Adenosine Triphosphatase Activity in the Neural Lobe of the Bovine Pituitary Gland

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(Received 11 February 1974)

1. Homogenates of neural lobes of bovine pituitary glands were fractionated by differential and density-gradient ultracentrifugation and the distribution of adenosine triphosphatase (ATPase) activity was studied. It was shown that all the activity was membrane-bound. 2. On the basis of ionic requirements the ATPase activity was grouped into three categories: (a) Mg^{2+} -dependent, (b) Ca^{2+} -dependent and (c) $Mg^{2+}+Na^{+}+K^{+}$ -dependent (ouabain-sensitive) ATPases. The activity in the absence of bivalent cations was negligible. The ratio between the activities of the three ATPases varied between the different subcellular fractions. 3. Preincubation of the subcellular fractions with deoxycholate increased the activity of the $Mg^{2+}+Na^{+}+K^{+}$ -dependent enzyme, whereas the Mg^{2+} - and Ca^{2+} -activated ATPases were either unaffected or slightly inhibited. Triton X-100 solubilized the Mg^{2+} - and Ca^{2+} -ATPases; however, the activity of the $Mg^{2+}+Na^{+}+K^{+}$ -ATPase was abolished by the concentration of Triton X-100 used. 4. All the subfractions displayed unspecific nucleotide triphosphatase activity towards GTP, ITP and UTP. These substrates inhibited the hydrolysis of ATP by all three ATPases. ADP also inhibited the ATPases. 5. Polyacrylamide-gel electrophoresis of extracts containing the Mg^{2+} - and Ca^{2+} -dependent ATPase activity solubilized by Triton X-100 revealed the presence of two enzymes; one activated by either Mg^{2+} or Ca^{2+} and the other activated only by Ca^{2+} . 6. In sucrose density gradients the distribution of vasopressin was different from that of all three types of ATPases. It is therefore suggested that the neurosecretory granules do not possess ATPase activity.

In the hypothalamo–neurohypophyseal system the hormones vasopressin and oxytocin are believed to be synthesized in the supraoptic and paraventricular nuclei of the hypothalamus (Sachs, 1963). The hormones are subsequently enclosed in subcellular vesicles, the neurosecretory granules. Beside hormones, these vesicles contain soluble proteins, the neurophysins (Hollenberg & Hope, 1968; Dean & Hope, 1968). The granules are transported from the hypothalamic perikarya within the axons to the nerve endings of the neurohypophysis, where considerable amounts of hormone are stored (Lederis & Jayasena, 1970; Vilhardt, 1970).

Secretion of hormone is believed to occur by exocytosis, i.e. a fusion of the membrane of the neurosecretory granules with the plasma membrane of the nerve ending, whereby the soluble contents of the vesicles gain access to the extracellular compartment (Douglas *et al.*, 1971; Uttenthal *et al.*, 1971; Santolaya *et al.*, 1972).

According to Pickering & Jones (1971) the intra-axonal transport of neurohypophyseal hormones occurs at a rate of 1–4 mm/h. Similarly Norström & Sjöstrand (1971) showed that rat neurophysin was transported within the hypo-

thalamo–neurohypophyseal tract at a minimum rate of 2 mm/h. These values are comparable with the so-called fast rate of transport of subcellular particles described in noradrenergic neurones by Dahlström & Häggendal (1966) and in sensory and motor fibres by Ochs & Ranish (1969). It is believed that the fast axonal flow of secretory particles is mediated by microtubules (Dahlström, 1971) and it has been suggested that the energy required for this process could be derived from the hydrolysis of ATP by ATPase* in the membrane of the secretory particles (Schmitt, 1968). Indeed ATPase activity has been described in association with synaptic vesicles from brain tissue (Hosie, 1965; Germain & Proulx, 1965; Berl *et al.*, 1972).

Although microtubules have been demonstrated in the hypothalamo–neurohypophyseal system (Barer & Lederis, 1966; Livingston, 1973) it is not known whether these structures are involved in the transport of neurosecretory granules. It is reasonable to assume, however, that this fast transport of particles is energy-dependent. Since ATPase activity has been demonstrated in subcellular fractions of

* Abbreviation: ATPase, adenosine triphosphatase.

granules (Poisner & Douglas, 1968*a,b*; Vilhardt & Højlmer, 1972; Pickup *et al.*, 1973) the source of energy required for the transport of granules might well be ATP.

The secretion of hormone is initiated by the depolarization of the plasma membrane of the nerve ending accompanied by an increased permeability of the membrane to extracellular Ca^{2+} (Douglas & Poisner, 1964*a,b*). It is not known, however, which mechanism ultimately makes the fusion of the granular membrane with the plasma membrane possible. ATP may be involved in this process, since inhibitors of ATP synthesis block the secretion of hormone from the neural lobe (Douglas *et al.*, 1965).

Since ATPase may thus be involved in the intraxonal transport as well as in the secretion of hormone, we decided to investigate some of the properties of the ATPases in the neural lobe and to study their distribution among subcellular fractions.

Materials and Methods

Biological material

Pituitary glands were removed from adult cattle of both sexes after the animals had been killed by shooting. The glands were transported to the laboratory in an ice-bucket. After removal of the meninges the anterior and posterior lobes were separated and a 1 mm layer of tissue was cut from the ventral and posterior surfaces of the posterior pituitary lobe to remove as much of the intermediate lobe as possible. The remaining neural lobe was freed from connective tissue and placed in a beaker with ice-cold 0.3M-sucrose. Usually five to eight glands were prepared at a time; the yield of neural lobe tissue was of the order of 1–2g wet wt.

Homogenization and fractionation

The neural lobes were placed on a Perspex board and chopped to a fine mince with a stainless-steel knife. Portions (600–1000mg wet wt.) were then homogenized in 12ml of 0.3M-sucrose by using a Teflon-glass homogenizer with a clearance of approx. 0.13mm. Three strokes of the pestle slowly up and down at 950rev./min was the usual procedure.

The homogenate was then centrifuged for 15min at 800*g*_{av.} in a MSE Mistral 6L refrigerated (4°C) centrifuge, yielding a pellet which was resuspended in 5ml of 0.3M-sucrose (fraction I). The supernatant was further centrifuged at 4000*g*_{av.} for 15min in an A-40 rotor by using a Spinco model L refrigerated (4°C) ultracentrifuge. The sediment was resuspended in 2ml of 0.3M-sucrose (fraction II), whereas the supernatant was centrifuged at 26000*g*_{av.} for 15min. The sediment was resuspended in 1–2ml of 0.3M-sucrose (fraction III), leaving the final super-

natant including the microsomal fraction (fraction IV).

Two types of density gradients were used. The first was a discontinuous gradient built up by layering 1ml of 2M-, 1.4M-, 1.35M- and 1.3M-sucrose on top of each other. The other gradient was a continuous, non-linear gradient ranging from 1.3M-sucrose at the top to 2M-sucrose at the bottom. Both types of gradients were made up in Beckman cellulose nitrate tubes (no. 305050) and placed in the cold-room for 18h before use. Fraction (III) (0.8ml) was layered on top of the gradients, which were then placed in a SW 50.1 rotor and centrifuged at 101000*g*_{av.} at 4°C in a Spinco model L-2 65B ultracentrifuge for 60min or longer. Subfractions were recovered either by cutting the gradients, in a Schuster centrifuge-tube cutter or by piercing the bottom of the tubes with a hypodermic needle and collecting fractions of five drops.

Analytical procedures

Protein. Samples (0.1ml) were incubated with 500μl of 1M-NaOH for 30min at room temperature, then 400μl of distilled water was added and the protein content of the mixture was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

ATPase activity. Samples (usually 0.1ml) were incubated in 1ml of medium containing 100mM-Tris-HCl, pH 7.5 (measured at 37°C), and either 2mM-MgCl₂ or 2mM-CaCl₂ or 2mM-MgCl₂+100mM-NaCl+10mM-KCl. Strophanthin-G (1mM) was used to inhibit the $\text{Mg}^{2+}+\text{Na}^{+}+\text{K}^{+}$ -dependent activity when appropriate. The tubes with enzyme (50–250μg of protein) were placed in a water bath at 37°C for 10min before addition of ATP to a final concentration of 3mM. The incubation was carried out for 20min with continuous shaking and the reaction was then terminated by addition of 0.1ml of 50% (w/v) trichloroacetic acid. The tubes were briefly placed in ice-water before centrifugation for 20min at 1500*g*_{av.}. The supernatants were recovered for estimation of P_i . A modification of the method of Fiske & SubbaRow (1925) was used. The sample (0.5ml) was added to 1.5ml of 0.8M-H₂SO₄; then 0.8ml of 1.25% (w/v) ammonium molybdate was added followed by 0.2ml of Fiske-SubbaRow reagent [0.25% (w/v) 1-amino-2-naphthol-4-sulphonic acid–15% (v/v) sodium hydrogen sulphite–0.5% (w/v) anhydrous sodium sulphite]. Exactly 20min later E_{820} of the mixture was read.

P_i formation during the enzyme reaction was linear with time up to about 30min, after which it declined somewhat. Suitable dilution of the samples ensured that ATP hydrolysis was kept below 10% of the total ATP concentration.

Vasopressin. This was measured by the method of Dekanski (1952) as modified by Dean & Hope (1967).

Vasopressor activity was extracted from the fractions by the addition of acetic acid to a concentration of 0.25% (v/v) and boiling on a water bath for 5 min. For the assay male albino rats were anaesthetized with urethane (1.25g/kg body wt.) given intraperitoneally and phenoxybenzamine (1mg/kg body wt.) was given intravenously.

Polyacrylamide-gel electrophoresis. Various subcellular fractions were extracted with 1% (v/v) Triton X-100 (5–10mg of protein/ml) for 3 h at room temperature and then for a further 21 h in the cold-room. The mixture was then centrifuged at 100000g_{av} for 60 min. Sucrose (5%, w/v) was added to the supernatant and samples (50 µl) were used for each gel.

The gels were made up by using a modification of the method of Clarke (1964). Glass tubes (7.5 cm × 0.5 cm internal diam.) were filled with 1.3 ml of 5.5% (w/v) Cyanogum 41 (95% acrylamide monomer and 5% *NN'*-methylene bisacrylamide) in 0.075% (w/v) Tris and 0.363% (w/v) glycine. Polymerization was catalysed by 0.07% (w/v) ammonium persulphate in the presence of 0.035% (v/v) *NNN'*-tetramethylethylenediamine. The tops of the gels were covered with 2-methylpropan-1-ol and left at room temperature for 24 h. The gels were then pre-run at 0.5 mA/tube in a buffer of 0.075% (w/v) Tris and 0.363 (w/v) glycine.

For the actual run 50 µl of sample was placed on top of each gel together with 5 µl of 0.05% Bromophenol Blue and electrophoresis was carried out at 0.5 mA/tube till the dye had entered the gel and then at 1 mA/tube. The electrode buffer contained 0.06% (w/v) Tris, 0.29% (w/v) glycine and 5 mM-HCl. Electrophoresis was stopped when the dye had reached to within 2 mm from the end of the gels.

The tubes were removed and some of the gels stained for 30 min in 0.1% (w/v) Amido Black in 7.5% (v/v) acetic acid followed by repeated washings with 7.5% (v/v) acetic acid. The gels to be used for

demonstration of ATPase activity were incubated at 37°C in a medium containing 100 mM-Tris-HCl, pH 7.7, 4 mM-MgCl₂ or 4 mM-CaCl₂ and 3 mM-ATP [a modification of the method of Wachstein & Meisel (1957)]. After 30 min Pb(NO₃)₂ was added to give a final concentration of 3 mM. After a further 30 min incubation at 37°C the gels were stored in the medium at room temperature.

Chemicals

Adenosine 5'-triphosphate (disodium or di-Tris salt) was obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Before use it was dissolved as a 30 mM solution in 50 mM-Tris-HCl at pH 6.5. GTP, ITP and UTP as trisodium salts and ADP as disodium salt were likewise obtained from Sigma Chemical Co. Triton X-100 was from Packard Instrument Co., Downers Grove, Ill., U.S.A., and sodium dodecyl sulphate from BDH Chemicals Ltd., Poole, Dorset, U.K. Deoxycholate was obtained as the Tris salt from sodium deoxycholate (BDH Chemicals Ltd.) by precipitating the acid with HCl and dissolving the precipitate in Tris. This procedure was repeated twice. Strophanthin-G (ouabain) and Cyanogum 41 were from BDH Chemicals Ltd. 2,3-Dimercaptopropan-1-ol (British Anti-Lewisite) from BDH Chemicals Ltd. was used as a fine emulsion in distilled water. Synthetic (8-arginine)-vasopressin (Studer, 1963) was used as a standard for the pressor assays. All chemicals used were of analytical grade.

Results

Distribution of ATPase activity in fractions from differential centrifugation

In subcellular fractions three types of ATPases could be distinguished, a Ca²⁺-, a Mg²⁺- and a Mg²⁺+Na⁺+K⁺-dependent enzyme. The four frac-

Table 1. *Specific activities of ATPases in subcellular fractions of bovine neural lobes*

The upper section of the Table shows the activity in the four fractions obtained by differential centrifugation (fractions I, II, III and IV). The lower part of the Table shows the activity in the three subfractions (A, B and C) obtained by discontinuous density-gradient centrifugation of fraction (III) for 60 min (see Fig. 3). The results are means ± s.d. (n = 4).

Fraction	Sp. activity (nmol of P _i released/h per mg of protein)			Ratio between ATPases	
	Mg ²⁺ -ATPase	Ca ²⁺ -ATPase	Mg ²⁺ +Na ⁺ +K ⁺ -ATPase	Ca ²⁺ /Mg ²⁺	Mg ²⁺ +Na ⁺ +K ⁺ /Mg ²⁺
(I)	2569 ± 1560	2482 ± 990	1317 ± 620	0.97	0.51
(II)	2871 ± 453	1809 ± 73	2320 ± 142	0.63	0.81
(III)	3524 ± 352	2710 ± 30	1289 ± 52	0.77	0.37
(IV)	1063 ± 45	1532 ± 315	220 ± 61	1.44	0.21
A	4163 ± 2398	2840 ± 1316	2040 ± 1112		
B	1106 ± 712	554 ± 205	<100		
C	985 ± 675	443 ± 225	<100		

tions all contained the three ATPases, but the ratio between the activities of the three enzymes varied from fraction to fraction (Table 1). Whereas the fractions sedimented first (I, II and III) were richest in Mg^{2+} - and $Mg^{2+}+Na^{+}+K^{+}$ -ATPase, the microsomal fraction (IV) predominantly contained the Ca^{2+} -activated enzyme. Table 1 also shows the absolute specific activities (nmol of P_i /h per mg of protein) in the fractions. The specific activities of the fractions could, however, be increased to various degrees by washing the pellets in dilute buffer. Resedimentation of the material then yielded a pellet with an increased specific activity, presumably because of loss of soluble proteins from the particles. The supernatants, even after repeated washings, were free of activity, showing that the enzymes were firmly attached to membranes. The ATPase activity

of the final supernatant (fraction IV) could be sedimented together with the microsomal fraction by centrifugation at $100000g_{av}$ for 60 min.

Ionic requirements

In the absence of bivalent cations the activity of subcellular fraction (III) was very low (Table 2). Addition of $Na^{+}+K^{+}$ to Mg^{2+} increased the ATP hydrolysis corresponding to the activation of the $Mg^{2+}+Na^{+}+K^{+}$ -ATPase. This effect was completely abolished by 1 mM-ouabain. In the presence of ouabain Na^{+} and K^{+} seemed to slightly inhibit the Mg^{2+} -ATPase. Addition of $Na^{+}+K^{+}$ to Ca^{2+} did not change the activity found with Ca^{2+} alone.

Table 2. Influence of ions on the ATPase activity of bovine neural lobes

Portions of fraction (III) were incubated with either Mg^{2+} (left column) or Ca^{2+} (right column). The activity found on addition to the medium of various ions is expressed as a percentage of the activity observed with either Mg^{2+} or Ca^{2+} alone. Values are means $\pm$ S.D. ($n=4$), except for the addition of EDTA (one expt.).

Addition (mm)	Activity (%)	
	Mg^{2+} (2mm) = 100	Ca^{2+} (2mm) = 100
Na^{+} (100)	87 ± 7	94 ± 9
K^{+} (10)	98 ± 6	99 ± 11
$Na^{+}+K^{+}$ (100+10)	131 ± 17	93 ± 6
$Na^{+}+K^{+}$ +ouabain (100+10+1)	89 ± 5	—
Ca^{2+} (2) or Mg^{2+} (2)	77 ± 10	136 ± 16
$Na^{+}+K^{+}+Ca^{2+}$ (100+10+2)	91 ± 9	—
EDTA (2)	3	5

Fig. 1. Influence of Mg^{2+} and Ca^{2+} on ATPase activity of bovine neural lobe

Portions of fraction (III) were assayed for ATPase activity in the presence of increasing concentrations of either Mg^{2+} (○), or Ca^{2+} (●) or 1 mM- Mg^{2+} +increasing concentrations of Ca^{2+} (■).

Table 3. Substrate specificity of ATPases from bovine neural lobes

The formation of P_i during incubation of portions of fraction (III) with various substrates is expressed as a percentage of the activity observed with ATP as substrate. Values are means $\pm$ S.D. ($n=3$), except for ATP+UTP (one expt.).

Substrate (mm)	Activity (%)		
	Mg^{2+} -ATPase	Ca^{2+} -ATPase	$Mg^{2+}+Na^{+}+K^{+}$ -ATPase
ATP (3)	100	100	100
ITP (3)	69 ± 9	98 ± 1	18 ± 4
GTP (3)	63 ± 11	96 ± 2	8 ± 3
UTP (3)	65 ± 12	96 ± 6	6 ± 3
ADP (3)	58 ± 12	50 ± 2	37 ± 1
Glucose 1-phosphate (3)	0	0	0
ATP (3)+ADP (0.5)	98 ± 2	94 ± 3	79 ± 5
ATP (3)+ADP (3)	68 ± 12	71 ± 26	41 ± 23
ATP (3)+UTP (0.5)	93	88	57

Addition to the incubation medium of equimolar amounts of Ca^{2+} and Mg^{2+} gave values intermediate between those found with either Ca^{2+} or Mg^{2+} alone. Ca^{2+} also inhibited the $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -dependent activity.

The activity of the enzymes increased with the concentration of bivalent cations up to about 3 mM (Fig. 1). Further increase in the concentration of Mg^{2+} slightly inhibited the ATPase; no inhibition was seen with increased concentrations of Ca^{2+} . Increasing the concentration of Ca^{2+} in the presence of 1 mM- Mg^{2+} indicated that the activation by these two cations was not additive and that some form of inhibition was involved.

Substrate specificity

Besides ATP other nucleoside triphosphates as well as ADP, but not glucose 1-phosphate were hydrolysed by the enzyme preparations (Table 3). GTP, ITP and UTP seemed to be equally good substrates especially in the presence of Mg^{2+} or Ca^{2+} . At low concentrations (0.6 mM) ADP inhibited the $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -ATPase. At higher concentrations of ADP (3 mM) the Ca^{2+} - and Mg^{2+} -activated ATPases were also inhibited.

Influence of detergents and other reagents

Deoxycholate even in low concentrations (0.025%) inhibited the ATPase activity. However, preincubation of the enzyme preparation with 0.08% deoxycholate as described by Jørgensen & Skou (1971) followed by a 20-fold dilution before incubation with ATP increased the activity of the $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -ATPase, whereas the Mg^{2+} - and Ca^{2+} -dependent activities were unaffected or even impaired (Table 4).

Preincubation with low concentrations (0.01%) of Triton X-100 inhibited the $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -ATPase but did not affect the Mg^{2+} - and Ca^{2+} -activated enzymes. Higher concentration of Triton X-100 (1%) solubilized the Mg^{2+} - and Ca^{2+} -ATPase, however, with some loss of activity; in fraction (III) $39 \pm 14\%$ of the Mg^{2+} -activated ATPase and $52 \pm 12\%$ of the Ca^{2+} -activated ATPase were solubilized by 1% Triton X-100 (four expts.). None of the detergents used solubilized appreciable amounts of $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -ATPase. Procedures for extracting actomyosin from muscle tissue (Szent-Györgyi, 1951) applied to the present subcellular fractions were likewise ineffective in solubilizing the enzymes.

Preincubation of the enzyme preparation with 0.1% sodium dodecyl sulphate inhibited the Mg^{2+} - and Ca^{2+} -dependent ATPases, whereas the $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -ATPase was activated. Higher concentrations of sodium dodecyl sulphate completely abolished all ATPase activity.

All three enzymes seemed dependent on SH groups, since incubation in the presence of $10 \mu\text{M}$ -*p*-chloromercuric benzoate (at pH 8.1) decreased their activity (Table 5). Subsequent addition of 2,3-dimercaptopropan-1-ol or cysteine restored the Mg^{2+} - and Ca^{2+} -dependent activities, whereas the inhibition of the $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -ATPase, which was complete, could not be reversed.

pH-dependence of ATPase activity

The ATPases are active over a rather wide range of pH values (Fig. 2). Mg^{2+} -ATPase had a small peak of activity close to pH 6 and a maximum between pH 8.5 and 9.5. The Ca^{2+} -ATPase seemed to have an optimum of activity close to pH 9.5, whereas the $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -ATPase displays a broad

Table 4. Influence of detergents on the ATPase activity of bovine neural lobes

The results are expressed as a percentage of the activity found without detergent in the incubation medium (means $\pm$ S.D.; $n = 4$). Upper part of the Table: portions (approx. $150 \mu\text{g}$ of protein) of fraction (III) were assayed for ATPase activity as described in the Materials and Methods section in the presence of sodium dodecyl sulphate or Triton X-100 at the concentrations indicated. Lower part of the Table: portions of fraction (III) were preincubated for 30 min at 20°C with the concentrations of deoxycholate, sodium dodecyl sulphate or Triton X-100 indicated. After a 20-fold dilution the ATPase activity was determined as described in the Materials and Methods section.

Detergent added (concentration during incubation)	Activity (%)		
	Mg^{2+} -ATPase	Ca^{2+} -ATPase	$\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ -ATPase
Control	100	100	100
Sodium dodecyl sulphate (0.1%)	0	0	0
Triton X-100 (0.01%)	94 ± 12	93 ± 8	55 ± 15
Triton X-100 (1%)	83 ± 8	85 ± 11	0
Deoxycholate (0.08%)	104 ± 7	88 ± 12	199 ± 32
Sodium dodecyl sulphate (0.1%)	11 ± 2	19 ± 4	183 ± 28
Triton X-100 (0.01%)	107 ± 5	92 ± 8	57 ± 13
Triton X-100 (1%)	115 ± 12	96 ± 8	0

Table 5. *ATPases from bovine neural lobes: influence of SH reagents*

Portions of fraction (II) were assayed for ATPase activity at pH 8.1, but otherwise as described in the Materials and Methods section. The total incubation time was 30 min. In some instances the enzyme preparation was incubated for 5 min with *p*-chloromercuric benzoate, after which 2,3-dimercaptopropan-1-ol or cysteine was added to the medium and the incubation was continued for a further 25 min. The activities observed are expressed as a percentage of the activity found in control incubations.

	Activity (%)		
	Mg ²⁺ -ATPase	Ca ²⁺ -ATPase	Mg ²⁺ +Na ⁺ +K ⁺ -ATPase
Control	100	100	100
<i>p</i> -Chloromercuribenzoate (10 μ M)	74	64	0
2,3-Dimercaptopropan-1-ol (1 mM)	117	93	115
Cysteine (10 mM)	124	95	113
<i>p</i> -Chloromercuribenzoate (5 min)+ 2,3-dimercaptopropan-1-ol (25 min)	104	112	0
<i>p</i> -Chloromercuribenzoate (5 min)+ cysteine (25 min)	105	120	0

Fig. 2. *pH-dependence of ATPases from bovine neural lobe*

Portions of fraction (II) were assayed for ATPase activity in buffered (Tris-HCl-succinate) media of various pH values. pH of the incubation mixtures was checked after the addition of ATP and again after the incubation. The activities of Mg²⁺-ATPase (○), Ca²⁺-ATPase (●) and Mg²⁺+Na⁺+K⁺-ATPase (△) were measured as described in the Materials and Methods section.

maximum of activity between pH 6 and 8. In all instances the activity declined sharply at pH values above 9.5.

Distribution of ATPases in fractions from density gradients

The discontinuous sucrose density gradient used was a modification of that described by Dean & Hope (1967). Centrifugation of fraction (III) on this gradient gave rise to two sharp bands of material

Fig. 3. *Profile of a discontinuous sucrose gradient*

The gradient was made up of layers of sucrose of various molarity as indicated on the schematic drawing and left for 18 h at 4°C. A sample (0.8 ml) of fraction (III) was layered on the gradient, which was then centrifuged at 101 000 *g*_{av}, usually for 60 min. The three particulate bands (A, B and C) were recovered by cutting the tube as indicated by the arrows, or the whole gradient was sampled in smaller fractions by piercing a hole in the bottom of the tube.

(bands A and C, Fig. 3). The intermediate zone contained particulate material apparently evenly dispersed. The corresponding fractions were recovered by cutting the gradient as indicated in Fig. 3.

The specific activities of ATPase in the three subfractions (A, B and C) are given in Table 1. Fraction C represents a purified preparation of neurosecretory granules (Dean & Hope, 1967; Vilhardt & Tøndevold, 1972). This fraction displayed no $Mg^{2+}+Na^{+}+K^{+}$ -dependent activity, but contained both Mg^{2+} - and Ca^{2+} -ATPase. Characteristically the amount of activity in the fraction varied considerably from one experiment to another. To obtain a more detailed picture of the distribution of hormone and enzymes on the gradient, fractions were collected by piercing the bottom of the tube with a hypodermic needle.

Fig. 4 shows the distribution of protein, vasopressin and Mg^{2+} -ATPase in fractions of discontinuous density gradients centrifuged for various

lengths of time. By using vasopressin as a marker for neurosecretory granules it can be seen that these particles gradually build up as a band in the lower part of the gradient (Fig. 3, band C), which also displays some ATPase activity. The profile of distribution of ATPase is, however, completely different from that of vasopressin. Ca^{2+} -activated ATPase was distributed in the same way as the Mg^{2+} -activated enzyme, whereas the $Mg^{2+}+Na^{+}+K^{+}$ -ATPase was only found in the top band (Fig. 3, band A) of the gradient. The difference between the distribution of vasopressin and ATPase became even more evident when the material was placed on a continuous density gradient (1.3–2.0M-sucrose) and centrifuged for 180 min. Fig. 5 shows that the accumulation of neurosecretory granules (vasopressin) in the lower part (band C) of the gradient is much more prominent than when a discontinuous gradient is used. The major part of the ATPase activity, however, is still found in the top band (A) of the gradient.

Fig. 4. Distribution of protein, vasopressin and Mg^{2+} -ATPase on a discontinuous gradient centrifuged for various lengths of time

Portions of fraction (III) were layered on the gradients, which were then centrifuged at $101\,000g_{av}$ for 20, 45, 60 or 180 min. Fractions were recovered by piercing a hole in the bottom of the tubes. In each diagram the top of the gradient is to the left (fraction 1). The amounts of protein (a), vasopressin (b) and Mg^{2+} -ATPase (c) in each fraction are expressed as a percentage of the total amount placed on the gradient.

Fig. 5. Distribution of protein, vasopressin and Mg^{2+} -ATPase on a continuous density gradient

The gradient ranged from 1.3M- to 2.0M-sucrose. A portion of fraction (III) was layered on the gradient, which was then centrifuged at $101\,000g_{av}$ for 180 min. Fractions were collected through the bottom of the tube. In each diagram the top of the gradient is to the left (fraction 1). The density (a) of each fraction was measured by weighing a sample in a $100\mu l$ constriction pipette. The amounts of protein (b), vasopressin (c) and Mg^{2+} -ATPase (d) in each fraction are expressed as a percentage of the total amount placed on the gradient.

Polyacrylamide-gel electrophoresis of ATPases

Proteins solubilized by 1% Triton X-100 from various fractions were run on 5.5% (w/v) polyacrylamide gels. After the run the gels were incubated with ATP and different ions. On addition

of Pb^{2+} white bands appeared in the gels owing to the precipitation of liberated P_i . When either ions, ATP or Pb^{2+} were omitted no bands were formed, and gels incubated with Mg^{2+} or Ca^{2+} differed (Plate 1). Band A did not appear regularly in the gels, band B seemed to be common to both ATPases, whereas band C was seen only in gels incubated with Ca^{2+} . Incubation of the gels with $Mg^{2+} + Na^+ + K^+$ medium gave a picture similar to the one obtained with Mg^{2+} alone, which is to be expected since the $Mg^{2+} + Na^+ + K^+$ -ATPase is not solubilized by Triton X-100. Incubation of the gels with ITP gave rise to bands in the same positions as when ATP was used.

Discussion

The presence of ATPase activity in the granular fraction obtained by sucrose-gradient centrifugation of homogenates of bovine posterior pituitary reported by Poisner & Douglas (1968a,b) has been confirmed in the present study. The possibility that ATPase is a constituent of the neurosecretory vesicles and may participate with ATP in the secretion of oxytocin and vasopressin from the pituitary gland led us to study the enzyme in greater detail. Subcellular fractionation of other endocrine and nervous tissues has shown that ATPase is associated with vesicles storing hormones and transmitters: adrenal chromaffin granules (Hillarp, 1958; Banks, 1965; Kirschner *et al.*, 1966; Trifaró & Warner, 1972), noradrenalin storage vesicles from splenic nerve (Klein & Lagercrantz, 1971) and synaptic vesicles from brain tissue (Germain & Proulx, 1965; Hosie, 1965; Kadota *et al.*, 1967). The properties of the ATPases found in these vesicles differ considerably from one tissue to another.

The neurosecretory granule preparation used by Poisner & Douglas (1968b) was prepared from whole posterior lobes of bovine pituitary glands by the method of Dean & Hope (1967). Granules prepared in this way are relatively free from mitochondrial and lysosomal contamination [according to Dean & Hope (1967) the amounts of succinate dehydrogenase and cathepsin found in the granule fraction constitute less than 1% of the activities placed on the gradient]. Whole posterior pituitary lobes, however, contain much non-neuronal tissue (pars intermedia) and it is possible that the ATPase is derived, at least in part, from subcellular particles other than neurosecretory granules (Howe & Maxwell, 1968). In the present work this form of contamination has been minimized as far as possible by dissecting away the pars intermedia and by using only the pars nervosa tissue.

In the work of Poisner & Douglas (1968b) the ATPase described in fractions containing neurosecretory granules was primarily activated by Mg^{2+} and to a lesser extent by Ca^{2+} . In the present study

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of ATPases from bovine neural lobe

Triton X-100 (1%) extracts of fraction (III) were electrophoresed on 5.5% (w/v) polyacrylamide gels. The gels were then incubated with ATP, Mg^{2+} (gels 1 and 3) or Ca^{2+} (gels 2 and 4) followed by addition of Pb^{2+} as described in the Materials and Methods section.

both Mg^{2+} and Ca^{2+} activated the hydrolysis of ATP, but the ratio between the two activities varied from one subcellular fraction to another. This led us to suppose that at least two different enzymes were present in the fractions. This view was further supported by the results of the polyacrylamide-gel electrophoresis of soluble-enzyme preparations. This technique demonstrated the presence of one enzyme which was activated solely by Ca^{2+} (Ca^{2+} -ATPase) and another enzyme activated by either Mg^{2+} or Ca^{2+} (Mg^{2+} -ATPase). The method was not suitable for quantitative purposes and therefore it was not possible from these results to estimate the extent of the activation of the latter enzyme by either Ca^{2+} or Mg^{2+} . However, the presence of this enzyme in subcellular fractions may explain why the effect of the two ions was not additive, if it is assumed that Mg^{2+} and Ca^{2+} compete for the activation of the enzyme. Similar effects of Ca^{2+} and Mg^{2+} have been reported in ATPase preparations of crab nerve (Skou, 1960), rabbit brain (Skou, 1962), bovine heart mitochondria (Pullman *et al.*, 1960), 5-hydroxytryptamine-storing cells (French *et al.*, 1970; Heinrich *et al.*, 1972) and microsomal fractions from the adrenal medulla (Goz, 1967).

The use of both discontinuous and continuous density gradients followed by fractionation by drop-collecting proved a valuable means for estimating and comparing the precise distributions of hormone and enzymes in the gradients. In both types of gradients the neurosecretory granules tended to accumulate between densities of 1.20 and 1.23, which agrees with the findings of Pickup & Hope (1971). The reason why a considerable amount of pressor activity is found at densities lower than 1.18 (band A of the gradient) is at present unclear. Part of the explanation might be that it takes several hours for most of the granules to reach equilibrium position in the density gradient as indicated by the present results.

The behaviour of ATPase activity on the gradients was completely different from that of vasopressin. Even after prolonged centrifugation the major part of the ATPase activity was found at a density of approximately 1.18 and only small amounts of activity could be demonstrated further down the gradient (less than 10% of the amount of Mg^{2+} -ATPase placed on the gradient was found in the granule fraction, corresponding to a specific activity of the enzyme of less than 1 μ mol of P_i released/h per mg of protein).

Besides the marked differences in the distribution of vasopressor and ATPase activities, it was striking that experimentally induced changes in the distribution of vasopressin (different gradients, various times of centrifugation) were not paralleled by similar changes in the distribution of ATPase.

It was not possible to separate completely the vasopressor and ATPase activities. However, the observed discrepancies in the behaviour of the two activities in our opinion suggest that the ATPase found in the granular fraction represents a contamination and that the neurosecretory granules themselves may not possess such an enzyme.

If ATPase is not a constituent of the neurosecretory granules the energy required for transport and secretion of hormone must come from other sources than ATP, or the hydrolysis of ATP must be catalysed by enzymes associated with other subcellular components involved in transport and secretion, e.g. the microtubular system and the plasma membrane. The presence of Mg^{2+} - and $Mg^{2+}+Na^{+}+K^{+}$ -ATPases in the plasma membrane of cells has been demonstrated both biochemically (Post *et al.*, 1965; Emmelot & Bos, 1966) and histochemically (Sluga & Tomonaga, 1970; Fernando, 1972). Nagayama & Dales (1970) reported that preparations of mouse fibroblast microtubular protein crystallized with vinblastine possess ATPase activity. It remains to be demonstrated whether microtubules of neurosecretory cells display such activity.

This work was supported by grants from the Medical Research Council, the British Council and the Danish Medical Research Council.

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