Subcellular localization of noradrenaline and ATP in C_{1300} mouse neuroblastoma[1]

N.H. Fraeyman, W.P. De Potter and A.F. De Schaepdryver

Heymans Institute of Pharmacology, Ghent University Medical School, De Pintelaan 135, B-9000 Ghent (Belgium), 23 February 1978

Summary. A density and velocity gradient centrifugation study of C_{1300} mouse neuroblastoma showed that ATP is nearly absent from noradrenaline-containing granules and is mainly localized in mitochondria, suggesting that in this tissue ATP is not involved in the storage of noradrenaline.

In the adrenal medulla[2,3], as well as in adrenergic nerves[4], catecholamines are stored in so-called storage vesicles. Chemical analysis of the chromaffin granules revealed that the catecholamines are stored together with ATP in a molar ratio of about 4/1, a finding which has led to the storage complex hypothesis[3]. Whereas earlier work on noradrenaline (NA) vesicles containing fractions from bovine splenic nerves also revealed a similar ratio[5-8], more recent work indicated that these fractions were heavily contaminated with mitochondria. When this was taken into account, ratios of 7/12[9] or more[10] were found.

We thought it of interest to determine the molar catecholamines/ATP ratio in the C_{1300} mouse neuroblastoma catecholamine storage vesicles which have a similar equilibrium density to those from bovine splenic nerve[11], and which seem to correspond to large dense cored vesicles[12].

Materials and methods. C_{1300} mouse neuroblastoma tissue was taken 2 weeks after s.c. innoculation into A/J strain mice of both sexes, ranging from 4 to 10 weeks of age. The tissue was rinsed 3 times in ice-cold 0.25 M sucrose. Homogenization was carried out after a 1/4 (w/v) dilution in ice-cold 0.25 M sucrose buffered with Tris-HCl 5 mM pH 7.3. The homogenate was submitted to differential gradient centrifugation in 2 subsequent steps: 1st at 3000 g_{av} for 10 min (Sorvall), followed by 88,000 g_{av} for 45 min (Spinco L50). This yields 3 fractions: a) sediment 1, mainly unbroken material, nuclei and larger mitochondria, b) sediment 2 or microsomal fraction, and c) a supernatant.

Sediment 2 was subfractioned using 2 types of gradient centrifugation: 1. equilibrium density gradient centrifugation: sediment 2 was resuspended in a 0.45 M sucrose solution, layered on top of a 0.5-1.7 M sucrose linear gradient in 5 mM Tris-HCl pH 7.3 and centrifuged in a SW-40 rotor in a Beckman ultracentrifuge at 40,000 rpm (190,000 g_{av}) for 150 min; 2. differential or velocity gradient centrifugation: sediment 2 was resuspended in a 0.25 M sucrose solution, layered on top of a 0.3-0.8 M sucrose linear gradient in 5 mM Tris-HCl pH 7.3 with a cushion of 1 ml 2 M sucrose and centrifuged in a SW-40 rotor in a Beckman ultracentrifuge at 20,000 rpm (50,000 g_{av}) for 20 min. After centrifugation, fractions of 1 ml each were obtained by pumping a 2 M sucrose solution through the bottom of the tube. 12 fractions were collected and the following determinations performed on each fraction: proteins according to Lowry[13], NA as described by Laverty and Taylor[14], ATP following the method of Stanley and Williams[15], and monoamine oxidase [monoamine oxygen oxidoreductase (deaminating); EC 1.4.3.4, MAO] as described by Wurtmann and Axelrod[16]. The NA/ATP ratio (both substances expressed in nmoles/g tissue) was calculated in each substep of the purification procedure for NA-containing granules.

Results. The absolute amounts of proteins, NA, ATP and MAO in the total tissue homogenates as well as the percentual distribution in the fractions obtained by differential centrifugation are given in table 1. From this a mean value of 28 can be calculated for the NA/ATP ratio in NA-enriched sediment 2, as shown in table 2.

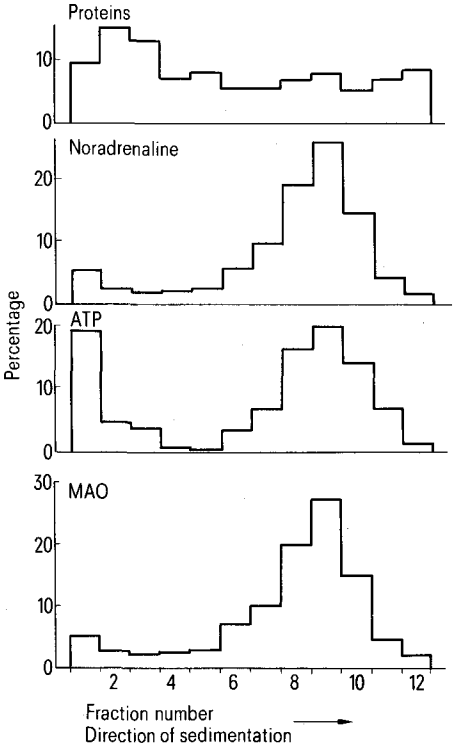

Fig. 1. Equilibrium density gradient centrifugation of a NA-enriched fraction of C_{1300} mouse neuroblastoma (n=3). The result of a typical experiment is presented.

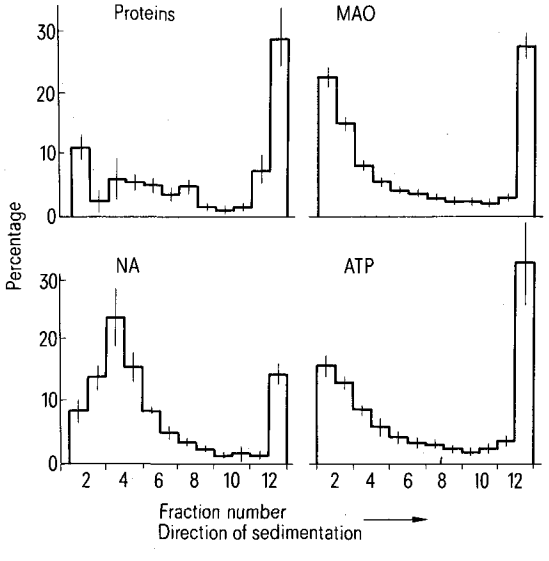

Fig. 2. Velocity gradient centrifugation of a NA-enriched fraction of C_{1300} mouse neuroblastoma (n=4). The result of a typical experiment is presented.

Table 1. Differential centrifugation data

	Protein	NA	ATP	MAO
Content	114.0±5.8	7.467±1.025	5.736± 0.327	0.538±0.091
Percentual distribution				
Sediment 1	51.6±0.9	17.1±3.0	61.0 ± 4.6	67.7 ±6.0
Sediment 2	8.8±0.9	64.5±2.1	3.3 ± 0.4	27.6 ±2.4
Supernatant	36.9±0.8	18.9±3.4	35.7 ± 3.1	4.5 ±1.9
Recovery (%)	92.4±5.3	64.0±8.3	89.0 ±10.2	90.5 ±7.9

Units. Proteins: mg/g tissue. NA: nmoles/g tissue. ATP: nmoles/g tissue. MAO: units/g tissue (1 unit = 1 µmole product/1 h incubation).

Table 2. NA/ATP ratios. Substances are expressed in nmoles/g tissue

	Tissue	Sediment 2	Density gradient	Velocity gradient
Ratio $\frac{NA}{ATP}$	1.3	25.3	31.7	111.4
Range	(1.0–1.6)	(24.4–32.9)	(27.6–36.8)	(95.5–131.4)

Equilibrium density gradient centrifugation of sediment 2 gives the distribution pattern shown in figure 1. The coincidence of NA and MAO, suggesting that NA granules and mitochondria band at the same density, does not permit conclusions concerning the NA/ATP ratio in the NA storage granules.

By submitting sediment 2 to velocity gradient centrifugation, a very satisfactory separation of NA granules and mitochondria was obtained, as shown in figure 2. Apart from the soluble ATP present in fraction 1 of the gradient, the largest amount of ATP accumulates, as MAO does, in fraction 12. As NA is mainly concentrated in fractions 2, 3 and 4 of the gradient, these fractions were used for the calculation of the NA/ATP ratio.

Discussion. The main problem arising with the kind of experiments described here is to obtain a good separation of NA vesicles and mitochondria. The differential centrifugation yields a vesicle preparation which contains 64% of the NA present in the C_{1300} mouse neuroblastoma and 27% of the MAO, suggesting that the contamination of the vesicle fraction with mitochondria is still considerable.

When the vesicle preparation was further subjected to equilibrium density gradient centrifugation, the distribution of NA and MAO coincided almost completely, indicating that the separation between NA storage vesicles and mitochondria had not been obtained under these conditions. It is therefore not surprising that the NA/ATP values calculated from this gradient are in fact not different from the values obtained in sediment 2 (table 2). It is only when sediment 2 was subjected to differential gradient centrifugation that a satisfactory separation between MAO and NA was obtained. Under those conditions, a value of 111 for the NA/ATP ratio could be calculated. This value is much higher than any value so far obtained for bovine splenic nerve vesicles (ranging from 7 to 12) and presumably was inversely correlated with the degree of contamination with mitochondria. We further observed that, in spite of this very high molar ratio, the rate of loss of NA upon incubation at 37 °C from isolated vesicles from the C_{1300} mouse neuroblastoma is not significantly different from that of bovine splenic nerve axon vesicles[17], suggesting that both types of NA storage vesicles have a similar stability.

The present results further indicate that the NA content of C_{1300} mouse neuroblastoma is similar to the same in peripheral noradrenergic tissue, e.g. splenic nerve[7]. This observation, together with the finding that only very small amounts of ATP are present in the fraction containing the bulk of NA storage granules – as reflected in the very high NA/ATP ratio observed (table 2) – suggest that the presence of a soluble NA/ATP complex is not a prerequisite for the storage of NA within the catecholamine granules of this tumor.

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