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THE pH DEPENDENCE OF THE EFFECTS OF Na^+, K^+ AND OUABAIN ON THE ATPase ACTIVITY OF NaI-TREATED BRAIN MICROSOMES WITH Mg^{2+}, Mn^{2+}, Ca^{2+} AND Zn^{2+} AS DIVALENT METAL ACTIVATORS

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SUMMARY

1. The influence of pH on the ATPase activities of NaI-treated microsomes from pig brain has been investigated with Mg^{2+}, Mn^{2+}, Ca^{2+} or Zn^{2+} as divalent metal cofactor. Some ouabain-inhibitable ATPase has been found with each of these cations even without addition of alkali metal cations.

2. The pH profile of ouabain-sensitive Mg^{2+}-ATPase varied in different preparations of NaI-microsomes. Some preparations had three pH optima at pH values 5.5, 7.4 and 9.0, corresponding to the pH optima for the K^+-, (Na^+–K^+)- and Na^+-ATPases of the same preparation. 30 mM K^+ inhibited Mg^{2+}-ATPase activity to an extent and with a pH dependence similar to ouabain.

3. ATPase activities with Mn^{2+} were usually similar to those with Mg^{2+}, except that at alkaline pH Mn^{2+} was inhibited by 100 mM Na^+. Na^+ also reduced ouabain-insensitive Mn^{2+}-ATPase activity at high pH.

4. Ca^{2+}-ATPase was optimal at pH 9 and was slightly inhibited by ouabain. Low concentrations of Na^+ stimulated Ca^{2+}-ATPase at pH 7.5, while joint addition of 100 mM Na^+ and 30 mM K^+ stimulated ouabain-inhibitable ATPase activity with a pH optimum of 7.7. 100 mM Na^+ alone inhibited Ca^{2+}-ATPase but the ouabain-sensitivity of Ca^{2+}-ATPase activity was increased in the presence of Na^+.

5. Zn^{2+}-ATPase activity was greatest at pH 5.5 and was partially inhibited by ouabain. 30 mM K^+ inhibited Zn^{2+}-ATPase with a pH-dependence and extent very similar to ouabain at pH values up to 7. Both 4 mM Na^+ and joint addition of 100 mM Na^+ and 30 mM K^+ stimulated ouabain-inhibitable Zn^{2+}-ATPase activity with pH optima of 7.4 and 6–6.5 respectively.

6. NaI-microsomes also had Mg^{2+}- and Ca^{2+}-ITPase activities, but Zn^{2+}-ITPase was very low. Mg^{2+}-ITPase was activated by Na^+ but not by K^+.

INTRODUCTION

The studies of Nakao *et al.*[1] and Fujita *et al.*[2,3] have shown that treatment of microsomal membranes from tissues such as pig brain and rat kidney cortex

Abbreviations: AMPD, 2-amino-2-methylpropane-1,3-diol; Me, stands for metal ion.

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with NaI yields membrane preparations with high ratios of (Mg^{2+}–Na^+–K^+)-ATPase to Mg^{2+}-ATPase activity. Additionally, Fujita *et al.*[2] demonstrated that NaI-treated pig brain microsomes also have ouabain-inhibitable Na^+- and K^+-dependent ATPase activities, each with a pH optimum different from that of the (Na^+–K^+)-dependent ATPase. Although the Na^+-ATPase activity of ox brain microsomes has been attributed to intrinsic bound K^+ (ref. 4), this paper provides evidence that with ATP concentrations in the mM range it is unlikely that either Na^+- or K^+-ATPase activities can be due to endogenous cations.

This paper also reports the occurrence of a new type of NaI-microsomes with ouabain-inhibitable Mg^{2+}-ATPase activity that does not appear to be attributable to endogenous Na^+ or K^+. The ouabain-sensitive Mg^{2+}-ATPase has three pH optima corresponding closely to those found for the K^+-, (Na^+–K^+)- and Na^+-dependent ATPases and it therefore appears that the transport ATPase turns over slowly even in the absence of alkali metal ions.

ATPase activities have also been studied in systems in which Mn^{2+}, Ca^{2+} or Zn^{2+} replaces Mg^{2+}. Ouabain-inhibitable and alkali metal ion-stimulated ATPase activities have been observed with all these abnormal divalent cations, but the pH dependences of these activities sometimes differ from those with Mg^{2+}. Comparison of the ATPase activities occurring in the presence of different divalent cations may be helpful in understanding the mechanism of the transport ATPase.

MATERIALS AND METHODS

Preparation of NaI-microsomes

Pig cerebral cortex was homogenized in 5 vol. ice-cold 0.25 M sucrose, 5 mM EDTA–Tris, 25 mM imidazole–HCl (pH 7.4, 2°) for 4 × 10 sec at maximum speed in an MSE Ato-mix. Microsomes were then isolated by differential centrifugation according to the method of Schwartz *et al.*[5], washed twice with 5 mM EDTA–Tris (pH 7.4) and suspended in distilled water at 2–5 mg protein per ml. Treatment with NaI was modified from the method of Nakao *et al.*[1]. 1 vol. of NaI reagent containing 4 M NaI, 1 mM EDTA–Tris, 4 mM ATP, 5 mM cysteine–HCl and 100 mM Tris–HCl (pH 8.1, 2°), was added at 0–4° to an equal volume of a suspension of microsomes (2–5 mg protein per ml.). After standing for 30 min at 0–4° the solution was diluted to 0.8 M NaI with 0.5 mM EDTA–Tris (pH 7.4) then centrifuged at 30000 × *g* for 30 min. The pellet (referred to below as NaI-microsomes) was washed three times by suspension in 5 mM EDTA–Tris (pH 7.4) and was stored frozen at —17° as a suspension of 1–5 mg protein per ml in distilled water. The yield of NaI-microsomes was 25–35 % of the starting microsomal protein. Neither rhodanese nor succinate dehydrogenase activity was detectable in NaI-microsomes.

Significant ATPase activity was observed with NaI-microsomes in the absence of added divalent cations. This was probably due to endogenous Mg^{2+}. For experiments with Ca^{2+} and Zn^{2+} NaI-microsomes were homogenized in 5 mM EDTA–Tris, 5 mM imidazole–HCl (pH 7.4, 2°) and stored at 0–4° for 12–24 h. The suspension was then centrifuged at 30000 × *g* for 30 min and the pellet of NaI-microsomes was washed twice with distilled water. This treatment reduced ATPase activity without added divalent cation to 0.00–0.10 μmole P_i released per mg protein per h in the presence or absence of 100 mM Na^+ and 30 mM K^+.

NaI-microsomes were assayed for ATPase activity either immediately after preparation or after one cycle of freezing and thawing, usually within 7 days of preparation.

Determination of ATPase activity

ATPase activity was measured at 37° in 1 ml incubation medium containing 25 mM buffer (see below), 3 mM ATP–Tris or 3 mM ATP–2-amino-2-methylpropane-1,3-diol (AMPD) at the pH of the buffer, and 40–500 μg NaI-microsomes. Either 3 mM $MgCl_2$, 2 mM $MnCl_2$, 0.05–3 mM $CaCl_2$, or 1.5 mM $ZnSO_4$ was always present and various concentrations of NaCl and KCl were added as required. 10^{-4} M ouabain was used for determination of ouabain-sensitive ATPase activities. The different concentrations of divalent cations were those for optimal ATPase activity with 3 mM ATP–Tris. Reactions were started by adding NaI-microsomes, and terminated with 0.5 ml. 1.25 M $HClO_4$ (0°). Precipitated protein was removed by centrifugation aud inorganic phosphate (P_i) determined in 1-ml samples by a modification of the method of Fiske and SubbaRow[6].

Buffers

Buffers used in the presence of Mg^{2+}, Mn^{2+} and Ca^{2+} were: acetate–Tris from pH 4.0 to 5.5, maleate–Tris from pH 5.2 to 7.2, Tris–maleate from pH 7.0 to 8.0, Tris–HCl from pH 7.0 to 8.5, AMPD–HCl from pH 8.0 to 10.0, and less frequently, imidazole–HCl from pH 6.2 to 8.0. Similar buffers were used with Zn^{2+}, which forms complexes with AMPD and Tris above pH 7.7, except as follows; Tris–HCl, pH 7.0–7.6; glycylglycine adjusted with NaOH or KOH, pH 7.5–9.0; glycine, adjusted with NaOH or KOH, pH 8.6–10.0. The last two buffers were adjusted to pH with NaOH for determination of Zn^{2+}-ATPase; and with NaOH and KOH for $(Na^+–K^+)$-dependent ATPase with Zn^{2+}, NaCl and KCl being added to give final concentrations of 100 mM Na^+ and 30 mM K^+. Unless otherwise stated the final concentration in the incubation medium was 25 mM with respect to the first named component, the second component being used to adjust to the pH applicable at 37°.

Na^+, K^+ and NH_4^+ in NaI-microsomes

Analysis of Na^+ and K^+ was by flame emission following digestion in concentrated HNO_3. Ammonia was analysed by the method of Vogel[7] after treatment of microsomes with 1% sodium dodecyl sulphate.

Protein

Protein was determined by the method of Lowry *et al.*[8] using crystalline bovine serum albumin, Fraction V (Sigma) as standard.

ATP and ITP

Na_2ATP or Na_2ITP (Sigma) was desalted by passage through a column of Dowex 50 (200–400 mesh, H^+-form), adjusted to the required pH with Tris or AMPD, and stored at —17°. ATP solutions routinely contained 5 mmoles Na^+ per mole ATP.

ATPase activities referred to below are defined as in Fujita *et al.*[2,3], where Me refers to metal ion:

(i) Me^{2+}-, (Me^{2+}–Na^+)-, (Me^{2+}–K^+)-, and (Me^{2+}–Na^+–K^+)-ATPases refer to total ATPase activities in the presence of the divalent cation, Me^{2+}, with the following additions: none, 10 or 100 mM NaCl (see text); 1 mM KCl, 100 mM NaCl *plus* 30 mM KCl.

(ii) Na^+- and K^+ -ATPases are (Me^{2+}–Na^+)- and (Me^{2+}–K^+)-ATPases *minus* the appropriate Me^{2+}-ATPase.

(iii) Ouabain-sensitive Me^{2+}-ATPases are Me^{2+}-ATPase activities *minus* the same activities in the presence of 10^{-4} M ouabain.

(iv) (Na^+–K^+)-ATPases are (Me^{2+}–Na^+–K^+)-ATPases *minus* ATPase activity in the same media with 10^{-4} M ouabain added.

(v) Where (Me^{2+}–Na^+)- or (Me^{2+}–K^+)-ATPases are less than the corresponding Me^{2+}-ATPase, reference is made to Na^+ or K^+ inhibition of the Me^{2+}-ATPase.

RESULTS

The Mg^{2+}-ATPase activity of NaI-microsomes had a complex pH profile and at least two types of NaI preparation were found, as follows. (i) Type α. This type was similar to that described by Fujita *et al.*[2] and was characterized by (a) ouabain-insensitive Mg^{2+}-ATPase with a broad optimum from pH 5 to 6, and (b) ouabain-inhibitable Mg^{2+}-ATPase at pH 9 of less than 5% of total Mg^{2+}-ATPase at that pH (Fig. 1a). (ii) Type β. These NaI-treated microsomes were characterized by (a) total Mg^{2+}-ATPase with three pH optima at pH values 5.5. 7.4 and 9.0 and

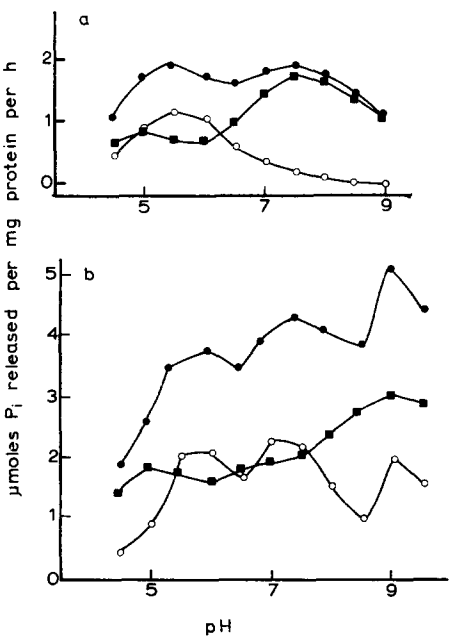

Fig. 1. The pH dependence and ouabain-sensitivity of the Mg^{2+}-ATPase activity of NaI-microsomes (Type α in (a) and Type β in (b)). Incubations were for 30 min at 37° with 310–410 μg protein, 25 mM buffer, 3 mM ATP, 3 mM $MgCl_2$ and 10^{-4} M ouabain when required. Mg^{2+}-ATPase (●); ouabain-inhibitable Mg^{2+}-ATPase (○); ouabain-insensitive Mg^{2+}-ATPase (■). Results are from 12 determinations and S.D. values were less than ± 5%.

(b) ouabain-inhibitable Mg^{2+}-ATPase actvity with the same three pH optima (Fig. 1b).

Among 19 preparations of NaI-microsomes, six were of Type α, 6 of Type β, and seven had intermediate properties and may have been mixtures of the α and β types. The appearance of the different types of NaI-microsomes was not a function of the starting microsomes since among 11 preparations made simultaneously from a single batch of microsomes, 4 were of Type α, 3 of Type β, and 4 were intermediate. Furthermore, neither variation in the time between microsome preparation from 2 h to 2 weeks, nor a single cycle of freezing and thawing of microsomes influenced the type of NaI-treated product.

Clearly, the Na^+ and K^+ contents of the enzyme preparations and assay media might contribute to ouabain-sensitive Mg^{2+}-ATPase activities in the absence of added alkali metal ions. NaI-microsomes always contained less than 65 nmoles Na^+, 15 nmoles K^+, and 10 nmoles NH_4^+ per mg protein and the combined contribution of NaI-microsomes and assay reagents led to final concentrations in the incubation medium not exceeding 0.1 mM Na^+, 0.028 mM K^+ and 0.075 mM NH_4^+. As can be seen in Table I, concentrations of Na^+, K^+ and NH_4^+ of this magnitude did not significantly increase Mg^{2+}-ATPase activity and it therefore seems unlikely that endogenous cations can account for the observed ouabain-sensitive Mg^{2+}- ATPase activities (see also ref. 3). Additionally, ouabain-sensitive Mg^{2+}-ATPase at pH 9.0 has been found to decrease on storage at —17° relative to both the similar activities at pH values 5.5 and 7.4 and to $(Na^+–K^+)$-dependent ATPase. Clearly

TABLE I

EFFECTS OF LOW CONCENTRATIONS OF MONOVALENT CATIONS ON Mg^{2+}-ATPase ACTIVITY

ATPase activities were measured at 37° using 97–411 μg NaI-microsomal protein (Type β) in the presence of 25 mM buffer, 3 mM ATP and 3 mM $MgCl_2$. Monovalent cations (Me^+) were added as chlorides. Results are from 8 determinations ± S.D.

Concentration of Me^+ (mM)	*Increase in ATPase on addition of Me^+ (μmole P_i released per mg protein per h)*			
Me^+:	Na^+ (*pH 7.7*)	Na^+ (*pH 9.0*)	K^+ (*pH 5.5*)	NH_4^+ (*pH 5.5*)
0.02	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.02	0.00 ± 0.01
0.10	0.00 ± 0.02	0.01 ± 0.01	0.02 ± 0.02	0.00 ± 0.01
0.50	0.16 ± 0.03	0.13 ± 0.02	1.00 ± 0.05	0.65 ± 0.04
10.0	7.28 ± 0.14	7.55 ± 0.17		
100	4.62 ± 0.10	2.03 ± 0.04		

Concentration of Na^+: K^+ or NH_4^+ (mM)	*Increase in ATPase activity on addition of Me^+ in the presence of Na^+*			
Me^+:	K^+ (*pH 7.7*)	K^+ (*pH 9.0*)	NH_4^+ (*pH 7.7*)	NH_4^+ (*pH 9.0*)
0.1 : 0.02	0.00 ± 0.02	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
0.1 : 0.1	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.00 ± 0.01
10 : 0.02	0.29 ± 0.02	0.17 ± 0.01	0.10 ± 0.02	0.05 ± 0.01
10 : 0.1	0.76 ± 0.04	0.34 ± 0.02	0.26 ± 0.01	0.14 ± 0.02
100 : 0.1	0.10 ± 0.01	0.08 ± 0.02	0.02 ± 0.02	0.00 ± 0.02

the loss of ouabain-sensitive Mg^{2+}-ATPase (pH 9.0) cannot have been paralleled by a loss of alkali metal ions.

The changes on storage of the Type β NaI-microsomes rendered them nearer in properties to Type α after about 50 days. The possibility that formation of disulphide bonds might be involved in this change has been considered, but the presence of 10 mM oxidized or reduced glutathione (replacing cysteine) in the NaI reagent did not appear to affect the type of NaI preparation obtained. However, it is possible that Type β NaI-microsomes contain a metastable form of either the transport ATPase or the membranes containing the enzyme, and that disulphide bonds are not involved.

Mn^{2+}-ATPase was generally similar to Mg^{2+}-ATPase in both microsomes and NaI-microsomes and the latter could be classified as α or β with Mn^{2+} in the same way as with Mg^{2+}.

(Na^+–K^+)-, Na^+- and K^+-ATPase activities with Mg^{2+} and Mn^{2+}

NaI-microsomes always had bell-shaped pH profiles for both Mg^{2+}- and Mn^{2+}-supported (Na^+–K^+)-ATPase activities, with pH optima at pH 7.7 with Mg^{2+}

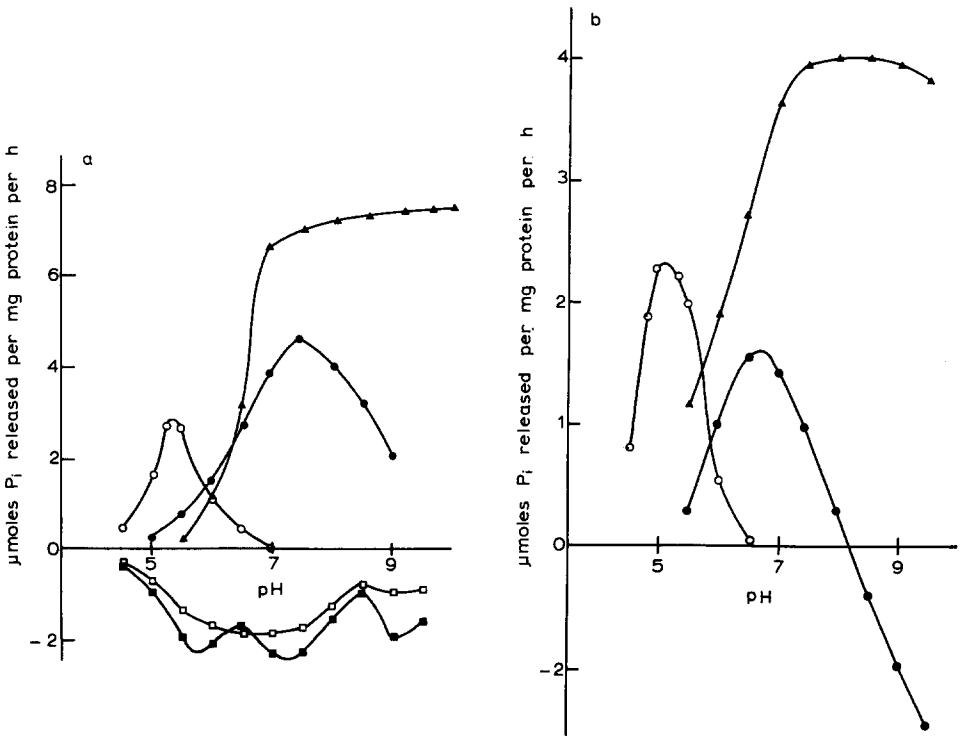

Fig. 2. The pH dependence of the Na^+- and K^+-stimulated, and ouabain- and K^+-inhibited ATPase activities of NaI-microsomes in the presence of Mg^{2+} (a) and Mn^{2+} (b). Incubations were for 30 min at 37° with 150–465 µg. NaI-microsomal protein (Type β). Media contained 25 mM buffer, 3 mM ATP, and 3 mM $MgCl_2$ (a) or 2 mM $MnCl_2$ (b). Results are from 8 determinations made within 5 days of NaI-microsome preparation. ATPase stimulated by addition of 1 mM KCl (○); 10 mM NaCl (▲); 100 mM NaCl (●); and inhibition of ATPase activity by 30 mM KCl (□); 10^{-4} M ouabain (■) is shown. S.D. values were less than ± 5%.

and pH 7.5–8.0 with Mn^{2+}. For a typical preparation maximum $(Na^+–K^+)$-ATPase activities were 87.0 μmoles P_i released per mg protein per h with Mg^{2+} and 34.5 μmoles P_i released per mg protein per h with Mn^{2+}.

Fig. 2a illustrates the modifying effects of Na^+ and K^+ on the Mg^{2+}-ATPase activity of Type β NaI--microsomes, when the cations were added singly. 1 mM K^+ stimulated a K^+-ATPase which was optimal at pH 5.5 and inhibited by 10^{-4} M ouabain. However, 30 mM K^+ partially inhibited Mg^{2+}-ATPase, the pH dependence and extent of this K^+ inhibition being similar to that of ouabain. These effects of K^+ were similar in Type α preparations.

Fujita *et al.*[2,3] reported that Na^+-dependent Mg^{2+}-ATPase was greatest at 100 mM Na^+ at pH 9.0. We have obtained similar results with Type α preparations, but with Type β NaI-microsomes the optimum Na^+ concentration was only 10 mM, 100 mM Na^+ giving less activity and having a pH optimum of 7.7 (Fig. 2a). The Na^+-ATPase of preparations of Types α and β also differed in that in Type β Na^+-ATPase was greater both in absolute magnitude and in relation to $(Na^+–K^+)$-ATPase activity.

Interestingly, increasing concentrations of Na^+ decreased ouabain-insensitive Mg^{2+}-ATPase activity was well as stimulating Na^+-ATPase. In one typical Type β preparation, Mg^{2+}-ATPase in the presence of 10^{-4} M ouabain was reduced by 100 mM Na^+ by 14% at pH 7.7 and by 21% at pH 9.0. This effect was barely detectable in type α preparations.

In the presence of Mn^{2+}, effects of Na^+ and K^+ on ATPase activity were generally similar to those with Mg^{2+}, except that K^+-ATPase was optimal at pH 5.0, not 5.5, and that 100 mM Na^+ inhibited Mn^{2+}-ATPase in the pH range 8.2–9.5 (Fig. 2b). Remarkably, even though 100 mM Na^+ substantially reduced Mn^{2+}-ATPase it increased ouabain-inhibitable Mn^{2+}-ATPase activity by about 30% (Table II). One explanation for this would be that Na^+ renders an otherwise ouabain-insensitive fraction of Mn^{2+}-ATPase sensitive to the glycoside, although it is also possible that Na^+ simultaneously stimulates a ouabain-sensitive $(Mn^{2+}–Na^+)$-ATPase and inhibits a second unrelated enzyme.

TABLE II

INHIBITION OF Mn^{2+}- AND ENHANCEMENT OF OUABAIN-SENSITIVE Mn^{2+}-ATPase ACTIVITIES BY Na^+

Incubations were with 463 μg NaI-microsomal protein (Type β) at 37° for 20 min. Media contained 25 mM AMPD–HCl (pH 9.0), 3 mM ATP–AMPD (pH 9.0), 2 mM $MnCl_2$ and additions as shown. Three experiments are given, each as the average of triplicate determinations.

Additions to assay media	*ATPase activities (μmole P_i released per mg protein per h)*		
	Expt. A	*Expt. B*	*Expt. C*
None	5.42	5.28	5.40
100 mM NaCl	4.40	4.64	4.73
10^{-4} M ouabain	3.53	3.81	3.81
100 mM NaCl, 10^{-4} M ouabain	1.98	2.61	2.63
Ouabain-sensitive activity without Na^+	1.89	1.47	1.59
Ouabain-sensitive activity with Na^+	2.42	2.03	2.10

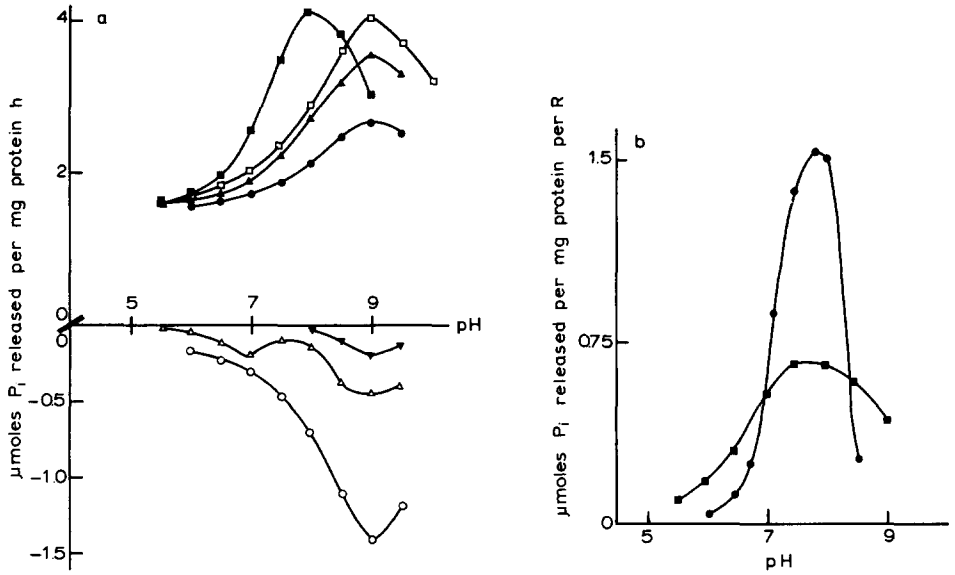

Fig. 3. Effects of Na^+, K^+, and ouabain on the Ca^{2+}-ATPase activity of NaI-microsomes (Type β). Incubations were for 30 min at 37° with 310 μg protein. Media contained 25 mM buffer, 3 mM ATP, and (normally) 3 mM $CaCl_2$. In (a) additions were none (□); 100 mM NaCl (●); 30 mM KCl (▲); or 100 mM NaCl *plus* 30 mM KCl (■). Inhibitory effects of added 30 mM KCl (△); 100 mM NaCl (○); and 10^{-4} M ouabain (▼) are also shown and represent activities in the presence of these agents after subtraction of Ca^{2+}-ATPase activity (□) in each case. (b) shows stimulation of Ca^{2+}-ATPase activity by addition of 2 mM NaCl (■) and ouabain-inhibitable ATPase activity in the presence of 100 mM NaCl, 30 mM KCl, and 0.05 mM $CaCl_2$ (●). Results are means of 6–10 determinations and S.D. values were less than ± 5%.

ATPase activities with Ca^{2+}

Fig. 3 illustrates the pH dependence of the Ca^{2+}-ATPase activity of Type β NaI-microsomes. Ca^{2+}-ATPase was inhibited by 100 mM Na^+, with maximum effect at pH 9.0 and, as with Mn^{2+}, the inhibitory effect of 10^{-4} M ouabain was greater with than without Na^+ (Table III).

Ca^{2+}-ATPase was slightly stimulated by low Na^+ concentrations, the greatest effect being with 2 mM Na^+ at pH 7.5 (Fig. 3b). This stimulation was abolished by 10^{-4} M ouabain (Table III) and could not be accounted for by contaminating K^+ or NH_4^+. K^+ inhibited Ca^{2+}-ATPase to a somewhat lesser extent than Na^+ in an effect which was saturated at 30 mM K^+ and showed two pH optima at pH values 7 and 9.

The joint presence of 100 mM Na^+ and 30 mM K^+ gave rise to a ouabain-sensitive (Na^+–K^+)-ATPase with Ca^{2+} with an optimum at pH 7.7. Lineweaver–Burk plots of the dependence of ATPase activities on Ca^{2+} concentration (at a constant 3 mM ATP) gave approximate K_m values for Ca^{2+} of 0.008 and 0.12 mM in activation of (Ca^{2+}–Na^+–K^+)- and Ca^{2+}-ATPases respectively, compared with K_m values for Mg^{2+} of 0.59 and 1.5 mM in the corresponding (Mg^{2+}–Na^+–K^+)- and Mg^{2+}-ATPases at pH 7.7. Surprisingly, at equimolar concentrations of divalent cation up to 0.015 mM (Na^+–K^+)-ATPase activity was greater with Ca^{2+} than Mg^{2+} (Fig. 4) indicating that the activity with Ca^{2+} cannot have been due to contaminating

TABLE III

EFFECTS OF Na^+, K^+ AND OUABAIN ON Ca^{2+}- AND Zn^{2+}-ATPase ACTIVITIES OF NaI-MICROSOMES (TYPE β)

Incubations were with 463 μg protein for 10 min at 37°. Media contained 25 mM buffer, 3 mM ATP, and 3 mM $CaCl_2$ or 1.5 mM $ZnSO_4$ with additions as shown. Results are means of 6 determinations and S.D. values were less than ± 5 %.

Divalent metal	*pH*	*10^{-4} M ouabain, absent (—), present (+)*	*ATPase activity (μmoles P_i released per mg protein per h)*			
			Additions: *None*	*2 mM NaCl*	*100 mM NaCl*	*30 mM KCl*
Ca^{2+}	9.0	—	4.14	4.72	3.18	3.71
	9.0	+	4.08	4.04	2.42	3.72
	7.4	—	2.29	3.05	1.87	2.15
	7.4	+	2.27	2.28	1.50	2.18
Zn^{2+}	5.5	—	3.85	4.46	4.13	2.27
	5.5	+	1.98	1.94	1.67	2.00
	7.0	—	2.68	4.72	2.89	1.61
	7.0	+	1.61	1.59	1.51	1.58

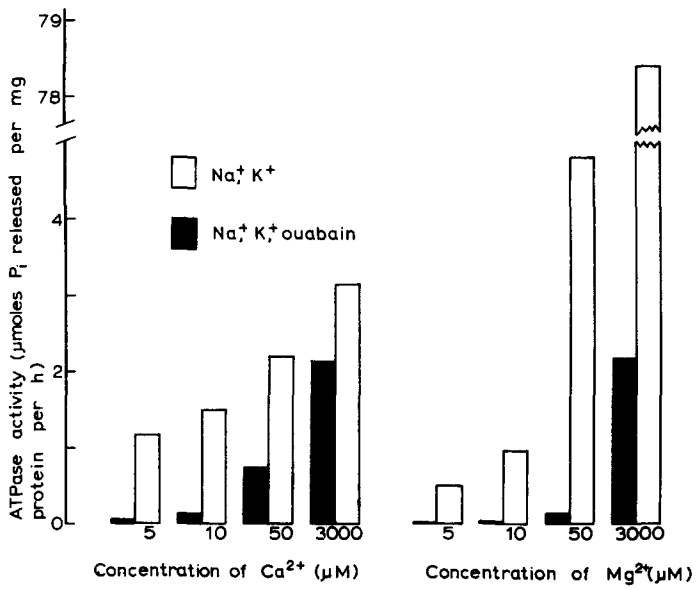

Fig. 4. ATPase activities of NaI-microsomes (Type β) in the presence of low concentrations of Ca^{2+} and Mg^{2+}. Incubations were with 10–4000 μg protein for 30 or 60 min at 37°. Media contained 25 mM Tris–HCl (pH 7.7), 3 mM ATP–Tris (pH 7.7), 100 mM NaCl, 30 mM KCl; $CaCl_2$, $MgCl_2$ and 10^{-4} M ouabain were present as indicated. Results are means of 6 determinations and S.D. values were less than ± 5 % except where activities were less than 1 μmole P_i released per mg protein per h.

Mg^{2+}. However, the extrapolated v_{max} for (Ca^{2+}–Na^+–K^+)-ATPase was substantially less than for the activity with Mg^{2+}.

The explanation for the low optimum Ca^{2+} concentration (0.02–0.05 mM) for (Na^+–K^+)-ATPase activity could lie in the low apparent K_m for Ca^{2+} in both

the (Na^+–K^+)-dependent and independent ATPase activities. At Ca^{2+} concentrations near the K_m for (Ca^{2+}–Na^+–K^+)-ATPase activity Ca^{2+}, presumably as Ca–ATP^{2-}, may be bound chiefly in a Na^+-dependent fashion yielding an enzyme–substrate complex susceptible to K^+-catalysed hydrolysis. However, at Ca^{2+} concentrations greater than the K_m for Ca^{2+}-ATPase Ca–ATP^{2-} may be bound in a Na^+-independent way leading to a K^+-insensitive enzyme–substrate complex. This suggestion is in agreement with the finding of Epstein and Whittam[10] that Ca–ATP^{2-} inhibits Mg^{2+}-dependent (Na^+–K^+)-ATPase by competing with Mg–ATP^{2-} and it is probably significant that the apparent K_m for Ca^{2+} in Ca^{2+}-ATPase (0.12 mM) is near to the apparent K_i for Ca^{2+} inhibition of (Mg^{2+}–Na^+–K^+)- ATPase (0.1 mM) in pig brain NaI-microsomes (unpublished results).

ATPase activities with Zn^{2+}

Zn^{2+}-ATPase activity of NaI-microsomes had a pH optimum of 5.5 with a shoulder at pH 7–7.5 (Fig. 5a). Addition of K^+ inhibited Zn^{2+}-ATPase, 30 mM K^+ having the maximum effect. The extent and pH dependence of inhibition by 30 mM K^+ corresponded closely to the effect of 10^{-4} M ouabain up to pH 7 (Fig. 5b).

4 mM Na^+ stimulated Zn^{2+}-ATPase with greatest effect at pH 7.5, while 100 mM Na^+ stimulated very slightly at pH values below 7 and inhibited at alkaline

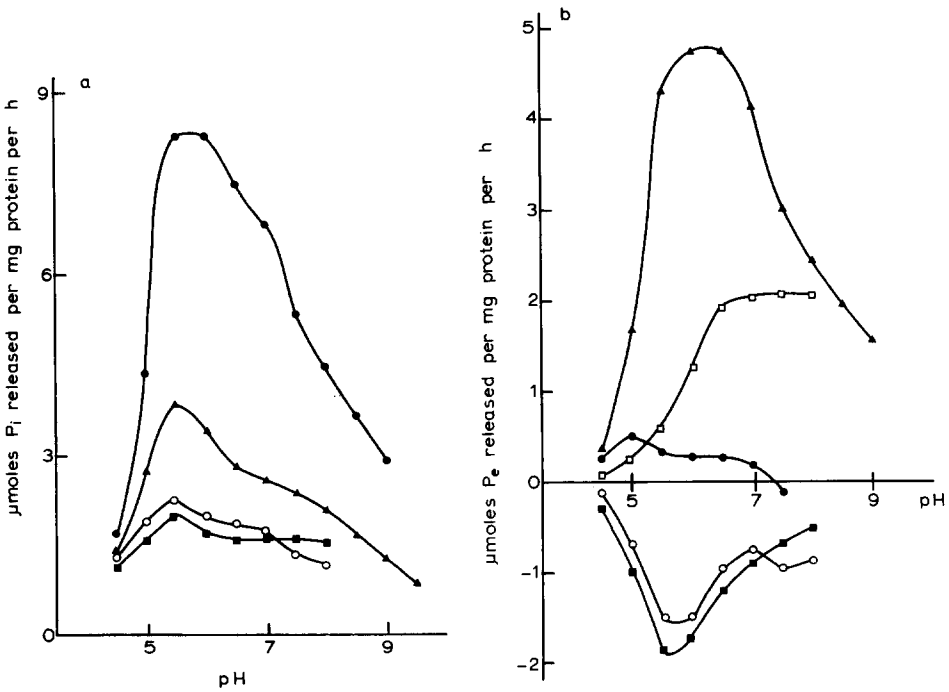

Fig. 5. Effects of Na^+, K^+, and ouabain on the Zn^{2+}-ATPase activity of NaI-microsomes (Type β). Incubations were for 10 or 15 min at 37° with 370 or 430 μg protein. Media contained 25 mM buffer, 3 mM ATP, and 1.5 mM $ZnCl_2$. In (a) additions were none (▲); 100 mM NaCl *plus* 30 mM KCl (●); 30 mM KCl (○); and 10^{-4} M ouabain (■). (b) shows the stimulation of ATPase activity on addition of 4 mM NaCl (□); 100 mM NaCl (●); or 100 mM NaCl *plus* 30 mM KCl (▲); together with ATPase activity inhibited by addition of 30 mM KCl (○); or 10^{-4} M ouabain (■). Results are means of 8 determinations and S.D. values were less than ± 5%.

pH values (Fig. 5b, Table III). In the presence of 100 mM Na^+, 10^{-4} M ouabain reduced ATPase activity to a level less than that in the absence of Na^+ at both pH 5.5 and pH 7.0 (Table II). This effect is similar to that seen with Mn^{2+} and Ca^{2+}.

The joint presence of 100 mM Na^+ and 30 mM K^+ stimulated Zn^{2+}-ATPase to a greater extent than Na^+ or K^+ alone. This effect was greatest at pH 6.0–6.5 (Fig. 5b) which is considerably more acidic than the optima for $(Na^+–K^+)$-ATPase activities with other divalent cations. $(Na^+–K^+)$-ATPase in the presence of Zn^{2+} has also been observed by Quigley and Gotterer[11] in membranes from intestinal mucosal cells.

TABLE IV

ITPase ACTIVITIES OF NaI-MICROSOMES WITH Mg^{2+}, Ca^{2+} AND Zn^{2+}

Incubations were with 100–700 μg NaI-microsomal protein (Type β) at 37°. Media contained 25 mM buffer, 3 mM ITP titrated with Tris or AMPD to the pH required, and 3 mM $MgCl_2$, 3 mM $CaCl_2$ or 1.5 mM $ZnCl_2$. 10 or 100 mM NaCl, 1 or 30 mM KCl and 10^{-4} M ouabain were added where indicated. Results are means of 6 determinations ± S.D. Concentrations of NaCl and KCl were 100 mM and 30 mM, respectively when present together, and 10 and 1 mM, respectively when only one alkali metal salt was present.

Divalent cation	*pH*	*Type of triphosphatase activity*	*Triphosphatase activity (μmoles P_i released per mg protein per h)*	
		Substrate:	*ITP*	*ATP*
Mg^{2+}	5.5	Mg^{2+}-	0.2 ± 0.1	3.5 ± 0.1
	7.4		4.0 ± 0.1	3.9 ± 0.1
	9.0		4.9 ± 0.2	4.6 ± 0.1
	5.5	Ouabain-sensitive Mg^{2+}-	0.0 ± 0.1	2.0 ± 0.1
	7.4		2.0 ± 0.1	2.2 ± 0.1
	9.0		1.9 ± 0.1	1.9 ± 0.1
	5.5	K^+-	0.0 ± 0.1	2.7 ± 0.1
	7.7	Na^+-	8.1 ± 0.3	7.3 ± 0.2
	9.0	Na^+-	8.8 ± 0.2	7.6 ± 0.2
	7.7	$(Na^+–K^+)$-	0.2 ± 0.2	80.4 ± 3.2
Ca^{2+}	7.4	Ca^{2+}-	2.4 ± 0.1	2.3 ± 0.1
	9.0		4.6 ± 0.2	4.1 ± 0.1
Zn^{2+}	5.5	Zn^{2+}-	0.2 ± 0.2	3.9 ± 0.2
	7.0		0.2 ± 0.1	2.6 ± 0.1

ITPase activities with Mg^{2+}, Ca^{2+} and Zn^{2+}

Table IV compares the ATPase and ITPase activities of NaI-microsomes in the presence of Mg^{2+}, Ca^{2+} and Zn^{2+}. Important differences are that whereas both triphosphatase activities are similar with Ca^{2+} and Mg^{2+} at pH 7.4 and 9.0, Mg^{2+}-ITPase is much less than the ATPase at pH 5.5. In addition, neither $(Na^+–K^+)$- nor K^+-ITPase activities were found with Mg^{2+}, but Na^+-ITPase was slightly greater than the Na^+-ATPase activity. Very little Zn^{2+}-ITPase was observed at pH 5.5 or 7.0.

The results probably indicate that Na^+ and ITP are handled by the transport ATPase in the same way as Na^+ and ATP, but that normal K^+-catalysed hydrolysis cannot occur with ITP. Schoner *et al.*[12] also found a reduced catalytic effect of K^+

with ITP as substrate, but these workers did find some (Na^+–K^+)-ITPase activity in their preparation.

DISCUSSION

Hydrolysis of ATP by the (Na^+–K^+)-dependent transport ATPase is generally thought to take place in at least two steps, the first dependent of Na^+, the second on K^+. The intermediate stage is probably a phosphorylated enzyme[13,14] although a change in conformation of the enzyme is no doubt also involved. Fujita *et al.*[3] have argued that since the Na^+-, K^+- and (Na^+–K^+)-dependent ATPases of NaI-microsomes are all similarly inhibited by ouabain and other agents, they are properties of the same enzyme. If this is so, then (Mg^{2+}–Na^+)-ATPase activity at pH 7–9 may represent normal Na^+-dependent substrate binding and change to the intermediate stage, followed by abnormal hydrolysis, while (Mg^{2+}–K^+)-ATPase activity may involve a normal K^+-dependent hydrolytic step preceded by abnormal substrate binding. The pH optimum for (Mg^{2+}–K^+)-ATPase is well below that for (Mg^{2+}–Na^+–K^+)-ATPase activity. This could be because a conformational change occurring on phosphorylation or simple binding of ATP, Na^+ and Mg^{2+} to the enzyme changes the pK of an amino acid residue at the catalytic centre. A transition that would fit the pH dependences observed would be separation of a thiol–imidazole system (with an acidic pK, ref. 15) to give a free imidazole group with a pK nearer to 7.

The three pH optima for ouabain-inhibitable Mg^{2+}- and Mn^{2+}-ATPase activities of Type β NaI-microsomes correspond remarkably well with the optima of the K^+-, (Na^+–K^+)- and Na^+-ATPases. Contaminating alkali metal ions do not appear to be responsible for these activities, therefore, they probably reflect a slow turnover of the transport ATPase in the absence of these ions. Similarly, the small ouabain-sensitive ATPases with Ca^{2+} and Zn^{2+} also probably represent operation of the enzyme under sub-optimal conditions. Restriction of Ca^{2+}-ATPase to alkaline pH values may indicate that Ca–ATP^{2-} can be bound to the enzyme in a similar way to Mg–ATP^{2-} with Na^+, while the low K^+- and (Na^+–K^+)-ATPases with Ca^{2+} presumably reflect the unsuitability of bound Ca–ATP^{2-} for K^+-catalysed hydrolysis. On the other hand, Zn-ATP^{2-} is apparently poorly bound by the enzyme at alkaline pH (K_m values for Me^{2+} in both Me^{2+}- and (Me^{2+}–Na^+–K^+)-ATPases decrease in the order, $Zn^{2+} > Mg^{2+} > Ca^{2+}$) but is readily hydrolysed at acid pH even in the absence of K^+. Inhibition of Zn^{2+}-ATPase by K^+ seems anomalous, but may occur because an inhibitory effect of K^+ on binding of Zn–ATP^{2-} to the enzyme outweighs any stimulatory effect of K^+ on hydrolysis. A similar inhibitory effect of K^+ on Mg^{2+}-ATPase was seen at neutral pH.

The different ATPase activities with the various divalent cations presumably reflect differences in the metal–ATP complexes. Evidence that such differences exist has been provided by Schneider *et al.*[16]. Their ultraviolet studies of aqueous solutions of ATP indicate that interaction with the N-7 of the adenine nucleus, to give a folded Me–ATP^{2-} complex, is much greater with Zn^{2+} than with Mg^{2+} or Ca^{2+}. Also infrared spectra of aqueous solutions led Brintzinger[17,18] to conclude that Zn^{2+} forms a predominantly 'inner sphere' complex with an oxygen atom of the γ-phosphate group of ATP, whereas 'outer sphere' complexes (with coordination of the metal ion *via* water to the oxygen atom) were present in greater proportion with Mg^{2+} and Mn^{2+} and particularly with Ca^{2+}.

It is possible that these inherent differences in $Me-ATP^{2-}$ complexes are recognized by the transport ATPase, the unfolded 'outer sphere' $Me-ATP^{2-}$ conformation being preferentially bound to the enzyme, and the folded form being more readily hydrolysed. The suitability of Mg^{2+} as cofactor would thus rest with its ability to support both ATP conformations during interaction with the enzyme.

Clearly the above suggestion is not the only possible explanation for our results. However, such a system could readily account for the transport of Na^+ and K^+ if the ATPase has access to both sides of the membrane and changes its conformation during activity in a way that exposes the alkali metal ion binding sites to alternate sides. Models of this type have been suggested by Jardetzky[19] and Lowe[20].

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