Distribution of Sodium-plus-Potassium-Stimulated Adenosine-Triphosphatase Activity in Isolated Nerve-Ending Particles

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1. A rapid method for the isolation of nerve-ending particles from brain is described. This involved the centrifugation of the large-granule fraction over a discontinuous density gradient consisting of 3% (w/v) and 13% (w/v) Ficoll dissolved in 0.32M-sucrose. The results of the biochemical as well as morphological identification of nerve-ending particles are given. 2. Approx. 20% of the ($\text{Na}^+ + \text{K}^+$)-stimulated adenosine-triphosphatase activity originally present in the cerebral grey-matter suspension was recovered in the fraction consisting principally of large nerve-ending particles (approx. 1μ in diameter). The activity of the adenosine triphosphatase/mg. of protein in the nerve-ending fraction approximated to that in the small-granule fraction after the treatment with glycol ether diamine-tetra-acetic acid. The conclusion was drawn that the synaptic structure, supposedly the limiting membrane of the nerve-ending particle, is one of the feasible sites of localization of the ($\text{Na}^+ + \text{K}^+$)-stimulated adenosine-triphosphatase activity in cerebral tissues. Adenosine triphosphatase in purified cerebral mitochondria was not stimulated by Na^+ . 3. No qualitative differences were found between the ($\text{Na}^+ + \text{K}^+$)-stimulated adenosine-triphosphatase activities exhibited by the nerve-ending particles and by the cerebral small-granule fraction with respect to pH-dependence, cation requirements and susceptibility to ouabain.

The activity of (NaK)-ATPase,* first demonstrated by Skou (1957) in leg nerve from *Carcinus maenus*, has further been examined in various tissues, and the highest activity reported in cerebral grey matter (Bonting, Simon & Hawkins, 1961). Although subfractionation studies of the mammalian cerebral cortex have shown that (NaK)-ATPase activity is mainly associated with the microsomal fraction (Deul & McIlwain, 1961; Schwartz, Bachelard & McIlwain, 1962), a considerable proportion of the total activity is also distributed in the crude mitochondrial fraction (Deul & McIlwain, 1961; Schwartz *et al.* 1962; Whittam & Blond, 1964). Because of the structural heterogeneity of the cerebral mitochondrial fraction (Whittaker, 1959; Gray & Whittaker, 1962), an attempt has now been made to examine the localization of ATPase activity in subfractions of the primary mitochondrial fraction. It is shown in the present

paper that the nerve-ending structure is one of the feasible sites of localization of (NaK)-ATPase activity. A rapid method for the isolation of nerve-ending particles (synaptosomes; Whittaker, Michaelson & Kirkland, 1964) is also presented. Preliminary accounts of this work have been given (Kurokawa, Sakamoto & Kato, 1964, 1965).

MATERIALS AND METHODS

Reagents. The following chemicals were obtained from the sources indicated: ATP (disodium, dipotassium and tris salts), tris (Trizma base), CoA, NADH, cytochrome *c* and NaBH_4 were from Sigma Chemical Co., St Louis, Mo., U.S.A.; acetylcholine chloride was from S. A. F. Hoffmann-La Roche and Co., Basle, Switzerland; physostigmine sulphate was from E. Merck A.-G., Darmstadt, Germany; Ficoll (chloride impurities less than 0.1%, as NaCl) was from Pharmacia, Uppsala, Sweden; glycol ether diamine-tetra-acetic acid was from Dojin Yakukagaku Kenkyusho, Kumamoto, Japan; Triton X-100 was from Rohm and Haas Co., Philadelphia, Pa., U.S.A.; Pereston N was from

* Abbreviation: (NaK)-ATPase, the component of adenosine triphosphatase that requires Mg^{2+} , Na^+ and K^+ .

Bayer A.-G., Leverkusen, Germany. Whenever possible other reagents were of the highest grade of purity commercially available. All solutions were prepared with deionized distilled water. Disodium ATP was converted into the tris salt by using batches of Dowex 50 resin (X8; H⁺ form; 50–100 mesh) as described by Järnefelt (1961). The yield in the conversion was 90–95% by spectrophotometric assay at 260 m μ . In some experiments, tris-ATP (Sigma) was used for comparison, with identical results. In the earlier experiments, Ficoll was purified by dialysis and freeze-drying according to the method of Holter & Møller (1958); this, however, did not prove essential within the limits of our present experiments.

Initial tissue dispersions. Preparations were made at 0–4°. Brains were taken from adult guinea pigs stunned by a blow on the neck, and cerebral white matter was removed. The grey matter, usually 1.3–1.5 g./animal, was placed within 3–4 min. of death into an ice-cold glass tube and, in batches of approx. 3 g., was immediately ground in 9 vol. of 0.32 M-sucrose by using a ball-ended plastic pestle (clearance 0.08–0.12 mm.; Dounce, Witter, Monty, Pate & Cottone, 1955, with modifications). The pestle was moved ten times up and down at approx. 2500 rev./min. during a total time of 2 min. In some experiments, as indicated, a more intense homogenization was applied; grinding in this case involved 25 passages of the pestle, rotating at 4000 rev./min., during 4 min.

A few preparations were made from guinea-pig kidney cortex. Batches (3–4 g.) were minced with a blade, homogenized as above (ten passages at 2500 rev./min. in 2 min.) and the suspension was strained through muslin.

Preparation of primary fractions. Centrifugal forces are expressed as $g_{av.}$; values calculated to the centres of the tubes. The 10% (w/v) homogenate was centrifuged at 2800 rev./min. (1000 g) for 10 min. (Kubota model KR-6PCW ultracentrifuge), and the residue was resuspended in the same volume of 0.32 M-sucrose and again centrifuged. The combined supernatant and washing was centrifuged at 11000 rev./min. (10000 g) for 15 min. (Hitachi model 40P ultracentrifuge), and the residue was washed twice with two-thirds of the original volume of sucrose. Finally, the combined supernatants from these centrifugings were centrifuged at 19000 rev./min. (31100 g) for 40 min. These produced fractions P₁ (1000 g), P₂ (10000 g), P₃ (31100 g) and S (supernatant) respectively. Pellets of P₁, P₂ and P₃ were dispersed in appropriate volumes of 0.32 M-sucrose and portions were used for assay.

Isolation of the nerve-ending particles. The unwashed P₂ pellet, sedimented either at 13000 rev./min. (14500 g) or at 11000 rev./min. (10000 g) as indicated, was stirred up in a few millilitres of 0.32 M-sucrose, disintegrated by sucking up and down several times in a Pasteur pipette with a small orifice, transferred to a glass homogenizer and resuspended by gentle homogenization by hand in a sufficient quantity of 0.32 M-sucrose to bring the suspension to a final volume of 2.8–3.2 ml./g. of the original tissue.

Separation from the P₂ suspension of the myelin (A), nerve-ending (B) and mitochondrial (C) fractions was carried out by using either sucrose-density-gradient centrifugation (Whittaker, 1959; Gray & Whittaker, 1962) or Ficoll-density-gradient centrifugation as described below. A discontinuous density gradient, consisting of 2 ml. of 3% (w/v) and 2 ml. of 13% (w/v) Ficoll dissolved in 0.32 M-sucrose, was kept for 60–90 min. at room temperature and

then for approx. 30 min. at 0–4° before use. A 1 ml. portion of the P₂ suspension was carefully layered on the Ficoll gradient, and three equivalent tubes were centrifuged at 16000 rev./min. (20900 g) for 15 min. in the Hitachi RPS40 swing-out head. This produced three distinct layers, as shown in Fig. 1. The liquid phases of A and C were nearly transparent, whereas that of B was hazy. Band A was white, B greyish white with a yellow tint underneath, and C (pellet) was brown. Layers A, B and C nearly corresponded in volume to the 0.32 M-sucrose, 3% (w/v) Ficoll and 13% (w/v) Ficoll layers respectively. Each layer was separated with bent-tip Pasteur pipettes and with suction. Equivalent layers from the three tubes were combined, diluted with 0.32 M-sucrose and centrifuged at 40000 rev./min. (105400 g) in the Hitachi RP40 head for 20 min. The pellet from the C layer was solid, whereas those from the A and B layers were loosely packed. Each pellet was suspended in an appropriate volume of 0.32 M-sucrose, or in other media as indicated, and portions were used for assay. Biochemical and morphological identifications of the A, B and C fractions are detailed in the Results section.

Separation of synaptic vesicles. This was carried out as described by Whittaker *et al.* (1964) and Eichberg, Whittaker & Dawson (1964), with minor modifications as given below. The P₂ pellet sedimented at 10000 g (15 min.) was washed once in 0.32 M-sucrose, resuspended in water (approx. 2.5 ml./g. of original tissue), kept for 20 min. at 0–4° and centrifuged at 10000 g for 15 min. A cloudy supernatant (W_s) was transferred (1 ml./tube) to Hitachi RPS40 tubes containing a continuous concentration gradient of sucrose ranging from 0.4 M at the top to 0.6 M at the bottom (3 ml./

Fig. 1. Appearance of subfractions A, B and C from the large-granule fraction (P₂). The unwashed P₂ pellet, sedimented at 14500 g after the removal of crude nuclear fraction, was resuspended in 0.32 M-sucrose. A 1 ml. portion of the P₂ suspension was layered on the top of the discontinuous density gradient, consisting of 2 ml. of 3% (w/v) and 2 ml. of 13% (w/v) Ficoll dissolved in 0.32 M-sucrose. This was centrifuged at 20900 g for 15 min. in the Hitachi RPS40 swing-out head, and three layers of A, B and C were produced.

EXPLANATION OF PLATE I

(a) and (b) Electron micrographs of fraction A, showing fragments of myelin sheaths (*my*) and other membranous and vesicular structures, including nerve-ending particles (*ne*) and rough endoplasmic reticulum (*er*).

EXPLANATION OF PLATE 2

(a) and (b) Electron micrographs of fraction B, made up principally of pinched-off nerve-ending particles (*ne*) containing numerous synaptic vesicles and occasional mitochondria (*m*). (c) Nerve-ending particle with synaptic cleft (*sc*) and the attachment of the postsynaptic membrane (*psm*); mitochondria (*m*) and synaptic vesicles (*sv*) are also shown. (d) Nerve-ending particle (*ne*) passing into a 'black body' (*bb*) (Gray & Whittaker, 1962); the postsynaptic membrane (*psm*) is indicated.

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EXPLANATION OF PLATE 3

(a) and (b) Electron micrographs of fraction C, showing free mitochondria in various states of preservation (*m*, mitochondrion; *m'*, partially degenerated mitochondrion); occasional nerve-ending particles are also present.

EXPLANATION OF PLATE 4

Electron micrograph of the P₃ fraction, showing rough endoplasmic reticulum (*er*), unattached ribosomes (*r*), small nerve-ending particles (*ne*) and other membranous and vesicular structures.

tube), which was layered over 1.2M-sucrose. After centrifuging at 35000 rev./min. (100000g) for 60 min., three well-defined layers were produced: a thin white band at the top of the continuous gradient (fraction I); a dense band, white but with a yellowish tint underneath, near the boundary between the continuous concentration gradient and the layer of 1.2M-sucrose (fraction II); a brown pellet at the bottom of the tube (fraction III). Each fraction was separated by bent-tip Pasteur pipettes and with suction, and diluted with water, and pellets were obtained after centrifugation at 40000 rev./min. (105400g) for 40 min. in the Hitachi RP40 head. Fraction I was taken as the synaptic-vesicle fraction.

Acetylcholine bioassay. Acetylcholine was assayed by using the eserine-treated rectus abdominis muscle of the frog. Non-specific effects of tissue extracts on the rectus contraction were controlled according to the method of Feldberg (1945). Interference with the rectus contraction of the iso-osmotic sucrose was minimized by halving the sucrose concentration, as described by Kurokawa, Machiyama & Kato (1963). Acetylcholine chloride was used as the standard throughout.

Choline acetyltransferase (EC 2.3.1.6). The reaction mixture (1.9 ml.) [containing (final concentrations) MgCl_2 (10 mM), KCl (30 mM), potassium acetate (10 mM), dipotassium ATP (5.1 mM), CoA (0.06 mM), NaBH_4 (0.5 mM), potassium phosphate buffer, pH 7.4 (10 mM), 0.2 ml. of aged pigeon-liver enzyme (Kaplan & Lipmann, 1948; extracted in 20 mM- KHCO_3) and 0.1–0.2 ml. of brain tissue suspension] was incubated aerobically at 37.5° for 20 min., and then choline chloride (10 mM) together with physostigmine sulphate (50 μM) were added (0.1 ml.). After incubation for 60 min. at 37.5°, portions of the reaction mixture were diluted in sufficient quantities of ice-cold K^+ -free frog Ringer solution [containing (final concentrations) NaCl (111.2 mM), KCl (1.9 mM), CaCl_2 (0.8 mM) and NaHCO_3 (2.4 mM), adjusted to pH 4 with HCl] to bring the K^+ concentration to approx. 2 mM. Acetylcholine in these samples was assayed as above. Tissue suspensions had been treated with ether to increase the transferase activity (Hebb & Smallman, 1956). Under the present assay conditions, the amount of acetylcholine increased with time at least up to 90 min. after the addition of choline plus physostigmine. The transferase activity was expressed in terms of μmoles of acetylcholine formed/60 min./g. of original tissue at 37.5°.

Lactate dehydrogenase (EC 1.1.1.27). This was assayed by the method of Johnson (1960) in the reaction medium (3.0 ml.) consisting of (final concentrations) triethanolamine-HCl buffer, pH 7.4 (50 mM), NADH (160 μM), sodium pyruvate (330 μM) and tissue suspensions (0.1 ml.). The activity was expressed in terms of $\Delta E_{340}/\text{min.}/\text{g.}$ of original tissue at 24°.

Succinate dehydrogenase (EC 1.3.99.1). This was determined spectrophotometrically by the reduction of cytochrome *c* at 550 μm (Kuff & Schneider, 1954). The reaction mixture (3.0 ml.) consisted of (final concentrations) potassium phosphate buffer, pH 7.4 (17 mM), KCN (200 μM), cytochrome *c* (26 μM), CaCl_2 (400 μM) and potassium succinate (33 mM). To obtain constant activity, the tissue suspension was diluted with water, and further treated with 0.1% (v/v) Triton X-100 at 0–4° for 60–90 min. The final concentration of Triton X-100 in the assay mixture was less than 0.003%, which did not interfere with the assay.

The dehydrogenase activity, expressed in terms of $\Delta E_{550}/\text{min.}/\text{g.}$ of original tissue at 24°, was usually doubled after this treatment.

Adenosine triphosphatase. Reaction mixtures [2.2 ml.; containing (final concentrations) MgCl_2 (6 mM), KCl (5 mM), NaCl (100 mM), tris-HCl buffer, pH 7.4 (30 mM), and the tissue suspension (0.2 ml., contributing 100–300 $\mu\text{g.}$ of protein/ml. of incubation mixture)] were prepared in tubes in an ice bath. NaCl was omitted where indicated. In the experiments indicated, other combinations of univalent cations were used. The mixtures were shaken at 37.5° for 6 min. and the reaction was initiated by the addition of tris-ATP (0.1 ml.) to give a final concentration of 3 mM. Shaking was continued for a further 15 min., and 1.0 ml. of ice-cold 24% (w/v) trichloroacetic acid solution then added. After 10–20 min. at 0–4°, the precipitated protein was spun down, and orthophosphate was measured in samples from the supernatants by the method of Takahashi (1955). Unless otherwise specified, the assay was carried out on the day of preparation without freezing and thawing of the samples. The unit of enzyme activity refers to μmoles of orthophosphate liberated/15 min. at 37.5°.

Protein. This was determined according to the method of Lowry, Rosebrough, Farr & Randall (1951), after the initial removal of lipid materials with ethanol-ether (11:9, v/v). Bovine serum albumin was used as standard.

Total nitrogen. This was determined by the micro-Kjeldahl method.

Relative specific activity. This was expressed as the percentage distribution of acetylcholine or of enzyme activity per percentage distribution of total nitrogen in subfractions A, B and C.

Electron microscopy. Pellets of subfractions A, B and C, and of fraction P_3 , were fixed at 0–4° for 15 min. with 1% (w/v) OsO_4 solution, containing Pereston N, sodium phosphate buffer, pH 7.4 (16.6 mM), and sufficient sucrose to give an approximately iso-osmotic solution. Tissues were dehydrated with several changes of ethanol solutions of increasing concentration and imbedded in Epon (Kushida, 1959, with minor modifications). Sections were cut with a JUM-5A microtome (Nihon Denshi Co.), stained with uranyl acetate and examined with a JEM-T6S microscope (Nihon Denshi Co.).

RESULTS

Electron-microscopic appearance of the subfractions A, B and C separated by the Ficoll-density gradient centrifugation. Subfraction A consisted mainly of fragments of myelin sheaths varying in size; there were also a few small nerve endings, vesicular membrane fragments (rough endoplasmic reticulum) and other membranous and vesicular structures (Plates 1a and 1b).

Subfraction B was made up principally of pinched-off nerve endings, but in addition a heterogeneous array of vesicular and membranous structures and occasional mitochondria were present (Plates 2a and 2b). Most of the nerve endings were spheroidal or elliptical in shape, with well-preserved limiting membrane, synaptic vesicles and one or more mitochondria inside. 'Black bodies', which were

described by Gray & Whittaker (1962), seemed to be less frequently encountered in the present preparations. In some instances, the synaptic cleft and the attachment of the postsynaptic membrane were clearly observed (Plates 2c and 2d).

Subfraction C consisted mainly of free mitochondria, but occasional nerve endings and assorted membranous structures were also present. Some of the mitochondria seemed to be morphologically intact, with respect to the state of preservation of double membranes, cristae and matrices. In some mitochondria, however, the cristae became fused and dense, and the basic structures tended to fade (Plates 3a and 3b). Swelling of mitochondria occurred less frequently in the Ficoll method than in the sucrose method.

Distribution in subfractions A, B and C of acetylcholine, choline acetyltransferase, lactate dehydrogenase, succinate dehydrogenase, total nitrogen and protein. The P₂ fraction sedimented at 14500g contained approx. 60% of the acetylcholine originally present in the brain suspension. Three-quarters of the acetylcholine in the P₂ fraction was concentrated in the subfraction B, which was highly populated with nerve endings (Table 1). Also, the percentage distribution as well as the relative specific activities on a nitrogen basis of choline acetyltransferase and of lactate dehydrogenase were highest in subfraction B. Thus the percentage distributions of acetylcholine, lactate dehydrogenase and succinate dehydrogenase in

the subfractions separated by the Ficoll method were in essential agreement with those obtained by the sucrose method (Johnson & Whittaker, 1963; Whittaker, 1959); generally, it appeared that there was increasing contamination in subfractions A to C in the Ficoll method, and increasing contamination in subfractions C to A in the sucrose method. The distribution of total nitrogen was somewhat at variance with that reported by Whittaker (1959), but was comparable with that reported by Eichberg *et al.* (1964). Approx. 5% of the lactate-dehydrogenase activity in the P₂ fraction was recovered in the supernatant from the A pellet. This indicates the extent of the cytoplasmic contamination, since the supernatants from the B and C pellets did not show any detectable lactate-dehydrogenase activity, and thus release of the activity from the synaptosomal cytoplasm was unlikely to occur during the preparation.

The hazy liquid layer of subfraction B contained very little acetylcholine, choline acetyltransferase and lactate dehydrogenase (less than 3% of the activities in the P₂ fraction), and may be disregarded where the quantitative recovery is not a matter of importance.

Washing or aging of the P₂ fraction caused an unsatisfactory separation of subfractions A, B and C, there being increasing contamination in subfractions A to C. Insufficient temperature equilibration of the Ficoll-density-gradient tubes, or aging of the Ficoll solution, occasionally caused

Table 1. *Distribution of acetylcholine, choline acetyltransferase, lactate dehydrogenase, succinate dehydrogenase, total nitrogen and protein in subfractions from the large-granule fraction (P₂)*

The P₂ fraction was the large-granule fraction sedimented at 14500g after the removal of crude nuclei and cell debris. Subfractions A, B and C were those indicated in Fig. 1 (see the Materials and Methods section). Units were: acetylcholine, mμmoles; choline acetyltransferase, mμmoles of acetylcholine formed/60 min. (37.5°); lactate dehydrogenase, ΔE₃₄₀/min. (24°); succinate dehydrogenase, ΔE₅₅₀/min. (24°); total nitrogen and protein, mg. Relative specific activity was expressed as the percentage distribution of acetylcholine or of enzyme activity per percentage distribution of total nitrogen in each fraction. Values are means ± s.d. of the numbers of experiments given in parentheses.

	Fraction P ₂ (units/g. of cortex)	Percentage distribution			Percentage recovery from fraction P ₂
		Subfraction A	Subfraction B	Subfraction C	
Acetylcholine	7.52 ± 0.17 (4)	7.5	74.2	18.3	78
Choline acetyltransferase	1500 (3)	9.5	75.2	15.3	85
Lactate dehydrogenase	20.5 ± 0.1 (4)	13.4	60.5	26.1	90
Succinate dehydrogenase	6.7 ± 0.1 (4)	0	24.1	75.9	93
Total nitrogen	6.4 ± 0.3 (4)	29.1	41.4	29.5	88
Protein	40.4 ± 1.0 (4)	23.8	46.4	29.8	89
Relative specific activity on a nitrogen basis					
	Fraction P ₂	Subfraction A	Subfraction B	Subfraction C	
Acetylcholine	1.00	0.26	1.80	0.62	
Choline acetyltransferase	1.00	0.33	1.82	0.52	
Lactate dehydrogenase	1.00	0.46	1.46	0.89	
Succinate dehydrogenase	1.00	0	0.58	2.57	

floating material to appear in the 13% (w/v) Ficoll layer. In practice, the Ficoll solution was replaced every 2 weeks, stored at 0–4°.

Release of particle-bound acetylcholine in dense media. The P₂ pellets, sedimented at 14500g, were dispersed in 15% (w/v) and 3% (w/v) Ficoll dissolved in 0.32M-sucrose, and also in 1.00M-, 0.80M- and 0.32M-sucrose, 0.16M-sodium chloride and water, kept at 0–4° for 30min. and centrifuged at 105400g for 20min. Except for the sodium

chloride tube, concentrated sodium chloride solution was added, immediately before the centrifugation, to give a final concentration of 50mM, to ensure the complete sedimentation of particles. The solid pellets were resuspended in appropriate volumes of acidic frog Ringer solution containing physostigmine sulphate (50μM), and were assayed for acetylcholine. Ficoll did not cause any appreciable release of particle-bound acetylcholine, by comparison with the acetylcholine value in the pellet treated with 0.32M-sucrose (Table 2). In contrast, a small portion of particle-bound acetylcholine was released in hyperosmotic sucrose solutions, although the amount released in this way was much less than that due to hypo-osmoticity (Table 2; Whitaker, 1959; Kurokawa *et al.* 1963). The iso-osmotic sodium chloride solution did not release acetylcholine from the particle (Table 2).

ATPase activities of primary fractions from guinea-pig cerebral cortex and kidney cortex. Although there is a general agreement as to the predominant occurrence of (NaK)-ATPase activity in the small-granule fraction (Schwartz *et al.* 1962; Bonting, Caravaggio & Hawkins, 1962; P₃ in Table 3), it seems that there is some discrepancy about the percentage distribution of the activity in primary fractions from the cerebral cortex. Thus Schwartz *et al.* (1962) found only 1% of the recovered activity in their nuclear fraction, whereas in the preparations obtained by Bonting *et al.* (1962) 33% of the activity was distributed in the nuclear fraction. In our results, 18% of the recovered activity was found in the P₁ fraction (Table 3). With respect to percentage distribution of the activity in the P₂ (crude mitochondrial) fraction, the results in Table 3 (32%) are comparable with that (26%)

Table 2. *Release of particle-bound acetylcholine in dense Ficoll and sucrose media*

The P₂ pellets, sedimented at 14500g, were dispersed in the dense media indicated, kept at 0–4° for 30min. and centrifuged at 105400g for 20min. Ficoll was dissolved in 0.32M-sucrose. With the exception of the NaCl tube, concentrated NaCl solution was added immediately before the centrifugation to give a final concentration of 50mM, to ensure the complete sedimentation of the particles. Solid pellets were resuspended in acid frog Ringer solution containing physostigmine (50μM). Mean values from three experiments are given.

Medium	Acetylcholine remaining in pellets after treatment (μmoles/g. of cortex)
3% (w/v) Ficoll	7.10
15% (w/v) Ficoll	7.20
0.32M-Sucrose	7.15
0.80M-Sucrose	6.10
1.00M-Sucrose	5.85
0.16M-NaCl	7.32
Distilled water	4.00

Table 3. *ATPase activities of primary fractions from guinea-pig cerebral cortex*

H represents the original homogenate of cerebral cortex. Fractions were P₁ (sedimented at 1000g for 10min.; washed once), P₂ (10000g for 15min.; washed twice), P₃ (31100g for 40min.; unwashed) and S, which was the supernatant from fraction P₃ (see the Materials and Methods section). Samples (0.2ml.) of each fraction were incubated for 6min. at 37.5° in 2.2ml. of medium containing (final concentrations) MgCl₂ (6mM), KCl (5mM), NaCl (100mM) and tris-HCl buffer, pH 7.4 (30mM); NaCl was omitted where indicated. The reaction was initiated by the addition of 0.1ml. of tris-ATP (3mM), and terminated after incubation for 15min. at 37.5° by adding 1.0ml. of 24% (w/v) trichloroacetic acid. Protein was removed by centrifugation, and orthophosphate was measured in samples from the supernatants. Enzyme activity was expressed in terms of μmoles of orthophosphate liberated/15min. at 37.5°. Mean values from three preparations are given.

Fraction	Protein (mg./g. of cortex)	ATPase activity					
		(μmoles/g. of cortex/15 min.)			(μmoles/mg. of protein/15 min.)		
		Na ⁺ present (a)	Na ⁺ absent (b)	(a)–(b)	Na ⁺ present (a)	Na ⁺ absent (b)	(a)–(b)
H	97.6	336	183	153	3.5	1.9	1.6
P ₁	9.9	40	15	25	4.0	1.5	2.5
P ₂	36.0	111	68	43	3.1	1.9	1.2
P ₃	17.1	119	51	68	7.0	3.0	4.0
S	32.7	19	21	–2	0.6	0.6	0

Table 4. *ATPase activities of primary fractions from guinea-pig kidney cortex*

Fractionation procedures were identical with those for cerebral cortex (see Table 3). Assay conditions were also as given in Table 3. Mean values from two preparations are given.

Fraction	Protein (mg./g. of cortex)	ATPase activity					
		(μ moles/g. of cortex/15 min.)			(μ moles/mg. of protein/15 min.)		
		Na ⁺ present (a)	Na ⁺ absent (b)	(a) - (b)	Na ⁺ present (a)	Na ⁺ absent (b)	(a) - (b)
H	135.0	108	49	59	0.80	0.36	0.44
P ₁	21.1	21	8	13	1.00	0.38	0.62
P ₂	29.4	18	13	5	0.61	0.44	0.17
P ₃	15.6	32	15	17	2.05	0.96	1.09
S	60.6	6	6	0	0.09	0.09	0

Table 5. *ATPase activities in subfractions A, B and C from the large-granule fraction (P₂)*

In method I, fractions were those given in Fig. 1 and in Table 1, except that the P₂ fraction was sedimented at 10000g instead of 14500g. In method II, subfractions A, B and C were separated according to the method of Whittaker (1959); this involved centrifugation of the P₂ suspension (approx. 3.0 ml./g. of original tissue) at 100000g for 60 min. over the discontinuous density gradient consisting of 2 ml. of 0.8 M- and 2 ml. of 1.2 M-sucrose. ATPase was assayed as described in Table 3. For both methods, mean values from three preparations are given.

Fraction	Protein (mg./g. of cortex)	ATPase activity					
		(μ moles/g. of cortex/15 min.)			(μ moles/mg. of protein/15 min.)		
		Na ⁺ present (a)	Na ⁺ absent (b)	(a) - (b)	Na ⁺ present (a)	Na ⁺ absent (b)	(a) - (b)
Method I							
P ₂	39.1	115	62	53	2.9	1.6	1.4
A	7.5	42	22	20	5.6	2.9	2.7
B	14.5	68	36	32	4.7	2.5	2.2
C	12.5	28	22	6	2.2	1.8	0.5
Method II							
P ₂	36.5	105	55	50	2.9	1.5	1.4
A	6.9	30	10	20	4.4	1.5	2.9
B	14.4	65	31	34	4.5	2.2	2.4
C	10.0	21	16	5	2.1	1.6	0.5

found by Schwartz *et al.* (1962); Bonting *et al.* (1962) found only 10% of the activity in their mitochondrial fraction. These may reflect small differences in homogenization and fractionation procedures; intense homogenization, as described in the Materials and Methods section, however, did not essentially alter the distribution of ATPase activity given in Table 3.

In the P₁, P₂, P₃ and S fractions of the kidney cortex, homogenized and fractionated in a manner identical with that used for cerebral cortex, the percentage distribution of (NaK)-ATPase activity was found to be 37, 14, 49 and 0% respectively (Table 4). The result was in essential agreement with that of Wheeler & Whittam (1964) in showing a limited distribution of the activity in the P₂ (mitochondrial) fraction.

ATPase activities in subfractions A, B and C from the large-granule fraction (P₂). When the P₂ fraction, sedimented at 10000g (15 min.) and re-suspended in 0.32 M-sucrose, was fractionated over the Ficoll density gradient, or over the sucrose density gradient, more than half of the (NaK)-ATPase activity was found in subfraction B, which was densely populated with nerve endings (Table 5). Subfraction C, which consisted mainly of free mitochondria, exhibited a very limited (NaK)-ATPase activity, although the Na⁺-independent ATPase was relatively more active. Subfraction A contained approx. 35% of the recovered (NaK)-ATPase activity.

Centrifugation over the Ficoll density gradient of washings from the large-granule fraction (P₂). Since, for the reason mentioned above, the P₂

pellet was not washed during the isolation of subfractions A, B and C, it was thought that membranous and vesicular structures with similar sedimentation properties to those of the P_3 organelles would contaminate the P_2 fraction. The following experiments were carried out to determine the sedimentation of these contaminants in the Ficoll density gradient.

The P_2 pellet sedimented at 10000g for 15 min. was washed twice, each time with two-thirds of the original volume of 0.32M-sucrose, and the combined washings were centrifuged at 31100g for 40 min. The sediment was resuspended in 0.32M-sucrose (0.7–1.0 ml./g. of original tissue) and taken as the P'_3 fraction. A 1 ml. portion of the P'_3 fraction was layered on the discontinuous Ficoll density gradient, identical with that used for the isolation of subfractions A, B and C, and three equivalent tubes were centrifuged at 20900g for 15 min. in the Hitachi RPS40 swing-out head. Nearly all the tissue material was retained in the 0.32M-sucrose layer, the 3% (w/v) Ficoll layer remaining practically transparent. The 0.32M-sucrose layer (A'), 3% (w/v) Ficoll layer (B') and 13% (w/v) Ficoll layer (C') were separated with bent-tip Pasteur pipettes and with suction, and centrifuged at 105400g for 20 min. The B' layer produced only a small pellet, and no pellet was produced from the C' layer. Pellets A' and B' were dispersed in appropriate volumes of 0.32M-sucrose, and portions were taken for the determination of ATPase activity and protein. It was shown that practically all the ATPase activity as well as the protein of the P'_3 fraction was recovered in subfraction A' (Table 6). The activity of (NaK)-ATPase in subfraction A' accounts for almost all that in subfraction A.

ATPase activities in subfractions I, II and III from the water-treated large-granule fraction. It was shown that subfraction I (synaptic-vesicle fraction) contributed only one-tenth of the (NaK)-ATPase activity originally present in the supernatant of the water-treated P_2 fraction (W_2). Because of the small amount of protein present, however, the specific activity/mg. of protein in subfraction I was higher than in the other subfractions (Table 7). Most of the (NaK)-ATPase activity was recovered in subfraction II; in view of the electron-microscopic observations by Whitaker *et al.* (1964), this fraction was believed to consist mainly of synaptosome 'ghosts', together with damaged synaptosomes and membrane fragments.

Properties of the (NaK)-ATPase activity observed in the nerve-ending fraction (B). (a) pH-dependence. The liberation of orthophosphate from ATP by the nerve-ending preparation was maximal at about pH 7.5; no difference in the optimum pH was found between the nerve-ending preparation and the P_3 preparation (Table 8).

(b) Cation requirements. The dependence of synaptosomal (NaK)-ATPase activity on the presence of 3–6 mM- Mg^{2+} , together with 5–10 mM- K^+ , is shown in Table 9. The K^+ could be replaced by NH_4^+ , Rb^+ , Li^+ or Cs^+ , although the extent of stimulation was different among these univalent cations. However, Na^+ could not be replaced by any of the cations examined. The (NaK)-ATPase activity was completely inhibited by Ca^{2+} at 3–6 mM. Again, no qualitative differences were found with respect to the cation requirements of (NaK)-ATPase activity between the nerve-ending preparation and the P_3 preparation (Table 9).

Table 6. *ATPase activities in washings of the large-granule fraction (P_2)*

The P_2 pellet, sedimented at 10000g (15 min.), was washed twice with 0.32M-sucrose, and the combined washings were centrifuged at 31100g for 40 min. The sediment, resuspended in 0.32M-sucrose (0.7–1.0 ml./g. of original tissue), was taken as the P'_3 fraction. A 1 ml. portion of the P'_3 dispersion was layered on the top of the discontinuous density gradient of Ficoll, which was identical with that used for the isolation of subfractions A, B and C from the P_2 suspension (see Fig. 1), and three equivalent tubes were centrifuged at 20900g for 15 min. The 0.32M-sucrose (A'), 3% (w/v) Ficoll (B') and 13% (w/v) Ficoll layers were separated with bent-tip Pasteur pipettes, and centrifuged at 105400g for 20 min. Almost all the tissue material was recovered in the A' pellet; the B' layer produced a small pellet, whereas no pellet was produced from the C' layer. Dispersions of P'_3 , A' and B' were assayed for ATPase in the media given in Table 3. Mean values from two preparations are given.

Fraction	Protein (mg./g. of cortex)	ATPase activity					
		(μ moles/g. of cortex/15 min.)			(μ moles/mg. of protein/15 min.)		
		Na ⁺ present (a)	Na ⁺ absent (b)	(a)–(b)	Na ⁺ present (a)	Na ⁺ absent (b)	(a)–(b)
P'_3	3.7	25	10	15	6.8	2.7	4.1
A'	2.8	26	12	14	9.3	4.3	5.0
B'	0.4	0.7	0.7	0	1.8	1.8	0
C'	0	—	—	—	—	—	—

Table 7. *ATPase activities in subfractions I, II and III of the water-treated large-granule fraction (P₂)*

The P₂ pellet, sedimented at 10000g (15 min.), was washed once in 0.32M-sucrose, resuspended in water (approx. 2.5 ml./g. of original tissue), kept for 20 min. at 0–4° and centrifuged at 10000g for 15 min. A cloudy supernatant (W_s) was transferred (1 ml./tube) to tubes fitting the Hitachi RPS40 swing-out head and consisting of a continuous concentration gradient of sucrose ranging from 0.4M at the top to 0.6M at the bottom (3 ml./tube), which was layered over 1.2M-sucrose (Eichberg *et al.* 1964; with minor modifications). After centrifuging at 100000g for 60 min., a thin white band at the top of the continuous gradient (fraction I), a dense band, white but with a brownish tint underneath, near the boundary between the continuous concentration gradient and the layer of 1.2M-sucrose (fraction II), and a brown pellet at the bottom of the tube (fraction III), were produced. Each fraction was separated by bent-tip Pasteur pipettes and diluted with water, and pellets were obtained after centrifugation at 105400g for 40 min. Dispersions of subfractions W_s, I, II and III were assayed for ATPase in the media given in Table 3. Mean values from three preparations are given.

Fraction	Protein (mg./g. of cortex)	ATPase activity					
		(μmoles/g. of cortex/15 min.)			(μmoles/mg. of protein/15 min.)		
		Na ⁺ present	Na ⁺ absent	(a) – (b)	Na ⁺ present	Na ⁺ absent	(a) – (b)
		(a)	(b)		(a)	(b)	
W _s	21.6	123	60	63	5.7	2.8	2.9
I	2.0	20	12	8	10.0	6.0	4.0
II	10.6	84	48	36	7.9	4.5	3.4
III	6.8	42	24	18	6.2	3.5	2.7

Table 8. *Effect of pH on the ATPase activities of the nerve-ending fraction (B) and small-granule fraction (P₃)*

The nerve-ending fraction (B) was obtained from the unwashed P₂ fraction sedimented at 10000g (15 min.) by use of the Ficoll method (see Fig. 1 and Table 1). The small-granule fraction (P₃) was separated as described in Table 3. The tris buffer (60mm) was adjusted to the required pH with HCl by using a glass electrode. Other assay conditions were as given in Table 3. Mean values from two preparations are given, determined after keeping for 4 days at –20°.

pH	ATPase activity (μmoles/g. of cortex/15 min.)					
	B fraction			P ₃ fraction		
	Na ⁺ present	Na ⁺ absent	(a) – (b)	Na ⁺ present	Na ⁺ absent	(a) – (b)
	(a)	(b)		(a)	(b)	
6.5	70	30	40	112	40	72
7.0	94	37	57	152	59	93
7.5	110	40	70	160	56	104
8.0	106	42	64	158	65	93
8.5	94	37	57	130	67	63
9.0	64	30	34	95	48	47
9.5	60	25	35	90	38	52

(c) Inhibition studies. The portion of the ATPase activity that was stimulated by the presence of 100mm-sodium chloride was nearly completely inhibited by 100–500 μM-ouabain. The action of 10 μM-*p*-chloromercuribenzoate was less typical, the Na⁺-independent portion of the ATPase activity also being inhibited to some extent. Inhibition by 10 μM-*p*-chloromercuribenzoate was reversed by the further addition of L-cysteine (10mm) (Table 10). Deoxycholate (0.5%, w/v) or Triton X-100 (0.1%, v/v) inhibited both the oua-

bain-sensitive and the ouabain-insensitive portions of the ATPase activity (Table 10). The ATPase activities in both the nerve-ending fraction and small-granule fraction behaved similarly to these inhibitors.

(d) Specific activity. To compare the specific activity of the ATPase in nerve-ending particles and in the small-granule fraction, the following experiments were carried out. Pellets of the A, B and P₃ fractions were dispersed in aq. 1mm-glycol ether diaminetetra-acetic acid that had been

Table 9. *Effects of cations on the ATPase activities of the nerve-ending fraction (B) and of the small-granule fraction (P₃)*

The B and P₃ fractions were obtained as described in Table 8. The basic medium contained tris-HCl buffer, pH 7.4 (30 mM), and tris-ATP (3 mM). Other assay conditions were as given in Table 3. In the experiments indicated by asterisks (*) samples were kept frozen at -20° for 2 days before use. Other assays were carried out on the day of preparation of each fraction. Mean values from two or three preparations are given.

Additions (final concn.)	ATPase activity (μmoles/g. of cortex/45 min.)	
	B fraction	P ₃ fraction
None	10	12
MgCl ₂ (3 mM)	34	52
MgCl ₂ (6 mM)	35	50
KCl (5 mM)	10	10
NaCl (100 mM)	15	15
MgCl ₂ (3 mM), KCl (5 mM)	36	60
MgCl ₂ (6 mM), KCl (5 mM)	40	55
MgCl ₂ (6 mM), KCl (5 mM)*	44	56
MgCl ₂ (3 mM), NaCl (100 mM)	46	90
MgCl ₂ (6 mM), NaCl (100 mM)	42	87
MgCl ₂ (6 mM), KCl (5 mM), NaCl (100 mM)	71	130
MgCl ₂ (6 mM), KCl (5 mM), NaCl (100 mM)*	92	138
MgCl ₂ (6 mM), KCl (20 mM), NaCl (100 mM)	92	138
MgCl ₂ (6 mM), NaCl (100 mM), LiCl (40 mM)*	89	119
MgCl ₂ (6 mM), NaCl (100 mM), KCl (40 mM)*	93	118
MgCl ₂ (6 mM), NaCl (100 mM), RbCl (40 mM)*	90	117
MgCl ₂ (6 mM), NaCl (100 mM), CsCl (40 mM)*	75	102
MgCl ₂ (6 mM), NaCl (100 mM), NH ₄ Cl (40 mM)*	100	120
MgCl ₂ (6 mM), KCl (5 mM), LiCl (100 mM)*	49	61
MgCl ₂ (6 mM), KCl (5 mM), RbCl (100 mM)*	47	61
MgCl ₂ (6 mM), KCl (5 mM), CsCl (100 mM)*	39	48
MgCl ₂ (6 mM), KCl (5 mM), NH ₄ Cl (100 mM)*	51	62
MgCl ₂ (6 mM), KCl (5 mM), NaCl (100 mM), CaCl ₂ (1 mM)	60	105
MgCl ₂ (6 mM), KCl (5 mM), NaCl (100 mM), CaCl ₂ (3 mM)	40	63
MgCl ₂ (6 mM), KCl (5 mM), NaCl (100 mM), CaCl ₂ (6 mM)	35	55

neutralized with m-tris. These were kept at 0-4° for 30 min. and centrifuged at 105 400g for 20 min. Pellets were resuspended in appropriate volumes of water, and portions were assayed for ATPase activity and protein. Under these conditions, no

solubilization or inhibition of the ATPase activity was observed, but 52 and 27% of the protein were released from the B subfraction and from the P₃ fraction respectively (Table 11). This nearly doubled the specific activity of the (NaK)-ATPase (per mg. of protein) in the subfraction B, bringing it close to that in the P₃ fraction. The change in the specific activity was less marked in subfraction A.

DISCUSSION

Isolation of nerve-ending particles in a discontinuous Ficoll density gradient. The extent of separation of subfraction A (myelin fragments), subfraction B (nerve-ending particles) and subfraction C (free mitochondria) given in Table 1 was comparable with that obtained by the sucrose method (Whittaker, 1959; Johnson & Whittaker, 1963), with respect to the distribution of acetylcholine, lactate dehydrogenase and succinate dehydrogenase in those fractions. One of the advantages of the Ficoll method is its rapidity; thus synaptosomes can be isolated in about 1 hr. after the separation of the large-granule fraction from cerebral cortex. Another advantage is the lack of spontaneous release of particle-bound acetylcholine in dense Ficoll media, presumably because of the low osmotic pressure exerted by Ficoll. In the Ficoll method, the formation of 'black bodies' was less often encountered than in the sucrose method. The formation of 'black bodies' in the sucrose method is attributed partly to the hyperosmolarity of concentrated sucrose solution (Gray & Whittaker, 1962). In contrast, concentrated Ficoll solution does not cause a rise in osmotic pressure, and the less frequent formation of black bodies in the Ficoll method may be partly due to the lack of hyperosmotic effect of concentrated Ficoll solution. Swelling of mitochondria was also less frequently observed in samples prepared by the Ficoll method.

One of the drawbacks of the Ficoll method was that the separation of band A from the liquid phase of B was less clear-cut than in the sucrose method, but this did not make consistent separation of the two fractions difficult. Although it has been reported that Ficoll would exert a harmful action on biological preparations such as isolated cell nuclei, most of the toxicity could be removed by prolonged dialysis of the Ficoll solution against distilled water (Allfrey, Littau & Mirsky, 1964). In our experiments, involving the assay of choline acetyltransferase, lactate dehydrogenase, succinate dehydrogenase and ATPases, the purification of Ficoll did not prove essential. However, this does not rule out the possibility of a toxic action of undialysed Ficoll on other kinds of enzyme activities. The advantages of using Ficoll in the

Table 10. *Effects of ouabain, p-chloromercuribenzoate, deoxycholate and Triton X-100 on the ATPase activities of the nerve-ending fraction (B) and the small-granule fraction (P₃)*

Assay conditions were as given in Table 3. Fractions were those given in Table 8. Mean values from three preparations are given, determined after keeping for 2 days at -20° .

Addition (final concn.)	Nerve-ending fraction (B)				Small-granule fraction (P ₃)			
	Protein (mg./g. of cortex)	ATPase activity (μmoles/g. of cortex/15 min.)			Protein (mg./g. of cortex)	ATPase activity (μmoles/g. of cortex/15 min.)		
		Na ⁺ present	Na ⁺ absent	(a) - (b)		Na ⁺ present	Na ⁺ absent	(a) - (b)
		(a)	(b)			(a)	(b)	
None	14.8	102	44	58	15.5	128	56	72
Ouabain (1 μM)	14.2	92	48	44	15.0	110	60	50
Ouabain (10 μM)		84	45	39		95	55	40
Ouabain (100 μM)		60	42	18		70	58	12
Ouabain (500 μM)		50	43	7		60	56	4
p-Chloromercuribenzoate (10 μM)	13.8	64	34	30	15.2	83	40	43
p-Chloromercuribenzoate (10 μM) + L-cysteine (10 mM)		86	40	46		120	50	70
Deoxycholate (0.5%, w/v)	12.1	21	3	18	14.9	30	6	24
Triton X-100 (0.1%, v/v)	13.4	18	16	2	14.0	35	30	5

Table 11. *ATPase activities in the myelin (A), nerve-ending (B) and small-granule (P₃) fractions treated with glycol ether diaminetetra-acetic acid*

Subfractions A and B were separated from the P₂ fraction sedimented at 10000g (15 min.) by the Ficoll-density-gradient method (see Fig. 1 and Table 1). The P₃ fraction was obtained as described in Table 3. Pellets of A, B and P₃ were dispersed in aq. 1mM-glycol ether diaminetetra-acetic acid (GEDTA) solution that had been neutralized with m-tris. These were kept at $0-4^{\circ}$ for 30 min. and centrifuged at 105400g for 20 min. ATPase activities were assayed, as described in Table 3, in portions both of supernatants and of pellets, which had been resuspended in appropriate volumes of water. Mean values from three preparations are given.

Fraction	Protein (mg./g. of cortex)	ATPase activity					
		(μmoles/g. of cortex/15 min.)			(μmoles/mg. of protein/15 min.)		
		Na ⁺ present	Na ⁺ absent	(a) - (b)	Na ⁺ present	Na ⁺ absent	(a) - (b)
		(a)	(b)		(a)	(b)	
No treatment:							
A	8.1	45	20	25	5.6	2.5	3.1
B	14.4	81	30	51	5.6	2.1	3.6
P ₃	13.7	111	47	64	8.1	3.4	4.7
With 1mM- GEDTA: (pellet)							
A	5.6	43	20	23	7.7	3.6	4.1
B	6.9	77	30	47	11.2	4.4	6.8
P ₃	10.0	115	51	64	11.5	5.1	6.4
(supernatant)							
A	2.0	< 1	< 1	—			
B	7.7	3	2	1			
P ₃	3.5	2	3	-1			

isolation of brain mitochondria have been discussed by Stahl, Smith, Napolitano & Basford (1963).

Presence of (NaK)-ATPase activity in isolated nerve endings. In contrast with the equivalent

fractions from kidney cortex (Wheeler & Whittam, 1964; Table 4) or from liver (Bonting *et al.* 1962), the P₂ fraction from the cerebral cortex invariably contained (NaK)-ATPase activity to an extent that

could not be explained as being due to contamination with other fractions (Deul & McIlwain, 1961; Schwartz *et al.* 1962; Table 3). On subfractionation of the P₂ fraction, more than half of the (NaK)-ATPase activity was recovered in subfraction B (Table 5), which was highly populated with nerve-ending particles (Gray & Whittaker, 1962; Plates 2a and 2b). Subfraction C contained only one-tenth of the recovered activity, which may be taken as being due to contamination of this fraction with synaptosomes. Thus free mitochondria did not seem to contain any appreciable (NaK)-ATPase activity, a finding in accord with observations obtained with brain mitochondria purified in another way (Jöbsis, 1963).

Subfraction A contributed a considerable proportion of the (NaK)-ATPase activity observed in the P₂ fraction, but this is largely attributable to the organelles that should have been sedimented in the small-granule fraction, and that inevitably contaminated the unwashed large-granule fraction (Table 6). However, the lack of suitable microsomal marker available for brain tissue made estimation of the extent of microsomal contamination difficult. A part of the (NaK)-ATPase activity in subfraction A would also be explained by the presence of small synaptosomes, which were occasionally observed in this subfraction on electron microscopy (Plates 1a and 1b). Although, on electron microscopy, myelin-sheath fragments were the predominant feature of subfraction A, a significant contribution of these structures to the (NaK)-ATPase activity exhibited by subfraction A seems unlikely, in view of the low activity of this enzyme system reported in experiments with isolated myelin (Adams, Davison & Gregson, 1963).

That the (NaK)-ATPase activity demonstrated in subfraction B is in fact to be explained by the presence of the activity in synaptosomes, and is not due to contamination by the organelles contained in the P₃ fraction, is substantiated by the experiments presented in Table 6. Equalization of the specific activity of (NaK)-ATPase/mg. of protein in subfraction B treated with glycol ether diamine-tetra-acetic acid to that in the P₃ fraction similarly treated (Table 11) gives indirect support to the conclusion of the presence of this enzyme activity in synaptosomes. However, the possibility that the (NaK)-ATPase activity observed in subfraction B is, in part, attributable to the membranous structures in this fraction cannot be ruled out, although the population of these structures was much less than that of the synaptosomes on electron microscopy.

Within the limits of the procedure employed, it was not conclusive whether the (NaK)-ATPase activity in nerve endings was located on the surface of the synaptosomal limiting membrane or on

synaptic vesicles. The results given in Table 7 seem to favour the localization of the activity on the synaptosomal limiting membrane rather than on the synaptic vesicles; a slight activity in subfraction I, believed to consist of synaptic vesicles, would indicate contamination from the P₃ fraction. However, the lack of evidence for a quantitative separation of synaptic vesicles from synaptosome 'ghosts' in the procedure employed leaves considerable room for uncertainty about the exclusive localization of (NaK)-ATPase activity on the synaptosomal limiting membrane.

The results given in Table 5 are at variance with those described by Tanaka & Abood (1964), who found a decrease, not an increase, in ATPase activity in their nerve-ending fraction on the addition of 150mM-sodium chloride to the incubation media, which contained magnesium chloride (3mM) together with potassium chloride (15 or 150mM). No reasonable explanation for this discrepancy is offered; the absence of data on the distribution of acetylcholine, choline acetyltransferase and lactate dehydrogenase in the paper of Tanaka & Abood (1964) makes comparison between their nerve-ending fraction and ours meaningless.

The demonstration of (NaK)-ATPase activity in the synaptosome fraction raises the possibility that a part at least of the activity that has been reported in the cerebral microsomal fraction is attributable to the smaller synaptosomes, which are invariably found in this fraction on electron microscopy (Laverty, Michaelson, Sharman & Whittaker, 1963; Plate 4). Whether there is any difference in the activity of (NaK)-ATPase, e.g. per unit area of membrane surface, among various types of synaptosomes is, however, open to question.

Comparison of the (NaK)-ATPase activities of the nerve-ending fraction and the small-granule fraction. It is not known whether the (NaK)-ATPase activities in nerve-ending particles and in the P₃ fraction represent a common sequence of enzymic reactions or not. The synaptosomal enzyme system behaved qualitatively in its relations to cations and to inhibitors, such as ouabain or *p*-chloromercuribenzoate, like the (NaK)-ATPase activity previously reported in peripheral nerves (Skou, 1957, 1960), in brain microsomes (Deul & McIlwain, 1961; Aldridge, 1962; Schwartz *et al.* 1962; Skou, 1962, 1963), in the electric organ (Glynn, 1963), in kidney cortex (Wheeler & Whittam, 1962; Skou, 1962), in erythrocytes (Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961; Whittam, 1962) and in the parotid gland (Schwartz, Laseter & Kraititz, 1963). It has been reported by Deul & McIlwain (1961) that the optimum pH for the (NaK)-ATPase activity of brain mitochondrial fraction is 9, in contrast with that of the brain microsomal fraction, which is 7.5. The alkaline

optimum pH was not substantiated for the nerve-ending fraction separated from the crude mitochondrial fraction (Table 8).

Possible significance of synaptosomal (NaK)-ATPase activity. Because of a marked heterogeneity of the cerebral tissues, there are particular difficulties in locating the (NaK)-ATPase activity at a cellular level on the basis of subfractionation studies. Nevertheless, studies with isolated nerve cells lend support to the idea that the (NaK)-ATPase activity is located at least on the neuronal and glial cell membranes (Cummins & Hyden, 1962). The extraordinarily high total activity of (NaK)-ATPase in brain, particularly in grey matter (Bonting *et al.* 1961), will be related to the presence of numerous synapses in this organ. It has been reported that the mean number of nerve endings of the postsynaptic neuron surface is $16.27/100\mu^2$, and the number of nerve endings/cell was estimated to be 3200 in a single mesencephalic neuron of the cat (David, 1957). It has also been estimated that 50–70% of the surface area of the cell and dendrites is devoted to synaptic contacts in the cat spinal cord (Illis, 1964). The distribution of (NaK)-ATPase activity in synaptosomes indicates the presence of the mechanism for the active transport of Na^+ across the synaptic membrane, which involves this enzyme activity.

After our present studies had been rounded off, a relevant article came to our attention. Hosie (1964) has demonstrated that synaptic vesicles, isolated according to the method of Whittaker *et al.* (1964), contained only Mg^{2+} -stimulated ATPase, whereas the fraction consisting mainly of large oval membranes, believed to be the external synaptosome membranes, showed a high specific activity of (NaK)-ATPase. Inhibition of this enzyme activity by 0.1 mM-ouabain, 10 μM -*p*-chloromercuribenzoate and 3 mM-calcium chloride was observed. These results seem to be in accord with ours, but a detailed comparison remains to be made.

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