The Cerebral Sodium-plus-Potassium Ion-stimulated Adenosine Triphosphatase of Bovine Brain and its Microsomal Matrix

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1. Adenosine triphosphatase activities of dispersions prepared from bovine cerebral cortex that had been frozen, were greater than those of dispersions prepared from fresh tissue. The subcellular distribution of components of the dispersion was not altered by freezing the tissue and a microsomal fraction enriched in $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase activity was prepared. 2. The bovine cerebral microsomes were further treated with a 2M-sodium iodide reagent to obtain a particulate preparation with minimal $\text{Na}^+ + \text{K}^+$ -independent adenosine triphosphatase activity. $\text{Na}^+ + \text{K}^+$ -stimulated activity was increased by the sodium iodide treatment and this preparation was shown to be enriched in lipid constituents. 3. Density-gradient centrifugation of the sodium iodide treated preparation gave three main subfractions each containing approximately equal amounts of phospholipid and protein. Further exposure of the sodium iodide-treated preparation to the 2M-sodium iodide reagent altered the distribution of protein and phospholipid among the fractions obtained by density-gradient centrifugation. Dissociation of phospholipids from protein in the sodium iodide-treated preparation was brought about also by high concentrations of arginine. Concentrated solutions of arginine and sodium thiocyanate brought about dissociation of phospholipids from protein of the microsomal preparation. 4. Many amino acids were found to inhibit $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase activity when present in high concentrations. The inhibition was complex but resulted, in part at least, from diminished affinity for ATP and Na^+ in the presence of the amino acids. 5. A non-ionic detergent, Lubrol W, solubilized up to 40% of the enzyme activity of the sodium iodide-treated preparation together with 30% of the protein and phospholipid in the preparation. Protein was released from the sodium iodide-treated preparation by pancreatic elastase but $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase activity of the residue was diminished. Ultrasonic treatment of the sodium iodide-treated preparation failed to release a significant proportion of $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase activity into a form not deposited by ultracentrifugation.

Microsomal fractions showing Na,K-ATPase* activity are insoluble associations of many molecular species and display several enzyme activities. They derive in part from the cell membrane across which Na^+ and K^+ movements take place and resolution of microsomal components has now been examined in seeking knowledge of the Na,K-ATPase which participates in active cation transport.

Previous investigations with surface-active
* Abbreviation: Na,K-ATPase, $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase.

agents (Swanson, Bradford & McIlwain, 1964), urea and ultrasonic irradiation (Cooper & McIlwain, 1967) have emphasized the importance of structural factors for Na,K-ATPase activity but failed to make any distinct separation of constituents. An enrichment of Na,K-ATPase activity from rabbit cerebral microsomes was achieved by using a sodium iodide reagent that yielded a particulate preparation with minimal Na,K-independent adenosine triphosphatase activity (Nakao, Tashima, Nagano & Nakao, 1965). This procedure is here applied to ox cerebral microsomes and the sodium iodide-treated

material is examined to determine the effects of the reagent on microsomal composition. Other potential dissociating agents have been examined, including sodium thiocyanate and amino acids (Wyman, 1936; Porath & Ui, 1964).

EXPERIMENTAL

Adenosine triphosphatase preparations. Shortly after the animal's death by bleeding, ox brains were collected and the brain stem, membranes and blood vessels were removed. Each cerebral hemisphere was sealed in a polythene bag and packed in ice for transport to the laboratory where they were frozen at -15°C and stored for up to three months. When required, 30–35 g of grey matter was obtained from one brain by bringing the hemispheres into room temperature and scraping with a spatula as the surface thawed. The tissue was rapidly transferred to ice-cold 0.32M-sucrose, pH 7.4 (adjusted with KOH), and when collection was completed more sucrose solution was added to give a 10% (w/v) tissue suspension.

The tissue dispersion and centrifugation sequence was that used by Schwartz, Bachelard & McIlwain (1962) for guinea-pig cerebral tissue but, because of the greater volumes involved, a combined nuclear and mitochondrial pellet was deposited by centrifuging at 10400g for 15 min in a Sorvall RC-2B centrifuge. Microsomes were deposited from the post-mitochondrial supernatants by centrifuging at 22600g for 1 h in a Spinco no. 21 rotor and washed once with the sucrose solution before resuspending in water.

Microsomes were further treated with a 2M-NaI reagent as described by Nakao *et al.* (1965) on a scale utilizing 300–500 mg of microsomal protein but further washings of the microsomes with 0.32M-sucrose–5 mM-EDTA before exposure to the NaI reagent were omitted.

Exposure to added agents and centrifugation. The adenosine triphosphatase preparations were generally at a protein concentration of 2–3 mg/ml in a final volume of 2 or 2.5 ml which included an added agent at the appropriate concentration and pH. After mixing, 30 min at 0°C elapsed before the mixture (2 ml) was centrifuged in the Spinco no. 50 rotor at 150000g for 1 h at 4°C . Supernatants were collected by Pasteur pipette after gently resuspending with a fine glass rod any floating material adhering to the top of the tube. Deposits were then resuspended in water and homogenized. Both supernatants and deposits were dialysed in the cold for 3–5 h against 4×1 litre changes of 5 mM-tris-hydrochloric acid buffer, pH 7.5. Contents of the dialysis bag were washed out with water, homogenized if necessary and diluted to 5 ml for analysis.

For density-gradient centrifugation a Spinco SW 39 rotor was used. The gradients were 1 ml each of cold 1.4-, 1.2-, 1.0- and 0.8M-sucrose adjusted to pH 7.4 with tris. Centrifugation was for 2 h at 50000g as described by De Robertis, De Iraldi, De Lores Arnaiz & Salganicoff (1962) for the resolution of a cerebral mitochondrial fraction. Fractions were obtained by aspiration through a platinum tube. All centrifugal forces are expressed as average g values calculated to the centres of the tubes.

Enzyme assays. Adenosine triphosphatase activity was assayed and P_i was determined as described by Swanson *et al.* (1964), except that the enzyme reaction was initiated by adding tris-ATP and was terminated by adding 2 ml

of 6% (w/v) trichloroacetic acid. Concentrations of the amino acids used, with the exception of γ -aminobutyric acid, interfered with the phosphate determination so that appropriate concentrations of the amino acids were included in the phosphate standard solutions.

Acetylcholinesterase activity was determined spectrophotometrically at 30°C with acetylthiocholine as substrate (Ellman, Courtney, Andres & Featherstone, 1961) at a concentration of 1.64 mM which is optimum for calf brain acetylthiocholinesterase in this procedure (Jackson & Aprison, 1966a). Succinate dehydrogenase was measured by using a tetrazolium salt as electron acceptor by the method of Pennington (1961) as modified by Porteous & Clark (1965).

Other determinations. Lipid extracts were prepared by shaking suspensions of the sample with 19 vol. of chloroform-methanol (2:1, v/v) and were filtered or centrifuged before partitioning against 0.1M-KCl and upper phase solvent (Folch, Lees & Sloane-Stanley, 1957). Combined upper phases containing gangliosides were dialysed against water and evaporated to dryness in a stream of air at 80°C and these residues were hydrolysed for 2 h in 0.1M- H_2SO_4 at 80°C . The lipid-extracted protein residues were treated similarly but for 1 h. Liberated *N*-acetylneuraminic acid was determined in these hydrolysates by the procedure described by Warren (1959). Lower-phase lipid extracts were evaporated to dryness and analysed for cholesterol (Schwartz *et al.* 1962) or phospholipid by digesting with 12M- HClO_4 before P_i determination.

RNA was measured spectrophotometrically by the method of Scott, Fraccastro & Taft (1956) as applied to subcellular fractions by Littlefield & Keller (1956) by using an extinction coefficient for RNA of $34.2 \text{ mg}^{-1} \text{ cm}^{-1}$. Protein was determined by the Miller (1959) modification of the Lowry determination (Lowry, Rosebrough, Farr & Randall, 1951) with crystalline bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard.

Reagents. Tris-ATP was prepared from the sodium salt of ATP by passing concentrated solutions through a column (1 cm $\times$ 7 cm) of Amberlite resin IR-120 (H^+ form) and neutralizing with tris. Pancreatic elastase was a twice recrystallized preparation with a solubilizing activity of 45–60 mg of elastin/h per mg of protein at pH 8.8 and 37°C and this, ATP (sodium salt), L-arginine and tris base were from the Sigma (London) Chemical Co., London S.W.6, U.K. Lubrol W was from Imperial Chemical Industries Ltd., Wilmslow, Cheshire, U.K. Other chemicals were of analytical grade, or the highest grade available and were from British Drug Houses Ltd., Poole, Dorset, U.K.

RESULTS

Microsomes from frozen ox cerebral cortex. Constituents of the microsomal fraction obtained from frozen ox cerebral cortex by procedures established for fresh guinea-pig and ox brain (Schwartz *et al.* 1962; Bradford, Brownlow & Gammack, 1966) are shown in Table 1. The average amount of protein obtained per g of tissue weight in the dispersion of frozen ox cerebral cortex was 5–25% greater than that obtained from fresh ox or guinea-pig cerebral

Table 1. *Constituents of the enzyme preparations*

Grey matter from frozen ox cerebral cortex was homogenized and centrifuged so as to obtain the microsomal fraction sedimenting between 10 400g for 15 min and 22 600g for 60 min. The microsome preparations were then treated with the sodium iodide reagent of Nakao *et al.* (1965) by using the procedure described in the Experimental section. Values quoted are means $\pm$ s.d. of eight preparations except where indicated in parentheses. Assays and analyses were performed after not more than 3 days' storage at 4°C.

Constituent	Measured in:		
	Dispersion	Microsomes	NaI-treated preparation
Protein (mg/g of tissue)	109.2 $\pm$ 7.9	13.3 $\pm$ 3.0	3.1 $\pm$ 0.9
Adenosine triphosphatase, with Na ⁺ :			
(μ mol/h per g of tissue)	3156 $\pm$ 134	562 $\pm$ 18	289 $\pm$ 104
(μ mol/h per mg of protein)	28.9 $\pm$ 1.7	42.3 $\pm$ 6.1	90.7 $\pm$ 16.4
Adenosine triphosphatase, without Na ⁺ :			
(μ mol/h per g of tissue)	1190 $\pm$ 190	150 $\pm$ 7	6 $\pm$ 4
(μ mol/h per mg of protein)	10.9 $\pm$ 2.4	11.3 $\pm$ 2.5	1.9 $\pm$ 0.9
Acetylcholinesterase (μ mol/h per g of tissue)	213 $\pm$ 24 (3)	47 $\pm$ 1.7 (3)	3.6 $\pm$ 1.8 (3)
Succinate-tetrazolium reductase (μ mol/h per g of tissue)	81.8 $\pm$ 11.9 (4)	1.6 $\pm$ 0.7 (4)	0
RNA (μ g/mg of protein)	11.9 $\pm$ 3.2 (3)	11.1 $\pm$ 2.2 (4)	6.7 $\pm$ 5.3 (4)
Lipid phosphorus (nmol/mg of protein)	529 $\pm$ 27 (5)*	704 $\pm$ 81 (5)	1062 $\pm$ 35
Cholesterol (nmol/mg of protein)	382 $\pm$ 21 (5)*	414 $\pm$ 54 (3)	767 $\pm$ 84 (3)
N-acetylneuraminic acid:			
Lipid (nmol/mg of protein)	—	10.1 $\pm$ 1.8 (3)	41.5 $\pm$ 17.1 (3)
Protein (nmol/mg of protein)	—	12.3 $\pm$ 5.6 (3)	20.1 $\pm$ 12.6 (3)

* Values calculated from the data of Bradford *et al.* (1964) for dispersions of fresh guinea-pig cerebral cortex.

cortex, which yielded 85–103 mg/g of tissue (Schwartz *et al.* 1962; Bradford, Swanson & Gammack, 1964; Kurokawa, Sakamoto & Kato, 1965; Bradford *et al.* 1966). Concentrations of other constituents from the frozen ox cortex were proportionately increased in the dispersion except for Na,K-ATPase activity which was increased to a greater extent, indicating an activation of this enzyme.

Specific activities of the adenosine triphosphatases of fresh ox cerebral dispersion were 18 μ mol of P_i/h per mg of protein in the presence of 100 mM-sodium chloride and 8 μ mol of P_i/h per mg in the absence of added sodium chloride (Bradford *et al.* 1966). The higher specific activity now found for the Na,K-ATPase in the dispersion of frozen ox cerebral cortex extended to the microsomal fraction with average adenosine triphosphatase activities of 42.3 and 11.3 μ mol of P_i/h per mg of protein in the presence and absence of 100 mM-sodium chloride (Table 1). In contrast, similar fractions prepared from fresh ox cerebral cortex had specific activities of 14–25 and 4–10 μ mol of P_i/h per mg of protein with and without 100 mM-sodium chloride (Gibbs, Roddy & Titus, 1965; Bradford *et al.* 1966; Stahl, Sattin & McIlwain, 1966; Cooper & McIlwain, 1967).

Cerebral dispersions prepared from frozen ox brain

by Schoner, von Ilberg, Kramer & Seubert (1967) contained 84.5 mg of protein/g of tissue. It is likely that the increased protein contents of dispersions obtained here arise from procedural differences in storage of the frozen brains and separation of grey from white matter. The increased adenosine triphosphatase activities are likely to result from the freezing process since a comparative study showed increased specific activity in dispersions obtained from frozen rat brain when compared with the fresh material (Samson & Quinn, 1967). Guinea-pig cerebral microsomal adenosine triphosphatase preparations have previously been shown to increase in activity on freezing (Schwartz *et al.* 1962).

Treatment of microsomes with a sodium iodide reagent. The procedure applied to rabbit cerebral microsomes by Nakao *et al.* (1965) included washing the microsomes with 0.32 M-sucrose containing 5 mM-EDTA before exposure to the 2 M-sodium iodide reagent. Results obtained by applying the same procedure to freshly prepared microsomes from frozen ox cortical tissue are shown in Table 2 (Expt. 1A). Recoveries of protein and Na,K-ATPase activity in the particulate material were such that the specific activity of Na,K-ATPase was increased by an average factor of 2.7. If aged or frozen microsomes were used the procedure was less effective since the sodium iodide-treated

Table 2. *Microsomal protein and adenosine triphosphatase after treatment with a sodium iodide reagent*

Microsome preparations (300–500 mg of protein) were treated with the NaI reagent of Nakao *et al.* (1965) by using the procedure described in the Experimental section except that for fraction (A) of Expt. 1 the microsomes were washed three times with 0.32 M-sucrose–5 mM-EDTA, pH 7.4 (adjusted with tris) before exposure to the NaI reagent. The microsome preparations for (A) and (B) have been grouped together for expression of the results, which are mean values $\pm$ s.d. of the number of preparations shown in parentheses. In Expt. 2 all supernatants were combined and dialysed before assay against 5 mM-tris-HCl, pH 7.4, until I⁻ was no longer detectable in the diffusate on addition of a solution of AgNO₃. The mean values of two preparations are given.

Expt.	Fraction	Adenosine triphosphatase (μ mol/h per mg of protein)		Recovery of constituents (% of initial values)		
		With		Protein	Na,K-ATPase (a-b)	Na ⁺ -independent ATPase (b)
		100 mM-NaCl (a)	Without Na ⁺ (b)			
1.	Microsomes (16)	41.0 $\pm$ 9.3	11.6 $\pm$ 2.3	(100)	(100)	(100)
	(A) NaI-treated deposit(8)	85.6 $\pm$ 14.1	4.5 $\pm$ 1.7	19.0 $\pm$ 4.5	57.6 $\pm$ 11.5	6.8 $\pm$ 2.9
	(B) NaI-treated deposit(8)	90.7 $\pm$ 16.4	1.9 $\pm$ 0.9	30.4 $\pm$ 5.9	84.4 $\pm$ 10.3	5.4 $\pm$ 2.0
2.	Microsomes	40.8	9.3	(100)	(100)	(100)
	NaI-treated deposit	90.5	1.6	30	84	5
	Supernatant and washings	39.9	10.3	65	60	71

material retains a greater proportion of protein and Na⁺-independent adenosine triphosphatase activity with less Na,K-ATPase activity.

When the intermediate washings with sucrose-EDTA were omitted, recoveries of protein and Na,K-ATPase activity were increased, without altering the removal of Na⁺-independent adenosine triphosphatase activity or the degree of Na,K-ATPase enrichment (Table 2, Expt. 1B). This modified procedure was adopted for subsequent preparations because it gave a greater yield of enriched Na,K-ATPase. Comparable results have been noted in other systems in which the intermediate washings were not omitted (Uesugi, Kahlenberg, Medzihradsky & Hokin, 1969; Fujita *et al.* 1966; Fujita *et al.* 1967).

Two of the sodium iodide-treated preparations included in Table 2 (Expt. 1B) contained more Na,K-ATPase activity than was present in the parent microsomes. Protein recovery in each case was 37%, with 124% and 114% of the original total Na,K-ATPase activity recovered. Still larger apparent recoveries of Na,K-ATPase, up to 300% of the original activity, were found in sodium iodide-treated microsomes of fresh ox or rat cortex that had been washed with MgATP²⁻ solutions (Stahl *et al.* 1966; Stahl, 1968).

Collection of the supernatant and washings obtained during the sodium iodide procedure and analysis of all fractions for protein and adenosine triphosphatase clearly showed activation of the Na,K-ATPase (Table 2). The sum of Na,K-ATPase activity recovered after treatment of the microsomes was 44% greater than the activity originally present whereas the Na⁺-independent adenosine

triphosphatase had lost activity. After sodium iodide treatment 10% of microsomal acetylcholinesterase activity remained in the deposit but only 25% was recovered in the supernatant and washings, showing partial inactivation of this enzyme. None of the succinate-tetrazolium reductase activity present in the microsomes was recovered in the fractions after sodium iodide treatment.

Cholesterol and phospholipids are major constituents of the microsomal fraction and their presence in the sodium iodide-treated material has been examined (Table 1). The sodium iodide procedure did not remove these lipids to the same extent as it removed protein, so that the treated material became enriched in lipids almost to the same extent as in Na,K-ATPase activity. Assuming the lipid phosphorus to be in compounds of average molecular weight 775, then the cholesterol plus phospholipid contributed 1.1 mg/mg of protein in the sodium iodide-treated preparation, in contrast with 0.7 mg/mg of microsomal protein. Enrichment of the Na,K-ATPase was less when related to the combined lipid and protein weights than when expressed per unit of protein.

Gangliosides and protein-bound *N*-acetylneuraminic acid are associated with membrane structures, particularly the external synaptosome membrane, as shown by their enrichment in morphologically characterized subfractions of rat brain (Brunngraber, Dekirmenjian & Brown, 1967; Lapetina, Soto & De Robertis, 1968). These compounds were also concentrated in the material remaining after sodium iodide treatment of the microsomes (Table 1).

Phospholipid-protein dissociation by sodium

Table 3. *Phospholipid and protein in subfractions of the sodium iodide-treated preparation*

The enzyme preparation was divided into two equal portions and these were suspended in the 2 M-NaI reagent or in sucrose of equal density. Samples (3×1 ml) of each suspension were covered with a sucrose density gradient and centrifuged for 2 h at 50000g in a Spinco SW 39 rotor. Centrifugation from sucrose gave three bands at distances of 6.5, 7.3 and 8.1 cm from the rotor centre whereas only two bands, at distances of 6.6 and 7.0 cm, were found after centrifuging the enzyme preparation from the NaI reagent. Equal fractions were taken from all tubes and samples were precipitated and washed free of sucrose with 6% (w/v) trichloroacetic acid before analysis for protein (after redissolving in M-NaOH) and phospholipid. The percentages of the total of each constituent recovered in the fractions are shown in parentheses.

Fraction (cm from rotor centre)	Centrifuged from:			
	Sucrose		NaI reagent	
	Protein (mg)	Lipid phosphorus (μ mol)	Protein (mg)	Lipid phosphorus (μ mol)
4.7-6.8	3.1 (24)	4.9 (32)	6.5 (50)	10.0 (66)
6.8-7.8	5.0 (38)	4.6 (30)	2.3 (18)	2.6 (17)
7.8-9.8	4.7 (36)	5.6 (37)	4.3 (33)	2.6 (17)

Fig. 1. Percentages of constituents of the NaI-treated preparation deposited from a reagent containing various concentrations of NaI, 25 mM-cysteine, 2.5 mM-MgCl₂, 2.5 mM-EDTA and 1.5 mM-ATP at pH 8 (adjusted with tris). NaI-treated material containing 5 mg of protein, 5.78 μ mol of lipid phosphorus with an Na,K-ATPase activity of 330 μ mol of P_i/h was suspended in 2 ml of the reagent and centrifuged at 105000g for 1 h. Deposits were dialysed before analysis for protein ($\square$), lipid phosphorus ($\blacksquare$) and Na,K-ATPase activity ($\circ$). Supernatants contained no Na,K-ATPase activity.

showed the sodium iodide-treated preparation to comprise three main fractions of differing densities, each of which had approximately the same proportions of phospholipid and protein (Table 3). Exposure to the 2 M-sodium iodide reagent immediately before separation altered the distribution. Phospholipid and protein were displaced to less dense particles, suggesting their dissociation by the 2 M-sodium iodide reagent.

Samples of the sodium iodide-treated preparation were also resuspended in reagents of different sodium iodide concentrations and centrifuged (Fig. 1). The phospholipid per unit of protein in the deposited material ranged between 36 and 75% of that in the original material showing that sodium iodide concentrations lower than 2 M can also cause dissociation. Centrifugation of a suspension of the same preparation in sucrose of density equal to that of the 1.2 M-sodium iodide reagent gave no deposit, but a floating pellicle that had the same phospholipid and protein composition as the original material. The amount of protein deposited decreased with increasing sodium iodide concentration partly because of the increasing solution density (1.162 g/ml at 20°C for the 1.4 M-sodium iodide reagent) but Na,K-ATPase activity in the deposit decreased more rapidly. No significant concentrations of Na,K-ATPase activity were found in the supernatants so that the enzyme was being inactivated. The inactivation was concluded to result from the centrifugal separation since exposure of the preparation to 2 M-sodium iodide and dialysis, as in Fig. 1, did not result in loss but in activation of Na,K-ATPase activity. Simple mixing of the supernatants and deposits of Fig. 1 before assay did not enhance Na,K-ATPase activity so that the inactivation was not easily reversible.

iodide. The stability of phospholipid-protein associations in the sodium iodide-treated preparation was examined as shown in Table 3. The preparation was resuspended in further 2 M-sodium iodide reagent or in a sucrose solution of equal density. Both suspensions were overlaid with a discontinuous sucrose density gradient and centrifuged. Centrifugation of the sucrose suspension

Fig. 2. Microsomal constituents floating from suspension in solutions of 0.4–1.2M-sucrose (●), 0.5–2M-arginine (○), or 1–4M-sodium thiocyanate (□), are shown plotted against the density of the solutions, which were pH 8 (adjusted with tris or HCl). Microsomes, equivalent to about 4 mg of protein, 0.61 μmol of lipid phosphorus/mg of protein and Na,K-ATPase activity of 22.3 $\mu\text{mol of } P_i/\text{h per mg of protein}$, were suspended in 2 ml of the solutions and centrifuged at 150000g for 1 h. Supernatant material was collected, homogenized and dialysed before analysis. (a) Sp. activity of Na,K-ATPase; (b) phospholipid content; (c) protein content.

Dissociation by sodium thiocyanate and arginine. Centrifugation of microsomes suspended in solutions of sodium thiocyanate, arginine hydrochloride or sucrose, at 150000g for 1 h, gave deposits and well-packed floating layers with intervening clear solutions. Less protein was deposited from the arginine and sodium thiocyanate solutions than from sucrose when the density of the solutions was less than 1.155 g/ml (Fig. 2). Material floating from the sucrose suspensions had a phospholipid/protein ratio very similar to that of the original microsomes, whereas this ratio was increased in supernatants from arginine solutions and decreased in supernatants from sodium thiocyanate solutions. Microsomal Na,K-ATPase activity was completely lost after exposure to sodium thiocyanate and although activity was retained after centrifugation from the arginine solutions there was no enrichment of Na,K-ATPase activity in either supernatant or deposited material.

The sodium iodide-treated Na,K-ATPase preparation was also centrifuged from arginine hydro-

Fig. 3. Constituents of the NaI-treated preparation deposited from suspension in 2 ml of arginine (●) or of sucrose (○) solutions, by centrifuging at 150000g for 1 h. Concentrations of the arginine solutions (adjusted to pH 8 with HCl) are shown on the abscissa and corresponding sucrose solutions (pH 8 with tris) were of equal density. The NaI-treated material contained 3 mg of protein, 1.096 μmol of lipid phosphorus and Na,K-ATPase activity of 98.4 $\mu\text{mol of } P_i/\text{h per mg of protein}$. Deposits were homogenized and dialysed before analysis. (a) Sp. activity of Na,K-ATPase; (b) phospholipid concentration; (c) protein content.

chloride solutions and from sucrose solutions of equal density. Analyses of deposited material showed (Fig. 3) little difference in the proportion of protein deposited from either sucrose or arginine solutions but the phospholipid/protein ratio of material deposited from arginine solution was decreased. The specific activity of Na,K-ATPase was decreased in the material deposited from suspensions containing arginine but was not increased in supernatant material.

Fig. 4. The Na,K-ATPase activity of the NaI-treated preparation was determined in the presence of, or after exposure to, arginine solutions (pH 7.4, adjusted with HCl) of the indicated concentration. O, Enzyme equivalent to 2mg of protein was exposed to arginine in a volume of 1ml for 30min at 0°C before 25 μ l was taken for assay. ●, Enzyme equivalent to 47 μ g of protein was assayed with arginine included in the assay media.

Fig. 5. Inhibition of Na,K-ATPase activity of the NaI-treated material when amino acids (pH 7.4 with tris or HCl) of the molarity shown were included in the assay media. The amino acids were; arginine (●), γ-aminobutyric acid (○), glycine (□), β-alanine (■) and 6-amino-n-hexanoic acid (Δ).

Amino acids and Na,K-ATPase activity of the sodium iodide-treated preparation. Arginine inhibited Na,K-ATPase activity and the inhibition was

readily reversed when the arginine concentration was decreased by dilution (Fig. 4). Other amino acids also inhibited Na,K-ATPase activity, arginine being the most effective when judged by molar concentrations of the amino acids (Fig. 5). The increase in bulk dielectric constant of the assay media, brought about by the amino acids (Wyman, 1936), was appraised as a contributing factor. Similar inhibitions of Na,K-ATPase activity were produced by either arginine or γ-aminobutyric acid solutions of equal dielectric constant. With the exception of 6-amino-n-hexanoic acid, the molar concentrations of each amino acid required to inhibit the Na,K-ATPase by 50% decreased in the order in which their dielectric increments, obtained from data of Edsall & Wyman (1958), increased. There was not a linear relationship between the logarithm of Na,K-ATPase activity and the reciprocal of the dielectric constant as there is for ester hydrolysis by trypsin and α-chymotrypsin in solutions containing glycine (Castaneda-Agulla & Del Castillo, 1962).

The concentration of ATP necessary for half maximum activity was increased by arginine and γ-aminobutyric acid, which also decreased Na⁺-activation of the enzyme. Double-reciprocal plots of these results were not linear but both the maximum velocity of the Na,K-ATPase and the K_m of the enzyme for Na⁺ and ATP appear to be changed. It is apparent that the amino acids have several effects on Na,K-ATPase activity and these are concluded to arise, in part at least, from diminution of electrostatic interactions between the enzyme, substrate and activators.

Other treatments: Lubrol W, elastase and ultrasonic irradiation. Na,K-ATPase activity of the sodium iodide-treated preparation was increased by including the non-ionic detergent Lubrol W in the assay medium. A Lubrol W concentration equivalent to 1.7mg of detergent/mg of protein increased activity by 50%. After exposure of the sodium iodide-treated preparation to increasing amounts of Lubrol W and centrifugation, increased amounts of protein, phospholipid and Na,K-ATPase activity were no longer deposited (Fig. 6). The maximum solubilization of protein and phospholipid was about 30%; that is, slightly less than the activity solubilized so that the solubilized material had a higher Na,K-ATPase specific activity than the starting material, despite an overall loss of Na,K-ATPase activity at the higher detergent concentrations.

Results of incubating the sodium iodide-treated preparation with pancreatic elastase for various periods of time and centrifuging are shown in Fig. 7. No Na,K-ATPase activity was released into the supernatant and Na,K-ATPase activity that remained in the deposit was diminished. Short

Fig. 6. The NaI-treated preparation, containing 3 mg of protein and $3 \mu\text{mol}$ of lipid phosphorus with an Na,K-ATPase activity of $63 \mu\text{mol of P}_i/\text{h per mg of protein}$, was mixed with the indicated amount of Lubrol W in a final volume of 2 ml. After being left in ice for 30 min the mixtures were centrifuged at $150000g$ for 60 min and the supernatants and deposits were analysed. (a) Specific activity of the Na,K-ATPase in the supernatant. (b) Percentage of total Na,K-ATPase activity ($\circ$), protein ($\square$) and lipid phosphorus ($\blacksquare$) in the supernatant and the percentage recovery of Na,K-ATPase activity in both supernatant and deposit ($\bullet$) are shown.

periods of incubation released Na,K-ATPase-inactive protein to a greater extent than Na,K-ATPase activity was destroyed, so that the specific activity of the Na,K-ATPase remaining in the particulate material was increased. After longer periods of incubation Na,K-ATPase activity was destroyed without a corresponding release of protein indicating that the inactivated Na,K-ATPase remains insoluble.

Conditions for ultrasonic treatment of cerebral microsomes were found in which up to 55% of the protein and Na,K-ATPase activity was no longer deposited by ultracentrifugation (Cooper & McIlwain, 1967). Ultrasonic treatment of the sodium iodide-treated preparation was carried out under a variety of conditions, including those of Cooper & McIlwain (1967), but in no case was more than 30% of the protein and 15% of the Na,K-ATPase activity found in the supernatants ob-

Fig. 7. Digestion of the NaI-treated Na,K-ATPase preparation with pancreatic elastase. Enzyme protein (2.22 mg) with an Na,K-ATPase activity of $78 \mu\text{mol of P}_i/\text{h per mg of protein}$ was incubated with $22 \mu\text{g}$ of elastase in 2 ml of 20 mM-tris-hydrochloric acid buffer, pH 8.8, at 25°C for the indicated times, and then centrifuged at $150000g$ for 30 min. Protein ($\square$), Na,K-ATPase activity ($\circ$) and the specific activity of the Na,K-ATPase in the deposit ($\bullet$) are shown as percentages of untreated material. The zero time value was obtained by omitting elastase and was independent of the time of incubation.

tained after centrifugation at $150000g$ for 1 h. Analyses for lipid phosphorus failed to show any alteration in the lipid phosphorus/unit of protein in the supernatant obtained after centrifugation.

DISCUSSION

The particulate Na,K-ATPase preparation obtained by sodium iodide treatment of bovine cerebral microsomes had lost 95% of its original Na^+ -independent adenosine triphosphatase activity, and this was largely recovered in the supernatant fluids. The enzymes responsible for Na,K-ATPase and Na^+ -independent adenosine triphosphatase activities were thus distinct and separable. Differential and density-gradient separation without the employment of an agent such as sodium iodide did not give comparable separation of the adenosine triphosphatases (Hosie, 1965; Kurokawa *et al.* 1965). In addition, the Na,K-ATPase activity of the sodium iodide-treated preparation was greatly increased and this resulted partly from a 70% removal of microsomal protein, and partly from enzyme activation. The Na,K-ATPase of the sodium iodide-treated preparation was further activated by Lubrol W, but to a lesser extent than given by Lubrol W acting on the Na,K-ATPase of cerebral microsomes (Swanson *et al.* 1964).

The sodium-iodide reagent and Lubrol W may thus share a common step in their activation of the Na,K-ATPase but the two procedures contrast in that acetylcholinesterase was activated by non-ionic detergents similar to Lubrol W (Jackson & Aprison, 1966b) whereas this enzyme was inactivated by the sodium iodide treatment.

The changes in lipid/protein ratios that were brought about by extracting microsomal preparations with sodium iodide solutions are also much beyond those obtained by differential or density-gradient separations (Wherrett & McIlwain, 1962; Seminario, Hren & Gomez, 1964; Lapetina *et al.* 1968). Dissociation of phospholipid, cholesterol, protein and *N*-acetylneuraminic acid derivatives had occurred during exposure to 2M-sodium iodide (Tables 1 and 3). When, subsequently, solutions were diluted to 0.8M-sodium iodide, some lipid-protein associations that lacked Na⁺-independent adenosine triphosphatase activity but were enriched in Na,K-ATPase activity presumably re-formed. The dissociation and re-association of phospholipid and protein that can take place with the sodium iodide reagent suggest that it does not simply separate preformed lipid-protein complexes from the microsomes. Current views of membrane structure emphasize the importance of hydrophobic bonding forces between proteins and lipids (Wallach & Gordon, 1968) and properties of the sodium iodide-treated Na,K-ATPase preparation suggest that it is enriched in non-polar proteins. The preparation was more aggregated than the microsomes and had a higher content, relative to protein, of cholesterol and phospholipids. The action of sodium thiocyanate is presumed to arise from its 'structure-breaking' effect on water, thereby weakening hydrophobic protein-protein and protein-lipid bonds (Hatefi & Hanstein, 1969). Solutions containing sodium iodide may be acting similarly.

The action of arginine as a phospholipid-protein dissociating agent appears also to involve the changes which it causes in dielectric constant. It is one of the more potent α -amino acids in increasing the bulk dielectric constant of aqueous solutions and may be expected to weaken ionic bonds. Also, it carries a guanidinium group which is considered to form stable bonds with carboxylate groups (Lewin, 1969) and may therefore displace other ionic bonds involving carboxylic acids. Electrostatic interactions in the Na,K-ATPase reaction itself may be involved in the enzyme inhibition shown to be produced by a variety of amino acids. The inhibition was related to, but not wholly explained by, the alteration in bulk dielectric constant of the medium caused by the amino acids. Effects of increased dielectric constant on the overall rate of the Na,K-ATPase are likely to be complex but to include changes in affinity of the enzyme for ATP

and Na⁺. Further, in relation to the inhibition by arginine, a carboxylate group of the adenosine triphosphatase active centre is involved in forming with ATP a phosphorylated intermediate in the Na,K-ATPase reaction (Hems & Rodnight, 1966).

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