarp's [29] value of 0.685, Laduron's [6] value of 0.67 or Kirshner et al.'s [30] value of 0.60. Complete exchange of this

space for ^2H_2O would give a granule density of approx. 1.19. Laduron's figure of 0.67 yields $\rho_g = 1.192$.

Therefore water/^2H_2O exchange in the osmotic space alone cannot account for the pelleting of the granules at the bottom of the Ficoll/sucrose/^2H_2O layer. It is possible that non-osmotically active water which is easily exchangeable for ^2H_2O could account for the remaining density change. Another plausible explanation is that enough Ficoll binds to the surface of the granules to shift their density to something greater than 1.205.

Ludox would not be expected to penetrate the granules or mitochondria. Therefore the increase in the density of both particles seen by Lagercrantz et al. [7] probably is due to adsorption of the silica gel to the particles. Such adsorption has been reported in the isolation of other subcellular organelles on similar gradients [31,32].

Acknowledgement

We wish to thank Dr. R. Schober, Max-Planck-Institute for Brain Research, Frankfurt, for the electron micrographs.

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