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CATECHOLAMINES AND NUCLEOTIDES IN PHAEOCHROMOCYTOMA

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Abstract—Isolated catecholamine (CA) storage granules from a phaeochromocytoma were studied *in vitro*. The tumour granules had a high NA content, while A amounted to only a few per cent of the total CA. Most of the amines were particle bound. The storage granules had a lower density and adenine nucleotide content than bovine adrenal medullary granules. Thus only a minor part of the CA in this tumour could be stored by a mechanism requiring stoichiometric relationship between amines and adenine nucleotides.

On incubation *in vitro* ATP was lost at a slightly higher rate than the CA, leading to an increase in the molar CA/ATP ratio. The ATP lost was quantitatively recovered as AMP in the extragranular medium. ATP-splitting activity occurred to some extent in the particle-free supernatant but was more evident in the high speed sediment.

When the tumour was stored in a refrigerator at +2° a gradual loss of CA occurred while the ATP remained unchanged. Thus from the third day and onwards a CA/ATP ratio giving an equivalence ratio close to unity was established. On incubation of such granules the rate of CA loss was found to be slightly increased while that of ATP loss was unchanged.

ATP or ADP and magnesium addition to the incubation medium partly prevented the spontaneous decrease of the storage granule CA. Phenoxybenzamine caused a profound fall in the CA and ATP content, while reserpine did not have any clearcut effects on the granules, in the concentrations used.

THE relationship between catecholamines and adenine nucleotides in chromaffin cell tumours has previously been studied by several authors.[1–3] The tumours described showed a catecholamine and ATP content varying from 1·1 to 60 μmol/g and from 0·07 to 2·57 μmol/g, respectively. The molar catecholamine to ATP ratio thus ranged from 10·2 to more than 30.

If catecholamines are bound to nucleotides in chromaffin cell granules, as has been assumed,[11, 12] they are apparently to a large extent stored differently in tumour granules and in the adrenal medullary granules.

The present investigation was intended to study the functional characteristics of these storage granules by exposing them to different *in-vitro* conditions.

MATERIALS AND METHODS

Clinical data. The phaeochromocytoma was removed from a 41-year-old woman who suffered from hypertension (blood pressure fluctuating during 24 hr between 210/135 and 175/105) with severe vascular retinal changes (fundus hypertonicus II–IV). The excretion of noradrenaline in the urine was 1055–2000 μg/24 hr before and

26 μg/24 hr after the operation. Adrenaline excretion was only moderately elevated (46–134 μg/24 hr).

Tumour material. The tumour was connected to the left adrenal gland. Microscopic examination confirmed the diagnosis phaeochromocytoma and indicated that the tumour was probably malignant.

The tumour was completely removed 1·5–2 hr after starting ligation of its vascular supply, and after an additional half hour at room temperature was kept at 0–2°. Part of the tumour was used for experiments on the day of operation. The remaining part was placed in the refrigerator at 2–4° and kept for similar experiments up to the ninth day after removal.

Extraction of tissue homogenate. A piece of tumour was homogenized with an Ultra Turrax apparatus and extracted with 10 vol. of ice-cold 0·4 M perchloric acid. After centrifugation the supernatant was brought to pH 5–6 with ice-cold potassium hydroxide, the precipitate removed by centrifugation and the final extract analyzed for catecholamines and nucleotides.

Preparation of storage granules

a. *Crude granules.* Another piece of the tumour was used for preparation of the catecholamine storage granules according to the method of Euler,[4] by squeezing the tissue between nylon rollers at 0° and diluting the press juice obtained with ice-cold neutral isotonic potassium phosphate buffer. After removal of nuclear débris and coarser tissue particles by centrifugation at 600–1000 *g* for 10 min, part of the supernatant, containing the 'crude' granules, was incubated at 37° to study the spontaneous release of amines and nucleotides.

b. *Fractionated granules.* Another part of the supernatant was centrifuged at 50,000 *g* for 30 min, and the sediment after resuspension in 0·3 M sucrose layered on top of a 1·2 M sucrose solution. After centrifugation at 50,000 *g* for 60 min a sediment was formed in the bottom of the tubes, while the rest of the particulate fraction accumulated in the boundary zone between the 0·3 and 1·2 M sucrose layers. Both the 'heavy' and the 'light' granules in this centrifugation were resuspended in neutral isotonic potassium phosphate buffer and incubated at 37°.

Incubation. The sedimented granules were incubated at 37° in neutral isotonic potassium phosphate buffer for varying times and the spontaneous release of amines and nucleotides studied. In some experiments 3 mM adenosine triphosphate (ATP) or adenosine diphosphate (ADP) + 1 mM $MgCl_2$, phenoxybenzamine $7{\cdot}5 \times 10^{-5}$ M to $1{\cdot}5 \times 10^{-4}$ M or reserpine 10^{-5} M, were added. After the incubation period th tubes were centrifuged at 50,000*g* for 30 min at 0°, the supernatant decanted and the sediment extracted with ice-cold 0·4 M perchloric acid as described above. Both the sediments and the supernatants were analyzed for catecholamines and nucleotides.

ATPase activity. The localization of the ATP-splitting activity of the preparation was determined by comparing the rate of breakdown at 37° of ATP in 1 mM concentration, added to an aliquot of the granule-free supernatant and to a corresponding amount of the resuspended crude granule fraction, a third series in potassium phosphate buffer serving as a control. The reaction was stopped by adding one volume of ice-cold 0·4 M perchloric acid. The extraction and adenine nucleotide assay were carried out as described above and below.

Catecholamine determination. Noradrenaline and adrenaline were determined directly in the extracts by the fluorimetric method of Euler and Lishajko.[5]

ATP assay. ATP was determined by a modification of the luciferase method of Strehler and Totter.[6]

Ion exchange chromatography. The neutral extract was passed through an anion exchange column (Dowex 2, 200 to 400 mesh, 150 × 4 mm, formate form). The elution (2·5-ml fractions, flow about 7·5 ml/hr) was performed using the gradient method of Hurlbert *et al.*[7] The eluant in the reservoir consisted of 4 M formic acid, 1 M ammonium formate. The mixer volume was 210 ml. The elution pattern was followed by reading the ultraviolet absorption at 260 mμ, using a Beckman DU instrument.

RESULTS

Total extract

When a slice of the fresh tumour was extracted and analyzed it was found to contain 20·7 μmoles noradrenaline, 1·0 μmole adrenaline and 0·83 μmole ATP/g tissue, wet wt, respectively, giving a catecholamine to ATP ratio of 26·2. On ion exchange chromatography (Fig. 1) ATP, ADP, and AMP (adenylic acid) were found to dominate the

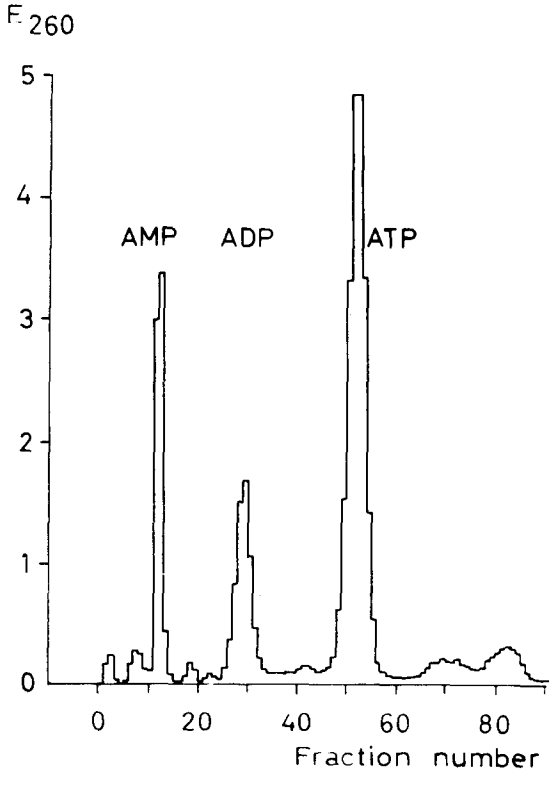

FIG. 1. Anion exchange chromatogram of nucleotides in total extract of phaeochromocytoma. Ordinate, extinction at 260 mμ; abscissa, fraction number.

nucleotide pattern, making up 61·7, 17·9 and 20·4% of the total amount of adenine nucleotides, respectively. No attempts were made to identify the smaller peaks in the chromatogram.

Granules

Most of the catecholamines were particle bound. A small amount, 12·1%, appeared in the slow speed sediment (1000 *g*), while 76·9% occurred in the high speed sediment (50,000 *g*). Only 11% were found in the particle-free supernatant of the last-mentioned centrifugation. On isolation of the specific storage granules by the sucrose gradient method it was found that no sediment was formed when the bottom sucrose layer was 1·6 M, while the particles sedimented readily if it was made 1·2 M. In the crude granule fractions as well as in the heavier granules obtained on a sucrose gradient the amine to ATP ratio was of the same order of magnitude as in the total tumour extract. The light granules in the boundary layer between 0·3 M and 1·2 M sucrose had a CA/ATP ratio of 8–10. This fraction only represented a few per cent of the total catecholamines, however, and was not further studied.

On incubation at 37° the granules slowly lost both amines and nucleotides. ATP disappeared at a slightly higher rate, which caused the ratio to increase during the first hour (Fig. 2). Ion exchange chromatography revealed that the ATP lost had

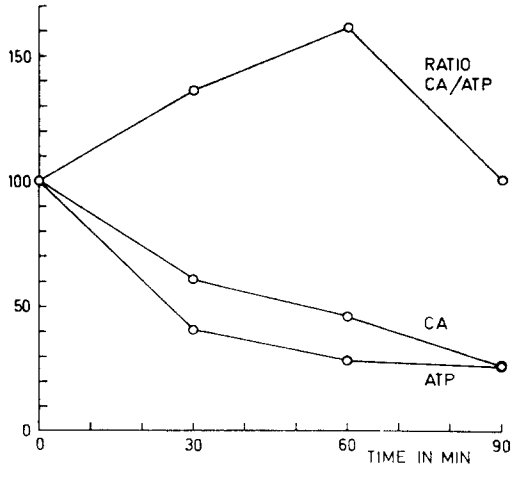

FIG. 2. Catecholamines (CA), ATP and ratio CA/ATP in tumour granules purified on sucrose gradient, incubated at 37°, in per cent of controls at 0°.

vanished entirely from the granules without giving rise to compensatory accumulation of any of its possible metabolites (Fig. 3).

ATPase activity

ATP added to the granule suspension was rapidly broken down and quantitatively recovered as AMP (Fig. 4). This ATP-splitting quality was partly present in the particle-free supernatant but was more evident in the high speed sediment (Fig. 5). Added ADP was split in a similar way.

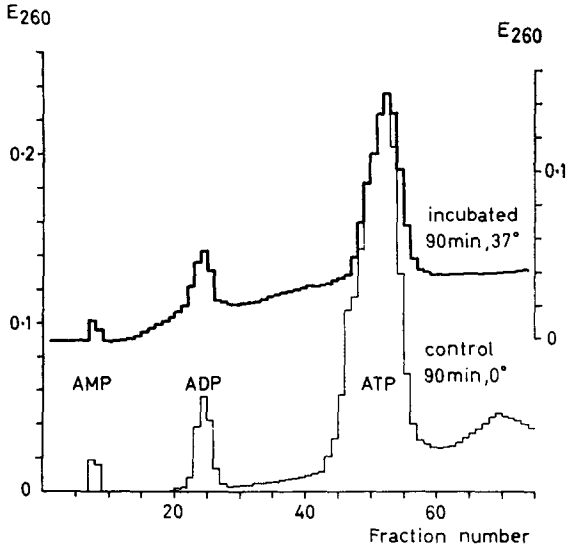

FIG. 3. Anion exchange chromatogram of nucleotides in tumour granules purified on sucrose gradient after incubation at 0° and 37° for 90 min. Ordinates, extinction at 260 mμ; abscissa, fraction number.

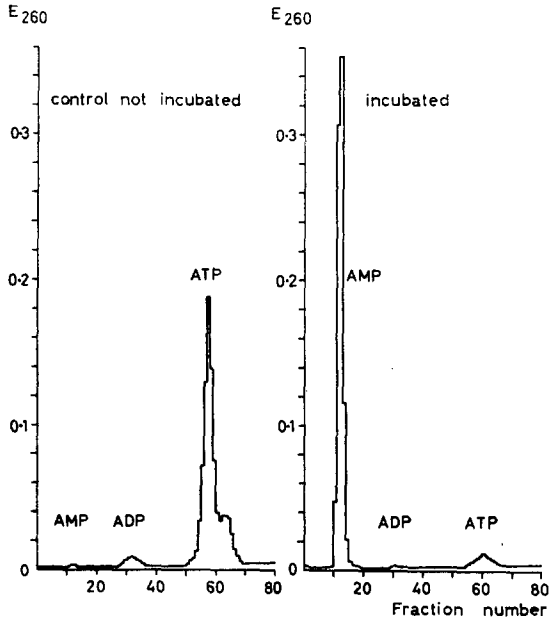

FIG. 4. Anion exchange chromatogram of tumour granule suspension with ATP added, 3 mM, before (left) and after (right) incubation for 60 min at 37°. Ordinate, extinction at 260 mμ; abscissa, fraction number.

'Ageing'

When the organ was kept in the refrigerator and samples were taken at intervals up to the ninth day after the operation, it was noticed that the catecholamine content steadily decreased, while ATP was maintained at about the starting level. This caused the ratio to drop from around 20 to 5·9 (Fig. 6). The reason for this fall in the

catecholamines appears to be destruction of part of the granule-bound amine fraction, since there was no corresponding increase in the catecholamine level in the particle-free supernatant.

On incubation of the granules from the 'aged' tumour, the rate of release of catecholamines increased with the degree of 'ageing', while there was no definite change in the rate of ATP loss from the granules (Fig. 7).

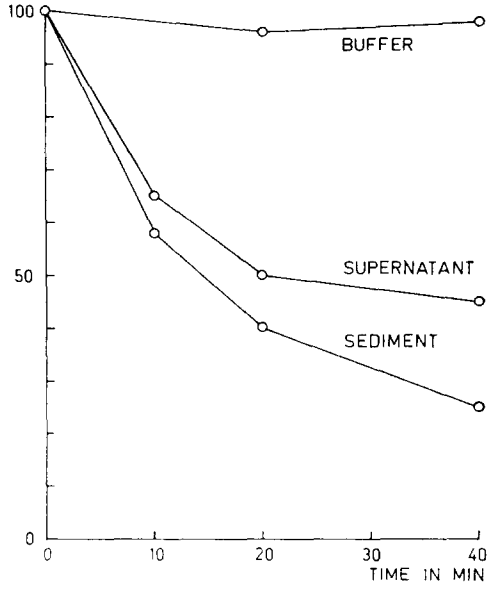

FIG. 5. ATP-splitting activity at 37° in particle-free supernatant and high speed sediment from tumour, ATP added to 1 mM. Control, ATP plus potassium phosphate buffer. Ordinate, per cent of starting ATP values.

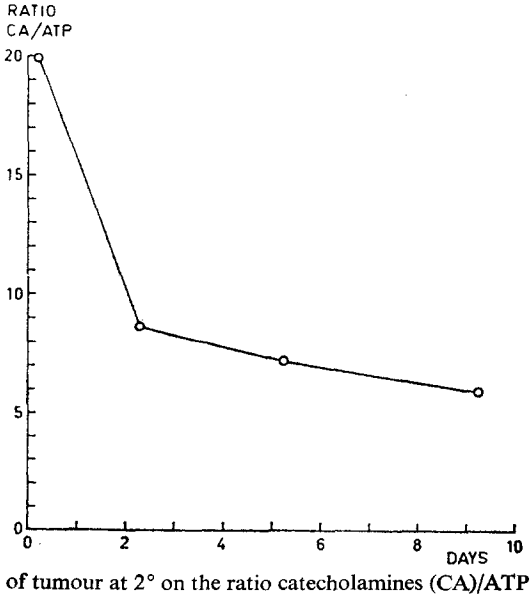

FIG. 6. Effect of ageing of tumour at 2° on the ratio catecholamines (CA)/ATP in granules. Ordinate, ratio CA/ATP; abscissa, days after operation.

Drug actions

ATP, 3 mM, added to the granules together with magnesium, partly prevented the spontaneous decrease in their catecholamine content on incubation at 37°. ADP at a similar concentration was about half as active.

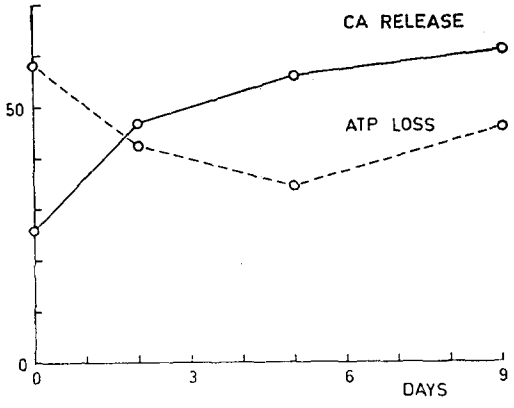

FIG. 7. Catecholamine (CA) and ATP loss in granules from fresh and aged tumour incubated for 60 min at 37°. Ordinate, CA and ATP loss, in per cent of control values at 2°; abscissa, days after operation.

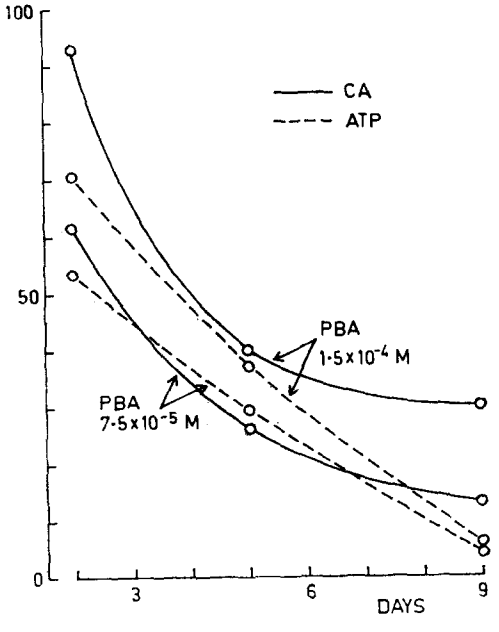

FIG. 8. Effect of ageing of tumour on catecholamine (CA) and ATP loss from granules on incubation with phenoxybenzamine (PBA) for 60 min at 37°. Ordinate, loss in per cent of control values (60 min, 37°); abscissa, days after operation.

Phenoxybenzamine, added to the granule suspension at a concentration of $7{\cdot}6 \times 10^{-5}$ to $1{\cdot}5 \times 10^{-4}$ M, caused a precipitous fall in the catecholamine and ATP content during the first days after removal of the tumour. This depleting effect became gradually weaker during the 'ageing' process (Fig. 8).

Reserpine at 10^{-5} M did not have any consistent effect of its own on the catecholamines or ATP of the granules. Nor did it change the 'protective' effect of ATP on the catecholamine content of the granules.

DISCUSSION

The catecholamines in the tumour studied, which was almost exclusively noradrenaline-producing, were to 88% contained in the microgranular fraction of the supernatant after the separation of coarse particles. This distribution is similar to that regularly found in the adrenal medulla[8] and is in general agreement with the findings in phaeochromocytoma by Schümann,[2] but at variance with the report of Burger and Langemann[9] who found only a minor portion of the catecholamines in the granule fraction.

The adenine nucleotide pattern in a total extract of the tumour was more similar to that found in bovine adrenal medullary tissue[10] than in the phaeochromocytoma described by Hillarp, Lindqvist and Vendsalu[3] in which the proportion of ATP was considerably smaller than that of ADP and AMP. The catecholamine to ATP ratio in the isolated granules of our tumour was much higher than that found in adrenal medullary tissue and in agreement with the values reported by Gélinas, Pellerin and D'Iorio[1] and by Schümann.[2] The high ratio was found not only in the 'crude' granules but in the 'purified' granules, obtained on sucrose gradient centrifugation, as well. Consequently, at the most 20% of the amines in the 'pure' storage granules could be bound to adenine nucleotides as suggested for adrenal medullary granules.[11–13]

It is of interest, in this connection, to notice the observations of Hillarp[14] that, at least in the 'crude' adrenomedullary granules, part of the amines appear to be stored unrelated to adenine nucleotides.

As pointed out by Schümann,[2] the parallel loss of catecholamines and ATP from the storage granules of the adrenal medulla may indicate that ATP is related not only to the storage, but also to the secretion of catecholamines. In the present experiments it was regularly observed that the ATP in the granules decreased when the amines were released. However, the rates of catecholamine and ATP loss were not parallel and therefore appear to be to some extent independent of each other. This applies both to the spontaneous loss on incubation and to the release caused by phenoxybenzamine. It was particularly striking during the process of 'ageing', which gradually depleted the granules of 68% of their catecholamines, leaving the ATP essentially unchanged, thus reducing the amine to ATP ratio from about 21 to 4·9, a value close to that of adrenal medullary 'pure' granules.[10] The decrease in the amine content of the granules during ageing was not accompanied by an increase in the 'free' catecholamine fraction in the particle-free supernatant. One would thus have to assume either that part of the amines were destroyed in the granules, leaving the ATP unaffected, or that amines were continuously leaking out of the granules, independently of ATP, and inactivated in the cytoplasm at a rate, which maintained a constant level of 'free' extragranular catecholamines.

An effect similar to the above mentioned selective loss of catecholamines during an 'ageing' process was described[14] for adrenal medullary tissue, which was kept at 37° for 3 hr.

The observation that addition of either ATP or ADP together with magnesium ions maintained the catecholamine content of the granules at a higher level than that found in the controls is in agreement with similar findings in nerve storage granules.[15] This effect may imply that adenine nucleotides, when added to the external medium, are able to block the outward movement of amines, or accelerate an inward movement of external catecholamines, which will tend to balance the spontaneously occurring flow in the opposite direction. A considerable additional uptake of external amines into the granules in the presence of ATP or ADP and magnesium has in fact been demonstrated both for adrenal medullary[11, 17] and nerve storage granules.[15] This suggests that the energy-rich phosphates may act by supplying the energy needed to force the amines across the granule membrane against a concentration gradient.

The presence of ATP- and ADP-splitting systems in the particulate fraction, leading to an accumulation of AMP, is in accordance with the findings in the sedimentable fractions from adrenal medullary[18] and nerve tissue (Euler, Lishajko and Stjärne, to be published). It may be interpreted either as indicating the presence, in the particulate fraction, of one enzyme, capable of splitting both the terminal phosphate bonds of ATP and ADP, or of two enzymes, one acting as a specific ATPase, and the other possibly representing an adenylate kinase, as has been suggested for adrenal medullary granules.[18] However, no attempt was made in the present experiments to find out whether the enzymatic activity was located in the specific catecholamine storage granules, or in other intracellular particles, i.e. mitochondria or microsomes.

The sympatholytic agent phenoxybenzamine was found to cause a depletion of both amines and ATP in the tumour granules. This is in accordance with its action on adrenal medullary granules [19, 20] while the opposite effect is found in nerve granules in the concentrations used. It also strongly inhibits the uptake of external amines into adrenal medullary granules,[16] and nerve granules.[19] The well-known fact that phenoxybenzamine is structurally similar enough to the catecholamines to be able to block specific catecholamine receptors suggests that its effects on the granules may be due to competition with the amines for some strategic sites, possibly involving an active transport mechanism. The differences observed in the gradually developing refractoriness of the tumour granules to the amine and ATP depleting action of phenoxybenzamine appear to indicate that the release mechanisms for amines and ATP may in part be independent of each other.

Reserpine, at the concentrations used in the present experiments, inhibits the spontaneous loss of catecholamines in nerve granules.[21] The fact that this drug did not change the release of amines from the tumour storage granules further emphasizes the differences between catecholamine storage granules of different origin.

Although the low ATP content in the tumour granules, relative to the catecholamines, indicates that only a minor portion of the amines could be stored by binding to the negative charges of the nucleotides, the present results, particularly those of the ageing experiments, are not incompatible with the concept[11–13] that the catecholamines in part occur in a storage complex, characterized by a stoichiometrical relationship between the amines and the adenine nucleotides. Such a role of ATP does not preclude that it may also have additional functions, possibly serving as some kind of 'template' during catecholamine synthesis, or somehow 'activating' the stored amines when they are being mobilized and extruded from the granules.

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