The Localization of Adenosine Triphosphatases in Morphologically Characterized Subcellular Fractions of Guinea-Pig Brain

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1. The distribution of adenosine triphosphatase was studied in morphologically characterized subcellular fractions of guinea-pig brain. The conditions of homogenization were selected so as to favour the survival of nerve endings as organized structures. 2. A fraction consisting mainly of the external membranes of nerve endings was rich in a ouabain-sensitive Na^+ - K^+ -stimulated adenosine triphosphatase which closely resembled that present in the classical microsomal fraction studied by other workers, but which showed a higher specific activity. 3. A dinitrophenol-stimulated adenosine triphosphatase was located in the nerve-ending mitochondria. 4. The synaptic-vesicle fraction contained a small amount of adenosine triphosphatase that differed in its response to several ions and other compounds from the membrane, myelin and mitochondrial fractions, indicating freedom from contamination by these elements.

The electrical excitability of nervous tissue depends on the maintenance of Na^+ and K^+ concentration gradients across the cell membrane, and thus any system involved in the active transport of these cations is of great interest. Much recent work indicates that a membrane-bound adenosine-triphosphatase system which is ouabain-sensitive and stimulated by Na^+ and K^+ is involved in active cation transport in nervous tissue as well as in erythrocytes (Dunham & Glynn, 1961; Post, Merritt, Kinsolving & Albright, 1960), kidney (Whittam, 1961; Wheeler & Whittam, 1964) and the electric organ of *Electrophorus electricus* (Glynn, 1963).

The distribution and properties of adenosine triphosphatase in brain tissue have been studied among others by Järnefelt (1961), Deul & McIlwain (1961), Aldridge (1962) and Schwartz, Bachelard & McIlwain (1962). The Na^+ - K^+ -activated adenosine triphosphatase was found mainly in the microsomal fraction sedimenting at forces greater than 10^6g -min. One of the limitations of this work is the heterogeneity of the brain microsomal fraction that is derived from membranes from many different cell types and includes the outer cell membrane as well as the endoplasmic reticulum. Cytochemical studies by Hess & Pope (1959), Lewin & Hess (1964) and Torack & Barnett (1964) have provided valuable information on the cytological localization of

adenosine triphosphatase. They have shown that the Na^+ - K^+ -stimulated enzyme is localized in plasma membranes and is particularly active at axo-dendritic synapses. Cummins & Hydén (1962) isolated nerve-cell membranes by micro-dissection and showed that adenosine triphosphatase is localized on the inside of the nerve-cell membrane. Because of the micro scale of this technique only a limited number of conditions could be studied, but the activity was stimulated by Na^+ and K^+ and inhibited by ouabain.

In the present study advantage has been taken of the isolation of presynaptic nerve terminals (synaptosomes) and their constituent organelles (Gray & Whittaker, 1962; Whittaker, Michaelson & Kirkland, 1964) to study the adenosine-triphosphatase activity of neuronal membranes of known morphological origin. In this way it is possible to obtain sufficient material to examine the activity under a variety of conditions. A preliminary account of this investigation has been reported (Hosie, 1964).

METHODS

Preparation of subcellular fractions

Primary fractions. Guinea-pig fore-brain (i.e. the whole brain rostral to the inferior colliculi) or cerebral neocortex was homogenized in 0.32M-sucrose in a Perspex-and-glass homogenizer (Aldridge, Emery & Street, 1960); the difference in diameter between tube and pestle was 0.025 cm.

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and the speed of rotation 840 rev./min. unless stated otherwise. The homogenate was separated into fractions P_1 (nuclei, large myelin fragments, tissue debris), P_2 (mitochondria, synaptosomes, small myelin fragments, some microsomes), P_3 (microsomes) and S_3 (supernatant) as described by Gray & Whittaker (1962) except that P_1 was washed only once. The two tissues gave similar results except that the lower myelin content of the cortex diminished the myelin contamination of certain fractions and led to cleaner separations. In some experiments the P_1 or P_2 pellets, suspended in 0.32M-sucrose, were further fractionated into subfractions P_{1A} , P_{1B} and P_{1C} , or P_{2A} , P_{2B} and P_{2C} , on a discontinuous density gradient, consisting of equal volumes of 0.8M- and 1.2M-sucrose, which was centrifuged at 53500g for 120 min. in the SW25 head of the Spinco model L preparative ultracentrifuge as described by Eichberg, Whittaker & Dawson (1964).

When it was required to disrupt fraction P_2 and separate the components on a discontinuous density gradient the pellet comprising this fraction was sedimented at 12000g for 20 min. to minimize microsomal contamination and to speed preparation.

Disruption of fractions containing synaptosomes

Procedure A. The P_2 pellet was disrupted by suspending it with gentle homogenization in water (2 ml./g. of original tissue). This suspension was centrifuged at 12000g for 20 min. to give a pellet of large mitochondria and myelin fragments and a supernatant, P_2W_s (Whittaker *et al.* 1964), containing synaptic vesicles, external synaptosome membranes, incompletely disrupted synaptosomes, microsomes and small mitochondria.

Procedure B. Fraction P_2B was diluted with an equal volume of water to bring the concentration of sucrose to approx. 0.4M and centrifuged at 105000g for 30 min. to give a supernatant, which was discarded, and a pellet containing mainly synaptosomes and some microsomes. This pellet was disrupted by suspending in water (2 ml./g. of original tissue) with gentle stirring to give fraction P_2BW_s .

Density-gradient separation of disrupted fractions. Suspensions of fraction P_2W_s or P_2BW_s (5 ml./tube) were placed on a discontinuous density gradient consisting of five successive layers of sucrose each of 5 ml. as described by Whittaker *et al.* (1964). After centrifuging at 53500g for 120 min. the bands were separated by slicing the tubes with a tube cutter. The seven fractions obtained were designated O, D, E, F, G, H and I in order of increasing density of sucrose.

Storage. Samples of fractions for analysis were stored at -20° .

Ultrasonic disruption of fractions. Fractions were submitted to ultrasonic vibrations at 20000 cyc./sec. from a 60W Mullard-MSE ultrasonic disintegrator (type 7685/2 with a titanium probe) for 4 min. at -6° .

Electron microscopy

Fractions were examined by (1) positive staining and thin sectioning and (2) negative staining, as follows.

(1) Suspensions were fixed by the addition of approx. 5 vol. of cold potassium permanganate fixative (Luft, 1956); after 30 min. particulate material was sedimented at

5000g for 10 min. The pellet was dehydrated in acetone and embedded in Araldite. Sections cut with the Porter-Blum MT-2 Ultra-microtome were mounted on carbon-coated collodion grids, and stained with lead hydroxide (Millonig, 1961).

(2) Small drops of suspensions were negatively stained with 2% (w/v) phosphotungstic acid previously neutralized with 2N-NaOH, as described by Whittaker *et al.* (1964).

Difficulties were experienced in obtaining satisfactory spreading with fraction G and its derivatives even with hydrophilic grids. This has been observed before but has not previously been reported. The effect was traced to the deposition of amorphous material that remained in suspension in the sucrose until the latter was diluted with negative stain. This material may be responsible for the sticky quality of the nerve-ending cytoplasm that has been inferred from the tendency of synaptic vesicles to remain clumped together after rupture of the external synaptosome membrane (Whittaker, 1961, 1962).

Reagents and analyses

Tris-adenosine triphosphate. ATP (disodium salt) (Pabst Laboratories, Milwaukee, Wis., U.S.A.) was converted into the free acid by passing it through a column of Dowex 50 resin (H^+ form; 200-400 mesh) in a cold room and adjusting it to pH 7.4 with 0.5M-tris. Deionized water was added to make the solution 60 mM with respect to ATP as determined by the extinction at 260 m μ . The concentration of Na^+ in this solution was 0.2 mM and was therefore about 10 μ M in the assay medium.

Adenosine-triphosphatase assay. The reaction medium in a final volume of 2 ml. contained tris-HCl buffer (160 mM), pH 7.4, and EDTA (tris salt) (1 mM) and the desired additions: $MgCl_2$ (3 mM), KCl (20 mM), NaCl (150 mM), $CaCl_2$ (3 mM) and 2,4-dinitrophenol (0.1 mM), and the fraction to be assayed. After 5 min. for temperature equilibration at 37° , tris-ATP (3 mM) was added to start the reaction. After 10 min. incubation the tubes were transferred to an ice bath and 0.5 ml. of cold 20% (w/v) trichloroacetic acid was added to stop the reaction. After 10 min. the tubes were centrifuged at 1000g for 5 min. and the inorganic phosphate was determined by the method of Berenblum & Chain as described by Lindberg & Ernster (1956) with modifications. A 2 ml. sample was added to 0.5 ml. of 2.5N- H_2SO_4 , 0.5 ml. of 10% (w/v) ammonium molybdate and 5 ml. of isobutanol-light petroleum (b.p. 100-120 $^\circ$) (4:1, v/v), in an ice bath. The tubes were shaken for 15 sec. and centrifuged at low speed. A sample (4 ml.) of the upper layer was added to an equal volume of N- H_2SO_4 in ethanol and 0.5 ml. of freshly prepared 0.05% (w/v) $SnCl_2$ in 2.5N- H_2SO_4 was added. After 15 min. the colour intensity was read at 650 m μ . The standard curve was constructed in the presence of the same concentration of trichloroacetic acid. One unit of activity was defined as the amount releasing 1 μ mole of inorganic phosphate from the substrate/hr.

Nitrogen. This was determined by the micro-Kjeldahl method.

Expression of results. Results are expressed: (1) in units of activity/volume of fraction derived from 1 g. of original tissue, (2) units of activity/mg. of nitrogen (specific activity) and (3) as percentages of total recovered activity.

RESULTS

Distribution of adenosine triphosphatase in primary fractions

The reaction mixtures were based on those described in the literature but conditions were chosen to determine the distribution of adenosine triphosphatase in the presence of Mg^{2+} or Ca^{2+} alone and on the addition of Na^+ and K^+ or 2,4-dinitrophenol to the medium containing Mg^{2+} . Table 1 gives the distribution of adenosine-triphosphatase activity in fractions derived from fore-brain. Under the conditions chosen the adenosine-triphosphatase activity of a homogenate of cerebral

cortex (Table 2) was higher than that of whole fore-brain (expressed /g. of original tissue or /mg. of nitrogen), amounting to 2700 units/g. of tissue with Na^+ , K^+ and Mg^{2+} present as compared with 2270 units/g. for fore-brain (Table 1). The Na^+ - K^+ -stimulated activity of cortex was as high as 1300 units/g. of tissue, which is comparable with the value of 1500 units/g. for rabbit cortex reported by Bonting, Caravaggio & Hawkins (1962). The distribution of activity in the subcellular fractions was very similar with both tissues. The distribution of Ca^{2+} -activated adenosine triphosphatase approximately paralleled that of the Mg^{2+} -stimulated enzyme: 50–60% of the activity was recovered in

Table 1. *Adenosine-triphosphatase activity of primary subcellular fractions from guinea-pig fore-brain*

For nomenclature of fractions and composition of reaction mixture see the text. The values in parentheses represent the distribution of activity as a percentage of the recovered value.

Fraction	Nitrogen (mg./g. of tissue)	Adenosine-triphosphatase activity (units/g. of tissue)				
		Mg^{2+} (a)	$Mg^{2+} + Na^+ + K^+$ (b)	$Mg^{2+} + \text{dinitrophenol}$ (c)	Ca^{2+} (d)	(b-a)
Homogenate	14.6	1220	2270	1420	1070	1050
P_1	4.03 (31)	235 (23)	547 (31)	288 (24)	235 (25)	312 (40)
P_2	4.96 (38)	553 (55)	846 (47)	678 (57)	498 (54)	293 (38)
P_3	0.98 (8)	200 (20)	360 (20)	210 (17)	184 (20)	160 (21)
S_3	3.00 (23)	22 (2)	30 (2)	22 (2)	13 (1)	8 (1)
Total	12.97	1010	1783	1198	930	773
Recovery (% of homogenate)	88	83	79	84	87	74

Table 2. *Adenosine-triphosphatase activity of subfractions from guinea-pig cerebral neocortex*

For nomenclature of fractions and composition of reaction mixtures see the text.

Fraction	Nitrogen (mg./g. of tissue)	Adenosine-triphosphatase activity (units/g. of tissue)			
		Mg^{2+} (a)	$Mg^{2+} + Na^+ + K^+$ (b)	Ca^{2+} (c)	(b-a)
Homogenate	14.8	1410	2720	1280	1310
P_1	3.52	254	592	305	338
P_1A	1.00	24	60	22	36
P_1B	1.83	133	266	100	133
P_1C	0.57	131	165	110	34
Total	3.40	288	491	232	203
Recovery (% of P_1)	97	113	83	76	60
P_2	4.56	597	1190	597	593
P_2A	1.18	69	133	64	64
P_2B	2.22	336	676	283	340
P_2C	0.64	160	166	131	6
Total	4.04	565	975	478	410
Recovery (% of P_2)	89	94	82	80	69
Purified myelin	0.54	12	38	10	26

fraction P_2 , and the highest specific activity was present in fraction P_3 . About 40% of the Na^+ - K^+ -stimulated adenosine-triphosphatase activity was recovered in fraction P_1 , but the highest specific activity was present in fraction P_3 with 20% of the total activity stimulated by Na^+ and K^+ . Under conditions of slightly more vigorous homogenization (speed of rotation of homogenizer 1400 instead of 840 rev./min.), 25% of the Na^+ - K^+ -stimulated adenosine-triphosphatase activity was recovered in fraction P_3 (activity, 407 units/g. of tissue).

With the gentle homogenization conditions used as a routine in this study large membrane fragments, incompletely homogenized cells, fragments of axons and smaller entrapped particles are carried down in the first centrifugation of 11000g-min.

Distribution of adenosine-triphosphatase activity in subfractions

Fractions P_1 and P_2 were separated into subfractions by centrifuging into discontinuous density gradients. Table 2 shows the distribution of adenosine triphosphatase in these subfractions. The morphological constituents of fraction P_1A are large myelin fragments, and those of P_1B are nuclei contaminated with myelin fragments and membranes. Both these fractions possessed adenosine-triphosphatase activity stimulated by Na^+ and K^+ . Purified myelin prepared from the P_1A fraction by the method of Autilio, Norton & Terry (1964), as modified by Eichberg & Dawson (1965), showed the same degree of stimulation by Na^+ and

K^+ as the parent fraction. The adenosine triphosphatase of fraction P_1C , which consisted of cell debris and blood cells, was less stimulated by Na^+ and K^+ .

The adenosine-triphosphatase activity of fractions P_2A (small myelin fragments) and P_2B (synaptosomes) was stimulated by Na^+ and K^+ , whereas the activity of fraction P_2C (mitochondria) was not stimulated by Na^+ and K^+ but was stimulated by 2,4-dinitrophenol.

Distribution of adenosine-triphosphatase activity in fractions from disrupted synaptosomes

Tables 3 and 4 show the distribution of adenosine-triphosphatase activity in fractions derived from water-treated fractions P_2 and P_2B after separation by density-gradient centrifugation according to procedure A or B described in the Methods section. The synaptic-vesicle fraction D possessed an Mg^{2+} -stimulated adenosine triphosphatase that was not activated by Na^+ , K^+ or Ca^{2+} . The membrane fractions E , F , G and H were characterized by an Na^+ - K^+ -stimulated adenosine triphosphatase. The distribution of the Na^+ - K^+ -stimulated adenosine triphosphatase resembled that of acetylcholinesterase (Whittaker *et al.* 1964); however, the fraction with the highest specific activity with respect to acetylcholinesterase was fraction E , whereas fraction F , G or H possessed the highest specific activity with respect to the Na^+ - K^+ -stimulated adenosine triphosphatase. Frequently Ca^{2+} stimulated slightly more than Mg^{2+} , and the distribution of activity in the presence of

Table 3. Adenosine-triphosphatase activity of subfractions of disrupted synaptosome preparations from guinea-pig fore-brain

For nomenclature of fractions and composition of reaction mixtures see the text. The values are the means of two experiments. The values in parentheses represent the distribution as a percentage of the recovered value.

Fraction	Nitrogen (mg./g. of tissue)	Adenosine-triphosphatase activity (units/g. of tissue)				
		Mg^{2+} (a)	$\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ (b)	$\text{Mg}^{2+} + \text{dinitrophenol}$ (c)	Ca^{2+} (d)	(b-a)
P_2W	3.56	546	905	656	501	359
P_2W_i	1.42	252	337	333	205	85
P_2W_p	2.17	310	574	340	264	264
O	0.519	3 (1)	4 (1)	3 (1)	2 (1)	1 (<1)
D	0.235	20 (8)	17 (3)	18 (6)	8 (3)	0 (0)
E	0.180	24 (10)	60 (12)	24 (9)	27 (12)	36 (14)
F	0.231	32 (13)	74 (15)	32 (12)	33 (15)	42 (17)
G	0.201	45 (18)	112 (23)	40 (15)	51 (23)	67 (27)
H	0.247	54 (22)	140 (28)	59 (22)	50 (22)	86 (34)
I	0.265	70 (28)	91 (18)	96 (35)	55 (24)	21 (8)
Total	1.88	248	498	272	226	253
Recovery (% of P_2W_i)	87	80	87	81	85	96

Table 4. *Adenosine-triphosphatase activity of subfractions of disrupted synaptosomes from guinea-pig cerebral neocortex*

For nomenclature of fractions and composition of reaction mixtures see the text. The values in parentheses show the distribution of activity as a percentage of the recovered values.

Fraction	Nitrogen (mg./g. of tissue)	Adenosine-triphosphatase activity (units/g. of tissue)			
		Mg ²⁺ (a)	Mg ²⁺ + Na ⁺ + K ⁺ (b)	Ca ²⁺ (c)	(b-a)
<i>P₂BW</i>	2.25	319	565	276	246
<i>O</i>	0.540	3 (1)	3 (1)	1 (<1)	0 (0)
<i>D</i>	0.061	11 (4)	8 (2)	7 (3)	0 (0)
<i>E</i>	0.067	11 (4)	18 (4)	8 (3)	7 (4)
<i>F</i>	0.256	39 (15)	101 (22)	29 (12)	62 (33)
<i>G</i>	0.290	50 (19)	116 (25)	58 (25)	66 (34)
<i>H</i>	0.536	130 (48)	177 (39)	114 (49)	47 (25)
<i>I</i>	0.172	25 (9)	32 (7)	17 (7)	7 (4)
Total	1.92	269	455	234	189
Recovery (% of <i>P₂BW</i>)	85	84	81	85	77

Ca²⁺ alone differed slightly from that in the presence of Mg²⁺. The effect of Ca²⁺ on the Na⁺-K⁺-stimulated activity is considered below.

In the electron microscope, fraction *E* was seen to consist mainly of membrane fragments about 0.1–0.3 μ in diameter similar to those seen in the microsomal fraction of brain homogenates. Some synaptic vesicles and myelin fragments were present. Fractions *F* and *G* resembled each other and contained membrane fragments of various sizes; those of *F* were on average smaller than *G*. Most of the membranes present in *G* were large ovals about the size of synaptosomes and are believed to be the external synaptosome membranes (Plates 1a and 1b). The amorphous material present in fraction *G* that often prevented the even spreading of negative stain is shown in Plate 2(a). Fraction *H* consisted mainly of large membranes similar to those seen in fraction *G*, with a few undisrupted synaptosomes and some small mitochondria about 0.5 μ long, similar to those seen in intact synaptosomes. Fractions prepared by procedure B from *P₂B* were more homogeneous than those prepared by procedure A and were free from myelin and mitochondria.

The mitochondrial fraction *I* was distinguished by its content of 2,4-dinitrophenol-stimulated adenosine triphosphatase. The distribution of the dinitrophenol-stimulated adenosine triphosphatase resembled that of succinate dehydrogenase, an enzyme characteristic of mitochondria. Na⁺ and K⁺ had little effect on the activity of this fraction, and Ca²⁺ gave less activation than the equivalent concentration of Mg²⁺.

Storage. The Na⁺-K⁺-stimulated adenosine triphosphatase was very stable; activity increased

to a maximum after about 5 days at –20°, then fell slightly, remaining fairly constant for 80 days. The 2,4-dinitrophenol-stimulated adenosine triphosphatase was relatively unstable.

Effects of ions and biogenic amines on the adenosine triphosphatase of the synaptic-vesicle fraction

Effect of ions. Mg²⁺ stimulated the activity of the adenosine triphosphatase of the *D* (synaptic-vesicle) fraction; maximum activity was achieved in the presence of 4mM-Mg²⁺ (1mM-EDTA also present). In the presence of Mg²⁺ the addition of either Na⁺ or K⁺ had little effect; Na⁺ and K⁺ together decreased the activity. Ca²⁺ alone stimulated activity to only half the value given by Mg²⁺, and when added with Mg²⁺ had no effect.

Effect of amines. In view of the relatively high content of acetylcholine, noradrenaline and possibly other transmitters in this fraction (Whittaker *et al.* 1964; Michaelson, Whittaker, Lavery & Sharman, 1964) and the role that ATP has been shown to play in the uptake and storage of pressor amines in peripheral storage granules, the effect of these compounds on the activity of this fraction was investigated. As shown in Table 5 the results obtained varied with the age of the preparation and the ion present. Although stimulation of adenosine-triphosphatase activity was obtained in the presence of acetylcholine and glutamate on several occasions, this effect was not obtained consistently. For comparison the effect of acetylcholine on membranal adenosine triphosphatase was determined. No effect was observed with 0.1mM- or 0.5mM-acetylcholine in the presence of Mg²⁺ and preparations of fraction *F*.

EXPLANATION OF PLATE 1

Fraction *G*, a large membrane fraction derived from disrupted synaptosomes by density-gradient separation and believed to consist largely of external synaptosome membranes. The membranes (*m*) are seen after permanganate fixation, lead staining and thin section in (*a*) and after negative staining in (*b*). The dense particles (*d*) in (*a*) may be cross-sections of the strands of amorphous material (*s*) in (*b*).

EXPLANATION OF PLATE 2

(a) Patch of amorphous material from fraction *G*, negatively stained. This material may be the same as that comprising the strands (s) in Plate 1(b). (b) Fraction *G* after ultrasonic treatment. The material was fixed, stained and sectioned as in Plate 1(a). Some of the particles may be the same material as in (a) but finely divided. (c) Fraction *D* similarly prepared for comparison.

Table 5. *Effects of acetylcholine and noradrenaline on the adenosine-triphosphatase activity of the synaptic-vesicle fraction from guinea-pig cerebral cortex*

The preparations were incubated with acetylcholine and eserine ($1\mu\text{M}$), or noradrenaline and nialamide ($1\mu\text{M}$), for 10 min. before the addition of ATP.

Prep. no.	Age (days)	Ion(s) present	Concn. of amine (mM)	Adenosine-triphosphatase activity	
				(units/g.)	(% of control)
				Acetylcholine	
1	1	Mg ²⁺	0.4	13.5	98
		Mg ²⁺ + Na ⁺ + K ⁺	0.4	5.1	88
		Ca ²⁺	0.4	7.9	100
2	7	Mg ²⁺	0.4	12.7	110
		Ca ²⁺	0.4	8.4	116
3	30	Mg ²⁺	0.1	20.7	95
		Mg ²⁺ + Na ⁺ + K ⁺	0.1	18.8	99
Noradrenaline					
4	1	Mg ²⁺	1.0	19.7	96
		Mg ²⁺ + Na ⁺ + K ⁺	1.0	17.9	95
		Ca ²⁺	1.0	4.1	96

Properties of the adenosine-triphosphatase activity of membrane fractions

Fractions *F*, *G* and *H* all contained membranes believed to be the external membranes of the nerve ending, but since fraction *G* was the most homogeneous preparation this fraction was selected for further study and comparison with fractions *E* (microsomes) and *P*₃ (the high-speed microsomal pellet).

Effect of univalent ions. In the presence of 3mM- Mg^{2+} the addition of Na^+ (150mM) increased the activity about 1.3-fold and the addition of K^+ (20mM) stimulated activity about 1.1-fold; however, the simultaneous addition of Na^+ (150mM) and K^+ (20mM) to mixtures containing Mg^{2+} (3mM) stimulated activity 2.5–3-fold, and the addition of these ions simultaneously to mixtures containing Mg^{2+} (6mM) stimulated activity about 4-fold. In the presence of 3mM- Ca^{2+} the addition of Na^+ (150mM) increased activity about 1.1-fold and the addition of K^+ (20mM) gave little further stimulation (Table 6).

Effect of bivalent ions. Both Mg^{2+} and Ca^{2+} alone stimulated adenosine triphosphatase; the stimulation was dependent on the concentration of the ions and was maximal at 4mM (1mM-EDTA and 3mM-ATP). The addition of Ca^{2+} (3mM) to mixtures containing Mg^{2+} (3mM) increased activity only a little compared with that obtained in the presence of Mg^{2+} alone, as shown in Table 6.

Effect of ouabain. The cardiac glycoside ouabain (strophanthin G) preferentially inhibited the Na^+ - K^+ -stimulated adenosine triphosphatase of both fractions *G* and *E* (Table 7). Ouabain inhibited the Na^+ - K^+ -stimulated activity by 80–90% at a

Table 6. *Effects of ions on the adenosine-triphosphatase activity of membrane fractions of guinea-pig brain*

The medium contained EDTA (1mM), and MgCl_2 (2–6mM), KCl (20mM) and NaCl (150mM) as indicated.

Expt. no.	Ion(s) present	Adenosine-triphosphatase activity (units/mg. of nitrogen)	
		Fraction <i>E</i>	Fraction <i>G</i>
1	Mg^{2+} (2mM) (no EDTA)	—	107
	Mg^{2+} (3mM)	103	118
	$\text{Mg}^{2+} + \text{Na}^+$	134	154
	$\text{Mg}^{2+} + \text{K}^+$	113	136
	$\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$	305	326
	Mg^{2+} (6mM) + $\text{Na}^+ + \text{K}^+$	355	390
	Ca^{2+}	118	—
	$\text{Ca}^{2+} + \text{Na}^+$	132	—
	$\text{Ca}^{2+} + \text{Na}^+ + \text{K}^+$	134	—
2	Mg^{2+} (3mM)	113	147
	Ca^{2+}	118	160
	$\text{Mg}^{2+} + \text{Ca}^{2+}$	133	214

concentration of 0.5mM, but had negligible effect on the activity in the absence of these cations.

Effect of p-chloromercuribenzoate. $10\mu\text{M}$ -p-Chloromercuribenzoate inhibited adenosine-triphosphatase activity in the presence of Mg^{2+} or Ca^{2+} alone and in the presence of Mg^{2+} , Na^+ and K^+ . The activities of fractions *E* and *G* were similarly affected (Table 8). The activity in the presence of Mg^{2+} or Ca^{2+} alone was inhibited to about the same extent but the inhibition of the Na^+ - K^+ -stimulated activity was greater.

Effect of Ca^{2+} and chelating agents on the Na^+ - K^+ -stimulated adenosine-triphosphatase activity. In

Table 7. *Effects of ouabain on the adenosine-triphosphatase activity of membrane fractions from guinea-pig fore-brain*

The preparations were incubated with or without ouabain (final concn. 0.5 mM) for 5 min. at 37° before the addition of ATP. The medium contained MgCl₂ (3 mM), CaCl₂ (3 mM), KCl (20 mM), NaCl (150 mM) as indicated, and EDTA (1 mM).

Ion(s) present	Adenosine-triphosphatase activity (units/mg. of nitrogen)					
	Fraction E		Fraction G		Fraction P ₃	
	No ouabain	With ouabain	No ouabain	With ouabain	No ouabain	With ouabain
Mg ²⁺	98	97	147	140	124	113
Ca ²⁺	111	109	160	150	138	130
Mg ²⁺ + Ca ²⁺	—	—	174	160	127	114
Mg ²⁺ + Na ⁺ + K ⁺	305	109	400	179	199	119
Ca ²⁺ + Na ⁺ + K ⁺	139	119	180	150	155	135
Ca ²⁺ + Mg ²⁺ + Na ⁺ + K ⁺	—	—	214	174	—	—

Table 8. *Effects of p-chloromercuribenzoate on the adenosine-triphosphatase activity of membrane fractions from guinea-pig fore-brain*

The preparations were incubated with or without p-chloromercuribenzoate (final concn. 10 μM) in the medium before the addition of ATP for 5 min. at 37° as indicated. The medium contained MgCl₂ (3 mM), CaCl₂ (3 mM), KCl (20 mM) and NaCl (150 mM) as indicated. PCMB, p-Chloromercuribenzoate.

Ion(s) present	Adenosine-triphosphatase activity (units/mg. of nitrogen)			
	Fraction E		Fraction G	
	No PCMB	With PCMB	No PCMB	With PCMB
Mg ²⁺	119	68	157	93
Mg ²⁺ + Na ⁺ + K ⁺	284	80	494	127
Ca ²⁺	—	—	226	150

the absence of EDTA or 1,2-bis-[2'-(bis(carboxymethyl)amino)ethoxy]ethane (EGTA), Ca²⁺ stimulated adenosine triphosphatase slightly in the absence of Na⁺ plus K⁺ but inhibited considerably in their presence (Table 9). In the presence of EDTA or 1,2-bis-[2'-(bis(carboxymethyl)amino)ethoxy]ethane the adenosine-triphosphatase activity increased slightly compared with the effect of equivalent concentrations of free Mg²⁺ and the inhibitory effect of low concentrations of Ca²⁺ in the presence of Na⁺ and K⁺ was prevented by EDTA or 1,2-bis-[2'-(bis(carboxymethyl)amino)ethoxy]ethane. These substances were only able partially to overcome the inhibition caused by higher concentrations of Ca²⁺.

Effect of ultrasonic disruption on fraction G

In view of a report by Campbell (1964), which appeared during the course of this work, that

Na⁺-K⁺-stimulated adenosine triphosphatase was associated with a synaptic-vesicle fraction prepared by the ultrasonic disruption of synaptosomes in hypo-osmotic sucrose, the effect of ultrasonic treatment on the membranes of fraction G was studied. The results varied somewhat according to whether the fraction was diluted before treatment or not; if applied to the undiluted fraction, most of the material present was disrupted to particles 0.03–0.3 μ in diameter (Plate 2b), most of which could be readily distinguished from synaptic vesicles (Plate 2c). If applied to fraction G after 1:4.5 (v/v) dilution with water to simulate more closely the conditions of Campbell's (1964) experiments, the ultrasonic treatment was less effective, many of the larger membranes escaping complete disruption. Separation of the ultrasonically treated fractions in a density gradient similar to that originally used to prepare fraction G showed faint bands of particulate material in the upper portion of the density gradient corresponding to fractions D to F as well as in the region corresponding to fraction G, whereas a control run with untreated fraction G gave a single band in the original position. In the run with ultrasonically treated fraction G Na⁺-K⁺-stimulated adenosine triphosphatase was now detected in the upper part of the gradient. It would thus appear that Campbell's (1964) results are due, as he suggested, to the presence in his synaptic-vesicle fraction of membrane fragments derived from the external synaptosome membrane.

DISCUSSION

The main result of the present study is the finding that fractions F, G and H, rich in the external membranes of nerve endings, possess a high concentration of Na⁺-K⁺-stimulated adenosine triphosphatase that closely resembles the much

Table 9. *Effects of Ca²⁺ and chelating agents on the adenosine-triphosphatase activity of fractions from guinea-pig cerebral cortex*

The medium contained MgCl₂ (3 mm), KCl (20 mm), NaCl (150 mm), CaCl₂ (0.1 or 3 mm), EDTA or 1,2-bis-(2'-[bis-(carboxymethyl)amino]ethoxy)ethane (1 mm) as indicated. In the absence of 1 mm-EDTA, 2 mm-MgCl₂ was present in the reaction mixture. EGTA, 1,2-Bis-(2'-[bis(carboxymethyl)amino]ethoxy)ethane.

Ion(s) present	Adenosine-triphosphatase activity (units/mg. of nitrogen)			
	Fraction <i>E</i>		Fraction <i>G</i>	
	No chelating agent	With chelating agent	No chelating agent	With chelating agent
EGTA				
Mg ²⁺ + Ca ²⁺ (3 mm)	104	103	137	139
Mg ²⁺ + Na ⁺ + K ⁺	206	227	399	410
Ca ²⁺ (0.1 mm) + Mg ²⁺ + Na ⁺ + K ⁺	146	206	369	416
Ca ²⁺ (3 mm) + Mg ²⁺ + Na ⁺ + K ⁺	100	106	178	220
EDTA				
Mg ²⁺ + Ca ²⁺ (3 mm)	93	106	139	141
Mg ²⁺ + Na ⁺ + K ⁺	193	216	394	401
Ca ²⁺ (0.1 mm) + Mg ²⁺ + Na ⁺ + K ⁺	168	201	327	367
Ca ²⁺ (3 mm) + Mg ²⁺ + Na ⁺ + K ⁺	113	132	184	246

studied but morphologically heterogeneous microsomal enzyme in its response to ions and inhibition by ouabain. The specific stimulated activities of these fractions, in particular *G* (208–337 units/mg. of nitrogen), were appreciably higher than those of the microsome fractions *P*₃ (75–164 units/mg.) and *E* (104–207 units/mg.), as is seen by comparing Tables 1, 3, 4 and 6–8. These findings implement earlier histochemical (Hess & Pope, 1959; Lewin & Hess, 1964; Torack & Barnett, 1964) and microdissection (Cummins & Hydén, 1962) studies, in showing that in brain tissue as in liver (Emmelot & Bos, 1962) and kidney (Wheeler & Whittam, 1964) the Na⁺–K⁺-stimulated adenosine triphosphatase is localized in cell membranes.

The synaptic vesicles (fraction *D*) were found not to contain the Na⁺–K⁺-stimulated enzyme. This suggests that the synaptic-vesicle membrane is biochemically distinct from the external synaptosome membrane, a conclusion supported by the relatively low cholinesterase (Whittaker *et al.* 1964) and ganglioside (J. Eichberg & G. H. C. Dowe, personal communication) content of this fraction as compared with fractions *F*, *G* and *H*. The implications of this for current theories of vesicle fusion and transmitter release have yet to be evaluated fully. The absence of the Na⁺–K⁺-stimulated enzyme from this fraction contrasts with the finding of Campbell (1964), but it would seem that his procedure of ultrasonic treatment before fractionation causes the fragmentation of external membranes and the contamination of the synaptic-vesicle fraction with minute portions of the external membrane. Apart from the occasional cases of stimulation by acetylcholine, the slightly inhibitory

effects of acetylcholine, glutamate and noradrenaline obtained with this fraction agree with the observations of Gore (1957) with guinea-pig brain homogenate and Bonting *et al.* (1962) with cat grey matter.

Another adenosine triphosphatase with a morphologically defined location is the dinitrophenol-stimulated enzyme. This was found to be associated with the mitochondrial fraction (*I*) and its distribution among the fractions derived from disrupted synaptosomes correlated closely with that previously found for succinate dehydrogenase (Whittaker *et al.* 1964). Again, the complete absence of this enzyme in fraction *D* indicates the freedom of the synaptic-vesicle fraction from contamination by mitochondrial fragments.

The distribution of Na⁺–K⁺-stimulated adenosine triphosphatase in the primary fractions differed from that obtained by Schwartz *et al.* (1962). These authors obtained 75% of the total stimulated activity in the microsomal pellet as compared with about 20% in the present study. This difference can probably be accounted for by differences in homogenization techniques. In the present study, the conditions of homogenization were relatively mild (clearance between pestle and mortar, 0.25 mm.; speed of rotation of pestle, 840 rev./min.) and were those that previous work in this Laboratory (see Whittaker & Dowe, 1965) has indicated for a maximum preservation of nerve endings. By contrast, Schwartz *et al.* (1962) employed a homogenizer with a more tightly fitting pestle (0.13–0.18 mm. clearance) and a higher speed of rotation (2300 rev./min.). Under these more rigorous conditions the cell membranes would be expected to

be very finely dispersed and would sediment in the microsomal fraction; whereas with my milder homogenization the cell membranes are less fragmented and a considerable amount of organized cellular material sediments in the nuclear fraction (Gray & Whittaker, 1962).

The 3-fold stimulation of the adenosine triphosphatase of the membrane fractions by Na^+ and K^+ was similar to that reported by Aldridge (1962) and Deul & McIlwain (1961). Skou (1962), however, obtained an 8-fold increase in the activity of brain microsomes after treatment with EDTA and deoxycholate. Doubling the concentration of Mg^{2+} , as shown by Skou (1960), gave a further increase in activity, although not to the extent achieved by Skou (1960). With different preparations differing ratios for Na^+ - K^+ -stimulation were obtained. Skou (1963) has proposed several possible explanations for this phenomenon based on the involvement of thiol groups. On storage, the activity in the presence of Mg^{2+} alone sometimes declined more than with Na^+ and K^+ in addition, giving rise to increased stimulation ratios and correspondingly increased sensitivity to ouabain. Schwartz *et al.* (1962) have carried out a detailed study of this change in activity with time and temperature of storage.

The cardiac glycoside ouabain inhibited the Na^+ - K^+ - Mg^{2+} -activated adenosine triphosphatase of all three membrane systems studied, as has been found with the adenosine triphosphatase of brain microsomes (Aldridge, 1962; Deul & McIlwain, 1961; Skou, 1962), crab nerves (Skou, 1960) and erythrocyte membranes (Dunham & Glynn, 1961; Post *et al.* 1960). It was found that Ca^{2+} inhibited the glycoside-sensitive Na^+ - K^+ -stimulated adenosine triphosphatase but stimulated the glycoside-insensitive activity. This agrees with the findings of Glynn (1964) with adenosine triphosphatase of the electric organ of *Torpedo*. There are some conflicting reports on the effect of Ca^{2+} ; Skou (1960, 1962), working with preparations of crab nerve and rat and guinea-pig brain, found that, in the absence of Na^+ and K^+ , Ca^{2+} inhibited the adenosine-triphosphatase activity in the presence of Mg^{2+} . This contrasts with the findings of Aldridge (1962), Glynn (1964) and the present investigation. Aldridge (1962) and Somogyi & Budai (1964) found that the inhibition of Na^+ - K^+ -stimulated activity caused by low concentrations of Ca^{2+} could be overcome by the addition of EDTA. This phenomenon was confirmed in the present investigation.

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