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INFLUENCE OF MAGNESIUM IN THE HOMOGENIZATION MEDIUM ON THE STATE OF A DIVALENT CATION-STIMULATED, DIETHYLSTILBESTROL-SENSITIVE ADENYLNUCLEOTIDYL PHOSPHATASE ACTIVITY FROM PEA STEM MICROSOMAL PREPARATIONS

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The amount of divalent cation-activated, diethylstilbestrol-sensitive adenylnucleotidyl phosphatase activity recovered in the 'microsomes' (13 000–80 000 × *g* sediment) from pea stem tissue is strongly influenced by the concentration of Mg^{2+} in the homogenization medium. The absence of Mg^{2+} during homogenization results in a marked decrease of the activity found in the microsomal fraction, compensated by its increase in the soluble fraction. Part of the solubilized activity becomes sedimentable at 80 000 × *g* upon addition of 5–10 mM Mg^{2+} (or Mn^{2+}, Ca^{2+}, Zn^{2+}) to the supernatant. This sediment shows a very high specific activity, and can be re-solubilized by treatment with either EDTA or 0.3 M monovalent salts, or deoxycholate. When the supernatant containing the solubilized activity is incubated together with low-adenylnucleotidyl phosphatase microsomes and with 10 mM $MgCl_2$ the activity recovered in the sediment is much larger than the sum of the activity of the microsomes plus that of the sediment obtained by incubating the same supernatant with Mg^{2+}. Microsomes prepared with Mg^{2+} in the homogenization medium do not show this effect. The supernatant/microsomes saturation curves as well as a change of the temperature coefficient of the activity following combination of the soluble preparation with the microsomal particles suggest an at least partial reconstitution of the original enzyme-membrane structure.

Introduction

Preliminary attempts to optimize the conditions for the study of the relationships between ATPase and fusicoccin binding in plant cell membrane preparations showed that the composition of the grinding medium influences the distribution of these activities among the homogenate fractions obtained by differential centrifugation [1,2]. In particular, large effects of the presence of Mg^{2+} and/or EDTA on the distribution of pea stem microsomal ATPase activity were observed [2].

Recent work in this laboratory also showed that the main ATPase present in the pea stem microsomal fraction (namely, the fraction sedimenting between 13 000 and 80 000 × *g*, containing plasmalemma together with other membranes but almost devoid of mitochondrial fragments) is a Mg^{2+} (or Ca^{2+} or Mn^{2+})-dependent, diethylstilbestrol and orthovanadate-sensitive enzyme, attacking, besides ATP, other nucleoside triphosphates, and, among nucleoside diphosphates, ADP [3,4]. Although the localization of this enzyme at the plasmalemma and its possible role in ion transport are still open to investigation, its very high activity in the tissue homogenates and some of its

Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium lauryl sulfate.

characteristics seem worthy of attention, and more information in this regards appears desirable *.

The present investigation deals with the effects of Mg^{2+} on the state of this enzyme in a cell free system. In fact, the importance of Mg^{2+} concentration in the medium in maintaining the original characteristics of membrane ATPase from a number of other materials is well established [5,6]. The results presented in this paper show that this is also true for the pea stem microsomal enzyme.

Materials and Methods

Pea (*Pisum sativum*, cv. Alaska) seeds were germinated in poplar sawdust at 26°C in the dark and, after 4 to 5 days, subapical internode segments approx. 1 cm long were harvested, washed in cold distilled water and immediately homogenized.

Homogenization and cell fractionation

The excised stem segments were ground in a mortar at 4°C in a medium (5 ml/g of tissue) consisting of 250 mM sucrose and 20 mM Hepes (pH 7), with or without the addition of 10 mM $MgCl_2$. The homogenate was strained through three layers of cheesecloth and, for routine preparations, the suspension was centrifuged at 13 000 × *g* for 10 min and the supernatant was centrifuged at 80 000 × *g* for 20 min to yield the 'microsomal' fraction (13–80 K pellet). The supernatant from the 80-K run was further centrifuged for 60 min at 220 000 × *g* to yield the 'soluble' fraction (220-K supernatant).

Otherwise, in order to obtain a more detailed cell fractionation, the original suspension was successively submitted to the following series of centrifugations: 1 000 × *g* for 10 min (0–1 K pellet), 8 000 × *g* for 10 min (1–8 K pellet), 13 000 × *g* for 10 min (8–13 K pellet), 80 000 × *g* for 20 min (13–80 K pellet), and 220 000 × *g* for 60 min (80–220 K pellet and 220-K supernatant).

In the following text the microsomal (13–80 K pellet) and the 220-K supernatant are referred to as 'high Mg^{2+}' microsomes, 'high Mg^{2+}' supernatant, or 'low Mg^{2+}' microsomes, 'low Mg^{2+}' supernatant, according to whether 10 mM Mg^{2+} was present or not in the homogenization medium.

The sedimentation of the enzyme from the 'low Mg^{2+}' supernatant was obtained as follows: 2–2.5 ml aliquots of 'low Mg^{2+}' supernatant were supplemented with $MgCl_2$ or, eventually, with other divalent cations, incubated for 30 min at 0°C and centrifuged at 80 000 × *g* for 20 min. The resulting sediment is referred to as Mg^{2+} sediment. When the precipitation by Mg^{2+} of the enzyme was performed in the presence of an aliquot of the 'microsomal' fraction the resulting sediment is referred to as microsomes-Mg^{2+} sediment.

The solubilization of the Mg^{2+} sediment was achieved by resuspending the sediment in a 250 mM sucrose, 20 mM Hepes solution (pH 7) containing 5 mM EDTA, or NaCl of KCl of $NaClO_4$ at a final 0.3 M concentration, or sodium deoxycholate at a 6 mM concentration. The mixtures were allowed to stand at 25°C for 30 min and were afterwards centrifuged at 80 000 × *g* for 20 min.

When the microsomal preparations and the Mg^{2+} sediment were to be prepared for the enzyme activity assay the pellets were resuspended in appropriate amounts of the same homogenization medium (Mg^{2+}-free) so as to obtain a final protein concentration 20 to 60 μg/ml for the 13–80 K pellets and 4 μg/ml for the Mg^{2+} sediment. The 'soluble' fractions were also diluted with the same buffer to give a final protein concentration of about 80 μg/ml.

Enzyme assays

The incubation mixture for the assay of the activity on ATP, or the other nucleoside phosphates was routinely composed of 100 μl of extract, 10 μmol KCl, 1 μmol $MgCl_2$, 1 μmol nucleoside phosphate (sodium salt) in a final 1 ml volume of 250 mM sucrose, 10 mM Hepes buffer (pH 6). The reaction was started by the addition of the substrate and was carried out for 20 min at 28°C. In the experiments on the effects of temperature the protein content in the assay mixture was 4- to 10-times higher and the reaction was stopped after incubation for 3 or 5 min at the different temperatures. The released phosphate

* Because of its higher activity in hydrolysing ADP than ATP, the enzyme investigated here is better described as an adenylnucleotidyl phosphatase than as an ATPase. In most of the experiments described in the text, however, the activity was measured with ATP as a substrate and the relative data are thus presented as values of ATPase activity.

was determined by the colorimetric procedure of Tausski and Shorr [7] partially modified: briefly, to the 1 ml incubation mixture 2 ml of 0.375 M H_2SO_4 containing 0.75% ammonium molybdate, 3% $FeSO_4$ and 0.75% SDS were added, and the increase in absorbance at 740 nm was measured.

The incubation mixture for *p*-nitrophenylphosphatase activity was the same as for the ATPase assay, but for the absence of magnesium and for the much higher amount of active preparation added (approx. 250 μg protein for the 'soluble' fractions, 30 to 45 μg for the 13–80 K pellets and 20 μg for the Mg^{2+} sediment). The reaction was started by the addition of 1 μmol *p*-nitrophenylphosphate and was carried out at pH 5 for 60 min in a 2 ml final volume at 28°C. The reaction was stopped by the addition of 1 ml 0.3 M NaOH and the *p*-nitrophenylphosphate hydrolyzed was measured by evaluating the absorbance at 400 nm [8].

Protein assay

Protein was determined according to Lowry et al. [9], with crystalline bovine serum albumin as a standard.

Results and Discussion

Effects of Mg^{2+} in the homogenization medium on the distribution of ATPase activity among the homogenate fractions

The data of Table I show that the presence of 5 mM Mg^{2+} in the grinding medium induces the following changes: (1) Mg^{2+} increases by approx. 30% the protein contents in the 'heavy' (0 to 13-K) fractions, and decreases it by an equivalent amount in the 'light' or 'microsomal' (13 to 220 K) fractions. Neither the amount of protein remaining in the cheesecloth (cell walls, fragments of various nature) nor that in the total particulate fraction (0 to 220-K) or the 220-K supernatant are significantly changed.

(2) The presence of Mg^{2+} in the grinding medium does not significantly influence the total (particulate plus soluble) ATPase activity. However it induces in the 220-K supernatant a dramatic decrease (−50%),

TABLE I

EFFECTS OF Mg^{2+} IN THE GRINDING MEDIUM ON THE DISTRIBUTION OF PROTEIN AND ATPase ACTIVITY AMONG THE HOMOGENATE FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION

Experimental conditions as described in Methods. Specific activity in μmol P_i per h per mg of protein. Data are the average of three experiments run in triplicate.

	Protein (mg per g fresh wt.)		ATPase activity			
			μmol P_i per h per g fresh wt.		Spec. act. [a]	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
Cheesecloth-retained fragments	0.93	0.90				
'Heavy' 0–1 K	0.29	0.41	81	64	279	156
(A) 1–8 K	0.34	0.47	46	36	135	76
8–13 K	0.13	0.16	34	48	261	300
'Light' 13–80 K	0.51	0.31	26	84	50	270
(B) 80–220 K	0.21	0.14	62	69	295	492
220-K supernatant (C)	2.24	2.19	146	75	65	34
Sum A	0.76	1.04	161	148		
Sum B	0.72	0.45	88	153		
Sum A + B + C	3.72	3.68	395	376		

[a] In μmol P_i per h per mg protein in the fraction considered.

almost completely accounted for by an increase in the 'light' particulate fractions, with little change in the 'heavy' fractions. The Mg^{2+}-induced shift of ATPase activity from the soluble to the particulate fractions is quantitatively more important than the shift of protein material. Consequently, the presence of Mg^{2+} in the grinding medium markedly increases the ATPase specific activity in the 'light' microsomal fractions and decreases it in the 'soluble' one (plus 200% and minus 50%, respectively).

Reversible effect of Mg^{2+} and other divalent cations in sedimenting the ATPase activity present in the supernatant from tissue ground in the absence of Mg^{2+}

The concentration of endogenous Mg^{2+} in the 220-K supernatant from the homogenate prepared without Mg^{2+} in the medium is approx. 0.9 mM. The data of Fig. 1 show that when this supernatant is supplemented with $MgCl_2$ a consistent fraction of

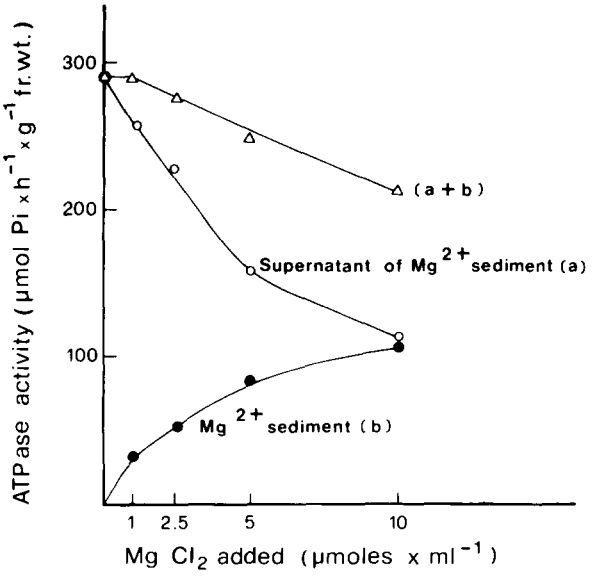

Fig. 1. Effects of the addition of $MgCl_2$ in making sedimentable the ATPase activity of the 'low Mg^{2+}' supernatant. The concentration of endogenous Mg^{2+} in the original 'low Mg^{2+}' supernatant, as determined by atomic absorption spectroscopy, was 0.9 mM and this value should be summed up with the values of the added Mg^{2+} (indicated in abscissa) to give the real final Mg^{2+} concentration. For other details and conditions see Materials and Methods. Data are the means of three separate experiments run in triplicate.

ATPase activity becomes insoluble and can be recovered by centrifugation at 80 000 × *g*. The amount of sedimented activity increases with the increase of Mg^{2+} concentration up to 10 mM. At higher Mg^{2+} concentrations an apparently irreversible inactivation of the enzyme becomes apparent. With 10 mM Mg^{2+} the fraction of ATPase activity sedimented is 30–50%. Differential centrifugation experiments show that approx. 80% of the Mg^{2+}-insolubilized ATPase activity sediments between 13 000 and 80 000 × *g*. The 0–80 K ATPase-rich sediment obtained by treatment with 10 mM Mg^{2+} is referred to in the following as Mg^{2+} sediment.

Table II allows a comparison of the protein contents and the ATPase activity of the Mg^{2+} sediment and of its supernatant with those of the microsomal (13–80 K) and of the 'soluble' fractions obtained by homogenization of the tissue either in the presence or in the absence of 10 mM Mg^{2+}. It is seen that the ATPase specific activity of the Mg^{2+} sediment is approx. 4-times higher than in the 'high Mg^{2+}' microsomes, 14-times higher than that of the 'low Mg^{2+}' microsomes and 12-times higher than that of the supernatant from which it has been obtained 'low Mg^{2+}' supernatant). The formation of the Mg^{2+} sediment seems thus to correspond to a rather specific aggregation of ATPase-rich components present in the 'soluble' fraction obtained by homogenization in the absence of Mg^{2+}. Also other divalent cations, such as Ca^{2+}, Mn^{2+} and Zn^{2+}, when added to the 'low Mg^{2+}' supernatant at 10 mM concentration, induced a sedimentation of the ATPase activity approximately equal or even larger than that induced by 10 mM Mg^{2+}; K^+ and Li^+ at concentration up to 100 mM were completely ineffective (data not shown).

The data of Table III show that a small fraction only of the Mg^{2+} sediment is solubilized when resuspended in plain buffer (Mg^{2+} free). However, substantial re-solubilized was observed upon resuspension of the Mg^{2+} sediment in 5 mM EDTA, or 0.3 M NaCl or $NaClO_4$, or KCl (data not shown), or (to a lesser extent), in 6 mM sodium deoxycholate. NaCl and deoxycholate induced a significant increase of total (soluble plus insoluble) activity, possibly due to the exposure of catalytic sites masked in the Mg^{2+} sediment particles. The data suggest that the Mg^{2+} sediment results from the formation of Mg^{2+}-protein complexes, which can be resolved in smaller (soluble)

TABLE II

PROTEIN CONTENTS AND ATPase ACTIVITY IN SOME FRACTIONS OBTAINED FROM PEA STEM HOMOGENATE AND PRECIPITATION OF HIGH SPECIFIC ACTIVITY ATPase FROM THE 'LOW Mg^{2+}' SUPERNATANT BY TREATMENT WITH Mg^{2+}

After homogenization of the tissue a 13–80 K pellet (microsomes) and a 220-K supernatant were obtained as described in Methods (routine preparation). Aliquots of the 'low Mg^{2+}' supernatant corresponding to approx. 400 mg initial fresh weight were incubated for 30 min at 0°C in the presence of 10 mM $MgCl_2$ and centrifuged at 80 000 × *g* for 20 min to give the Mg^{2+} sediment. Data are averages of at least eight experiments run in triplicate.

	Protein (mg per g fresh wt.)		ATPase activity		
			μmol P_i per h per g fresh wt.		Spec. act.
		Sum		Sum	
'High Mg^{2+}' microsomes	0.54	2.89	305	413	379
'High Mg^{2+}' supernatant	2.35		108		46
'Low Mg^{2+}' microsomes	0.75	3.14	79	375	105
'Low Mg^{2+}' supernatant	2.39		296		124
Mg^{2+} sediment	0.06	2.31 [a]	89	244 [a]	1483
Supernatant of Mg^{2+} sediment	2.25		155		69

[a] Compare with the values of the 'low Mg^{2+}' supernatant, from which these two fractions have been obtained.

components either by removal of Mg^{2+} (treatment with EDTA), or by an increase in the concentration of monovalent ions in the medium. On the other hand, the solubilizing effect of deoxycholate suggests that also hydrophobic interactions can play a role in maintaining the Mg^{2+}-sediment particles in the aggregated state.

Relationship between the adenylnucleotidyl phosphatase activity of the microsomal fractions and that of the Mg^{2+} sediment

To answer the question whether the ATPase activity of the Mg^{2+} sediment was due to the same enzyme present in the microsomes, we compared in our preparations the specificity for some substrates, the sensi-

TABLE III

SOLUBILIZATION OF THE ATPase ACTIVITY SEDIMENTED BY TREATMENT OF THE 'LOW Mg^{2+}' SUPERNATANT WITH $MgCl_2$

The Mg^{2+} sediment was obtained as described in Methods. Aliquots of the Mg^{2+} sediment corresponding to 400 mg initial fresh weight were resuspended in two ml Mg^{2+}-free buffer (pH 7) or in the same medium containing the different solubilizing agents; the mixtures were allowed to stand for 30 min at 20°C and centrifuged at 80 000 × *g* for 20 min to give the supernatant (A) and the precipitate (B). Data are averages from experiments carried out in triplicate and repeated twice. DOC, deoxycholate.

Treatment	ATPase activity (μmol P_i per h per g fresh weight)				
	Plain buffer (no Mg^{2+})	EDTA (5 mM)	DOC (6 mM)	$NaClO_4$ (0.3 M)	NaCl (0.3 M)
Supernatant (A)	10.9	54.9	38.2	40.4	63.3
Precipitate (B)	46.5	2.9	30.2	6.0	2.0
Total (A + B)	57.4	57.8	68.4	46.4	65.3
Supernatant/total × 100	19	95	56	87	97

TABLE IV

ATPase, ADPase, *p*-NITROPHENYLPHOSPHATASE ACTIVITY AND EFFECT OF DIETHYLSTILBESTROL ON THE ATPase ACTIVITY OF FRACTIONS OBTAINED FROM PEA STEM HOMOGENATES

Microsomes, supernatants and Mg^{2+} sediment were obtained as described in Table II. Reaction mixture for the enzyme assay contained 1 mM substrate at pH 6 (ATP and ADP) or at 5 (*p*-nitrophenylphosphate). The effect of diethylstilbestrol on ATPase activity was tested at $3 \cdot 10^{-4}$ M inhibitor concentration in the presence of 10 mM $MgCl_2$ and 50 mM KCl. Data are the means of at least three experiments run in triplicate. *p*-NPP, *p*-nitrophenylphosphate.

	Enzyme activity (μmol P_i per h per g fresh. wt.)			Ratio ATPase / ADPase	% Inhibition of ATPase activity by diethylstilbestrol
	ATP	ADP	*p*-NPP		
'High Mg^{2+}'.microsomes	238	280	0.096	0.85	–87
'Low Mg^{2+}' microsomes	90	118	0.105	0.76	–75
Mg^{2+} sediment	80	89	0.012	0.90	–85
'High Mg^{2+}'. supernatant	102	112	0.944	0.91	–45
'Low Mg^{2+}' supernatant	263	293	0.482	0.90	–46
Supernatant of the Mg^{2+} sediment	117	152	0.497	0.77	–41

tivity to divalent cations and to the inhibitor diethylstilbestrol, and the effect of pH. Recent results by Tognoli and Marrè [4] had shown that pea stem microsomal preparations as well as an enzyme extracted and purified from the same are able to hydrolyze ATP, GTP, UTP, CTP and among the nucleoside diphosphates, ADP, with an ATPase/ADPase ratio between 0.75 and 0.85, while very little (for the microsomes) or no activity (for the purified enzyme) was observed towards *p*-nitrophenylphosphate, nucleoside monophosphates and other phosphomonoesterase substrates. The pH optimum was 6 for the activity with ATP and 7 for that with ADP. The activity on both substrates was strongly stimulated by Mg^{2+}, or Ca^{2+} of Mn^{2+} (optimum with 0.5–1 mM Mg^{2+}) and inhibited by diethylstilbestrol and orthovanadate [4].

In our experiments, all of these activity characteristics were found not only for the microsomal preparations, independently of the presence of Mg^{2+} in the grinding medium, but also, essentially identical for the Mg^{2+} sediment: (a) for the activities with ATP, ADP and *p*-nitrophenylphosphate and for the inhibition by diethylstilbestrol (Table IV); (b) for the activation by divalent cations (Table V); (c) for the effect of pH on the ATPase and the ADPase activities (Fig. 2); other data not shown. These results strongly suggest that the activities of both the microsomal preparations and the Mg^{2+} sediment depend on the presence of the same enzyme.

TABLE V

EFFECTS OF DIVALENT CATIONS ON THE ATPase ACTIVITY OF THE 'HIGH Mg^{2+}' MICROSOMES AND OF THE Mg^{2+} SEDIMENT

Experimental conditions as described in Table II and in Methods. Data are the averages of two experiments run in triplicate.

Divalent cation (1 mM)	Percent stimulation of ATPase activity [a]	
	'low Mg^{2+}' microsomes	Mg^{2+} sediment
Mn	431	470
Ca	328	360
Mg	290	224
Co	78	137

[a] The ATPase activity of the controls (without divalent cations), taken = 100, was 20 and 24 μmol P_i per h per g fresh wt. for 'low Mg^{2+}' microsomes and Mg^{2+} sediment, respectively.

The data of Table IV and Fig. 2 also show that the soluble fraction contains, in addition of that sedimentable by Mg^{2+}, a second type of quite different, non Mg^{2+}-sedimentable activity attacking ATP, ADP and *p*-nitrophenylphosphate and probably due

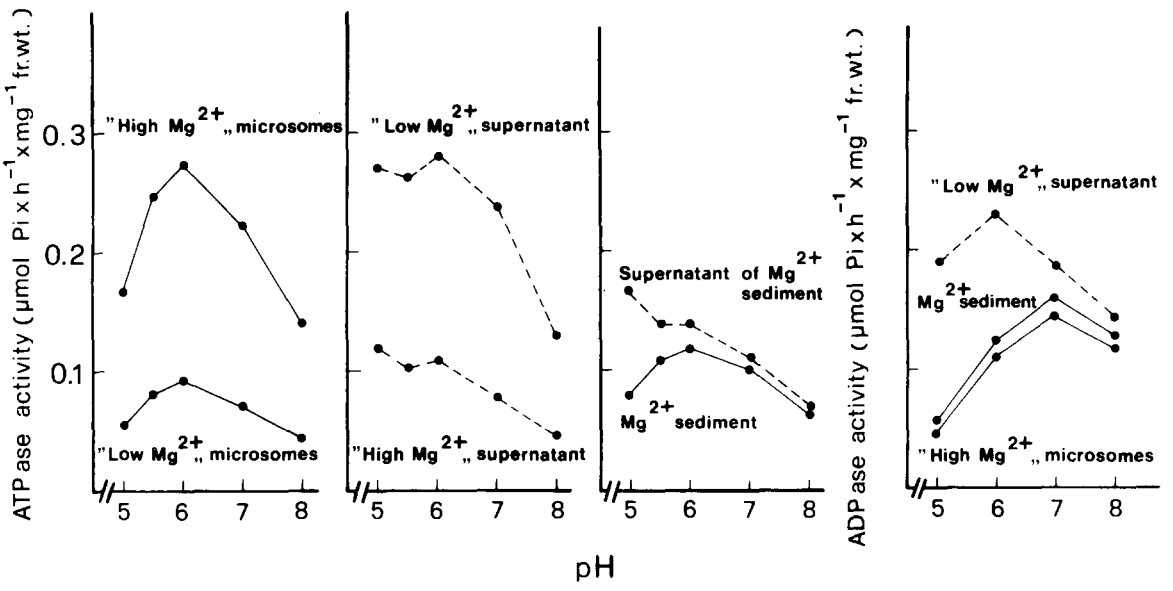

Fig. 2. Effect of pH of the reaction mixture on the ATPase and ADPase activities of different fractions from pea stem homogenates. Microsomes, supernatants and Mg^{2+} sediment were obtained as described in Materials and Methods. Data are the means of two separate experiments run in triplicate.

to the presence of an aspecific, soluble acid phosphatase described in various plant materials [8,10]. In fact, the activity against *p*-nitrophenylphosphate is much higher, while the sensitivity to diethylstilbestrol is much lower in all of the three supernatants considered in Table IV than in either the microsomal preparations or in the Mg^{2+} sediment (the *p*-nitrophenylphosphatase activity of these preparations is not significantly inhibited by diethylstilbestrol).

This conclusion is confirmed by the behaviour of the pH-actvity curves of Fig. 2. These show that, with ATP as a substrate, the activity profiles are very similar for the Mg^{2+} sediment and for the microsomes with a single optimum at pH 6, while all of the three supernatants show a tendency to a rise in the acidic region (pH 5.5 to 5), more marked for the supernatant of the Mg^{2+} sediment. Also with ADP as a substrate, the profile of the Mg^{2+} sediment coincides with that of the microsomes, with an optimum at pH 7, while the optimum is shifted towards acidity for the 'low Mg^{2+}' supernatant. These data, together with that of the very high specific activity of the Mg^{2+} sediment, indicate that the treatment with Mg^{2+} leads to a rather specific insolubilization of only one of the phosphohydrolases present in the soluble fraction.

Solubilization of the microsomal adenylnucleotidyl phosphatase activity by treatment with EDTA in the absence of Mg^{2+}

The results mentioned above indicated that the Mg^{2+}-sedimentable adenylnucleotidyl phosphatase activity of the supernatant prepared without Mg^{2+} in the grinding medium belongs to the same enzyme present in the microsomal preparations. The known tendency of other membrane ATPase from other materials to become soluble upon removal of Mg^{2+} suggested that also in our case the 'soluble', Mg^{2+}-sedimentable adenylnycleotidyl phosphatase was originally located in the microsomal particles, and had been released during grinding in the Mg^{2+}-lacking medium. The finding that even in this condition a large fraction of the ATPase activity remains in the microsomes might depend on the presence in the grinding medium of a small amount of endogenous Mg^{2+}; in fact, the total (free plus bound) concentration of this ion in the pea tissue is approx. 5 mM, and its concentration in the original homogenate (1 : 5 tissue/buffer ratio) is 0.85 mM, approx. 10% of total Mg^{2+} being 'bound' to the sedimentable microsomal particles. Thus, we investigated whether the further removal of Mg^{2+} (and/or eventually of Ca^{2+}) from the microsomal preparations should solubilize the remain-

TABLE VI

SOLUBILIZATION OF THE MICROSOMAL ATPase ACTIVITY BY EDTA IN THE ABSENCE OF Mg^{2+}

'High Mg^{2+}' microsomes or 'low Mg^{2+}' microsomes from 150 mg initial fresh weight were resuspended in 2 ml plain buffer (Mg^{2+}-free, pH 7) and supplemented or not with 10 mM $MgCl_2$ or 5 mM EDTA. The resuspensions were allowed to stand at 20°C for 30 min and centrifuged at 80 000 × *g* for 20 min to give the supernatants (A) and the precipitates (B). The pellet resulting from a first EDTA treatment of the 'low Mg^{2+}' microsomes was submitted to a second EDTA treatment in the same conditions as above. The initial ATPase values of the treated pellets were 218, for the 'high Mg^{2+}' microsomes, and 76 μmol P_i per h per g fresh weight for the 'low Mg^{2+}' microsomes. Data are the averages of three experiments run in triplicate.

	Resuspended in plain buffer plus:	ATPase activity (μmol P_i per h per g fresh wt.)		
		Supernatant (A)	Precipitate (B)	Total (A + B)
'High Mg^{2+}' microsomes	Mg^{2+}	6	194	200
	nil	14	236	250
	EDTA (1 wash)	212	32	244
'Low Mg^{2+}' microsomes	nil	6	66	72
	EDTA (1 wash)	68	7	75
	EDTA (2 wash)	77	2	79

ing particle-bound activity.

The data of Table VI show that this is the case. In fact, when the microsomes prepared either in the presence or in the absence of Mg^{2+} in the grinding medium are resuspended in a Mg^{2+}-free medium containing 5 mM EDTA, and then re-sedimented by centrifugation, a solubilization of 85 to 96% of the ATPase activity originally present is observed. The sensitivity of the ATPase activity thus solubilized to diethylstilbestrol remains practically unchanged (70 to 80% inhibition by $3 \cdot 10^{-4}$ M diethylstilbestrol, data not shown).

Recombination of the solubilized adenylnucleotidyl phosphatase with the 'low Mg^{2+}' microsomes

If the soluble, diethylstilbestrol-sensitive adenylnucleotidyl phosphatase found in the 'low Mg^{2+}' supernatant was really originally membrane-bound, one would expect that under appropriate conditions it might again recombine with the ATPase-depleted microsomes at the original site. Our work in this direction is still in a preliminary stage; some indications, however, encourage the attempt of a reconstruction of the ATPase-microsomes system. These indications can be summarized as follows:

TABLE VII

EFFECTS OF THE PRESENCE OF MICROSOMES ON THE AMOUNT OF ATPase ACTIVITY SEDIMENTING FROM THE 'LOW Mg^{2+}' SUPERNATANT WHEN SUPPLEMENTED WITH Mg^{2+}

'Low Mg^{2+}' or 'high Mg^{2+}' microsomes from 500 mg initial fresh weight were resuspended in plain buffer without Mg^{2+}; the resuspended microsomes and aliquots of 'low Mg^{2+}' supernatant corresponding to 330 mg initial fresh weight were incubated, together or separately, for 30 min at 0°C in the presence of 10 mM $MgCl_2$, then centrifuged at 80 000 × *g* for 20 min to give the microsome-Mg^{2+} sediment (see Methods). The activity in the sediments is expressed as μmol P_i per h. Data are the averages of three experiments run in triplicate.

	ATPase activity (μmol/h)			
	Mg^{2+} sediment (a)	Microsomes only (b)	Microsomes-Mg^{2+}sediment (c)	Increment [c − (a + b)]
	29.5			
'High Mg^{2+}' microsomes		108.6	140.0	1.9
'Low Mg^{2+}' microsomes		40.2	94.6	24.9

(1) The data of Table VII show that when the 'low Mg^{2+}' supernatant is incubated with the 'low Mg^{2+}' microsomes and with 10 mM $MgCl_2$, and then centrifuged at 80 000 × *g*, consistently more ATPase activity is recovered in the sediment (microsomes-Mg^{2+} sediment, see Methods) than the sum of the activity of the microsomes plus that of the Mg^{2+} sediment. This increment in activity is fully accounted for by a corresponding decrease in the supernatant (data not shown), hence it does not depend on activation following recombination of the microsomes with the supernatant. No similar increment in the activity of the sediment is observed when the supernatant is incubated with the Mg^{2+} microsomes, which have not been depleted of the originally 'bound' activity.

(2) The increment in the sedimentable adenylnucleotidylphosphatase activity observed when the 'low Mg^{2+}' microsomes are present seems due to the same enzyme made sedimentable by Mg^{2+} in the Mg^{2+} sediment. In fact it shows the same characteristics of activity towards ATP and ADP, pH optimum and sensibility to diethylstilbestrol as the Mg^{2+} sediment. Moreover, its amount is strongly decreased (by approx. 50%) when the microsomes are incubated with a supernatant previously treated with 10 mM Mg^{2+} and centrifuged so to eliminate the Mg^{2+} sediment (Note that the 'insolubilization' of the ATPase of the 'low Mg^{2+}' supernatant is still incomplete even at the 10 mM Mg^{2+} concentration, as shown in Fig. 1.).

(3) A defined fraction only of the ATPase activity of the supernatant is capable of binding to microsomes. In fact the data of Fig. 3 show that as the amount of 'low Mg^{2+}' supernatant is kept constant, and the amount of added microsomes is progressively increased, the increment of sedimentable activity rise at first linearly with the concentration of microsomes. With the further increase of the microsomes the increment increases more slowly and reaches a consistent value when the microsomes/supernatant ratio (as g tissue original fresh wheight) approached a value of 1. This may suggest that in the microsomes the sites binding the soluble enzyme are those set free by its release during homogenization in the absence of Mg^{2+}.

(4) Determinations of the temperature coefficients (Q_{10}) of the ATPase activity in the 10 to 30°C interval (Table VIII and Fig. 4) show a clear difference

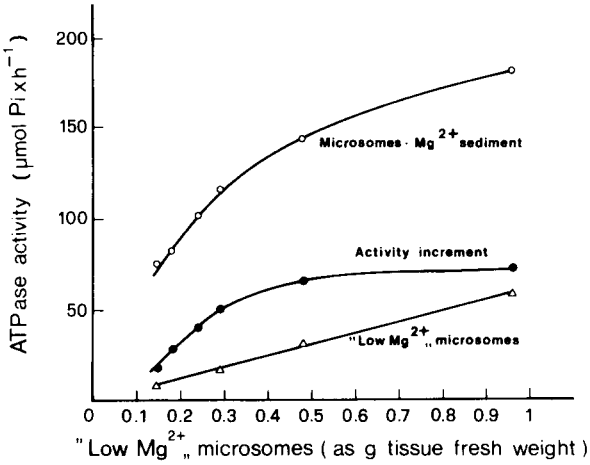

Fig. 3. Increment of Mg^{2+}-sedimentable ATPase activity as a function of the presence of increasing amounts of 'low Mg^{2+}' microsomes. Microsomes prepared in a Mg^{2+}-free medium were resuspended in the same medium, split into aliquots corresponding to different amounts of initial fresh weight (from 144 to 960 mg) and combined (○———○) or not (△———△) with aliquots of the Mg^{2+}-less supernatant corresponding to 800 mg initial fresh weight, in the presence of 10 mM $MgCl_2$. An equal amount of the same supernatant was added with 10 mM $MgCl_2$ to give the Mg^{2+} sediment (with an ATPase activity of 50 µmol P_i per h per aliquot). All the suspensions (4.8 ml final volume) were then incubated for 30 min at 0°C and centrifuged at 80 000 × *g* for 20 min. The 80 000 × *g* sediments were then assayed for ATPase activity. The activity increment (●———●) was calculated by subtracting the values corresponding to the sum of the ATPase activity of the 'low Mg^{2+}' microsomes alone, plus that of the Mg^{2+} sediment alone (48 µmol P_i per h) from the values of the activity of the microsomes-Mg^{2+} sediment. Data are the means of two separate experiments run in triplicate.

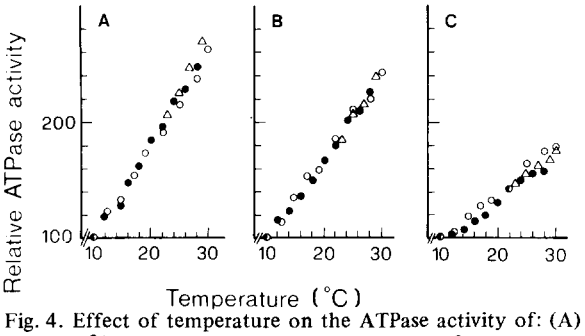

Fig. 4. Effect of temperature on the ATPase activity of: (A) 'low Mg^{2+}' microsomes; (B) microsomes-Mg^{2+} sediment; (C) Mg^{2+} sediment. All three samples were prepared under the same experimental conditions as in Table VIII. The activity at 10°C was taken as = 100 in comparing the relative activities at the various temperatures. Arrhenius plots suggested (in the limit of variability) some break at approx. 24°C for all of the three samples. The three symbols in A, B and C indicate data obtained in triplicate experiments.

TABLE VIII

EFFECTS OF TEMPERATURE ON THE ATPase ACTIVITY OF THE Mg^{2+} SEDIMENT, OF THE 'LOW Mg^{2+}' MICROSOMES AND OF THE MICROSOMES-Mg^{2+} SEDIMENT

'Low Mg^{2+}' microsomes from 500 mg initial fresh weight combined or not with aliquots for 'low Mg^{2+}' supernatant corresponding to 300 mg initial fresh weight in the presence of 10 mM $MgCl_2$ were prepared as in Table VII. The total ATPase activity in the 80-K sediment is expressed as μmol P_i per h. Data are the averages of three separate experiments run in triplicate.

	ATPase activity in the 80-K sediment			Q_{10}	
	at 10°C	at 20°C	at 30°C	10–20°C	20–30°C
Mg^{2+} sediment (A)	18.3	24.9	31.2	1.36	1.25
'Low Mg^{2+}' microsomes (B)	16.0	28.9	41.9	1.80	1.45
(Sum A + B)	(34.3)	(53.8)	(73.1)	(1.57)	(1.36)
Microsomes-Mg^{2+} sediment	38.9	66.5	95.1	1.71	1.43

between the low Q_{10} of the Mg^{2+} sediment and the higher one of the 'low Mg^{2+}' microsomes. The Q_{10} of the sedimentable ATPase activity recovered upon combination of the 'low Mg^{2+}' supernatant with the 'low Mg^{2+}' microsomes in presence of Mg^{2+} does not show the intermediate value which should be expected if the microsomes had not interacted with the soluble enzyme, but it is only slightly lower than that of the microsomes. This is what should be expected if a large fraction of the soluble activity had reestablished its original relationships with the microsomal membranes.

These results seems thus consistent with the hypothesis that the increment in sedimentable adenylnucleotidyl phosphatase activity corresponds to an at least partial reconstruction of the original membrane-enzyme structure.

Conclusion

The results presented in this paper lead to the following conclusions:

(1) Even in the absence of chelating agents for divalent cations the absence of Mg^{2+} in the homogenization medium solubilizes a large fraction of the microsomal, diethylstilbestrol-sensitive, divalent cation-dependent pH 6 optimum adenylnucleotidyl phosphatase of pea stem tissues. Washing the microsomes with 5 mM EDTA almost completely solubilizes the residual activity.

(2) Approx. 50% of the adenylnucleotidyl phosphatase activity present in the supernatant of homogenates prepared without Mg^{2+} in the brinding medium becomes insoluble, and can be sedimented at 80 000 × g, when incubated with 5–10 mM Mg^{2+} (or, also, Ca^{2+}, Mn^{2+} or Zn^{2+}). This sediment shows a very high specific activity with characteristics apparently identical to those of the microsomal enzyme as far as pH optimum, substrate specificity, sensitivity to the inhibitor diethylstilbestrol and activation by divalent cations are concerned. The sediment activity can be re-solubilized by treatment with either the divalent cation-chelating agent EDTA or with high concentration of monovalent salts, or, also to a lesser extent, with deoxycholate. This suggests that the aggregation of the Mg^{2+}-sediment particles mainly depend on ionic interactions between soluble macromolecular components and Mg^{2+} (or other divalent cations), with a minor contribution by hydrophobic bonds.

(3) Preliminary evidence suggests that in the presence of Mg^{2+} the soluble activity released by the microsomes as a consequence of the low Mg^{2+} treatment can re-bind to specific sites present in those only the microsomes which have previously been depleted of bound adenylnucleotidyl phosphatase by low Mg^{2+} treatment.

Further investigations are presently under way in this laboratory concerning the mechanism of the release of the adenylnucleotidyl phosphatase from, and its recombination to the microsomes, as well as the biochemical characterization of the enzyme and of the membrane components of the system.

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