A Characterization of the Nucleotide Uptake by Chromaffin Granules of Bovine Adrenal Medulla

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Chromaffin granules isolated from bovine adrenal gland were incubated with ^3H -labelled nucleotides and ^{14}C noradrenaline to study the uptake of these substances. ^3H ATP, ^3H ADP and ^3H AMP are taken up by these organelles by the same temperature-dependent mechanism. The apparent K_m for ATP and ADP is 1.4 mM, and for AMP it is 2.9 mM. The uptake of ATP has a flat pH optimum, whereas the catecholamine uptake increases with more alkaline pH. Atractyloside and carboxyatractyloside are competitive and specific inhibitors of nucleotide uptake, whereas reserpine inhibits only that for catecholamines. Mg^{2+} ions activate uptake of both catecholamine and nucleotides, whereas EDTA and *N*-ethylmaleimide inhibit these processes. Nucleotide and catecholamine uptakes are inhibited by uncouplers of oxidative phosphorylation and by two ATP analogues. NH_4^+ ions and nigericin in the presence of KCl inhibit only catecholamine uptake. It is concluded that nucleotide uptake, as proposed previously for catecholamine uptake, depends on an electrochemical proton gradient produced by a proton-translocating adenosine triphosphatase localized in the membrane of chromaffin granules. Furthermore, as suggested by the effect of NH_4^+ and nigericin, catecholamine uptake apparently depends on the chemical part of this gradient, whereas the results for nucleotide uptake are consistent with its dependence on the electrical component.

The catecholamine-storing organelles of adrenal medulla, i.e. the chromaffin granules, contain a high concentration of catecholamines, Ca^{2+} and nucleotides (Smith, 1968; Winkler, 1976). The accumulation of these substances during the biogenesis of these organelles and their subsequent maintenance requires the presence of efficient uptake mechanisms. Evidence for an uptake process for catecholamines was obtained several years ago (Kirshner, 1962; Carlsson *et al.*, 1963; Taugner, 1972). These amines are accumulated by a temperature-dependent process which is activated by ATP and Mg^{2+} and inhibited by *N*-ethylmaleimide. These findings indicate an involvement of the thiol-dependent Mg^{2+} -activated ATPase localized in the granule membranes (Banks, 1965). Furthermore, later observations have convincingly shown that this ATPase is a proton-translocating enzyme, and therefore that catecholamine uptake is driven by an electrochemical proton gradient (Bashford *et al.*, 1975, 1976; Casey *et al.*, 1976, 1977).

In contrast with the well-characterized catecholamine uptake, evidence for an uptake of ATP by chromaffin granules was slow to appear. When adrenal glands were perfused with radioactively labelled precursors of nucleotides, then the ATP pool of chromaffin granules became labelled (Stjärne *et al.*, 1970; Stevens *et al.*, 1972; Winkler *et al.*, 1972).

Furthermore, it was demonstrated that this ATP had been synthesized outside the chromaffin granules and was only subsequently taken up by these organelles (Peer *et al.*, 1976). On the basis of these findings we attempted to demonstrate an ATP uptake by isolated chromaffin granules. A temperature-dependent process was discovered which appeared to be carrier-mediated, since it was inhibited by a blocker of mitochondrial nucleotide uptake, i.e. atractyloside (Winkler *et al.*, 1975; Kostron *et al.*, 1977). A first indication of an interrelationship between the uptake of ATP and that of catecholamines was provided by the finding that both processes were inhibited by *N*-ethylmaleimide and by uncouplers of oxidative phosphorylation.

The present paper gives a detailed analysis of the uptake of ATP, ADP and AMP by isolated chromaffin granules. Evidence is presented that nucleotide uptake, like that of catecholamines, is dependent on a proton-translocating ATPase.

Methods and Materials

Isolation of chromaffin granules

Bovine adrenal medullae were homogenized in (unbuffered) 0.3M-sucrose (see Smith & Winkler, 1967a, for details). After removal of a first sediment (800g_{max} for 20min), a large-granule fraction was

Abbreviation used: ATPase, adenosine triphosphatase.

sedimented at $10000g_{\max}$ for 20 min. The brown upper layer of this sediment, enriched in mitochondria, was removed by swirling with 0.3 M-sucrose. The remaining pellet was resuspended and sedimented again (see Taugner & Hasselbach, 1966; Kostron *et al.*, 1977). After removal of the top layer, the remaining partly purified chromaffin granules were used for further experiments.

Incubation procedures

Partly purified chromaffin granules (1.8–4.0 mg of protein) were incubated in 1 ml of buffered (0.01 M-Tris/HCl, pH 7.2) 0.3 M-sucrose solution. Unlabelled nucleotides and various other compounds were present in the concentrations given in the Results section. Usually $10\mu\text{Ci}$ of [^3H]ATP, $10\mu\text{Ci}$ of [^3H]ADP or $20\mu\text{Ci}$ of [^3H]AMP was added. The stock solutions (The Radiochemical Centre, Amersham, Bucks., U.K.) of the labelled nucleotides contained 50% (v/v) ethanol. Since ethanol in concentrations above 0.5% produced some inhibition of the uptakes of catecholamines and nucleotides, the ethanol was evaporated off before higher concentrations of labelled nucleotides were used for incubation. In most experiments the incubation tubes also contained $0.25\mu\text{Ci}$ of [^{14}C]noradrenaline (dissolved in 1 mM-HCl containing unlabelled 2 mM-noradrenaline). After incubation, usually for 5 min, the suspension was diluted with 3 ml of cold (0°C) buffered 0.3 M-sucrose, containing 20 mM-disodium EDTA. After standing for 20 min at 0°C , the granules were sedimented at $33000g_{\max}$ for 10 min. The sediment was resuspended in 4 ml of the EDTA/sucrose solution and left for 20 min on ice before again being sedimented. In some experiments this washing procedure was again repeated, but this did not result in any significant advantage. In some experiments using [^3H]AMP, non-radioactive AMP (2 mM) was added to the washing solution. This addition was necessary, since otherwise variable uptakes were found for the incubation at 0°C ; the latter uptake in some cases even exceeded the values found during incubation at 37°C . On the other hand, the uptake rates at 37°C were not changed by the inclusion of AMP in the washing solution. Apparently, some unspecific adsorption of AMP to the granules occurred at 0°C . After the washes the resuspended particles were centrifuged through unbuffered 1.5 M-sucrose (see Smith & Winkler, 1967a) at $120000g_{\max}$ for 60 min. The pellet of purified chromaffin granules thus obtained was resuspended in Tris/sodium succinate buffer (pH 5.9, 10.015, see Smith & Winkler, 1967b) and samples were taken for radioactivity counting in a liquid-scintillation spectrometer (see Kostron *et al.*, 1977). Uptake rates for the nucleotides were calculated on the basis of the specific radioactivity of the added nucleotide. No corrections were

made for any endogenous nucleotides, i.e. those released from the granules during incubation. In any case, the high concentration (2 mM) of nucleotides usually used for incubation is unlikely to be significantly influenced by the presence of endogenous nucleotides. Uptake rates for catecholamines were expressed only by the radioactivity (c.p.m.) of [^{14}C]noradrenaline found in the granules.

Thin-layer chromatography

The separation of nucleotides by t.l.c. (Stevens *et al.*, 1972) was described by Kostron *et al.* (1977).

Chemical assays

Protein was determined by the biuret method as described by Winkler *et al.* (1970). Catecholamines were measured colorimetrically (Von Euler & Hamberg, 1949).

Materials

[2- ^3H]ATP, ammonium salt (15–30 Ci/mmol), [2- ^3H]ADP, ammonium salt (15–25 Ci/mmol), [2- ^3H]AMP, ammonium salt (15–22 Ci/mmol), and DL-[methylene- ^{14}C]noradrenaline DL-bitartrate (50 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. ATP (disodium salt), ADP and AMP (sodium salt), atractyloside, carboxyatractyloside, *N*-ethylmaleimide, carbonyl cyanide *m*-chlorophenylhydrazone, oligomycin and antimycin A were purchased from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. 2,4-Dinitrophenol was from Serva, Heidelberg, Germany. The ATP analogues adenylyl imidodiphosphate (adenosine 5'-[β -imido]triphosphate; p[NH]ppA) and adenylyl [β -methylene]diphosphonate (adenosine 5'-[β -methylene]triphosphate; p[CH₂]ppA) were obtained from Boehringer Mannheim, Germany. Thin-layer plates (Polygram Cell 300 PEI) came from Macherey-Nagel, Düren, Germany. Nigericin and reserpine phosphate were presents from Hofmann-La Roche (Basle, Switzerland) and Ciba-Geigy (Basle, Switzerland) respectively.

Results

Uptake of ^3H -labelled nucleotides by chromaffin granules

Partly purified chromaffin granules were incubated with various ^3H -labelled nucleotides together with [^{14}C]noradrenaline. After incubation the granules were washed and purified by a simplified gradient procedure and the radioactive material that had been taken up was determined and characterized. Kostron *et al.* (1977) established that this procedure allows

Fig. 1. pH-dependence of catecholamine and nucleotide uptake

Chromaffin granules were incubated for 5 min at 37°C in Tris/HCl (0.01 M)-buffered 0.3 M-sucrose of various pH values. The results for the uptake of [³H]ATP (●) are mean values of three experiments (±S.E.M.); those for catecholamines (▲) are mean values of two well-agreeing experiments.

Table 1. Characterization of radioactively labelled material in chromaffin granules after incubation with ³H-labelled nucleotides

The labelled material that had been taken up by chromaffin granules after incubation with ³H-labelled nucleotides (2 mM) for 5 min at 37°C was analysed by t.l.c. The Table gives the percentages of the total radioactive material found in the nucleotide and adenosine spots.

Incubation with ...	Percentage of total radioactivity in granule	
	[³ H]ADP (n = 6)	[³ H]AMP (n = 4)
[³ H]ATP	46 ± 4	5 ± 1
[³ H]ADP	40 ± 3	6 ± 1
[³ H]AMP	9 ± 1	72 ± 1
[³ H]Adenosine	5 ± 2	17 ± 1

one to measure ATP uptake without any interference from other cell particles.

The pH-dependence of the [³H]ATP uptake is shown in Fig. 1 and compared with that for nor-adrenaline uptake. There is a relatively small dependence of nucleotide uptake on external pH, with a slight optimum around pH 7.0, whereas catecholamine uptake increases from pH 6.5 to 8.0. More extreme pH values were not tested, since instability of the granules at such pH values would have made the interpretation of the results difficult.

Chromaffin granules took up not only [³H]ATP but also [³H]ADP and [³H]AMP. The radioactive material that had been taken up by chromaffin granules after incubation with these nucleotides was

characterized by t.l.c. It has been previously shown (Kostron *et al.*, 1977) that after incubation with [³H]ATP more than 60% of the radioactivity in the granules was confined to ATP. After incubation with [³H]AMP this nucleotide was also the major radioactively labelled component appearing inside the granules. On the other hand, after incubation with [³H]ADP a considerable part of the labelled material inside the granules was found to be [³H]ATP (see Table 1). This cannot be due to the appearance of [³H]ATP in the medium during incubation and its subsequent uptake into granules, since, as shown by t.l.c., only insignificant amounts (less than 10% of the total radioactivity) of [³H]ATP appeared in the medium during incubation for 5 min.

Table 2 compiles several features of the uptake of [³H]ADP and [³H]AMP. The uptake of [³H]ADP is higher than that of [³H]AMP, but still lower than that of [³H]ATP (0.268), as reported previously (Kostron *et al.*, 1977). In the presence of ATP, catecholamine uptake was also highest. When this uptake was taken as 100% catecholamine uptake in the presence of ADP was 52 ± 2% (±S.E.M., n = 19) and in the presence of AMP 56 ± 5% (±S.E.M., n = 8).

The rates of uptakes of the three nucleotides became saturated at higher substrate concentrations. Lineweaver-Burk plots revealed an apparent K_m of 1.4 mM for ATP (see Fig. 2) and ADP (see Fig. 3), whereas the K_m for AMP was 2.9 mM. The V_{max} of the uptake was highest for ATP (0.476 nmol/min per mg of protein; see Fig. 2). For AMP (0.198) and for ADP (0.256) the V_{max} values were similar (see Fig. 3).

After hypo-osmotic lysis of the granules, which releases their soluble contents, the labelled ADP and AMP were quantitatively recovered with the lysate (see Table 2) in agreement with previous results on ATP (Kostron *et al.*, 1977). Apparently, no significant amount of nucleotides was bound to the membranes during uptake.

When granules which had previously taken up ³H-labelled nucleotides were left for 18 h at 0°C, a considerable part of the labelled material was still retained in them (see Table 2). This stability was lower for [³H]AMP. For [³H]ATP a value of 86% was reported previously (Kostron *et al.*, 1977).

Activation and inhibition of the uptake of nucleotides and catecholamines

Table 3 compiles the results for the effect of various substances on catecholamine and nucleotide uptake. In general, the uptake of the three nucleotides was always affected in the same manner, whereas the uptake of catecholamines showed some similarities to, but also significant differences from, the nucleotide uptake. The uptakes of both substances were activated by Mg²⁺, but inhibited by EDTA and N-ethylmaleimide. Two uncouplers of

Table 2. Uptake of ^3H -labelled nucleotides by chromaffin granules

Chromaffin granules were incubated with ^3H -labelled nucleotides for 5 min. The rate of uptake at 37 and 0°C was determined at a nucleotide concentration of 2 mM. For hypo-osmotic lysis, purified chromaffin granules, which had been incubated with ^3H -labelled nucleotides, were extracted twice (together with one cycle of freezing and thawing) with 5 ml of Tris/sodium succinate buffer (pH 5.9, 10.015). The ^3H in the soluble and sedimentable material (130000g for 45 min) was determined. For stability measurements partly purified chromaffin granules which had been incubated with ^3H -labelled nucleotides were left after the usual washes for 18 h at 0°C suspended in 4 ml of 0.3 M-sucrose. The granules were then sedimented and purified by the usual gradient procedure. The remaining ^3H radioactivity in the granules was compared with that of control granules treated as usual. The results are means $\pm$ S.E.M. for the numbers of experiments in parentheses.

	Incubation with:	
	^3H ADP	^3H AMP
Uptake rate at 37°C (nmol/min per mg of protein)	0.159 ± 0.006 (13)	0.132 ± 0.009 (6)
Uptake rate at 0°C (% of uptake at 37°C)	37 ± 3.2 (16)	28.4 ± 3.7 (7)
Percentage released by hypo-osmotic shock	98 ± 0.2 (3)	98.9 ± 0.2 (3)
Stability at 0°C for 18 h (% of control)	66 ± 7.2 (8)	51 ± 3.7 (8)

Fig. 2. Lineweaver-Burk plots for ^3H ATP uptake. ^3H ATP (0.5–6 mM) was incubated with chromaffin granules for 5 min at 37°C in the absence (control, Δ) and in the presence of attractylsidoside (0.2 mM, $\circ$) and carboxyatractylsidoside (0.4 mM, $\bullet$). The experimental points are mean values of four experiments. In no case did the S.E.M. exceed 15%.

oxidative phosphorylation, dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazine, inhibited the uptake of both types of components. The ionophore nigericin, in the presence of 10 mM- K^+ , inhibited only the catecholamine uptake. A similar result was obtained when ammonium acetate was used. This inhibition can be attributed to the NH_4^+ ion, since sodium acetate slightly activated both uptakes. An activation of catecholamine uptake and ATPase activity *in vitro* by several ions such as Na^+ has been reported (Taugner, 1971b). Reserpine,

Fig. 3. Lineweaver-Burk plot of ^3H -labelled nucleotide uptakes.

Chromaffin granules were incubated with 0.5–6 mM- ^3H ADP ($\circ$) and ^3H AMP (Δ) at 37 and 0°C for 5 min. The washing solution for ^3H AMP incubation contained 2 mM-AMP (see the Methods and Materials section). The uptake observed at 0°C was subtracted from the values found at 37°C before plotting. The experimental points are mean values of three experiments. The S.E.M. did not exceed $\pm 12\%$ of the mean values.

oligomycin and antimycin inhibited only catecholamine uptake, whereas attractylsidoside and carboxyatractylsidoside were specific inhibitors of nucleotide

Table 3. *Activation and inhibition of nucleotide and catecholamine uptake*

The influence of various compounds was tested during incubation of granules with 2mM-nucleotides for 5 min at 37°C. In each experiment control tubes were incubated. The rates of uptake in these controls (see the text and Table 2 for absolute values) were taken as 100%. [^{14}C]Noradrenaline was present in the experiments with all of the three nucleotides. However, only the values for ATP incubation are given, since analogous results were obtained during incubation with ADP and AMP. Nigericin, antimycin and oligomycin were added in ethanol solutions (5–10 $\mu\text{l/ml}$ of incubation medium). Ethanol in the same concentration was added to control tubes. The results are mean values (n is given in parentheses) $\pm$ S.E.M. The values for [^3H]ATP (marked with asterisks) are taken from the paper by Kostron *et al.* (1977). To check that the addition of the various compounds did not lead to any lysis of the chromaffin granules, the endogenous catecholamine contents of incubated granules were measured in representative experiments. No significant decrease was found with any of the compounds tested.

Effect of	Incubation with			
	[^{14}C]Noradrenaline (+ ATP)	[^3H]ATP	[^3H]ADP	[^3H]AMP
Control	100	100	100	100
Mg $^{2+}$ (1 mM)	134 $\pm$ 7.7 (8)	133 $\pm$ 3.5 (8)*	135 $\pm$ 1 (4)	109 $\pm$ 4 (6)
Disodium EDTA (1 mM)	54 $\pm$ 2 (8)	82 $\pm$ 3 (10)	71 $\pm$ 4 (7)	—
N-Ethylmaleimide (1 mM)	37 (2)	21 $\pm$ 2 (11)*	56 $\pm$ 6 (6)	77 $\pm$ 3 (4)
Dinitrophenol (0.5 mM)	81 (2)	51 (2)	57 $\pm$ 3 (6)	—
Carbonyl cyanide <i>m</i> -chloro-phenylhydrazone (0.25 mM)	38 (2)	26 $\pm$ 4 (5)*	56 $\pm$ 7 (6)	81 $\pm$ 3 (15)
Nigericin (5 $\mu\text{g/ml}$) (plus 10 mM-KCl)	36 $\pm$ 2 (3)	115 $\pm$ 3 (3)	—	—
Ammonium acetate (5 mM)	57 $\pm$ 3 (6)	135 $\pm$ 5 (6)	—	—
Sodium acetate (5 mM)	128 $\pm$ 2 (6)	122 $\pm$ 2 (6)	—	—
Reserpine (0.1 mM)	26 (2)	98 $\pm$ 5 (4)	100 $\pm$ 2 (5)	—
Antimycin (20 μM)	73 $\pm$ 3 (11)	99 $\pm$ 4 (11)	—	—
Oligomycin (50 μM)	64 $\pm$ 2 (7)	101 $\pm$ 3 (7)	—	—
Atractyloside (0.2 mM)	92 $\pm$ 5 (4)	23 $\pm$ 2 (10)*	50 $\pm$ 4 (8)	56 $\pm$ 2 (9)
Carboxyatractyloside (0.4 mM)	94 $\pm$ 4 (3)	59 $\pm$ 4 (4)	76 $\pm$ 2 (4)	—

Table 4. *Influence of ATP analogues on uptake of nucleotides and catecholamines*

ATP analogues were added to an incubation mixture consisting of chromaffin granules, ^3H -labelled nucleotides (2 mM) and [^{14}C]noradrenaline. Incubation was for 5 min at 37°C. The results are expressed as percentages of controls ($\pm$ S.E.M.) which were incubated at the same time. The numbers of experiments are given in parentheses.

	Incubation with					
	ATP		ADP		AMP	
	[^{14}C]Noradrenaline	[^3H]ATP	[^{14}C]Noradrenaline	[^3H]ADP	[^{14}C]Noradrenaline	[^3H]AMP
Adenylyl imidodiphosphate (4 mM)	36 $\pm$ 3 (4)	68 $\pm$ 9 (4)	62 $\pm$ 1 (8)	64 $\pm$ 2 (8)	54 $\pm$ 1 (3)	67 $\pm$ 2 (3)
Adenylyl [$\beta\gamma$ -methylene]diphosphonate (4 mM)	55 $\pm$ 1 (3)	67 $\pm$ 2 (3)	76 $\pm$ 2 (8)	71 $\pm$ 1 (8)	60 $\pm$ 2 (3)	67 $\pm$ 2 (3)

accumulation. These latter two compounds appear to be competitive inhibitors of ATP uptake, as shown by a Lineweaver–Burk plot (see Fig. 2).

Effects of ATP analogues on uptake of nucleotides and catecholamines

Two analogues of ATP, adenylyl imidodiphosphate and adenylyl [$\beta\gamma$ -methylene]diphosphonate,

were tested for their effect on the two uptake processes. Adenylyl [$\beta\gamma$ -methylene]diphosphonate has been shown to inhibit active catecholamine uptake (Phillips, 1974), and both are inhibitors of the granule ATPase (Hoffman *et al.*, 1976). Table 4 shows that the analogues inhibited the uptake of all three nucleotides. Catecholamine uptake was also inhibited irrespective of whether ATP, ADP or AMP was present in the incubation mixture.

Discussion

Uptake of nucleotides by chromaffin granules

In a previous study (Kostron *et al.*, 1977) it was established that isolated chromaffin granules are able to take up [^3H]ATP from an incubation medium. The present paper now demonstrates that ADP and AMP are taken up by the same process. All these nucleotides are apparently incorporated into the soluble content of these organelles, since they are quantitatively released by hypo-osmotic shock. Furthermore, various activators and inhibitors (see below) affect the uptake of all three nucleotides in an analogous manner. Thus atractyloside, a well-known blocker of the carrier-mediated mitochondrial ATP/ADP exchange (see Klingenberg, 1970; Vignais, 1976), inhibits not only the granule uptake of ATP and ADP but also that of AMP. This latter nucleotide has no affinity for the mitochondrial nucleotide carrier (see Klingenberg, 1970; Vignais, 1976). This clear difference from mitochondria can be taken as further evidence, in addition to that already presented (Kostron *et al.*, 1977), that the uptake observed here with a chromaffin-granule fraction cannot be attributed to contaminating mitochondrial particles. In this context it should be added that carboxy-atractyloside is a competitive inhibitor of ATP uptake into granules, whereas the inhibition in mitochondria is of the non-competitive type (Vignais *et al.*, 1973).

The uptake of the various nucleotides in granules seems to be based on the same process; however, there are some differences, as revealed by kinetic analysis. Whereas the affinity of [^3H]ATP and [^3H]ADP appears similar, with a K_m of 1.4 mM, the K_m for AMP is much higher (2.9 mM). Furthermore, the uptake of nucleotides occurring during incubation at 0°C, presumably by diffusion, increases from ATP to ADP and AMP. There is only insignificant accumulation of ATP (4.5%; see Kostron *et al.*, 1977) at 0°C, whereas for the other two nucleotides uptake amounts to about 30% of that occurring at 37°C. Apparently, the lesser charge on the ADP and AMP molecules increases the ability of these nucleotides to cross membranes by simple diffusion. It is interesting that the ability to retain nucleotides in the granules after uptake decreases from ATP to ADP and AMP, since here again the less-charged molecules appear to diffuse more readily. This leakage is, however, likely to be slowed down by the binding of these nucleotides to the storage complex with catecholamines, which is present in these organelles. This should be true, at least for ATP and ADP, which are capable of forming such complexes with catecholamines (Goetz *et al.*, 1971).

Previous experiments using doubly labelled ATP (Kostron *et al.*, 1977) have shown that ATP is probably taken up by the granules as an intact

molecule. The same can now be suggested for AMP. For ADP the interpretation of the data is more difficult, since about half of the radioactive material found in the granules after incubation with [^3H]ADP is present as [^3H]ATP. It seems unlikely that this [^3H]ATP is taken up from the incubation medium, since only insignificant amounts of this nucleotide are formed there during incubation with [^3H]ADP. Therefore it is probable that the [^3H]ADP is converted into [^3H]ATP after its uptake into the granules. This apparently does not occur by the action of an adenylate kinase (EC 2.7.4.3), since no equivalent amounts of [^3H]AMP are formed, and secondly this enzyme is reported to be absent from chromaffin granules (see Winkler, 1976). On the other hand Taugner (1974) has demonstrated that the soluble proteins from chromaffin granules can transphosphorylate ADP in the presence of ATP, a finding that we have recently confirmed (E. Huber, W. Aberer & H. Winkler, unpublished work). Thus part of the [^3H]ADP taken up by the granules may be phosphorylated by the endogenous ATP. At present it remains obscure what kind of functional implication this process may have.

Relationship between the uptake of nucleotides and catecholamines

Considerable evidence has been presented that the uptake of catecholamines into granules depends on an ATPase localized in the membranes of these organelles (Kirshner, 1962; Carlsson *et al.*, 1963; Banks, 1965; Taugner, 1971a, 1972; Phillips, 1974; Bashford *et al.*, 1976). It now appears that this enzyme is also involved in ATP accumulation. Uptake of both amines and nucleotides is activated by Mg^{2+} and inhibited by *N*-ethylmaleimide and EDTA to a similar degree. In addition, catecholamine uptake requires the presence of ATP to serve as a substrate for the ATPase. It is, of course, rather complicated to prove that the uptake of nucleotides also depends on the presence of ATP. In fact, the observation that the uptake of ADP and AMP occurred without the addition of exogenous ATP seemed to contradict such an idea. However, experiments with ATP analogues demonstrated that the ATPase of the granules was working even without the addition of exogenous ATP, since these two analogues inhibited catecholamine uptake in the presence of ADP and AMP. The most likely explanation of this finding is that enough endogenous ATP released from granules was available for driving catecholamine uptake via the ATPase. The incubated granules (2 mg of protein) contain at least 1 μmol of ATP (see Winkler, 1976). If only 10% of this nucleotide is released during resuspension and incubation this would give a 100 μM -ATP concentration in the medium which is quite sufficient to drive catecholamine uptake.

Therefore the uptake of ADP and AMP by the granules can be driven by the same ATP in the incubation medium which also stimulates catecholamine uptake. Thus it can be proposed that the similarities between the properties of the enzyme ATPase and of the uptake of catecholamines and nucleotides are an expression of the fact that both uptakes are driven by ATP breakdown. A further argument for this concept is the fact that the uptake rates for catecholamines, which were about twice as high in the presence of ATP as in the presence of ADP and AMP, correspond well to the relative maximal uptake rates of the nucleotides, since the rate for ATP was about twice as high as the rate for AMP and ADP. The lower rates in the presence of ADP and AMP can be explained by a driving force (ATPase) not optimally working owing to the insufficient provision of endogenous ATP.

Although the driving force for the two uptake systems appears to be the same, the carriers involved seem to be different. Reserpine inhibits only catecholamine uptake. This is consistent with the view (Slotkin, 1973) that it interferes specifically with the catecholamine carrier. Oligomycin, as suggested by Bashford *et al.* (1976), may act in a similar manner, whereas antimycin may interfere with the membrane cytochrome (Bashford *et al.*, 1976), whose involvement in catecholamine uptake is, however, far from clear. The three nucleotides, on the other hand, seem to depend on a specific carrier which is blocked by atractyloside and carboxyatractyloside. Thus the observation that the uptake of catecholamines and nucleotides can be inhibited independently strongly supports the existence of two separate carrier systems which, however, are driven by energy derived from a single source.

Mechanism of nucleotide and catecholamine uptake

As elaborated recently by elegant experiments (Bashford *et al.*, 1975, 1976; Casey *et al.*, 1976, 1977) the uptake of catecholamines depends on an electrochemical proton gradient ($\Delta\mu_{H^+}$) which is built up by the membrane ATPase. The same mechanism can now also be considered responsible for nucleotide uptake. In addition to the dependence of nucleotide uptake on ATPase discussed above, it has been shown that two uncouplers of oxidative phosphorylation, which can release H^+ gradients, inhibit nucleotide uptake just as they block catecholamine transport. These results are consistent with a dependence of both uptakes on an electrochemical proton gradient. Our experiments, however, allow a further differentiation. A proton gradient actually consists of two components, a difference in electrical charge on the two sides of the granule membrane (electrical portion) and a decreased pH inside the granule (chemical portion). Catecholamines, as weakly basic

amines, are likely to be taken up as a result of the chemical part (ΔpH) of the proton pump (Casey *et al.*, 1977). Consistent with this view is the fact that an increase in the pH differential between medium and granule interior leads to an increase in the uptake of catecholamines (see also Taugner, 1972). Furthermore, NH_4^+ ions, which can abolish pH gradients across membranes (Deamer *et al.*, 1972), were found to be strong inhibitors of catecholamine uptake, which confirms previous results (Carlsson *et al.*, 1963). Finally, the ionophore nigericin, in the presence of external KCl, releases the pH gradient in granules (Johnson & Scarpa, 1976) by leading to an exchange of exogenous K^+ for H^+ from within the granules. In accordance, catecholamine uptake is strongly inhibited under these conditions.

ATP uptake, on the other hand, does not appear to be dependent on the chemical portion of the electrochemical proton gradient. Its uptake is only slightly affected by changes in external pH. Furthermore, neither NH_4^+ nor nigericin influences this uptake. It is noteworthy, however, that both of these agents abolish only the chemical part of the proton gradient, but do not affect the electrical portion, which provides the granule interior with a positive charge. We therefore suggest that the nucleotide uptake is driven by the electrical component ($\Delta\psi$) of the proton gradient built up by the ATPase. It has been shown that the existence of an electrical gradient can lead to the accumulation of the membrane-permeant ion chloride in the granules under conditions *in vitro* (Casey *et al.*, 1977). We now propose that the physiological function of the electrical gradient is to assist in the accumulation of the membrane-impermeable negatively charged nucleotides which are carried via an atractyloside-sensitive transport molecule. Thus chromaffin granules, just like bacteria (see, e.g., Ramos & Kaback, 1977a,b), can use an electrochemical proton gradient for transporting quite different substances. Such a mechanism provides them with an elegant solution to their uptake problems, which require the accumulation of high concentrations of both positively charged amines and negatively charged nucleotides.

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