Adenosine Triphosphate

A CONSTITUENT OF CHOLINERGIC SYNAPTIC VESICLES

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(Received 13 September 1973)

1. Synaptic vesicles separated by density-gradient centrifugation from extracts of the cholinergic nerve terminals of the electric organ of *Torpedo marmorata* were found to contain appreciable amounts of ATP as well as acetylcholine. 2. Vesicular ATP was stable in the presence of concentrations of apyrase and myokinase that rapidly destroyed equivalent amounts of endogenous or added free ATP; pre-treatment of cytoplasmic extracts of electric tissue with these enzymes destroyed endogenous free ATP, but did not affect the vesicular ATP. 3. When [U-¹⁴C]ATP was added to electric tissue at the time of comminution and extraction of the vesicles, all the radioactivity was associated with soluble components in the subsequent fractionation: none was associated with vesicles or membrane fragments; thus it is unlikely that vesicular ATP can be accounted for by the sequestration of endogenous free ATP within any vesicles formed during comminution and extraction of the tissue. 4. When synaptic vesicles were passed through iso-osmotic columns of Bio-Gel A-5m, which separates vesicles from soluble proteins and small molecules, all the recovered ATP and acetylcholine passed through together in the void volume. 5. Regression analysis showed that vesicular ATP content was highly correlated with vesicular acetylcholine content in different experiments, the molar ratio acetylcholine/ATP being $5.32 \pm (\text{S.E.M.}) 0.45$ (21 expts.) for the peak density-gradient fraction. The ratio varied, however, somewhat across the density-gradient peak suggesting some degree of chemical heterogeneity in the vesicle population.

In recent work (Whittaker, 1971, 1972; Whittaker *et al.*, 1971, 1972a,b, 1974) methods have been developed for the relatively large-scale isolation of cholinergic synaptic vesicles, with zonal rotors, from the electric tissue of species of *Torpedo*. This has made possible a detailed investigation of the composition of these cell organelles. Preliminary investigations of the proteins of the synaptic-vesicle fraction led to the identification of a low-molecular-weight acidic protein ('vesiculin'), which is released by hypo-osmotic treatment of the fraction and which we believe is derived from the core of the vesicle. Spectroscopic and chemical analysis of this component suggested that it was associated with adenine nucleotides.

Since a variety of amine-storage granules [chromaffin granules, Hillarp (1958); splenic-nerve granules, Schümann (1958); and platelet granules, Da Prada & Pletscher (1968)] contain ATP, it was decided to investigate the occurrence of ATP in cholinergic vesicles by using the specific and sensitive firefly luciferin-

luciferase system. This study has shown that ATP is also a constituent of the cholinergic vesicles isolated from *Torpedo* electric tissue. A preliminary account of some of this work has been published (Whittaker *et al.*, 1972a).

Materials and Methods

Materials

Fish. Live specimens of *Torpedo marmorata* were obtained from the Institut de Biologie Marine, Arcachon, France. The fish were transported in sealed plastic bags containing sea-water under slight oxygen pressure. They were kept in a sea-water aquarium at 10–12°C until used. Live specimens of *Torpedo californica* (Pacific Bio-Marine Co., Venice, Calif., U.S.A.), a gift from Dr. M. V. L. Bennett, were housed in tanks of running filtered sea-water at 12–15°C until required.

Chemicals. Unless otherwise stated all chemicals used were of analytical grade. The following chemicals were purchased from Sigma (London) Chemical Co., London S.W.6, U.K.: catalase (EC 1.11.1.6), purified from ox liver; ATP disodium salt, from equine muscle,

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desiccated firefly tails and freeze-dried firefly tail extract; potato apyrase (EC 3.6.1.5) (grade I); rabbit muscle myokinase (EC 2.7.4.3) (grade III) and 5'-AMP deaminase (EC 3.5.4.6) (grade IV); and calf intestinal mucosa adenosine deaminase (EC 3.5.4.4). Acetylcholine perchlorate was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., and [^{14}C]-ATP from The Radiochemical Centre, Amersham, Bucks., U.K.

Extraction and isolation of synaptic vesicles. This was carried out essentially as described by Whittaker *et al.* (1972b) with minor modifications. Unless otherwise stated fish were killed by pithing at cold-room temperatures. In a few experiments fish were anaesthetized with Tricaine (ethyl-*m*-aminobenzoate methanesulphonate) (final concn. 0.01% in seawater) before pithing or severance of the electric nerves. After pithing or severance of the electric nerves, the electric tissue was rapidly removed and weighed, frozen in liquid Freon 12 (dichlorodifluoromethane), and finally crushed to a coarse powder in a mortar. In some experiments tissue was kept in temporary storage (up to 8 h) by immersion in liquid N_2 before comminution. In two experiments the tissue was not frozen, but comminuted by blending with vesicle-extraction medium by using an MSE Atomix apparatus operating at full speed for three periods of 30 s interrupted by two 30 s rest periods.

Vesicles were extracted from powdered tissue by suspension in 0.2 M-sucrose–0.3 M-NaCl (0.5–2 vol.), and then filtration through muslin to remove coarse tissue debris. The resultant cytoplasmic extract was then fractionated into fractions P_{12} and S_{12} , as described by Whittaker *et al.* (1972b). Synaptic vesicles were isolated from fraction S_{12} by using density-gradient centrifugation. In some of the preliminary experiments with *T. californica*, discontinuous gradients in a swinging-bucket rotor (Beckman SW25) were used as described by Israël *et al.* (1970). In all other experiments vesicles were isolated from fraction S_{12} by zonal centrifugation with a Beckman Ti-14 rotor in an L2 65B preparative ultracentrifuge housed in a cold-room as described by Whittaker *et al.* (1972b).

During the course of the work, the extraction and isolation media were modified by the inclusion of 10 mM-Tris-HCl buffer (pH 7.0 at 0–4°C), and since this probably results in more stable preparations it has been adopted in our laboratory as standard procedure. Experiments in which buffered media were used have been so indicated.

Analytical methods

Protein. This was determined by using the method of Lowry *et al.* (1951), with bovine plasma albumin as standard.

Acetylcholine. This was extracted from whole tissue with ice-cold 10% (w/v) trichloroacetic acid, and from suspensions of subcellular particles by heating at pH 4.0 and 100°C for 10 min; it was assayed on small slips of leech dorsal muscle (for details see Whittaker *et al.*, 1972b).

ATP. The assay procedures were based on the method of Stanley & Williams (1969) with modifications as described below. Method I was used for some of the early experiments, but was superseded by method II, which was found to be more reliable.

Method I. ATP was extracted from small pieces (0.5–1 g) of whole tissue and suspensions of subcellular particles (0.2–1 ml) with 2 vol. of ice-cold 0.2 M-HClO₄. For whole tissue it was necessary to mince the tissue suspended in acid with a fine pair of scissors. After allowing at least 10 min for extraction, the insoluble material was removed by centrifugation at 3000g for 10 min at 0–4°C, and samples (10–50 μl) of the supernatant were assayed by using the luciferin-luciferase enzyme system. The enzyme was prepared from dried firefly lanterns as described by Stanley & Williams (1969). For preparations of the highest activity it was necessary to prepare the enzyme extract on the day of assay. Light produced by the enzyme in the presence of ATP was measured at ambient temperature with a Beckman LS 150 liquid-scintillation spectrometer as described below. The sample was mixed with 3 ml of buffer [prepared by mixing equal volumes of 0.1 M-sodium arsenate buffer containing 40 mM-MgSO₄ (pH 7.4), 0.01 M-potassium phosphate buffer containing 4 mM-MgSO₄ (pH 7.4), and water] contained in a glass scintillation vial (20 ml). Luciferin-luciferase extract (50 μl) was added and 40 s later the light emission in the wide-window ^{14}C channel was measured for 30 s. Calculation of the ATP content of the unknowns was made either by reference to standard curves constructed with each batch of assays with authentic ATP in 0.15 M-HClO₄, or by assaying light production for unknowns in the presence and absence of added ATP (internal standardization). The assay was sensitive over the range 0.1–10 nmol.

Method II. This was used principally for the assay of ATP in zonal fractions. Samples (normally 0.5 ml) were extracted by the addition of 50 μl of ice-cold 0.25 M-HClO₄ and where necessary insoluble material was removed by centrifugation as described in method I. The luciferin-luciferase enzyme was prepared by addition of 2.5 ml of water to each vial of freeze-dried firefly lantern extract, and insoluble material was removed by filtration through Whatman no. 1 filter paper. Light production was measured by using samples of HClO₄ extract (50 μl) and enzyme (50 μl) as described in method I. Freshly prepared solutions of authentic ATP (1–10 μM) in 0.2 M-sucrose–0.3 M-NaCl (+10 mM-Tris-HCl, pH 7.0, where applicable; see 'Extraction and isolation of vesicles') were ex-

tracted with 0.1 vol. of 0.25M-HClO₄ and samples (50 µl) were used for the construction of standard curves. The assay was sensitive over the range 0.05–0.5 nmol. In experiments where tissue fractions were treated with an enzymic ATP-hydrolysing mixture (see below) the extraction of ATP with HClO₄ did not irreversibly terminate the ATPase (adenosine triphosphatase) activity of the added enzymes. Thus adjustment of a sample of the HClO₄ extract to neutral pH resulted in a progressive loss of ATP. Therefore for samples of this nature it was necessary to add the HClO₄ extract just before the luciferin-luciferase (20 s was found to be a sufficiently short time for the conditions employed in our experiments).

Other procedures

ATP-hydrolysing mixture. This consisted essentially of apyrase–myokinase [1:8, with respect to standard units of activity (1 unit of apyrase liberates 1 µmol of P_i from ATP/min at 30°C and pH 6.5; 1 unit of myokinase converts 2 µmol of ADP into ATP+AMP/min at 37°C and pH 7.6)]. The enzymes were normally prepared in 0.2M-sucrose–0.3M-NaCl buffered with 10mM-Tris-HCl buffer, pH 7, and had a final activity for apyrase of 50–100 units/ml. This mixture is referred to as the 'ATP-hydrolysing mixture'. In some experiments the ATP-hydrolysing mixture also contained 0.48 unit of 5'-AMP deaminase/ml (1 unit deaminates 1 µmol of 5'-AMP to 5'-IMP/min at pH 6.5 and 25°C) and/or 3.15 units of adenosine deaminase/ml (1 unit deaminates 1 µmol of adenosine to inosine/min at pH 7.5 and 25°C).

Gel filtration of isolated vesicles. A sample (2 ml) of isolated vesicles was loaded on to a column (30 cm × 1.5 cm) of Bio-Gel A-5m (50–100 mesh) (Bio-Rad Laboratories, Bromley, Kent, U.K.) equilibrated with 0.2M-sucrose–0.3M-NaCl and eluted with the same medium. Fractions were collected and analysed for E₂₆₀, E₂₈₀, acetylcholine and ATP. The column was pre-calibrated with the following markers; Blue Dextran 2000 (mol.wt. ≈ 2 × 10⁶), catalase (mol.wt. 2.5 × 10⁵) and ATP (mol.wt. 5 × 10²).

Measurement of ¹⁴C. Samples of zonal fractions (0.5 ml made up to 1 ml with water) were counted for

radioactivity in 10 ml of toluene-Triton X-100 (2:1, v/v) liquid-scintillation fluid (Patterson & Greene, 1965) in a Beckman LS 150 liquid-scintillation spectrometer at ambient temperature. The efficiency of counting was monitored by using the external-standardization procedure and found to be constant at about 70%; the counts have been left uncorrected.

Results

Subcellular distribution of ATP

Preliminary experiments were carried out with *T. californica* and Table 1 shows the concentrations of ATP and acetylcholine in extracts of whole tissue, tissue homogenates and the subcellular fractions (P₁₂ and S₁₂) of these homogenates. As in previous studies (Sheridan *et al.*, 1966; Israël *et al.*, 1968, 1970; Whittaker *et al.*, 1972b) with electric tissue from Torpedinidae, homogenization caused a considerable loss of acetylcholine, the so-called 'free pool' (thought to represent cytoplasmic acetylcholine). However, as before, the acetylcholine which survived homogenization ('bound' = vesicular acetylcholine) was stable under the conditions used for the preparation of subcellular fractions. Homogenization also caused a marked fall in tissue ATP concentrations, but unlike the acetylcholine, the surviving ATP was not stable during subsequent subcellular fractionation and this led to a low overall recovery. This suggested that a high proportion of the total tissue ATP was released as 'free' ATP after homogenization, and was subsequently destroyed by tissue ATPases (but probably more slowly than 'free' acetylcholine is destroyed by tissue esterases). Density-gradient centrifugation of fraction S₁₂ with the swinging-bucket discontinuous gradients developed by Israël *et al.* (1970) showed that although most of the ATP in fraction S₁₂ behaved essentially as a cytoplasmic component, a significant proportion was associated with the synaptic vesicle fraction (fraction 2, Fig. 1). Since the concentration (nmol/ml of fraction) of ATP was higher in fraction 2 than in fraction 1, it cannot have been due to diffusion of soluble ATP from fraction 1 to fraction 2. Attempts to study the distribution of the ATP in fraction S₁₂ on

Table 1. ATP and acetylcholine contents of cytoplasmic extracts and subcellular fractions prepared from *Torpedo electric* tissue

Values are the means ± s.d. (3 expts.) or range (2 expts.) and are for *T. californica*. ATP was measured by method I (see the Materials and Methods section).

Tissue sample	Units	ATP	Acetylcholine
Whole tissue	nmol/g	64 ± 41 (3)	192
Cytoplasmic extract	nmol/g	12.1 ± 4.5 (3)	21.0 ± 0.6 (2)
	% of whole tissue	21.8 ± 7.9 (3)	11.0
Fraction P ₁₂	% of cytoplasmic extract	9.4 ± 5.1 (3)	27.8 ± 5.3 (2)
Fraction S ₁₂		21.2 ± 10.6 (3)	69.0 ± 3.0 (2)
P ₁₂ +S ₁₂ (recovery)		30.6 ± 11.6 (3)	96.8 ± 2.2 (2)

Fig. 1. Density-gradient centrifugation of fraction S_{12} prepared from *T. californica*

A sample (5 ml) of fraction S_{12} equivalent to 1.6 g of original tissue was layered on to the density gradient and centrifuged at 25000 rev./min for 3 h in a Beckman SW25 rotor in a refrigerated Beckman L2 ultracentrifuge. The appearance of the centrifuge tube after centrifugation is shown (left), together with the distribution of ATP (■) (measured by method 1) and acetylcholine (□) (right). Fractions were removed with a Pasteur pipette. Recoveries from the gradient were 71% and 113% respectively for ATP and acetylcholine. For further details of the various fractions see Israël *et al.* (1970).

Fig. 2. Distribution of ATP after zonal centrifugation of fraction S_{12} from *T. marmorata*

(a) Fraction S_{12} prepared with Tris-HCl-buffered sucrose-saline (see the Materials and Methods section), and equivalent to 61 g of original tissue was centrifuged at 48000 rev./min for 3 h in a Ti-14 rotor. The gradient was prepared essentially as described by Whittaker *et al.* (1972b) except that it was buffered throughout with 10 mM-Tris-HCl, pH 7.0, contained a 25 ml underlay of 0.2 M-sucrose-0.3 M-NaCl and 300 ml of gradient, with 0.5 M-sucrose-0.15 M-NaCl as the dense solution, was run in before the change to 1.45 M-sucrose. The distribution of acetylcholine (●) shows the vesicle peak; ATP (○) was measured by using method 1. (b) Insert comparing distribution of protein (cross-hatched profile) from a similar zonal run with that of ATP (clear profile) of the experiment in (a) to show that most of ATP is in fraction SP.

zonal gradients by using the technique of Whittaker *et al.* (1972b) proved unsuccessful because of the very poor yield of vesicles (as judged by bound-acetylcholine concentrations) from the electric organ of this species of *Torpedo*. All further experiments were therefore carried out with *T. marmorata*, which seems to be particularly well suited for vesicle isolation by subcellular fractionation (see Whittaker *et al.*, 1972b).

Fig. 2 shows the distribution of ATP after zonal centrifugation of an S_{12} fraction from *T. marmorata*. Most of the ATP was recovered from the low-density region of the sucrose gradient along with soluble protein in a fraction designated SP (see inset to Fig. 2). Two other, much smaller, peaks of ATP were found further down the gradient at densities equivalent to about 0.38M-sucrose and 1.0–1.2M-sucrose. The peak of ATP at a density equivalent to 0.38M-sucrose was reasonably well separated from the ATP in fraction SP and was coincident with the peak of bound acetylcholine, the synaptic-vesicle marker. The ATP in this region was therefore designated vesicle-peak (VP) ATP. The peak of ATP recovered from the denser regions of the gradient was always much smaller than the ATP in fraction VP and was associated with membranous material [see inset to Fig. 2 and also Whittaker *et al.* (1972b)]. The nature of the ATP in this fraction is described more fully below.

Occluded nature of vesicular ATP

The ATP associated with fraction VP was very small (<5%) compared with that in fraction SP, and might possibly have arisen by adsorption or uptake of ATP from fraction SP by particles (either synaptic vesicles or other particles of equal density) collected in fraction VP. To test the state of the ATP, samples of fractions SP and VP were subjected to enzymic attack by incubation with an ATP-hydrolysing mixture (for details see the Materials and Methods section). Comparison of the effects of this treatment on the ATP in each of the fractions with equivalent amounts of authentic ATP in free solution are shown in Fig. 3(a). The ATP in fraction SP, like the ATP in free solution, was rapidly destroyed by enzymic treatment, but the ATP in fraction VP was largely immune to the enzymic mixture, just as vesicular acetylcholine is immune to the action of acetylcholinesterase. This finding strongly suggests that the ATP associated with the vesicles is in a tightly bound state. When vesicles from fraction VP were subjected to gel filtration by passage under iso-osmotic conditions through a column of Bio-Gel A-5m, all the ATP was associated with the void-volume fraction (Fig. 3b), i.e. with constituents of greater than 5×10^6 daltons. Thus the ATP in fraction VP behaved exactly the same as the bound acetylcholine of this fraction (see also Whittaker *et al.*, 1972b). Experiments with porous

Fig. 3. Evidence for the bound state of ATP in the synaptic-vesicle fraction

Subcellular fractions were prepared from *T. marmorata* by zonal centrifugation. (a) Fractions VP (●), SP (○) and MP (▲) and 20 μ M-ATP (□) were treated with ATP-hydrolysing mixture (containing 5'-AMP deaminase and adenosine deaminase; see the Materials and Methods section) (25 μ l/ml) at 5°C. Incubation was terminated by addition of 0.1 vol. of 0.25M-HClO₄ and ATP assayed for by using method II. The original concentrations of ATP in the subcellular fractions were 6.74 μ M, 34.3 μ M and 1.1 μ M respectively for fractions VP, SP and MP. Fractions VP and SP were from the same zonal run; fraction MP was from a separate but similar run. (b) A 2 ml sample of fraction VP [same fraction as in (a)] was subjected to gel filtration with a column of Bio-Gel A-5m; for details see the Materials and Methods section. ■, E_{260} ; □, E_{280} ; ○, ATP; ●, acetylcholine. Recovery of ATP from the column was 60% and for acetylcholine 100%. Note that both ATP and acetylcholine emerge in the void volume together with most of the material absorbing at 260 and 280 nm. The positions of the molecular-weight markers, Blue Dextran 2000, catalase and ATP are indicated by the vertical lines.

Fig. 4. Separation, from a cytoplasmic extract treated with an ATP-hydrolysing mixture, of a synaptic-vesicle fraction from the electric organ of *T. marmorata*

A sample (50 ml) of fraction S_{12} equivalent to 65.4 g of tissue was treated with 0.8 ml of ATP-hydrolysing mixture (containing 5'-AMP deaminase and adenosine deaminase; see the Materials and Methods section) for 30 min at 0°C and then subjected to zonal centrifugation as described for Fig. 2, but with 0.2 ml of the ATP-hydrolysing mixture also in the 25 ml underlay. ATP was measured by using method II. Note that free ATP is hydrolysed, leaving one main peak of ATP (○) coinciding with that of acetylcholine (●) in the expected position of the synaptic vesicles. The second, much smaller, peaks of ATP and acetylcholine in the position of the membrane peak indicate the presence in the latter of fragments of incompletely disrupted terminals. There is now no ATP in fraction SP, here indicated by the initial peak of material absorbing at 256 nm (---).

glass beads, which are capable of resolving synaptic vesicles from larger membrane fragments, have also shown that ATP and acetylcholine are present in particles of the same size (Morris, 1973). Uptake of ATP into the vesicle fraction during tissue fractionation was investigated by pre-treatment of fraction S_{12} with the ATP-hydrolysing mixture before zonal centrifugation. This resulted in the complete destruction of the ATP of fraction SP, whereas the ATP of the vesicles (and the membrane fraction, MP) remained (Fig. 4). Thus uptake of soluble ATP by constituents of the vesicle and membrane fractions seems unlikely to have occurred, at least during zonal centrifugation. Attempts to destroy the free ATP at the time of tissue comminution, by using extraction medium containing the ATP-hydrolysing mixture, were unsuccessful, probably because of adsorption of the enzymes to coarse tissue fragments. Therefore other experiments were designed to test for ATP uptake by constituents of the vesicle fraction between the time of tissue comminution and vesicle isolation; these are described below.

Control experiments

When tissue was comminuted in the presence of added [U- 14 C]ATP and the resultant tissue extract used for the preparation of vesicles, none of the added ATP could be detected in the vesicle fraction (apart from a slight overlap from the soluble fraction) (Fig. 5a). Note that in this experiment, to define precisely the distribution of bound ATP fraction S_{12} was pre-treated with ATP-hydrolysing mixture before centrifugation. Therefore the radioactive counts in fraction SP are actually those of ATP-hydrolysis products (i.e. AMP and IMP) and not ATP; but are presumably representative of the localization of ATP in the absence of pre-treatment. The lack of association of the 14 C with the vesicle peak shows that the ATP associated with this fraction is unlikely to have arisen as an uptake artifact, between the time of tissue comminution and the preparation of fraction S_{12} .

The absence of 14 C from fraction MP (Fig. 5a) also deserves comment, since this fraction contains microsome-like membranous particles (150–500 nm

Fig. 5. Failure of added $[\text{U-}^{14}\text{C}]\text{ATP}$ to be associated with vesicular ATP

$[\text{U-}^{14}\text{C}]\text{ATP}$ was added either to frozen powdered tissue (a) or to whole tissue at the time of comminution (b) and after the preparation of vesicles by zonal centrifugation ^{14}C (●) and ATP (○) (method II) were determined in gradient fractions. (a) Same experiment as shown in Fig. 4; 1 μmol of ATP (5 μCi) was added with the tissue extraction medium. (b) Similar experiment to that shown in (a), but the tissue (170 g) was comminuted by blending (see the Materials and Methods section) with Tris-buffered extraction medium (60 ml) containing added $[\text{U-}^{14}\text{C}]\text{ATP}$ (10 μmol and 2.5 μCi). The zonal-centrifugation conditions for this experiment were similar to those given for Fig. 2 except that the volume of underlay was 50 ml. Note that in both (a) and (b) the added ^{14}C was recovered only from those regions of the gradients containing soluble protein. Arrows indicate the peak tubes for acetylcholine in each run.

diam.), together with more complex synaptosome-like bodies (H. Zimmermann, personal communication). The origin of the microsome-like particles is unknown [recent results (Zimmermann & Whittaker, 1973) suggest that they might arise in part from the nerve-terminal membrane], but it seems probable that they are produced in part by vesiculation of larger membranes during tissue comminution and extraction. If so, the results shown in Fig. 5(a) indicate a lack of equilibrium between the added [^{14}C]-ATP and endogenous cytoplasmic ATP. Membranous particles of the type recovered in fraction MP were also present as a minor contaminant of the vesicle fraction (Plate 1). Since the ATP recovered from fraction MP was always much less than that recovered from fraction VP (see Figs. 2 and 4), it seems unlikely that much of the vesicle-peak ATP was caused by membrane contaminants. Nevertheless attempts were made to determine the degree of entrainment of cytoplasmic ATP at the time of tissue comminution and its effect on the ATP recovered from the vesicle peak. For this purpose tissue was again comminuted in the presence of added [^{14}C]-ATP, but by a blending procedure, rather than the normal method, in an attempt to increase the membrane vesiculation. This treatment did not markedly change the amount of ATP associated with the vesicle peak (Fig. 5b) and as with the standard procedure (Fig. 5a) all the [^{14}C]-ATP in fraction S_{12} was recovered in the soluble protein fraction. However, the blending conditions used actually decreased the amount of material recovered from fraction MP (subsequent experiments confirmed this, but also showed an increased acetylcholinesterase activity throughout the vesicle-peak region of the gradient, suggesting increased membrane contamination of the vesicle fraction). Therefore although this experiment provides little evidence either for or against the possibility that the ATP of the vesicle peak is an entrainment artifact, it does confirm the belief that bound ATP in fraction VP (and fraction MP) represents a tightly bound, inaccessible cellular compartment both before and after tissue comminution and extraction. No bound ATP could be detected in S_{12} fractions prepared from 'cytoplasmic extracts' of *Torpedo* muscle tissue.

Yield of vesicular acetylcholine

During the course of the present series of experiments on the isolation of synaptic vesicles from *Torpedo*, several modifications have been introduced into the procedure(s) developed by Whittaker *et al.* (1972b). The most important of these include the use of Tris-HCl-buffered extraction and isolation media, the treatment of tissue extracts with an enzymic ATP-hydrolysing mixture, and also from time to

time the use of anaesthetized fish and the temporary storage of tissue in a frozen state before comminution. The effects of these various modifications on the yield of synaptic vesicles have not been systematically evaluated, but the available results are shown in Table 2. Statistical evaluation of these results, by using Snedecor's F test (with Bessel's correction for small sample sizes) and then, where applicable, Student's *t* test (two-tailed) showed that at the $P = 0.05$ level with unanaesthetized fish neither temporary storage in liquid N_2 nor buffering of the extraction and isolation media had any effect on the concentration of acetylcholine in the isolated vesicle peak. There is reason to believe, however, that the vesicles prepared from buffered media may be more stable after isolation than vesicles prepared from unbuffered media. Although the concentrations of acetylcholine in vesicles prepared from enzyme-treated fraction S_{12} were significantly lower ($P < 0.02$) than corresponding concentrations in vesicles prepared from untreated fraction S_{12} (Table 2; cf. lines 5 and 1 in column 3), subsequent studies on the stability of acetylcholine in fraction S_{12} indicate that this is not a genuine effect of the enzymic treatment (H. Zimmermann & M. J. Dowdall, unpublished work), and probably arises from independent causes (see legend to Table 2). Finally, vesicles prepared from anaesthetized fish contained the highest concentrations of acetylcholine found in the present series of experiments; this has now been more fully established in a separate study (Zimmermann & Whittaker, 1973).

Relationship between vesicular acetylcholine and ATP

In addition to the yields of vesicle acetylcholine, Table 2 also contains values for the corresponding yields of ATP associated with the vesicle fraction. The yields of ATP are co-variant with those of acetylcholine, i.e. the molar ratio of acetylcholine in ATP is reasonably constant over a wide range of absolute values. With the exception of the values from vesicles prepared from unbuffered media this molar ratio is between 3 and 6. The correlation is best seen when the individual paired results are plotted against each other in a scattergram (Fig. 6). Statistical analysis by linear regression revealed a highly significant correlation between these two components of the vesicle peak [$r = 0.941$, $P(r > 0) = 0.001$], the line of linear regression having a slope of $0.188 \pm (\text{s.e.m.}) 0.016$, i.e. the average molar ratio acetylcholine/ATP was $5.32 \pm (\text{s.e.m.}) 0.45$. The intercept of this line on the *y* axis (ATP) was $0.028 \pm (\text{s.e.m.}) 0.106$, i.e. it was not significantly different from zero. The simplest explanation of these results is that acetylcholine and ATP are present in the same particles, namely synaptic vesicles, in the molar ratio of about 5:1.

EXPLANATION OF PLATE I

Electron micrograph of synaptic vesicles from fraction VP showing some membrane contaminants

Preparation was by a glutaraldehyde–osmium–lead–uranyl acetate–Epon method. Magnification: $\times 42\,600$. Note the presence of membrane contaminants (MC) among an otherwise uniform population of synaptic vesicles (SV). Electron micrograph kindly provided by Dr. H. Zimmermann.

Table 2. *Acetylcholine and ATP concentrations in VP fractions prepared under different conditions*

Values are for peak acetylcholine fractions and are given in nmol/ml, having been normalized with respect to the concentration of the parent fraction, S₁₂; the normal being 1 ml of S₁₂ = 1 g of original tissue. For details of the procedures used see the Materials and Methods section. The measurements of ATP for the values in column 3 were by method 1. Values for more than one experiment are means $\pm$ s.e.m. with the number of experiments in parentheses.

	State of fish before removal of tissue		Unanaesthetized				Anaesthetized	
	...		Unbuffered		Buffered		Buffered	
	Extraction and isolation medium		...		...		...	
	Method of tissue comminution		Crushing		Crushing		Blending	
No treatment of fraction S ₁₂								
Acetylcholine								
ATP			24.5 $\pm$ 4.9 (11)		30.8 $\pm$ 3.1 (5) *		17.9	
Acetylcholine/ATP molar ratio			2.9 $\pm$ 2.1 (5)		9.1 $\pm$ 1.1 (13) }		4.5	
Mean			8.4		3.4		—	
Paired results			11.0 $\pm$ 3.4 (5)		4.9 $\pm$ 0.4 (7)		4.0	
Enzyme-treated fraction S ₁₂								
Acetylcholine			—		9.9 $\pm$ 1.8 (4) *		18.2	
ATP			—		2.3 $\pm$ 0.7 (4) }		4.0	
Acetylcholine/ATP molar ratio			—		4.3		—	
Mean			—		4.9 $\pm$ 1.2 (4)		4.6	
Paired results							5.4	
							5.8 $\pm$ 0.5 (3)	

* Results suggest that the enzyme treatment resulted in a lower yield of vesicular acetylcholine and ATP; however, it is possible that the lower yield was due to poor temperature regulation in the cold-room at the time of these four experiments; see the text for discussion.

Fig. 6. Scattergram of vesicular ATP and acetylcholine concentrations

Paired values for the peak vesicle fraction (as defined by acetylcholine) in different zonal runs are plotted against each other. Values have been normalized with respect to the tissue concentration of each parent S_{12} fraction as described in Table 2. The continuous line represents the line of best fit by linear regression. Results are for runs with unbuffered medium (■) and one with phosphate-buffered medium (△). All other results are for Tris-HCl-buffered media as follows: normal extraction procedure (○), blending (▲) and anaesthetized fish (●). Experiments in which the ATP-hydrolysing mixture was used on fraction S_{12} are denoted by the bars tagged to each symbol (i.e. ○, ▲ and ●).

Fig. 7. Variation in molar ratio of acetylcholine to ATP across the vesicle peak

Vertical bars are $\pm$ range (2 expts.) or $\pm$ S.E.M. (3 or more expts.) of mean values indicated by the points (●). Small numbers by the bars indicate the number of individual values meaned.

Evidence for vesicular heterogeneity

Although there was good correspondence between the peaks of acetylcholine and ATP associated with the isolated synaptic vesicles (Figs. 2, 4 and 5) the molar ratio acetylcholine/ATP was found to vary across the vesicle peak (Fig. 7), being at a maximum in the fraction with the maximum acetylcholine concentration, and declining to lower values either side of this fraction. A varying ratio across the peak might be taken as an indication that the acetylcholine and ATP are in different particles, which are partially, but not completely, resolved by the density gradient, or that synaptic vesicles comprise a heterogeneous population differing in their acetylcholine/ATP ratio. This might be determined by the age of the vesicle and might be accompanied by physical differences (size, density) that would permit partial separation in a density gradient. A decrease of vesicle size on stimulation has been observed (Zimmermann & Whittaker, 1973).

Discussion

Evidence that ATP is a constituent of the synaptic vesicles

We believe that the present paper is the first indication of the presence of ATP in synaptic vesicles, other than noradrenaline-storage particles, isolated from any source. Two important questions therefore arise: (1) is the measured activity really ATP? and (2) if so, is the ATP really a synaptic-vesicle constituent? It is well known that the luciferin-luciferase enzyme system is highly specific for ATP, i.e. there is negligible light production with other naturally occurring mono-, di- or tri-phosphonucleotides or with ADP or AMP (see McElroy & Green, 1956). In addition, u.v. spectral analyses (M. J. Dowdall, A. F. Boyne & V. P. Whittaker, unpublished work) have confirmed the presence of the adenine moiety in the synaptic-vesicle fraction in quantities consistent with the concentration of ATP as measured by the luciferin-luciferase assay procedure. It is therefore concluded that the 'measured ATP' was indeed due to ATP.

The following observations provide good evidence that ATP is a constituent of the synaptic vesicles: (1) the peak of acetylcholine is in most cases exactly coincident with a peak of ATP; (2) like acetylcholine the 'vesicular' ATP is present in an enzymically resistant state both during and after preparation of the synaptic vesicle; (3) the 'vesicular' ATP is associated with particles larger than 5×10^6 daltons.

In addition the amount of ATP associated with the acetylcholinesterase-enriched membrane fraction was smaller than the amount of ATP associated with the acetylcholinesterase-poor vesicle fraction; this eliminates the possibility that 'vesicular' ATP is

caused by membrane contamination in the synaptic-vesicle fraction. Rather this indicates the presence of vesicles in the membrane fraction, perhaps because of a small amount of synaptosome formation.

The most conclusive evidence that ATP is a vesicle constituent is the correlation between ATP and acetylcholine concentrations in the isolated vesicle fraction (Fig. 6). In particular, the finding that the correlation line intersects both axes at zero is difficult to reconcile with any model where ATP and acetylcholine are present in different particles. More recent studies (Zimmermann & Whittaker, 1973), in which vesicle stores of acetylcholine have been depleted to varying degrees by electrical stimulation, have shown that the depletion of vesicular acetylcholine is followed *pari passu* by depletion of vesicular ATP, and here also the correlation line of the plot of ATP concentration against acetylcholine concentration passes through the origin. Thus there can be little doubt that ATP is a constituent of the cholinergic vesicles of the *Torpedo* electric organ.

Vesicle heterogeneity

As shown above, the variation in molar ratio (acetylcholine/ATP) across the vesicle peak (Fig. 7) might indicate that the acetylcholine and ATP are in different particles, those containing ATP having a broader range of densities, but the same mean density as those containing acetylcholine. Since all the other evidence concerning the relationship between acetylcholine and ATP is in favour of both being synaptic-vesicle constituents the observed variation is probably best explained by assuming the existence of a sub-population of synaptic vesicles, especially rich in acetylcholine and sedimenting in the central region of the vesicle peak. Further studies will be required to test this interesting possibility.

Comparison with other storage granules

The results of the present study have shown that like the storage granules for other pharmacologically active amines, i.e. chromaffin granules (adrenaline), splenic-nerve granules (noradrenaline) and platelet granules (5-hydroxytryptamine and histamine), cholinergic synaptic vesicles from *Torpedo* also contain ATP. It is of considerable interest to compare the amine/ATP molar ratios with those in these various storage particles (Table 3). The fourth column in the Table gives the specific concentration of each amine per mg of protein and has been included as an indication of the purity of each preparation. On this basis the cholinergic-vesicle preparation compares favourably with both chromaffin granules and platelet storage granules. The amine/ATP molar ratios for these three different preparations are very similar,

Table 3. Comparison of amine and ATP contents of various storage particles

Amine	Genus	Storage particle	Amine content (nmol/mg of protein)	Amine/ATP molar ratio	Source of information	Occurrence of ATP originally by :
Adrenaline	Ox	Chromaffin granule	3450	4.55	Smith (1968) (review)	Hillarp (1958)
Noradrenaline	Rat	Stellate ganglia		3.12		
		Heart		2.9-4.2		
		Vas deferens		1.3-3.7		Schümann (1958)
	Ox	Stellate ganglia	—	3.12	Quoted by Geffen & Livett (1971) (review)	
		Splenic nerve (1)	—	3-5.2*		
		Splenic nerve (2)	9	7.5-12*	Quoted by de Potter <i>et al.</i> (1970)	
5-Hydroxytryptamine	Rabbit	Platelet granules	210	3.5 (total amine)	Da Prada & Pletscher (1968)	Da Prada & Pletscher (1968)
Histamine	Rabbit	Platelet granules	90			
Acetylcholine	<i>Torpedo</i>	Electric organ synaptic vesicles	680-1200	5.3	This paper	Whittaker <i>et al.</i> (1972a)

* Corrected for mitochondrial contamination.

ranging from 3 to 6. They are also similar to the ratio found for noradrenaline-storage particles from a variety of sources, although it must be borne in mind that these preparations are relatively impure (see de Potter *et al.*, 1970) and the ratios are therefore regarded as provisional. Indeed there is good reason to believe that preparations of noradrenaline-storage particles from the mammalian peripheral nervous system may also contain cholinergic synaptic vesicles, since many of these sources are also relatively rich in cholinergic nerve endings. It is surprising that this important point has never been checked, and in light of the present results this question becomes even more important for a reliable estimate of the amine/ATP ratio in these granules.

We can at the moment only speculate as to the function of ATP in cholinergic synaptic vesicles, but the acetylcholine/ATP molar ratio (Table 3) is certainly very suggestive of a common general function for ATP in all the amine-storage granules so far studied. Current opinion about the role of ATP in catecholamine-storage granules is divided. On the one hand there is evidence that adrenaline, noradrenaline and 5-hydroxytryptamine *in vitro* can form stable high-molecular-weight aggregates with ATP (see Berneis *et al.*, 1970) in the molar ratio of about 3.5:1, and this suggests that similar complexes may also exist in amine-storage granules. However, there is evidence that ATP provides the energy for 'driving' the uptake into, and maintaining the concentration of catecholamines within, these storage particles (von Euler & Lishajko, 1963; Stjärne, 1964). Similar considerations obviously also apply to the role of ATP in cholinergic vesicles, and the results obtained with this preparation so far (M. J. Dowdall, A. F. Boyne & V. P. Whittaker, unpublished work) certainly indicate that the ATP might well be involved in the binding of acetylcholine. The precise mechanism of this binding and the possible involvement of vesicular ATP in the uptake and release of acetylcholine must await further work with the preparation.

This work was supported in part by grants nos. G.969/488/8 from the U.K. Medical Research Council and B/SR/6474 from the U.K. Science Research Council (to V. P. W.) and a Wellcome Trust travel grant (to M. J. D.). A. F. B. gratefully acknowledges a Science Research Council Fellowship. We also thank our colleagues Mr. W. Edwards and Dr. H. Zimmermann, for assistance with electron microscopy and valuable discussion, Miss F.

Henderson, Mr. G. H. C. Dowe and Mr. C. R. Denston, for skilled technical assistance, and the Directors of the Marine Biological Laboratories, Woods Hole and Archachon, for research facilities and the supply of *Torpedos*.

References

- Berneis, K. H., Pletscher, A., DaPrada, M. (1970) *Brit. J. Pharmacol. Chemother.* **39**, 382-392
- Da Prada, M. & Pletscher, A. (1968) *Brit. J. Pharmacol. Chemother.* **34**, 591-597
- de Potter, W. P., Smith, A. D. & de Schaepdryver, A. F. (1970) *Tissue & Cell* **2**, 529-546
- Geffen, L. B. & Livett, B. G. (1971) *Physiol. Rev.* **51**, 98-157
- Hillarp, N. Å. (1958) *Acta Physiol. Scand.* **42**, 321-332
- Israël, M., Gautron, J. & Lesbats, B. (1968) *C. R. Acad. Sci. Ser. D* **266**, 273-275
- Israël, M., Gautron, J. & Lesbats, B. (1970) *J. Neurochem.* **17**, 1441-1450
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- McElroy, W. D. & Green, A. (1956) *Arch. Biochem. Biophys.* **64**, 257-271
- Morris, S. J. (1973) *J. Neurochem.* **21**, 713-715
- Patterson, M. S. & Greene, R. C. (1965) *Anal. Chem.* **37**, 854-857
- Schümann, H. J. (1958) *Naunyn-Schmiedeberg's Arch. Pharmacol. Exp. Pathol.* **233**, 296-300
- Sheridan, M. N., Whittaker, V. P. & Israël, M. (1966) *Z. Zellforsch.* **74**, 291-307
- Smith, A. D. (1968) in *The Interaction of Drugs and Subcellular Components on Animal Cells* (Campbell, P. N., ed.), pp. 239-292, J. and A. Churchill, London
- Stanley, P. E. & Williams, S. G. (1969) *Anal. Biochem.* **29**, 381-392
- Stjärne, L. (1964) *Acta Physiol. Scand. Suppl.* **228**, 1-97
- von Euler, U. S. & Lishajko, F. (1963) *Acta Physiol. Scand.* **59**, 454-461
- Whittaker, V. P. (1971) *Ann. N.Y. Acad. Sci.* **183**, 21-32
- Whittaker, V. P. (1972) in *Le Système Cholinergique en Anesthésiologie et en Réanimation* (Nahas, G. G., Salamagne, J. C., Viars, P. & Vourc'h, G., eds.), pp. 45-60, Librairie Arnette, Paris
- Whittaker, V. P., Dowe, G. H. C. & Scotto, J. (1971) *Abstr. Int. Meet. Int. Soc. Neurochem.* 3rd p. 266
- Whittaker, V. P., Dowdall, M. J. & Boyne, A. F. (1972a) *Biochem. Soc. Symp.* **36**, 49-68
- Whittaker, V. P., Essman, W. E. & Dowe, G. H. C. (1972b) *Biochem. J.* **128**, 833-846
- Whittaker, V. P., Dowdall, M. J., Dowe, G. H. C., Facino, R. M. & Scotto, J. (1974) *Brain Res.* in the press
- Zimmermann, H. & Whittaker, V. P. (1973) *J. Neurochem.* in the press