The Sodium-plus-Potassium Ion-Activated Adenosine Triphosphatase of Cerebral Microsomal Fractions : Treatment with Disrupting Agents

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1. The Na^+ -plus- K^+ -stimulated adenosine triphosphatase [$(\text{Na}^+, \text{K}^+)\text{-ATPase}$] of microsomal preparations from ox brain was inactivated or diminished in activity by exposure to 2–8 M-urea. Similar concentrations of urea diminished the turbidity of the suspensions. 2. Low concentrations (about 2.5 mM) of NaATP with the urea gave partial or complete protection of the ATPase, without altering the concomitant change in turbidity. Some protection of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ was afforded by tris ATP, but the greatest protection was found with NaATP and in its presence the change in $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ with 3 M-urea included a phase in which activity was enhanced by 40%. 3. The protective effect was specific to NaATP: KATP, NaADP, NaAMP and sodium pyrophosphate were without protective effect and in some cases they augmented the action of urea. 4. The turbidity of cerebral microsomal suspensions was diminished also by ultrasonic irradiation; NaATP did not alter this change. After ultrasonic treatment up to 55% of the protein and of the ATPase activity were no longer deposited by centrifugal forces of $4.5 \times 10^6 \text{ g} \cdot \text{min}$. 5. Ultrasonic treatment and centrifugation could be carried out with little or no loss of ATPase and ammonium sulphate flocculation of the supernatant then afforded in the first material precipitated a three- to five-fold enrichment of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity. 6. Sodium borohydride and dimethyl sulphoxide also diminished the turbidity of the microsomal fraction but enrichment of the ATPase was not effected by these reagents; ten other compounds were without action on the ATPase. 7. Acetyl phosphate was hydrolysed by the microsomal preparation and this activity was increased by added K^+ . Acetyl-phosphatase activity persisted in the ultrasonically treated and ammonium sulphate-fractionated preparations, which were more exacting in their requirements for K^+ . 8. The findings are discussed in relation to the mechanism of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$.

Microsomal fractions showing $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ † activity, though often referred to as an enzyme (see Skou, 1964), are more correctly to be regarded as morphological entities. Processes of disruption or extraction are thus a necessary part of the biochemical study of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$. A previous investigation (Stahl, Sattin & McIlwain, 1966) showed that simple washing procedures could extract from ox-brain microsomal preparations an ADP-ATP exchange system, which earlier investigators had regarded as an essential part of the

$(\text{Na}^+, \text{K}^+)\text{-ATPase}$; yet its extraction did not diminish the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity of the preparations.

In the present study, cerebral microsomal fractions have been subjected to more extensive treatments, including ultrasonic irradiation and exposure to high concentrations of urea, with the object of differential extraction and of learning more about the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ and its relationship to other microsomal constituents. Such relationships are inherently likely to be important to the participation of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ in active movements of Na^+ and K^+ across cell boundaries (see Bonting, 1964; McIlwain, 1963; Skou, 1964). While the present investigations were

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† Abbreviations: ATPase, adenosine triphosphatase; $(\text{Na}^+, \text{K}^+)\text{-ATPase}$, Na^+ -plus- K^+ -stimulated ATPase.

in progress Skou & Hilberg (1965) reported the use of urea in examining a differently prepared microsomal ATPase and this receives comment below.

EXPERIMENTAL

Microsomal suspensions. Ox brains were placed in a plastic bag in ice, within 15 min. of the animals' slaughter. About 45 min. later, grey matter was snipped from the cerebral hemispheres and about 70 g. of cortex from two brains was blended with 9 vol. of 0.32 M-sucrose, adjusted to pH 7.4 with KOH. The centrifuging sequence of Stahl *et al.* (1966) was followed, except that for washing the mitochondrial fraction was resuspended in a test-tube homogenizer and that the combined supernatant yielded a microsomal pellet that was suspended in 0.32 M-sucrose at a concentration corresponding to 10–15 mg. of protein/ml. This was either used immediately, or was kept for up to 3 days at 6° or at –20° for up to 3 weeks.

Exposure to urea. This was normally carried out at room temperature (16–20°) with mixtures containing microsomal suspensions equivalent to 1–2 mg. of protein/ml., and the concentrations of urea and other reagents that are specified in individual experiments. When extinction values were measured the mixtures were made in cells of a Unicam SP.600 spectrophotometer with which readings were taken periodically at 450 m μ (Fig. 1). After chosen times, measured portions of the mixtures were pipetted to tubes for enzyme assays; this involved at least tenfold dilution of the urea.

Ultrasonic treatment. Microsomal suspensions containing 7–18 mg. of protein/ml. of 0.32 M-sucrose (2 ml.) were placed in a 10 ml. cup, cooled in ice-water, into which dipped a 1 cm. diam. probe of a 60 W MSE ultrasonic unit. The ultrasonic irradiation was carried out intermittently, for periods of 30 sec. each followed by a 30 sec. interval, until the irradiation periods totalled 3 min. After treatment the samples were centrifuged (Spinco preparative machine L2) at $1.5 \times 10^5 g$ for 30 min. or 60 min. and the supernatants and deposits collected. When specified (Table 2) the deposits were resuspended in 2 ml. of 0.32 M-sucrose and the ultrasonic treatment was repeated.

Ammonium sulphate fractionation. Saturated aqueous $(\text{NH}_4)_2\text{SO}_4$ neutralized with aq. NH_3 was added to the supernatants from ultrasonic treatment, to give the concentrations specified in Table 3. The resulting suspensions, turbid when the $(\text{NH}_4)_2\text{SO}_4$ was 30% saturated or above, were centrifuged at $1.5 \times 10^5 g$ for 30 min. and the supernatants removed. The solid that separated formed a plaque-like deposit with the higher $(\text{NH}_4)_2\text{SO}_4$ concentrations that adhered to a side of the tubes. It was resuspended in water for assay.

ATPase assay. Activity was determined by the method of Schwartz, Bachlard & McIlwain (1962) except that normal concentrations of reagents for $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ were 50 mM-tris-HCl, pH 7.6, 3 mM-tris ATP, 3 mM-MgCl₂, 100 mM-NaCl and 30 mM-KCl. For ATPase as quoted in Tables 1–4, the NaCl and KCl were omitted. Also, after incubation the reaction was terminated by the addition of 1 ml. of ice-cold 7.2% (w/v) HClO_4 . The extract was then neutralized with 1 ml. of 1.3 N-KOH, refrigerated for 10 min. and centrifuged. A 2 ml. portion of the supernatant was taken for phosphate determination. Since pyridithiamine and Persantin interfered with the determination, the following modification was employed when these drugs were tested.

After incubation the reaction was stopped by the addition of 0.1 ml. of N-HCl, and the samples were placed in a boiling-water bath for 1 min., cooled and neutralized with 0.5 M- NaHCO_3 . The tubes were centrifuged and 0.5 ml. of the supernatant was placed on a column (1 cm. $\times$ 7 cm.) of the cation-exchange resin Amberlite IRC-50 (Na^+ form). The effluent and subsequent water wash were collected and made to 3 ml., and a portion was taken for phosphate determination.

Acetyl-phosphatase assay. Incubation media contained 50 mM-tris-HCl, pH 7.6, 3 mM-MgCl₂ and 30 mM-KCl, and to 0.5 ml. of the media was added the sample to be assayed, in 0.3 ml. After preincubation at 37° for 5 min. the reaction was initiated by adding 0.2 ml. of 50 mM-acetyl phosphate as either the lithium or tris salt. The sample was incubated for 20 min., and the reaction stopped by the addition of 1 ml. of neutralized hydroxylamine. Control samples without the enzyme were carried through the procedure, and where specified (Table 4) mixtures lacking K^+ ions were included. The acetyl phosphate remaining was then determined photometrically as described by Lipmann & Tuttle (1945). Examination of reaction mixtures buffered between pH 7.2 and 8.2 showed greatest activity at pH 7.6; the reaction progressed linearly with time for 30 min.

Other reagents and determinations. NaATP was obtained as the crystalline hydrated disodium salt of 99–100% purity from the Sigma Chemical Co., London, S.W.6. The tris or potassium salts when required were prepared from the sodium salt by passing concentrated solutions through a 1 cm. $\times$ 7 cm. column of Amberlite resin IR-120 (H^+ form), and immediately neutralizing with either tris base or KHCO_3 . Tris base and hydrochloride and lithium acetyl phosphate were also obtained from the Sigma Chemical Co. Urea (ultra pure) was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Diethylaminoethyl diphenylpropyl acetate hydrochloride, SKF 525A, was a gift from Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A. Persantin (dipyridamole) was supplied by Geigy Pharmaceuticals, Ardsley, N.Y., U.S.A. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

The microsomal fractions that formed the starting material of the present investigations were similar in preparation and properties to those of Swanson, Bradford & McIlwain (1964) and Stahl *et al.* (1966), including their requirements for Na^+ and K^+ ions. Their rate of hydrolysis of ATP in solutions containing magnesium salts was 0.3–0.5 that obtained with further addition of Na^+ and K^+ (see Tables 1 and 2). These Tables indicate also the variations typically seen between different preparations, in enzyme activity and in degree of Na^+ - and K^+ -activation.

Treatment with urea

Optical effects. The actions of urea in unfolding and dissociating protein chains (see Koshland, 1958; Nozaki & Tanford, 1963) were applied to myosin ATPase by Stracher (1961), with 2–10 M-urea. In

Fig. 1. Urea and the extinction value of microsomal suspensions. (a) The differently marked points refer to different microsomal preparations. In each case the suspensions (12 mg. of protein/ml., 0.1 ml.) were added to 0.05 ml. of 50 mM-NaATP, 10 M-urea of volume needed for the final concentrations quoted, and water to 1 ml. Readings were taken at 450 m μ 20 min. after mixing. In parallel experiments (not quoted) NaATP was omitted and the extinction values were found to be the same. (b) Microsomal suspensions were exposed as just described, with the following additions: □, none; ○, 4 M-urea; ●, 4 M-urea + 2.5 mM-NaATP.

the present study a similar range of concentrations was used, and these brought about physical changes in the microsomal suspensions, as well as alterations in their ATPase activity (Fig. 1). Pronounced clearing of the originally opalescent microsomal suspension took place within a few seconds of admixture with urea. The change progressed for several minutes and required the high concentrations of urea shown in Fig. 1(a). It was not altered by the presence of 2.5 mM-NaATP.

ATPase inactivation and protection. At 3 M-urea, when change in the extinction of the suspensions had undergone about half its maximal change, the suspensions had also changed greatly in ATPase. Their activity, when tested either in the presence or absence of Na⁺ and K⁺, had fallen by 50–80%; examination was carried out in reaction mixtures in which the urea was diluted 30-fold, and thus the effect of urea was an inactivation of the ATPase rather than an inhibition. This situation was greatly altered when ATP also was present during the exposure to urea (Fig. 2). Thus after exposing the microsomal suspension at room temperature to 2.5 mM-NaATP plus 3 mM-urea for 20 min., and subsequently diluting it 30-fold for assay in the

standard reaction mixture, the (Na⁺,K⁺)-ATPase activity exhibited had increased by 40%. This increase in the Na⁺- and K⁺-activation was accompanied by diminution in the ATPase activity in the absence of Na⁺ and K⁺ (Table 1), though this activity remained at a higher level than after exposure to urea only. The augmentation in (Na⁺,K⁺)-ATPase could occur as a result of the presence of urea and ATP independently of Na⁺, but the greatest activities were obtained when both Na⁺ and ATP were present with urea (Fig. 2). The protective effects of Na⁺ and ATP were shown towards all concentrations of urea examined, i.e. up to 6 M, though only at the lower concentrations was (Na⁺,K⁺)-ATPase activity enhanced.

Specificity of protection by sodium ATP. Only NaATP among several related compounds protected the microsomal ATPase from inactivation by urea (Table 1). The KATP and tris ATP were ineffective; the protective effect of NaATP was shown at a range of concentrations (Table 1, Expt. C) and was maximal at 2.5 mM with urea at 4 M. NaAMP and NaADP at 2.5 mM did not protect against urea, though sodium pyrophosphate augmented the loss of activity (Table 1, Expt. B).

Fig. 2. ATPase of microsomal suspensions, previously exposed to urea in the presence (O) and absence (●) of NaATP. Mixtures were prepared containing the different amounts of urea and with or without 2.5 mM-NaATP. The microsomal suspension was then added and the mixtures were kept at 20° for 10 min.; portions were then taken for assay of ATPase (A) and (Na⁺,K⁺)-ATPase (B) under the standard conditions stated in the Experimental section.

In Table 1 are quoted ATPase estimations in the presence and absence of Na⁺ and K⁺ (independently of the presence or absence of Na⁺ during exposure to urea). The protective effects described were exhibited in both circumstances: i.e. the 'MgATPase' as well as the '(Na⁺,K⁺,Mg²⁺)-ATPase' was protected, though to a smaller degree. Certain of the added substances, it will be noted, augmented the action of urea under one or both of these circumstances rather than opposing it. Sodium pyrophosphate and KATP were most potent in this respect. After treatment of microsomal suspensions with urea in the presence or absence of NaATP, centrifugation under the conditions used for originally preparing the microsomes gave a deposit that again carried all the (Na⁺,K⁺)-ATPase activity of the suspensions.

Ultrasonic irradiation

Exposure of cerebral microsomal suspensions to ultrasonic irradiation was noted by Bradford, Swanson & Gammack (1964) to be deleterious to their ATPase activity. However, about 12% of the ATPase or (Na⁺,K⁺)-ATPase of the suspensions was no longer deposited by the centrifugal forces used in preparing the microsomes. By choosing the conditions described in the Experimental section, involving intermittent treatment of well-cooled suspensions, the procedure has now been carried out with minimal loss; Table 2 indicates that

Table 1. Protection of (Na⁺,K⁺)-ATPase against inactivation by urea

In all experiments microsomes were exposed to urea at 4 M; the nucleotide or phosphate salts were added at 2.5 mM except in Expt. C. For each exposure, reagents were added to 0.1 ml. of microsomal preparation (10–15 mg. of protein/ml.) with 0.05 ml. of adenine nucleotide or pyrophosphate (50 mM), 0.4 ml. of urea (10 M) and 0.45 ml. of water. The suspensions were kept for 10 min. at room temperature and then 0.1 ml. portions were taken for ATPase assay.

Expt.	Added compounds	ATPase activity (μmoles of P _i /mg. of protein/hr.)		
		(a) No Na ⁺ or K ⁺	(b) Na ⁺ (100 mM) + K ⁺ (30 mM)	(b) – (a)
A	None	11.8	22.8	11.0
	Urea	1.6	5.1	3.5
	Urea, NaATP	5.8	20.7	14.9
	Urea, KATP	0.3	2.3	2.0
	Urea, tris ATP	2.2	2.5	0.3
B	None	3.9	12.6	8.7
	Urea	0.9	4.9	4.0
	Urea, NaATP	1.4	10.9	9.5
	Urea, NaADP	1.3	5.4	4.1
	Urea, NaAMP	0.3	1.2	0.9
	Urea, sodium pyrophosphate	0	0	0
	Urea, 0.5 mM-NaATP	2.9	16.5	13.6
C	None	13.0	29.6	16.6
	Urea	1.0	3.1	2.1
	Urea, 0.5 mM-NaATP	2.9	16.5	13.6
	Urea, 1.25 mM-NaATP	2.1	14.8	12.7
	Urea, 2.5 mM-NaATP	1.7	20.2	18.5
	Urea, 5 mM-NaATP	4.8	12.4	7.6

Table 2. *Ultrasonic treatment of cerebral microsomal preparations*

The different microsomal preparations were derived from the grey matter of different ox brains. Each preparation contained 15–40 mg. of protein, and was exposed to ultrasonic irradiation as described in the Experimental section. After centrifuging, preparations I, II and IV were resuspended in sucrose and the irradiation and centrifuging repeated. Determinations of protein and activity were made with each supernatant and residue immediately after their preparation.

Prep. no.	Determination	Material examined										Activity (% of original)			
		Original microsomes		First supernatant		Second supernatant		Residue		Recovered		Remaining supernatant			
		With- out	With	With- out	With	With- out	With	With- out	With	With- out	With	With- out	With	With- out	With
I	μmoles of P _i /fraction/hr.	125	378	31	109	16.8	5.2	6.6	42.3	44	54	39	44		
II	μmoles of P _i /mg. of protein/hr.	6.2	18.7	3.5	12.5	4.2	13.0	2.2	14.1	—	—	—	—		
	μmoles of P _i /fraction/hr.	86.7	325	23.1	74.9	19.3	66	22.9	73.5	75	68	49	43		
III	μmoles of P _i /mg. of protein/hr.	4.3	16.2	2.4	7.5	3.7	12.8	4.4	15.1	—	—	—	—		
	μmoles of P _i /fraction/hr.	55.7	135.5	18.7	50.4	—	—	25.7	82.6	81	98	—	—		
IV	μmoles of P _i /mg. of protein/hr.	7.5	18.5	5.7	15.3	—	—	7.6	24.3	—	—	—	—		
	μmoles of P _i /fraction/hr.	102	365	39.2	118	16.0	50.3	—	—	53	46	55	47		
V	μmoles of P _i /mg. of protein/hr.	5.7	20.4	5.7	17.1	4.4	14.0	—	—	—	—	—	—		
	μmoles of P _i /fraction/hr.	55.7	135	15.7	37.3	—	—	23.2	94.0	70	96	—	—		
	μmoles of P _i /mg. of protein/hr.	7.5	18.5	4.8	11.5	—	—	5.9	23.8	—	—	—	—		

recoveries of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ averaged 72% and reached 98%.

Solubilized material and salt fractionation

The ultrasonic treatment caused major changes in the microsomal suspensions shown in Tables 2 and 3; their extinction values diminished, e.g. in the suspensions of Table 2 by about 40%. Moreover, much of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ originally present in the suspensions was now no longer deposited by centrifugal forces greater than those used in preparing the microsomes. Some 40–50% of the original activity now remained supernatant after centrifuging at $4.5 \times 10^6 \text{g-min.}$ (Table 2). After the ultrasonic treatment, a similar proportion of the total microsomal protein was also no longer deposited and thus the specific activity of the ATPases was little changed, in supernatant or in residual fractions (Table 2). Opportunity was, however, offered by the presence of ATPase activity in the supernatant for application of different methods of fractionation.

Enrichment of the enzyme was first afforded by the ammonium sulphate fractionation of Table 3. Addition of increasing concentrations of ammonium sulphate could precipitate almost all the ATPase and 80% of the protein of the supernatant from ultrasonic treatment. Selectivity was, however, shown in the ATPase being more readily brought out of solution than were admixed proteins; increase in specific activity or ATPase per unit of protein, was

then obtained. With the first separation of quite small amounts of protein with 20% saturated solution of ammonium sulphate, some 40% of the activity was associated. Almost all the activity was flocculated with 30%-saturated ammonium sulphate, at similarly high specific activity. Higher concentrations of ammonium sulphate gave precipitates that carried increasing proportions of protein inactive in the ATPase assay.

Treatment of microsomal suspensions with a sodium iodide reagent has been investigated by Nakao, Yashima, Nagamo & Nakao (1965). Ultrasonic treatment and centrifugation of this preparation also has now been found to yield a supernatant with ATPase activity (Table 4).

Acetyl-phosphatase activity. Microsomal fractions from kidney cortex carry a K^+ -activated acetyl-phosphatase activity, which is considered to arise from their $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (Bader & Sen, 1966), though fractionation methods were not employed in reaching this conclusion. As the procedures just described had afforded a separation of $(\text{Na}^+ \text{K}^+)\text{-ATPase}$ from admixed protein, the resulting preparations were examined for acetyl-phosphatase activity.

Such activity was found (Table 4); the original microsomes liberated phosphate from ATP and acetyl phosphate at about the same speed. Ultrasonic treatment of the microsomal suspensions increased their acetyl-phosphatase activity and gave preparations in which acetyl phosphatase was activated by Mg^{2+} and K^+ and inhibited by Na^+

Table 3. *Enrichment of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity by ammonium sulphate treatment*

Microsomes were ultrasonically treated and centrifuged as described in the Experimental section. To separate portions of the supernatant, neutralized saturated $(\text{NH}_4)_2\text{SO}_4$ was added at 0° to give the final saturation value shown in the Table. After the addition of $(\text{NH}_4)_2\text{SO}_4$, the samples were centrifuged at 150000g for 30 min. The supernatant fraction was aspirated and discarded; the $(\text{NH}_4)_2\text{SO}_4$ precipitates were suspended in water and their protein and activities determined.

Preparation	(a) Protein (mg./ml.)	(b) Phosphate liberated ($\mu\text{moles of P}_i/\text{mg. of protein/hr.}$)		(a) $\times$ (b) Enzyme units	
		No Na^+ or K^+	With Na^+ and K^+	No Na^+ or K^+	With Na^+ and K^+
A: Supernatant from ultrasonic treatment	8.17	1.64	10.9	13.4	89
Precipitates with $(\text{NH}_4)_2\text{SO}_4$ at:					
20% satn.	0.60	3.83	59	2.3	35
40% satn.	1.80	1.90	48	3.4	87
60% satn.	6.0	0.74	15.5	5.0	93
80% satn.	6.8	0.59	13.8	4.0	94
B: Original microsomes	19.3	4.4	15.0	84.3	290
Supernatant from ultrasonic treatment	5.9	4.8	14.0	28.4	83
Precipitates with $(\text{NH}_4)_2\text{SO}_4$ at:					
30% satn.	2.2	7.7	50.5	16.9	111
35% satn.	3.0	5.8	28.8	17.3	87
40% satn.	3.5	5.2	26.2	18.1	92

Table 4. *Acetyl-phosphatase activity of different ATPase preparations*

Preparations (i), (ii), (iv) and (vi) were made as described in Table 3, with (NH₄)₂SO₄ at 30% saturation to obtain (iv). Preparation (iii) was the material deposited by the centrifugation, which yielded (ii), and corresponded to the 'residue' of Table 2. We are indebted to Mr I. Pull for the NaI-treated microsomal suspension that was made according to Nakao *et al.* (1965) and that, after ultrasonic treatment and centrifugation as described in Table 2, yielded the supernatant (v). When specified, Na⁺ was present at 100mM and K⁺ at 30mM.

Preparation	ATPase (μmoles of P _i /mg. of protein/hr.)		Acetyl phosphatase (μmoles of P _i /mg. of protein/hr.)	
	No Na ⁺ or K ⁺	With Na ⁺ and K ⁺	No K ⁺	With K ⁺
(i) Microsomal suspension	7.7	24.7	16.8	23.8
(ii) Supernatant from ultrasonic treatment	9.4	24.1	31.1	40.1
(iii) Residue from ultrasonic treatment	10.2	25.5	29.9	34.8
(iv) (NH ₄) ₂ SO ₄ precipitate from supernatant	7.8	22.6	2.8	27.6
(v) Supernatant from ultrasonic treatment of NaI-treated microsomes	1.0	28.9	5.5	53.8
Additions to acetyl phosphatase reaction mixture				
(vi) As preparation (ii)	None		11.3	11.3
As preparation (ii)	3mM-MgSO ₄		18.6	49.5
As preparation (ii)	3mM-MgSO ₄ , 100mM-NaCl		14.5	29.3

ions. On centrifugation the acetyl phosphatase was distributed between supernatant and deposit in about the same proportion as was the (Na⁺,K⁺)-ATPase. Moreover, the acetyl phosphatase of the supernatant was deposited by ammonium sulphate together with the ATPase; its activation by K⁺ ions greatly increased after this treatment. The preparation obtained by ultrasonic treatment and centrifugation of microsomes treated with sodium iodide reagent (see above and Table 4) also showed increased activation by added K⁺ ions.

Miscellaneous agents

Of other disruptive or solubilizing agents, detergents have been examined previously (Schwartz *et al.* 1962; Swanson *et al.* 1964). Among a number of reagents tested in the present study (Table 5) dimethyl sulphoxide and sodium borohydride caused pronounced diminution in the extinction value of cerebral microsomal suspensions, but on centrifuging the treated solutions all (Na⁺,K⁺)-ATPase was again deposited. Further optical clearing with higher concentrations of the sulphoxide or borohydride than quoted in Table 5 caused loss of (Na⁺,K⁺)-ATPase. The other reagents of Table 5 were examined in exploring possible cofactor requirements of the system. Bruchhausen (1964) reported actions of pteridine derivatives on an erythrocyte ATPase, but action was not found in the amethopterin examined in the present study. The nicotinamide nucleotides were

Table 5. *Agents examined for effect on (Na⁺,K⁺)-ATPase*

Potassium borohydride was added to microsomal suspensions in 50mM-tris, pH 8, and kept at 37° for 10 min. before taking a portion of the suspension for assay. Dimethyl-sulphoxide was added to the microsomal suspension and kept at 18° for 20 min. before taking samples for assay. The other agents were added to ATPase reaction mixtures before the microsomal suspensions were added.

Agent	Concentra- tions examined (mM)	Inhibition of of (Na ⁺ ,K ⁺)- ATPase (%)
KBH ₄	20	0
KBH ₄	60	46*
KBH ₄	240	77*
NAD ⁺ , NADH, NADP ⁺ or NADPH	0.66	0
Thiamine pyrophosphate	1	0
Pyriethamine	0.5, 5	0
GSH	1	0
Yeast extract (10mg./ml.)	—	0
Amethopterin	0.3	0
o-Phenanthroline	1	0
Persantin	0.5	0
SKF 525A	0.3	0
Dimethyl sulphoxide	420	0
Dimethyl sulphoxide	1400	21*
Dimethyl sulphoxide	4200	37*

* In these instances the extinction value of the microsomal suspensions was diminished: by 60mM-KBH₄ to 81% and by 240mM-KBH₄ to 49% of its original value.

included in view of observations of Siekevitz (1965), thiamine pyrophosphate and pyrithiamine in view of those of Arnett & Cooper (1965), SKF 525A because of its effects on other microsomal systems (Cooper, Axelrod & Brodie, 1954), and Persantin because of similarities in its action to that of ouabain.

DISCUSSION

Conceivably an agent ideal for the present investigation would separate the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ unchanged from its microsomal matrix. Those examined, urea and ultrasonic irradiation, as well as the detergents of a previous study (Swanson *et al.* 1964), have modified ATPase activity during its separation. This was the result also of a distinct type of enrichment employing sodium iodide and cysteine, which extracted associated material, leaving an enriched $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (Nakao *et al.* 1965). It is encouraging that the alterations in $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ encountered after each of these treatments have made the enzyme more exacting in its requirement for Na^+ and K^+ for ATPase activity, or have included an increase in rate of hydrolysis. This makes it more likely that the Na^+ and K^+ requirements are properties of the enzyme system itself rather than being imposed by independent microsomal systems that, for example, functioned by allowing access of ions to the enzyme.

Features in the action of urea. (i) Comparison of Figs. 1 and 2 shows that the concentrations of urea that caused optical clearing of microsomal suspensions were similar to those that (in the absence of NaATP) diminished the suspensions' $(\text{Na}^+, \text{K}^+)\text{-ATPase}$. Both of these actions of urea are presumably to be ascribed to its property of forming new linkages of the hydrogen-bond type with peptide chains, while dissociating those native to the microsomal components (see Kauzmann, 1959; Nozaki & Tanford, 1963). That the loss of activity caused by moderate concentrations of urea can be opposed by NaATP, whereas that of greater concentrations cannot, suggests the affinities involved between substrate and enzyme, and enzyme and urea, are similar in magnitude. (ii) Further, NaATP not only protects the microsomal ATPase from inactivation by urea: joint action of NaATP and 3–4 M-urea can enhance the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$. This comes about not by an increase in the total ATPase activity observed (Table 1) but by diminishing the contribution made by the component not requiring added Na^+ and K^+ . This result is compatible with the suggestion that these two enzyme activities are interconvertible, a view supported by Skou & Hilberg (1965), but as a result of investigating microsomes exposed to deoxycholate, EDTA and histidine during their preparation. The

properties of this material differed from those of the microsomal preparation now examined, for deoxycholate itself modifies the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (Schwartz *et al.* 1962; Skou & Hilberg, 1965). With the deoxycholate-EDTA-histidine-treated preparation, in contrast with the present results, a protective role for Na^+ was not found: Na^+ increased an inactivation caused by *N*-ethylmaleimide both in the presence and absence of ATP. Effects of Na^+ in the presence of urea were stated to be similar to those in the presence of the imide, but without data. Na^+ did, however, protect a similar cerebral microsomal ATPase from inactivation brought about by incubation with chlorpromazine (Squires, 1965).

The potency of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ makes its control mandatory *in vivo*. In the derived microsomes, relevance to control mechanisms may thus be seen in the finding that the extent of activation by Na^+ and K^+ , and also the resulting $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity, can both be increased by urea. Comparable features were previously reported in the action of detergents and of ouabain and their relevance to control of the enzyme's functioning was appraised (Swanson, *et al.* 1964; Swanson & McIlwain, 1965).

(iii) Properties of metal salts of ATP in purely chemical systems appear relevant to the specificity shown by NaATP as protective agent. Combination of ATP with Na^+ in ATPase reaction mixtures independently of the enzyme was noted earlier (McIlwain, 1963; Schwartz *et al.* 1962). Melchior (1954) emphasized that firm complexes existed in aqueous solutions between ATP and Mg^{2+} , Na^+ or K^+ , and that the preferred configuration taken up by ATP differed in the different salts. In the present system, Na^+ is without protective action unless ATP is present; therefore, although the two could still be acting separately, the simplest assumption would regard NaATP itself as the protective agent. This view is supported by the greater protective action shown by NaATP in comparison with the sodium salts of the other phosphates examined. In dilute aqueous solution, orthophosphate, AMP and ADP had less ability to bind Na^+ than had ATP, which could attach 2 Na^+ ions/mol. (Hickling & White, 1965). This parallels the protective effects shown in Table 1.

(iv) The chemical properties of KATP often differ little from those quoted above for NaATP, but in protecting the microsomal ATPase from urea the two salts differ greatly. The diminution of ATPase induced by urea was augmented by KATP. In judging aspects of the ATPase related to these phenomena, proposed intermediates and steric factors appear relevant. Analogous ATPase systems of giant axons and erythrocytes required Na^+ and ATP to be together on the inside of the cell mem-

brane (Glynn, 1962; Whittam, 1962); K⁺ acted at the other side and catalysed breakdown of a labile phosphate derivative that was formed when Na⁺, ATP and Mg²⁺ were present (Post, Sen & Rosenthal, 1965; Hems & Rodnight, 1965). In the present experiments protection is afforded by the simultaneous presence of two reactants that are needed on the same side of the cell membrane but that themselves are inadequate for enzymic change. The urea experiments thus support formulations of (Na⁺,K⁺)-ATPase action that permit substrate-addition compounds of the type Enzyme·NaATP.

Acetyl phosphatase and ultrasonic treatment. It is a feature of the (Na⁺,K⁺)-ATPase mechanisms that propose successive Na⁺- and K⁺-activated steps (see above) that the phosphorylated intermediate has the properties of an acyl phosphate. The second, K⁺-activated, stage is then an acyl phosphatase for which acetyl phosphate is concluded to be a relevant substrate (Bader & Sen, 1966; Hokin, Sastry, Galsworthy & Yoda, 1966). The present investigations give additional support to this, for the microsomal suspensions that formed the starting material carried acetyl-phosphatase activity approximately equal to their (Na⁺,K⁺)-ATPase activity, and the two activities remained associated after ultrasonic treatment and ammonium sulphate fractionation (Table 4). It is especially noteworthy that activation of the acetyl phosphatase by K⁺ was shown throughout, and increased after the fractionation. The inhibition caused in the acetyl phosphatase by Na⁺ may also be relevant to the functioning of the enzyme in its normal cellular environment.

The extent to which ultrasonic treatment may have given solution of the ATPase is uncertain. Centrifugation demonstrated diminution in particle size to a degree often associated with solution and ammonium sulphate indicated that over 75% of microsomal protein was not deposited on centrifuging solutions that were 40% saturated with ammonium sulphate. The (Na⁺,K⁺)-ATPase was, however, flocculated by relatively low ammonium sulphate concentrations. The enrichment achieved by removing in this way most of the microsomal protein indicates that the process merits further study, which is in progress.

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