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SYNTHESIS OF NUCLEOTIDES IN ADRENAL MEDULLA AND THEIR UPTAKE INTO CHROMAFFIN GRANULES*

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Abstract—Perfused bovine adrenal glands were labelled with ^{3}H-adenosine or ^{32}P-phosphate. The time course of the labelling and the nature of the labelled products in the various subcellular fractions were investigated. The catecholamine storing vesicles (chromaffin granules) took up labelled ATP, but significant amounts of ^{3}H-ATP did not appear until 30 min after the injection of the isotope. Four hours after the injection of the isotope the predominantly labelled nucleotide in adrenal medulla was ^{3}H-ATP which was specifically confined to chromaffin granules. There was no evidence that chromaffin granules accumulated significant amounts of ^{3}H-ADP and ^{3}H-AMP. Similar results were obtained with ^{32}P-phosphate as a precursor. Inhibitors of mitochondrial oxidative phosphorylation (cyanide, dinitrophenol) almost completely prevented the uptake of ^{3}H-ATP in chromaffin granules, whereas iodoacetic acid was less effective. It is concluded that in intact cells chromaffin granules obtain newly synthesized ATP mainly from a mitochondrial pool. The mechanism of ATP uptake into chromaffin granules now warrants a detailed study on isolated granules incubated *in vitro*.

Catecholamines in adrenal medulla, sympathetic nerve and brain are stored in specialised subcellular organelles, the best characterized being those of the adrenal medulla, the so-called chromaffin granules [1,2]. The large dense-core vesicles of sympathetic nerves are very similar to chromaffin granules in their biochemical composition [3]. The membrane-limited chromaffin granules contain macromolecular components and small molecules [1, 2]. The main macromolecules are the acidic chromogranins, the enzyme dopamine β-hydroxylase and mucopolysaccharides. The small molecules are the catecholamines, calcium and nucleotides. The nucleotides, mainly ATP, are found in a concentration corresponding to a 0·13 M solution [4]. However it now seems quite likely that these nucleotides are present inside the vesicles in the form of a polymerized complex involving nucleotides, calcium, catecholamines and possibly the chromogranins [5]. Nucleotides are also required for the binding of acetylcholine [6] and 5-hydroxytryptamine in the respective storage organelles [5].

Recent studies have concentrated on the biogenesis of chromaffin granules [7–10]. Results relevant to the biosynthesis of the chromogranins, the mucopolysaccharides and the catecholamines have been obtained. The present study deals with the synthesis of nucleotides in adrenal medulla and their uptake into the catecholamine storing organelles in an attempt to clarify how chromaffin granules obtain and maintain their high concentration of nucleotides.

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MATERIALS AND METHODS

Bovine adrenals were perfused as previously described [7, 9, 10]. The perfusion medium was Tyrode's solution plus 10% (v/v) single-strength tissue culture medium 199 (TC45). Thirty minutes after the start of the perfusion the glands were stimulated once with 2·4 ml of a 15 mM carbamoylcholine chloride solution. If a good secretory response was obtained perfusion was continued, but with the omission of medium 199. Ten minutes later ^{3}H-adenosine (1 mCi) or ^{32}P-phosphate (5 mCi) was injected over 4 min into the perfusion medium. Four min after the injection, medium 199 with the addition of unlabelled adenosine (final concentration in perfusion fluid 2 mM) or sodium phosphate (final concentration in perfusion fluid 5 mM) was again added to the perfusion medium for up to 30 min. At various times after the radioactive pulse the medullas were dissected out and homogenized with a Potter-Elvehjem homogenizer. A large granule fraction (mitochondria, chromaffin granules, lysosomes and some contaminating microsomes) was isolated by differential centrifugation and then subjected to sucrose density gradient centrifugation (5 hr at 120,000 *g*) as already described in detail [11, 12]. To the fractions from the gradient 0·1 ml of a solution containing ATP, ADP, AMP and adenosine (2 mg of each/ml) was added, followed by perchloric acid (final concentration 3%). The precipitate was spun off and the supernatant immediately neutralised with 20% K_2CO_3. Aliquots of the supernatant were then counted in a liquid scintillation spectrometer or spotted on polygram thin layer plates. The plates were developed according to Stevens *et al.* [13], first with H_2O and then with 1 M lithium chloride. The nucleotide and adenosine spots were visualized in ultraviolet light, marked and scraped off into scintillation vials. The material was treated with 0·5 ml of *N*-ethyl, *N*-dodecyl-*N*,*N*-dimethylammonium

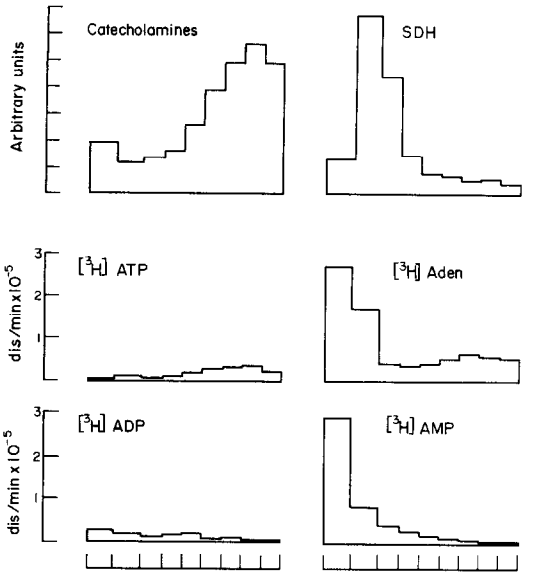

Fig. 1. Density gradient centrifugation of large granule fraction of bovine adrenal medulla labelled with ^{3}H-adenosine. Ten minutes after the injection of ^{3}H-adenosine the perfusion was stopped and a large granule fraction was isolated by differential centrifugation. This fraction was then subjected to sucrose density gradient centrifugation (1·3 to 2·0 M sucrose). The results of the analysis of the density gradient fractions are expressed by histograms. In each histogram the columns from left to right correspond to the fractions of the gradient from top to bottom. The width of the column gives the relative volume of the fraction. The ordinates for catecholamines and succinate dehydrogenase (SDH) are divided by arbitrary units. The actual value of one unit is 0·25 μmole/ml fraction/g adrenal medulla for catecholamines and 1 μmole substrate breakdown/ml fraction/g adrenal medulla for succinate dehydrogenase. The amounts of labelled nucleotides are expressed as dis/min/ml of fraction/g adrenal medulla. Recoveries from the gradient ranged from 70 to 87%.

hydroxide in methanol for 30 min and then 14 ml Bray's scintillation fluid [14] was added. From the total counts present in the gradient fraction and the relative distribution of counts between the nucleotides the absolute counts for nucleotides and adenosine could be calculated.

In experiments with inhibitors of ATP synthesis the compounds were dissolved in Tyrode's solution and then added to the perfusion fluid (10%, v/v) with a syringe delivering a constant volume. Dinitrophenol (5 mM) was dissolved by adding sodium hydroxide to the Tyrode's solution till pH 9 was reached, then the pH was adjusted with HCl to pH 7·4.

Catecholamines were determined colorimetrically [15]. Succinate dehydrogenase (EC 1.3.99.1) was measured according to Porteous and Clark [16].

Materials. 2-^{3}H-Adenosine (20–23 Ci/m-mol) and ^{32}P-Na_2HPO_4 (200 mCi/m-mol) were obtained from Radiochemical Centre, Amersham. *N*-Ethyl, *N*-dodecyl, *N*,*N*-dimethylammoniumhydroxide in methanol and potassium cyanide were purchased from Merck, Darmstadt, Germany. Thin-layer plates (Polygram Cel 300 PEI) were from Macherey-Nagel, Düren, Germany. Tissue culture medium 199 was obtained from Wellcome Reagents, Beckenham, England, 2,4-dinitrophenol from Serva-Heidelberg, Germany and iodoacetic acid, sodium salt from Sigma, London.

RESULTS

Experiments with ^{3}H-adenosine. Perfused bovine adrenal glands were labelled with ^{3}H-adenosine (1 mCi). At various times after the injection of the radioactive compound the adrenal medullae were homogenized. Large granule fractions which contain the bulk of the mitochondria, lysosomes and chromaffin granules and part of the microsomes were isolated and subjected to density gradient centrifugation [11, 12]. After centrifugation mitochondria were concentrated in the top fractions of the gradient (Fig. 1); a similar distribution, as shown previously [7], was demonstrated for glucose-6-phosphatase, a microsomal marker. The catecholamine storing vesicles were found in the dense regions of the gradient (Fig. 1). These fractions also contain the bulk of the endogenous ATP [17]. The distribution of several other marker enzymes in this gradient has already been established [7,11].

In the experiment shown in Fig. 1 the perfusion of the gland was stopped 10 min after the injection of the isotope. Most of the label was found in adenosine and AMP. The bulk of these components was present in the soluble fraction on the top of the gradient and in the mitochondrial fraction. There was a very small peak of labelled adenosine and of ^{3}H-ATP in the dense fractions of the gradient.

When the perfusion was stopped 30 min after the isotope injection, the most significant change was the accumulation of ^{3}H-ATP in the dense fraction of the gradient, where the chromaffin granules containing the catecholamines are concentrated (Fig. 2). This labelling of the more dense fractions is even more pronounced 4 hrs after the isotope (Fig. 3). After this time interval ^{3}H-ATP was the predominantly labelled compound. It was almost entirely confined to chromaffin granules since the distribution of catecholamines was very similar to that of this nucleotide. The distribution of ^{3}H-ADP and ^{3}H-AMP was characterized by a peak in the middle of the gradient, whereas

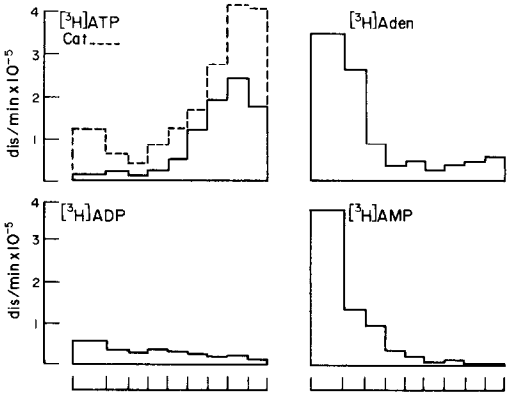

Fig. 2. Density gradient centrifugation of large granule fraction isolated 30 min after ^{3}H-adenosine. The results are expressed as in Fig. 1. The distribution of catecholamines (see dashed line) is given as a percentage of total amount per ml of fraction. Recovery of total dis/min from the gradient was 98%.

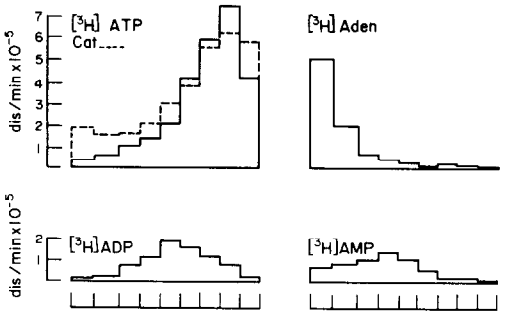

Fig. 3. Density gradient centrifugation of large granule fraction isolated 4 hr after ^{3}H-adenosine. The results are expressed as in Fig. 1. Recovery of total dis/min from the gradient was 70%.

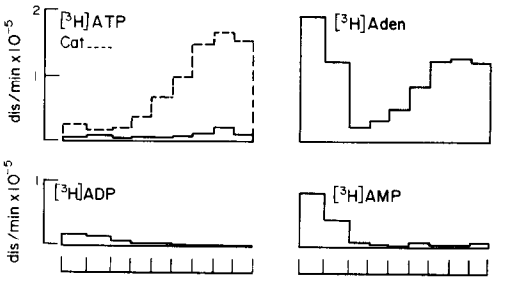

Fig. 4. Labelling of adrenal medulla with ^{3}H-adenosine in the presence of cyanide. Potassium cyanide was added to the perfusion medium in a final concentration of 1 mM 20 min before the injection of the isotope. Cyanide was present in the medium until the perfusion was stopped 30 min after the injection of isotope. The results are expressed as in Fig. 1. Recovery of total dis/min from the gradient was 71%.

^{3}H-adenosine remained in the top fractions. These results are expressed quantitatively in Table 1, which demonstrates that there was a marked increase in ^{3}H-ATP in chromaffin granules between 10 and 30 min after the injection of the isotope.

The localisation of the ^{3}H-nucleotides within the chromaffin granules was investigated by hypotonic lysis and freeze–thawing of these organelles (bottom fractions of the gradient from 30 min experiments) followed by removal of the membranes by high speed centrifugation (40 min at 120,000 *g*). Ninety-five percent of the label was recovered in the soluble lysate (2 extractions combined) indicating that the nucleotides are confined to the soluble part of these organelles without significant binding to the membrane.

In a further series of experiments we used various inhibitors of ATP biosynthesis to obtain information on the origin of the labelled nucleotides found in chromaffin granules. Fig. 4 presents one experiment with cyanide (1 mM), a blocker of mitochondrial oxidative phosphorylation. In the presence of this compound accumulation of ^{3}H-ATP in chromaffin granules was almost completely absent (compare Fig. 4 with Fig. 2; see also Table 1). Under these conditions chromaffin granules accumulate a significant amount of ^{3}H-adenosine as indicated by the peak in the dense regions of the gradient, however there is no accumulation of AMP and ADP. Practically identical results were obtained in a second experiment with cyanide. In another experiment using dinitrophenol (0·5 mM), the results were very similar to those with cyanide (see Table 1). Iodoacetic acid, on the other hand, in a final concentration of 1 mM had a less dramatic influence on ATP uptake into chromaffin granules; the ATP uptake was reduced by about a half in 2 experiments (see Table 1).

Experiments with ^{32}P-phosphate. To confirm the results with ^{3}H-adenosine one experiment with ^{32}P-phosphate was performed. Fig. 5 demonstrates that a very similar result is obtained with this isotope. 30 min after the injection of ^{32}P-phosphate significant amounts of ^{32}P labelled ATP were found in chromaffin granules, whereas ^{32}P-ADP and ^{32}P-AMP did not accumulate in these organelles (see Fig. 5).

DISCUSSION

Three investigations have shown that it is possible to label the nucleotides of chromaffin granules either with ^{32}P-phosphate [10, 28] or ^{3}H-adenosine [13]. In the present study the time course of the labelling, the nature of the labelled products and their subcellular distribution have been determined in perfused ox adrenal glands. It has been shown previously that this preparation functions well as far as the synthesis and secretion of catecholamines, chromogranins and mucopolysaccharides is concerned [7, 9, 10].

After a pulse of ^{3}H-adenosine chromaffin granules start to take up labelled nucleotides. However, a significant concentration of ^{3}H-nucleotides is reached only after 30 min whereas after ten minutes only small amounts are present. The concentration of labelled ATP and ADP in the mitochondrial fraction is always very low. This may well be an artefact since it is likely that during subcellular fractionation the nucleotides in these organelles may diffuse out or become degraded. The ^{3}H-nucleotides of chromaffin granules appear to be present inside these organelles, where they are protected against breakdown by the granule membrane. Nucleotides of chromaffin granules do not diffuse out when they are kept at 0° [18].

Table 1. Uptake of ^{3}H-nucleotides in chromaffin granules

	10 min (n = 4)	30 min (n = 3)	30 min (n = 2) (+cyanide)	30 min (n = 1) (+dinitrophenol)	30 min (n = 2) (+iodoacetic acid)
^{3}H-ATP	105,200 ± 8,000	434,000 ± 103,000	28,300	14,900	260,000
^{3}H-ADP	21,110 ± 3,125	47,000 ± 5,500	6,300	5,200	16,800
^{3}H-AMP	11,800 ± 3,450	10,000 ± 3,540	4,100	12,700	12,000
^{3}H-Adenosine	174,000 ± 14,000	166,000 ± 23,000	250,000	244,000	220,000

Chromaffin granules were isolated by density gradient centrifugation 10 and 30 min after the injection of ^{3}H-adenosine. The three fractions from the gradient containing the highest amount of catecholamines were taken as the chromaffin granule fraction. The values represent dis/min/g adrenal medulla (mean ± S.E.).

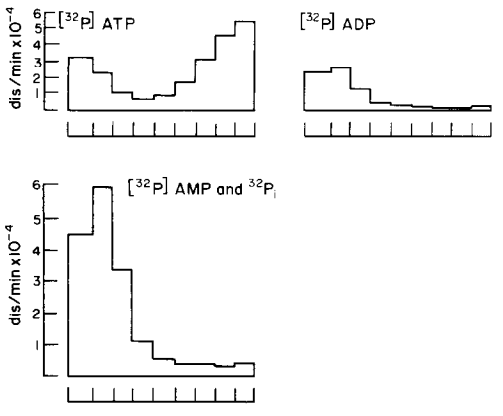

Fig. 5. Density gradient centrifugation of large granule fraction isolated 30 min after ^{32}P-phosphate. The results are expressed as in Fig. 1. ^{32}P-AMP and ^{32}P-phosphate were not separated on the thin layer system used and are given together. Recovery of total dis/min from the gradient was 83%.

Four hours after the injection of labelled adenosine the predominantly labelled nucleotide in adrenal medulla is ATP which is specifically confined to chromaffin granules. There is no evidence that these organelles accumulate significant amounts of ADP and AMP. There is a peak of ADP in the middle of the gradient above the fractions where chromaffin granules accumulate. The significance of this finding is at present obscure.

What is the origin of the labelled ATP in chromaffin granules? ATP could be synthesised either within these organelles or outside followed by an uptake into them. Our results favour the latter possibility. If the nucleotides were synthesised within chromaffin granules one would expect some accumulation of labelled intermediate products, i.e. ^{3}H-AMP and ^{3}H-ADP, in these organelles. However there was no evidence for this as shown by the distribution of the labelled nucleotides in the density gradient fractions. Furthermore, with the synthesis of ATP occurring in chromaffin granules most of the labelled ATP should appear in them shortly after the injection of the labelled adenosine precursor, when followed by an excess of unlabelled adenosine. However there was a considerable increase of labelled ATP within granules between 10 and 30 min after the injection of ^{3}H-adenosine. Similar results were obtained previously with ^{32}P-phosphate [10]. There was no significant appearance of ^{32}P-labelled nucleotides in chromaffin granules 3 min after the radioactive pulse, whereas after 45 min significant amounts were present. This time lag between the injection of the radioactive precursor and the appearance of labelled ATP in chromaffin granules is consistent with a synthesis of this compound outside these organelles followed by a subsequent transport into them. Such a mechanism is supported by the experiments with cyanide, dinitrophenol and iodoacetic acid. All these compounds led to a significant reduction in the appearance of ^{3}H-ATP in chromaffin granules, the two inhibitors of oxidative phosphorylation being more effective. These results point to the mitochondria and the cytoplasmic sap as the locus of ATP-synthesis for chromaffin granules. It is unlikely that chromaffin granules have an enzymatic setup which could be blocked by these three inhibitors and in fact subcellular fractionation studies have provided no evidence for this [19]. However, the results obtained with cyanide deserve some further comment. In the presence of this compound chromaffin granules accumulated significant amounts of ^{3}H-adenosine in contrast to the experiment without cyanide (compare Fig. 4 with Fig. 2). What is the explanation for this finding? Two possibilities should be considered. (i) The precursor ^{3}H-adenosine accumulates in chromaffin granules since cyanide blocks the synthesis of nucleotides inside these organelles. (ii) The presence of cyanide reduces the phosphorylation of ^{3}H-adenosine in the cytoplasm which allows some ^{3}H-adenosine to diffuse into the granules. Actually, in the presence of cyanide less ^{3}H-AMP was present in the soluble fraction (compare Fig. 4 with Fig. 2). This suggestion is further supported by experiments with atractyloside (Peer, Kostron and Winkler, unpublished experiments) which showed that in the presence of this specific inhibitor of ATP release from mitochondria chromaffin granules did not accumulate either ^{3}H-ATP or ^{3}H-adenosine. On the other hand considerable amounts of ^{3}H-ADP and ^{3}H-AMP appeared in the soluble fraction, since there was no inhibition of phosphorylation of ^{3}H-adenosine.

From the results discussed in the preceding paragraph we can conclude that ATP is synthesised outside the chromaffin granules and is subsequently taken up by these organelles. This raises the question of how chromaffin granules accumulate exogenous nucleotides. There are two possibilities. Firstly, the membranes of chromaffin granules are permeable for nucleotides and the nucleotides are taken up directly into the storage complex and stored in the granules. This would explain how mature granules take up nucleotides. However newly formed granules which have not yet acquired a store of catecholamines and ATP would be unable to accumulate nucleotides. Furthermore, chromaffin granules take up labelled ATP preferentially whereas the storage complex can also incorporate ADP [20]. This preferential uptake may however be a simple expression of the intracellular concentration of ATP and ADP, e.g. in brain the ATP concentration is 4 times higher than that of ADP [21]. In this connection it is interesting to note that chromaffin granules of some species, in contrast to those of bovine adrenal medulla, contain quite high concentrations of ADP. For example in chromaffin granules of hen the ADP concentration is as high as that of ATP [22]. However, our experiments gave no evidence for an accumulation of ADP. This high ADP content of chromaffin granules in some species might be an expression of a high ADP concentration in the cytoplasm. Alternatively, in agreement with the present labelling experiments, only ATP may enter the granules but then is degraded to ADP during prolonged storage *in vivo*. Similar problems have arisen in 5-hydroxytryptamine storage granules of thrombocytes which contain significant amounts of ADP but accumulate only labelled ATP in radioactive precursor studies [23].

A second possibility for the uptake of nucleotides

in the chromaffin granules is a carrier mechanism for nucleotides localized in a membrane relatively impermeable for them. This would be a mechanism similar to that for catecholamines. There is now substantial evidence that the membranes of chromaffin granules can actively transport catecholamines from the outside to the inside of these organelles [1, 5, 24]. Such an uptake of nucleotides would enable these organelles to replenish any losses of nucleotides from the storage complex which may be caused by outward diffusion or spontaneous breakdown. Further, this mechanism would enable newly formed chromaffin granules to accumulate nucleotides providing a core for the storage complex. Evidence suggests that nucleotides are concentrated in new granules before catecholamines can be accumulated [8, 25].

Our study on intact cells does not enable us to decide between the two possible mechanisms of nucleotide uptake in chromaffin granules. *In vitro* studies on isolated chromaffin granules are required to provide the final answer. Previous results on isolated granules were inconclusive. Kirshner [26] could not demonstrate any uptake of nucleotides into chromaffin granules. A small uptake of nucleotides was reported by Carlsson *et al.* [27] however no further studies were performed.

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